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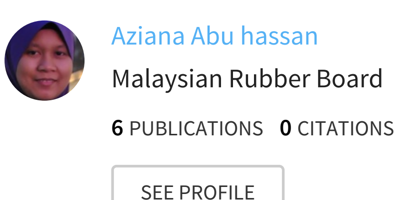

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Preface

The current volume communicates aspects of beneficial microorganisms in relation to agriculture and aquaculture. In agriculture, microorganisms have been proven useful in a wide array of potential applications ranging from biofertilizers to increasing crop productivity and from production of biomass and biofuels to enhanced quality of poultry production. Similarly in aquaculture, microorganisms have been applied in treatment and purification of wastewater from biofarming, applications of bioflocculation, and biopreservation of sea harvests. New innovations have also been observed, including the microbial production of surfactants, colorants, and biodegradable plastics, all providing insights of the unlimited potentials of microorganisms in these areas.

Penang, Malaysia | Min-Tze Liong

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Probiotics in Poultry

Sri Harimurti and Widodo Hadisaputro

Abstract The main pathogens associated with poultry farming and production are various serotypes of *Salmonella enterica*, *Escherichia coli*, and *Campylobacter jejuni*. These enteric pathogens are the most common causes of diarrhea in the poultry flock in Indonesia. Their spread is due to poor sanitation and drainage as well as improper litter management, which all lead to bacterial infection in birds. Antibiotics have previously been used for many decades by veterinarians and poultry farmers before they were banned in many countries due to concerns with increased antibiotic resistance in pathogens and antibiotic contamination in food. In the search for an alternative to antibiotics in poultry feed, the addition of probiotics is proposed. Among all the probiotics utilized in poultry production, lactic acid bacteria (LAB) are the most commonly used. The addition of LAB replaces enteric pathogens by means of competitive exclusion in the poultry intestinal tract, thereby increasing the intestinal health of poultry. This chapter discusses the increased growth and performance of poultry due to the application of native LAB.

1 Introduction

The main pathogens associated with poultry farming and production are various serotypes of *Salmonella enterica*, *Escherichia coli*, and *Campylobacter jejuni*. These enteric pathogens are the most common causes of diarrhea in the poultry flock. Bacterial infection with extraneous pathogens can be avoided when poultry are reared intensively in good environmentally controlled housing and all appropriate biosecurity measures are followed. However, many small-scale farmers in Indonesia lack the knowledge and skill to deal with biosecurity measures often resulting in an outbreak of pathogen-associated disease and the death of birds. The main causes of pathogen spread are due to poor sanitation and drainage, as well as improper litter management, which all lead to bacterial infection in birds. As a

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solution, farmers usually utilize antibiotics as a growth-promoting feed supplement which is targeted either to eradicate bacterial infection or to promote the growth of birds by increasing the feed efficiency.

Antibiotics have been used for many decades by veterinarians and poultry farmers before they were banned in many countries due to concerns with increased antibiotic resistance in pathogens and antibiotic contamination in food. According to Jones and Ricke (2003), about 30 kinds of antibiotics are used in poultry feed as additives or administered as drugs, and it is estimated that more than 13.7 % of the antibiotics used in animal production are used at subtherapeutic (growth promoting) levels. The prohibition of antibiotic use is because the residues of these drugs present in meat and eggs constitute a potential health hazard to consumers. The major health risks associated with antibiotics are the development of antibiotic resistance in exposed individuals, hypersensitivity reactions, and the development of microorganisms resistant to antibiotics in humans (Dipeolu et al. 2005). When tetracycline is provided at 200 mg/kg feed to birds, it is eventually released in the eggs, while the metabolism of tetracycline continues in the body of the layer. Birds provided with an antibiotic-supplemented diet produced eggs with a tetracycline residue level of about 0.017 μg/g. These tetracycline residues started appearing in the egg from the second week after the birds were fed a tetracycline-supplemented diet. The tetracycline residues continued to present in the eggs even after 1 week of supplementation. The Codex's recommended maximum residue level (MRL) for tetracycline in eggs is 0.02 μg/g.

In the search for an alternative to antibiotics in poultry feed, the addition of probiotics has been proposed (Fuller 1989, 2001). Among all the probiotics utilized in poultry production, lactic acid bacteria (LAB) are the most commonly used. The addition of LAB replaces enteric pathogens by means of competitive exclusion in the poultry intestinal tract and subsequently reduces bacterial contamination in poultry products, thereby increasing the safety of chicken meat and eggs. This chapter discusses the application of native LAB and their application in poultry to increase growth and performance.

2 Native Microflora in the Digestive Tract of Poultry

Under normal circumstances, the microflora that colonizes the poultry gastrointestinal tract from a few days after birth consists of 400–500 different bacterial strains for a total count of 10^{14} colony forming units (CFU) per gram of intestinal contents (Baba et al. 1991). This microflora consists of transient bacteria which reside in the tract temporarily and indigenous bacteria that colonize the intestinal tract permanently. The major component of the bacterial population of the cecal microflora in 2-day-old chicks includes strains of *Lactobacillus* spp., while *Bacteroides* spp. and *Bifidobacterium* spp. do not colonize 2-day-old chicks. Small numbers of *Clostridium* spp. and fungi are also detected. Total counts of the cecal microflora in 2-day-old chicks include *Lactobacillus* spp. ($\log_{10}$) 3.60–4.59, *Escherichia coli*

($\log_{10}$) 9.71, and Gram-positive aerobes ($\log_{10}$) 9.34; *Bacteroides* spp. and *Bifidobacterium* spp. counts were less than ($\log_{10}$) 3. However, the total counts of *Lactobacillus* spp. in adult chickens is increased to ($\log_{10}$) 9.34, while *Bacteroides* spp. and *Bifidobacterium* spp. are found at ($\log_{10}$) 9.00–9.61. Except for *Escherichia coli*, total counts of Gram-positive aerobes are decreased in adult chickens to ($\log_{10}$) 4.87 and 8.60 (Baba et al. 1991).

When feed is consumed, it is temporarily stored inside the crop (the part of the chicken digestive system after esophagus and before the gall bladder) where lactic acid fermentation takes place. The pH in this fermentation process is low, around 4–5 in a healthy chicken, and the predominant microorganisms are lactobacilli. The pH of the proventriculus and gizzard (the digestive system after the gall bladder) is much lower (pH 1–2) caused by the establishment of a surviving microbe population. The relatively high flow rate of the fluid content in the intestine inhibits the multiplication of microorganisms. In the ceca, which contains a thick viscous fluid, the highest viable bacterial count (around 10^{11} per gram of fluid) is observed, consisting of the most complex microflora. These microorganisms are mostly obligate anaerobes, i.e., Gram-positive anaerobic cocci (up to one-third of the total), while others are Gram-negative nonspore rods such as the *Bacteriodaceae* (one-fifth of the total); *Clostridium* spp. and bifidobacteria only represent one-tenth of the total. A lower number of facultative anaerobes including *E. coli*, *Salmonella*, and *Klebsiella* spp. are also present (Vila et al. 2010). *Escherichia coli* are the major competitor to *Salmonella* in newly hatched chickens. *E. coli* and *Salmonella* belong to the same family of *Enterobacteriaceae*. It is likely that these bacteria are competitive against each other in establishing themselves in the chicken intestine.

3 Natural Mechanisms of Pathogenic Control

Studies on pathogenic control by intestinal microflora have garnered great interest in poultry production in recent years. The enteric microflora of poultry has evolved according to the anatomy and physiological characteristics of the digestive tract, resulting in specific host–microbe interactions (Rinttila and Apajalahti 2013). The ability of specific bacteria in the digestive tract to degrade some dietary components into high-energy metabolites is an example of the importance of the host–microbe connection that increases nutrient availability and enhances the health of the host (Rinttila and Apajalahti 2013).

The microbial types which are first established, in all cases, are those that colonize and grow in the digestive tract of poultry throughout their adult life. The various types of colonizing bacteria are sensitive to changes which may occur in the digestive tract of the host. The genus *Lactobacillus* colonizes the avian enteric tract on the first day of life. Lee et al. (2002) reported that more than 68.8 % of sequences, from all of the tested ages, were related to those of *Lactobacillus*. However, the sequences of the bacterial population varied significantly according to the age of the birds. The community structure of 3-day-old chicks shows

homologous to *Lactobacillus delbrueckii*, *Clostridium perfringens*, and *Campylobacter coli*. From 7 days of age to 21 days, a similar community structure was maintained with dominant sequences related to *Lactobacillus acidophilus*, *Enterococcus* spp., and *Streptococcus* spp. Lactobacilli are among the few bacteria found in the acidic environment of digestive tract, but numbers rarely reach more than 10^3 CFU/ml or CFU/g. In the ileum, they range from 10^2 to10^5 CFU/ml and in the colon from 10^4 to 10^9 CFU/ml or per CFU/g (Lee et al. 2002).

The beneficial effects of lactobacilli have been attributed to their ability to suppress the growth of pathogens, probably by the secretion of antibacterial substances such as lactic acid, peroxide, and bacteriocins (Salminen and von Wright 1998). Mead (2005) reported that the size and complexity of the overall microbial community are significant factors related to controlling pathogens. Possible control mechanisms of pathogens include (1) competition between pathogens and native organisms for adhesion sites, (2) production of inhibitory metabolites by specific microorganisms, (3) competition for limiting nutrients, (4) establishment of environmental conditions that are inhibitory to the pathogen, (5) coaggregation between pathogens and certain native gut bacteria, and (6) stimulation of the immune system (Otutumi et al. 2012). Competition for adhesion sites is crucial because bacteria have to overcome peristalsis in the small intestine and subsequently will adhere tightly and withstand movement during the digestion of food. Adherence to the mucosa of the digestive tract allows probiotic colonization, immune stimulation, and competition with pathogens.

The production of inhibitory metabolites by specific microorganisms as shown by lactobacilli and *Bacillus cereus* has been reported due to various types of antibiotics. *Lactobacillus acidophilus* produces acidophilin, lactocidin, and acidolin, whereas *L. plantarum* produces lactolin. Nisin and diplococcin are among the antibiotics produced by streptococci. *Bacillus cereus* produces a bacteriocin-like substance that inhibits closely related *Bacillus* spp. and species such as *Staphylococcus aureus* and *Micrococcus luteus*, which show high activity in the pH range of 2.0–9.0 (Risoen et al. 2004). Additionally, Vila et al. (2010) reported that some lactobacilli produce sufficient hydrogen peroxide to inhibit various microorganisms. Lyzozyme produced by *Bifidobacteria* has been reported to alter the pathogenic activities of bacteria, reduce antibiotic-induced side effects, inhibit mammary and liver tumors, and in conjunction with oligofructose decrease 1,2-dimethylhydrazine which induces carcinogenesis (Chichlowski et al. 2007).

The chicken digestive tract is a natural environment that consists of diverse microbial species. In this environment, bacteria compete with each other to obtain both space for attachment sites and nutrition for their growth (Hibbing et al. 2010). The establishment of environmental conditions that are inhibitory to pathogens involves lowering the pH of the intestine.

Butyrate is known to decrease the colonization of *Salmonella*, partly mediated by a specific suppression of the *Salmonella* Pathogenicity Island I genes (Gantois et al. 2006). Increases in LAB counts in the digestive tract were correlated with increases in butyric acid in a rat model. Although LAB do not produce butyric acid themselves, they increase butyric acid concentrations in the digestive tract

indirectly, and this stimulate the proliferation of butyric acid producing bacteria. This mechanism is called cross-feeding. It has been shown that lactic acid, produced in vitro by *Bifidobacterium adolescentis* with starch as the sole carbon source, is used by *Anaerostipes caccae* and *Eubacterium halii* (in co-culture) for the production of high concentrations of butyric acid. The same mechanism of cross-feeding is most likely present in the digestive tract of chicken, as LAB are highly prevalent and anaerobic butyric acid-producing bacteria in the chicken's cecum, taxonomically classified as part of *Clostridium* cluster IV and XIVa, are present (Van Immerseel et al. 2009).

Stimulation of the immune system is a part of the natural mechanism of pathogen control. The normal microflora in an animal has a significant impact on the body's immune system. The number of intraepithelial lymphocytes, plasma cells, and Peyer's patches is lower in germ-free animals than in conventional animals. The immune-stimulant effect associated with probiotic administration is related to the ability of these microorganisms to interact with Peyer's patches and intestinal epithelial cells (IEC), thereby activating mucosal immunity by stimulating plasma cells, IgA secretion, and the migration of intestinal T cells (Eto et al. 2012).

In the intestine, probiotics interact with enterocytes, goblet cells, M cells from Peyer's patches, and isolated follicles that are extended through the mucosa and submucosa in the small intestine, forming the gut-associated lymphoid tissue (GALT) (Fig. 1). These interactions result in an increase in the number of IgA-producing cells accompanied by the production of secretory IgM and IgA that are particularly important to the immunity of the mucosa, as they contribute to the barrier against pathogenic microorganisms (Otutumi et al. 2012).

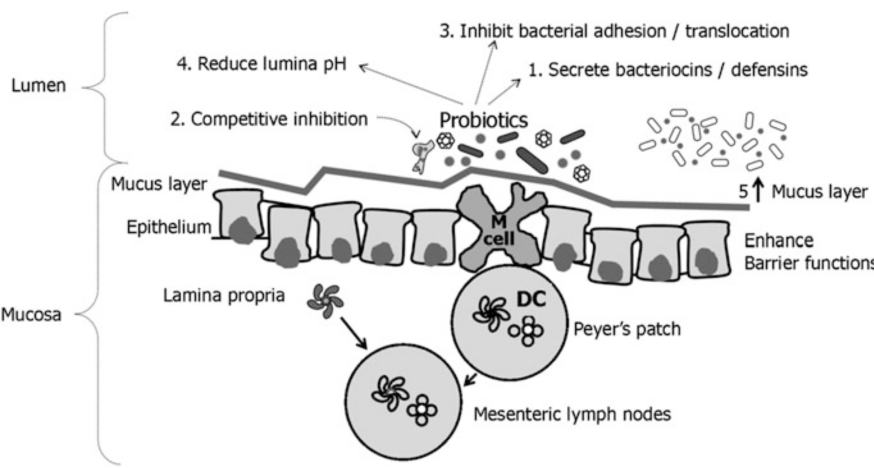

Fig. 1 Inhibition of enteropathogenic bacteria and enhancement of barrier function by probiotic bacteria. Schematic representation of the crosstalk between probiotic bacteria and the intestinal mucosa (Otutumi et al. 2012)

The mechanisms by which microflora can contribute to the intestinal health of poultry are growth promotion, improvement of the mucosal architecture, degradation of unfermentable substrates into digestable components, improvement of intestinal and general health, breakdown of cytotoxic substances, production of vitamins, suppression of pathogens, competition for nutrients, competition for adhesion sites at the mucosal epithelium, stimulation of intestinal motility, stimulation of the immune system, production of volatile fatty acids, and production of antimicrobial substances (for review see Yang et al. 2009).

Mucosal barrier systems are formed by a mucus layer, epithelial cells connected by tight junctions, and leukocyte activity. These systems play important roles in preventing microbial infection in mucosal tissues. Tight junctions (TJ) of the mucosal epithelium form a paracellular barrier that protects the underlying tissue against toxic materials or pathogens (Goto and Kiyono 2012). Claudins are proteins that form the main components of the TJ. Each family member of the claudin interacts laterally with another claudin protein located in the adjacent cell in a homotypic or heterotypic manner. They assemble in the plasma membrane together with other proteins of the TJ complex, such as occludin and tricellulin. If microorganisms cross an epithelial barrier and begin to replicate in mucosal tissues, phagocytic cells including monocytes/macrophages and polymorphonuclear leukocytes (PMNs) recognize, ingest, and destroy them (Murphy et al. 2007). Sri-Harimurti and Ariyadi (2014) reported that the relative expression of *claudin-1*, *-3*, *-5* mRNA in the ileum is higher in birds supplemented with indigenous LAB probiotics compared to unsupplemented birds. These results suggest that probiotics such as LAB play a role in regulating the mucosal barrier formed by tight junctions in the intestine of broiler chickens.

4 Application of Probiotic Microorganisms

4.1 Definition of the Probiotic Concept

Many external factors, such as bird stress, dietary changes, vaccinations, the addition of antibiotics and drugs, or improper management occur on farms and contribute to an imbalance in the poultry's intestinal microflora. This condition may lead to increased populations of potentially harmful microorganisms, such as clostridia and certain Enterobacteriaceae, or extraneous pathogens that enable them to colonize the intestinal tract. One strategic way to overcome these problems is to add beneficial microorganisms to the host orally, using the normal route of the digestive tract of the bird. These added beneficial microbes are known as probiotics.

Probiotics have been redefined throughout the years with the acquisition of better understanding and more scientific knowledge on its relationship between intestinal health and better performance. Fuller (1989) defined probiotics as "a live microbial feed supplement which beneficially affects the host animals by improving

its microbial balance." Schrezenmeir and de Vrese (2001) improved the probiotic definition as "a preparation of a product containing viable, in sufficient numbers, which alter the microflora in compartment of the host and by that exert beneficial health effects in this host." Timmerman et al. (2006) provided evidence that multispecies probiotics are more effective than monospecies probiotics and also that species-specific probiotics elicit different health effects than do probiotics derived from another host species. Furthermore, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have redefined probiotics based on health purposes; according to the FAO and WHO, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Lee and Salminen 2009).

Nowadays, probiotics have been marketed containing single or multimicrobial strains of known identities. Many criteria have been proposed to determine suitable probiotics in the poultry industry, including being suitable for preparation of a viable product on a commercial scale, remaining viable and stable for long periods during storage and field use, and able to survive but not necessarily multiply in the host intestine. Sri-Harimurti et al. (2014) reported that indigenous LAB probiotics consisting of *Lactobacillus murinus*, *Streptococcus thermophilus*, and *Pediococcus acidilactici* attached firmly to the intestine of broiler chickens, and a number of these species were detected in the ileum of laying quails 3 days after stopping probiotic supplementation. This means that probiotics originally isolated from chicken culture strains can survive and establish within the gastrointestinal tract of broiler chickens and laying quails (poultry).

4.2 Selection of the Ideal Probiotic Microorganisms

Some genera of bacteria have been applied as probiotic cultures, but the most commonly used are *Lactobacillus* and *Bifidobacterium* species. The selection of the strain to be used as an ideal probiotic is a complex process. It begins with choosing the source of bacteria; the natural intestinal environment is the most suitable source. When the objective of the work is to obtain the ideal bacterial probiotics for poultry, the best source is the digestive tract of healthy adult poultry. The next procedure for the characterization of potential probiotics usually involves in vitro tests to determine functional aspects and a safety assessment. Functional aspects of a probiotic include the ability to (1) resist digestive tract conditions (acid and bile), (2) attach to IEC in birds and trigger pathogen displacement (competitive exclusion), (3) inhibit the growth of potential pathogens by producing antimicrobial substances, and (4) modulate the immune system (antibody titer tests to measure the plasma antibody activity of the host). When all of the functional capabilities of the probiotic candidate have been met, the subsequent evaluations for probiotics are done using in vivo studies. The in vitro performance can be further evaluated using in vivo studies with animals (poultry) in laboratory test or on a poultry farm. In this stage, it is important to use untreated birds as the control to measure the efficacy of

the selected strain of probiotic bacteria compared with that of treated birds and also to determine the possible adverse effects.

4.3 Positive Influences of Probiotics in Poultry

4.3.1 Effect on Broiler Productivity

Although probiotics are considered to promote poultry health, the actual mechanisms involved have not yet been fully elucidated. The most important advantage of probiotic application in poultry is that, unlike antibiotics, they leave no residues in the meats and eggs, which may have serious health implications for consumers.

Nowadays, it is well recognized that probiotics are strain-specific, live microbial cultures that produce beneficial effects on the host's body. These cultures could be a single species of bacteria or a combination of many microbes. They are commonly isolated from the digestive tract of a healthy adult animal, typically from the same species to which the probiotics will be administered. As an ideal probiotic, these bacteria should become a part of normal microbial flora in the intestine, survive gastrointestinal passage, and be able to adhere and colonize the intestinal tract.

Sri-Harimurti et al. (2010) reported that indigenous LAB isolated from the digestive tract of healthy adult Indonesian native chickens (*ayam kampung*), including *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kp6, proved to be efficient as a feed supplement to improve the live performance of broiler chickens (Table 1).

Table 1 shows that supplementation of probiotics benefited the host animals. Oral supplementation of LAB probiotics to broilers at 10^7 (T1), 10^8 (T2), and 10^9 CFU/ml/bird/day (T3) had a significant effect ($P < 0.05$) on the productivity, including cumulative body weight gain, cumulative feed consumption, and the feed conversion ratio (Table 1).

The beneficial effects of probiotics are thought to occur due to the production of secreted factors that stimulate cytokines and cell-mediated immunity (Britton and Versalovic 2008), thereby stimulating appetite (Asli et al. 2007) and improving intestinal balance, which shortens the period required to stabilize the microflora (Vila et al. 2010). Table 1 shows that supplementation of indigenous LAB as probiotics to broiler chickens benefited the host by stimulating appetite. Cumulative feed consumption of broiler chickens in all the probiotic-supplemented groups was higher than in the control (unsupplemented birds). Higher cumulative feed consumption in this study was in agreement with greater body weight gain. The cumulative body weight gain of broiler chickens on T1 and T3 (1892.28 ± 9.74 and 1886.70 ± 1.46 g/bird, respectively) was greater than T2 (1840.45 ± 4.58 $6#g/bird). These results confirmed the fact that supplementation of probiotics during 35 days of growth enhanced broiler productivity performance. These findings are also in agreement with those of Angel et al. (2005) reported that the addition of direct-fed microbes to broilers improved N, Ca, and P retention. Probiotics

Table 1 The effect of probiotic supplementation on the productivity of broiler during a 35-day experimental period (Sri-Harimurti et al. 2010)

	Dosage of supplementation (CFU/ml/bird/day)			
	T0 (unsupplemented)	10^7 (T1)	10^8 (T2)	10^9 (T3)
Cumulative feed consumption, g/bird/35 days	2845.33 ± 68.2^{a}	$3112.33 \pm 47.24^{b,c}$	3011.00 ± 66.77^{b}	3149.00 ± 36.14^{c}
Cumulative body weight gain, g/bird/35 days	1689.62 ± 25.19^{a}	1892.28 ± 9.74^{c}	1840.45 ± 4.58^{b}	1886.70 ± 1.46^{c}
Feed conversion ratio	1.61 ± 0.01^{b}	1.58 ± 0.02^{a}	$1.60 \pm 0.06^{a,b}$	1.58 ± 0.01^{a}

T0: unsupplemented birds. T1, T2, and T3: supplemented with a probiotic mixture of *L. murinus* Ar3, *S. thermophilus* Kp2, and *P. acidilactici* Kd6 provided orally at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively

^{a,b,c}Values within a row with no common superscripts differ significantly ($P < 0.05$)

Table 2 Effects of probiotic supplementation on live weight (g), carcass yield (g), carcass (%), breast portion weight (g), abdominal fat weight, and abdominal fat percentage in broiler chickens aged 35 days (Sri-Harimurti et al. 2013a, b)

	T0	T1	T2	T3
Live weight (g)	1748.20 ± 19.52^{a}	1966.20 ± 38.05^{d}	1824.50 ± 38.89^{b}	1899.00 ± 43.50^{c}
Carcass yield (g)	1173.63 ± 32.79^{a}	1360.78 ± 40.81^{d}	1245.51 ± 27.08^{b}	1304.41 ± 59.42^{c}
Carcass (%)	67.60 ± 1.46	69.20 ± 0.90	68.26 ± 0.60	68.66 ± 1.86
Breast portion weight (g)	504.24 ± 20.61^{a}	573.57 ± 19.56^{c}	523.26 ± 16.33^{a}	551.85 ± 13.22^{b}
Abdominal fat weight (g)	35.16 ± 1.64^{c}	35.40 ± 2.25^{c}	30.52 ± 1.34^{b}	27.28 ± 1.60^{a}
Abdominal fat percentage (%)	2.00 ± 0.08^{d}	1.80 ± 0.08^{c}	1.67 ± 0.06^{b}	1.43 ± 0.05^{a}

T0: unsupplemented birds. T1, T2, and T3: supplemented with probiotic mixture of *L. murinus* Ar3, *S. thermophilus* Kp2, and *P. acidilactici* Kd6 provided orally at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively

[a,b,c,d]Values within a row with no common superscripts differ significantly ($P < 0.05$)

enhanced the absorption of vitamins, especially the B-vitamin group, and increased amylolytic activities which improved feed utilization.

Sri-Harimurti et al. (2013a, b) also reported that the application of a mixture of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kp6 had significant effects on live weight, carcass yield, breast portion weight, abdominal fat weight, and abdominal fat percentage but not in carcass percentage when supplemented to broilers orally at 10^7 (T1), 10^8 (T2), and 10^9 CFU/ml/bird/day (T3) (Table 2). The carcass percentages in this experiment were 68.26 ± 0.60 (T2), 68.66 ± 1.86 (T3), and 69.20 ± 0.90 (T1), similar to 70.40 observed with the application of a commercial Lactina probiotic. However, the live body weights with the supplementation of indigenous LAB at 1824.50 ± 38.89 (T2), 1899.00 ± 43.50 (T3), and 1966.20 ± 38.05 (T1) were higher compared to 1688.9 g in broilers supplemented with a commercial Lactina probiotic (Djouvinov et al. 2005a, b).

4.3.2 Effect on Intestinal Morphology

After 35 days of treatment with a mixture of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kp6, the villi height, villi width, and crypt depth of the duodenum, jejunum, and ileum were statistically different ($P < 0.05$) from the control group (unsupplemented with probiotics), as presented in Table 3.

Table 3 shows that probiotic supplementation in broiler chickens increases the villus height and villus width. Previous findings reported that villus height, villus width, and crypt depth in the jejunum, ileum, and cecum were significantly increased in 28- and 42-day-old broiler chickens fed with a probiotic mixture

Table 3 Effect of probiotic supplementation on the intestinal morphology of broiler chickens aged 35 days (Sri-Harimurti et al. 2013a, b)

	T0	T1	T2	T3
Duodenum				
Villus height (μm)	497.23[a]	697.20[b]	713.87[b]	688.87[b]
Villus width (μm)	73.33[a]	104.47[b]	122.20[b]	111.13[b]
Crypt depth (μm)	90.53[a]	141.70[b]	125.03[b]	134.46[b]
Jejunum				
Villus height (μm)	558.33[a]	811.32[b]	791.66[b]	775.56[b]
Villus width (μm)	75.53[a]	136.10[c]	119.43[b]	122.23[b]
Crypt depth (μm)	92.80[a]	113.90[b]	120.57[b]	114.43[b]
Ileum				
Villus height (μm)	516.66[a]	738.90[b]	747.23[b]	722.23[b]
Villus width (μm)	69.97[a]	132.20[b]	113.90[b]	121.67[b]
Crypt depth (μm)	76.10	108.33	114.43	123.86

T0: unsupplemented birds. T1, T2, and T3: supplemented with probiotic mixture of *L. murinus* Ar3, *S. thermophilus* Kp2, and *P. acidilactici* Kd6 provided orally at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively
[a,b,c,d]Values within a row with no common superscripts differ significantly ($P < 0.05$)

Table 4 Effect of indigenous probiotic lactic acid bacteria supplementation on ileal propionic and butyric acid production (%) in male laying quails at 42 days of age (Sri-Harimurti and Widodo 2014)

Short chain fatty acids	T0	T1	T2	T3
Propionic acid	0.027[a]	0.035[a]	0.033[a]	0.072[b]
Butyric acid	0.015[a]	0.022[b]	0.033[c]	0.028[c]

T0: unsupplemented birds. T1, T2, and T3: supplemented with probiotic mixture of *L. murinus* Ar3, *S. thermophilus* Kp2, and *P. acidilactici* Kd6 provided orally at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively
[a,b,c]Values within a row with no common superscripts differ significantly ($P < 0.05$)

(protexin) (Gunal et al. 2006). The increase was likely due to enhanced short chain fatty acid formation induced by the probiotic (Table 4).

Table 4 shows the production of propionic and butyric acids in the ileum and cecum of laying quails after supplementation with probiotics consisting of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kd6. Short-chain fatty acids (SCFAs), including propionic and butyric acids, are by-products of bacterial fermentation that stimulate the proliferation of the bowel epithelium. The most promising targets for probiotics are gastrointestinal functions, including those that control transit time, bowel habits, and mucosal motility as well as those that modulate the epithelial cells of the gastrointestinal tract, as presented in Fig. 2.

The in vitro adhesion assay showed that *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kp2, and *Pediococcus acidilactici* Kd6 had a good ability to adhere to IEC, as revealed by phase-contrast microscopy (Fig. 3). Scanning electron

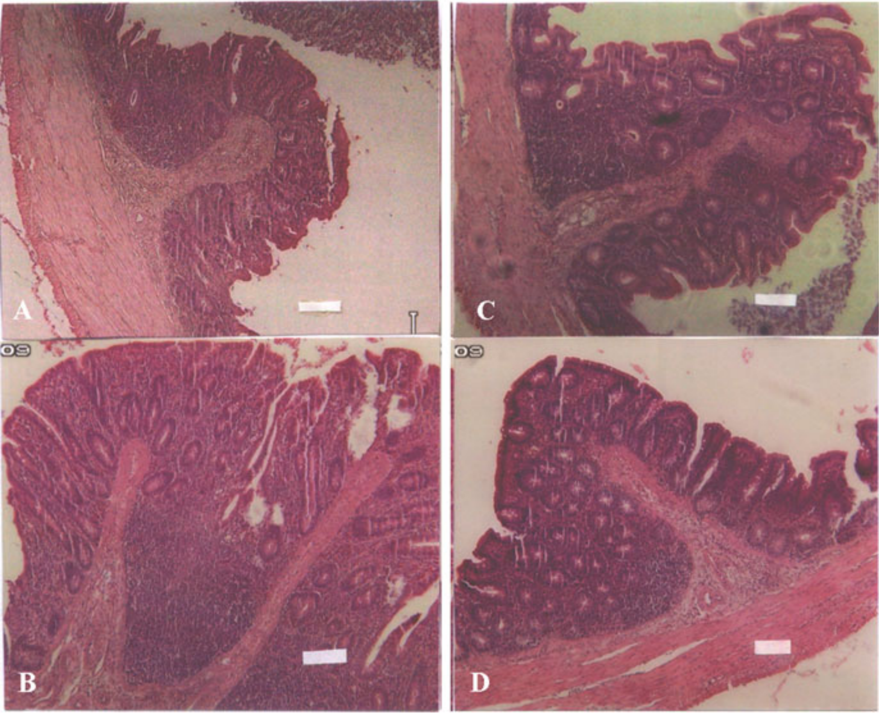

Fig. 2 Histology of the villi (ileum) of broilers after supplementation with probiotic LAB. *Scale bar* = 10 μm. (**a**) Villi of control (unsupplemented); (**b**) supplemented with probiotics at 10^8 CFU/ml/chick/day; (**c**) supplemented with probiotics at 10^7 CFU/ml/chick/day; and (**d**) supplemented with probiotics at 10^9 CFU/ml/chick/day

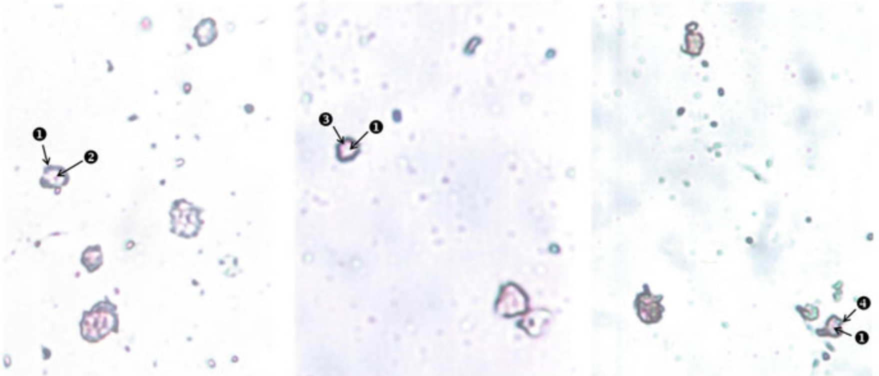

Fig. 3 Phase-contrast microscopy of adherence of lactic acid bacteria on intestinal epithelial cells (IEC) of the chicken. (1) IEC; (2) *L. murinus* (Ar3); (3) *S. thermophilus* (Kp2); and (4) *P. acidilactici* (Kd6)

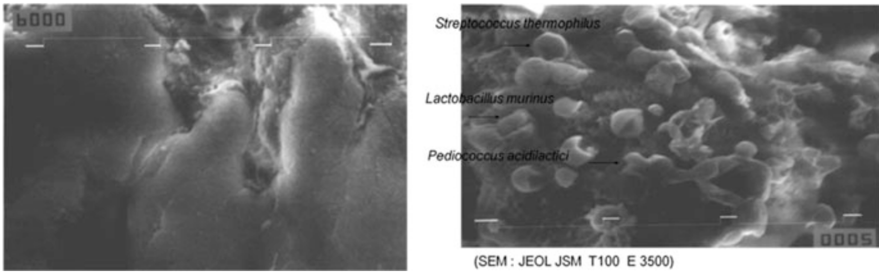

Fig. 4 Scanning electron micrograph of *Lactobacillus murinus* (Ar3), *Streptococcus thermophilus* (Kp2), and *Pediococcus acidilactici* (Kd6) in the chicken intestine. *Left*: unsupplemented chicken intestine; *right*: supplemented by those three strains. *Scale bar* = 1 μm

micrographs showed a clear appearance of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kp2, and *Pediococcus acidilactici* Kd6 attached firmly to the intestine of the supplemented broiler, but there was no attachment on the intestine of unsupplemented chickens (Fig. 4). It is assumed that lactobacilli adhere to epithelial surfaces by interactions occurring between specific molecules on bacterial cells and on the gastrointestinal surface of the host (Gusils et al. 2003). It has been reported that *Lactobacillus* strains adhere to the intestinal epithelium through specific proteins located on the bacterial surface. Tannock (1990) reported the contribution of carbohydrate-specific molecules (lectins) to lactobacillus adherence to epithelial surfaces.

A clear appearance of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kp2, and *Pediococcus acidilactici* Kd6 attached firmly to the intestine of a supplemented broiler, but there was no attachment on the intestine of the unsupplemented chicken, as shown in Fig. 4 (Sri-Harimurti et al. 2012). The adherence of these probiotics improved the intestinal morphology of broiler chickens (Sri-Harimurti et al. 2013a, b), as seen in Table 3 and Fig. 2. It has been previously reported that the adherence of LAB to IEC is able to modulate some characteristics of digestive physiology, such as intestinal permeability, mucosal immunity and the mucus layer (Awad et al. 2008), and promote intestinal health by improving intestinal enzyme activity and nutrient availability (Angel et al. 2005; Carvalho and Hansen 2005). Sieo et al. (2005) have shown that changes in GIT morphology by the adherence of LAB provide a greater surface area for the absorption of nutrients by the host.

Probiotics enhance the absorption of vitamins, especially the B-vitamin group, and increase amylolytic activity, which improves feed utilization. Other studies have found increased intestinal health as a result of greater intestinal enzyme activity and nutrient availability (Gunal et al. 2006). The improved intestinal morphology of the host induced by probiotics increases the nutrient absorption area (Smirnov et al. 2005). These findings support this report that oral supplementation with indigenous probiotics consisting of a mixture of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kp2, and *Pediococcus acidilactici* Kd6 improved the feed conversion ratio of broiler chickens.

Table 5 The effect of indigenous LAB supplementation on intestinal length, liver weight, and gizzard weight of broilers (Sri-Harimurti and Widodo 2014)

	Dosage of supplementation (CFU/ml/bird/day)			
	T0 (unsupplemented)	10^7 (T1)	10^8 (T2)	10^9 (T3)
Intestinal length, cm/bird/35 days	149.32 ± 0.95^a	165.00 ± 2.13^c	155.58 ± 3.55^b	163.26 ± 1.08^c
Liver weight, g/bird/35 days	39.05 ± 0.95^a	51.75 ± 12.37^d	43.00 ± 19.33^b	47.02 ± 11.55^c
Gizzard weight, g/bird/35 days	19.10 ± 4.62^a	23.53 ± 2.73^c	21.67 ± 9.33^b	23.32 ± 7.6^c

T0: unsupplemented birds. T1, T2, and T3: supplemented with probiotic mixture of *L. murinus* Ar3, *S. thermophilus* Kp2, and *P. acidilactici* Kd6 provided orally at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively

[a,b,c]Values within a row with no common superscripts differ significantly ($P < 0.05$)

Intestinal length, liver weight, and gizzard weight were significantly affected ($P < 0.05$) by the supplementation of indigenous probiotics to broilers (Table 5).

These results (especially regarding intestine length) were the consequence of the adherence ability and colonization of LAB to the ileal epithelial cells of broiler chickens, as presented in Figs. 3 and 4 (Sri-Harimurti et al. 2012). The adherence of these probiotics improved the intestinal morphology of broiler chickens (Table 5). Synthesis of butyric acid and propionic acid (Table 4) could play a role in gut morphology. LAB do not produce butyric acid themselves; rather they increase the butyric acid concentration in the gut indirectly, as they stimulate the proliferation of butyric acid producing bacteria. This mechanism is called cross-feeding (Van Immerseel et al. 2009).

The liver is the largest glandular organ in broiler chickens as it maintains homeostatic functions in the body (Diot and Douaire 1999). Hence, a larger liver is an indicator of the maximum metabolic activities in the chicken (Zhao et al. 2009). Djouvinov et al. (2005a, b) reported that probiotics administered in the broiler diet improved the live weight, weight of carcass, liver, gizzard and heart, although it had no effect on feed consumption. The increased gizzard size was due to the presence of indigestible materials and greater density of muscle tissue. In this study, the size of the gizzard in T1 was statistically similar with that in T3, possibly related to the patterns of greater body weight gain and the amount of feed consumption in those birds, as shown in the Table 5.

4.3.3 Effect on the Immune System

The diameter of Peyer's patches in the intestine, as well as the weight of the bursa and spleen, as indicators of immune responses in chickens following probiotic supplementation is presented in Table 6.

Table 6 Effects of probiotic supplementation on the diameter of Peyer's patches in the ileum, as well as bursa and spleen weight in broiler chickens aged 35 days

	T0	T1	T2	T3
Diameter of Peyer's patches of the ileum (μm)	339.83 ± 21.85^{a}	539.50 ± 23.68^{c}	467.67 ± 49.28^{b}	$505.67 \pm 26.59^{b,c}$
Bursa weight (g)	1.93 ± 0.10^{a}	2.61 ± 0.07^{c}	2.26 ± 0.23^{b}	$2.51 \pm 0.12^{b,c}$
Spleen weight (g)	1.01 ± 0.11^{a}	1.73 ± 0.17^{c}	1.31 ± 0.10^{b}	$1.55 \pm 0.15^{b,c}$

T0: unsupplemented birds. T1, T2, and T3: supplemented with probiotic mixture of *L. murinus* Ar3, *S. thermophilus* Kp2, and *P. acidilactici* Kd6 provided orally at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively
[a,b,c,d]Values within a row with no common superscripts differ significantly ($P < 0.05$)

Table 7 The effect of LAB supplementation on the total LAB count in the ileum of male quails at 42 days of age

	Total LAB count in the ileum (log CFU)			
Replication	T0	T1	T2	T3
1	5.54	6.22	6.93	8.24
2	5.47	6.16	6.96	8.27
3	5.51	6.27	6.99	8.19
Average	5.51^{a}	6.22^{b}	6.96^{c}	8.23^{d}

T0: unsupplemented birds. T1, T2, and T3: supplemented probiotics at 10^7, 10^8, and 10^9 CFU/ml/bird/day, respectively
[a,b,c,d]Values within a row with no common superscripts differ significantly ($P < 0.05$)

Ahmad (2006) proposed a mode of action of probiotics in stimulating the immunity of chickens in two ways: (a) flora from probiotics migrates throughout the gut wall and multiply to a limited extent, or (b) antigens released by dead microorganisms are absorbed and thus stimulate the immune system. It is believed that there is some relationship between the ability of a strain to translocate and its ability to be immunogenic. Havenaar and Spanhaak (1994) explained the improvement in immune system in three different ways: (a) enhanced macrophage activity and the ability to phagocytose microorganisms or carbon particles, (b) increased production of antibodies, particularly the IgG and IgM classes and interferon (a nonspecific antiviral agent), and (c) increased local antibody levels at mucosal surfaces such as the gut wall (usually IgA). The increased diameter of Peyer's patches of the ileum, as well as bursa weight and spleen weight, as seen in Table 6 could increase the interaction of microorganisms with Peyer's patches and IEC, thereby activating mucosal immunity by stimulating plasma cells, IgA secretion, and the migration of intestinal T cells.

The beneficial effects of probiotics have also been attributed to their ability to suppress the growth of enteric pathogens (data not shown) and increase LAB probiotic microbes in the ileum of chickens (Table 7).

Probiotic supplementation of the broiler diet significantly increased the total LAB in the ileum of chickens at the age of 42 days (Table 7). The higher the

concentration of LAB added, higher the total LAB counts in the ileum. The total LAB counts in the ileum of chickens provided with a mixture of probiotics at 10^9 CFU/ml/bird/day 3 days after stopping probiotic supplementation were even higher than that in birds unsupplemented with LAB (data not shown). The higher concentration of probiotics inside the ileum of chickens contributed to competitive exclusion and the growth inhibition of *Salmonella pullorum* (data not shown).

5 The Future of Probiotic Application in the Poultry Industry

With increasing concern about antibiotic resistance and the ban on subtherapeutic antibiotic usage in Europe and the United States, it has become important to find alternatives to antibiotic use in poultry production. Probiotics are an alternative with the potential to reduce pathogenic infections in poultry, which is relevant in improving the productivity and immunity of the host.

Until now, there has been limited knowledge on the effective dosage of probiotics when they are administered continuously. The application of probiotics for broiler chickens is different than that for laying hens due to differences in their lifespan. Broiler chickens have a short lifespan (about 35 days), while laying hens are commonly raised for 80–100 weeks. Apart from that, the effects of probiotics on poultry health and productivity vary greatly depending on many factors: (1) the type of probiotic (lactobacilli, bifidobacteria, yeasts, enterococci, etc.), (2) the daily dose (10^7–10^{10} CFU/ml/bird/day), (3) the frequency of daily administration (1–4 times), (4) the timing of administration (before, during, and after a meal), (5) the method of delivery (fermented feed, orally via drinking water, in the feed), (6) the viability of the probiotic bacteria, and (7) the duration of administration (short term or longer term).

By definition, probiotic is a beneficial microbial preparation or direct-fed live microbes given to poultry. Therefore, it is important to maintain the viability of probiotic bacteria. One method for maintaining the viability of microbial biomass is by protecting them from damaging external factors through microencapsulation. When microencapsulated, the viability and functional activities of the probiotics can be maintained. In the future, the poultry feed mill industry and the large-scale poultry industry will require encapsulated probiotic products supplemented in the feed.

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Utilization of Microorganisms for Biopurification of Wastewaters (Agricultural and Industrial): An Environmental Perspective

Jeremiah David Bala, Japareng Lalung, and Ismail Norli

Abstract Microbial biotreatment of wastewaters is a concern in recent years. Discharge of toxic pollutants to wastewater collection systems has increased concurrently with society's progressive industrialization. Although industrialization is inevitable, various devastating ecological and human disasters which have continuously occurred implicate industries as major contributors to pollution problems and environmental degradation of various magnitudes. Organic and inorganic substances which were released into the environment as a result of agricultural and industrial water activities lead to organic and inorganic pollution. It stands to reason that an effective treatment of these wastewaters is necessary. Microorganisms have been tested primarily as an approach for the removal of organic pollutants from wastewaters and have been proven effective at reducing chemical oxygen demand (COD) and toxicity. Biological treatment in the study provides some of the most viable options for the treatment of wastewaters. Microbial degradation of industrial wastewaters involving the application of a variety of microorganisms has demonstrated effective degradability of wastewaters which has attracted attention in recent time. The utilization of these microorganisms for bioremediation of toxic industrial wastewaters offers a very efficient tool for biopurification of contaminated effluents. Bacterial and fungal strains in this study have huge capability of treating wastewaters discharged from various industries. They are ubiquitous in nature and their adaptability to extreme conditions makes them good biodegraders. Their enzyme producing activity makes them effective decolorizers and they remove toxic metals by adsorption ultimately rendering the wastewaters more ecofriendly. Noteworthy, the bacterial and fungal biomasses present many assets for the biopurification of wastewaters.

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1 Introduction

Water pollution through industrial discharges, which is mainly in the form of effluents or wastewaters, is one of the biggest problems. These effluents have strong concentrations of chemical oxygen demand (COD), phenol and its derivatives, and often contain metals, inorganic nutrients, organic compounds, proteins, cyanides, chlorinated lignin, and dyes (Tripathi et al. 2007).

Industrial processes produce large amount of toxic and stable pollutants, which are all collected into the water coming out from the plant (Spina et al. 2012). The disposal of these contaminated effluents into receiving waters can cause environmental damages and further influence the aquatic ecosystem and human health (Prigione et al. 2008). It stands to reason that an effective treatment of these wastewaters is necessary.

Pollution has been defined in various ways. It is considered as the release of unwanted substances to the environment by man in quantities that damage either the health or the resources itself. Water pollution involves the release of small amounts of substances directly through point sources or indirectly through nonpoint sources. Industrial effluents or wastewaters from various industries like textile, dyestuffs, paper and pulp, distillery, olive oil mills, palm oil mills, pharmaceutical, crude oil production, oil refinery, petrochemical industry, metal processing, compressor condensates, lubricant and cooling agents, car washing, restaurants, metal industries, and so on are the major contributors to water pollution as they create more subtle effects on behavior, reproduction, or even survival of biotic communities (Tripathi et al. 2007; Lan et al. 2009).

The physical and chemical methods of industrial effluent .treatment processes remove organic pollutants at low level; they are highly selective to the range of pollutants removed and prohibitively expensive. Control of wastewater pollution is one of the prime concerns of society today with economic constraints on pollution control processes, affordable and effective methods have become a necessity (Tripathi et al. 2007). Untreated or partially treated wastewaters and industrial effluent discharged into natural ecosystems pose a serious problem to the ecosystem and the life forms. Microbial treatment systems have advantage of being simple in design and low in cost (Banat et al. 1996).

The biological processes for wastewater treatment. consist of mixed communities with a wide spectrum of microorganisms, including bacteria, protozoa, fungi, rotifers, and possibly algae (Sethupathi 2004). Biological treatment of wastewaters depends greatly on consortium of active microorganisms, which utilize the organic substances present in the wastewater as nutrients and eventually degrades these organic matters into simple byproducts such as methane, carbon dioxide, hydrogen sulfide, and water (Jameel et al. 2011). The exploitation of these microorganisms for treatment and bioremediation purposes offers a very efficient tool for biopurification/biotreatment of contaminated wastewaters or effluents (Glazer and Nikaido 1995). In addition, Maygaonkar et al. (2012) reported that the physical and chemical treatments of industrial wastewaters are found to be insufficient whereas

the biological treatment is most often found to be effective. Biological systems have shown attractive success in the removal of contaminated industrial wastewaters (Gantzer et al. 1989; Fogarty and Kelly 1990; Gehara 1999).

Microbial degradation of wastewaters .involving the application of variety of microorganisms has demonstrated effective degradability of wastewaters which has attracted attention in recent time (Erguder et al. 2000; Kissi et al. 2001; Ettayebi et al. 2003; Ammar et al. 2005; Dhouib et al. 2006; El-Bestawy et al. 2005). Microorganisms have been used to remove organic matter and toxic chemicals from domestic and industrial wastes discharged for many years (Gupta and Mukerji 2001).

The use of microorganisms to destroy, or reduce the concentration of, hazardous waste on a contaminated site is called bioremediation. Such a. biological treatment system has various applications, including cleanup of contaminated sites such as water, soils, ground water, lagoons, sludges, waste streams/wastewaters, and process-waste streams. The treatment of the Alaskan shoreline of Prince Williams Sound after the oil spill of Exxon Valdez in 1989 is one common example in which bioremediation methods got public attention. Although the Alaska oil spill cleanup represents the most extensive use of bioremediation on any one site, there have been many other successful applications on a smaller scale. There are numerous other success stories of bioremediation in cleaning up chemical spills, leaking underground storage tanks of gasoline, and many toxic industrial effluents (Boopathy 2000). Crude oil degradation by *Bacillus* sp. IOS1-7, *Corynebacterium* sp. BPS2-6, *Pseudomonas* sp. HPS2-5, and *Pseudomonas* sp. BPS1-8 has been reported based on their efficiency for crude oil utilization (Sathishkumar et al. 2008). Besides, several genera of microorganisms have been found to be active in the biodegradation of crude oil, but the genus *Pseudomonas* stand out as most versatile (Miklosovicova and Trzilova 1991). On the other hand, photosynthetic bacteria such as *Rhodobacter sphaeroides* S (S) and *R. sphaeroides* NR-3 (NR-3) have been used for the removal of oil from domestic and industrial wastewaters (Takeno et al. 2005).

In this study, we describe the potentiality and capabilities of microorganisms in the biotreatment of the wastewaters. This study has, with some degree of success, employed different types of bacterial and fungal strains in the biopurification of wastewaters.

2 Compositions of Typical Wastewaters

Watercourses receive pollution from many different sources, which vary both in strength and volume. The composition of wastewater is a reflection of the life styles and technologies practiced in the producing society (Gray 1989). It is a complex mixture of natural organic and inorganic materials as well as man-made compounds. Organic and inorganic substances which are released into the environment as a result of domestic, agricultural, and industrial water activities lead to organic

and inorganic pollution. Three quarters of organic carbon in wastewaters are present as carbohydrates, fats, proteins, amino acids, and volatile acids (Abdel-Raouf et al. 2012). The inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulfur, phosphate, bicarbonate, ammonium salts, and heavy metals (Tebbutt 1983; Horan 1990; Lim et al. 2010).

Pollution is a man-made phenomenon, arising either when the concentrations of naturally occurring substances are increased or when nonnatural synthetic compounds (xenobiotics) are released into the environment (Abdel-Raouf et al. 2012). Different sources of pollutants include discharge of either raw or treated wastewaters from towns and villages; discharge from manufacturing or industrial plants; runoff from agricultural land; and leachates from solid waste disposal sites (Horan 1990; De la Noüe and De Pauw 1988).

3 Microbiological Composition of Wastewaters

Wastewater environment is an ideal media for a wide range of microorganisms especially bacteria, fungi, viruses, and protozoa. The majority is harmless and can be used in biological wastewater treatment, but some wastewaters also contain pathogenic microorganisms (Abdel-Raouf et al. 2012). Bacteria which cause cholera, typhoid and tuberculosis; fungi which cause dermatophytosis; viruses which cause infectious hepatitis; protozoa which cause dysentery; and the eggs of parasitic worms are all found in various wastewaters depending on their sources (Glynn-Henery 1989; Shaaban et al. 2004). Bala et al. (2012) have reported the isolation of potential pathogens of man capable of causing a variety of diseases from pharmaceutical wastewater in Nigeria.

4 Advantages of Biological Treatment of Wastewaters

1. Biological processes for the treatment of wastewaters have seen worldwide applications. They are considered environmentally friendly, reliable and, in most cases, cost-effective. Biological treatment is able to remove organic matter and inorganic nutrients (Paraskeva and Diamadopoulos 2006).
2. Biodegradation of oily wastes is the cheapest and surest means of managing wastewaters to prevent the attendant environmental impacts (Ohimain et al. 2012).
3. Many workers have revealed the role of microorganisms for degradation of pollutants and are very well known for their decomposition property. Microorganisms have tremendous capacity for treating industrial hazardous waste in support of an environment. Many experiments were carried out with different varieties of microorganisms, and it was observed that they are highly effective against industrial waste (Assadi and Jahangiri 2001).

4. Microorganisms are involved in the biodegradation of undesirable materials or compounds and convert them into harmless, tolerable, or useful products. Many organisms are involved in the biodegradation of organic waste, which has resulted in the production of novel substances of biotechnological importance. (Tripathi et al. 2007).
5. Since most wastewaters contain a very high organic matter, which is generally biodegradable, this facilitates the application of biological treatment based on aerobic processes (Chin and Wong 1983). The biological treatment depends greatly on consortium of active microorganisms, which utilize the organic substances present in the palm oil mill effluent (POME) as nutrients and eventually degrades these organic matters into simple by-products such as methane, carbon dioxide, hydrogen sulfide, and water (Jameel et al. 2011).
6. The exploitation of microorganisms for treatment and bioremediation purposes offers a very efficient tool for purifying contaminated effluents (Glazer and Nikaido 1995).
7. Microorganisms, than any other class of organisms, have an unique ability to interact both chemically and physically with a huge range of both man made and naturally occurring compounds leading to a structural change to, or the complete degradation of, the target molecule (Chakrabarty et al. 1988; Gordon 1994).
8. Biological processes appear to perform well compared to the chemical and physical processes, which are in general too costly to be implemented in most places and which may lead to secondary pollution.

5 Microorganisms

5.1 Bacteria

Different types of microorganisms have been tested in aerobic processes to treat wastewaters, including *Bacillus pumilus* (Ramos-Cormenzana et al. 1996), *Arthrobacter* sp. (Knupp et al. 1996), *Azotobacter vinelandii* (Papadelli et al. 1996; Ehaliotis et al. 1999; Piperidou et al. 2000), *Pseudomonas putida* and *Ralstonia* sp. (Di Gioia et al. 2001a, b), and various bacterial consortia (Ranalli 1992; Borja and Banks 1995a; Zouari and Ellouz 1996a; Benitez et al. 1997).

Aerobic bacteria have been tested primarily as an approach for reduction of organic pollutants from oil wastewaters, although some studies have also focused on the reduction of COD (McNamara et al. 2008). Ramos-Cormenzana et al. (1996) reported reduction in the organic pollutant content of oily wastewaters by *B. pumilus*. Also, *A. vinelandii* has been shown to effectively reduce the organic load of wastewaters (Constantinos et al. 1999; Piperidou et al. 2000). Zouari and Ellouz (1996a) reported a 50 % reduction in COD using an enrichment culture of organisms isolated from industrial and municipal wastes and agricultural soil.

Several studies of bioremediation of oily wastewaters in Greece have focused on *A. vinelandii*. Papadelli et al. (1996) isolated a strain of *A. vinelandii* from soil

treated with olive mill wastewater (OMWW). Eventually, over 90 % removal of organic pollutants from OMWW was achieved using this strain (Ehaliotis et al. 1999; Piperidou et al. 2000). Di Gioia et al. (2001a, b) used two bacteria, *Ralstonia* sp. and *P. putida*, to treat Italian OMWW. Cultured together, these bacteria were able to degrade many phenolic compounds found in OMWW $6# (McNamara et al. 2008).

A number of studies have utilized bacterial consortia for bioremediation of oil wastewaters. The consortia have come from activated sludge (Borja and Banks 1995a; Benitez et al. 1997), commercial communities (Ranalli 1992), soil, and wastewater (Zouari and Ellouz 1996a). Bioremediation of OMWW using aerobic consortia has been quite successful in these studies, achieving significant reductions in COD (up to 80 %). In a similar study, Bala et al. (2015) have also reported the use of *Micrococcus luteus*101PB, *Stenotrophomonas maltophilia* 102PB, *Bacillus cereus* 103PB and *Bacillus subtilis*106PB for the reduction of organic load from POME in Malaysia.

5.2 Fungi

Fungi, their biology, economic value, and pathogenic capabilities are not new to human society. They have been used from fermentation of foods to production of pharmaceuticals. Fungi thrive well in inhospitable habitats with environmental extremes because of their enzyme system (Cooke 1979).

Many researchers have revealed the role of microbes for degradation of pollutants, among microbes the fungi are very well known for their decomposition property. Fungi have tremendous capacity for treating industrial hazardous waste in support of an environment. Many experiments were carried out with different varieties of fungi, and it was observed that fungi are highly effective against industrial waste (Assadi and Jahangiri 2001; AbdulKarim et al. 2011).

Fungi have been attracting a growing interest for the biotreatment of wastewater ingredients such as metals, inorganic nutrients, and organic compounds (Akthar and Mohan 1995; Field et al. 1993; Feijoo et al. 1995; Palma et al. 1999; Coulibaly 2002). Fungi are involved in the biodegradation of undesirable materials or compounds and convert them into harmless, tolerable, or useful products. Many organisms are involved in the biodegradation of organic waste, which has resulted in the production of novel substances of biotechnological importance (Tripathi et al. 2007).

Fungi are recognized for their superior aptitudes to produce a large variety of extracellular proteins, organic acids and other metabolites, and for their capacities to adapt to severe environmental constraints (Lilly and Barnett 1951; Cochrane 1958).

Fungal remediation of oily wastewaters has been studied using three types of organisms: white rot fungi (including the edible mushrooms *Lentinula* and *Pleurotus*), *Aspergillus* sp., and several different yeasts. In addition to reduction

of COD and removal of simple phenolics, fungi are also effective at reducing coloration of oily wastewaters (McNamara et al. 2008).

A variety of white rot fungi have been used for remediation of oily wastewaters including *Coriolus versicolor* and *Funalia trogii* (Yesilada et al. 1995, 1998), *Geotrichum candidum* (Assas et al. 2000), *Lentinula* (*Lentinus*) *edodes* (Vinciguerra et al. 1995, 1997; D'Annibale et al. 1998), and *Phanerochaete* sp. (Sayadi and Ellouz 1992; Ben Hamman et al. 1999) A number of studies have examined the ability of the edible white rot fungus *Pleurotus* to degrade OMWW (Sanjust et al. 1991; Tomati et al. 1991; Kissi et al. 2001; Tsioulpas et al. 2002). The white rot fungi appear quite effective, achieving removal rates as high as 88 % for COD, 100 % for phenolics, and 81 % for coloration. A variety of fungi also have been used for remediation of POME, including *Yarrowia lipolytica* (Oswal et al. 2002) and *Trichoderma harzianum* (AbdulKarim et al. 2011) achieving COD reduction rate of more than 80 %.

Aspergillus has been used for the bioremediation of wastewaters in previous studies. Hamdi and coresearchers, in a number of studies, characterized the ability of *Aspergillus niger* to degrade oily wastewaters (Hamdi et al. 1991a, b; Hamdi and Ellouz 1992a, b, 1993). Reduction of COD of greater than 60 % was achieved using *A. niger*. Similar reductions in COD were found using *Aspergillus terreus*. In a previous work by the authors, *Aspergillus fumigatus* 107PF and *Aspergillus niger* 109PF have been used for the bioremediation of POME (Bala et al. unpublished work).

Yeasts used in the bioremediation of wastewaters include *Trichosporon cutaneum* (Chtourou et al. 2004), *Candida tropicalis* (Ettayebi et al. 2003), *Yarrowia lipolytica* NCIM 3589 (Oswal et al. 2002), and *Saccharomyces* sp. (Gharsallah 1993). Like the other fungi, these yeasts were effective at reduction of COD.

5.3 Combined Bacterial–Fungal Strains

De Felice et al (1997) used a combination of bacteria and yeast to degrade OMWW. The yeast, *Yarrowia lipolytica*, reduced the COD of OMWW by 80 %. Effluent from the yeast fermentation was then treated with *P. putida*, producing further reduction of COD. Oswal et al. (2002) have used the combination of *Yarrowia* with a consortium of bacteria and algae developed from garden soil, achieving COD reduction rate of 99 % for the treatment of POME. El-Bestawy et al. (2005) also reported the combination of *Pseudomonas* sp. and *P. diminuta* as mixed cultures which produced the highest activity in reducing COD from contaminated industrial effluents. The use of mixed cultures of bacterial strains has been previously reported by Bala et al. (2015). AbdulKarim et al. (2011) in a similar study have also reported the use of *Trichoderma harzianum* and *Penicillium* in the treatment of POME.

5.4 Anaerobic Microorganisms

Anaerobic bioremediation of wastewaters has employed, almost exclusively, uncharacterized microbial consortia derived from municipal and other waste facilities (Rozzi et al. 1989; Martin et al. 1991; Borja et al. 1996b; Dalis et al. 1996; Fiestas Ros de Ursinos and Borja-Padilla 1996; Zouari and Ellouz 1996b; Marques et al. 1997; Marques 2001). A significant advantage of anaerobic processes over aerobic may be the generation of methane, which could potentially be used in remediation or as an energy source for other processes (Fiestas Ros de Ursinos and Borja-Padilla 1996).

Reductions in COD from 70 to 89 % have been reported for anaerobic processes (Borja et al. 1996a; Marques et al. 1997; Marques 2001). In addition to a substantial reduction of COD, Dalis et al. (1996) reported large reductions of more than 75 %.

6 Wastewater Treatment

6.1 Anaerobic Digestion Treatment of Wastewaters

Anaerobic filter has been applied to treat various types of wastewater including soybean processing wastewater (Yu et al. 2002), wine vinases (Nebot et al. 1995; Pérez et al. 1998), landfill leachate (Wang and Banks 2007), municipal wastewater (Bodkhe 2008), brewery wastewater (Leal et al. 1998), slaughterhouse wastewater (Ruiz et al. 1997), drug wastewater (Gangagni Rao et al. 2005), distillery wastewater (Acharya et al. 2008), beet sugar water (Farhadian et al. 2007), and wastewater from icecream manufacturing (Hawkes et al. 1995; Monroy et al. 1994). Borja and Banks (1994b, 1995b) have also utilized anaerobic filter for POME treatment. In general, anaerobic filter is capable of treating wastewaters to give good effluent quality with at least 70 % of COD removal efficiency with methane composition of more than 50 % (Poh and Chong 2009).

Investigations have been done on the application of anaerobic fluidized bed reactor to treat cutting-oil wastewater (Perez et al. 2007); real textile wastewater (Sen and Demirer 2003); wine and distillery wastewater (Garcia-Calderon et al. 1998; Sowmeyan and Swaminathan 2008); brewery wastewater (Alvarado-Lassman et al. 2008); icecream wastewater (Borja and Banks 1995a; Hawkes et al. 1995); slaughterhouse wastewater (Toldrá et al. 1987); pharmaceutical effluent (Saravanane et al. 2001); and POME (Borja and Banks 1995b). Studies using fluidized bed to treat icecream wastewater showed different COD removal efficiencies when different support materials were used. Hawkes et al. (1995) found that fluidized bed using granular activated carbon (GAC) gave about 60 % COD removal, while Borja and Banks (1995a) obtained 94.4 % of COD removal using

ovoid saponite. Thus, suitable support material needs to be selected to obtain high COD removal efficiency in the system.

The anaerobic contact digestion process has been implemented in POME (Ibrahim et al. 1984), icecream wastewater, alcohol distillery wastewater (Vlissidis and Zouboulis 1993), and fermented OMWW treatment (Hamdi and Garcia 1991). Concentrated wastewaters are suitable to be treated by anaerobic contact digestion since relatively high-quality effluent can be achieved (Leslie Grady et al. 1999). This system has been able to remove COD efficiently, achieving up to 80 % removal efficiency (Vlissidis and Zouboulis 1993).

Upflow Anaerobic Sludge Blanket (UASB) reactor has been applied for the treatment of potato wastewater (Kalyuzhnyi et al. 1998; Lettinga et al. 1980; Parawira et al. 2006); domestic wastewater (Barbosa and Sant'Anna 1989; Behling et al. 1997); slaughterhouse wastewater (Sayed et al. 1984); icecream wastewater (Hawkes et al. 1995); POME (Borja and Banks 1994a); pharmaceutical wastewater (Stronach et al. 1987); instant coffee wastewater (Dinsdale et al. 1997); and sugar beet wastewater (Lettinga et al. 1980). In general, UASB is successful in COD removal of more than 60 % for most wastewater types (Hawkes et al. (1995). POME treatment has been successfully conducted with UASB reactor achieving COD removal efficiency up to 98.4 % (Borja and Banks 1994a).

Upflow Anaerobic Sludge Fixed Film (UASFF) reactor is more efficient compared to UASB and anaerobic filter in the treatment of wood fiber wastewater. Other investigations of wastewater treatments using UASFF include sugar wastewater (Guiot and van den Berg 1985); dairy wastewater (Córdoba et al. 1995); slaughterhouse wastewater (Borja et al. 1995, 1998; Lo et al. 1994); wash waters from purification of virgin olive oil (Borja et al. 1996b); coffee wastewater (Bello-Mendoza and Castillo-Rivera 1998); brewery wastewater (Yu and Gu 1996); and POME (Najafpour et al. 2006). UASFF is also able to achieve COD removal efficiency of at least 70 % and above. Methane production for UASFF is also at a satisfactory level (Poh and Chong 2009).

6.2 *Agroindustrial Wastewaters*

Industries of olive oil, distillery (molasses), cotton bleaching, pulp, and paper processing produce billion liters of colored, often toxic, and harmful wastewaters all over the world. These effluents have strong concentrations of COD, phenol, and its derivatives and often contain proteins, cyanides, chlorinated lignin compounds, and dyes (Bengtsson and Triet 1994; Yesilada et al. 1998; Kahmark and Unwin 1999). High levels of lignin peroxidase have been linked with high decolorization efficiency of olive mill effluents (Sayadi and Ellouz 1995). Distillery wastewater causes many environmental problems and colored substances must be removed from it before discharge into the environment. Locally isolated *Aspergillus fumigatus* has been found to be effective for decolorization of anaerobically treated distillery wastewater (Mohammad et al. 2006). Benito et al. (1997) studied the

decolorization of molasses wastewater using *Trametes versicolor*. Dhamankar described the possibility of using dead yeast cells for decolorization of distillery wastewaters as dry yeast powder decolorized the effluents of biomethanation plant by more than 70 %.

Fungi especially the white rot fungi produce enzymes laccase, Mn peroxidase (manganese peroxidase), and lignin peroxidase (LiP), which are involved in the degradation of lignin in their natural lignocellulosic substrates (Tripathi et al. 2007). This ligninolytic system of white rot fungi is directly involved in the degradation of various xenobiotic compounds and dyes. The ability of the white rot fungi to degrade dye can be directly linked with its ability to degrade lignin; the dye molecules are degraded along with lignin. Use of white rot fungi is the most unique technology of bioremediation as their ability to degrade structurally diverse xenobiotic organopollutants is enormous (Christian et al. 2005).

Eaton et al. (1980) used *Phanerochaete chrysosporium* and found it effective for color removal through microbial degradation of polymeric lignin molecules from pulp and paper industrial effluents. The large amount of lignin derivatives in these effluents is responsible for their dark-brown color (Calvo et al. 1995). *Trametes versicolor* is one of the white rot fungi known to decolorize kraft mill effluents from sulfate pulping (Livernoche et al. 1983). Another white rot fungus *Phanerochaete chyrsosporium* produces isoenzymes, including lignin peroxidases and Mn-dependent peroxidases that are capable of degrading not only lignin but also chlorinated lignins found in pulp bleaching effluents (Kirk et al. 1986; Lankinen et al. 1990). The mechanisms of decolorization of agroindustrial wastewaters by fungi are reported to include biosorption and/or biodegradation (Christov et al. 1999; Nagarathnamma et al. 1999). Ligninolytic enzymes are also involved in the degradation of organic compounds, including dyes, within these effluents (Chivukula et al. 1995).

Other agroindustrial wastewaters that are relatively nontoxic (e.g., dilute lignocellulosics, starch, rice and mussels processing, sauce production, palm oil mill wastewaters, etc.), fungal growth on them has been reported to produce single cell protein (SCP), enzymes, chitosan, amylolytic preparations, and a good reduction of COD (up to 97.8 %) (Jin et al. 1999, 2001).

6.3 Dyed Wastewaters

The wastewaters of pharmaceutical industries, dyeing, printing, photographs, textile, and cosmetics contain dyes (McMullan et al. 2001). Effluents from textile industries are a complex mixture of many polluting substances such as organochlorine-based pesticides, heavy metals, pigments, and dyes. Among the various types of organics present, color is the most difficult to remove; color indicates an increased biological oxygen demand (BOD) and COD. Azo dyes are used extensively in the textile and dyestuff industries; these dyes have a complex structure and some of them are carcinogenic and mutagenic. *Phanerochaete*

chyrsosporium a major wood rotting fungus is capable enough to degrade a wide range of recalcitrant xenobiotic compound, including azo dyes (Aust 1990; Cripps et al. 1990).

Phanerochaete chyrsosporium degrades a wide variety of structurally diverse organopollutants (Bumpus et al. 1985) through its nonspecific H_2O_2-dependent extracellular lignin degradation enzyme system (Cripps et al. 1990). Abadulla et al. (2000) have assessed the potential of *Trametes hirsuta* and a laccase from this organism to continuously degrade textile dyes and found that both water consumption and effluent toxicity in textile dyeing could be reduced by enzyme remediation with laccases. Dead biomass of *Aspergillus niger* has been found to be an effective biosorbent of dyes like methyl violet and basic fuchsin (Bhole et al. 2004). Mohorcic et al. (2004) also reported the use of *Bjerkandera adusta* to decolorize synthetic textile dye Reactive Black 5 from blue black to a yellow color. Vyas and Molitoris (1995) have shown good results of *Pleurotus ostreatus* through its enzymatic activity to decolorize Remazol Brilliant Blue R dye.

Other fungi have also shown some capabilities to remove dyes from industrial effluents. Dyes are removed by fungi by biosorption (Zheng et al. 1999; Fu and Viraraghavan 2000), biodegradation (Nigam et al. 1995; Conneely et al. 1999), and enzymatic mineralization (LiP, MnP, manganese independent peroxidase (MIP), and Lacc) (Ferreira et al. 2000; Pointing and Vrijmoed 2000; Wesenberg et al. 2003). However, one or more of these mechanisms could be involved in color removal depending on the fungus used.

6.4 Heavy Metal Containing Wastewaters

Metallurgical industries, mining surface cleaning, and waste incinerators produce large wastewater polluted by metals. Dissolved metals escaping into the environment pose a serious health hazard as they accumulate in living tissues throughout the food chain, which has human at its top(Lacina et al. 2003). There is a need to remove heavy metals before they enter the complex ecosystem. Utilization of biomasses in general (McKay et al. 1999; Gupta et al. 2000) and particularly that of fungi are considered to be best alternatives for wastewater purification (Kratochvil and Volesky 1998; Mogollon et al. 1998; Tobin and Roux 1998). The purification of the water containing metals by fungal biomass is cheaper and it has the following advantages: (1) production of residual small volume; (2) possibility of valorisation of fungal waste biomasses from industrial fermentations; (3) fast removal; and (4) easy installation of the process (Lacina et al. 2003).

Fungal biomasses walls are composed of macromolecules (chitin, chitosan, glucan, lipid, and phospholipids), which contain carboxyl groups (R–COOH), amino groups (R_2NH, R–NH_2), phosphates, lipids, melanin, sulfates (R–OSO_3^-), and hydroxides (OH^-) (Fogarty and Tobin 1996; Kapoor et al. 1999). These functional groups are metal sorption sites (Mashitah et al. 1999; Tereshina et al. 1999; Zhou 1999). Fungi remove metals essentially by adsorption,

chemisorptions (ion exchange), complexation, coordination, chelation, physical adsorption, and microprecipitation (Kapoor and Viraraghavan 1997; Sarret et al. 1998).

Akthar and Mohan (1995) used biomass of *Aspergillus niger* to remove Zn^{2+} and Cd^{2+}. Massaccesi et al. (2002) have used filamentous soil fungi like *Aspergillus terreus*, *Cladosporium cladosporioides*, *Fusarium oxysporum*, *Gliocladium roseum*, *Penicillium* spp., and *Trichoderma koningii* isolated from industrially polluted sediments for the removal of Cadmium. The fungus *Penicillium frequentans* has been found to effectively remove phenanthrene in soil (Amezcua-Allieri et al. 2005).

6.5 Microalgae for Wastewater Treatment

The history of the commercial use of algal cultures spans about 75 years with application to wastewater treatment and mass production of different strains such as *Chlorella* and *Dunaliella* (Abdel-Raouf et al. 2012). Currently, significant interest is developed in some advanced world nations such as Australia, the USA, Thailand, Taiwan, and Mexico (Borowitzka and Borowitzka 1989a, b; Wong and Chan 1990; Renaud et al. 1994).

Biotreatment with microalgae is particularly attractive because of their photosynthetic capabilities, converting solar energy into useful biomasses and incorporating nutrients such as nitrogen and phosphorus causing eutrophication (De la Noüe and De Pauw 1988). This fascinating idea launched some 55 years ago in the USA by Oswald and Gotaas (1957) has since been intensively tested in many countries (Shelef and Soeder 1980; De Pauw and Van Vaerenbergh 1983).

Palmer (1974) surveyed microalgal genera from a wide distribution of waste stabilization ponds. In order of abundance and frequency of occurrence the algae found were *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium*, and *Golenkinia*. Algal systems have traditionally been employed as a tertiary process (Oswald 1988). They have been proposed as a potential secondary treatment system (Tam and Wong 1989).

Algae can be used in wastewater treatment for a range of purposes, some of which are used for the removal of coliform bacteria, reduction of both COD and BOD, removal of N and/or P, and also for the removal of heavy metals (Abdel-Raouf et al. 2012). Reports in the literature revealed that considerable coliform bacteria removal is achieved in stabilization ponds. Thus, Malina and Yousef (1964) reported a reduction of 88.8 % in 11.4 days. Meron et al. (1965) reported a reduction of 99.6 %. Another supported study was performed in this respect (Oswald et al. 1967; Parhad and Rao 1976). The biotreatment of wastewaters with algae to remove nutrients such as nitrogen and phosphorus has been previously reported (Shi et al. 2007; Zhu et al. 2008).

Field experiments reported by Gale (1986) indicated that live photosynthetic microalgae have an effective role in metal detoxification of mine wastewater. By using cyanobacteria in a system of artificial pools and meanders, 99 % of dissolved and particulate metals could be removed. Soeder et al. (1978) showed that *Coelastrum proboscideum* absorbs 100 % of lead from 1.0 ppm solution within 20 h at 23 °C and about 90 % after only 1.5 h at 30 °C. Microalgae are known to sequester heavy metals (Rai et al. 1981). Microalgae are efficient absorbers of heavy metals (Abdel-Raouf et al. 2012). Recently, Hernández et al. (2013) reported the treatment of agroindustrial wastewaters using microalgae–bacteria consortium combined with anaerobic digestion of the produced biomass.

6.6 Aquatic and Microbial Systems for Wastewater Treatment

Serious interests in natural methods for wastewater treatment have reemerged. The use of aquaculture systems as engineered systems in wastewater (domestic and industrial) treatment and recycling has increased enormously over the past few years; they are designed to achieve specific wastewater treatment and can simultaneously solve the environmental and sanitary problems and may also be economically efficient (Hussein et al. 2004; Deng et al. 2007).

Aquatic treatment systems consist of one or more shallow ponds in which one or more species of water tolerant vascular plants such as water hyacinths or duckweed are grown (Tchobanoglous 1987). Water hyacinth systems are capable of removing high levels of BOD, suspended solids (SS), nitrogen, and refractory trace organic matter while phosphorus removal seldom exceeds 50–70 % in wastewater, as it is mainly limited to the plant uptake (Dinges 1976; Bastian and Reed 1979).

A system consisting of a pond covered with duckweed mat seems to be able to purify the wastewater jointly with bacteria. The bacterial decomposition causes anaerobiosis in the water. It is maintained by the duckweed mat as it prevents reaeration (Abdel-Raouf et al. 2012). It has been shown that duckweed species such as *Spirodela* and *Lemna* even reduce the oxygen content of water (Culley and Epps 1973) but this anaerobiosis does not seem to affect the plants. The main minerals C, N, and P in turn will be converted into protein by duckweed, also, it has the ability to remove the organic materials because of their ability to use simple organic compounds directly and assimilate them as carbohydrates and various amino acids (Hillman 1976).

7 Conclusion

This article highlighted the capabilities of microorganisms (bacteria, fungi, and microalgae) for biotreatment of raw wastewaters. However, essential works on this subject are still laboratory tests and they are of less industrial scale application. Hence, most research on bacterial and fungal capabilities to purify polluted wastewaters has been performed on a laboratory scale; thus, there is a need to extend such research to pilot scale and apply it to industrial processes.

Consequently, work on pilot and the development of treatment plants are to be encouraged. Importantly, works on bacterial and fungal utilization for raw wastewater biopurification have been laboratory scale. This situation can be elucidated by the fact that bacterial and fungal utilization in environmental biotechnology is still under investigation to assess information on process implementation. Thus, more technically advanced research efforts are required for searching, exploiting new bacterial and fungal species, and improvement of practical application to propagate the use of bacteria and fungi for bioremediation of industrial effluents. Noteworthy, the bacterial and fungal biomasses present many assets for biopurification of wastewaters.

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Production of Violacein by *Chromobacterium violaceum* Grown in Liquid Pineapple Waste: Current Scenario

Wan Azlina Ahmad, Chidambaram Kulandaisamy Venil, and Claira Arul Aruldass

1 Pineapple Plantation Scenario in Malaysia

The production of pineapples in Malaysia increased by 5 % from 332,736 MT in 2011 to 335,488 MT in 2012. The revenue generated from the pineapple industry, categorized under tropical fruits production, amounts to RM 110.5 (LPNM 2012). About 70 % of the world's pineapple produced is consumed raw in the pineapple producing countries. The production and consumption of pineapples in the world was 15 million MT per annum with an annual production of 200,000 MT per year. The world's pineapple market is dominated by Thailand, Brazil, Costa Rica, Philippines, Indonesia, India, Nigeria, China, Mexico, and Vietnam. Malaysia ranks 15 among the 87 pineapple producing countries.

Pineapple production by the small-scale farmers showed an increase of 30.38 % with a production of 61,198 MT in 2012 compared to 46,937 MT in 2011. The production of pineapples in the estate sector remained constant at 43.15 % for 2012 compared to the previous year. The increase in pineapple production by the small-scale farmers is related to the increase in land acreage for pineapple plantation for the year 2011/2012 (LPNM 2011) and due to grants disbursed under the Pakej Tanaman Baru (New Planting Package) and Pakej Tanaman Semula (Replanting Package).

The pineapple industry in Malaysia certainly looks bright in the years to come with the Government's many incentives with MD2 variety listed as one of the seven tropical fruits in focusing the output production via the National Key Economic Area (NKEA) initiative under the Malaysian Permanent Food Production Areas (LPNM 2012).

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2 Bacterial Pigments

2.1 Introduction

The awareness in human safety and environmental conservation has kindled fresh enthusiasm for the use of colors from natural sources. Natural colorants or dyes derived from flora and fauna are believed to be safe because of nontoxic, noncarcinogenic, and biodegradable in nature (Cristea and Vilarem 2006). Natural pigments are sourced from ores, insects, plants, and microbes. Among microbes, bacteria have immense potential to produce diverse bioproducts and one such bioproduct is pigments. The production and application of bacterial pigments as natural colorants has been investigated by many researchers (Joshi et al. 2003; Venil and Lakshmanaperumalsamy 2009; Ahmad et al. 2012a, b). Bacterial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications (Venil et al. 2013). Most of the bacterial pigment production is still at the research and development stage. Hence, work on the bacterial pigments should be intensified which can reduce the cost and increase its applicability for industrial production (Ahmad et al. 2012a, b).

The quest of biotech industries for the discovery of novel bacterial pigments with superior productivity to retain their global competitiveness is persisting. Research investigations via purified bacterial pigments have exposed their potential for applications in food, cloth, painting, cosmetics, pharmaceuticals, plastics, etc. As a result, bacterial pigments have become one of the key bioproducts of the day with immense industrial implications which could be exploited optimistically in a sustainable environment. In addition to their potential for industrial applications, bacterial pigments also possess various functions and taxonomic significance as shown in Fig. 1.

2.2 Synthetic Dyes and Natural Pigments

Synthetic dyes are severely criticized and consumers show inhibition toward these products (Koes et al. 1994). In the 1960s in US, the environmental activists demonstrated against the use of such food additives, and this attitude was spread out widely (Chattopadhyay et al. 2008). Activists campaigned for natural colorants highlighting their nutritional characteristics as a sales tool. Thus, a worldwide tendency to use natural colorants was generated. Currently, people interpret the content of synthetic products as contaminants and the tendency has been reinforced (Krishnamurthy et al. 2002). Because of their pharmacological properties, the number of advantages of using natural pigments, over synthetic colorants, has further boosted.

Natural pigments and synthetic dyes are extensively used in various fields of everyday life such as food production, textile industries, paper production, and

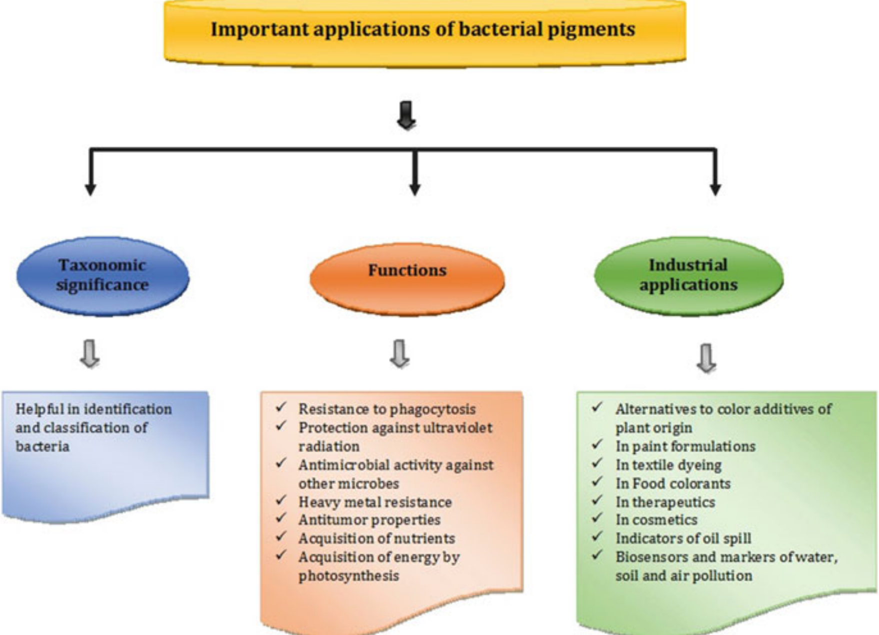

Fig. 1 Aspects of bacterial pigments (Venil et al. 2014)

agricultural practices (Cserhati 2006). According to green technology policy, less toxic products and more natural starting material are favorable for today's production lines. It is well known that some synthesized dye manufacturing is prohibited due to the carcinogenicity of the precursor or product and also the effect of disposal of their industrial wastes in the ecosystem. Natural pigments not only have the capacity to increase the marketability of products, but also display advantageous biological activities as antioxidants and anticancer agents (Venil et al. 2013). It is, therefore, essential to explore various natural sources of colorants and their applications. The industry is now able to produce some bacterial pigments for applications in food, cosmetics, or textiles (Dufosse 2009).

Since time immemorial, natural products such as isoprenoids, alkaloids, and flavonoids have been used by humans as colorants, flavors, and fragrances. Due to the scarce availability and high price of natural products, synthetic compounds have gained more and more importance in the food industry in the last decades. However, nowadays customers demand natural products as a consequence of proven toxicological effects of some synthetic compounds (Sowbhagya and Chitra 2010). Natural pigments are also of great importance as pharmaceutical drugs. For example, anthocyanins are involved in a wide range of biological activities that affect positively the health properties and decrease the risk of cancer, reduce inflammatory insult, and modulate immune response (Kong et al. 2003). However, due to the limited chemical diversity and structural complexity of such synthetic libraries, as

well as great success of natural pigments on the market, screening of untapped novel bacteria for new pigments is expected to be continued in the future (Li and Vederas 2009; Venil et al. 2014).

2.3 Technology and Challenges

The pigmented bacteria can be sourced from various environmental sources (soil and water from different geographic locations) which can be cultured and purified. Various growth media (nutrient broth) can be used to isolate different types of bacteria producing pigments. However, due to the high cost of using synthetic medium, there is a need to develop new low cost process for the production of pigments. The use of agroindustrial residues would provide a profitable means of reducing substrate cost. Pigment produced by bacteria can be separated using solvent extraction and further characterized using various instrumental based analytical techniques such as thin layer chromatography (TLC), ultraviolet–visible spectroscopy (UV–VIS), Fourier transform infrared spectroscopy (FT-IR), electrospray ionization mass spectrometry (ESI-MS), nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), and gel permeation chromatography (GPC). The utility of a pigment for industrial applications is dictated not only by its inherent properties, but also by the ability to produce it in sufficient quantities. However, extraction of bacterial pigments in relatively pure and concentrate forms is the main technological challenge.

There are several challenges associated with scaling up of pigment production; much of the technology to overcome these challenges is already in place, which provides a potential route for reintroducing bacterial pigments into a cost-sensitive world. A more significant challenge lies in the need to increase the production of the pigments from bacteria to make its manufacture economically viable. Here, recent developments in molecular biology could be used. The genes responsible for the biosynthesis of numerous pigments have been cloned, and recombinant DNA technology has been harnessed to overproduce these pigments (Pfeifer and Khosla 2001).

Finally, the bacterial pigments must have acceptable stability when exposed to environmental stresses, especially under UV light. UV light initiates undesirable free-radical reactions in industry that ultimately lead to their degradation. A variety of UV absorbers (such as benzotriazole- and triazine-based molecules) and free-radical scavengers (such as hindered amines) are already used in the industry and are commercially available (Charkoudian et al. 2010), whereas their effectiveness in conjunction with bacterial pigments remains to be studied.

Recent developments in molecular biology could be of use. The genes responsible for the biosynthesis of numerous pigments have been cloned, and recombinant DNA technology has been harnessed to overproduce these pigments (Pfeifer and Khosla 2001). For example, scientists at Amgen, Inc. were able to engineer a widely used nonhazardous strain of *Escherichia coli* to overproduce indigo

(which at one point was exclusively derived from the woad plant) in fermentation tanks (Venil et al. 2013). The current technology (biosynthetic pathways to engineer a pigment's molecular structure) will have the potential to enhance the utility of bacterial pigments besides its challenges.

2.4 Economics for Pigment Production

The market for the bacterial pigments produced by bioprocesses is hard to estimate. This is due to the lack of statistics of the regional, low-technology products such as annatto extracts, or the fact that the production is pulverized over many small companies worldwide (Carvalho et al. 2003; Babitha et al. 2004, 2007). At one side, there is a growing preference for the bacterial pigments in food industry, textile dyeing, pharmaceuticals, and cosmetics; from other side, in some cases, natural pigments may be several times more expensive than synthetic analogs. An unique example is the β-carotene produced by bacteria which has an approximate cost of US$1000/kg against US$500/kg by synthetic means; although more costly, β-carotene produced by the bacterial means competes in a market segment where it is important that all the pigments be "natural" (Venil et al. 2013; Ruijter 1998).

Increasing globalization, restructuring, and internationalization have been the key trends shaping the pigment industry over the past several years. Global demand for organic pigments and dyes is expected to reach almost 10 million tons by 2017 according to Global Industry Analysis (Venil et al. 2013). The global dye manufacturing industries were originally dominated by suppliers from UK, Switzerland, and Germany and later shifted to Asia over the past 20 years. Textile industries remain the largest consumer of synthetic dyes, while faster growth is expected to occur in other industrial sectors such as printing inks, paints, coatings, as well as plastic production. It is well known that some synthesized dye manufacturing is prohibited due to the carcinogenicity of the precursor or product and also the effect of disposal of their industrial wastes in the ecosystem. Thus, there is an increasing thrust toward the use of natural dyes due to the forbidden use of synthetic compounds (banning of azo dyes in Europe) (Venil et al. 2013). Market value will benefit from consumer preferences for environmentally friendly products.

2.5 Industrial Production of Bacterial Pigments

Nowadays, the importance of biotechnological processes has increased due to the benefits that they provide such as high yields, low costs, and less waste disposals. These benefits are dependent on nutrients and culture conditions and many factors such as bioreactor design, raw materials used, bacteria, pigment, and type of fermentation (batch, feed batch, or continuous) must be taken into consideration.

These features play a very important role to achieve the desired yields of a target pigment (Hu et al. 2012). The configuration and volume of the bioreactor are important factors to be considered in the production of bacterial pigments. A fully functional bioreactor offers advantages such as perfect integration of several components, ensuring that cultures will reach to the desired productivity of microbial pigments or other microbial compounds, through an efficient and rigorous control of some parameters such as temperature, agitation, aeration, pH, and dissolved oxygen among others. Industrial production of bacterial pigments generally employs genetically modified strains to maximize pigment production and metabolic engineering being an interesting field triggers pigmentation.

2.6 Commercialization/Market Potential

Successful development of bacterial pigments coupled with economic feasibility of using agroindustrial residues minimizes the cost for commercial production and marketing. Enabling successful advancement and commercialization of bacterial pigments will require the confidence and engagement of key public and private stakeholders so that they can make necessary investments to reduce the technical risks and overcome the challenges associated with developing bacterial pigments in industry (John and Reed 2010). The bacterial pigments will offer good opportunities due to their enhanced environmental acceptability and superior performance characteristics and are expected to continue dominate the organic market (Venil et al. 2013).

2.7 Future Perspectives

The preference for natural colorants over synthetics started with the green movement of the 1960s and shows no sign of decreasing. This may result from a perceived uneasiness with the safety of the colorants on the part of the consumer, but another factor, perhaps more important, is that most governments allow more flexibility and leniency in the use of natural colorants. Production of colors by fermentation has a number of advantages: cheaper production, possibly easier extraction, higher yields, no lack of raw materials, and no seasonal variations. There is an increasing interest involving bacteria as a possible alternate source of colorants used in foods, textile, pharma, etc. In this direction, biotechnology may play a crucial role for large fermentation of biocolorants.

2.8 Conclusions

Bacterial pigments have economic potential and industrial importance offering opportunities for applications in textile, food, pharmaceuticals, cosmetics, etc. But their current volume of production still has not attained the optimum level to meet the demand aroused due to the recent awareness for natural products. The current novel strategies like genetic engineering, molecular biology techniques, and fermentation technologies are greatly contributing to higher production of bacterial pigments. For cost-competitive and higher production of bacterial pigments, these current processes of screening of new pigmented bacteria should continue in order to support the discovery and application of novel bacterial pigments that possess high activities and useful properties from less expensive sources.

3 Liquid Pineapple Waste as Low Cost Growth Medium for Production of Violet Pigment by *Chromobacterium violaceum* UTM5

3.1 Materials

Nutrient agar (NA; 20 g L^{-1}), nutrient broth (NB; 8 g L^{-1}), deuterated dimethyl sulfoxide-d_6 (DMSO-d_6; 99.8 %), silica gel 0.04–0.063 mm, and Silica gel 60 PF 254 containing gypsum were purchased from Merck (Germany). Potassium bromide (KBr; 99 %), L-tryptophan (98 %), and Antifoam A were purchased from Sigma-Aldrich (St. Louis, USA). Chloroform (99.5 %) and acetone (99.5 %) were obtained from Qrec Asia (Malaysia).

3.2 Methods

3.2.1 Culture

A violet pigmented bacterial strain was isolated from soil sample collected from the vicinity of a wastewater treatment plant in an oil refinery premise in Negeri Sembilan (Ahmad et al. 2012a, b). The strain was deposited as *Chromobacterium violaceum* UTM5 (Fig. 2) with GenBank accession number of HM132057. Nutrient broth, NB, and nutrient agar, NA, were used to grow and maintain the strain.

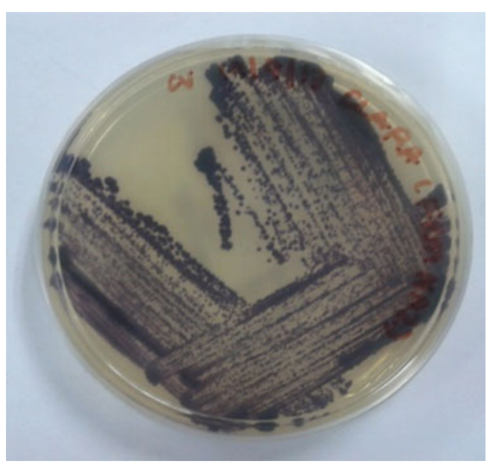

Fig. 2 *C. violaceum* UTM5 grown in NA plate

3.2.2 Production of Violet Pigment in Liquid Pineapple Waste

Lab Scale Pigment Production in Shake Flask

A loopful of 24 h *C. violaceum* UTM5 was inoculated into a 250 mL Erlenmeyer flask containing 62.5 mL NB followed by incubation at 30 °C for 24 h in the dark under static condition (Fig. 3). After incubation, the active culture of *C. violaceum* UTM5, 10 % (v/v), was inoculated in a 500 mL Erlenmeyer flask containing 125 mL NB supplemented with 150 mg L^{-1} L-tryptophan (from stock solution: 1000 mg L^{-1}). The culture was incubated at 30 °C for 24 h in the dark under static condition. After 24 h incubation, seed culture 10 % (v/v) was inoculated into a 2 L Erlenmeyer flask containing 500 mL liquid pineapple waste (10 % (v/v); at pH 7.0) followed by incubation at 200 rpm, 30 °C for 24 h in the dark. Violet pigment was extracted using ethyl acetate and concentrated using rotary evaporator at 50 °C and the pigment was evaporated to dryness. Based on the dry weight of violet pigment, the pigment yield (mg L^{-1}) was calculated according to Eq. (1).

$$\text{Yield of pigment}(\text{mg/L}) = \frac{(A_1 - A_0)\,\text{g}}{20\,\text{mL}} \times \frac{1000\,\text{mg}}{\text{g}} \times \frac{\text{mL}}{0.001\,\text{L}} \tag{1}$$

Key:

A_0: Weight of empty vial
A_1: Weight of dried pigment

Fig. 3 Structures of violacein (**1**) and deoxyviolacein (**2**)

Pilot Scale Pigment Production in a 5 L Bioreactor

A loopful of 24 h *C. violaceum* UTM5 was inoculated into a 250 mL Erlenmeyer flask containing 62.5 mL NB followed by incubation at 30 °C for 24 h in the dark under static condition. Active culture of *C. violaceum*, 10 % (v/v), was inoculated in a 2 L Erlenmeyer flask containing 500 mL NB supplemented with 150 mg L^{-1} L-tryptophan (from stock solution: 1000 mg L^{-1}). The culture was incubated at 30 °C for 24 h in the dark under static condition. After 24 h incubation, the 500 mL seed culture was inoculated into a 5 L bioreactor containing 4.5 L liquid pineapple waste (10 % (v/v); at pH 7.0) followed by 24 h incubation under the following conditions: 200 rpm, 30 °C, aeration rate 3 L min^{-1}, initial pH of 7.0, and with addition of Antifoam A (Sigma, Germany). Violet pigment was extracted using ethyl acetate and concentrated using rotary evaporator at 50 °C and pigment was evaporated to dryness. Based on the dry weight of violet pigment, the pigment yield (mg L^{-1}) was calculated [Eq. (1)]. In order to monitor the reliability and producibility of the optimized methods the fermentation was carried out three times in the 5 L bioreactor to check for consistency of the pigment yield.

3.2.3 Isolation and Characterization of Major Compounds in Violet Pigment

The extracted crude violet pigment was fractionated using vacuum liquid chromatography (VLC) with column size of 7.0 × 5.0 cm and packed with silica gel 0.04–0.063 mm. The violet fraction was purified using preparative TLC (Silica gel 60 PF 254 containing gypsum; plates: 20 × 20 cm) with a solvent system of chloroform: acetone (5:5). Two compounds were isolated as purple powder with R_f values of 0.43 and 0.58 on TLC, representing violacein (**1**) and deoxyviolacein (**2**) (Fig. 3), respectively. FT-IR absorption in KBr at v_{max} and ESI-MS *m*/*z* (relative intensity) for (**1**) and (**2**) were recorded in Table 1.

Table 1 FT-IR absorption and ESI-MS analysis for (**1**) and (**2**)

Compound	FT-IR absorption (cm^{-1})	ESI-MS *m/z* (relative intensity)
(**1**)	3421 (s) 3237 (br) 1689 (s) 1669 (s) 1621 (s) 1279 (s) 1219 (s)	343 $[(M-H)^-, C_{20}H_{13}N_3O_3]$
(**2**)	3425 (br s) 1670 (br w) 1620 (br w) 1279 (br w) 1214 (br w)	327 $[(M+H)^+, C_{20}H_{13}N_3O_2]$

3.3 Results and Discussion

3.3.1 Violet Pigment Production in Liquid Pineapple Waste

Large amounts of pineapple pulp are normally disposed with other types of residues during the mechanical peeling of pineapple (Barretto et al. 2013). Since pineapple waste has not been utilized for obtaining other highly aggregated value byproducts, it seems necessary to use liquid pineapple waste as an alternative low cost growth medium for *C. violaceum* UTM5. From this study, it is found that *C. violaceum* UTM5 showed good adaptability in growing and producing violet pigment in 10 % (v/v) liquid pineapple waste (Fig. 4).

High pigment yield of 367 ± 2 mg L^{-1} was obtained when *C. violaceum* UTM5 was cultured from static to shaking condition in liquid pineapple waste in shake flask and supplemented with 150 mg L^{-1} L-tryptophan (Table 2). However, the yield was 16 times higher (i.e. 5790 ± 10 mg L^{-1}) when *C. violaceum* UTM5 was cultivated in a 5 L bioreactor under similar condition. Wang et al. (2006) reported that pineapple waste serves as carbon and nitrogen sources for cell growth and by-product production as they are composed of various types of organic substances. It was also reported that pineapple syrup (food processing waste) was used as low cost substrate for the production of lactic acid using *Lactobacillus lactis* and enzyme invertase to hydrolyze sucrose into glucose and fructose (Ueno et al. 2003; Idris and Suzana 2006). Similarly, *C. violaceum* UTM 5 might successfully utilize the carbon and nitrogen sources in liquid pineapple waste to produce high yields of the violet pigment.

Besides that, the culture condition used in this study (from static to shaking) also plays a role in increasing the pigment production where it was reported that high agitation rate (high shear stress) decreases the pigment production (Wei et al. 2005; Aruldass et al. 2014). It was reported that agitation rate influences pigment (violacein) production from *Pseudoalteromonas luteoviolacea* (Yang et al. 2007). In this study, initial adaptation to the media might occur as *C. violaceum* UTM5 formed clumps during static condition. However, occurrence of minimal shear

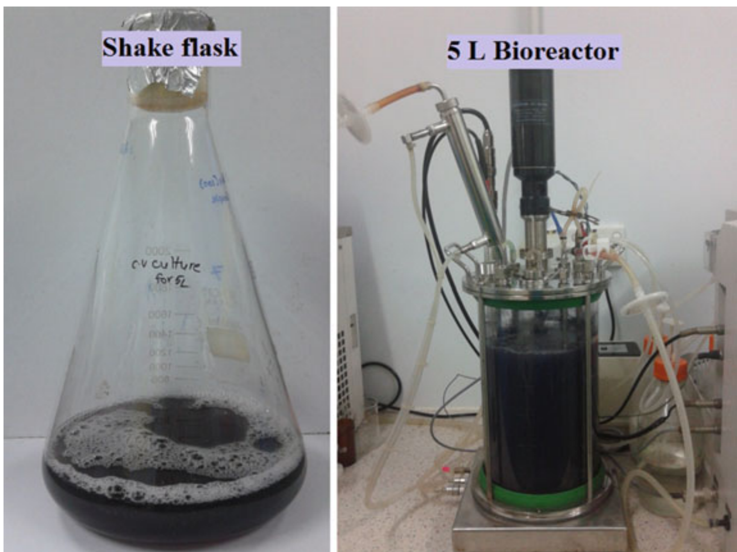

Fig. 4 *C. violaceum* UTM 5 grown in liquid pineapple waste

Table 2 Comparison of violet pigment yield in lab scale and pilot scale production

Violet pigment production	Violet pigment yield (mg L^{-1})
Shake flask	367 ± 2
5 L bioreactor	5790 ± 10

stress during the cultivation in liquid pineapple waste may increase the yield of the pigment. Besides that, supplementation of L-tryptophan also plays an important role in increasing the pigment production as it was reported that L-tryptophan is a precursor for the basic structure of violacein (**1**) (Fig. 3). Two molecules of L-tryptophan contribute all the carbon, nitrogen, and hydrogen atoms of violacein (**1**). Oxygenation of indole rings of intermediate violacein (**1**) compound contributes the oxygen atoms (Momen and Hoshino 2000). Other approaches used by researchers for the production of violet pigment are summarized in Table 3. However, the approach in utilizing liquid pineapple waste as an alternative low cost medium in the present study recorded highest yield of violet pigment as compared to Nakamura et al. (2003) and Yang et al. (2011).

3.3.2 Characterization of Violacein and Deoxyviolacein

The FT-IR spectrum for violacein (**1**) exhibited several absorption bands at 3421 for amine (NH) group, 3237 for hydroxyl (OH) group, 1669 and 1689 for carbonyl amide (NH–C=O), 1621 for olefin (C=C) group, and 1279 cm^{-1} for amine (C–N) group. Similarly, for deoxyviolacein (**2**), absorption bands at 3425 for amine

Table 3 Comparison analysis of violacein production in different media

Culture medium	Condition	Yield of pigment (mg L^{-1})	Reference
Glucose, casein, yeast extract, K_2HPO_4, and $MgSO_4$	At 20 °C in a 3 L bioreactor	3700	Nakamura et al. (2003)
E2 medium (minimal salt medium supplemented with metal traces), glycerol, NH_4Cl and L-tryptophan	At 20 °C in a 5 L bioreactor	4130	Yang et al. (2011)
Liquid pineapple waste (10 % (v/v)) supplemented with L-tryptophan	At 30 °C in a 5 L bioreactor	5790	Present study

(NH) group, 1670 for carbonyl amide (NH–C=O), 1620 for olefin (C=C) group, and 1279 cm^{-1} for amine (C–N) group were recorded. However, the absorption for hydroxyl group was absent for (**2**). The mass spectrum of (**1**) and (**2**) shows the molecular ion of 343 $[(M-H)^-$ and 327 $[(M+H)^+$ representing violacein and deoxyviolacein, respectively. Both of the compounds' absorption bands in FT-IR and mass spectrum are similar to that reported by Rettori and Durán (1998), Brady et al. (2001), and Wille and Steglich (2001).

4 Conclusion

This study demonstrates the potential application of liquid pineapple waste as alternative growth substrate for the production of violet pigment (violacein and deoxyviolacein) by *C. violaceum* UTM5. Culture condition (static to shaking) and supplementation of L-tryptophan enhanced the violet pigment production and the isolated major compounds were identified as violacein and deoxyviolacein. Utilization of low cost agricultural waste (liquid pineapple waste) is a substitute for conventional complex medium which produces the same pigment of interest from the bacteria. This violet pigment may be utilized in other applications such as textile dyeing, soap making, food colorant, and pharmaceutical industries.

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Yeast (*Saccharomyces cerevisiae*) as a Probiotic of Choice for Broiler Production

Chuka Ezema and Clifford Chidozie Ugwu

Abstract A good probiotic should be a strain capable of exerting beneficial effects on the host, be nonpathogenic and nontoxic, be present as viable cells, and be stable in storage and field conditions. The beneficial effects of probiotics in animal production have been related to different modes of action. The effects will depend on a number of factors, including the strain chosen, level of consumption, duration and frequency of exposure, and the physiological condition of the host. Probiotics are growth promoters and their mechanism of action are varied with some not yet clearly understood. *Saccharomyces cerevisiae* exerts probiotic influence in broilers by promoting the metabolic processes of digestion and nutrient utilization. It has also been demonstrated that *S. cerevisiae* improved the immune status of broilers. Part of the probiotic mechanism of action is to maintain the microbiota of the gastrointestinal tract by excluding pathogens and increasing the population of useful microbes. *Saccharomyces cerevisiae* cells respond to oxidative stress by altering their transcriptional program in a complex manner. Probiotic *S. cerevisiae* generally improved the health status of broilers and positively affected the meat quality of broiler chicken.

1 Introduction

1.1 What Is Probiotics?

The term probiotic originated from the Latin word *pro* (“for” or “in support”) and the Greek adjective (biotic) from the noun *bios* (“life”) meaning “for life” or “in

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support of life" and has had several different meanings over the years. Lilly and Stillwell (1965) described probiotics as substances secreted by one microorganism which stimulated the growth of another. Probiotics have been differently defined by many authors. However, the most widely accepted definition is the one suggested by FAO/WHO expert committee which described probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002). This definition implies that a health effect must be demonstrated for the probiotic. The beneficial modes of action include: regulation of intestinal microflora, stabilization of the gastrointestinal barrier function (Salminen 1996), increased enzymatic activity which improved digestion and absorption (Hooper et al. 2002; Timmerman et al. 2005), immunomodulation (Salzman et al. 2003), inhibition of procarcinogenic enzymes, and interference with the ability of pathogens to colonize and infect the mucosa (Gill 2003) which results in improved health and better growth performance of animals (Ezema 2007).

Box 1: Summary of the Evolutionary Definitions of Probiotics

- Substances secreted by one microorganism that stimulate another microorganism (Lilly and Stillwell 1965).
- Tissue extracts that stimulate microbial growth (Sperti 1971).
- Organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance (Parker 1974).
- A live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance (Fuller 1989).
- Direct fed source of live naturally occurring microorganisms in animal production and this includes bacteria, fungi, and yeast (Miles and Bootwalla 1991)
- A viable mono or mixed culture of microorganisms that, applied to animals or humans, beneficially affects the host by improving the properties of the indigenous microflora (Havenaar and Huis In't Veld 1992).
- A live microbial culture of cultured dairy product that beneficially influences the health and nutrition of the host (Salminen 1996).
- Single or mixed viable bacterial culture that has beneficial effects on the health status of the host (Donohue and Salminen 1996)
- Living microorganisms that on ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Guarner and Schaafsma 1998).
- A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity as well as improving nutritional and microbial balance in the intestinal tract (Naidu et al. 1999).
- A preparation of or a product containing viable, defined microorganisms in sufficient numbers that alter the microflora (by implantation or colonization) in a compartment of the host and exert beneficial health effects in this host (Schrezenmeir and De Vrese 2001).

(continued)

Box 1: continued

- Specific live or inactivated microbial cultures that have documented targets in reducing the risk of human disease or their nutritional management (Isolauri et al. 2002).
- Preparation of viable microorganisms that is consumed by humans or other animals with the aim of inducing beneficial effects by qualitatively or quantitatively influencing their gut microflora and/or modifying their immune status (Fuller 2004).
- Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (FAO 2009).

1.2 Historical Perspectives of Probiotics

Although the use of the word probiotic in relation to feed supplements only dates from 1974, the history of live microbial feed supplements goes back thousands of years. Probably, the first foods that contained living microorganisms were the fermented milks that are recorded in the Bible.

Metchnikoff is regarded as the godfather of probiotic. In his book entitled *Essais optimistes*, he discussed the philosophy, literature, religion, folklore, and science of aging. Only a small part of this discourse contained his views on the lower gut flora and the beneficial effects that fermented milk might have on it in humans. At the end of this section of the book, in the English edition, he concludes:

> If it be true that our precocious and unhappy old age is due to poisoning of the tissues (the greater part of the poisoning coming from the large intestine inhabited by numberless microbes), it is clear that agents which arrest intestinal putrefaction must at the same time postpone and ameliorate old age. This theoretical view is confirmed by the collection of facts regarding races which live chiefly on soured milk and amongst which great ages are common. However, in a question so important, the theory must be tested by direct observations. For this purpose, the numerous infirmaries for old people should be taken advantage of and systematic investigation should be made on the relation of intestinal microbes to precocious old age and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body. It can only be in the future, near or remote, that we shall obtain exact information upon what is one of the chief problems of humanity.

In spite of these guarded statements, he is always quoted as having established a relationship between consumption of fermented milk and long life. This reputation was seemingly endorsed by the English translation of his book which was given the little “The Prolongation of Life”. The consumption of fermented milk was given an added support by the publication in 1911 of a book by Londen Douglas called “*The Bacillus of Long life*”. In the book, the author reiterated the connection between fermented milks and longevity. He also summarized what was known at that time as the bacteriology of fermented milks (Douglas 1911).

One of the most convincing demonstrations of the role of the gut microflora in resistance to diseases was provided by Collins and Carter (1978). They showed that the germ-free guinea pig was killed by ten cells of *Salmonella enteritidis*, but it required 10^9 cells to kill a conventional grade animal with a complete gut microflora. It is therefore natural that animal intestines contain microbial population that offer protection against diseases. If that is the case, why do we need probiotics? Under normal conditions there would be no need for probiotics. In the wild, the young animal rapidly acquires a protective flora from its mother and the environment. However, modern husbandry practices tend to limit the duration of contact between newborn animals and their mother, especially in poultry where after the egg is laid, the chick is permanently separated from the hen. The result is that the gut microflora is deficient in some of the normal components that are responsible for resistance to diseases. Tannock (1983) reported that even the gut microflora of an adult can be affected by diet, antibacterial drugs, and stress. The use of probiotic supplements seeks to repair these deficiencies. The development of probiotics has been in part stimulated by the public misgivings about the side effects that often follow the use of antibiotics as therapeutic agents and growth promoters. There is therefore, a growing demand for an effective alternative to the antibiotic growth promoters and probiotics could fill the gap. Recently, interest in the use of probiotics to improve the product performance and general health status of livestock animals has been rekindled by legislations to curtail the use of subtherapeutic doses of antibiotics in animal diets (Cook 2000; Langhout 2000; Mellor 2000; Gill 2001; Plail 2006).

1.3 Characteristics of Good Probiotics

A good probiotic should possess some characteristics. According to Fuller (1989), good probiotic should be a strain, which is capable of exerting a beneficial effect on the host animal, e.g., increased growth or resistance to disease, be nonpathogenic and nontoxic, be present as viable cells, preferably in large numbers, and be stable and capable of remaining viable for long periods under storage and field conditions. However, it is also expected that a good probiotics should possess accurate taxonomic identification, be normal inhabitant of the targeted species, and be capable of surviving, colonizing, and being metabolically active in the targeted site, which implies resistance to gastric juice and bile, persistence in the gastrointestinal tract (GIT), adhesion to epithelium or mucus membrane, competition with the resident microbiota, production of antimicrobial substances, antagonism toward pathogenic bacteria, and modulation of immune responses (Gaggìa et al. 2010). It is also expected to be able to exert at least one scientifically supported health-promoting property, be genetically stable, and have desirable organoleptic and technological properties when included in industrial processes.

1.4 Benefits/Advantages of Probiotics

The use of probiotics has many potential benefits which include exclusion and killing of pathogens in the intestinal tract, reduced bacterial contamination on processed broiler carcasses, and enhanced nutrient absorption (Ferket et al. 2002).

Intestinal probiotics play an important role in determining the digestive mechanisms and general health in all animals and humans (Fuller 1992). The beneficial effects of probiotics will depend on a number of factors, including the strain chosen, level of consumption, duration and frequency of exposure, and the physiological condition of the individual (Koop-Hoolihan 2001).

1.4.1 Some of the Beneficial Effects of the Practical Uses of Probiotics

- Growth promotion in farm animals (Chang et al. 2001)—hydrocarbons are broken down by probiotic bacteria which means the food is being split into its most basic elements. This allows almost total absorption through the digestive system. In this way, probiotics dramatically increase overall nutrition and enhance rapid cellular growth and development. For instance, *Lactobacillus* and *Bifidobacteria* increased weight gain and reduced mortality in young piglets (Abe et al. 1995). Also, piglets fed *Bacillus coagulans* had lower mortality, improved weight gain, and feed conversion than unsupplemented piglets and did as well as or better than piglets fed subtherapeutic antibiotics (Adami and Cavazzoni 1999).
- Protection of host from intestinal infections (Nurmi and Rantala 1973; Pascual et al. 1999; Oyetayo et al. 2003). The intestinal tract is cleansed by probiotics. They go under the layer of crud on the intestinal walls, attach themselves, and dislodge the accumulated decay. This waste is then flushed out naturally. They also apply competitive exclusion which involves adherence to intestinal mucosa thereby preventing attachment of the pathogens.
- Production of antimicrobial compounds (bacteriocins and organic acids).
- Competition with pathogenic microorganisms for nutrients and stimulation of intestinal immune response (Ellin 2001).
- Alleviation of lactose intolerance (Garvie et al. 1984; Jiang et al. 1996). In humans, majority become lactase deficient during the 10–20 years of life. The inability to digest lactose causes a decrease in milk product consumption, eliminating a high-quality source of protein and calcium. *L. acidophilus*, *L. bifidus*, and *S. boulardii* participate in the hydrolytic digestion of ingested lactose. Therefore, ingestion of milk product with these live organisms is better tolerated and may actually alleviate malabsorption in lactose-intolerant people (Fuller 1992).
- Relief of constipation (Graf 1983). Constipation is quickly relieved by probiotics and the bowel movements become normalized.

- *Lactobacillus* can be taken both during and after antibiotic treatment. This helps in alleviating antibiotic-induced diarrhea caused by the indiscriminate killing off of both "good" and "bad" bacteria in the gastrointestinal tract (Fuller 1992).
- Anticarcinogenic effect (Walker and Duffy 1998; Zabala et al. 2001). *Lactobacillus* inactivates carcinogenic intestinal beta-glucuronidase and nitroreductase. Studies at the Sloan Kettering Institute for Cancer Research and the University of Nebraska showed *Lactobacillus* to possess a definite antitumor activity and to inhibit tumor proliferation (Fuller 1992). Animal studies have suggested that some lactic acid bacteria might help protect against colon cancer, but more research is still needed.
- Anticholesterolaemic effects (Tahri et al. 1995). *Lactobacillus* species possess anticholesterolemic and antilipidemic factors, which aid in cholesterol reduction. People who consume probiotics have experienced lowered cholesterol (Fuller 1992).
- Nutrient synthesis and bioavailability (Koop-Hoolihan 2001). Probiotic bacteria synthesize certain amino acids, which are directly assimilated, e.g., lysine from specific strains of *L. plantarum*. They produce B vitamins, such as folic acid, niacin, riboflavin, B_{12}, B_6, and pantothenic acid, which are biocatalysts in food metabolism and help to fight stress (Fuller 1992).
- Probiotics have a protein-sparing effect—the *Lactobacillus* primarily uses carbohydrates as a growth medium, while the pathogens use protein. By increasing the probiotic population and decreasing the pathogen population more protein is spared (Fuller 1992)
- Prevention of genital and urinary tract infections (Redondo-Lopez et al. 1990). *Candida albicans* which is the primary pathogenic yeast responsible for candidiasis has been shown to be inhibited by some probiotics (Fuller 1992).
- Immunostimulatory effects (Aattouri et al. 2001). It has been discovered that conventional animals with a complete gut flora have increased phagocytic activity and immunoglobulin levels compared with germ-free animals. *Lactobacilli casei*, in particular, was found to be active in the stimulation of phagocytic activity when administered to mice (Perdigon et al. 1986).

2 Mechanisms of Action

Probiotics carry out its functions including inhibition of pathogenic microorganisms both in vivo and in vitro through different mechanisms which in poultry includes:

1. Maintaining normal intestinal microflora by competitive exclusion and antagonism (Jin et al. 1998; Line et al. 1998; Kabir et al. 2005; Schneitz 2005)
2. Altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production (Jonvel 1993; Han et al. 1999; Yoon et al. 2004)

3. Improving feed intake and digestion (Dierck 1989; Nahaston et al. 1993; Awad et al. 2006)
4. Stimulating the immune system (Huang et al. 2004; Haghighi et al. 2006; Apata 2008; Brisbin et al. 2008)
5. Reduction of pH of colon (Ezema and Ugwu 2014)
6. Provision of nutrients (Ezema 2007)

Enhancements of colonization, resistance, and/or direct inhibitory effects against pathogens are important factors that had helped probiotics to reduce the incidence and duration of diseases. Probiotic and competitive exclusion approaches have been used as one method to control endemic and zoonotic agents in poultry. Nurmi and Rantala (1973) and Rantala and Nurmi (1973) first applied the concept when they attempted to control a severe outbreak of *Salmonella infantis* in finishing broiler flocks. In their studies, it was determined that very low challenge doses of *Salmonella* (1–10 cells into the crop) were sufficient to initiate salmonellosis in chickens. Additionally, they determined that it was during the 1st week posthatch that the chick was most susceptible to *Salmonella* infections. Use of a *Lactobacillus* strain did not produce protection and this forced them to evaluate an unmanipulated population of intestinal bacteria from adult chickens that were resistant to *S. infantis*. On oral administration of this undefined mixed culture, adult-type resistance to *Salmonella* was achieved. This procedure later became known as the Nurmi or competitive exclusion concept. The competitive exclusion approach of inoculating day-old chicks with an adult microflora successfully demonstrated the impact of the intestinal microbiota on intestinal function and disease resistance (Stern et al. 2001). Although competitive exclusion fits the definition of probiotics, the competitive exclusion approach instantaneously provides the chick with an adult intestinal microbiota instead of adding one or a few bacterial species to an established microbial population. Inoculating day-old chicks with competitive exclusion cultures or more classical probiotics serves as a nice model for determining the modes of action and efficacy of these microorganisms. Because of the susceptibility of day-old chicks to infection, this practice is also of commercial importance. By using this model, a number of probiotics have been shown to reduce colonization and shedding of *Salmonella* and *Campylobacter* (Jin et al. 1998; Line et al. 1998). Competitive exclusion is a very effective measure to protect newly hatched chicks, turkey poults, quails, and pheasants and possibly other game birds, too, against *Salmonella* and other enteropathogens (Schneitz 2005).

Upon consumption of the adult flora, many probiotic organisms are delivered into the gastrointestinal tract, which modify the intestinal milieu and produce enzymes and other beneficial substances (Marteau and Rambaud 1993). Supplementation of *L. acidophilus* or a mixture of *Lactobacillus* cultures to chickens significantly increased ($P < 0.05$) the levels of amylase after day 40 of feeding (Jin et al. 2000). The lactobacilli colonizing the intestine may secrete the enzyme, thus increasing the intestinal amylase activity (Duke 1977; Sissons 1989).

The effect of *Aspergillus oryzae* on macronutrients metabolism in laying hens was observed (Schneitz 2005), of which findings might be of practical relevance.

They postulated that active amylolytic and proteolytic enzymes residing in *Aspergillus oryzae* may influence the digested nutrients. Similarly, it was reported that an increase in the digestibility of dry matter was closely related to the enzymes released by yeast (Han et al. 1999). In addition, probiotics may contribute to the improvement of health status of birds by reducing ammonia production in the intestines (Chiang and Hsieh 1995). Probiotic is a generic term and products can contain yeast cells, bacterial cultures, or both that stimulate microorganisms capable of modifying the gastrointestinal environment to favor health status and improve feed efficiency (Dierck 1989). Mechanisms by which probiotics improve feed conversion efficiency include alteration in intestinal flora, enhancement of the growth of nonpathogenic facultative anaerobic and gram positive bacteria forming lactic acid and hydrogen peroxide, suppression of growth of intestinal pathogens, and enhancement of digestion and utilization of nutrients (Yeo and Kim 1997). Therefore, the major outcomes from using probiotics include improvement in growth (Yeo and Kim 1997), reduction in mortality (Kumprecht and Zobac 1998), and improvement in feed conversion efficiency (Yeo and Kim 1997). These results are consistent with the previous experiment of Tortuero and Fernandez (1995), who observed improved feed conversion efficiency with the supplementation of probiotic to the diet.

The manipulation of gut microbiota via the administration of probiotics influences the development of the immune response (McCracken and Gaskins 19g99). The exact mechanisms that mediate the immunomodulatory activities of probiotics are not clear. However, it has been shown that probiotics stimulate different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response (Christensen et al. 2002; Lammers et al. 2003). Probiotics could modulate the systemic antibody response to antigens in chickens (Kabir et al. 2004; Huang et al. 2004; Apata 2008).

Since healthy animals utilize and convert nutrients of ingested feedstuff effectively into constant growth, the beneficial impact of probiotic on the intestinal microbiota could lead to improved daily weight gain and feed conversion (Ezema and Ugwu 2014). It is a fact that the pH of the intestine influences the enzyme activity which directly affects the digestibility of feed nutrients. *Saccharomyces cerevisiae* cells respond to oxidative stress by altering their transcriptional program in a complex manner (Hossain et al. 2005; Khaksefidi and Ghoorchi 2006). Zhang et al. (2005) reported of improved oxidative stability in male broiler chickens supplemented with *S. cerevisiae*.

One of the metabolites of anaerobic fermentation of *S. cerevisiae* in the gastrointestinal tract of chicken is alcohol which is acidic. This is responsible for the reduced pH in the intestine of the *S. cerevisiae* supplemented broilers. The presence of alcohol makes the microecology of the GIT unfavorable for pathogenic organisms. It is well established that probiotics alter the gastrointestinal pH and flora to favor an increased activity of intestinal enzymes and digestibility of nutrients (Dierck 1989; Ezema and Ugwu 2014).

S. cerevisiae continuously divides by binary fusion and when the concentration of the yeast organism in an aliquot gets to 10^8 times they will clinically die and

the digestive enzymes of the host animal will utilize them as microbial proteins (Ezema 2007).

3 Applications of Probiosis to Broilers

Probiotics for chicken are designed for two main reasons namely:

(a) To place beneficial organisms that are not present in the alimentary tract.
(b) To provide the chicken with the effects of beneficial organisms and subsequently enhance growth performance.

Such beneficial organisms may be absent possibly because the present methods of husbandry prevent contact between the newly hatched chicks and its parents preventing rapid vertical transfer of beneficial microorganisms or by management practices which may disturb intestinal microecology (Barrow 1992). It is believed by most investigators that there is an unsteady balance of beneficial and nonbeneficial bacteria in the tract of normal, healthy, and nonstressed poultry. If stress occurs, the beneficial microbes tend to decrease in number leading to increase in the pathogenic ones and subsequently disease. This occurrence may predispose to disease, i.e., diarrhea, or be subclinical and reduce production parameters of growth, feed efficiency, etc. The protective flora which establishes itself in the gut is very stable, but it can be influenced by some dietary and environmental factors. The three most important factors are excessive hygiene, antibiotic therapy, and stress. In the wild, the chicken would receive a complete gut flora from its mother's feces and would consequently be protected against infection. However, commercially reared chickens are hatched in incubators which are clean and do not usually contain organisms commonly found in the chicken gut. The chicken is an extreme example of a young animal which is deprived of contact with its mother or other adults and which is, therefore, likely to benefit from supplementation with microbial preparations designed to restore the protective gut microflora (Fuller 2001). Therefore, the use of probiotic at hatch is more important for poultry than other animals. There could be effects of shell microbiological contamination which may influence gut microflora characteristics. Moreover, gastric secretion of HCl, which starts at 18 days of incubation, has a deep impact on microflora selection.

According to Barrow (1992), there are two major groups of probiotic preparations for chicken based on their site of action: those which are primarily intended to be effective in the crop and the anterior regions of the alimentary tract and those whose effects are directed at the caeca. However, it is likely that both types of preparation are to some extent effective throughout the gut.

Among the first group are the *Lactobacillus* cultures and preparations which are thought to colonize the crop and small intestine in ways described by Fuller (1978). They are thought to exert antibacterial effects against potential pathogens (Fuller 1974, 1978) and are also considered to increase performance by an unknown mechanism. The assumption is that once pathogen burden is reduced, the animal

will naturally perform better. Intestinal colonization is essential for the efficacy of probiotics. Fuller (1986) reported that many criteria are needed to ensure colonization of probiotics in the gut. These criteria may include adhesion to the crop epithelium, ability to grow in the nutritional environment of the gut, and ability to resist innate or microbial produced inhibitory mechanisms.

From work with monocontaminated and decontaminated gnotobiotic chicken, Morishita et al. (1971) found that whereas avian strains of *L. acidophilus*, *L. plantarum* and *L. fermentum* in addition to the nonintestinal *L. plantarum* and *L. casei* colonized well, a human *L. acidophilus* strain, *L. helveticus*, and *L. brevis* were rapidly eliminated from the alimentary tract. This indicated the importance of choosing both the right species and strain. A number of technical and experimental points must be considered in assessing the value of probiotic preparations. Barrow further stated that statistical significance must be aimed for, but even if it cannot be attained the result may nevertheless be of biological significance. A critical review of the available literature on the application of probiosis to poultry performance and health is essential in assessing its value.

4 *Saccharomyces cerevisiae* as a Probiotic of Choice for Broilers

Several yeast species have been used as probiotics for human and animals. However, a strain of *Saccharomyces cerevisiae* has proved to be the best probiotic for broilers. Probiotic research and application originated from human medicine. For these earlier research workers, their interests were simply to use probiotics to promote good health. Hence, their choice of probiotics was mainly of bacteria origin. Weight gain and efficiency of feed utilization are not issues in human beings. In fact, human researchers are more interested in those products that could cause weight loss. But in livestock production, weight gain and feed efficiency are the crucial issues that will determine the productivity and profitability of the livestock enterprise. This is why we considered *S. cerevisiae* as the probiotic of choice for animal production. *S. cerevisiae* also known as "baker's yeast" is one of the most widely commercialized species and one of the effective adsorbents which is rich in crude protein (40–45 %) and also rich in vitamin B complex, biotin, niacin, pantothenic acid, and thiamine and its biological value is high (Reed and Nagodawithana 1999). *Saccharomyces cerevisiae* is arguably one of the most studied microorganisms. However, its mechanism of action as a probiotic has not been fully understood (Ahmad 2006). It has been reported that it elaborates enzymes which aid in digestion (Martin et al. 1989) and produces lactic acid which increases acidity and reduces pH of the digestive tract, thereby preventing the growth of pathogenic organisms and aiding in enzymatic activity. This increases digestibility and utility of minerals, proteins, and amino acids (Gunal et al. 2006). *Saccharomyces cerevisiae* has mannan oligosaccharide (MOS) in its cell wall

which is a natural feed additive that is involved in helping the growth of beneficial microbes and inhibiting that of the pathogenic organisms (Yang et al. 2008).

Saccharomyces is a genus of budding yeast which is also part of the residual microbial system of the intestinal microbiota. *Saccharomyces cerevisiae*is widespread in nature and can be found in plants, fruits, and soil. It is included in foods and beverages for its key role in fermentation processes and in health foods. Strain known as *S. boulardii* was isolated from the skin of lychees grown in Indochina. This species does not have a taxonomic status and is considered a biotype of *S. cerevisiae* (van der Aa Kühle and Jespersen 2003). *S. boulardii* is used as probiotic especially in ruminants and pig feeding as well as in humans.

The adaptation to the posthatching period and the increased stress derived from practices used in modern broiler production, e.g., feed changes or imbalances, transportation, processing at the hatchery, and high stocking densities (Pinchasov and Noy 1993), may weaken immune functions and thus predispose broilers to colonization of the gastrointestinal tract by bacterial pathogens, posing a threat to bird's health and food safety. Among pathogens, Salmonella spp. has been the most studied because of its ability to easily infect chickens and hens thereby increasing the risk of contamination through the food chain (Humphrey 2006). Probiotics could be a possible strategy to control pathogen shedding and thus maintain a healthy indigenous gut microbiota. The application of probiotics in poultry is strictly associated with the concept of competitive exclusion (CE). Since the first applications on newly hatched chicks, several experiments with defined and undefined probiotic cultures have been developed and successfully applied to control and reduce Salmonella colonization. It has also been shown to protect chicks against *C. jejuni*, *Listeria monocytogenes*, pathogenic *E. coli*, *Yersinia enterocolitica* and *C. perfringens* (Nisbet et al. 1998; Schneitz 2005).

It is important to note that not all strains of *S. cerevisiae* can be used as a probiotic for broilers. A probiotic strain must be a fast growing strain as the growth rate is a measure of its bioactivity.

4.1 *Yeast (*Saccharomyces cerevisiae*) as a Growth Promoter*

S. cerevisiae was used for brewing and baking purposes, and was also employed extensively in poultry industry where encouraging results were achieved with respect to weight gain, feed efficiency, dressing percentage, egg production, egg weight, inhibition, and containment of proliferation of *Escherichia coli* in the intestines, phosphorus utilization and counteracting the effect of aflatoxins in the diet (McDaniel and Sefton 1991). Swamy and Upendra (2013) reported significant increase in body weight of broilers fed 0.1 % *S. cerevisiae* supplementation at the rate of 24.5 %, 19.1 %, 16.4 %, and 20.7 % on day 21, 28, 35, and 42, respectively. Krueger et al. (1990) observed improved feed efficiency by 3.16 % upon feeding of 454 and 908 g *S. cerevisiae* per ton of feed in broiler chickens from 28 to 49 days of age. Stanely et al. (1993) reported that the use of *Saccharomyces cerevisiae*

provided a source of proteins, vitamins, enzymes, and growth factors which improved nutrients absorbed in the digestive tracts of broilers. They reported that even if chicks were fed aflatoxin contaminated feed, weight gain could improve by 13 % when supplemented with 0.1 % *Saccharomyces cerevisiae*. Bradley et al. (1994) observed increased weight gain at 7, 14, and 21 days of age in poults fed diet containing *S. cerevisiae*. Maiorka et al. (2001) conducted an experiment in which they fed broilers with feed added antibiotics (Olaquindoxa and Nitrovina), prebiotic (0.2 % *S. cerevisiae* cell wall), probiotic (300 ppm *Bacillus subtilis*), and symbiotic (prebiotics and probiotics) and observed better live weight gain in broilers up to 45 days of age, fed with symbiotics followed by antibiotics, prebiotics, and probiotics. The total absence of additives in the diets worsened broiler chicken performance in that study. Santin et al. (2001) reported the efficacy of *S. cerevisiae* cell walls, obtained from the brewery industry, added at 0.1 and 0.2 % to broiler chicken diets, on performance and intestinal mucosa development (tropic effect). Kompiang (2002) conducted an experiment to evaluate the effect of marine yeast and *S. cerevisiae* as probiotic supplements on poultry performance for 5 weeks. Their effects on bird performance were better than the treatment of negative control and concluded that marine yeast or *S. cerevisiae* could replace the function of antibiotic as a growth promoter.

In another study, Ezema (2007) used a total of 140 day-old broiler chicks (Anak 2000) which were randomly divided into seven groups of 20 birds each. Each group was subdivided into four replicates of five birds each. Group 5 had no yeast (control 1). Group 7 had no yeast (control 2). All the groups were fed ad libitum. Daily feed intake and weekly weight gain were determined. The duration of the study was 10 weeks. Apparent digestibility and hematological parameters were also evaluated. There was no significant difference in feed intake. Group 2 weighed significantly heavier ($P < 0.05$) than the rest. Groups 2 and 3 had the highest apparent crude fiber digestibility of 30.86 % and 30.87 %, respectively. The cost of feed to produce 1 kg live weight gain of group 2 was 129.85 ± 2.17/kg, group 5 was 154.00 ± 2.08/kg, and group 7 was 192.28 ± 6.84/kg. Group 2 performed significantly better than others in weight gain, carcass weight, and economic gain. Based on their finding, supplementation to the level of 0.8 g yeast/kg of feed was recommended by them for optimum broiler production in the tropics. Studies on the beneficial impact on poultry performance have indicated that probiotic supplementation can have positive effects. It is clearly evident from the result of Kabir et al. (2004) that the live weight gains were significantly ($P < 0.01$) higher in experimental birds compared to control. This result is in agreement with many investigators (Kalavathy et al. 2003; Zulkifli et al. 2000; Islam et al. 2004; Kamruzzaman et al. 2005) who demonstrated increased live weight gain in probiotic fed birds. On the other hand, Lan et al. (2003) found higher ($P < 0.01$) weight gains in broilers subjected to two probiotic species.

Probiotic cultures have also been administered to turkeys and other poultry. In a study using 72 (15-day-old) white hybrid converter turkey poults, Cetin et al. (2005) investigated the effects of MOS and probiotic supplementation on hematological and immunological parameters of turkeys. This study also revealed

that both the probiotic and MOS supplementation resulted in significant increases ($P < 0.05$) in the serum IgG and IgM levels. This trial suggests that MOS and probiotics that enhanced immunoglobulin levels will have more positive effects on growth performance and turkeys' ability to resist diseases. It has been demonstrated that direct-fed microbial (DFM) may offer an effective alternative to antibiotic growth promoter in turkeys.

4.2 Probiotic Effects of S. cerevisiae on the Intestinal Microbiota and Intestinal Morphology

Huang et al. (2004) reported that yeast increased resistance to *E. coli* infection and helped microbial balance in the gastrointestinal tract. In a study carried out by Saadia and Nagla (2010), they found out that intestinal makeup of broilers on yeast supplementation reduced significantly. In that report, total bacterial count was 15, 12.5, 12.7, 10.1, and 5.4 for birds fed 0 %, 0.4 %, 0.8 %, 1.2 %, and 1.6 % *S. cerevisiae* in their diet. *E. coli*, *Staphylococcus* spp., *Campylobacter* spp. and *C. perfringens* reduced significantly while *Lactobacillus* spp. increased with the addition of yeast in the diet of the birds. The result indicated that feeding yeast to broilers increased the survival of beneficial microbes like *Lactobacillus* in the intestine.

Yaman et al. (2006), Mountzouris et al. (2007), and Higgins et al. (2007) demonstrated that probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have a potential effect on the modulation of intestinal microflora and pathogen inhibition.

4.3 Probiotic Effects of S. cerevisiae on Immune Response

Chitin, mannan, and glucan, derivatives of the cell wall of *Saccharomyces cerevisiae*, are known to stimulate the immune system, increase globulin protein levels (Abaza et al. 2008), and improve leukocyte count (Abdollahi et al. 2002). Kabir et al. (2004) evaluated the dynamics of probiotics on immune response of broilers and they reported significantly higher antibody production ($P < 0.01$) in experimental birds compared to control ones. In addition, Haghighi et al. (2006) demonstrated that administration of probiotics enhances serum and intestinal natural antibodies to several foreign antigens in chickens. Ezema (2012) observed a significant increase in absolute lymphocyte count in *S. cerevisiae* supplemented birds compared to the control.

4.4 *Probiotic Effects of* S. cerevisiae *on Broiler Meat Quality*

Kabir et al. (2005) evaluated the effects of probiotics on the sensory characteristics and microbiological quality of dressed broiler meat and reported that supplementation of probiotics in broiler ration improved the meat quality both at prefreezing and postfreezing storage. Mahajan et al. (2000) stated that the scores for the sensory attributes of the meat ball appearance, texture, juiciness, and overall acceptability were significantly higher and those for flavor were lower in the probiotic (Lacto-Sacc) fed group. The reduced flavor could be due to the decreased lipid content as *S. cerevisiae* has been reported to reduce the fat content of meat (Ezema 2007). On the other hand, Loddi et al. (2000) reported that neither probiotic nor antibiotic affected sensory characteristics (intensity of aroma, strange aroma, flavor, strange flavor, tenderness, juiciness, acceptability, characteristic color, and overall aspects) of breast and leg meats. Zhang et al. (2005) conducted an experiment with 240, day-old, male broilers to investigate the effects of *Saccharomyces cerevisiae* (SC) cell components on the meat quality and they reported that meat tenderness could be improved by the whole yeast (WY) or *Saccharomyces cerevisiae* extract (YE). In a work carried out by Shareef and Al-Dabbagh (2009), they reported that addition of *Saccharomyces cerevisiae* at 1, 1.5, and 2 % reduced the serum glycerides while only at 2 % significantly reduced serum cholesterol. Earlier, Mohan et al. (1996) and Yusrizal (2003) reported reduced cholesterol in the meat of chicks fed *Saccharomyces cerevisiae* in their diet. These all make the meat healthier for the consumer. Ashayerizadeh et al. (2009) also reported a lower cholesterol and triglyceride concentration of chicken meat among probiotic fed birds when compared to controls and antibiotic fed birds. Paryad and Mahmoudi (2008) reported that feeding broiler chicks 1.5 % *S. cerevisiae* reduce ($P < 0.05$) plasma cholesterol and triglycerides compared with broiler chicks fed control diets. Microbial meat quality of birds was reported to be affected by yeast supplementation as Mahajan et al. (2000) found lowered total viable microbial count from birds fed Lact-Sacc probiotics in their diet.

4.5 *Probiotic Effects of* S. cerevisiae *on Stress in Broilers*

Aluwong et al. (2013) reported a reduced glutathione peroxidase among 200 broilers fed *Saccharomyces cerevisiae* in their diet which caused much lower oxidative stress. Probiotic *S. cerevisiae* has been found to enhance an integrated antioxidant defense system in broiler chickens which maintains the physiological grade of oxidative stress needed for a number of biofunctions like growth (Aluwong et al. 2013). *S. cerevisiae* has been reported by many researchers to be rich in or enhance the production of B complex vitamins, which increase digestibility and reduce stress (Abaza et al. 2008).

4.6 *Probiotic Effects of* S. cerevisiae *on pH of GIT of Broilers*

From our recent study involving 100 broilers, the pH of colon was reduced significantly for the broilers fed diets supplemented with *S. cerevisiae*. Fermentation carried out in the colon by yeast produces alcohol and lactic acid which reduce the pH (Gunal et al. 2006; Philips et al. 2008). These acids prevent the development of pathogenic microorganisms and increase enzymatic activity which subsequently increases digestibility and utilization of minerals, proteins, and amino acids (Gunal et al. 2006; Karademir and Karademir 2003). Aksu et al. (2005) reported a decrease in the pH of carcass of broilers fed yeast supplement. In another study, the pH of ileum was 6.00 and 6.31 for broilers fed 0.8 and 1.2 % yeast in their diet. This was reduced when compared to the pH of 6.58 recorded for birds on control diet.

4.7 *Probiotic Effects of* S. cerevisiae *on Nutrient Digestibility in Broilers*

In a recent study, there was a significant ($P < 0.05$) increase in apparent digestibility coefficient of organic matter, crude protein, and crude fiber in the probiotic supplemented groups in contrast to the control (Ezema 2012). The increase in digestibility could be due to activities of digestive enzymes that are released by the probiotics. Digestive enzymes aid in the breakdown of food particles into smaller portions which can be easily absorbed by the host. Matsui et al. (1990) reported that *Saccharomyces cerevisiae* elaborates digestive enzymes, which help the host to digest fibrous feed. The fibrous walls in the feed make the nutrient unavailable for utilization by the bird. Other researchers had found out that *Saccharomyces cerevisiae* increases nutrient digestibility, thereby improving the growth of birds (Ferket et al. 2002; Sandikci et al. 2004; Silversides et al. 2006).

5 Conclusion

In broiler nutrition, probiotic strain of *Saccharomyces cerevisiae* had beneficial effects on weight gain, broiler performance, nutrient digestibility, and efficiency of feed utilization. It also improved the health status and the meat quality of broiler chicken. Hence, yeast *S. cerevisiae* is proposed as a probiotic of choice for broiler production.

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Bacillus for Rice Cultivation in Thailand

Mana Kanjanamaneesathian

Abstract Rice (*Oryza sativa*) is the most important cereal in the tropical region. It has been the main source of energy and nutrition for approximately 2.9 billion people. Because of its significance, rice has been subjected to a thorough scientific investigation. Internationally, the International Rice Research Institute (IRRI), based in the Philippines, has played a leading role in producing the improved rice varieties with better yield and resistance to insect pests and plant diseases. In Thailand, Rice Department, Department of Agriculture, and Department of Agricultural Extension have been responsible in conducting research and disseminating research outcomes (such as new rice varieties, knowledge in cultural practice for rice, and technology for controlling pests in rice) to the Thai rice farmers. The concern about both environmental deterioration as a result of overusing the chemical agents in rice production and health well-being of the farmers due to an exposure to toxic chemical agents has prompted the shift in the focus on researching in rice, and subsequent extension work conducted by various government agencies in Thailand. With the trend in sustainable production, natural-based products have been recommended to the farmers for use for both rice growth promotion and rice pest control. The natural-based products include crude botanical plant extract, wood vinegar, simple biomass of effective microorganisms (EM), biomass of antagonistic fungus *Trichoderma harzianum* growing on sterile cereal grains, and formulation of antagonistic *Bacillus* spp. Some of these natural-based products, such as biomass of EM, are recommended by the local agricultural officials and are commonly used by the farmers and their continual usage is a matter of trial and error. Formulations of antagonistic fungus and particularly antagonistic *Bacillus* spp. have been devised and have been subjected to rigorous laboratory, greenhouse, and field tests. Essential role of the stakeholders, such as local agricultural staffs, the farmers, and the researchers has been identified and highlighted as it will provide the model for further research and extension work in other rice growing areas.

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1 Introduction

Rice feeds more than two billion people in the developing world and is considered to be one of the most important food crop in the world (Datta 2004; Brar and Khush 2013). In Thailand, rice has been the backbone of the Thai society and its significance still remains up to the present time. Growing rice is the integral part of the Thai culture as well as the main source of food and income for the rural Thai farmers. In 2014, Thailand has exported approximately 5.6 million ton (MT) of rice with the total value around US$3 billion (Thai Rice Exporters Association 2014).

In the wet season, rice cultivation is predominant in the northeast region of the country accounting for 57 % of rice growing area, followed by the north region (22 %), the central region (17 %), and the south region (4 %). In the dry season, rice production is concentrated in the central region with 46 % of rice growing area, followed by the north region (41 %), the northeast region (10 %), and the south region (3 %) (Rice Department, Thailand 2014).

The increase of rice growing area in the dry season in both the Central and the North regions occurs as a result of the irrigated water availability in these areas. With the access to irrigated water, rice has been cultivated intensively in both the central and the north regions resulting in the occasional but widespread outbreak of both insect pests and plant diseases of rice (Bureau of Rice Research and Development, Thailand 2015) (Table 1 and Fig. 1). As a consequence, chemical

Table 1 Key pests causing severe and widespread damage to rice productivity

Pests	Common and scientific names	Main control measures
Insects	Brown planthopper (*Nilaparvata lugens*) Rice leaffolder (*Cnaphalocrocis medinalis*) Rice gall midge (*Orseolia oryzae*)	Plant resistance and chemical pesticides
Diseases caused by plant pathogenic fungi	Rice blast (*Pyricularia grisea*) Sheath blight (*Rhizoctonia solani*)	Plant resistance and chemical fungicides
	Grain discoloration (*Curvularia lunata*, *Cercospora oryzae*, *Helminthosporium oryzae*, *Fusarium semitectum*, *Trichoconis padwickii* and *Sarocladium oryzae*) Brown spot (*Helminthosporium oryzae*) Narrow brown spot (*Cercospora oryzae*)	Chemical fungicides
Diseases caused by plant pathogenic bacteria	Bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) Bacterial leaf streak (*X. oryzae* pv. *oryzicola*)	Plant resistance and cultural practice
Disease caused by plant pathogenic virus transmitted by insects	Rice tungro disease (tungro bacilliform and spherical virus) Rice ragged stunt disease (rice ragged stunt virus)	Plant resistance and insect–vector control

Source: Rice Department, Ministry of Agriculture and Cooperatives, Thailand (2014)

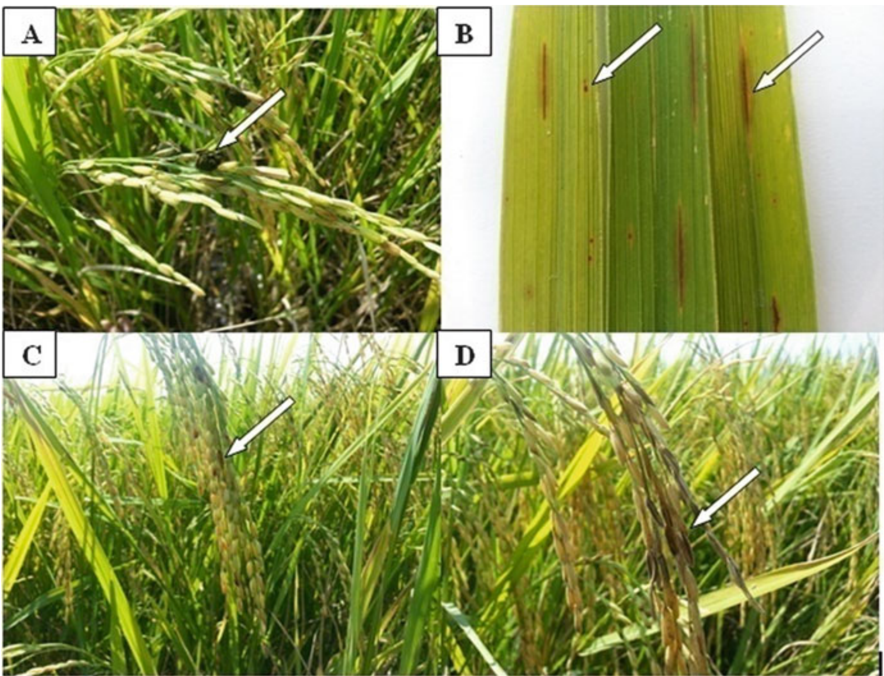

Fig. 1 Some diseases of rice (var. RD 31) in the rice paddy field from Thayang, Phetchaburi province, Thailand. (**a**) False smut of rice caused by *Ustilaginoidea virens*, showing mature *greenish black* spore ball (*arrow*) in some grains of the rice panicle. Disease is prevalent when N-fertilizer has been over used. There has been no report with respect to its significance to yield loss in rice. (**b**) Narrow leaf spot of rice caused by *Cercospora oryzae*, showing early small spot (*arrow*) and more developed larger symptom (*arrow*) on rice leaves. (**c**) Grain discoloration of rice, showing mild disease symptom. (**d**) Grain discoloration of rice, showing severe disease symptom. Yield loss has been reported in rice as a result of an epidemic of narrow leaf spot and grain discoloration diseases (Bureau of Rice Research and Development, Thailand 2015)

pesticides have been the main tool for controlling rice pests adopted by the farmers in these areas. The concern with respect to health and ecological issues as a result of chemical pesticides' overuse in producing rice has initiated the interest of using integrated pest management (IPM) approach to deal with pests of rice not only in Thailand, but also around the world (Savary et al. 2000b; IRRI 2002; Norton et al. 2010).

Basic research to accumulate enough knowledge on pests to create IPM program and technological development to invent applicable tool to implement IPM on plant pests are thus crucial to ensure that the farmers should adopt IPM in their agricultural production. Ecological friendly tools for controlling rice pests such as crude botanical plant extract, wood vinegar, and aqueous part of fermented food waste—containing a myriad of microorganisms—have been used by the Thai farmers although their use may be still very limited to a small area but scattered throughout the country. This may be because their usage is by the initiative of an individual

Table 2 Some activities which are needed to conduct in order to reduce the gap among the stakeholders in rice production (Garforth 1993; Norton 1993)

Main initiators	Activities	Objectives
Researchers	Disseminating research outcome via various media	To get the message across to the public so that every stakeholders in rice production have a chance to get to know the up-to-date information on research outcome
Researchers and local agricultural staffs	Interacting with the local agricultural staffs and the rice farmers who are interested with the research outcome	To transfer the knowledge and to establish both understanding and trust among the researchers, local agricultural staffs and the rice farmers
Local agricultural staffs	Conducting follow up focus group with the rice farmers	To determine the continual use of the research outcome and the possible adoption as a normal practice
Rice farmers	Using acquired knowledge in growing rice and provide useful feedback information to both local agricultural staffs and researchers	To learn the areas what need to be improved and to identify the areas what need future researching

local agricultural staff, whose role is to pass on the knowledge for its use to the rice farmers. To enhance rice farmers' access to the comparatively more reliable product, biological products developed based upon a well-plan research are needed, as the products are likely to be adopted by private entrepreneur for production, distribution, and marketing (Table 2).

Formulation of an effective antagonistic fungus *Trichoderma harzianum* has been produced and commercialized in Thailand. This formulation has been initially used to control *Pythium* spp. and *Phytophthora* spp., the causal agent of various economically important diseases in vegetables and fruit trees, respectively, in Thailand (Intana 2003). The formulation has been subsequently recommended to the rice farmers with an expectation that it may be able to suppress sheath blight disease caused by *Rhizoctonia solani*. The antagonistic fungus suppresses sheath blight disease by degrading rice plant tissue colonized by the pathogen resulting in the reduction of the primary inoculum. The added benefit of applying the biomass of *T. harzianum* to the rice field is that it plays a role in recycling essential nutrients to soil (Shukla et al. 2012).

The bacterium especially the *Bacillus* spp. has been used in agriculture for controlling insect pests and promoting plant growth (Mew and Rosales 1986; Mew et al. 2004; Vijay Krishna Kumar et al. 2009). Formulation of *B. thuringiensis*—commonly known as Bt—is a biological product widely used for controlling insect pests worldwide (Gupta and Dikshit 2010). In Thailand, Bt has been used extensively to control insect pests of the vegetables (Pinsri et al. 2012). Its usage in rice production focuses on the control of rice leaf folder insect (*Cnaphalocrocis medinalis*) (Kandibane et al. 2010). For disease control and growth promotion in rice, at least three species of *Bacillus* spp., such as *B. subtilis*

(Charigkapakorn et al. 1992), *B. megaterium* (Kanjanamaneesathian et al. 1998), and *B. cereus* (Jetiyanon and Plianbangchang 2010), either in the form of simple product from the liquid fermentation or in the formulations, have been used under both greenhouse and field trials to determine their efficacy with respect to disease control and growth promotion in rice. With the fundamental knowledge from these researches and the availability of basic fermentation technology, only a dried powder of *B. subtilis* has been produced and has been marketed for controlling diseases in rice and other crops in Thailand (Maketon 2004; Maketon et al. 2008). The dried powder of *B. subtilis* has been recommended to control rice blast and grain discoloration diseases, and chemical fungicide would be recommended for disease control only when these diseases are expected to be very severe (Arunyanart et al. 2001, 2008).

Nonetheless, other type of formulations with improved characteristics and new strains of the *Bacillus* spp. possessing multiple beneficial traits should be continually devised. The new formulations will provide a platform for other public organizations working with the rice farmers to consider accepting this technical know-how and transfer the appropriate technology to the rice farmers. Alternatively, the research in inventing the novel bacterial formulations should induce the interest of a local agricultural private entrepreneur in embarking upon commercializing the new products (Wiwattanapatapee et al. 2004, 2007, 2013a).

This chapter lays out major biotic factors which affect rice productivity. The content highlights the constraints in rice production but subsequently emphasizes on opportunity for research and development in beneficial microbes for use in rice production. The research and development of the formulation of the *Bacillus* spp. for plant growth promotion and disease control is presented in the context of the collaborative activities among agricultural staffs, academic team, and rice farmers.

2 Constraints for Rice Cultivation in Thailand

From the global estimate, animal pests (mostly insects) come first as the causal agents of crop losses in rice at approximately 15 %, followed by plant pathogens (at 12 %) and weeds (at 10 %) of the attainable yield (Oerke 2006). In southeast Asia, rice yield loss due to pests is estimated at 37 % (Savary et al. 2000a). In a survey in Thailand, the vast majority of Thai farmers have expected the losses to pests of more than 50 %. These data highlight the immensity of crop loss due to pest problems which are perceived to be at intolerable levels (Waibel 1990). Insect pests have been perceived as the agents causing significant damage to rice crop, followed by plant diseases and weeds. Because of this perception, rice farmers tend to purchase chemical pesticides, especially insecticides and fungicides, and spray them as recommended on the label on the calendar basis. Weeds, however, can be controlled by human labor and other cultural practices.

Rice farmers normally apply chemical agents out of habit. Injudicious and unnecessary use of both chemical fertilizers and chemical pesticides has a negative

effect, contributing to not only promoting a conducive environment for more pest infestation, but also wasting time and money for chemical applications. The biological constraints, particularly insect pests and plant diseases, can be obtained and identified through research. The outcome of this research can be made available publicly with an expectation that the rice farmers will use it for cultivating rice. Some key insect pests and plant diseases of rice in Thailand, which should be targeted for continuing research and development of biological products of *Bacillus* spp., have been highlighted (Table 1 and Fig. 1). Should these environmental friendly products be available, they can be utilized as a tool in managing the rice pests, in conjunction with other control measures which are available and have been recommended for use to control rice pests.

3 Opportunity for Developing the Products of Beneficial Microbes for Use in Rice Cultivation in Thailand

In Thailand, chemical fertilizers and chemical pesticides are the main tool for promoting plant growth and controlling key pests in rice production, respectively. In 2012, approximately 24,000 tons of chemical pesticides have been imported to Thailand in comparison with 103 tons of biopesticides (Office of Agricultural Economics 2014). The import of the biopesticide, although in quite a comparatively small quantity, indicates that there is a demand for its use and there is an opportunity for research and development regarding inventing the new bioproducts for use in Thailand. At present, biological products of the microbes may have been used as a supplementary tool for use in growing rice. The demand for these environmental friendly products by the rice farmers, however, should increase in the future when more rice farmers should opt to cultivate rice organically to respond to an increased demand for high-quality rice produce from both local and overseas consumers. This requires more research and development with respect to using beneficial microbes for producing rice.

Table 3 summarizes key components which are conducive for research and development with respect to utilization of beneficial microbes in agriculture in Thailand. Thailand Institute of Scientific and Technological Research (TISTR), for example, has undertaken research and development in producing biofertilizer for enhancing the growth and yield in rice production, based on N-fixing cyanobacteria such as *Anabaena* sp., *Calothrix* sp., *Nostoc* sp., and *Tolypothrix* sp. The technology has been transferred to the local private entrepreneur prior to commercialization recently. Public organizations within the Ministry of Agriculture and Agricultural Cooperatives may have different approach in technology transfer. For instance, Department of Agriculture and Department of Agricultural Extension have produced a fresh preparation of insect pathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* and distributed them to rice farmers for use to control *N. lugens*. The basic method for the preparation of these fungi has been transferred

Table 3 Key components which create the favorable environment for conducting research and development in utilizing beneficial microbes in agriculture sector in Thailand (Thailand's Biotechnology 2013)

Components	Contributory characteristics
Source of beneficial microbes	Diverse ecosystems with the wealth of biodiversity such as • Coral reefs • Mangrove and coastal vegetation • Tropical evergreen forests • Wetlands, coastal marshes and swamps
Source of skilled workforce	• At least 28 academic institutes providing knowledgeable and skilled graduates for public sector and private companies doing business in the areas of biotechnology • Most of these academic institutes, if not all, offers postgraduate degrees based on research
Sources of funding for research grant and laboratory facility	• National Research Council of Thailand (NRCT) • National Science and Technology Development Agency (NSTDA) • Biodiversity-Based Economy Development Office (BEDO) • National Biopharmaceutical Facility (NBF) • Thailand Institute of Scientific and Technological Research (TISTR) • Thailand Research Fund (TRF) • Agricultural Research Development Agency (ARDA)
Source of business opportunity	• Thailand Center of Excellence for Life Sciences (TCELS) • Board of Investment (BOI) of Thailand
Source of access to market	• Domestic demand • Overseas clients as a result of the establishment of the ASEAN Economic Community (AEC) in 2015

directly to the farmers nationwide for use to control *N. lugens* (National Science and Technology Development Agency 2015).

4 *Bacillus* spp. Used in Rice Cultivation in Thailand

4.1 *Source of the Effective* Bacillus *spp.*

Beneficial physical and physiological traits of the cells of *Bacillus* spp. are the focal point if they should be developed into the biological products for use in rice production. *Bacillus* spp. possesses two systems, nonribosomal synthesis of cyclic lipopeptides and polyketides, which synthesize various beneficial substances with the properties of either inhibiting plant pathogens or promoting plant growth (Mongkolthanaruk 2012). Various ecosystems as a potential habitat of the

undiscovered *Bacillus* spp.—which possesses the useful substances—should be investigated (Table 3).

Agricultural soil is the common source of *Bacillus* spp. This *Bacillus* spp. has been reported to be a good producer of cellulase enzyme (Akaracharanya et al. 2014). *Bacillus* spp. from paddy rice field soil has also been found to be effective in controlling rice diseases (Kanjanamaneesathian et al. 2009). Nevertheless, the soil sample is bulky, requiring labor and space for processing the sample particularly when soil sample collection covers a large area (Kanjanamaneesathian et al. 1998). Furthermore, special treatment to the soil samples is needed to get rid of other unwanted microbes before they should be used for isolating *Bacillus* spp. as the soil usually harbors a myriad of microorganisms. *B. megaterium* is obtained by diluting the soils in water and treating the samples with hot water at 80 °C for 20 min. This isolate has the inhibitory effect to the mycelial growth of *Rhizoctonia solani* (Kanjanamaneesathian et al. 1998). Recently, *B. thuringiensis* R 176, isolated from rhizosphere paddy soil, has been demonstrated to be an efficient producer of chitinase and this isolate has a prospect in application for controlling rice diseases caused by fungal pathogens (Chaiharn et al. 2012). With various soil types in different agroecosystems of rice cultivation in Thailand, it is expected that many more isolates of beneficial *Bacillus* spp. should be discovered.

Infertile soil in the areas with high salinity is a potential source of beneficial *Bacillus* spp. *B. megaterium* isolate no. A12ag, isolated from saline soil in Mahasakham province in the northeast of Thailand, promotes seed germination of rice var. KDML 105 and increases the growth and yield of rice cultivated in saline soil (Sapsirisopa et al. 2009). As a phosphate-solubilizing bacterium, *B. megaterium* A12ag and other beneficial *Bacillus* spp. from other adverse soil conditions have potential for use in rice cultivation in the areas where soil conditions are poor. This will provide an opportunity to capitalize land use in some areas where rice farmers have been growing rice var. KDML105—a rice variety with high grain quality—in the northeast of Thailand.

Rice seed is also a habitat of beneficial isolates of *Bacillus* spp. which can be utilized for controlling bacterial disease in rice. Two isolates of *B. subtilis* NSRS 89-24 and NSRS 89-26 have been originally isolated from an indigenous rice seed, var. Choa Boa from Nakornsrithamarat Rice Experiment Station. These bacteria inhibit *Xanthomonas oryzae* pv. *oryzae*, a causal agent of bacterial leaf blight of rice, in vitro and reduce disease incidence in the greenhouse test (Charigkapakorn et al. 1994). *B. amyloliquefaciens*, the endophytic bacterium isolated from the seeds of rice (var. Chai Nat and var. SuphanBuri), is capable of promoting seed germination and seedling growth (Suebphankoy et al. 2013). A vast number of novel *Bacillus* spp. should be found from the seeds of approximately more than 100 varieties of rice grown for commercial purpose in Thailand (Bureau of Rice Research and Development 2015).

Bacillus spp.—which possesses multiple beneficial characteristics such as nitrogen fixation, phosphate solubility, and IAA production—has been demonstrated to be a major component of microbes inside tissue of rice plants (Nhu and Diep 2014). Nonetheless, both epiphytic and endophytic *Bacillus* spp. can be isolated from the

main parts of rice plants in Thailand (Phetcharat and Duangpaeng 2012; Hongrittipun et al. 2014; Nongkhlaw and Joshi 2014). Among 123 isolates, two isolates of endophytic *Bacillus amyloliquefaciens* (isolate no. 25R14 from roots of rice seedling var. MueyNong 25) and *B. amyloliquefaciens* (isolate no. SR1 from roots of rice seedling var. Suphan Buri 1), have been found to possess high nitrogenase activity, indicating that they are potential candidates for use as biofertilizer in rice cultivation (Hongrittipun et al. 2014). The other 100 rice varieties in Thailand, with each variety having the unique morphological and physiological characteristics, may harbor other novel isolates of beneficial *Bacillus* spp.

Whilst various potential sources of beneficial *Bacillus* spp. are plentiful and contribute to the prospect of providing beneficial *Bacillus* spp., the isolated *Bacillus* spp. will be useful only when they are subject to the robust screening procedures which are efficient enough to identify both positive and negative traits. *B. amyloliquefaciens* FZB42 has been reported to stimulate root exudation, but limits phosphorus uptake in *Triticum aestivum* (Talboys et al. 2014). The efficient screening protocol should be developed to detect a negative effect of beneficial *Bacillus* spp. intended for use in rice, excluding the unwanted isolates which should not be used for mass production and formulation. Should such negative traits of beneficial *Bacillus* spp. have been overlooked in the screening for selection for commercialization, the tremendous loss in investment should occur as the products may cause detrimental effect to the end-users resulting in the rejection of using biological products.

4.2 Mode of Action

Mode of action of the *Bacillus* spp., such as *B. subtilis*, has been attributed to antibiosis (Bais et al. 2004: Chen et al. 2013; Vijay Krishna Kumar et al. 2013), hyperparasitism (Vijay Krishna Kumar et al. 2013), competition in the rhizosphere (Bais et al. 2004; Chen et al. 2013), induced resistance by activation of defense gene in plant (Kilian et al. 2000), and promotion of plant growth (Kilian et al. 2000; Idris et al. 2004; Kumar et al. 2011). Nevertheless, some researches indicate that effective *Bacillus* sp. strain possesses multiple mechanisms in disease suppression (Bais et al. 2004; Chen et al. 2013). Both biofilm formation and surfactin production by *B. subtilis* are contributory to the biocontrol efficacy against *Pseudomonas syringae* (Bais et al. 2004). Recently, Chen et al. (2013) reported that tomato plants are protected from *Ralstonia solanacearum*, a causal agent of tomato wilt disease, by *B. subtilis* strain which possesses the ability to form biofilm robustly and produces antimicrobial agents.

In rice, *B. megaterium* produces heat-stable toxin which is highly effective in inhibiting the mycelial growth of *R. solani* (Pengnoo et al. 2000; Wiwattanapatapee et al. 2004, 2007, 2013a; Chumthong et al. 2008). More recently, *B. subtilis* MBI 600 (Integral®) has been reported to cause loss of structural integrity, shriveling,

abnormal coiling, and lysis of *R. solani* hyphae due to antibiosis and hyperparasitism (Vijay Krishna Kumar et al. 2013). *B. subtilis* AUBS1 has been reported to induce resistance characteristics in rice plants against sheath blight disease caused by *R. solani*, possibly due to the accumulation of pathogenesis-related proteins (Jayaraj et al. 2004). More recently, it has been reported that a total of 31 differentially expressed proteins associated with disease resistance characteristics are induced in rice after exposure to *B. cereus* NMSL 88 (Wang et al. 2013). Gopalakrishnan et al. (2012) reported that *B. altitudinis* isolate SRI-178 and other six isolates, isolated from rice rhizosphere of a system of rice intensification (SRI), possess multiple mechanisms of actions including antibiosis, production of cell wall degrading enzymes and plant growth-promoting hormones. These broad spectrum bacteria offer an effective strategy in controlling multiple pathogens and insect pests of rice (Gopalakrishnan et al. 2012).

4.3 Biomass Production of the Beneficial **Bacillus** *spp.*

For commercial purpose, biomass of beneficial *Bacillus* spp. must be produced on the industry scale. Scientific research with respect to the industrial fermentation technology has been extensively undertaken in Thailand, supporting the operation and contributing to the success of the private entrepreneur in agriculture, energy, food, and pharmaceutical sectors (Valyasevi and Rolle 2002). Industrial by-products, such as sugarcane molasses and corn steep liquor which are abundant in Thailand, have been demonstrated to support the growth of *B. megaterium* and the accumulation of polyhydroxybutyrate (PHB), a highly crystalline thermoplastic polymer (Chaijamrus and Udpuay 2008). Recently, rice straw has been demonstrated to be the best substrate for the production of chitinase by *B. thuringiensis* R 176, an ideal candidate for biological control of rice pathogens, under solid-state fermentation (Chaiharn et al. 2012). These low cost substrates from agriculture and industry could underpin the success of large-scale production of beneficial *Bacillus* spp. intended for use in rice cultivation, reducing the cost of investment for the private entrepreneur to commercialize the biological products.

Nevertheless, size of the market and demand from the consumers determine the amount of investment and the degree of advanced technological know-how adopted by the private sector. In this context, production and commercialization of the products of beneficial *Bacillus* spp., which aim for use in the agricultural sector, do not receive enough attention for investment from the private company although the support for a technological know-how to produce the products is available (National Center for Genetic Engineering and Biotechnology 2009).This scenario dictates the current state of the scale of operation (for instance, volume of production, degree of advanced technological know-how used, and amount of investment used for marketing) which in turn affects the state of research and development with respect to biomass production of the beneficial *Bacillus* spp. for use in rice.

Fermentation facility to produce *Bacillus*-based feed additives for animal industry has been set up in 2009 under the joint venture between private entrepreneur, researchers, and research institute (National Center for Genetic Engineering and Biotechnology 2009). Such facility can produce beneficial *Bacillus* spp. for use in rice cultivation with minor modification if there is enough demand for the *Bacillus*-based products for promoting rice plant growth and yield and controlling rice pests. Industrial by-product from the factory producing monosodium glutamate (MSG) has been utilized to produce the biomass of *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* (Dharmsthiti et al. 1985). This industrial by-product should be tested and utilized to produce other beneficial *Bacillus* spp. which aims to use in rice cultivation.

Effective microorganisms (EM), which is a mixed culture of beneficial microbes presumably including *Bacillus* spp., have been introduced to the farmers for use to increase crop productivity. Bokashi, which is an organic substrate fermented with EM, has been recommended to the rice farmers in the Asia-Pacific region (EM Application Manual for Apnan Countries 1995). EM has been recommended for use in soaking rice seeds before planting and applying to rice field after planting. Bokashi amended with EM has also been recommended to use as supplementary fertilizer to rice field during rice cultivation. The preparations of Bokashi have been modified based upon the availability of both EM inoculums and organic substrates (such as rice straw). Simplicity for its preparation and ease for its use in rice are the main positive aspects of EM and Bokashi.

4.4 Formulation and Delivery

Formulation is essential for commercialization of the biological products because it is necessary to stabilize the microorganisms during production, distribution, and storage and facilitate in the handling and application of the product. Formulation also protects the biological entity from harmful environmental factors and enhances the activity of the microorganisms (Slininger et al. 2003; Schisler et al. 2004; Leggett et al. 2011). Leggett et al. (2011) state that one of the key components to the commercial success of a biological control agent for plant pathogens is formulation.

In Thailand, technological know-how to formulate the biological products has been available in the food and pharmaceutical industries (Thailand's Biotechnology 2013). Nevertheless, its application for producing the products for use in agriculture, particularly in pest control, and for local use has received little attention. Spray dry products of *B. subtilis* have been developed and produced in the laboratory scale for use in research to control rice diseases (Charigkapakorn et al. 1992, 1994). These works can be cited as the first attempt to utilize formulation technology to produce the products with bacterium as an active ingredient for controlling diseases in rice in Thailand. Spray dry products may have short shelf life, particularly when

vegetative cells of the bacterium have been subjected to high temperature regime during production.

Pseudomonas sp., a gram negative and nonspore-forming bacterium, has been formulated by wet granulation technique, a method requiring moderate temperature regime (at 35–40 °C for drying the product) (Fig. 2a). However, the viability of the bacterial cells still declines drastically after 3 months of storage (Kanjanamaneesathian et al. 1998). With *B. pumilus* and *B. megaterium* as an active ingredient, the finishing end products of these formulations produced by the wet granulation technique differ in particle size (Fig. 2b, c). Particle of the formulation containing *B. megaterium* isolate No. 16 does not form aggregate (Fig. 2b), while that of the formulation containing *B. pumilus* isolate No. 36b does coalesce (Fig. 2c) forming large aggregate and entrapping the bacterium (Kanjanamaneesathian et al. 2000). This undesirable physical characteristic may reduce the efficacy of the formulation containing *B. pumilus* isolate No. 36b in suppressing sheath blight disease in the greenhouse (Pengnoo et al. 2000). Since 1998, some improved formulations have been developed for controlling rice diseases in Thailand using *Bacillus* spp. as an active ingredient (Table 4). *B. megaterium* (Fig. 2d), an endospore-forming bacterium, is resistant to high temperature and other environmental fluctuations, making them an ideal candidate for industrial-scale production. The demand for the use of biological products may thus be a major bottleneck, hindering—if not preventing—the possible commercialization of the products.

The research in formulating the biomass of *Bacillus* spp. summarized in Table 4 involves the production of the cells of *Bacillus* spp. in flasks or small (up to 30 L capacity) fermentation facility in the laboratory. This biomass has been subsequently formulated using various techniques such as spray dry, mixing-extrusion and encapsulation. These formulation techniques, in conjunction with the use of dormant structure such as endospores of the bacterium as an ingredient, provide the end products with a long shelf life (more than 2 years) (Wiwattanapatapee et al. 2004, 2007). The simple liquid formulation, on the other hand, may have short shelf life and requires immediate use (Arunyanart et al. 2001). The variety of intrinsic characteristics of each formulation should provide an option for further development and use. The formulations produced with pharmaceutical techniques may possess appropriate characteristics which are suitable for commercialization (Leggett et al. 2011). Those which are produced with simple techniques may be transferred to both local agricultural staffs and rice farmers so that they can produce the formulation and use it for rice cultivation.

Most of the formulation research aims for controlling some major rice diseases (such as sheath blight and dirty panicle), overlooking other economically important rice diseases such as rice blast and brown spot and insect pests in rice. Research in formulating *Bacillus* spp., such as *B. cereus* with growth promoting capacity in rice, should also receive more attention (Jetiyanon and Plianbangchang 2010) as the formulation and delivery of this growth enhancement in rice should reduce the use of chemical fertilizers. Nevertheless, most of the formulation research ends up at the greenhouse and field trials (either at the experiment research stations or at the small rice field of the farmers), requiring the need to overcome the hurdle of

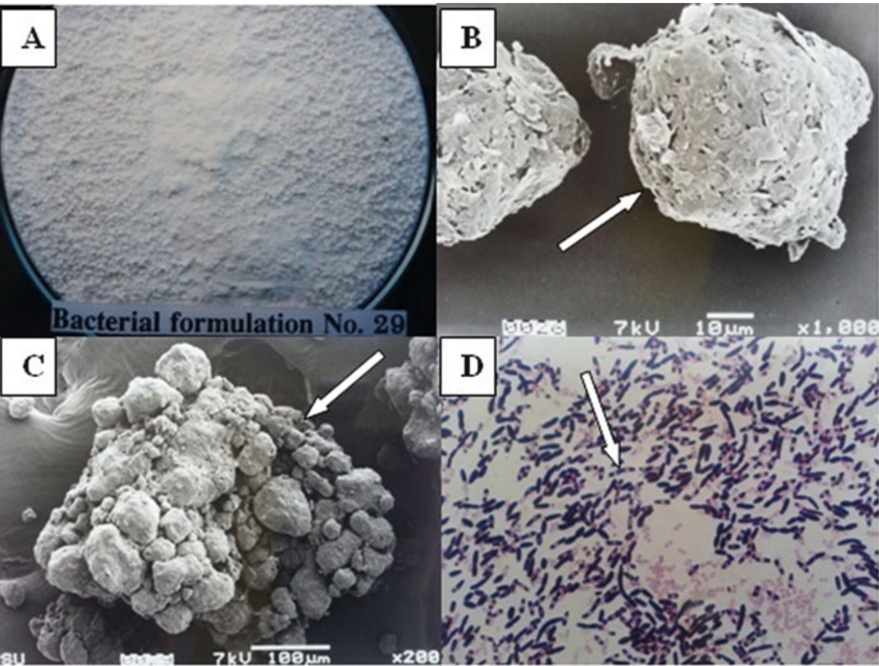

Fig. 2 (**a**) Formulation of *Pseudomonas* sp. isolate no. 29 produced by the wet granulation method. The formulation composes of suspension of *Pseudomonas* sp. isolate no. 29, hydrogenated vegetable oil, monohydrate lactose, polyvinyl pyrrolidone K 30 and crosslinked sodium carboxymethyl cellulose. (**b**) Scanning electron microscope (SEM) image of an individual small particle (*arrow*) of the formulation of *Bacillus megaterium* isolate no. 16. (**c**) SEM image of an aggregate of particle (*arrow*) of the formulation of *Bacillus pumilus* isolate no. 36b. (**d**) Endospore-forming bacterium *B. megaterium* isolate no. 16 (*arrow*) (×1000 magnification) which has been used in the later formulations, replacing nonspore-forming bacterium *Pseudomonas* sp. *B. megaterium* isolate no. 16 produces heat-stable toxin which is inhibitory to the mycelial growth of *Rhizoctonia solani*, a causal agent of rice sheath blight disease. Endospores of *B. megaterium* provide comparatively longer shelf life of the developed formulations (Kanjanamaneesathian et al. 1998, 2000; Pengnoo et al. 2000)

transferring the research to the private entrepreneur should more use and application of the biological products by the rice farmers are intended (Table 4). Alternatively, if the size of market for the biological products is considered as too small to warrant commercialization, an ad hoc collaboration between local agricultural staffs, rice farmers and the researchers should be established to provide a platform for the possible dissemination of the use of the biological products.

The arrival of nanoscience and nanotechnology will, however, move the formulation technology to another level, shifting the paradigm of objectives from formulating the products, which are easy to apply and have prolonged shelf life, to increasing their efficacy. Both chemical pesticides and plant-derived natural products have been formulated using nanotechnology and have been demonstrated to possess increased efficacy against various pests, including rice pest (Rai and Ingle

Table 4 Key formulations based upon *Bacillus* spp. developed mainly for controlling rice diseases since 1998 in Thailand

Types of formulation/ mode of delivery	Target	Stage of development	Source of research fund/reference
Pellet formulation/broadcast	Sheath blight	Greenhouse and preliminary field trials	National Research Council of Thailand (NRCT)/Pengnoo et al. (2000)
Liquid formulations/spray	Sheath blight	Greenhouse trials	National Center for Genetic Engineering and Biotechnology (BIOTEC)/ Wiwattanapatapee et al. (2007); Chumthong (2009)
Pellet formulation/broadcast or spray	Sheath blight	Greenhouse/field trials/introduced for farmer use	NRCT/Wiwattanapatapee et al. (2004); Kanjanamaneesathian et al. (2007, 2009); Chumthong et al. (2008)
Effervescent tablet/broadcast or spray	Sheath blight	Greenhouse trials	National Center for Genetic Engineering and Biotechnology (BIOTEC)/ Wiwattanapatapee et al. (2007); Chumthong (2009)
Powder formulation/spray	Dirty panicle	Preliminary field trials	Rice Department/Arunyanart et al. (2008)
Alginate microcapsule/spray	Sheath blight	Greenhouse trials	Agricultural Research Development Agency (ARDA)/Wiwattanapatapee et al. (2013a)

2012). Microorganisms, such as *Bacillus* spp., may also be used as a source for producing nanoparticles for controlling diseases in rice (Khan and Rizvi 2014). The nanoscience should be applied to develop the formulation of live-cell of the bacteria or cell-derived biological products which are applicable in rice cultivation. Nevertheless, the safety issue still remains very important as the nanoscale formulations may pose a hazard to health and needs serious attention during the product development (Schuler 2004).

4.5 Safety Issue

Safety issue should also be addressed when biological control agents have been considered to be used for controlling plant pests in rice. Numerous *Bacillus* spp. such as *B. cereus*, which have been reported to be an effective growth promotion in rice (Jetiyanon and Plianbangchang 2010), are reported to be possible opportunistic pathogens in human and animals (Logan 1988). *B. cereus* has been reported to cause food poisoning and its toxin has been associated with the diarrheal syndrome (Schoeni and Wong 2005; Kim et al. 2010). Recently, *B. cereus* has been reported to be present in the ready-to-eat cooked rice in Malaysia (Sandra et al. 2012). An efficient technique to detect the pathogenic strains of *Bacillus* spp., which possess

beneficial traits for use in rice production, is thus needed to eliminate the hazard of using health-threatening strains.

5 Research to Real Use: an Ad Hoc Approach

To deal with the complexity of production in agriculture, a clear objective with respect to research outcome is required collectively among farmers, agricultural staffs, and researchers if the outcome is anticipated to be utilized by the farmers. Farmers as the end users and a legitimate stakeholder of research outcome are often exempted from participating in formulating research project, leaving the question of how outcome of the research should be delivered to them only after the research has ended. To complicate the issue, the funding agencies, even with the prospect of either transferring the outcome to the private sector for possible commercialization or disseminating the know-how directly to the farmers, may focus on research outcome in terms of academic output, such as report and publication. This leaves a gap between those involved in funding and executing research and those involved in investing and using research in the field.

Participatory approach, with continuous communication through dialog and concrete activity among farmers, local agricultural staffs and research team, is obligatory in order to reduce this gap, thence facilitating the transfer of research into application and practice (Garforth 1993). In Uttaradit province, Thailand, the reception of research may have minimal barrier as some rice farmers are aware of the danger and negative effects of chemical pesticides, contributing to their willingness to replace chemical pesticides with environmental friendly biological products for cultivating rice. Local agricultural staffs tasked with extension service play a crucial role in acting as a liaison between the rice farmers and the research team.

Nonetheless, farmers would consider accepting the biological product and recommending it to other farmers when the efficacy of the products containing *B. megaterium* in controlling rice diseases in the repeated field trials has been demonstrated (Kanjanamaneesathian et al. 2009). They are ready to adopt the biological product for long-term use if the application of biological product not only contributes to promoting rice growth, but also having no other side effects as well (Kanjanamaneesathian et al. 2009). The proven efficacy of the product in the rice field will be a platform in which the local agricultural staffs will be used to disseminate the practice of using the biological products to other rice farmers (Table 2). The incentive for using the safe biological products in rice cultivation is that the farmers will get higher price for their produce.

To ensure that the rice farmers, who have abandoned using chemical pesticides in rice cultivation, will continue using the biological product, it is necessary to make the products available to them. To achieve this goal, it can be envisioned that rice farmers should form a so-called “rice production cooperatives”, a group of farmers who has long-term commitment in producing rice without chemical

pesticide input, necessitating the application of alternative safer biological product and requiring allocation of the group resource for setting up facility to produce the formulation for the group. Researchers and other experts from various agencies (listed in Table 3) can give advice on the degree of sophistication of the laboratory and the formulation facility which should be put in place for such purpose. The project has been proposed to set up the laboratory in an agricultural office, Uttaradit province, Thailand to produce the simple but applicable formulations for use in the rice fields in Uttaradit and adjacent provinces (Wiwattanapatapee et al. 2013b).

6 Perspectives and Conclusion

In Thailand, some rice farmers have turned away from using conventional approach, which depends upon chemical input, to adopting more environmental friendly approach such as organic farming of rice (Pornpratansombat et al. 2011). Organic rice farming has received more attention as a result of the inauguration of Thailand's National Agenda on Organic Agriculture in 2005 (USDA 2006). Agriculture Certification Thailand (ACT), a nonprofit foundation under the umbrella of International Federation of Organic Agriculture Movements (IFOAM), provides basic organic standards for producing organic products and offers organic certification services in Thailand (Organic Agriculture Certification Thailand 2012). Researchers working with *Bacillus* spp. should explore the way in which this bacterium should be utilized effectively in cultivating rice in compliance with this organic standard. *Bacillus* sp., isolated from the tissue of rice grown organically, has been reported to be a good producer of indole acetic acid (IAA), a plant growth hormone (Phetcharat and Duangpaeng 2012).

Private entrepreneur also plays a crucial role in both investing for the production of the biological products and commercializing them for use in the conventional production where the use of biological products are not as strictly controlled as those used in organic farming. This would happen if the government has a clear and proactive role, streamlining the registration process which is required to undertake by the private entrepreneur prior to commercializing the products. The efficient process would put more products to the market, making them readily available on the shelf and giving the rice farmers a safer product to use instead of chemical pesticides. With a comparatively stringent requirement for the importation, registration, marketing, and acquisition of chemical pesticides, environmental friendly biological products should be in a position to compete with chemical products, shifting the paradigm of reliance on hazardous chemical agents to environmental friendly products in rice cultivation. This scenario should become a reality only if the government has a clear policy in promoting the use of safe biological products for rice production.

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Microbial Surfactant for Preservation of Natural Rubber Latex

Saad Wan Zuhainis, Aziana Abu Hassan, Manroshan Singh, and Rosfarizan Mohamad

Abstract Surfactant is referred to as a surface active substance due to its ability to modify the surface properties between two immiscible liquids. In the preservation of natural rubber latex (NRL), surfactant assists in enhancing the NRL stability by maintaining the electrostatic repulsion on the surface of the rubber particles and therefore prevents flocculation of rubber molecules. Currently, chemical-based surfactants are widely being used. From an environmental view point, the approach on searching for microbial surfactant was due to the current focus on generating biological processing system. In this chapter, the vital role of surfactant for NRL preservation is discussed. Furthermore, an investigation on searching for potential microorganisms that have an advantage in excreting surfactant-like substances that could be used as surface active agent to preserve the NRL stability is also highlighted.

1 Introduction

Natural rubber latex (NRL) is derived from latex ducts which are in a layer outside the cambium of rubber trees (*Hevea brasiliensis*) (John 1982). When the tree bark is tapped, a milky fluid which comprises 30–40 % of rubber hydrocarbon particles with a few percent of nonrubber particles such as proteins, lipids, carbohydrates,

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and sugars is immediately flowed through the tapping panel on the tree bark and into the collecting cup (Angrove 1964). The remaining major component of NRL is water. NRL is contaminated with microorganisms after being tapped (Taysum 1960). The main sources of microbial contamination are derived from the tree lace, tapping panel bark, and the cup which receives the latex (Soeseno and Mansjoer 1975). Destabilization of NRL that occurs from a build-up of volatile fatty acids (VFA; primarily acetic, formic, and propionic acids) due to microbial metabolic activities consuming the nonrubber phase caused latex coagulation (Juntarachat et al. 2013). During latex coagulation, the negatively charged protein membranes that surround many rubber molecules were neutralized by oxidation reaction due to the presence of microorganisms and cause the rubber particles to repel each other, and therefore, flocculation of latex occurred (Booten et al. 2011).

Surfactant is referred to as a surface active substance due to its ability to modify the surface properties between two immiscible liquids. The surfactant molecule consists of a hydrophilic component (head) and a hydrophobic component (groups and chains) which make up the lipophilic tail (Christofi and Ivshina 2005). In general, the presence of a surfactant helps to increase the solubility of a solution, lower surface tension between liquid/liquid, liquid/solid, and liquid/vapor interfaces, enhances wet ability of a solution, and increases foaming capacity. The presence of a surfactant also enhances emulsion formation and solubilization between two liquids such as oil and water which subsequently increases the concentration of oil in the water phase (Christofi and Ivshina 2002). Although chemical-based surfactants are widely being used, in the recent years a new class of surfactant referred to as biosurfactants have emerged. These biosurfactants are the products of microorganisms mainly from bacteria, yeast, and fungi. As surfactant, biosurfactant is an amphipathic compound that exhibits distinct surface activity like other chemically synthesized surfactants (Eddouaouda et al. 2012). Furthermore, it also exhibits a broad diversity of chemical structures such as glycolipids, lipopeptides, lipoproteins, lipopolysaccharides, phospholipids, fatty acids, and polymeric lipids (Maier and Soberon-Chavez 2000).

The use of surfactants is so diverse that it is also used in the stabilization of NRL. However, unlike the synthetic lattices such as polyurethane and acrylonitrile butadiene latex which require the addition of man-made surfactants, NRL which is biologically produced in the *Hevea brasiliensis* tree already contains biologically produced surface active materials made of phospholipid–protein complexes distributed on the surface of the rubber particles which enhance the colloidal stability of NRL (Sansatsadeekul et al. 2011). However, due to their biological nature, the phospholipids are usually destroyed by bacteria from the environment upon tapping the rubber tree bark (Manroshan et al. 2007). Therefore, in order to replace the lost phospholipid layer on the rubber particles, synthetic surfactants are added. Adsorption of these surfactants onto the surface of the rubber particles helps to increase electrostatic repulsion between rubber particles and therefore prevents further flocculation of the rubber particles.

Although there is much literature on the use of synthetic surfactants with NRL, there is, however, not much work on the use of biosurfactants, especially the ones

related to products from microorganisms isolated from the NR environment. Therefore, herein we will discuss on the preparation of microbial extracts from the bacteria and fungi isolated from the NR environment and screen them for their surfactant activity. The surfactant activity of the microbial extracts was determined by measuring the emulsification capability and the critical micelle concentration (CMC). The effectiveness of the microbial extracts in NRL as surface active agent was carried out based on the enumeration of bacterial population, measurement of VFA number, and viscosity measurements which were performed to evaluate the stability of the latex.

2 Natural Rubber Latex

NRL is a stable dispersion of cis-1,4-polyisoprene rubber encapsulated in spherical particles with an average size of 0.15–3 μm in aqueous phase (Kroschwitz 1990). The dispersed phase of NRL is mainly rubber hydrocarbon and the dispersion medium is water. Freshly tapped NRL as obtained from the tree is a whitish fluid of density 0.975–0.980 g/ml and with a pH from 6.0 to 7.0 (Blomfield 1951). Being a natural product, its composition varies between wide limits. Typically, NRL consists of 36 % of total solid content (including dry rubber content of 33 %), proteinaceous substances about 1 %, resinous substances 1–2.5 %, ash and sugar 1 %, and 60 % of water content (Blackley 1966).

These compositions are distributed between three principal phases, rubber particles (35 % of latex by weight), the aqueous phase (55 %), and the remaining lutoid phase (10 %) (Angove 1964). There are also many minor phases present. The aqueous nonrubber phase is made up of carbohydrates (quebrachitol, sucrose, glucose, fructose, and raffinose), proteins and amino acids (α-globulin and hevein) and other serum constituents (free nitrogenous bases, lipids, organic acids, inorganic anions, and metallic ions), and a range of enzymes (Hasma and Subramaniam 1986). The inorganic ions in fresh latex are about 0.5 %. The principal substrates for VFA formation are carbohydrates, a glucose–amino acid complex, alanine, glutamic acid, and organic acids (citric and malic acids) (Zyska 1981). Of the protein content, about 60 % is dissolved in the aqueous phase, approximately 20 % is adsorbed on the surface of rubber particles and the remaining 20 % is associated with the larger particulate bodies such as lutoids Frey-Wyssling particles (Archer et al. 1966).

According to Tata (1980), fresh NR contains 0.95 % proteins, of which 27.2 % is in the rubber fraction, 47.5 % in the serum fraction, and 25.3 % on the bottom fraction (Fig. 1). The rubber in NR is cis-1,4-polyisoprene with a molecular weight ("sol" fraction) of 1×10^7 (Gazeley et al. 1988). It has a double bond for every 68 units of molecular weight and in the molecule the isoprene unit can be repeated 20,000 times (Brydson 1978). The molecular weight cannot be determined precisely because there is always a proportion of the rubber (gel) which is insoluble.

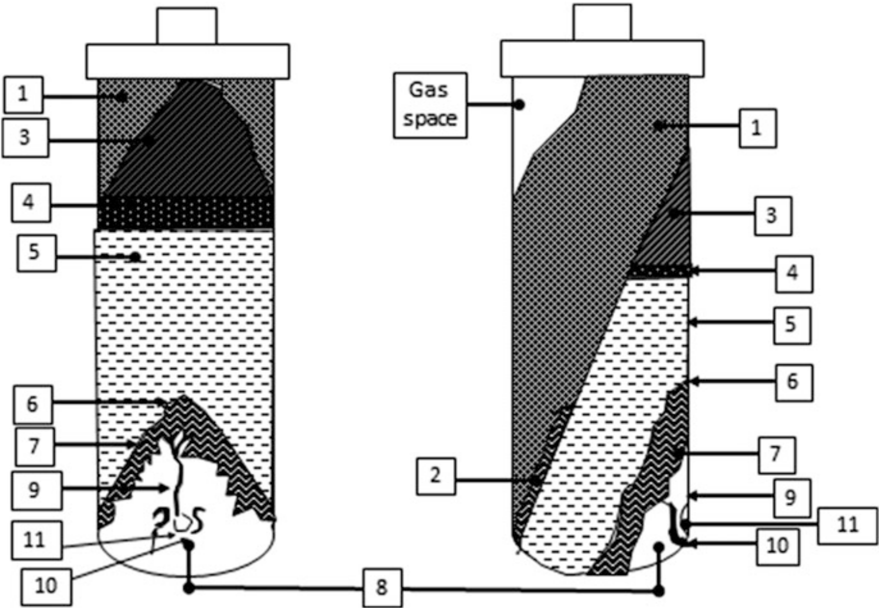

Fig. 1 Separation of fresh NRL by ultracentrifugation (21,880 × g in 40 min). Fraction 1–3: white rubber phase; fraction 4: yellow orange layer consists of Frey-Wyssling particles; fraction 5: C-serum; and fraction 6–11: bottom fraction (source: Moir 1959)

Latex collected from the rubber tree is processed in two different forms, either as concentrated latex or dry rubbers. These two separate processes produce raw materials for various kinds of NR-based products (Yip and Cacioli 2002). Latex concentrate is produced via one of these three methods: centrifugation, creaming with sodium alginate, and evaporation (Gazeley et al. 1988). But more than 90 % of commercial latex concentrates are produced by centrifugation method (Afreen et al. 2013). Nonrubber substances are denser than rubber particles therefore, the centrifugal force will speed up separation process, by removing the nonrubber particles, dissolved substances in serum, and excess water as skim latex (Blackley 1997). Concentrated latex contains almost 60 % of dry rubber content.

NRL collected from the field is preserved with ammonia to maintain the latex stability during shipping. Ammonia also added at factory during bulking and processing (John 1976). Concentrated latex is either preserved with low percentage of ammonia coupled with secondary preservative or solely preserved with high percentage of ammoniated (Wong et al. 1986). Concentrated latex is the starting materials for NRL products produced by dipping (glove, condoms) or extrusion (latex thread) (Yip and Cacioli 2002). Meanwhile, dry rubber is obtained after the latex was coagulated by assisted biological coagulation by microbes or chemically using formic acid. Dry rubber is the raw material for the production of tyres, tubings, hoses, footwear, and automotive components (Bhowmick et al. 1994).

3 Microorganisms in Natural Rubber Environment

Microorganisms coexist in almost all natural environments, particularly in soils, water, and sewage. The variety of nonrubber substrates in NRL provides an excellent growth medium for the diversity of microbial species encountered. The main sources of contamination are derived from the tree lace, tapping panel bark, and the cup which receives the latex. A floristic list had been published by Taysum (1958) and John (1977) as tabulated in Table 1. According to Taysum (1958), higher initial microbial population in latex will lead to lower maximum population, suggesting that growth of bacteria may be inhibited by the toxic products of previous metabolism (waste accumulation) or even other limitation, for example, oxygen supply, rather than by the limited amount of substrates.

The bacterial population in fresh latex is always large in any routine collection and many early workers were familiar with microorganisms in latex and in rubber products. A wide variety, mostly strong acid producers are present in field latex. They represent species of *Bacillus*, *Bacterium*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Streptococcus*, *Serratia*, *Sarcina*, *Klebsiella*, *Listeria*, *Azotobacter*, *Proteus*, and *Pseudomonas* (John 1977, 1982). Taysum (1958) used the modified Kliger's iron agar media to record the presence of more than 100 species of bacteria that belong to the families of Nitrobacteriaceae, Pseudomonadaceae, Azotobacteriaceae, Micrococcaceae, Lactobacteriaceae, Corynebacteriaceae, Achromobacteriaceae, Enterobacteriaceae, Salmonella and Shigella, Bacteriaceae, Bacillaceae, Actinomycetaceae, Streptomycetaceae, and Mycobacteriaceae. The Enterobacteriaceae such as *Escherichia*, *Erwinia*, *Serratia*, and *Proteus* predominates early in fresh latex and can survive under 0.3 % ammonia. The Lactobacteriaceae such as *Streptococcus*, *Lactobacilli*, *Microbacterium*, and *Propionibacterium* are strong acid producers and can survive ammoniation up to 0.8 %. The Micrococcaceae have preference for high pH conditions and is widespread in ammoniated latex (Taysum 1958).

Fungi in other aspect did not give any effect toward NRL due to insignificant amount of fungi population in latex. According to Gazis and Chaverri (2010), 175 isolates of endophytic fungi were successfully isolated from foliage, leaves, and sapwood from natural rubber tree that have the potential to be used as biological control agents. Another finding claimed that fungi are commonly found in NRL serum waste from rubber processing factory (Atagana et al. 1999). Serum waste produced after coagulation of NRL was found to be a suitable source of growth for fungi. Most of the fungi were capable of utilizing proteinaceous compounds, pseudo-sugars, and other nonrubber substances present in the serum waste.

Table 1 Part of the 1000 strains of bacteria isolated from *Hevea* latex systems

Family	Species
Family Nitrobacteriaceae	*Nitrosomonas monocella* *Nitrosococcus nitrous*
Family Pseudomonadaceae	*Pseudomonas fluorescens* *Acetobacter aceti* *Acetobacter suboxydans* *Vibro* sp. *Pseudomonas* sp. *Desulfovibrio desulfuricans* *Spirillum serpens*
Family Azotobacteriaceae	*Azotobacter chroococcum*
Family Micrococcaceae	*Micrococcus luteus* *Micrococcus flavus* *Micrococcus candidus* *Micrococcus pyogenes var. aureus* *Micrococcus citreus* *Micrococcus aurantiacus* *Micrococcus cinnabareus* *Micrococcus roseus* *Sarcina lutea* *Sarcina aurantiaca*
Family Lactobacteriaceae	*Streptococcus pyogenes* *Streptococcus agalactiae* *Streptococcus faecalis* *Lactobacillus lanceolatus* *Lactobacillus casei* *Microbacterium lacticum* *Propionibacterium shermanii*
Family Corynebacteriaceae	*Corynebacterium michiganense*
Family Enterobacteriaceae	*Escherichia coli* *Aerobacter aerogenes* Intermediate Type I *Coliform* *Serratia marcescens* *Proteus vulgaris*
Family Bacteriaceae	*Bacterium fulvum* *Bacterium zenkeri*
Family Bacillaceae	*Bacillus subtilis* *Bacillus megaterium* *Bacillus cereus* var. *mycoides* *Clostridium perfringens*
Family Actinomycetaceae	*Nicordia flava* *Nicordia salmonicolor*
Family Streptomycetaceae	*Streptomyces albus* *Streptomyces coelicolor*

Source: Taysum (1958); John (1977)

4 Theory on Natural Rubber Latex Destabilization

There are several postulations on the theory of latex coagulation. The most prominent are bacterial and enzymatic theories (Altman 1947; John et al. 1986; Chaikumpollert et al. 2007; Booten et al. 2011). In bacterial theory, acids which were produced by bacterial action were the direct cause of natural coagulation. NRL is naturally contaminated with microorganisms after being tapped (Taysum 1960). The destabilization of NRL occurs from a buildup of VFA (primarily acetic, formic, and propionic acids) due to microbial metabolic activities consuming the rich substrates that constitute the nonrubber phase (Taysum 1958; John 1968; Booten et al. 2011). Rubber particle is made up of a protein membrane that surrounds many rubber molecules. The negatively charged protein membranes prevent the rubber particles from repelling each other and therefore latex remains stable (Ho et al. 1996). However, due to the presence of microorganisms, negative charges of rubber particles were neutralizing by oxidation reaction (Vivayganathan et al. 1998). Partial completion of sugar breakdown by bacteria caused the latex pH to decrease by producing acidic by-product and therefore made the latex more favorable for bacterial growth (Jayachandran and Chandrasekaran 1998). Acid which consists of H^+ ion will neutralize the negatively charged protein membrane and cause the protein membrane of the rubber particles to break (Rippel and Galembeck 2009). Rubber particles will colloid with each other and clump together to form coagulum.

Enzymatic theory of NRL coagulation is mediated by the activation of certain enzymes by the presence of bacteria. Acids that were produced by the bacteria had activating influence on the enzyme indirectly. According to Gidrol et al. (1994), hevein a lectin-like protein from NR tree was involved in the coagulation of latex by interacting with the rubber particles under suitable physiological conditions. Coagulation of latex has been correlated with the rubber tree self-defensing systems (Broekaert et al. 1990). Coagulation of latex that occurs in the rubber tree is important for plant defense against possible pathogenic invasion. The coagulated latex will block the casual wound or those created during the frequent tapping of trees for latex. This autogenerated coagulated latex is believed to be mediated by hevein which is compartmentalized in lutoids (Wititsuwannakul et al. 2008).

According to Karunanayake and Perera (2006), the presence of Mg^{2+} ions is another major factor that causes latex destabilization. Removal of carboxylate ions from the protective layer of the rubber particles that caused by direct interaction between Mg^{2+} and the carboxylate ions produce insoluble, and un-ionized magnesium soaps which latter may precipitate in the aqueous phase (Blackley 1966). These actions may decrease the stability of colloidal dispersion and lead to the destabilization of latex.

Based on the theories, the major determining factor of latex coagulation is charge neutralization. Cation proteins from the lutoid were liberated when the lutoids in the rubber latex burst upon wounding and later the cation protein will interact with the negatively charged rubber particles (Southorn and Yip 1968).

Rubber particle is made up of a negatively charged protein membrane that surrounds many rubber molecules. The protein membrane prevents the rubber particles from repelling each other by having an electrostatic repulsion (Booten et al. 2011). Reduction in electrostatic repulsion between rubber particles resulted in latex destabilization. Hydrolysis, charge neutralization, and decrease of latex pH can reduce the electrostatic repulsion between these particles (Southorn 1960; Hasma and Subramaniam 1986; Manroshan et al. 2008).

5 Preservation of Natural Rubber Latex

Latex preservation refers to the ability of the rubber particles to avoid from repelling each other by chemical or physical means and therefore latex remains stable in liquid form (McGavack 1959). According to Cook (1960), preservation of latex can be divided into two types based on the storage time. Long-term latex preservation is a preservation system that permits the shipment and storage of liquid latex until being processed by the consuming countries. The second type of latex preservation is the short-term preservation that maintains the NRL in a liquid condition for a few hours or days before being processed. Short-term latex preservation is particularly being practiced in dry rubber production. Anticoagulant agents are practically used term for short-term latex preservative agent.

Latex preservation systems were widely being studied since the early years of latex emerging in the commodity industry. In general, the ideal preservation system must have the following criteria:

- Free of *N*-nitrosamines
- Works alone without ammonia
- Soluble in water
- Suitable to be used both for latex concentrate and block rubber/sheet production
- Able to preserve latex concentrates for at least 1 year and field latex for dry rubber production for 1–2 weeks with satisfactory properties
- Low toxicity
- Locally available

The main function of a preservative system is to arrest microbial growth for a period of time and yet obtain rubber end products with acceptable properties. In commercial industry, preservative agents are used to maintain the viscosity of concentrated latex liquid for storage purposes (Yip and Cacioli 2002).

The oldest known preservative for NRL is ammonia. It became the most favorable anticoagulant mainly due to its alkalinity and biocidal characteristic. It is established that one of the crucial criteria for an effective preservation agent is the ability to keep the VFA number of NRL at low levels while maintaining the stability of latex on storage (Taysum 1958). Ammonia inhibits bacterial growth and therefore it can control the VFA number level (Fig. 2). It also acts as an ion stabilizer in NRL by forming complexes with metal ions, especially Mg^{2+} and

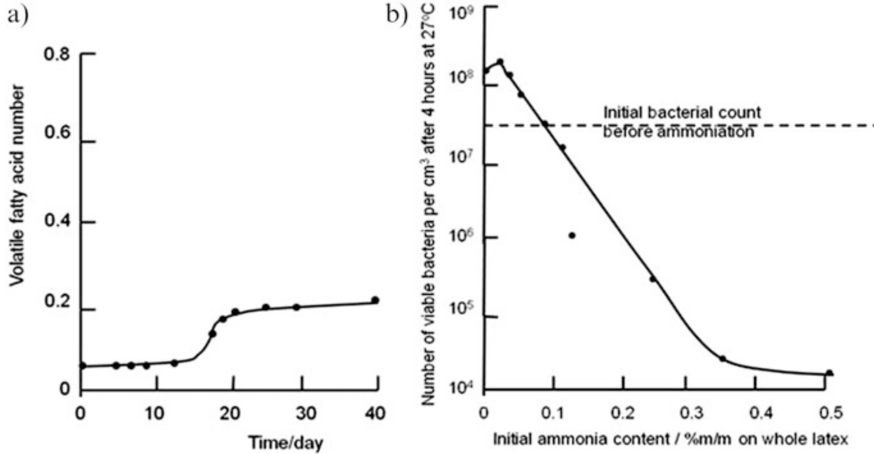

Fig. 2 Development of VFA anions (**a**) and number of viable bacteria (**b**) in ammonia preserved NRL (Lowe 1961)

Table 2 Secondary preservative systems used in natural rubber latex

Designation	Abbreviation	Preservative system (% by weight)
High or full ammonia	HA	0.7 % ammonia
Low ammonia TZ	LA-TZ	0.2 % ammonia, 0.025 % zinc oxide
		0.025 % tetramethylthiuram disulphide
Low ammonia pentachlorophenate	LA-SPP	0.2 % ammonia, 0.2 % sodium pentachlorophenate
Low ammonia boric acid	LA-BA	0.2 % ammonia, 0.24 % boric acid

Source: Gazeley et al. (1988)

helps to deposit the destabilizing magnesium from the protective layer of the rubber particles (Cook 1960). Furthermore, ammonia is easily available for the intended application.

Although ammonia seems harmless to humans, exposure of high concentrations for prolonged times could trigger burn and inflammation of the respiratory tract system and skin (Agency for Toxic Substances and Disease Registry 2011). Similarly, ammonia also affects the processing of natural rubber. Apart from the smell, ammonia has the tendency to slightly discolor latex products (Perera et al. 1981). The cost of producing dry rubber products from NRL also increases due to the higher amounts of acid used to coagulate the latex and in handling the resulting acidic waste (Vivayganathan et al. 2008). Due to the drawbacks of ammonia, researchers have developed new preservative systems dating back to 1950s. However, the presence of ammonia was still needed although in low amounts. These low ammonia (LA) systems are summarized in Table 2. Similar to ammonia, these systems also have their drawbacks. Vivayganathan et al. (2008) reported that the LA-TZ system caused discoloration in latex-based products. Rudzki and Rebandal

(1998) reported that the residual TMTD in latex products from the LA-TZ system caused Type IV contact dermatitis. Another study by Rama Rao et al. (1976) showed that ZnO is the major industrial pollutant in waste water and sludge although ZnO assists in inhibiting the growth of microorganisms and reacts with the enzymes responsible for NRL decomposition.

In NRL industry, surfactant is used as colloidal stabilizer (Manroshan et al. 2007). Production of latex concentrated from NRL preserved with ammonia required the addition of surfactant because higher shear forces were used to separate the NR serum. In the presence of surfactant, electrostatic repulsion between rubber particles increases and therefore prevents flocculation of rubber molecules (Booten et al. 2011). Surfactant is also used for removing proteins that bound to the surfaces of rubber particles by solubilizing particle-bound proteins (Schloman 2002). Removal of proteins from NRL helps to reduce the allergenic cases due to the application of NRL-based products. In general, surfactant helps to enhance the stability of NR during latex stage.

6 Microbial Surfactant

Biosurfactants are mainly synthesized extracellularly or as part of the cell membrane by aerobic microorganisms and a few are produced by anaerobic microorganisms (Mulligan 2005). Biosurfactant, also referred as microbial surfactant, is an amphipathic compound that exhibits indistinguishable surface active properties as other chemically synthesized surfactants (Karanth et al. 1999). Biosurfactants exhibited a broad diversity of chemical structure such as glycolipid, lipopeptides, lipoproteins, lipopolysaccharides, phospholipid, fatty acid, and polymeric lipids (Maier and Soberon-Chavez 2000). Herein, we summarized several types of microbial biosurfactant with the producer in Table 3.

Biologically derived surfactant served as biological compounds that are significantly less toxic and biodegradable (Mulligan et al. 2001). Due to these criteria, biosurfactant has been the most preferable substance to be used in environmental pollution control as bioremediation agent. Biosurfactant helps in removing contaminants from water or soil by solubilization dispersal and desorption of the pollutants from particulates and subsequently returns the cleaned water or soil to their original site (Pacwa-Plociniczak et al. 2011). Biosurfactant also exhibits diverse properties and physiological functions such as controlling the bioavailability of toxicants in soil and other environments, remediating contaminated soil by binding to heavy metal and assists in the solubilization to ease extraction of the contaminants, exhibits bacterial pathogenesis activity and has potential applications in the medical field as antibacterial, antifungal, and antiviral agents (Herman et al. 1997; Banat et al. 2000).

Although microbial surfactants have been applied in various industries, applications of such surfactants in NRL industry yet have not being discuss extensively. According to Sansatsadeekul et al. (2011), the presence of surfactant in NRL could

Table 3 Microbial surfactants and the producers

Type of surfactant	Microorganisms		References
	Bacteria	Fungi/yeast	
Trehalose lipids	*Arthrobacter paraffineus*		Franzetti et al. (2010)
	Corynebacterium spp.		Desai and Banat (1997)
	Mycobacterium spp.		
	Rhodococcus erythropolis		
Rhamnolipids	*Pseudomonas aeruginosa*		Zhang and Miller (1992)
	Pseudomonas spp.		
	Serratia rubidaea		
Sophorose lipids		*Candida apicola*	Rau et al. (1996)
		Candida bombicola	
		Candida lipolytica	
		Candida bogoriensis	
Glycolipids	*Alcanivorax borkumensis*		White et al. (2013)
	Arthrobacter spp.		Arora and Jain (2013)
	Corynebacterium spp.		Thavasi et al. (2011)
	Rhodococcus erythropolis		Abdel-Megeed et al. (2011)
	Serratia marcescens		Anyanwu et al. (2010)
	Tsukamurella spp.		Cortés-Sánchez et al. (2012)
Cellobiose lipids		*Ustilago maydis*	Hewald et al. (2005)
Polyol lipids		*Rhodotorula glutinis*	Mulligan et al. (2001)
		Rhodotorula graminus	
Diglycosyl diglycerides	*Lactobacillus fermentum*		Saharan et al. (2011)
Lipopolysaccharides	*Acinetobacter calcoaceticus*		Pacwa-Płociniczak et al. (2011)
	Pseudomonas spp.		
		Candida lipolytica	
Arthrofactin	*Arthrobacter* spp.		Morikawa et al. (1993)
Lichenysin A	*Bacillus licheniformis*		Yakimov et al. (1995)
Lichenysin B			
Surfactin	*Bacillus subtilis*		Eyéghé-Bickong (2011)
	Bacillus pumilus		
Viscosin	*Pseudomonas fluorescens*		Hildebrand et al. (1998)

(continued)

Table 3 (continued)

Type of surfactant	Microorganisms		References
	Bacteria	Fungi/yeast	
Ornithine, lysine peptides	*Thiobacillus thiooxidans*		Mulligan et al. (2001)
	Streptomyces sioyaensis		
	Gluconobacter cerinus		
Phospholipids	*Acinetobacter* spp.		Rosenberg and Ron (1999)
Sulfonyl lipids	*Tsukamurella thiooxidans*		Bodour and Maier (2002)
	Corynebacterium alkanolyticum		Crosman et al. (2002)
Fatty acid		*Penicillium spiculisporum*	Brown (1991)
	Corynebacterium lepus		
	Arthrobacter paraffineus		
		Talaromyces trachyspermus	
	Nocardia erythropolis		
Alasan	*Acinetobacter radioresistens*		Ron and Rosenberg (2002)
Streptofactin	*Streptomyces tendae*		Richter et al. (1998)
Particulate surfactant (PM)	*Pseudomonas marginalis*		Banat et al. (2000)
Biosur PM	*Pseudomonas maltophilla*		Cameotra and Bollag (2003)

Source: Mulligan (2005)

replace the protein–phospholipid layers surrounding the surface of the rubber particles that have been removed either biologically by microbial activity or physically by enzyme treatment or saponification. In NR field latex, the major causing effect of removal of protein–phospholipid layer might be due to the microorganism's metabolic activities that utilize the latex proteins. Natural rubber particles, enclosed with a monolayer membrane, consist of lipids, proteins, and other nonrubber components that attribute to the satisfied mechanical properties of NR (Intapun et al. 2009). Proteins absorbed on rubber particles by anchoring themselves on the phospholipid layer, contribute to the electrophoretic charges (Gong et al. 2013). Removal of protein–phospholipid complexes reduces the electrostatic repulsion of the rubber particles and eventually, flocculation occurs. Microbial surfactant has a distinguished characteristic; it exhibits same effect as chemically synthesized surfactant (Mulligan 2005). Therefore, presumably, by introducing microbial surfactant in NRL these molecules could arrange themselves on rubber particle surfaces, thus increasing the electrostatic repulsion and colloidal stability of

the NRL. Work on searching for biological surfactants from microorganisms that are suitable for NRL preservation perhaps could provide the industry with an alternative approach of practicing biologically based NRL processing systems.

7 In Search for Microbial Derived Surfactant for NRL Preservation

In the current work, searching for potential microorganisms that have an advantage in excreting surfactant-like substances that could be used as surface active agent to preserve the NRL stability is the main interest. Microorganisms' isolation was obtained from fresh NRL, coagulated NRL, and rubber plantation soil. Sampling was performed in Rubber Research Institute (RRIM), in Sungai Buloh, Malaysia. A total of 28 isolates comprising 20 bacterial isolates and 8 fungi were successfully obtained. However, out of the 28 isolates, only four bacterial and one fungal isolates namely MRB02, MRB12, MRB16, MRB18, and MRB25, respectively, were selected. The experiments involved in the screening for surfactant activity from the microbial isolates are discussed in the following section.

7.1 Preparation of the Microbial Extracts

7.1.1 Preparation of Microbial Extracts from Fungal Isolates

Crude extracts were prepared from isolated fungi using a modified procedure as previously described by (Hazalin et al. 2009). Fungi cultures (10 plates per fungus) were allowed to grow in a Petri dish with growth media (PDA) for 7 days or until the fungal mycelium completely covered the growth media surfaces. The conidial count of fungal isolates was calculated using a hemocytometer counting chamber (Hausser Scientific, Pennsylvania) to be within $1–5 \times 10^8$ spore/ml. The fungal mycelium was removed from the Petri dish by cutting it into smaller pieces and homogenized, before being transferred into a 500 ml capped bottle (Schott Duran®, Germany) filled with 250 ml methanol. The mixture was then left overnight in an incubator shaker at room temperature after which it was separated by filtration through a sieve. The sieved mycelium was soaked again with another 250 ml methanol, whereas filtrate was kept in a chiller at 4 °C. This filtration step was repeated a few times until the resulting filtrates became colorless. All filtrates were collected and filtered again through a Whatman No. 1 filter paper before transferring them to a round bottom flask connected to a rotary evaporator to remove methanol. The resulting extract was then dissolved in distilled water and kept at −40 °C before subjecting it to overnight freeze drying for further drying process. It was dissolved in 1 % dimethyl sulphoxide (DMSO) and kept at 4 °C until further used.

7.1.2 Preparation of Microbial Extracts from Bacterial Isolates

Preparations of extract from bacterial isolates were carried out based on previously described procedure by Bussaman et al. (2012). The bacterial isolates were grown on TSA for 24 h at 35 °C. Single colonies from each isolates were then transferred into 250 ml flasks containing 50 ml of TSB. The isolates were incubated in an incubator shaker, at 35 °C with speed of 130 rpm overnight. The inoculums were then serially diluted to tenfold (10^1–10^7). Standard growth curve for each isolate was obtained by measuring the quantity of bacteria (cfu/ml) in the serially diluted inoculums and the cell optical density (OD) using a UV–Vis Spectrophotometer at 600 nm wavelength. Each of the isolated bacteria was grown in TSB and incubating in an incubator shaker at 35 °C with speed of 130 rpm overnight. The optical density (OD) of the inoculums was adjusted in order to get the required number of bacteria (10^6–10^7 cfu/ml) as calculated based on the standard growth curve. Once the bacterial inoculums were prepared, the bacterial cells in the broth were separated by centrifugation at 2856 × *g* at 4 °C for 15 min. The recovered supernatants were then filtrated using 0.22 μm membrane filters and concentrated with rotary evaporator. It was then stored at 4 °C until further used.

7.2 Screening for Surfactant Activity from the Microbial Extracts

The screening assay was carried out based on the method described by Benincasa and Accorsini (2008) and Bilski et al. (1997). The surfactant activity of the isolates was screened through the formation of emulsion obtained by mixing the microbial extracts (in 0.05 g/l Rose Bengal solution) and mineral oil in a 1:1 ratio and vortexing the mixture. Strong surfactant activity was indicated by the formation of uniform sized oil droplets. On the other hand, oil droplets of large and variable sizes indicated moderate surfactant activity, whereas weak surfactant activity was indicated by low or no oil droplet formation. From 28 isolates, only four bacterial and one fungal isolates namely MRB02, MRB12, MRB16, MRB18, and MRB25, respectively, showed strong surfactant activity. Their designation, source, and microscopic images are shown in Table 4.

The mixture containing these microbial extracts had the emulsifying power resulting in an emulsion that is stable throughout the experimental period of 4 h. It is evident that surfactant helps in lowering the surface tension between two immiscible liquids, e.g., water and oil (Jönsson et al. 2003). In the presence of surfactant molecules, the hydrophobic chains of surfactant were attached to the oil surfaces and eventually increase its solubility in distilled water. Without surfactant, phase separation between water and oil would be impossible even after vigorous shaking. As for microbial extracts from other isolates either the absence or weak surfactant activity was detected.

Table 4 Morphological characteristics of bacteria and fungi that exhibited surfactant activity

Isolate designation	Isolates name	Source	Macroscopic image	Microscopic image ×100 magnification
Bacteria				
MRB02	–	Fresh latex	Yellow colonies	Gram positive, coccus shape
MRB12	*Myroides odoratus*	Coagulated latex	Circle, cream and yellowish colonies	Gram positive, coccus shape
MRB16	*Enterococcus feacalis*	Coagulated latex	Pin point and cream-white colonies	Gram positive, coccus shape
MRB18	*Bacillus pumilus*	Soil	Lobate edge, white/cream smooth surface colonies	Gram negative, rod shape

(continued)

Table 4 (continued)

Isolate designation	Isolates name	Source	Macroscopic image	Microscopic image ×100 magnification
Fungi				
MRB25	*Aspergillus* sp.	Fresh latex	(A) front view (B) back view	Microscopic image of the septate hyphae

7.2.1 CMC of the Microbial Extracts

CMCs of the microbial extract exhibiting surfactant activity from the five isolates were performed as the function of the concentration and its effects in changes of distilled water surface tension. The obtained results are summarized in Table 5.

The effectiveness of a biosurfactant produced by microorganisms in reducing the surface tension depends on the behavioral properties of the substance (Van Hamme et al. 2006). Each microbial surfactant has a unique and diverse physiological property. According to Mulligan (2005), biosurfactants produced by bacterial strains are more effective in reducing the surface tension although the biosurfactants produced by yeasts are also showing good capacity to reduce surface tension. Furthermore, study by Burgos-Díaz et al. (2012) claimed that biosurfactants with low molecular weight are effective in reducing the surface tension of water to 25–30 mN/m. In this study, surfactant activity from fungal isolate **(MRB25)** was better than the bacterial isolates. It is of interest that only a few fungi are reported to produce biosurfactant which include *Candida bombicola*, *Candida lipolytica*, *Candida ishiwadae*, *Candida batistae*, *Aspergillus ustus*, *Ustilago maydis*, and *Trichosporon asahii* (Bhardwaj et al. 2013). Another possibility on the gradual surface tension reduction could be due to the weak effect on the surface tension exhibited by the microbial extract with surfactant activity which can be classified as bioemulsifier. Bioemulsifiers are often categorized with biosurfactant because it exhibited emulsification properties but may not lower the surface tension (Karanth et al. 1999).

Meanwhile, CMCs are the measurement for the concentration of the surfactant solution to associate and organize their molecules into spherically shaped structure called micelles (Tadros 2006). The CMC values of the isolated fungi and bacterial isolates in this study were occasionally higher compared to the chemically synthesized surfactant of SDS and other biosurfactant compounds. Relatively, the CMC of SDS is 0.2 wt% and the CMC of biosurfactant is between 0.0002 and 0.15 wt% as described by Mulligan (2005). According to Rosen and Kunjappu (2012), there are a number of factors which affect the CMC values, namely the structure of the hydrophobic groups, nature of the hydrophilic group, type of counter ion, addition of electrolytes, and effect of temperature (McClements and Dungan 1993). A

Table 5 Critical micelle concentration (CMC) value and surface tension of distilled water in the presence of the microbial extract from respective isolates

		Concentration of CMC (wt %)	Surface tension at CMC (mN/m)
Control	SDS	0.2	~35
Isolate	Designation		
Bacteria	MRB02	1.7	~59.5
	MRB12	1.3	~68.5
	MRB16	0.91	~61
	MRB18	1.4	~68
Fungi	MRB25	0.85	~58

balance between the hydrophobic and hydrophilic components of the molecules is, therefore, required to determine the properties of a surfactant. In more hydrophobic molecules, micelles will be formed at lower solution concentration resulting in low number of CMC. If the molecules are more hydrophilic, the CMC value will be increased (Radulovic et al. 2009). Based on these facts, there was a possibility that the surfactant activities exhibited by the isolated fungi and bacteria in this study consisted of molecules which were more hydrophilic.

7.2.2 Evaluation of Emulsification Capability

Disperse systems consisting of two immiscible liquids are classified as emulsions (Tadros 2013). An emulsion consists of polar and nonpolar substances in which either one of these substances is dispersed in another substance in the presence of emulsifier. Formation of emulsion is characterized by the formation of liquid droplets. The disperse phase mainly consists of liquid droplets which were dispersed in a liquid medium or the continuous phase. On the microscopic level, the aggregation, diffusion, and creaming of the droplets are due to continual motion of droplets caused by Brownian, gravitational, or applied external forces (McClements and Dungan 1993).

As extract from MRB25 gave the highest surface tension reduction at the lowest concentration out of the five isolates, it was, therefore, selected as the emulsifier for oil in water emulsion to further evaluate the emulsification capability. The resulting emulsion was compared to another with SDS by measuring the oil droplet size and distribution formed. The resulting microscopic images of the two emulsions in the presence of SDS (A) and MRB25 (B) are shown in Fig. 3. From these images, it is evident that MRB25 was able to produce emulsion droplets similar to SDS. Furthermore, the emulsion containing MRB25 extracts also had a very thick interfacial layer. Figure 3 also shows that a large number of emulsion droplets with a wide distribution were produced for emulsion with MRB25 compared to emulsion with SDS.

Due to surfactant's amphiphilic character, droplets of the dispersed phase were covered with the interfacial layers with different thickness. The thickness of the interfacial layer is determined by the amount of amphiphiles at the interface by quantifying the surface excess concentration ($T_i =$ molecules per surface unit) (Ferragut and Chiralt 1996). The surface excess concentration determines the requirement for complete droplet surface coverage with the surfactant molecules (Bhardwaj and Hartland 1998). The interfacial layer assists in stabilizing the emulsion by acting as a barrier against coalescence. According to Jönsson et al. (2003), thicker interfacial layers have more tendencies to reduce the interaction potential between the droplets and consequently increase the emulsion stability.

There are several factors that affect the interfacial layer which include heavy polar fractions in the dispersed substance, solid materials including organic and inorganic, temperature, droplet size and droplet-size distribution, and pH (Kokal and Al-Juraid 1999). Thus, the thickness of the interfacial layer depends on the

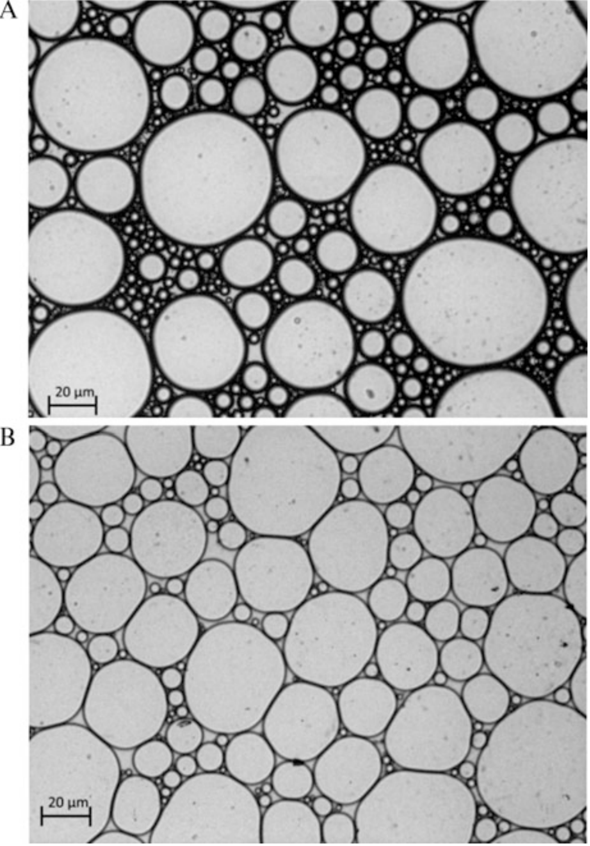

Fig. 3 Microscopic images of droplets oil in water emulsion in the presence of MRB25 extract (**a**) and **SDS** (**b**)

concentration of amphiphiles at the interface which directly translates to the stability of the emulsion. Therefore, the stability of emulsion in the presence of MRB25 extract could be higher than SDS containing emulsion. However, in order to confirm this claim, more work on the emulsion stability as a function of storage time still needs to be conducted in the presence of the MRB25 extract.

ImageJ software was used in order to gauge the information pertaining to the size and distribution, and the diameter of each droplet was calculated. The results of droplet frequency as a function of droplet diameter are shown in Fig. 4. The analysis showed a distribution which was skewed to the left with a higher number of smaller particles located for both emulsions at diameters less than 5 µm. However, compared to SDS, the average number of small oil droplets in the emulsion in the presence of MRB25 extract was higher than SDS, reaching a maximum droplet number of 160 at a size of 2 µm. This was almost three times higher than the oil droplets with SDS at the same diameter.

Size distribution of the droplets is one of the parameter in characterizing an emulsion. An emulsion with larger droplets will have lower solubility when

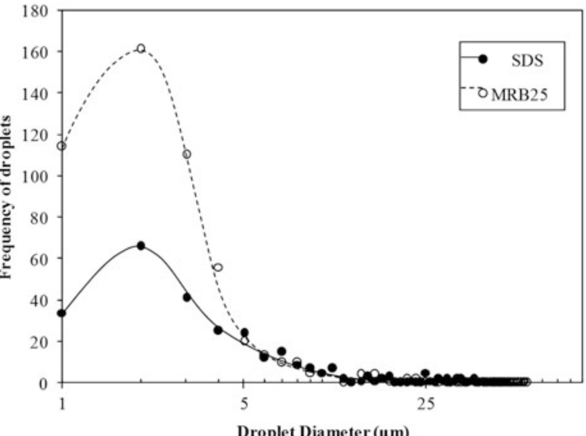

Fig. 4 Frequency of the droplets as a function of diameter for oil in water emulsions in the presence of SDS and MRB25 extract

compared to the smaller droplets (Tadros 2013). An effective surfactant could avoid significant changes on the size of the droplets over time. The droplet size distribution may shift to larger value over time due to the breakdown process of an emulsion (flocculation, creaming, coalescence, and Ostwald ripening). Evaluation of the droplet size distribution can be performed through a wide variety of mathematical and empirical distribution functions, such as the normal distribution, lognormal distribution, Rosin–Rammler equation, and Nukiyama–Tanasawa equation (Lefebvre 1989). According to Sood and Ashwati (2003), the actual distribution is rarely symmetrical therefore, experimental results presented as normal distribution usually show weak correlation. The lognormal distributions gave satisfactory representation for positively skewed distribution. When the droplet size distribution is broad with high number of small size droplets, the distribution will be significantly to the left side of the mean as shown in Fig. 4.

7.3 Stability of NRL in the Presence of Microbial Extracts with Surfactant Property

Stable NRL is defined by the ability to increase the electrostatic repulsion between the rubber particles and thus prevent the rubber particles from coalesces. NRL with good stability remained in liquid condition for certain period of time depending on the efficiency of a particular preservative agent. The effectiveness of a particular preservative agent that is being tested in NRL is characterized based on the number of bacterial population in NRL and that indicates the level of microbial contamination. Another characterization is on the level of VFA. Level of VFA is also one of the crucial criteria in assessing the quality of NRL which indicates the degree of spoilage. NRL with good stability shows slower increment or minimal changes of VFA number (Taysum 1958; Wren 1961; Kuriakose 1992). Flocculation of rubber

Table 6 Formulation for natural rubber latex with microbial extracts exhibiting antimicrobial and surfactant activities

	Treatment of field natural rubber latex in the absence and presence of the microbial extracts (g)						
Natural rubber latex (35 %)	100	100	100	100	100	100	100
Ammonia (%)	–	0.3	–	–	–	–	–
Isolates with surfactant activity							
Bacterial extracts							
MRB02	–	–	1.7	–	–	–	–
MRB12	–	–	–	1.3	–	–	–
MRB16	–	–	–	–	0.91	–	–
MRB18	–	–	–	–	–	1.4	–
Fungal extract							
MRB25	–	–	–	–	–	–	0.85

molecules indicates the reduction of electrostatic repulsion on the rubber particle surfaces. Concomitant with flocculation, a process called gelation or referred as storage hardening also hampered NRL especially during storage and transportation. Field NRL contained a small fraction of gel phase in the range of 7–30 % (Blomfield 1951). Gel content in NRL affects the original viscosity of the resulting rubber. Thus, to inspect the quality of NRL, viscosity measurement based on Mooney viscosity outlined in ISO 1652-2011(E) was applied. As a rule of thumb, a stable NRL must have minimal bacterial activity that will result in minimal changes of VFA number and thus the viscosity of the NRL will be remained constant with time.

In this study, extracts from the isolated microorganisms with surfactant property was added individually into NRL. The formulations of NRL with the microbial extracts are shown in Table 6. The critical micelle concentration (CMC) values of the isolates with surfactant property as tabulated in Table 5 were used as the concentration needed to be added into the NRL. Mixtures of treated latex were kept in transparent beaker and sampling was carried out every two hours until flocculation of NRL occurred.

Extracts from five isolates with surfactant property namely MRB02, MRB12, MRB16, MRB18, and MRB25 were evaluated for their capability in controlling the NRL stability. Compared to the other four isolates, MRB25 performed well in hindering the growth of bacteria in the NRL (Fig. 5). Surfactant property in the MRB25 extracts improved the stability of the NRL by maintaining the latex viscosity (Fig. 6). It is evident that the microbial extract from MRB25 showed better potential as surface active agent in NRL. The surfactant molecules from MRB25 extract probably carried an anionic charge that has the tendency to bind to the metal ions such as Mg^{2+} or Ca^{2+} on rubber particles. With the removal of metal ions, electrostatic forces between the rubber particles were increased and eventually delayed the flocculation of NRL (Booten et al. 2011). The minimal changes in

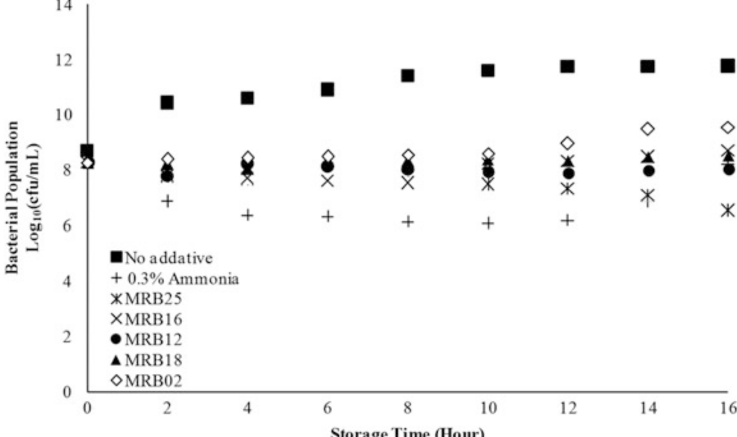

Fig. 5 Effect of fungal (MRB25) and bacterial (MRB02, MRB12, MRB16, and MRB18) isolates on bacterial population in NRL

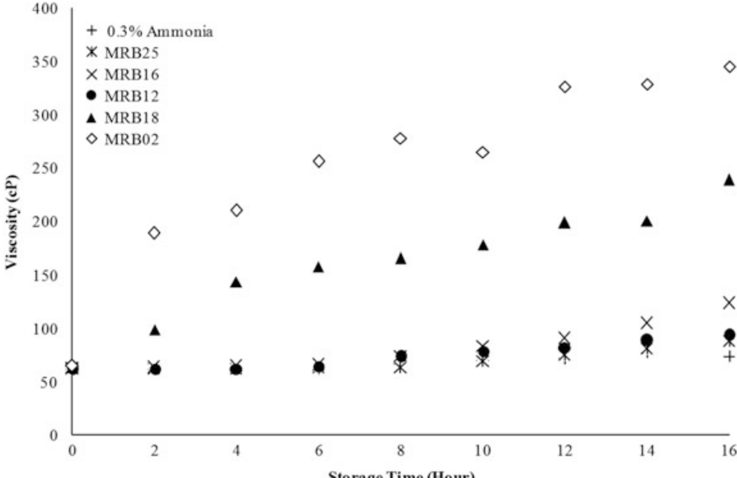

Fig. 6 Effect of fungal (MRB25) and bacterial (MRB02, MRB12, MRB16, and MRB18) isolates on viscosity of NRL

viscosity were also contributed by the minimal bacterial activity and gradual increment of VFA number (Fig. 7) which indicated good NRL stability.

From the results, it was confirmed that MRB25 performed well in hindering the growth of bacteria in the NRL. As a result, the VFA number was kept at a minimum with no changes in the viscosity of the latex indicating the absence of rubber particle–particle interaction. Further identification for MRB25 was carried out

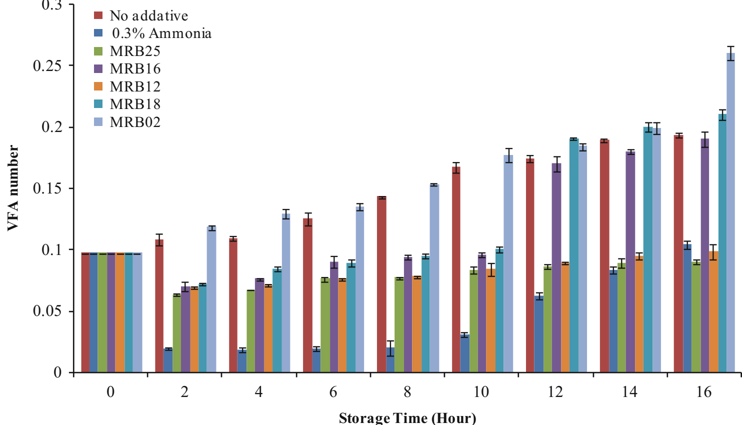

Fig. 7 Effect of fungal (MRB25) and bacterial (MRB02, MRB12, MRB16, and MRB18) isolates on volatile fatty acid number of NRL

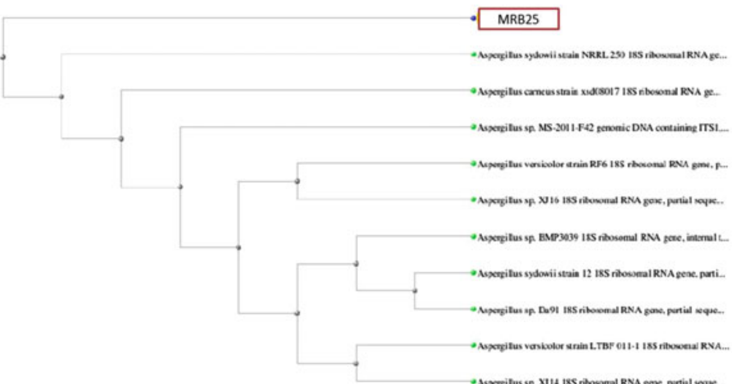

Fig. 8 Phylogenetic position of the MRB25 isolate

using 18sRNA sequencing technique. Results showed that the MRB25 was closely related with *Aspergillus sydowii* strain as shown in Fig. 8.

8 Conclusion

The finding from this study suggested that the microbial extracts have the potential to be used as an anticoagulant agent for NRL preservation. Further study on the biochemical and chemical characteristics of the microbial extracts would be necessary for better understanding in the mode of action of the biological compounds from the microbial extract of the isolated microorganisms against NRL stability. A

more comprehensive investigation will provide useful information in studying the effect of the biological compounds toward the nonrubber substances and existence of microbial metabolism in NRL. Eventually, this study has opened up the first step of introducing a biological based processing system in NRL industry.

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Microbial Production of Polyhydroxyalkanoates for Agricultural and Aquacultural Applications

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Abstract Current agriculture and aquaculture industries require the usage of large quantities of plastics, which contribute to a significant increase of plastic waste to be disposed. In order to overcome the serious drawback of huge quantities of plastic, researches on innovative biodegradable materials have been developed. Biomaterials are natural products catabolized and synthesized by different microorganisms. Bioplastics are known as special type of biomaterials that can be assimilated by many species of microorganisms and do not cause toxic effects to the host. The most widely produced bioplastics are polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides, and polylactides. PHAs are environmentally friendly biodegradable polymers which can be employed in a wide range of agricultural, industrial, medical, and pharmaceutical applications. This chapter addresses the microbial production of PHAs and the usage of short-chain-length-PHAs: poly(3-hydroxybutyrate) [P(3HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] for various applications in agricultural and aquacultural industries.

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1 Introduction

Plastic, an important component of our life has gained its place as one of the most efficient, versatile, convenient, and cheap material that can be used for various purposes (Luengo et al. 2003). In agriculture, the usage of plastic material provides a more efficient use of land for farming that resulted in healthier environment. In addition to that, plastic piping and drainage system have the ability to cut the cost of production of agricultural products (Picuno et al. 2012). The wide usage of plastic in agricultural field has resulted in the accumulation of plastic waste and this has geared the researchers to find biodegradable polymer to be applied in this field (Scarascia et al. 1997). The usage of plastic in aquaculture industry has also contributed to pollution (Emerenciano et al. 2013).

The ever-growing accumulation of petrochemical based non-biodegradable plastic waste in the environment has always been an important issue of global concern. Besides its detrimental effects, constant production of these products also results in the exhaustion of finite petroleum resources. A practical solution to this problem would be the use of biodegradable polymers derived from biobased resources. However, complete substitution of established common plastics with biodegradable materials is still a challenge. This is mainly due to the production cost and material properties. Nevertheless, constant research regarding these issues has yielded many breakthroughs and brought forward some potential substitutes with compatible material properties and lowered production cost (Shantini et al. 2012).

Polyhydroxyalkanoates (PHAs) is such a potential candidate to replace non-renewable resources. The renewability of PHA reduces the dependence on fossil fuel (Sudesh et al. 2000). PHAs are biopolymer that are accumulated by certain microorganisms under stress condition. This biodegradable polymer is non-water soluble and nontoxic (Reddy et al. 2003). Byrom (1987) reported that these materials exhibit material properties similar to common plastics. PHA polymers are produced through chemical synthesis, bacterial fermentation, or through the use of transgenic plants. PHAs can be accumulated in various forms and types depending on the feedstock provided and the bacterial strain. These PHAs are degraded by naturally occurring bacteria.

PHAs can be divided into three categories based on the number of carbon atoms present and their different proportions of monomer units (Zinn et al. 2001; Pantazaki et al. 2003; Chen et al. 2006; Ayub et al. 2007). The first category is the short-chain-length PHAs (SCL)-PHAs, polymerizing C3–C5 carbon length monomers and the second category is medium-chain-length (MCL)-PHAs whose carbon length ranges from C6 to C12. The third category is the copolymer of short-chain-length and medium-chain-length whose monomer contains C3–C14 carbon atoms (SCL-MCL)-PHAs (O'Leary et al. 2005). The composition of PHAs produced depends on the substrate used (Huisman et al. 1989). An example of (SCL)-PHAs is P(3HB) which is the most common PHA (Ayub et al. 2007). P(3HB) can be found accumulated in granular form in many bacteria (Akiyama et al. 1992). P(3HB) is also known to be optically active (Lenz and Marchessault 2005).

The (MCL)-PHAs are elastomers that have poor tensile strength but high extension at break (Sujatha et al. 2007). This (MCL)-PHAs are also sticky materials which can be modified to make rubbers (Suriyamongkol et al. 2007). The (MCL)-PHAs have a lower level of crystallinity and higher elasticity compared to P(3HB) or P(3HB-*co*-3HV) (Preusting et al. 1990)., the usage of PHAs in agricultural and aquacultural industry was mainly reported by using (SCL)-PHAs.

The application of PHAs has been reported in various fields such as industrial, medical, and pharmaceutical. Due to their biodegradability, these polymers can be applied in the agricultural and aquacultural industries to replace the non-renewable plastics being used. In this chapter, microbial production of PHAs and the usage of PHAs in agriculture and aquaculture field will be discussed.

2 Microbial Production of PHAs

PHAs are simple macromolecules produced naturally by many species of microorganisms. It is an example of nonpetroleum based biodegradable plastic that does not cause toxic effect to the host (Chien et al. 2007; Suriyamongkol et al. 2007). The building block of PHAs is 3-hydroxyalkanoic acid monomer unit (Fig. 1). More than 75 genera are known to have the ability to synthesize and accumulate PHAs (Koller et al. 2011). PHAs are synthesized and accumulated inside numerous bacteria to a level as high as 80 % of the cell dry weight (Ojumu and Solomon 2004). This storage polymer acts as carbon and energy storage materials or as a sink for reducing power (Doi 1990; Madison and Huisman 1999). Due to the insolubility of PHAs inside the bacterial cytoplasm, insignificant increase in osmotic pressure was observed. This prevents the leakage of the polymer compound out of the cell (Verlinden et al. 2007).

PHAs are synthesized under stress conditions, especially when the nutrient supplies are imbalanced, for instance, depletion of nitrogen, phosphorus, sulphate, oxygen or other essential elements, but in the presence of excessive carbon sources (Reddy et al. 2003). Besides, limitation of iron, magnesium, potassium, and sodium are also found to stimulate the accumulation of PHA. This is to prevent starvation if an essential element becomes unavailable. Bacteria that accumulate PHAs showed enhanced tolerance toward environmental conditions such as heat, osmotic shock, and UV irradiation (Kadouri et al. 2005).

Fig. 1 Chemical structure of PHA. R refers to side chain and *n* refers to the number of repeating units (Koller et al. 2011)

The monomeric units of PHAs are enantiomerically pure and in the (*R*)-configuration. The monomers are all in the *R*-configuration due to the stereospecificity of PHA synthase, the polymerizing enzyme. The *S* configuration monomers are detected only in rare cases (Haywood et al. 1991). The alkyl side chain of PHAs can be saturated, unsaturated, straight, branched chain containing aliphatic, aromatic, halogenated, epoxidized, or nitrile side chain. Different types of functional group and the length of the side chains directly influence the properties of the polymer such as crystallinity and melting point (Eggink et al. 1995).

In prokaryotes, PHAs can be found in the cell cytoplasm as small insoluble inclusions which usually have 0.2–0.5 μm diameter in size (Sudesh et al. 2000; Dennis et al. 2008). Observation under the electron and phase contrast microscopes shows the granules to be highly refractile inclusions (Lee 1996). The molecular weight of the polymer ranges from 2×10^5 to 3×10^6 Da and the polydispersity index is around 2.0 (Lee 1996; Madison and Huisman 1999; Agus et al. 2006). Different species have different number and size of granules. The number of granules of *Cupriavidus necator* varies between 8 and 12 granules whereas *Pseudomonas oleovorans* is estimated to have about one or two large granules (Zinn et al. 2001). Common producers of PHA are *Pseudomonas* species, *Rhodospirillum rubrum*, *Bacillus* species, *Aeromonas* species and *Cupriavidus necator* (previously known as *Alcaligenes eutrophus*, *Ralstonia eutropha*, and *Wautersia eutropha*) (Anderson and Dawes 1990).

The (SCL)-PHAs constitutes 3–5 carbon atoms in the monomer. The (SCL)-PHAs are thermoplastics that have higher melting temperature, stiffer in nature (Sujatha et al. 2007), and have high degree of crystallinity and rigidness (Chen et al. 2006; Liu and Chen 2007). The mechanical properties of P(3HB) is comparable to polypropylene, however, it has poor elongation at break compared to polypropylene (Iwata et al. 2003). This homopolymer also exhibited high melting temperature of 170 °C which is slightly lower than its degrading temperature (Sudesh et al. 2000). This makes the polymer difficult to be processed and thus limits its applications. It is also brittle and highly hydrophobic (Pachence et al. 2007). Copolymers can be formed by feeding precursors and this may result in the formation of copolymer containing P(3HB) with 3-hydroxyvalerate (3HV) monomer or 4-hydroxybutyrate (4HB) monomer (Huisman et al. 1992).

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] is a type of (SCL)-PHAs which is a strong and malleable thermoplastic with tensile strength comparable to that of polyethylene. These copolymers are usually produced using carbon substrate such as 1,6-hexanediol, 1,4-butanediol, 4-chlorobutyric acid, or gamma-butyrolactone (Doi et al. 1989). P(3HB-*co*-4HB) copolymer is mainly used in medical and pharmaceutical fields due to its biocompatibility and elastomeric properties (Martin and Williams 2003). Production of PHAs consisting of 4HB monomer by various microorganisms has been investigated since the early 1990s. Wild-type strains capable of biosynthesizing P(3HB-*co*-4HB) copolymer from different carbon sources are *Cupriavidus necator* (Doi 1990; Nakamura and Doi 1992; Valentin et al. 1995; Saito et al. 1996; Lee et al. 2000; Kim et al. 2005; Chanprateep et al. 2008, 2010; Rao et al. 2010; Volova et al. 2011), *Alcaligenes*

latus (Hiramitsu et al. 1993; Saito et al. 1996; Kang et al. 1995), *Comamonas testosteroni* (Renner et al. 1996), *Delftia acidovorans* (Kimura et al. 1992; Saito et al. 1996; Sudesh et al. 1999; Lee et al. 2004; Mothes and Ackermann 2005; Hsieh et al. 2009; Ch'ng et al. 2012), *Hydrogenophaga pseudoflava* (Choi et al. 1999), and *Chromobacterium* sp. (Zhila et al. 2011).

Saito et al. (1996) reported similar observation as made by Kunioka Masao in 1988 who demonstrated the production of random copolymer of P(3HB-*co*-4HB) by *Cupriavidus necator* using γ-butyrolactone, 4-hydroxybutyric acid, and alkanediols of even carbon number (1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol). Molar fraction of 4HB ranging from 9 to 34 mol% was produced using various carbon sources. Decrease in the 4HB molar fraction was observed when fructose or butyric acid was added into the nitrogen-deficient medium containing 4-hydroxybutyric acid or γ-butyrolactone. Similar synthesis of P(3HB-*co*-4HB) copolymer using various carbons was also carried out using *Delftia acidovorans* DS-17, isolated from activated sludge.

According to Kim et al. (2005), bacterial growth was inhibited by high concentration of fructose (>20 g/L) and γ-butyrolactone (>6 g/L) in the biosynthesis of P (3HB-*co*-4HB) copolymer by *Cupriavidus necator*, suggesting that a controlled feeding rate of fructose and γ-butyrolactone should be employed as one of the strategies in the fed-batch fermentation. Acetate as well as propionate was also used as a stimulator at a concentration of 2 g/L to increase the 4HB monomer incorporation from 38 to 54 mol%.

High proportions of 4HB unit (60–100 mol%) were also produced by *Cupriavidus necator* using 4-hydroxybutyric acid supplemented with additives such as ammonium sulphate and potassium dihydrogen citrate, however, the polyester content was found to decrease (Saito et al. 1996). Regulation of 4HB molar fraction through supplementation of propionate was also reported by Lee et al. (2000), suggesting the increment of 4HB monomer composition from 12 to 52 mol% through the addition of propionate in small amount together with γ-butyrolactone.

The feasibility production of P(3HB-*co*-4HB) copolymer by *Cupriavidus necator* using the spent palm oil left after frying activities and 1,4-butanediol was demonstrated by Rao et al. (2010) who reported high PHA yield (0.75–0.8 g/g of spent palm oil) with constant accumulation of 4HB monomer (15 mol%) that led to a conclusion that 4HB monomer accumulation was not influenced by the cultivation period and the existence of polar solids in the spent palm oil.

Cavalheiro et al. (2012) presented the first report on the production of P(3HB-*co*-4HB) copolymer from waste glycerine using high-cell density fed-batch cultures of *Cupriavidus necator* DSM 545. Incorporation of 4HB monomers was initiated by adding γ-butyrolactone. P(3HB-*co*-4HB) copolymers with 11–22 mol% of 4HB monomer were attained by manipulating the dissolved oxygen concentration and cultivation time. 4HB monomer increased by twofold using propionic acid as a stimulator and it had resulted in the formation of P(3HB-*co*-3HV-*co*-4HB) terpolymer because propionic is a precursor for the formation of HV monomer.

Delftia acidovorans possesses the most efficient metabolic pathway for the biosynthesis of P(3HB-*co*-4HB) copolymer and generally, PHAs extracted from this bacterium are safe as tested based on cytotoxicity, genotoxicity, and implant tests. Even though *Delftia acidovorans* is a potential strain for the production of P (3HB-*co*-4HB) copolymer for medical applications, but it has inferior ability of controlling the monomer compositions in a wide range (Ch'ng et al. 2012; Siew et al. 2009). According to the study carried out by Hsieh et al. (2009), P(3HB-*co*-94 %4HB) copolymer was achieved with cell concentration and PHA content of 2.5 g/L and 13 wt%, respectively, using *Delftia acidovorans* cultivated under optimal conditions as follows: 1,4-butanediol (10 g/L), pH (7), incubation time (72 h), and temperature (26 °C). *Delftia acidovorans* was also reported to produce P (3HB-*co*-4HB) copolymers with extremely high 4HB compositions (93–99 mol%) with PHA content of 12–18 wt% using 1,4-butanediol (5 g/L) as sole carbon source through two-stage cultivation process (Kimura et al. 1992).

According to Lee et al. (2004), besides adjusting the concentration of carbon, increase in 4HB monomer composition accumulation by *Delftia acidovorans* could also be achieved by adjusting pH and aeration. It was reported that influence of pH was greater in the incorporation of 3HB monomers rather than on 4HB monomers. The authors also reported that molar fraction of 4HB could be significantly enhanced without adversely affecting the PHA content by reducing the aeration as the conditions prevent the incorporation of 3HB monomer.

Effect of Mg^{2+} concentrations on the compositions of P(3HB-*co*-4HB) copolymer produced by *Delftia acidovorans* using glucose and 1,4-butanediol had been reported by Lee et al. (2007). The authors demonstrated that 3HB monomer decreased with the increase of Mg^{2+} which was due to the decrease in the uptake of glucose by the bacteria as the stability of membrane was disrupted by the ionic interactions within the phosphonyl group. P(3HB-*co*-4HB) copolymer content was also very low in the absence of Mg^{2+}, suggesting that enzymes involved in the conversion of glucose and 1,4-butanediol to 3HB and 4HB monomers require Mg^{2+} as a cofactor which will bind to the substrate to orient them properly for the reaction.

Choi et al. (1999) reported that 4HB contents up to 66 mol% could be produced by *Hydrogenophaga pseudoflava* cultivated using the combination of glucose and γ-butyrolactone through one-step cultivation process. Higher 4HB monomer compositions (89–95 mol%) were achieved through two-step cultivation processes. Random P(3HB-*co*-4HB) copolymers having broad range of 4HB monomer compositions from 0 to 83 mol% were produced by *Alcaligenes latus* using combination of 3-hydroxybutyric and 4-hydroxybutyric acids (Kang et al. 1995).

Bacterial strain, *Comamonas testosteroni* had been investigated for its potential of producing P(3HB-*co*-4HB) copolymer using various carbon sources and precursors which yielded molar fraction of 4HB monomer above 90 mol%. The remarkably high 4HB monomer composition accumulated by this strain was attributed to the very low degradation of 4HB-CoA into 3HB-CoA via acetyl-CoA (Renner et al. 1996).

Cupriavidus sp. USMAA1020 was able to produce 4HB molar fractions ranged from 6 to 14 mol% with high PHA content of 47–60 wt% using γ-butyrolactone when C/N ratio was increased from 10 to 60 through one-stage cultivation. Higher 4HB molar fraction of 60 mol% was achieved using γ-butyrolactone (20 g/L) through two-stage cultivation (Amirul et al. 2008). Effect of culture conditions such as phosphate ratio, cell concentration, and aeration on the P(3HB-*co*-4HB) copolymer accumulation had been studied using *Cupriavidus* sp. USMAA1020 through two-stage cultivation. It was demonstrated that 4HB monomer accumulation was enhanced from 33 to 57 mol% with the increase of cell concentration from 0.33 to 2.50 g/L. However, further increase in the cell concentration above 1.67 g/L had resulted in the decrease of PHA content from 45 to 25 wt%. The effect of different K_2HPO_4 to KH_2PO_4 ratios and its buffering capacity on the biosynthesis of P(3HB-*co*-4HB) copolymer showed that PHA content increased with the increase of K_2HPO_4 to KH_2PO_4 mol ratio up to 2.45 and remained constant above that ratio. However, incorporation of 4HB monomer showed insignificant changes (Vigneswari et al. 2009).

Separate plasmids harboring the PHA biosynthesis genes from *Cupriavidus necator* and the succinate degradation genes from *Clostridium kluyveri* have been introduced into *Escherichia coli* DH5α to develop a recombinant strain that has the ability to produce P(3HB-*co*-4HB) copolymer using unrelated carbon source, glucose. The recombinant strain of *Escherichia coli* was found to produce 50 wt % of P(3HB-*co*-3 %4HB) copolymer (Valentin and Dennis 1997). Similar experiment was conducted by Li et al. (2010) but with some improved techniques to enhance the 4HB monomer composition by the recombinant *Escherichia coli* strain. P(3HB-*co*-11 %4HB) copolymer was attained with 9.4 g/L and 66 wt% of cell dry weight and PHA content, respectively, using glucose at 48 h. The 4HB monomer accumulation was also enhanced from 11 to 20 mol% through addition of α-ketoglutarate or citrate.

Another example of (SCL)-PHAs is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] @ PHBV which is less stiff and brittle compared to P(3HB). It is produced by the incorporation of 3-hydroxyvalerate (3HV) monomer into P(3HB). There are various microorganisms that have the ability to produce copolymer P(3HB-*co*-3HV). Due to the ability of *Alcaligenes eutrophus* to accumulate PHA up to 80 % (w/w) of dry cell mass, therefore, it has been studied most extensively for copolymer P(3HB-*co*-3HV) production. This strain has the ability to produce copolymer P(3HB-*co*-3HV) from a mixture of glucose and propionic acid in large scale by applying fed-batch method (Byrom 1992). The carbon source and carbon precursor were fed during the accumulation of polymer. Different molar compositions of the copolymer could be obtained by varying the ratio of propionic acid and glucose (Byrom 1987, 1992). Feeding a mixture of glucose and propionic acid has resulted in more than 110 g/L of P(3HB-*co*-3HV). About 90 mol% of 3HV monomer was produced by cultivating this strain with butyric and pentanoic acids (Doi et al. 1988). Besides, this strain also has the ability to produce copolymer P(3HB-*co*-3HV) by feeding ethanol with propanol or pentanol (Alderete et al. 1993; Park and Damodaran 1994).

Zakaria et al. (2010) reported the usage of acetic and propionic acids for the production of copolymer P(3HB-*co*-3HV) by *Comamonas* sp. EB172. It was reported that the highest PHA content and CDW at 30 wt% and 2.8 g/L, respectively, were obtained when the acetic:propionic acid ratio of 2:3 was used. When the ratio of acetic to propionic acid was modulated to 1:4, the highest 3HV monomer of 17 mol% was obtained.

In another study by Bhubalan et al. (2010) the copolymer P(3HB-*co*-3HV) was produced by using *Chromobacterium* sp. USM2 cultivated in sodium propionate, sodium valerate, or valeric acid. Addition of 1.0 % sodium valerate resulted in the production of P(3HB-*co*-3HV) with PHA content of 32 wt% and 3HV monomer composition of 98 mol%. Addition of valeric acid has resulted in lower PHA content (10 wt%) and 3HV composition (88 mol%) compared to the addition of sodium valerate. According to Kolibachuk et al. (1999), addition of heptanoic acid to the culture of *Chromobacterium violaceum* resulted in the copolymer content of 15 wt% with 95 mol% 3HV monomer composition.

Addition of propionic acid and glucose to the culture medium containing glucose-utilizing mutant of *A. eutrophus* resulted in the production of random copolymer containing 3HB and 3HV monomers. The usage of sodium propionate as the sole carbon source produces copolymer containing 43 mol% 3HV with 35 wt % PHA content (Doi et al. 1986).

Recombinant *E. coli* was also found capable in producing copolymer P(3HB-*co*-3HV) by using sucrose (Lee and Chang 1993). This strain has the ability to produce 40 mol% 3HV monomer compositions by using glucose and propionate (Slater et al. 1992). Choi and Lee (1999) reported that the recombinant *E. coli* harboring *Alcaligenes latus* biosynthesis genes could produce 74 wt% P(3HB-*co*-3HV) with 18.1 mol% 3HV monomer composition by using acetic acid induction and oleic acid supplementation.

In another study by Choi et al. (2003), the mutant of *Alcaligenes* sp. SH-69 that has the ability to overproduce threonine was reported to produce 41.5 wt% P (3HB-*co*-3HV) with 46.3 mol% 3HV monomer unit when 10 mM threonine was added with glucose. Addition of other amino acids to glucose such as 10 mM isoleucine resulted in the production of 35.8 wt% P(3HB-*co*-3HV) with 42.7 mol% 3HV monomer unit.

Most bacteria synthesize copolymer P(3HB-*co*-3HV) only when supplied with external carbon precursor such as valeric acid or propionic acid. However, some strains such as *Rhodococcus ruber* produce copolymer with 75 mol% 3HV when grown on simple sugar (Haywood et al. 1991). *Bacillus cereus* was found to accumulate 50 mol% 3HV monomer units with 13 wt% PHA when cultivated with 0.1 % (wt/vol) propionic acid (Ramsay et al. 1990). Lee et al. (1995) reported the production of high molecular weight P(3HB-*co*-3HV) from structurally unrelated carbon source such as glucose or fructose by *Agrobacterium* sp. SH-1 and GW-014. When the strain was cultivated in glucose, 67.4 % P(3HB-*co*-3HV) of CDW with 6.1 mol% 3HV was produced. The same strain produced 74 % P (3HB-*co*-3HV) of CDW with 3.6 mol% 3HV when cultivated in fructose as sole carbon source.

The biodegradability of a polymer depends on the physical and chemical properties of the polymer. Polymers with low molecular weight are more prone to degradation. The rate of biodegradation of PHA depends on environmental conditions such as temperature, moisture, pH, nutrient supply, monomer composition, crystallinity, and surface area (Philip et al. 2007). Besides that, melting temperature of the polymer also influences the biodegradation whereby the enzymatic degradability decreases as the melting point increases (Tokiwa and Suzuki 1977). The important role of crystallinity in the biodegradation was claimed by Nishida and Tokiwa (1992). They mentioned that materials with high crystallinity have lower biodegradability.

3 Application of PHAs in Agriculture and Aquaculture Industry

One of the specialized applications of PHAs in agriculture is as mulch films. It has also been used as bacterial inoculants to enhance nitrogen fixation in plants and as controlled release of pesticides.

Mulch film is used in agricultural industry as a protective layer that is spread on top of the soil. The use of plastic mulch in agriculture has increased in the last 10 years due to the benefit of mulching for the plants. Mulching is used to increase fruit crop and vegetable productivity. It also increases the horticulture products, prevents water evaporation from the soil and soil erosion, reduces water consumption, and controls weeds which indirectly helps the presence of product for longer period and it also contributes to clean products (Espi et al. 2006). Management of the soil's temperature, physical structure, nutrients, and moisture level could also be enhanced by using mulch in agriculture (Espi et al. 2006). It was introduced to agricultural field in the 1950s (Shogren and Hochmuth 2004; Rudnik and Briassoulis 2011; Kasirajan and Ngouajio 2012). Natural mulches from animal and plant materials were used in maintaining soil organic matter (Tindall et al. 1991). Later it was found that net radiation in plastic mulches was higher compared to nonplastic mulch environment.

Then, petroleum derived plastic films, such as low density polyethylene (LDPE), linear low-density polyethylene and high-density polyethylene were used as mulch in agricultural field (Fleck-Arnold 2000). Plastic mulch film should be rigid and flexible so that it can be removed easily from various growing environments (Lamont 2005). This plastic was used as mulches in agricultural industries in cold regions. However, nowadays plastics are used in all kinds of soils and climates. It has been widely used in peanut production, corn, cotton, sugarcane, and rice plantations (Kasirajan and Ngouajio 2012). The usage of polyethylene mulches is an added advantage because it has the ability to aid in the retention of nutrients within the root zone indirectly allowing more efficient nutrient utilization by the crop (Cannington et al. 1975).

The usage of this petroleum derived materials has major drawbacks due to the post-consumption of plastic materials which causes environmental problem due to their disposal (Lemieux 1997; Lamont 2005; Levitan 2005; Corbin et al. 2013). Due to their low biodegradability in environment, petroleum derived plastics can cause serious land pollution. Therefore, researchers have focused on developing biodegradable polymers that do not generate toxic product upon degradation to be applied in agricultural field. This biodegradable polymer must be able to have suitable characteristic to be used from planting to harvesting (Briassoulis 2004; Scarascia-Mugnozza et al. 2004). Biodegradable mulches such as starches, polybutylene adipate terephthalate (PBAT), polycaprolactone (PCL), and polybutylene succinate (PBS) were developed in the 1980s (Hayes et al. 2012). However, these biodegradable mulches were reported to have low biobased content and their cost of production was high (Olsen and Gounder 2001; Tullo 2012).

Therefore, the researchers targeted for completely biobased agricultural mulches. In the end, the biodegradable materials can be integrated into the soil and later degraded to carbon dioxide, methane, and water (Immirzi et al. 2003). This indirectly reduces the costs associated with their removal after harvest. These lead to the production of agricultural mulches by using polylactic acid (PLA) and blends of PLA and PHAs (Wadsworth et al. 2012). PHAs known as "green" polymers are a promising biodegradable plastics because they are made from renewable resources through bacterial fermentation of sugars and lipids (Kaihara et al. 2005; Hayes et al. 2012)., Steinbuchel (2005) reported the application of PHAs in the field of agriculture as mulch.

In order to prepare the mulches, two methods have been employed. One is known as meltblown and the other spunbond. Meltblown mulches are prepared by melting the thermoplastic polymer through an extruder dye. These mulches were reported to have low tensile strength and high degradation compared to the mulches prepared by using spunbond. Spunbond mulches are prepared in a three continuous stages. First stage involves the spinning and extrusion of polymer melt; this is followed by collection of the fibers on the conveyer belt then heat bonding to produce continuous filaments (Dugan 2001; Li et al. 2011; Khan et al. 1995).

Hablot et al. (2014) reported in their study the preparation and characterization of meltblown nonwoven mulches using blend of copolymer P(3HB-*co*-4HB) and PLA at 75/25 w/w. This P(3HB-*co*-4HB)-PLA feedstock was first dry mixed and then extruded into pellets to ensure uniformity. Higher melt temperature was employed compared to spunbond mulches because low melt viscosity was required in achieving smaller diameter fibers in meltblown. P(3HB-*co*-4HB) is more thermally unstable compared to PLA, therefore, lower temperature was employed for the nonwoven processing of meltblown P(3HB-*co*-4HB)-PLA. Stimulated weathering of the mulches by UV irradiation and water spray was carried out in an accelerated weathering chamber. Black panel temperature of 63 °C was used to irradiate the samples with UV rays for 102 min. This was followed by 18 min under UV rays and water spray while the temperature decreased inside the weatherometer. Biodegradability testing was carried out for the mulches before and after stimulated weathering in a controlled experiment. The obtained compost was sieved using a

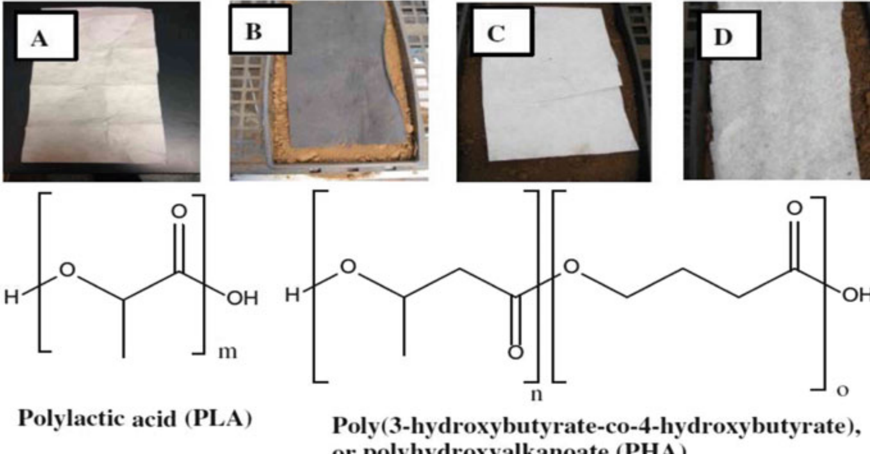

Fig. 2 Fully biobased agricultural mulches from (**A**, **B**) Spunbond PLA, (**C**) Meltblown PLA, (**D**) Meltblown P(3HB-*co*-4HB)-PLA (Hablot et al. 2014)

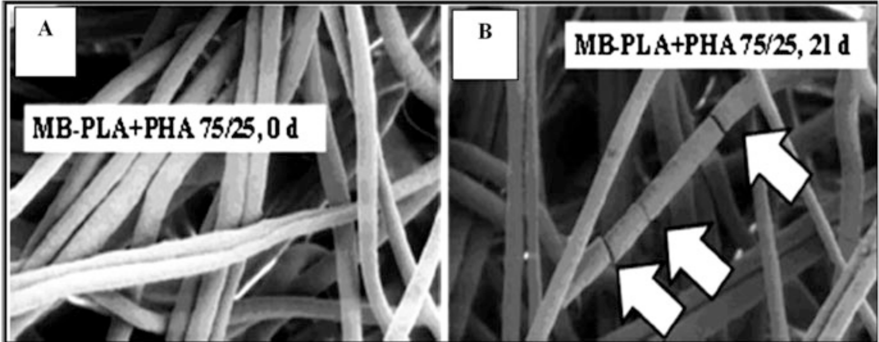

Fig. 3 SEM micrographs) of meltblown PLA + P(3HB-*co*-4HB)—75/25 w/w mulches (**A**) before and (**B**) after simulated weathering. *Arrows* shows the breakage of the fiber after 21 days (Hablot et al. 2014)

5 mm sieve in order to reduce the size and all the inorganic materials such as metal, stones, and glass were removed. This was followed by mixing the dry vermiculite and adjusting the moisture level to 60 %. The mulches were cut into 1×1 cm^2 squares and were added to the compost. The average percentage of biodegradation versus time was stimulated. Figure 2 shows the agricultural mulches of PLA and blends of P(3HB-*co*-4HB)-PLA.

The incorporation of P(3HB-*co*-4HB) into the meltblown mulches led to twofold increase in fiber diameter. However, simulated weathering slightly decreased the average fiber diameter for meltblown P(3HB-*co*-4HB)-PLA mulches. This author reported that weathering treatment led to fiber breakage. This is shown in SEM images of the meltblown mulches before and after weathering treatment (Fig. 3).

Wadsworth et al. (2012) reported that meltblown mulches underwent a large decrease in tensile strength compared to spunbond mulches due to the smaller fiber size of the meltblown mulches. They also reported that meltblown P (3HB-*co*-4HB)-PLA mulches lost 95 % tensile strength. Hayes et al. (2012) reported the tensile strength for P(3HB-*co*-4HB)-PLA mulches was 4 N and commercial polyethylene and biodegradable mulch films possessed tensile strength of 8–12 N. The incorporation of P(3HB-*co*-4HB) was also found to decrease the average molecular weight during the preparation of the nonwoven material. This was reported due to the loss of tensile strength and fiber breakage. The decrease of tensile strength leads to the opening of the supramolecular structure that enables slow degradation of the individual fibers.

As for the thermal properties, the incorporation of P(3HB-*co*-4HB) into the PLA mulches was found to decrease the melting enthalpy of PLA. This P(3HB-*co*-4HB)-PLA mulch was also reported to decrease the T_g from 65 to 57 °C that represent the decrease in molecular weight and crystallization enthalpy. Incorporation of P (3HB-*co*-4HB) to meltblown PLA mulches resulted in slightly broader and less intense peak due to the P(3HB-*co*-4HB) carbonyl stretch (Rudnik and Briassoulis 2011). Observation of chemical structure of P(3HB-*co*-4HB) using ATR-FTIR revealed the presence of less intense peak at 1751 cm^{-1} (C=O) carbonyl stretch, due to the maximum position for P(3HB-*co*-4HB) carbonyl stretch reported to be lower, 1919 cm^{-1}. P(3HB-*co*-4HB) was also reported to undergo random chain scission to produce double bonds and carboxylic acids (Grassie et al. 1984).

It was reported that meltblown P(3HB-*co*-4HB)-PLA undergoes the highest rate of biodegradation (Kim et al. 2000; Maiti et al. 2007). Simulated weathering increased the extent of degradation of meltblown P(3HB-*co*-4HB)-PLA mulches due to the lowest crystallinity observed and this facilitated the breakdown of molecules present in the blend. Therefore, Hablot et al. (2014) concluded in their study that the addition of P(3HB-*co*-4HB) to PLA mulches enhanced the biodegradability of the film and this is a viable candidate for the production of biodegradable agricultural mulch.

PHAs are also used as bacterial inoculants to enhance nitrogen fixation in plants. It was reported that the bacterial culture used as inoculants need to withstand stressful environments whenever it is used in agricultural field. This bacterial cell also must be stored for longer periods and this cell must be able to endure dessications and hot conditions. The bacterial inoculants also must possess the ability to sustain high survival rates within the carrier. Therefore, the usage of PHAs in *Azospirillum brasilense* inoculants promotes the plant growth (Reddy et al. 2003; Philip et al. 2007). Kadouri et al. (2003) stated that the production of PHA is important for improving shelf life, efficiency, and reliability of the inoculants. Fallik and Okon (1996) in their study reported that plant growth promoting effects were more consistent when using inoculants containing *A. brasilense* cells that had accumulated the storage polymer PHA.

A rapid and exponential growth of human population has greatly increased the global demand for agricultural based crop products. An improvement of the agricultural productivity is therefore inevitable to accommodate and fulfill such need.

To achieve favorable quantities of crops, a standard quality control in agricultural management is required. This leads to an integration of intensive technologies in agricultural development, which involves the massive use of vast variety of chemicals used as herbicides and pesticides.

The development of controlled release system of the chemicals is one of the efficient and improved methods which aim to increase the effectiveness of their action toward the target organisms; while minimizing the detrimental effect on nontarget organisms. This is regarded as one of the soil management strategies which enable the production of desirable quantity and quality of crops to fulfill the demands, without significant impact to health and environment. Controlled release systems have been tremendously used in food and pharmaceutical industries for the release of active substances such as drugs, nutrients, aroma, and medicine (El Bahri and Taverdet 2007; Melo et al. 2008; Moraes et al. 2010; Parajo et al. 2010; Natarajan et al. 2011). The principle lies behind this system is that the active substances were encapsulated in biodegradable polymeric materials which serve as coating and/or transport media. The substances are released slowly and continuously depending on the degradation rate of the polymeric materials used. This allows the release of substances in smaller but sufficient quantities, which subsequently ensures greater efficiency, with a reduced threat to nontarget organisms (Sinha et al. 2004; Sopeña et al. 2009).

Among all the controlled release technologies under development, the use of polymeric micro or nanoparticles is of special interest in agricultural context. In literature, there is a wide variety of materials used as the coating for the bioactive substances. These include the materials made up of silica (Barik and Sahu 2008), clays such as sepiolite and bentonites (Fernández-Pérez et al. 2004; Maqueda et al. 2008), polymeric substances such as lignin, alginate, ethyl cellulose, and polyurethane (Kulkarni et al. 2000; Perez-Martinez et al. 2001; El Bahri and Taverdet 2007; Fernández-Pérez 2007; Garrido-Herrera et al. 2009). Several studies have also described the use of PHA which is of microbial origin (Salehizadeh and Van Loosdreacht 2004). The materials which are used as carrier substances in this application are important as this determine the release of the desire amount over a period of time. There are some key features of choosing the materials in designing the coating and/or transport carrier. This includes the biodegradability, long-term retention, safety for both living and nonliving nature, formation of nontoxic by-products upon degradation, and the feasible transformation into different forms to meet current methodologies and technologies (Voinova et al. 2009). In this context, PHA comes to its rational position as one of the potential candidates to solve the issue addressed because it is completely biodegradable. Since it is one of the products from a commonly found survival mechanism, this material (which originally functions as energy reserved material) is easily available by fermentation of a wide variety of microorganism. Depending on the fermentation conditions, this material can also be obtained in an inexpensive way. Besides, PHA is not prone to rapid hydrolysis by chemicals but through biological degradation by intracellular or extracellular PHA depolymerases secreted by microorganism (Suyama et al. 1998). Subsequently, the complete degradation in nature takes longer period, and this is

desirable for designing a prolonged release action of bioactive substances. Finally, the degradation of PHA leads to by-products which are innocuous to the environment. PHA is degraded to carbon dioxide and water under aerobic conditions, whereas under anaerobic condition, it is degraded to form methane and water (Prudnikova et al. 2013). In the past decade, Procter & Gamble have commercially produced Nodax™, which is for multipurpose use. In agricultural application, Nodax™ is not only used to manufacture biodegradable mulch films, but also used for the controlled release of herbicides and insecticides. Particularly, the substances are integrated into P(3HB-*co*-3HV) containing pellets and sown along the plantation (Philip et al. 2007).

Grillo et al. (2011) developed a release system for ametryn by encapsulating the active substance in microparticles made of homopolymer poly(hydroxybutyrate) (PHB) and poly(hydroxybutyrate-valerate) (PHBV), respectively. The polymeric microparticles were prepared by forming oil in water emulsions using emulsion solvent evaporation technique (Lionzo et al. 2007). Both PHB:AMT and PHBV:AMT were prepared by dissolving 100 mg of the respective biodegradable polymers with 10 mg of ametryn in chloroform to form organic phase. It was then transferred to stirring aqueous phase made of 0.5 % (m/v) polyvinyl alcohol. The chloroform was evaporated using rotary evaporation system. Each process was carried out at 50 °C. The resulting microparticle suspension contained a final ametryn concentration of 50 mg/L.

In this study, ametryn-containing microparticles were evaluated in terms of the encapsulation efficiency and herbicide loading. The study demonstrated similar finding to those found in the literature for the formulations involving the encapsulation of different compounds but with the microparticles using PHB and PHBV (Bazzo et al. 2009; Sendil et al. 1999; Grillo et al. 2010). The encapsulation efficiencies (EE, %) of 34.3 % and 38.2 % were obtained for PHB:AMT and PHBV:AMT, whereas the herbicide loading were 13.2 and 11.1 %, respectively. In general, both microparticles possess low encapsulation efficiency. This is due to the use of higher concentration of emulsifier (polyvinyl alcohol in this case) during microparticle preparation. Polyvinyl alcohol enhances the solubility of ametryn in aqueous phase, which consequently reduces the association of the substance to the biopolymers (Sopeña et al. 2007; Fernandez-Urrusuno et al. 2000). Since lower percentage of the herbicide is associated or incorporated within the polymeric carrier, nonassociated portion of the substance will be released immediately at the plantation site, while the remaining portion will be released gradually with time. Slow release of herbicide was found useful in the agricultural practices.

The effect of encapsulation of ametryn into the polymer microparticles was physically examined by scanning electron microscope (SEM). The finding shows that PHB microparticles possessed a smooth surface with lower porosity, whereas PHBV microparticles showed an opposite outcome, a rough surface with higher porosity. Surface porosity could be related to the release mechanism of herbicide. Larger pores, will aid in the release of the herbicide molecules (Costa and Lobo 2001). The SEM micrographs were also analyzed and compared between the PHB and PHBV microparticles, as well as between PHB:AMT and PHBV:AMT

microparticles. In the comparison of PHB and PHBV microparticles (without microparticles), SEM micrograph analysis shows no significant difference in the average size distributions of 5.92 ± 0.74 μm and 5.63 ± 0.68 μm, respectively. This demonstrates a similar size distribution although the polymers used are different, which leads to the distinct characteristics of particle surface. However, there was an increase in size when the size distribution analysis was performed for both microparticles with ametryn. The average size of PHB:AMT and PHBV:AMT were 10.6 ± 0.43 μm and 20.5 ± 0.93 μm, respectively. This indicates an alteration of the size when the microparticles were associated with bioactive substances (Bazzo et al. 2009).

In vitro release assays were conducted for pure ametryn and ametryn encapsulated in PHA and PHBV microparticles. The release profiles as a function of time for each assay were plotted and compared. Kinetics analysis shows that pure ametryn was released at higher rate as compared to the one encapsulated in the microparticles. In 1.2 days, nearly 100 % of pure ametryn was released, meanwhile there were only 75 % and 87 % of ametryn released, respectively, for PHB:AMT and PHBV:AMT. The release efficiencies (RE, %) were further calculated for a better quantification of the release profiles (Khan and Rhodes 1975). The finding revealed that ametryn was released at a faster rate from the PHBV microparticles (84.6 %) as compared to the release from PHA microparticles (73.9 %). Grillo et al. (2011) suggested that the difference is due to the presence of plastification effect in the microparticles when PHB is associated with hydroxyvalerate (HV) monomer in PHBV. The plastification lowers the diffusion inside the microparticles, which latter increases the speed of the release of the associated ametryn. Gangrade and Price (1991) reported that even for PHBV with higher HV monomer contents, a reduced polymer crystallinity leads to a regular break in interchain, increasing the porosity which fasten the speed of the compound released. This is supported by the higher value of release constant, k, for PHBV:AMT (1.00 min^{-1}) calculated from Peppas model as compared to k of PHA:AMT (0.64 min^{-1}). Obtaining the release coefficient (n) of 0.83 and 0.90 for PHA and PHBV microparticles, the authors suggested that the diffusion process is not governed by Fick's law of diffusion but by the relaxation of polymer chains due to anomalous transport.

Lobo et al. (2012) also demonstrated the controlled release system for ametryn encapsulated in both PHB and PHBV polymers. In this study, both polymers were either used as individual or as a mixture. A total of 200 mg polymers were used in this study, rather than 100 mg as described by Grillo et al. (2011). The final concentration of ametryn was fixed at 50 mg/L after the end of microparticle preparation using emulsion solvent evaporation technique. The PHBV:PHB microparticles were prepared based on the different ratio of polymers in weight (mg): 200/0, 150/50, 100/100, 50/150, 0/200, which, respectively, corresponds to formulation A, B, C, D, and E. The finding shows that formulation A which purely used PHBV contributed to the highest encapsulation efficiency of 76.5 %. Whenever the PHVB portion in the polymer microparticles decreased, this is followed by the decrease of the efficiency. The microparticle which is made up of 100 % PHB was found to have the lowest encapsulation efficiency of 26.2 %. In this study, the

authors demonstrated that there was a positive polynomial relationship between the encapsulation efficiency of ametryn with respect to the PHBV concentration. Since the ratios used were different during the preparation, this is expected as the structures between the microparticles were different.

SEM analysis shows that all of the microparticles formed were spherical, with a clear distinction on the surface structures. This study is parallel with Grillo et al. (2011) which revealed that rough and high porosity was found on PHBV microparticles surface, but the surface of PHB microparticles is smooth with few pores. In this case, the former is true for the pure PHBV and PHBV rich microparticles, whereas the latter is similar with PHB and PHB rich microparticles. Since PHBV possessed higher encapsulation efficiency and formed more pores on the resulting microparticles surface, it was concluded that ametryn probably has greater affinity toward PHBV polymer. This is supported on the basis of the chemical structure in which both molecules possess alkyl branches, contributing to the porosity which enhances the interaction. The size distribution analysis shows that as the PHBV portion decreases, the average size of the microparticles increased. The pure PHBV microparticles have the lowest average microparticles size of 24.14 ± 1.61 μm, whereas the largest mean size of 110.2 ± 3.88 μm was obtained from pure PHB microparticles. It was suggested that since ametryn has higher affinity toward PHBV polymer, formation of linkage between the polymer chains leads to the shrinkage of the microparticles.

In the experiments of ametryn release profile, the free ametryn was almost released after 360 min. As compared to the microparticles with various formulations, the herbicide is not completely released at 360 min but the release percentage decreased from formulation A to E. This indicates that the presence of the pores on the PHBV microparticles surface contribute to the outflow of ametryn molecules as the contact with solvent increased. In this study, the kinetics of ametryn release was further analyzed and compared by using four different mathematical models, namely zero order, first order, Higuchi, and Korsmeyer–Peppas models. The analysis of release curve provides detail information on the release mechanisms of the herbicide from microparticles, which includes desorption from the microparticle surface, diffusion passing the pores or wall, and the degradation of the microparticle which release the compounds and also the dissolution and erosion of the polymeric materials (Polakovic et al. 1999). Therefore, the mathematical modeling provides better characterization analysis of the compounds released from the polymeric systems. Based on the comparison of various models, Korsmeyer–Peppas model was found to be the best model describing the release mechanism, according to the correlation coefficient obtained. The release exponent (n) was different for all formulations. Therefore, the release mechanisms were not similar. However, all the values obtained fall in the range of 0.43–0.85, which indicates that the release occurred does not obey Fick's law, but is due to anomalous transport, similar with the result obtained by Grillo et al. (2011).

Grillo et al. (2010) prepared atrazine-PHBV microspheres with 100 mg of PHBV with 10 mg atrazine using solvent evaporation technique. The prepared microparticles were then characterized by SEM, differential scanning calorimetry

(DSC), and in vitro release experiment. Besides, *Lactuca sativa* test was also performed to screen the genotoxicity of the prepared microspheres. It was found that the microparticles came with spherical and smooth surfaces. The encapsulation efficiency was about 32 %. The size distribution analysis of the SEM micrographs shows that there was no significant difference on the average size variation of PHBV and ATZ-PHBV, with the respective values of 5.97 ± 2.80 μm and 6.57 ± 3.10 μm. The melting point of ATZ, ATZ-PHBV, and PHBV microspheres were analyzed using DSC. The melting points of PHBV microsphere and pure atrazine were 170.4 and 181.9 °C, respectively. ATZ-PHBV microspheres showed a shift of melting point to a lower value of 167.2 °C, in relative to the pure PHBV microsphere. It was suggested that the distribution of herbicide in the polymer matrix has resulted in a decrease of the PHBV fusion temperature. The absence of the atrazine associated peak in the DSC chromatogram indicates that the herbicide is well dispersed in the polymeric matrix of the microparticles. To examine the sensitivity of the technique, DSC analysis was also performed for the physical mixture of atrazine and PHBV microspheres at 1:1 ratio. Since both atrazine and PHBV peaks were found on the DSC thermogram, the disappearance of endothermic peak of ATZ in ATZ-PHBV shows the interaction between two molecules, either by van der Waals interaction or by the hydrogen bonding that occurred between the carbonyls of the PHBV with the amino group of atrazine. The atrazine release experiment shows that the pure atrazine was completely released in aqueous medium after 1300 min. On the other hand, atrazine loaded in PHBV microparticles shows a significant difference on the amount of atrazine released. There was only 25 % left on the microspheres after 1300 min. It was suggested that a good dispersion of the molecules in the polymer matrix leads to a modification of the release kinetics. To determine the type of the mechanism involved in the release of atrazine, the release profile was fitted to Korsmeyer–Peppas model. A linear regression of the plot was obtained with the correlation coefficient of 0.992. The diffusion exponential, n was 0.16, whereas the release constant of ATZ-PHBV was 0.671 min^{-1}. Obtaining a lower value of diffusion exponential, the release of atrazine was influenced by diffusion through polymeric matrix and through water-filled pores on the PHBV microparticles.

In this study, Grillo et al. (2010) also performed the genotoxicity of atrazine on the *Lactuca sativa* (root meristem cells). It was found that the atrazine induces genotoxic effect on root meristem cells of *L. sativa*. The PHBV encapsulated with atrazine has inhibited the mitotic index and induced some chromosome aberrations such as bridges, fragments, and also chromosome lagging. However, the formulation still has lesser toxicity effect comparatively to free ATZ. Recently, Prudnikova et al. (2013) demonstrated the incorporation of herbicide Zellek Super into PHA, which functions as a carrier. Instead of using homopolymer P(3HB), copolymer P(3HB-*co*-10 %3HV) was chosen in this study since it was previously proven that the 3HV containing copolymer are more readily degraded in soil (Volova et al. 2007). Two different forms of herbicide delivery systems were demonstrated in this study, in microgranules and also in films. The microgranules were prepared by pouring the mixture solution of the copolymer and herbicide

dissolved in dichloromethane, into isopropanol to precipitate the copolymer in microgranules. The microcapsules were prepared with different polymer to herbicide mass proportion: 60:40 and 90:10. For the films, copolymer and herbicide were mixed at the proportion of 25:75 and 75:25, respectively, in dichloromethane solution.

The biodegradation of PHA microgranules in soil revealed the variation of weight loss which depends on the proportion of the herbicide incorporated. For polymer/herbicide ratio of 60:40, the residual weight was 87 % of initial but it was 56 % for the one of polymer/herbicide at the ratio of 90:10. With the experiment period of 42 days, it was found that a similar residual weight of 28 % of the initial mass was obtained. The molecular weight of the polymer decreased as the degradation of the polymer matrix occurred from time to time. There was an increase of the polydispersities, indicating an increase of fragments number with a difference in the degree of polymerizations. The X-ray structural analysis shows that there was an increase on the degree of crystallinity, suggesting that the amorphous phase (disordered phase) of the polymer is disintegrated at a faster rate, compared to the ordered phases. The reason of the phenomenon is suggested to have been due to the preference of the depolymerases to attack the amorphous regions of the polymer matrix, rather than the ordered phases (Jendrossek and Handrick 2002; Tokiwa and Calabia 2004). Biodegradation of the microgranules also revealed that the release rate of the herbicide is proportional to the portion of the herbicide. Apart from that, it was found that when the herbicide was loaded into polymer film (polymer to herbicide ratio of 75:25), the degradation of the polymer film was higher than that of the microgranules. Throughout the experiment, it was suggested that the carrier geometry greatly affects the release rate of the herbicide.

The study has also evaluated the effectiveness of the Zellek Super herbicide delivery system in killing the weeds, *Agrostis stolonifera* L. This study suggested that the herbicide loaded into microgranules works more effective than the conventional spraying methods on the weeds. Herbicide loaded into polymer films has resulted even better effectiveness on controlling the weed growth as compared to the herbicide loaded microgranules. The growth of the weeds was completely suppressed when the polymer films (polymer to herbicide ratio 25:75) were used. As a conclusion, this experiment demonstrated the construction of both microgranules and polymer films loaded with Zellek Super herbicide can be used to suppress weed's growth. As the final stage of the study, the abundance of the microorganisms in the soil was investigated when different herbicide delivery systems were used. The study is significant since the plant growth is associated with the root exudates which serve as nutrients for bacteria. Authors suggested that direct spraying method of the herbicide has caused the most significant inhibition on the rhizosphere bacteria; meanwhile, the herbicide loaded microgranules exerted least effect on the microorganism's abundance. This study shows an important construction of biodegradable herbicide delivery systems to reduce the effect on soil microorganism but at the same time it is able to maintain an optimum effectiveness against the target weed plants.

Biodegradable PHA was also applied in the controlled release system for pesticide. In the study carried out by Voinova et al. (2009), copolymer P (3HB-*co*-11 %3HV) was used as pesticide carrier for both α-hexachlorcyclohexane (HCCH) and lindane. There is a difference on the preparation in which the pellets were produced instead of microgranules and films. Although the polymers were ground in laboratory mill to form particles, the ground polymer was mixed with pesticides to obtain homogeneous powder, and subsequently pressed to form bulky pellets. The polymers to pesticide ratios for the polymeric matrices used were 60:40 and 40:60, respectively, for HCCH and lindane.

The microbiological analysis revealed that the soil containing the polymeric carriers consisted of higher titer of aerobic microflora. This is due to the presence of the copolymer which functions as nutrient which stimulates the bacterial growth activities. The abundance of microorganism in the soil samples with HCCH and lindane with polymeric material was 10.0×10^6 CFU/g soil, whereas only 8.7×10^6 CFU/g soil was found in soil samples without polymeric materials. For the pellets with HCCH, it was found that only 2–3 % of the insecticide was released for the first 30–40 days. However, the compounds started to release in larger amount on day 70–80 in which a concentration of 30 μg/g soil was found during this period, accounted for 10–12 % of release from the initial content. The maximum release of HCCH was achieved on day 40–55, with the maximum rate of 0.73 μg/day per gram soil. Generally, the study showed that the release of the pesticide is dependent on the types of polymer and also the polymer to pesticide ratio. Besides, the release rates of the particular compounds are affected by the pesticide content in the copolymer pellets. Since the abundance of microflora will affect the degradation of the copolymer in pellets, it was suggested that the release rate of the pesticide can be regulated by manipulating the polymer to pesticide ratio.

Suave et al. (2010) prepared microspheres of mixtures of homopolymer P(3HB) and poly(ε-caprolactone) (PCL), with pesticide malathion by emulsion solvent evaporation technique, at different weight to weight ratios: 0:100, 97:3, 95:5, 90:10, 80:20, 70:30, and 100:0. SEM was first used to observe the morphology of the microspheres unloaded with malathion. The analysis of SEM micrographs shows that rough and spherical surface was observed for both neat PCL and neat P(3HB). Meanwhile interwoven was presented on the surface of the P(3HB)/PCL with 70:30, 80:20, and 90:10. Among them, the ratio of 80:20 showed the most visible interwoven feature. It was suggested that the presence of the interwoven surface is related to the phased separation of the polymers, which is attributed by the low miscibility of P(3HB) and PCL at this composition, leading to the formation of the particular morphology. At the composition ratio of 95:5 and 97:3, the surface of the microspheres was similar to the neat P(3HB) which presented homogeneous and rough surface. In a comparison with the microspheres loaded with malathion, the morphology was found similar for neat P(3HB) and PCL. The analysis of microsphere size distribution shows a broad range of sizes (ranging between 75 and 1200 μm) obtained for the unloaded neat and blend microspheres. The effect of malathion can be seen in such a way that more smaller and homogeneous

microspheres were obtained, regardless of the polymer composition. The microspheres formed were in the size range from 200 to 500 μm. The DSC analysis of unloaded blend microspheres showed an influence toward the T_m, with a reduction from 64 to 58 °C when P(3HB) was added into PCL. The influence of malathion on the thermal properties of the blend microspheres was also observed. Reduction of T_m of PCL suggested that malathion and P(3HB) contributed to synergistic effect, due to the presence of the affinity between malathion and P(3HB).

In this study, authors reported the use of release experiment of malathion from the blend microspheres. The results showed that the composition ratio of 70:30 of P (3HB):PCL blend microspheres has the fastest kinetics of release; meanwhile the ratios of 95:5 and 97:3 possessed slower rate of release profile. The 80:20 blend microspheres showed faster release at similar stage, yet become slower after 100 h of the assay. The fastest kinetics release obtained for 70:30 blends was most probably due to the presence of greater degradation of the microspheres. As the polymers are immiscible, the degradation was facilitated by the amorphous region of the blends. The P(3HB)/PCL blend microspheres with the ratio of 90:10, 95:5, and 97:3 have resulted in the slow release of malathion. In general, it was observed that the duration of malathion release was proportional to PCL concentration found in the blend microspheres.

Besides, copolymer P(3HB-*co*-3HV) can also be used in agriculture as controlled release of insecticides. The process involves integrating the insecticides into P(3HB-*co*-3HV) pellets and it will be sown along with the farmer's crop. This insecticide then will be released at a rate related to the level of pest activity. This is because the bacteria breaking the polymer would be affected by the same environmental conditions as that of the soil pests (Yogesh et al. 2012). Therefore the usage of biodegradable polymer as pesticides and insecticides has the ability to control the slow release of this chemical on the plants.

PHAs have also been used in aquaculture industry especially as a solid substrate and biofilm carrier for denitrification. Besides, it has also been applied as antibacterial agent and act as biomimetic adsorbents for wastewater purification.

Nowadays, application of PHAs in the modern aquaculture is intensively in progress. One of the applications of PHAs in aquaculture is as solid substrate and biofilm carrier for denitrification in recirculated aquaculture systems (Rijn 1997; Boley et al. 2000). Solid and carbon waste removal and stringent control of pH and CO_2 are focused in the modern technology of water treatment in recirculating systems (Fig. 4).

Conversion of ammonia to nitrite and nitrite to nitrate is a biological process which is known as nitrification whereas process of denitrification involves removal of nitrate through reduction to nitrogen gas. Denitrifying filter is one treatment system used for denitrifying wastewater and to remove suspended solids from the effluent. A conventional technique that involves addition of organic carbon source such as ethanol and acetic acid to a denitrification reactor is not feasible because this treatment requires a close, sophisticated, and costly process control. The risk of overdosing and the need for an in-depth knowledge about the operation of this

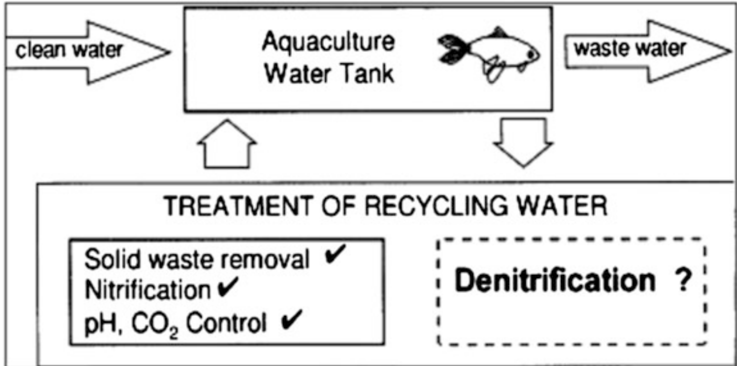

Fig. 4 Scheme of a modern recirculated aquaculture system (Boley et al. 2000)

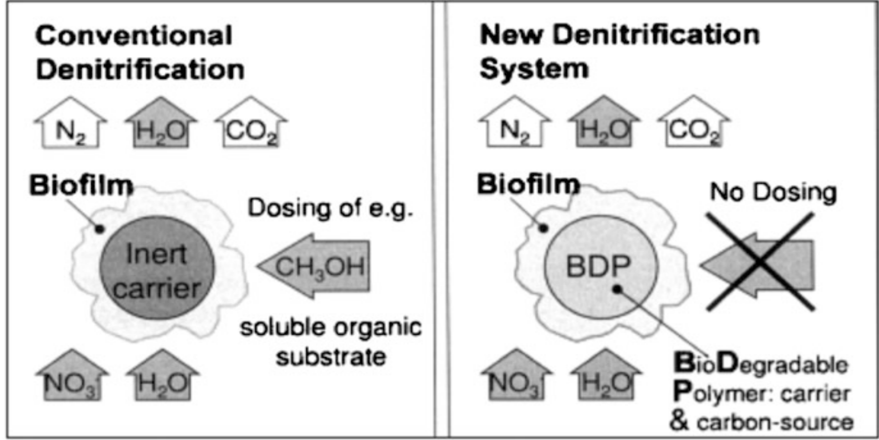

Fig. 5 Denitrification processes with different organic substrates (Boley et al. 2000)

biological system also hampered the development of conventional treatment (Rijn 1997; Boley et al. 2000; Rijn et al. 2006).

Therefore, researchers have attempted to develop a simpler denitrification process whereby biodegradable polymer pellets are used as solid substrate and biofilm carrier by microorganisms. In this system, external supply of soluble organic substrate is not required for the denitrification process (Boley et al. 2000). Figure 5 elucidates the difference between conventional and new denitrification systems.

Boley et al. (2000) had presented a comparison of the polymer-based denitrification with conventional denitrification processes. It was reported that poly (3-hydroxybutyrate) [P(3HB)] and polycaprolactone (PCL) showed lower volumetric and surface related denitrification rates compared to the use of methanol and ethanol as carbon sources. However, a system based on insoluble solid substrates as carbon source still holds a promising application in aquaculture system

as it is an easy to handle process and will reduce clean water requirement, waste water production and energy consumption. According to Hiraishi and Khan (2003), the potential risk of releasing organic carbon source with the resultant deterioration of effluent water quality could also be prevented by using PHAs as solid matrices. Application of PHAs in denitrification process will become more feasible if the PHA production cost is more economical.

PHAs are more preferred as solid substrate in solid-phase denitrification compared to other biopolymers because PHAs serve as microbial storage materials and are expected to be metabolized by a wide variety of PHA-degrading denitrifying microorganisms. Majority of the PHA-degrading denitrifying bacteria are clustered under β-proteobacteria. Detailed studies on the biodiversity of PHA-degrading denitrifiers are necessary to develop an efficient nitrogen-removal process using a particular microorganism. In addition to that, biodegradation characteristic of the polymers are of primary importance in the solid-phase denitrification process. *Paracoccus denitrificans* is one of the well-known denitrifiers that has intracellular PHA depolymerase and had been identified as suitable model to study the metabolic and regulatory relationship between PHA degradation and denitrification. It was reported that P(3HB) and P(3HB-*co*-3HV) polymers were degraded faster under denitrifying conditions compared to poly(butyrene succinic acid), poly (e-caprolactone), and poly(lactic acid). Higher denitrification rates were also observed for co-immobilized cells with PHA granules than suspended cells (Hiraishi and Khan 2003).

Although the effect of environmental conditions on solid-phase denitrifications using PHAs was not studied in detail, laboratory experiments conducted using *Diaphorobacter nitroreducens* strain NA10B by Hiraishi and Khan (2003) revealed that denitrification with P(3HB-*co*-3HV) copolymer as the substrate took place at 15–40 °C and pH range of 6–9. Strain NA10B is also capable of denitrifying using PHAs under aerobic conditions. Physicochemical properties of the PHA such as crystallinity, content of additives, and surface structure are also important factors affecting the PHA denitrification process. However, significant correlation between the denitrification activity and the melting temperature of the PHAs which was used as measurement for the degree of crystallinity was not observed. P(3HB-*co*-3HV) copolymer pellets with HV monomer composition range from 7 to 22 mol% were used to study the denitrification process using *Acidovorax* sp. strain 2nIII. Highest denitrification rate was observed for P(3HB-*co*-7 %3HV). However, a clear correlation between denitrification efficiency in polymers with different composition of HV monomers could not be obtained. Although the surface area of PHAs could possibly affect denitrification efficiency, but there was no difference in microscopic images of the uncolonized areas of P(3HB-*co*-7 %3HV) copolymer among different lots of PHA pellets. Microbial colonization on PHA surfaces could also be affected by other factors such as hydrophobicity, structure of the cell surface, and extracellular polymer substance (Biedermann et al. 1997).

Qin et al. (2005) had attempted to investigate the potential role of microbial PHA granules storage in denitrification process by studying its behavior under aerobic–anaerobic conditions using sequencing batch reactor (SBR). Scarce amount of

information is available on the role of PHAs as carbon source for denitrification process. SBR provides flexible operation, easy shifts among aerobic, anoxic, and anaerobic conditions as well as efficient selection of desired microbial population which makes it desirable for the intensive studies involving biological nitrogen removal. Five batches of experiments with various availabilities of external carbon source and nitrate were carried out under anaerobic conditions after microbial granules were successfully developed and matured in the alternating aerobic–anaerobic SBR. It was revealed through microscopic observation that microbial granules exhibited more compact with a spherical outer shape and denser structure than conventional activated sludge. In the aerobic reaction, about 95 % of influent chemical oxygen demand (COD) was removed within the first 30 min which was accompanied by gradual consumption of ammonium. The concentration of ammonium started to decline abruptly after the COD depletion. However, only about 14 mg nitrate-nitrogen/L was denitrified in the anaerobic phase in the absence of external carbon source. PHAs were utilized only for cell maintenance in the absence of external carbon and nitrate. Addition of sufficient ethanol as the external carbon source into the SBR at the beginning of the anaerobic phase had led to complete denitrification. This finding indicated that availability of organic carbon source had significant influence on the denitrification efficiency of PHA microbial granules. Increase in the denitrification rate in activated sludge had been reported with the supply of an easily degradable carbon sources.

Qin et al. (2005) also reported that PHA microbial granules were utilized at a constant rate when external organic carbon and nitrate were not provided in the anaerobic phase. This finding could be attributed to the anaerobic endogenous respiration of microbial granules. PHA microbial granules were utilized at a higher degradation rate when nitrate was added in the medium but with the absence of external organic carbon source. This observation implied that PHA microbial granules could serve as carbon and energy source for microbial functions and denitrification. In the presence of excessive organic carbon and limited or without nitrogen, cell growth would be suppressed and carbon would be used to synthesize PHA as intracellular storage energy. Under this condition, simultaneous actions took place whereby external carbon source was consumed for denitrification whereas PHA storage was used for cell growth and maintenance. According to the published data by Qin et al. (2005), PHA microbial granules were found to serve as carbon and energy source only when external carbon was no longer available. A readily biodegradable carbon source, ethanol exhibited 2–5 times higher denitrification rate compared to PHA. It was also reported that only less than 28 mg nitrate-nitrogen could be denitrified using PHA as internal carbon source in the batch experiments as well as in the parent SBR. Preference of microorganisms to utilize PHA microbial granules as internal carbon and energy source for cell functions rather than for denitrification was observed when the microbial PHA granules decreased to below a critical level. This finding was further affirmed by prolonging cultivation of batch 4 from 5 to 25 h. Medium in the batch 4 was added with ethanol and nitrate. Oxidation of total organic carbon (TOC) was coupled with the accumulation of PHA until all TOC was depleted after 2 h of anaerobic reaction. The

reaction was followed by reduction in nitrate which was coupled with the degradation of PHA until 8 h of anaerobic reaction. However, nitrate could hardly be reduced to nitrogen during 8–25 h and PHA microbial granules were gradually degraded solely for anaerobic endogenous respiration. When PHA microbial granules were required to survive under starvation condition, anaerobic degradation of PHA was dissociated from denitrification. Therefore, the efficiency of PHA as reducing power for denitrification would not be overvalued.

Gutierrez-Wing et al. (2007) had carried out experiments to determine the efficiency of PHAs as nonsoluble carbon source for bacterial denitrification with particular emphasis in recirculating water systems. Acclimation times at different salinities, effect of PHA molecular weight on denitrification rates, and bioplastic consumption were also analyzed in this experiment. PHAs used were P(3HB) supplied by Metabolix Inc. and identified as MBX-A, MBX-B, MBX-C, and MBX03-03-04-00. It was revealed that the oxygen removal was significantly different but the polymers showed no apparent difference in the removal of nitrate. Higher surface area of the polymers (552–930 m^2/m^3) might attribute to the differences in the oxygen removal. It was suggested that the molecular weight of the polymers could support the denitrification process at a similar rate. As the biofilm thickened, material hydrolyzed by the bacteria would be captured by biofilm and tend to reduce their growth. This action offered advantage in the passive denitrification and also reduced the need for aggressive cleaning.

MBX03-03-04-00, a P(3HB) homopolymer was selected to study the effect of different salinities on the denitrification rates. Although the average denitrification rate was slightly higher for 15 ppt and lower for 0 and 30 ppt salt concentration but the differences were not statistically different. However, higher denitrification rate was observed when the nitrate concentration was high usually above 100 mg nitrate-nitrogen/L but not for the 0–50 mg nitrate-nitrogen/L. Theoretically, 2.54 g of P(3HB) polymer consumed per gram of nitrogen conversion. The experimental results showed higher value (2.92 g) which could be attributed to the consumption of residual oxygen in the water. It was also reported that high PHA consumption during deoxygenation suggested that reducing the amount of oxygen in the denitrification bed would increase the efficiency of the denitrification process. At oxygen concentration of 4.5 mg/mL, denitrification could still occur which might be due to lower oxygen concentrations near the bead or in the media bed. Some authors have reported denitrification at oxygen concentration as high as 5 mg/mL, but it occurs at a very slow rate (Gutierrez-Wing et al. 2007).

Gutierrez-Wing et al. (2014) had developed a model of the denitrification on a PHA-based reactor for recirculating aquaculture. Various factors that control the denitrification process such as nitrate concentration, dissolved oxygen, organic carbon, biomass concentration, temperature, and salinity have been taken into consideration in the modeling of PHA-based reactor. The model was based on a maximum volumetric denitrification rate (VDR_{max}), maximum oxygen consumption rate (VOR_{max}), and a limitation on the maximum bacterial growth rate. The denitrification process and nitrate consumption rate were inhibited by the oxygen concentration. Facultative anaerobic bacteria which can use oxygen and nitrate as

electron acceptor were used as denitrification biomass. Since oxygen is a more favorable electron acceptor, it would be utilized first followed by nitrate.

It was reported that the length of bacterial exponential phase was affected by the oxygen half saturation constant K_o, but negligible effect was observed on the denitrification slope or the PHA consumption. The slope of the nitrate removal and PHA consumption curves were affected by the nitrate half saturation constant, K_n. In the soluble carbon denitrification systems, bacterial growth was promoted away from the support medium by the carbon supplied from the water. Diffusion efficiency would influence the transference to the internal part of the biofilm. In contrast to the soluble carbon denitrification system, PHA-based system allowed hydrolysis of carbon in the surface of the support substrate. The bacteria would be trapped within the biofilm if they hydrolyzed more carbon than needed. A thinner biofilm was formed due to distance limitation whereby bacterial growth was not visible away from the support. The variability on the denitrification rates was reflected on the model. Changes in the bacterial growth parameters as well as abundance of PHA-denitrifying bacteria contributed to the variation on denitrification rates. Denitrification rate was not significantly affected by the PHAs but it was suggested that the differences in efficiency and substrate consumption could be observed if other solid carbon source with different hydrolysis behavior or configuration was used in the place of PHAs. The developed model would serve as a tool to predict the denitrification rates with different size reactors, the required size of denitrification units and PHA recharging time depending on the expected nitrate loading and the desired time between PHA recharges. The simulation of the developed model predicted removal rate of 600 mg NO_3^- N d/L with 201 $cm^3$of PHA as initial volume for a volumetric rate of 2.97 kg NO_3^- N m^{-3} d/L (Gutierrez-Wing et al. 2014).

One of the main hurdles in the development of aquaculture sector is disease outbreaks especially infection by *Vibrios* which gives negative impact on the rearing of molluscs, finfish, lobsters, and shrimp. It was revealed that the short-chain fatty acids (SCFAs) PHAs were able to inhibit the growth of a virulent *Vibrio campbellii* strain in vitro. In addition to that, PHAs could also be added to the animal feed because few studies had proven that this biodegradable polymer was degraded when it passed through the gastrointestinal tract of animals. Therefore, inhibitory effects of PHAs on pathogenic *Vibrio campbellii* strain and its positive effect on the survival of infected *Artemia* nauplii were evaluated. The protective effect of the polymers was tested by delivering the particles of PHAs into the gut of *Artemia* (Brine shrimp). The experiments showed that inhibition of *Vibrio campbellii* was pH-dependent and the strain was completely inhibited at pH 6. Besides that, the survival of infected *Artemia* nauplii was also improved through the addition of 10 g/L of PHAs into the culture water. The effectiveness of PHAs particles in improving the survival of infected *Artemia* nauplii was observed at 100 mg/L, indicating that PHA particles were 100 times more efficient. It was assumed that fatty acids were released in the guts of *Artemia* when the PHA particles were degraded into β-hydroxybutyrate. The survival of infected *Artemia*

nauplii could be improved more efficiently if PHA particles with smaller size were used (Defoirdt et al. 2007).

Nhan et al. (2010) had studied the effect of PHA on larvi culture of the giant freshwater prawn *Macrobrachium rosenbergii* which is a commercially important aquaculture species. The expansion of this prawn farming is always hampered by the quality of hatchery-reared seed and mass mortality of larvae in the hatcheries. Numerous outbreaks of disease in prawn are caused by opportunistic pathogenic bacteria such as *Vibrio* which resulted in the disruption of the regular production of high-quality post-larvae. The use of antibiotics and disinfectants to control bacterial growth in the prawn hatcheries are getting less attention due to public health and environmental concern. Currently, SCFAs have been employed as eco-friendly biocontrol agents to maintain the animals' health and it has been reported that SCFAs could significantly increase the survival of challenged brine shrimp *Artemia* nauplii. However, high concentration of SCFAs which is required to protect the animals had led to the excessive heterotrophic bacterial growths that negatively affect the animal health.

Therefore, PHAs have been considered as an alternative to antibiotics and short-chain fatty acids. In this study, the effect of PHAs on the survival and growth of larvae of the giant freshwater prawn *M. rosenbergii* and on the microbiota (total bacteria and *Vibrio* spp.) was explored by administering the PHAs through the live food (*Artemia* nauplii). A lipid enrichment technique which usually employed in many fish and crustacean hatcheries was combined in the loading of the *Artemia* nauplii with PHA. Lipid enrichment technique involves feeding the larvae with a lipid emulsion rich in highly unsaturated fatty acids. Apart from serving as major source of metabolic and storage energy, lipid also supplies essential fatty acids required for the maintenance and integrity of cellular membranes as well as precursors of steroid and moulting hormones (Nhan et al. 2010).

Fluorescent microscopy analysis affirmed the accumulation of PHA particles in the gut of *Artemia* (Fig. 6). Larval stage index revealed significant improvement in the growth of larvae through PHA treatment compared to treatment without addition of PHAs. However, highest survival rate of larvae was observed through the treatment with both PHAs and lipid enrichment. The treatments with either PHAs or lipid enrichment alone also improved the larvae survival rate but less pronounced than the combined treatments and exhibited almost similar survival rate.

The author also concluded that larval survival rate was not influenced by the interaction between PHAs and lipid enrichment. However, significant interaction between feeding with PHA and lipid emulsion was revealed with respect to the microflora population density inside the larval gut. The number of bacteria and *Vibrio* spp. in the larval gut was initially 3.2×10^4 and 0.2×10^2 CFU/larva, respectively. At the end of the experiment, the lowest growth of total bacteria and *Vibrio* spp. of 5.6×10^4 and 0.3×10^2 CFU/larva, respectively, was observed for combined treatment of PHA and lipid. In the control treatment (without addition of PHAs and lipid), the final total bacterial growth was 6.8×10^4 CFU/larva, whereas for *Vibrio* spp. it was 13.3×10^2 CFU/larva. The highest total bacterial growth and *Vibrio* spp. was observed in the treatment with lipid alone which supported the

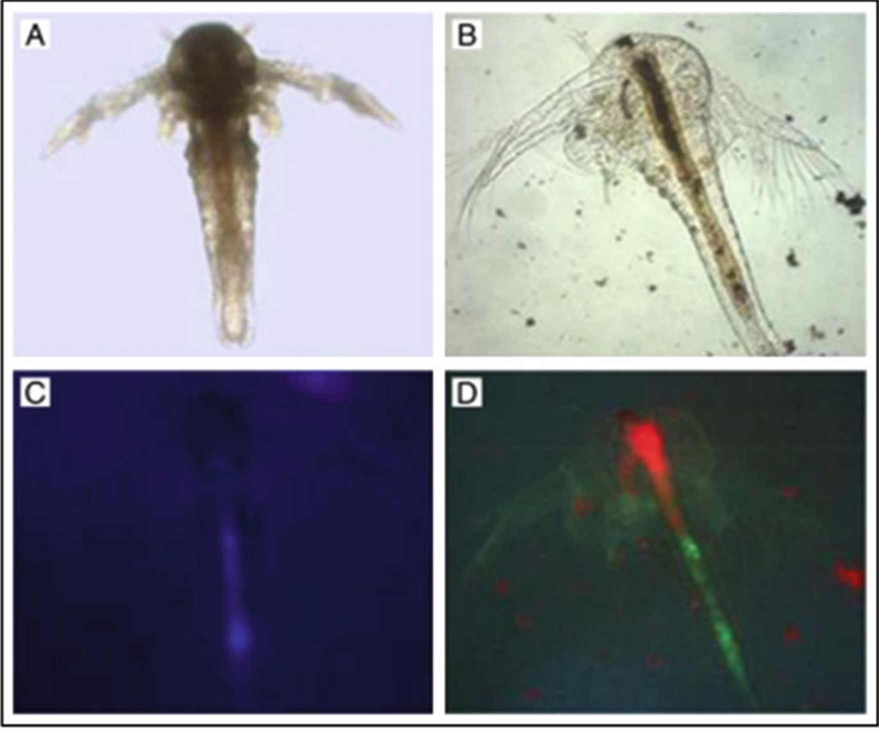

Fig. 6 Representative light (*upper row*) and epifluorescence microscopy (*lower row*) images of Nile Blue A-stained *Artemia* nauplii after 15 min without (panels **A** and **C**) and with (panels **B** and **D**) PHB added to culture water at 5 g/L (Nhan et al. 2010)

claim that SCFAs encouraged excessive heterotrophic bacterial growth. Finally, it was concluded that overall best culture performance was obtained using addition of PHAs and lipid emulsion as it resulted in the significantly improved larval survival as well as larval development. Optimal PHA concentration and formulation for encapsulation into *Artemia* should be explored further to improve economical efficiency of the larval production (Nhan et al. 2010).

Various articles have reported about the controlling effect exhibited by PHAs on the gastrointestinal microbiota which positively impacts the aquaculture industrial particularly in the rearing of prawn and brine shrimp. Despite that, the mechanism that involved in the action of PHAs on the intestinal microbial community of the treated animals has not yet been explored in depth. In the aquaculture industry, manipulation of the gut microbiota has been given considerate attention to enhance growth, feed digestion, immunity, and disease resistance of the host organism. Therefore, the effect of PHAs on the behavior of the gut microbial community in juvenile European sea bass had been studied in detail to provide greater insight on the increased resistance of aquatic organisms against infections; whether the

resistance against pathogen was due to antimicrobial effect of PHA monomers or development of a core group of bacteria feeding on PHAs (De Schryver et al. 2011).

According to De Schryver et al. (2011), PHAs and its degradation products had created an unfavorable intestinal environment for the long-term establishment of invading microorganisms. The experimental data obtained also revealed that similar microbial composition had been observed in the PHA-treated fish, although the rate of changes was higher. Equal abundance within the core group of microorganisms stimulated by PHAs increased the resistance of fish against invasion of pathogen as each member had equal chance to prominently function in the microbial community. In respect to the convergence of the gut microbiota composition and relative abundances, it was concluded that PHA compounds warrant more attention to provide resistance against infections in aquaculture rearing.

"Biofloc technology (BFT)" is an environmental friendly aquaculture system which is based on the growth of microorganisms in the culture medium. It is one of the efficient alternative systems because minimum water exchange is required and the nutrients could be recycled continuously. The microorganism which is known as biofloc plays two major roles: (1) to maintain quality of water through utilization of nitrogen compounds and (2) to improve culture feasibility through reduction of feed conversion ratio. Biofloc could also be employed as one of the novel strategies for the management of disease. The "natural probiotic" effect in BFT could act against pathogenic *Vibrio* sp. either internally or externally. About 80 % of the bacteria's cell dry matter and up to 16 % of biofloc dry weight comprises PHA polymer. The PHA polymer itself could prevent or protect against infection of *Vibrio* sp. and stimulate the growth as well as the survival of shrimp and fish larvae. However, the mechanism that involves in the antibacterial activity of PHA was not well understood. Therefore, further research was required to characterize and analyze biocontrol efficacy of PHA in different host–microbe systems and to maximize PHA content in bioflocs applied, such as in fish or shrimp feed (Emerenciano et al. 2013).

The adsorption technique has been found to be effective in the purification of the wastewater containing trace amounts of organic contaminants (TAOCs) because the adsorbents have high enrichment capacity toward the contaminants. Enrichment of adsorbents allowed the decomposition and detoxification of the contaminants using biological and chemical methods. The idea of developing biomimetic adsorbents was derived from the enrichment of TAOCs in natural water by aquatic organisms such as fish and shellfish. Most of the enriched organic contaminants were found in the brain tissue which is composed of lipid, suggesting that excellent adsorption capacity toward TAOCs was attributed to the materials of biological lipids. A new biomimetic adsorbent with high porosity was successfully prepared using lipid derived P(3HB) homopolymer produced by pollutant degrading microorganisms and named as PHBBMA. Chlorobenzene (CB) and *o*-nitrochlorobenzene (*o*-NCB) are among the organochlorine compounds that are always released to the environment as a carrier, solvent, or intermediates. They are easily enriched in lipid of organisms due to their strong hydrophobic and lipophilic properties. Adsorption capacity of PHBMMA was evaluated using CB and *o*-

NCB at low concentration. It was reported that the adsorption amount of CB was always greater than that of *o*-NCB under the same conditions. The absence of hydrophilic group (–NO_2) in CB contributed to its stronger affinity toward PHBMMA. The process of PHBBMA adsorption is a physical adsorption which is indicated by the presence of van der Waals force and hydrophobic force between the PHBBMA and CB or *o*-NCB (Wei et al. 2013).

4 Conclusion

The contribution of plastic materials to the development of agricultural and aquacultural sector is stimulated by their increasing use. The solution to the problem of agricultural and aquacultural plastic waste was solved through the development of biodegradable plastics produced by microorganisms. Industrial research based on biodegradable polymers for agricultural and aquacultural applications has induced the introduction of several materials in the market. In agriculture, the usage of biodegradable plastic mulch has become very important in mineralization of the fragments buried into soil. The usage of PHAs as pesticides and as bacterial inoculants has also geared the development of biodegradable polymer in these industries. The development of bioplastic industry is crucial for the adaptation of this polymer as a carbon source for denitrification processes. The usage of PHAs in aquaculture has advantages such as user friendly simplicity and the process is known to be safe. Besides, usage of PHAs as anti-infective would provide ecologically and economically sustainable alternative strategy to fight infections in aquaculture industry. In conclusion, the PHAs produced by microorganisms have a wide variety of applications in agricultural and aquacultural industries and it is emerging as the next generation of environmentally friendly materials.

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Application of Biofloc in Aquaculture: An Evaluation of Flocculating Activity of Selected Bacteria from Biofloc

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Abstract Over the last decade, synthetic flocculants such as polyacrylamide, polyethylene amine, and aluminum sulfate have been widely used as flocculating agents in wastewater treatment process. Despite their effective flocculating activity, there are problems regarding their safety status in terms of high potential to cause carcinogenic and neurotoxic effects on human health. Therefore, natural flocculation process, a new biological method known as bioflocculation, offers an alternative way for safety water treatment especially in aquaculture industry. Bioflocculation (or biofloc) is a dynamic process resulting from the synthesis of extracellular polymers by flocculant producing microorganisms. Hence, this study was aimed to assess the bioflocculant production by 13 marine bacteria which were isolated from the Integrated Shrimp Aquaculture Park owned by Blue Archipelago Berhad at Setiu, Terengganu. In this study, screening of bioflocculant-producing bacteria was carried out and the flocculating activity of bioflocculant-producing bacteria was measured based on the day of culture (DOC): 0, 30, and 70 days. A total of 5 g/L kaolin suspension was used to measure the flocculating activity of each bacteria species using kaolin clay suspension method. Isolates of bioflocculant-producing bacteria were identified using Gram staining method and a series of biochemical test. There were dominant species of bioflocculant-

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producing bacteria such as *Corynebacterium kutscheri*, *Corynebacterium xerosis*, *Klebsiella pneumoniae*, *Lactobacillus fermenti*, *Staphylococcus aureus*, *Bacillus azotoformans*, *Staphylococcus* spp., *Streptococcus* sp., *Bacillus* sp., *Vibrio* sp., *Neisseria* sp., *Serratia* sp., and *Yersinia* sp., which were identified in the biofloc samples. Among all identified bioflocculant-producing bacteria, *Streptococcus* sp. showed the highest (14 %) flocculating activity. Therefore, the bacteria that show best flocculating performance in terms of the highest flocculating activity could be suggested as potential bioflocculant agents in treating wastewater from aquaculture industry.

1 Introduction

Aquaculture industry has always been identified as one of the major contributor in environmental pollutions, particularly in aquatic environments. To meet human demands for seafood consumption, rapid growth of aquaculture industry had caused an increase in the environmental pollution along Malaysian coastal area for decades and may cause defects in aquaculture industry (Norazida et al. 2011). The farming of aquatic organisms such as mollusks, fishes, shrimps, crustaceans, and seaweeds requires a huge number of chemicals and nutrients which will give a serious impact to the surrounding environment. Aquaculture production can generate considerable amounts of effluent, such as waste feed and feces, medications, and pesticides, which can have undesirable impacts on the environment (Gowen and Bradbury 1987; Ackefors and Enell 1994; Wu 1995; Axler et al. 1996; Kelly et al. 1996; Read and Fernandes 2003). For instance, nitrogenous compounds such as ammonia (NH_3), nitrite (NO^{2-}), and nitrate (NO^{3-}) are usually considered as major contaminants derived from the aquaculture wastewater (Cao et al. 2007). To minimize the risks of downstream pollutions, large quantities of wastes that contain solid forms such as feces and uneaten feeds can be used by beneficial microbes and will easily settle down in the production pond.

Instead of massive construction of closed systems for aquaculture production, open systems such as cage farming may contribute to more serious environmental effects on the receiving water bodies. Being an essentially open system, cage farming is usually characterized by a high degree of interaction with environment and cage systems produce large bulk of wastes that are released directly into the environment (Cao et al. 2007). The increased amount of waste and nutrient might increase the potential for eutrophication phenomenon. Eutrophication problem resulting from the discharge of nutrients and organic matter derived from feces and uneaten feeds in the aquaculture ponds are common (Alabaster 1982; Eng et al. 1989). Many researchers and government authority departments are trying to solve the problem of environmental pollution. There is a conventional method known as Recirculating Aquaculture System (RAS) that had been applied

previously by farmers to solve this problem. This RAS is commonly used to manage the wastes from the aquaculture ponds by removing fish wastes and uneaten feeds by recirculating the water back into the ponds. Daily flows emanating from the fish farms coupled with cleaning events that are performed to reduce suspended solids and improve water quality within an aquaculture system can result in significant discharge of waste material (Summerfelt et al. 1999; Sharrer et al. 2007). To construct a RAS, however, is costly and its capacity to concentrate the particulate waste materials is relatively low. Thus, an environmentally friendly method was introduced in this study, i.e., biofloc technology as an alternative way to overcome this problem.

Biofloc technology has recently become an effective and sustainable method to enhance water quality through the addition of extra carbon sources into the aquaculture pond (Crab et al. 2010, 2012). Their addition will stimulate the growth of heterotrophic bacteria which will then promote the nitrogen uptake through the production of microbial protein. As a consequence, the ammonium concentration will decrease more rapidly as compared to natural nitrification process. Feeding on organic matter that is dissolved in water in the presence of oxygen together with bacteria decomposes organic matter to carbon dioxide and water will transform a part of organic matter into its own biomass (Fig. 1). As a result, the particulate matters in the culture system such as fish excreta, unfed food particles, and bioflocs can be easily removed. These solid particles are usually separated from the fish culture water using physical filtration process and then will be discharged as sludge (Suh et al. 1997).

In biofloc system, bioflocculation is the most widely used process for treating the wastewater from aquaculture industries (Rossini et al. 1999; Zaki et al. 2011). Bioflocculation process is an effective and convenient technique to remove suspended solids, colloids, and cell debris (Zhang et al. 2012). Recently, bioflocculant has widely attracted and obtained attention from researchers around the world where this technology can be considered as cheap and simple technique which can also act as an agent that triggers the aggregation of microorganisms to form bioflocs. Most of the bioflocculant is derived from the natural secretions of bacteria or cell lysis (Liu et al. 2009; Cosa 2010) such as extracellular biopolymers of macromolecular substances (e.g., glycoproteins, proteins, polysaccharides, and

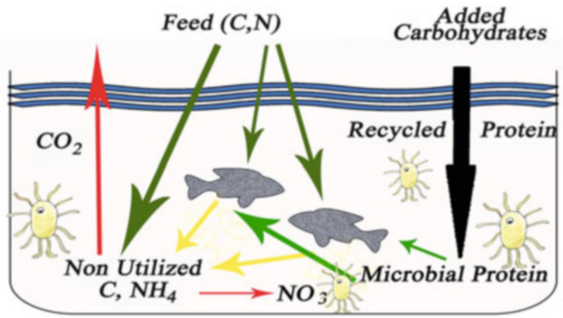

Fig. 1 Scheme of biofloc technology in aquaculture pond. Photo credited to Avnimelech (2013)

nucleic acids) (Lian et al. 2007; Zheng et al. 2008; He et al. 2010; Cosa 2010). Types of bioflocculants that are produced by the bacteria have different flocculating activities. Bioflocculants have been reported to have special advantages such as safety, strong effect, biodegradable, and harmless to humans and the environment (Kurane et al. 1986; Deng et al. 2005; Abd-El-Haleem et al. 2008). However, there is still lack of study on finding the best bioflocculant substances that do not pose risk to the environment and might be potentially applied for wastewater treatment especially in aquaculture system. Therefore, to begin the search, it is crucial to isolate and identify various types of bacteria from biofloc samples with high bioflocculant-producing ability and can be suggested as potential bacteria to be used as bioflocculants in aquaculture industry.

2 Methodology

2.1 Biofloc Sample Collection

Biofloc samples were collected from the Integrated Shrimp Aquaculture Park (iSHARP) of Blue Archipelago Berhad at Setiu, Terengganu (Fig. 2). The iSHARP production area encompasses the total area of 1000 ha which have been constructed in two phases: Phase One and Phase Two. Phase One of the iSHARP comprises

Fig. 2 Location of Integrated Shrimp Aquaculture Park (iSHARP) of Blue Archipelago Berhad at Setiu, Terengganu, Malaysia (in the *red circle*)

216 module ponds, whereas Phase Two comprises 400 module ponds. The biofloc samples were collected from the ponds during the day of culture (DOC) of 0, 30, and 70 days for a complete cycle of Whiteleg shrimp, *Penaeus vannamei* production. Two liters of samples were collected from the subsurface of *P. vannamei* ponds using 1 L Imhoff cone (Hargreaves 2006; Hargreaves 2013). The samples were put into sterilized pre-acid washed plastic bottles in order to minimize contamination and were brought back to the laboratory for further process.

2.2 Preparation of Biofloc Samples and Isolation of Bacteria

In the laboratory, biofloc samples were poured into centrifuge tubes for centrifugation at 6000 rpm for 3 min (Vijayalakshmi and Raichur 2002) to ensure all bacteria settled at the bottom of the tubes. The pellets which contain biofloc samples were collected and were used for bacterial isolation purposes.

All bacteria that might exist in the pellets were isolated using nonselective marine agar as the cultivation medium as described by Zaki et al. (2013). Firstly, the sample of bioflocs was streaked on marine agar plates using inoculation loop to allow the growth of different types of bacterial colonies. All the plates were then incubated at 30 °C for 24 h. After 24 h, the bacterial colonies which appeared on the plates were further subcultured in zig zag lines (Fig. 3) until the pure culture of single colony of bacteria was obtained. Pure cultures of these bacteria were obtained by a series of subcultivation on fresh marine agar plates (Fig. 4). Each different colony morphology was maintained on marine agar slants as stocks. The stocks were kept in refrigerator at 4 °C as described by Thiel (1999a, b) for further process.

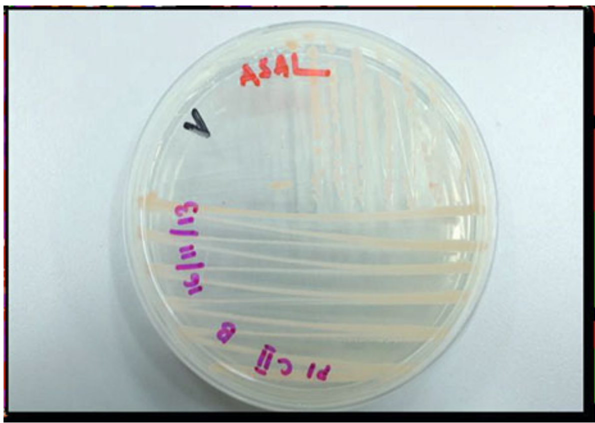

Fig. 3 Subculturing of biofloc samples in zig zag lines as a part of process to obtain pure culture bacteria using marine agar plate

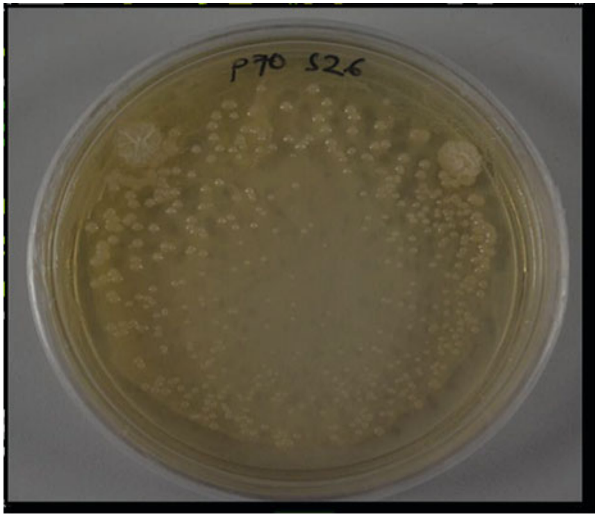

Fig. 4 Growth of pure bacteria isolates after several subcultivation in marine agar medium

2.3 Identification and Screening of Bioflocculant-Producing Bacteria

Identification of each bioflocculant-producing bacteria was carried out including differentiation between Gram-positive and Gram-negative bacteria. All bacteria isolates were undergone a series of biochemical tests in order to identify the bacteria (Rachel 2013). In particular, the Gram-positive bacteria were tested for spore staining, acid fast test, catalase test, starch hydrolysis test, citrate test, strict anerobes test, mannitol fermentation test, hemolysis test, and bile esculin test, while the Gram-negative bacteria were tested for oxidase test, indole test, MR-VP test, motility test, SIM test, and urease test following standard protocol by Begey's Manual of Determinative Biology.

The screening of bioflocculant-producing bacteria was carried out using method as described by Abd-El-Haleem et al. (2008). The pure isolates of bacteria were transferred into Yeast extracts Peptone Glycerol (YPG) solid medium which contains of 10 g peptone, 10 g yeast extract, 20 g glucose, and 15 g agar powder in a liter of deionized water at pH 6.5 (Chen and Zhao 2003). The bioflocculant-producing bacteria were identified based on their mucoid and ropy characteristics of colonies on agar (Fig. 5). The bacterial colonies whose morphology did not fit the criteria described by Abd-El-Haleem et al. (2008) are considered as nonbioflocculant-producing bacteria.

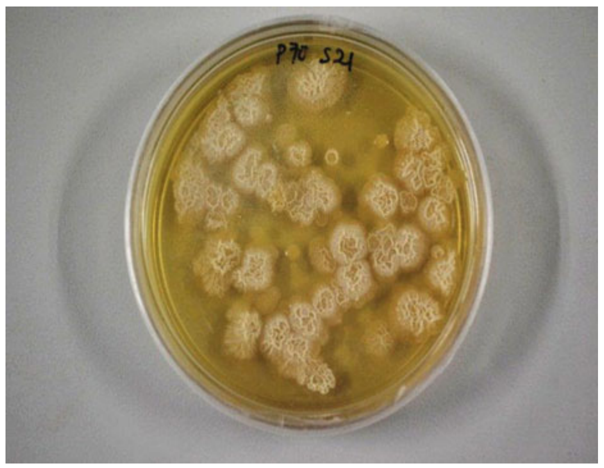

Fig. 5 Morphological characteristic of bioflocculant-producing bacteria which shows mucoid and ropy structure

2.4 Determination of Flocculating Activity of Bioflocculant-Producing Bacteria

Mass cultures of each bioflocculant-producing bacteria were prepared to produce a large volume of bacteria in suspension. Mass cultures of the isolated strains were prepared in 50 mL of YPG broth medium on a rotary shaker at 120 rpm/min at 25 °C for 3 days and were used for flocculation activity measurements (Lin and Harichund 2011). After incubation, the mass culture of bacteria in the YPG broth medium was centrifuged at 7000 rpm for 5 min. The supernatant part was used to measure the flocculating activity of the bacteria.

Flocculating activity of these bacteria was measured using kaolin clay suspension method as described by Kurane et al. (1986). Flocculation activity was determined using Jar test equipment which comprises six-paddle rotor (24.5 mm and 63.5 mm), equipped with six beakers for each paddle. The testing mixtures contain of 190 mL kaolin clay suspension (5 g/L, pH 7.0) and 0.5 mL free-cell supernatant. Then, the mixture was stirred with rapid mixing at 200 rpm for 2 min, followed by slow mixing at 80 rpm for 3 min and left standing for 5 min. Then, the free-cell supernatant and a control (distilled water) were measured for absorbance at 550 nm using a UV–VIS spectrophotometer (Shimadzu). The flocculating activity was calculated according to the following equation:

$$\text{Flocculation activity}\,(\%) = [A - B/A] \times 100$$

where

A and B are the absorbances at 550 nm for control and bioflocculant-producing bacteria sample, respectively.

3 Results

3.1 *Isolation and Identification of Bioflocculant-Producing Bacteria*

The isolation process of the mixture colonies from the biofloc samples to a single pure colony was conducted using a zig zag streaking method. Over 147 bacteria isolates were obtained from the bioflocs at the Integrated Shrimp Aquaculture Park, Blue Archipelago Berhad at Setiu, Terengganu. Out of this, a total of 51 - bioflocculant-producing bacteria were detected during DOC 0, DOC 30, and DOC 70 (Fig. 6) based on their morphological characteristics as mentioned by Abd-El-Haleem et al. (2008).

Gram staining process followed by a series of biochemical testing was conducted on all 51 isolates for further identification purposes. The results obtained are presented in Table 1. Gram staining observation for all isolates was shown in Figs. 7 and 8, respectively.

A series of biochemical tests were conducted in order to identify each type of bacteria isolates. The biochemical test includes spore staining, acid fast test, catalase test, starch hydrolysis test, citrate test, strict anerobes test, mannitol

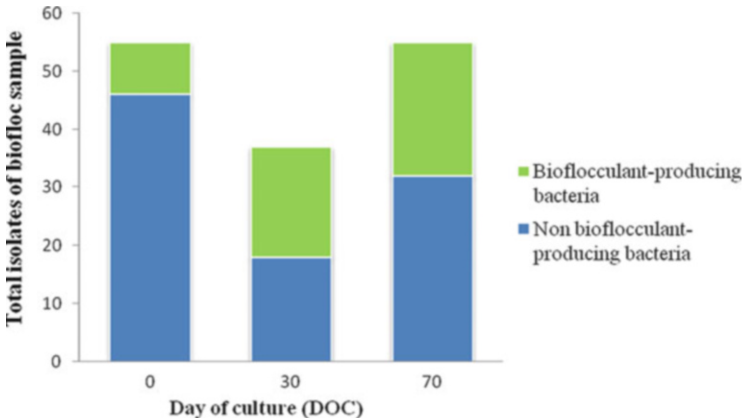

Fig. 6 Total bioflocculant-producing bacteria and nonbioflocculant-producing bacteria during DOC 0, DOC 30 and DOC 70

Table 1 Total number of Gram-positive and Gram-negative bioflocculant-producing bacteria isolates detected during DOC 0, DOC 30, and DOC 70

Day of culture (DOC)	Positive bacteria	Negative bacteria
0 day	7	2
30 day	11	8
70 day	18	5

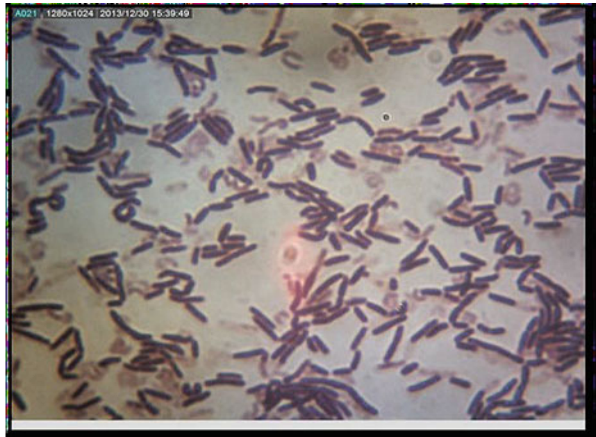

Fig. 7 One of Gram-positive bioflocculant-producing bacteria under ×1000 magnification. Gram-positive bacteria are *purple* in color

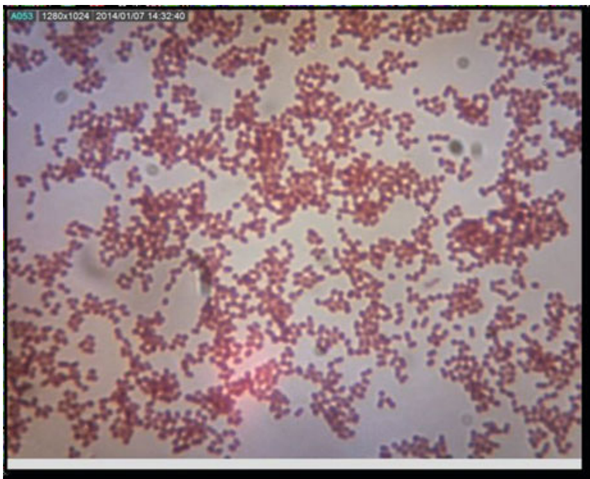

Fig. 8 One of Gram-negative bioflocculant-producing bacteria under ×1000 magnification. Gram-negative bacteria are *red* in colour

fermentation test, hemolysis test and bile esculin test, oxidase test, indole test, MR-VP test, motility test, SIM test, and urease test and data obtained are presented in Tables 2, 3, and 4. Out of the 51 isolates of bioflocculant-producing bacteria from DOC 0, DOC 30, and DOC 70, a total of 13 dominant species were identified as *Corynebacterium kutscheri*, *Corynebacterium xerosis*, *Klebsiella pneumoniae*, *Lactobacillus fermenti*, *Staphylococcus aureus*, *Bacillus azotoformans*, *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Vibrio* sp., *Neisseria* spp., *Yersinia* spp., and *Serratia* spp. (Figs. 9, 10, and 11).

During DOC 0 that was used as a control study, the highest number of bioflocculant-producing bacteria species was identified as *Corynebacterium kutscheri*, *Corynebacterium xerosis*, and *Staphylococcus* spp. (Fig. 9). During DOC 30, more species were present as compared to the biofloc sample which was

Table 2 Observation of biochemical test of bioflocculant-producing bacteria during DOC 0 from biofloc samples

Test	*Corynebacterium kutscheri*	*Corynebacterium xerosis*	*Bacillus azotoformans*	*Staphylococcus* spp.	*Klebsiella pneumoniae*	*Neisseria* spp.
Gram staining	+	+	+	+	–	–
Spore staining	–	–	+			
Add-fast	–	–				
Staining						
Catalase test	+	+	–	+		
Starch	+	–	–			
Hydrolysis						
Citrate test			+			
Mannitol				–		
Fermentation						
Yellow pigment (colony)				–		
Oxidase test					–	
Lactose					+	
Fermentation						
Indole test					–	
MR-VP test					+/–	
H_2S production					–	

Table 3 Observation of biochemical test of bioflocculant-producing bacteria during DOC 30 from biofloc samples

Test	*Corynebacterium kutscheri*	*Corynebacterium xerosis*	*Staphylococcus* spp.	*Streptococcus* spp.	*Staphylococcus aureus*	*Serratia* spp.	*Yersinia* spp.
Gram	+	+	+	+	+	–	–
Staining							
Spore	–	–					
Staining							
Acid-fast	–	–					
Staining							
Catalase test	+	+	+	–	+		
Starch	+	–					
Hydrolysis							
Citrate test							
Mannitol			–		+		
Fermentation							
Yellow pigment (colony)			–				
Hemolysis				γ			
Bile esculin				–			
Oxidase test							
Lactose							
Fermentation							
Indole test							
MR-VP test							
Urease test							
Motility							

γ gamma

Table 4 Observation of biochemical test of bioflocculant-producing bacteria during DOC 70 from biofloc samples

Test	*Lactobacillus fermenti*	*Corynebacterium xerosis*	*Bacillus* sp.	*Streptococcus* spp.	*Staphylococcus* spp.	*Staphylococcus aureus*	*Vibrio* spp.	*Neisseria* spp.
Gram staining	+	+	+	+	+	+	–	–
Spore staining	–	–	+					
Acid-fast								
Staining	–	–						
Catalase test	–	+	+	–	+	+		
Starch								
Hydrolysis		–	–					
Citrate test								
Mannitol								
Fermentation					–	+		
Yellow pigment (colony)					–			
Hemolysis				γ				
Bile esculin				–				
Oxidase test							+	
Lactose								
Fermentation								
Indole test								
MR-VP test								
Urease test								
Motility								
Glucose ferm.								
Activity	AG		–				+	
Na^+ required							+	

AG: acid and gas, γ: gamma

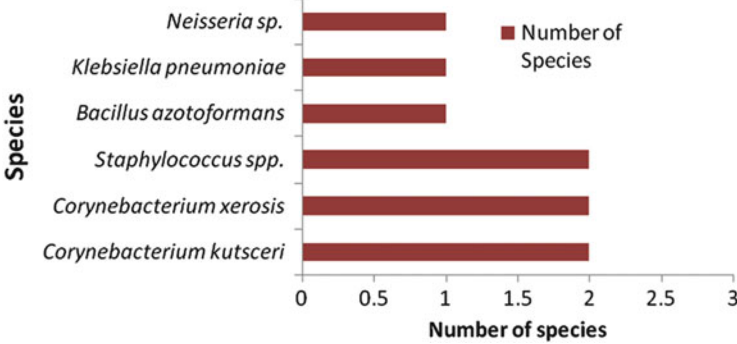

Fig. 9 Number of bacteria isolates of bioflocculant-producing bacteria from biofloc sample during DOC 0

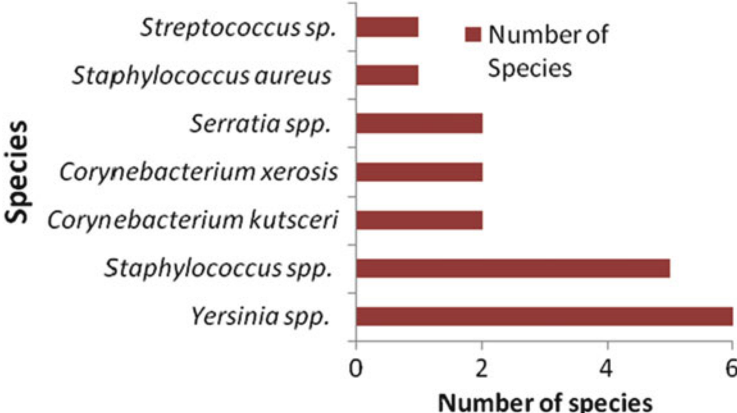

Fig. 10 Number of bacteria isolates of bioflocculant-producing bacteria from biofloc sample during DOC 30

collected during DOC 0. The most dominant bioflocculant-producing bacteria species were identified as *Yersinia* spp., *Staphylococcus* spp., *Corynebacterium kutscheri*, *Corynebacterium xerosis*, *Serratia* spp., *Staphylococcus aureus*, and *Streptococcus* sp. (Fig. 10). Among all the sample collection day, the highest number of bioflocculant-producing bacteria species was observed during DOC 70 (Fig. 11). The most dominant species of *Streptococcus* spp., *Neisseria* spp., *Lactobacillus fermenti*, *Staphylococcus* spp., *Staphylococcus aureus*, *Corynebacterium xerosis*, *Vibrio* sp., and *Bacillus* sp. were successfully identified.

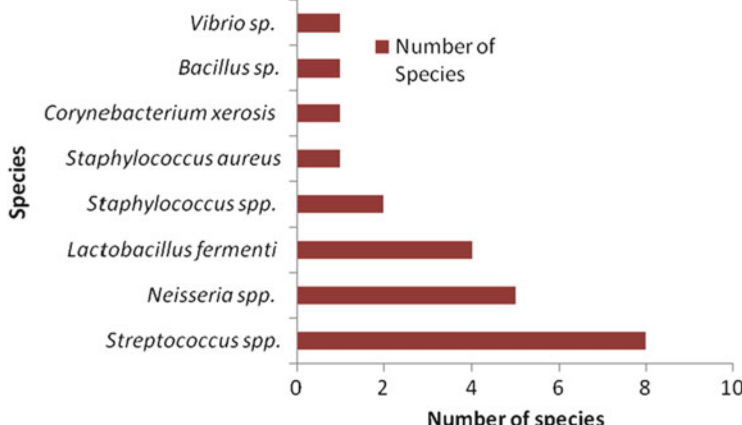

Fig. 11 Number of bacteria isolates of bioflocculant-producing bacteria from biofloc sample during DOC 70

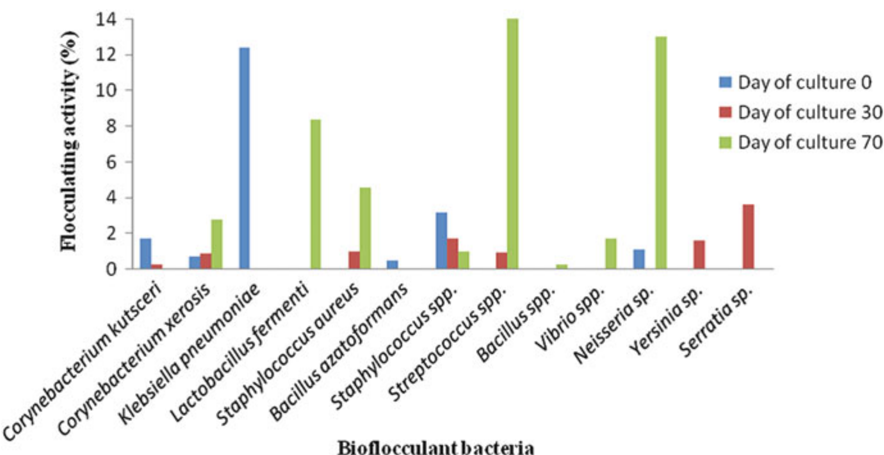

Fig. 12 Measurement of flocculating activity of 13 bioflocculant-producing bacteria from biofloc samples during DOC 0, DOC 30, and DOC 70

3.2 Analysis of Flocculating Activity of Bioflocculant-Producing Bacteria

Different species of bioflocculant-producing bacteria showed different performances in terms of flocculating activity at different periods of culture periods (Fig. 12). Over 13 identified bioflocculant-producing bacteria, *Streptococcus* sp. produced the highest flocculating activity during DOC 70 (14 %) followed by *Neisseria* sp. (13 %). The other bioflocculant-producing bacteria are *Lactobacillus*

fermenti (8 %), *Staphylococcus aureus* (5 %), *Vibrio* sp. (2 %), and *Staphylococcus* spp. (1 %) and the lowest was observed by *Bacillus* sp. (0.3 %).

4 Discussion

A total of 147 isolates of bacteria from biofloc samples were successfully isolated from the Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago Berhad, Setiu, Terengganu. Screening of bioflocculant-producing bacteria was based on the morphology of bacterial colonies such as slimy or mucoid appearance, cream colored, smooth, viscous, round, and convex edge when cultivated on YPG agar plates (Abd-El-Haleem et al. 2008; Cosa et al. 2011). The constituents of the YPG culture medium and culture conditions have a profound effect on bioflocculant production by some microorganisms (He et al. 2004; Xia et al. 2008). Bacteria can utilize the nutrients in the YPG culture medium to synthesize high molecular weight polymers internally within the cells under the action of specific enzymes (Gao et al. 2006). Thus, the YPG medium was used as the most suitable medium for the isolation of bioflocculant-producing bacteria.

In this study, 51 isolates were bioflocculant-producing bacteria, in which 13 identified bacteria species were recognized to potentially produce bioflocculant. The highest number of bioflocculant-producing isolates was found during DOC 70 as compared to the isolates during DOC 0 and DOC 30. This may be due to the ability of different bacteria species to produce their exopolymeric substances known as bioflocculant depending on the duration of culture periods (Deng et al. 2005). On the other hand, high molecular weight polymers that were synthesized internally can be excreted and exist in the medium or on the surface of the bacteria (Abd-El-Haleem et al. 2008). Hence, the action of bacteria during converting the simple substance that is present in their environment into a complex polymer that can be used as a bioflocculant may attract more microorganisms which start to aggregate.

In this study, the flocculating activity of the bioflocculant-producing bacteria was determined using kaolin clay suspension as their flocculation material (Kurane et al. 1986; Deng et al. 2005). Kaolin clay was used as a flocculation material because the charge on kaolin particles in solution are in negative form, thus whenever the bioflocculant is added on kaolin suspension, it will approach the particles in the solution and attract the electrostatic repulsion force (Li et al. 2008; Desouky et al. 2008). The highest flocculating activity of *Streptococcus* sp. at DOC 70 showed significant uniqueness of the chemical composition of bioflocculant produced by this bacteria. The different types of bioflocculant produced by different bacteria species might enhance the flocculating activity. This is possible because microbial bioflocculants are polymers which are produced by certain microorganisms and are based on their flocculating activity and the characteristics of the flocculants (Deng et al. 2005; Desouky et al. 2008; Gong et al. 2008).

The percentage of flocculating activity produced by different species of bacteria observed in the present study had different flocculating activities during DOC

0, DOC 30, and DOC 70. The percentage of flocculating activity produced by *Streptococcus* sp., *Neisseria* sp., *Vibrio* sp., *Staphylococcus aureus*, *Lactobacillus fermenti*, and *Corynebacterium xerosis* increased with the increase of DOC. It is presumably due to that each bacteria produces exopolymeric substance known as bioflocculant that accumulates during their growth (Deng et al. 2005; Cosa 2010). In addition, the absence of flocculating agents such as calcium chloride may stimulate flocculating activity by neutralizing and stabilizing the residual charge of different functional groups of the flocculants (Desouky et al. 2008). Therefore, in this study, only potential bioflocculant-producing bacteria were selected to examine their performance to flocculate without the use of other flocculating agents to initiate the bacteria to flocculate.

5 Conclusions

The establishment of bacterial isolation from biofloc that has the potential to produce bioflocculant and the determination of the flocculating activity by different species of bacteria were successfully conducted. This study had shown that the isolated bioflocculant-producing bacteria have the capability to remove suspended particles such as kaolin clay effectively. Different bioflocculant-producing bacteria were observed at different culture periods in terms of DOC; the longer culture periods, the higher flocculating activity. Therefore, microbial bioflocculants have the potential to be used as an alternative way to remove toxic wastes from the aquaculture systems. The bioflocculant produced by these bioflocculant-producing bacteria could be highly recommended to be used in aquaculture industry to solve pollution toward green environment.

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Biopreservation of Seafood by Using Bacteriocins and Bacteriocinogenic Lactic Acid Bacteria as Potential Bio-control Agents

Noraphat Hwanhlem and Aran H-Kittikun

Abstract This chapter reviews the use of bacteriocins and bacteriocinogenic lactic acid bacteria (LAB) as biopreservative in seafood and seafood products. The application is to control the growth of spoilage bacteria and/or to inhibit the growth of pathogenic bacteria in order to extend shelf life and to enhance the product safety. The genera of LAB, classification of bacteriocins, mode of action of bacteriocin, food-borne pathogenic, and spoilage bacteria in seafood are addressed. Several applications of LAB and their bacteriocin for biopreservation of seafood and seafood products are elaborated.

1 Introduction

Seafood has been recognized as a high-quality, healthy, and safe food and is one of the most important food commodities consumed worldwide. However, seafood, likes other foods, can be contaminated with spoilage bacteria and food-borne pathogens, resulting in spoilage and disease outbreaks with potential impact on human health. So most governmental and health authorities are highly concerned with seafood quality and safety. The assessment of safety issues related to nonregulated priority contaminants and the evaluation of their impact on public health and environment have become mandatory (Cruz et al. 2015; Mizan et al. 2015).Therefore, developing methods to ensure safety and to extend the shelf life of seafood is obviously a priority (Fall et al. 2012; Ghanbari et al. 2013). In recent years, a new approach called biopreservation using lactic acid bacteria (LAB) and their products specially bacteriocins in preventing the

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growth of unwanted microorganisms in seafood, has been increasingly investigated as an alternative to the use of chemical preservatives (Chahad et al. 2012; Fall et al. 2012; Garnier et al. 2010; Ghanbari et al. 2013).The seafood biopreservation by lactic acid bacteria has been intensively reviewed by Ghanbari et al. (2013).

Biopreservation is a powerful and natural tool to control the growth of spoilage and pathogenic bacteria in order to extend the shelf life of foods and/to improve the safety of foods. Thanks to naturally occurring microorganisms and/or their inherent antibacterial products. LAB present a major potential for use in biopreservation (Hwanhlem et al. 2015).They are also used throughout the world for manufacture of a wide variety of traditional fermented foods. They are involved in numerous food fermentations and it is assumed that most of the LAB do not pose any health risk to man, and most of them are classified as generally recognized as safe (GRAS) and may dominate the microflora of many foods (Azhari Ali 2010 Deegan et al. 2006; Ghanbari et al. 2013; Holzapfel et al. 1995; Pinto et al. 2009; Saito 2004; Stiles 1996; Takeshi et al. n.d.; Vignolo et al. 2012).

LAB, generally considered as "food grade" organisms, show special promise for selection and implementation as protective cultures. There are many potential applications of protective cultures in various food systems (Holzapfel et al. 1995; Stiles 1996; Vignolo et al. 2012). LAB are often present in fermented foods and may act as powerful competitors to spoilage and pathogenic microorganisms since they produce a wide range of antimicrobial metabolites during sugar fermentation such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides, and bacteriocins (Ghanbari et al. 2013; Mandal et al. 2011). These compounds are either bactericidal or bacteriostatic. Among these, bacteriocins have some specialty in that they are ribosomally synthesized proteinaceous compounds that are able to inhibit to a wide variety of organisms, mostly closely related to the producer organisms (Mandal et al. 2011).

Bacteriocins of LAB are considered as safe natural biopreservatives since they are degraded by the proteases in gastrointestinal tract (Cleveland et al. 2001). The inhibitory spectrum of some bacteriocins also includes food spoilage and/or food-borne pathogenic microorganisms (Schillinger et al. 1996). Bacteriocins or bacteriocin producers were applied as biopreservatives in many kinds of food products such as shrimp (Einarsson and Lauzon 1995), cooked meat (Vermeiren et al. 2004), canned fruit and vegetable (Lucas et al. 2006), cooked ham (Alves et al. 2006), fresh meat (Castellano et al. 2008), vacuum-packaged cold-smoked salmon (Tomé et al. 2008a, b), fermented meat sausage (Albano et al. 2009), artisanal dry sausages (Castro et al. 2011), etc.

2 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, nonspore forming cocci, coccobacilli or rods with a DNA base composition of less than 53 mol % G+C. They generally have anaerobic respiration and lack catalase enzyme (Makarova

et al. 2006; Medina et al. 2004; Slover and Danziger 2008). They ferment glucose primarily to lactic acid, or to lactic acid, CO_2, and ethanol. All LAB grow anaerobically, but unlike most anaerobes, they grow in the presence of O_2 as "aerotolerant anaerobes". Although they lack catalase, they possess superoxide dismutase and have alternative means to detoxify peroxide radicals, generally through peroxidase enzymes (Todar 2008).

LAB occur naturally in several raw materials like milk, meat, and flour used to produce foods. They are also used as natural or selected starters in food fermentations in which they perform acidification due to production of lactic and acetic acids. Protection of food from spoilage and pathogenic microorganisms by LAB is through producing organic acids, hydrogen peroxide, diacetyl, antifungal compounds such as fatty acids or phenyl lactic acid, and/or bacteriocins. LAB play an important role in food fermentation as the products obtained with their aid is characterized by hygienic safety, storage stability, and attractive sensory properties (Savadogo et al. 2006).

LAB are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid. As agents of fermentation, LAB are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut, but some species may spoil beer, wine, and processed meats (Todar 2008). LAB isolated from fish are *Aerococcus* spp., *Carnobacterium* spp., *Enterococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Vagococcus* spp., and *Weissella hellenica* (Ghanbari et al. 2013). Many kinds of ready-to-eat (RTE) seafood products also had LAB such as brined shrimp, cold-smoked fish, fermented fish, salt or marinated or dried fish, as well as sugar-salted fish and seafood salad (Ghanbari et al. 2013).

2.1 Metabolism of LAB

The essential feature of LAB metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. Adenosine triphosphate (ATP) generated is subsequently used for biosynthesis. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is lactic acid (>50 % of sugar carbon). However, LAB adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end product patterns (Davis et al. 1986; Kandler 1983; Ramos et al. 2002). Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative. The first category, homofermentative LAB, includes some lactobacilli and most species of enterococci, lactococci, pediococci, streptococci, tetragenococci, and vagococci that ferment hexoses by the Embden–Meyerhof (E–M) pathway. The second category, heterofermentative LAB, includes leuconostocs, some lactobacilli,

oenococci, and weissella species. The apparent difference on the enzyme level between these two categories is the presence or absence of the key cleavage enzymes of the E–M pathway (fructose 1,6-diphosphatekinase) and the pentose phosphate pathway (phosphoketolase) (Todar 2008).

2.1.1 Homofermentative LAB

Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize 1 mol of glucose in the E–M pathway to yield 2 mol of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields 2 mol of ATP per glucose consumed. Representative homofermentative LAB genera include *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Pediococcus* species. The transport and phosphorylation of sugars occur by (1) transport of free glucose and phosphorylation by an ATP-dependent hexose kinase (other sugars such as mannose and fructose enter the major pathways at the level of glucose-6-phosphate or fructose-6-phosphate after isomerization or phosphorylation or both); or (2) the phosphoenolpyruvate (PEP) sugar phosphotransferase system (PTS), in which PEP is the phosphoryl donor for the uptake of sugar. Some species of LAB use the PTS for transport of galactose only; others use the PTS for all sugars (Bulut et al. 2005; Damiani et al. 1996; McDonald et al. 1987; Todar 2008).

2.1.2 Heterofermentative LAB

Heterofermentative LAB utilize the pentose phosphate pathway to assimilate sugars. One mole of glucose-6-phosphate is dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield 1 mol of CO_2. The resulting pentose-5-phosphate is cleaved into 1 mol of glyceraldehyde phosphate (GAP) and 1 mol of acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates (McDonald et al. 1987; Zaunmüller et al. 2006). Theoretically, end products (CO_2, lactate, and ethanol) are produced in equimolar quantities from the catabolism of 1 mol of glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and certain lactobacilli.

2.2 Genera of Lactic Acid Bacteria

The genera that comprise LAB at its core are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as the more peripheral are *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*; these belong to the order Lactobacillales.

2.2.1 *Lactobacillus*

Lactobacillus is a genus of Gram-positive rod, facultative anaerobic, or microaerophilic bacteria. They are a major group of the LAB, commonly found and usually benign. Many species are prominent in decaying plant materials. Several members of the genus have their genome been sequenced (Ljungh and Wadström 2009).

2.2.2 *Leuconostoc*

Leuconostoc is a genus of Gram-positive coccus often forming chains. *Leuconostoc* spp. are intrinsically resistant to vancomycin and are catalase-negative (which distinguishes them from staphylococci). All species within this genus are heterofermentative and are able to produce dextran from sucrose. They are generally slime-forming (Björkroth and Holzapfel 2006).

2.2.3 *Pediococcus*

The genus *Pediococcus* is homofermentative and generally produce DL-lactate from glucose. However, *Ped. claussenii* and *Ped. dextrinicus* produce only L (+) lactic acid (Haakensen et al. 2009).

2.2.4 *Lactococcus*

Lactococcus was formerly included in the genus *Streptococcus* Group N1 (Schleifer et al. 1985). It is homofermentative. Some strains are known to grow at or below 7 °C (Jay et al. 2005; Teuber and Geis 2006).

2.2.5 *Streptococcus*

Streptococcus is a genus belonging to the phylum Firmicutes (Ryan et al. 2010) and the LAB group. Streptococci are oxidase- and catalase-negative, and many are facultative anaerobes. In 1984, many organisms formerly considered *Streptococcus* were separated out into enterococci and lactococci (Facklam 2002).

2.2.6 *Enterococcus*

Modern classification techniques resulted in the transfer of some members of the genus *Streptococcus*, notably some of the Lancefield's group D streptococci, to the

new genus *Enterococcus*. Some enterococci of food origin produce bacteriocins that exert anti-*Listeria* activity. Most enterococcal species are able to grow in the presence of 6.5 % NaCl, 40 % bile salts, at pH 9.6, and can survive 30 min at 60 °C. They are also commonly isolated from foods, plants, water, and soils, probably as a result of dissemination from fecal sources and their natural tolerance to adverse environmental conditions (Ogier and Serror 2008).

2.2.7 *Oenococcus*

The only species in the genus used to be *Oenococcus oeni* (which was known as *Leuconostoc oeni* until 1995). In 2006, the species *O. kitaharae* was identified. *O. oeni* is important in the field of oenology. It is the primary bacterium involved in completing the malolactic fermentation (Björkroth and Holzapfel 2006; Costantini et al. 2009).

2.2.8 *Weissella*

Weissella is a genus of Gram-positive bacteria, placed within the family of Leuconostocaceae. The morphology of weissellas varies from spherical or lenticular cells to irregular rods (Björkroth and Holzapfel 2006).

3 Bacteriocins Produced by LAB

Bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides (Cintas et al. 2001; Cuozzo et al. 2001; Pitt and Gaston 1995). In general, these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity (Risøen et al. 2004; Zacharof and Lovitt 2012).

3.1 Classification of Bacteriocins

Most bacteriocins of LAB are small (<10 kDa) cationic, heat stable, amphiphilic, and membrane permeabilizing peptides. Many of these bacteriocins appear to exhibit relatively little adsorption specificity. Zacharof and Lovitt (2012) divided bacteriocins of LAB into three major classes.

3.1.1 Class I: Lantibiotics

Class I, the lantibiotics, is a class of small peptide substances (<5 kDa) that contains polycyclic thioether amino acids, lanthionine, or methyl lanthionine, as well as the unsaturated amino acids, dehydroalanine and 2-aminoisobutyric acid. They are divided into two types based on structural similarities. Type A lantibiotics are relatively elongated, screw shaped, positively charged, amphipathic molecules. They generally act through pore formation by depolarization of the cytoplasmic membrane of the sensitive species. Nisin and lacticin 3147 are the major representatives of this group. Type B lantibiotics are globular in structure and interfere with cellular enzymatic reactions. Their molecular mass lies between 2 and 3 kDa and have no net charge.

3.1.2 Class II: Non-lantibiotics

Class II bacteriocins are also small (<10 kDa) relatively heat stable, non-lanthionine containing membrane active peptides. They are divided into two subclasses. Subclass II a, pediocin-like or listeria active bacteriocins subclass, possess an N-terminal consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys, for example, pediocin PA-1 and sakacin A. Subclass II b refers to two-component (two separate peptides) bacteriocins. They require two peptides to work synergistically in order to have an antimicrobial activity. Lactacin F and lactococcin G are members of this group.

3.1.3 Class III: Large Bacteriocins

This group consists of heat labile proteins, which have large molecular weight (>30 kDa), e.g., helveticin I produced by *Lactobacillus helveticus* and enterolysin produced by *Ent. faecium*.

3.2 Mode of Action of Bacteriocin

Due to the great variety of their chemical structures, bacteriocins affect different functions of the living cell, but most of them act by forming membrane channels or pores that destroy the energy potential of sensitive cells (Oscáriz and Pisabarro 2001).

LAB bacteriocins can be grouped on the basis of structure as well as the basis of mode of action. Most bacteriocins of LAB inhibit Gram-positive bacteria. They can bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell

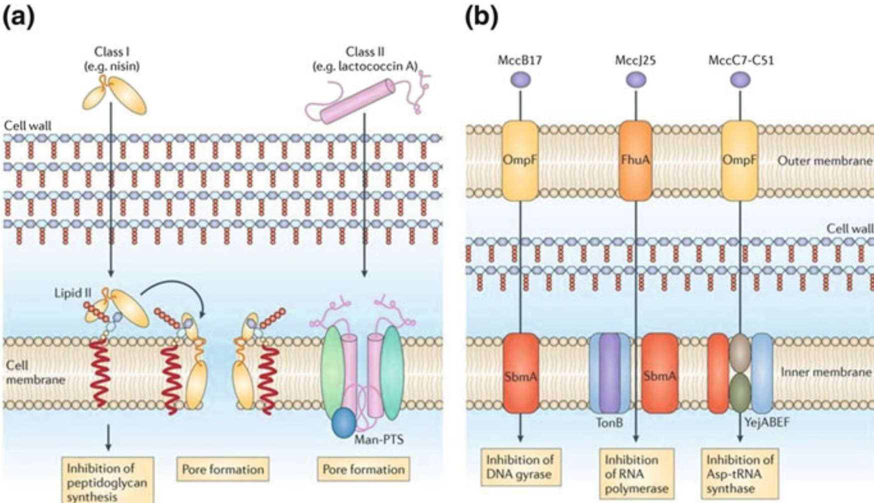

Fig. 1 Mechanism of action of representative bacteriocins. (**a**) Gram-positive targets. (**b**) Gram-negative targets. Source: Cotter et al. (2012)

death. Furthermore, they can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death (Figs. 1a and 2a). Class I bacteriocins such as nisin and lacticin 3147 have these dual activities distributed across two peptides, whereas mersacidin has only the lipid II binding activity, but does not form pores (Cotter et al. 2005, 2012; Zacharof and Lovitt 2012). The lantibiotic disturbs the lipid bilayer organization when it binds to the membrane. A wedge-like model for bacteriocin-induced pore formation may involve a proton motive force driven co-insertion of lipids and bacteriocin domains (Abee et al. 1995; Chen and Hoover 2003; Cotter et al. 2005, 2012; Moll et al. 1999; Zacharof and Lovitt 2012). The hinge(s) in the bacteriocin molecule might allow bending of the C-terminal part and thus its insertion into the membrane. Multiple inserted bacteriocin molecules may give rise to a large local disturbance of the lipid bilayer organization causing formation of transient lipid–protein pores. Such structures are intrinsically unstable due to the hydrophobic forces that will drive the rearrangements of the lipids into their original bilayer organization (Moll et al. 1999).

Class II bacteriocins such as lactococcin A bind to the pore-forming receptor mannose phosphotransferase system (Man-PTS) (Cotter et al. 2012). In general, the class II peptides have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarization and death (Cotter et al. 2005). Upon membrane insertion, the class II bacteriocins are thought to form a bundle of α-helical peptides much akin a barrel-stave like pore or a carpet mechanism (Fig. 2a, b) (Abee et al. 1995; Chen and Hoover 2003; Cotter et al. 2005, 2012; Moll et al. 1999; Oscáriz and Pisabarro 2001). According to this model, single peptide molecules might be oriented parallel to the membrane

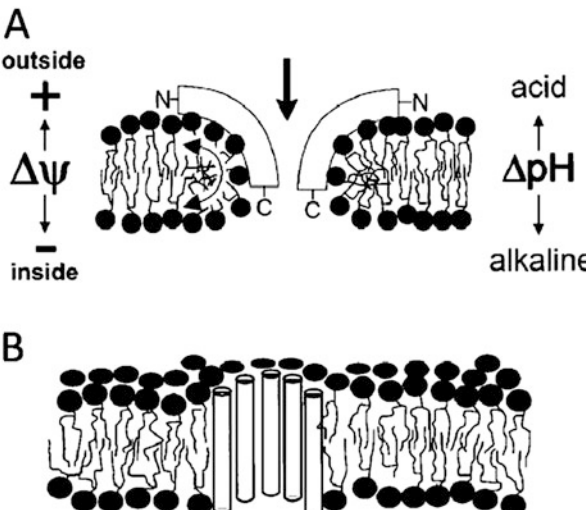

Fig. 2 Models of pore formation. (**a**) Wedge-like pore. (**b**) Barrel-stave pore. Source: Moll et al. (1999)

surface and interfere with the membrane bilayer organization without forming a peptide aggregate. Once there are sufficient peptides in each other's vicinity, the membrane will temporarily collapse due to a strong phospholipid mobilizing activity that cooperatively results in local and transient permeability. The negative charge of the membrane lipids has been suggested to confer cation selectivity to such pores (Moll et al. 1999).

Class III bacteriocin—large bacteriolytic proteins or bacteriolysins, such as lysostaphin, can function directly on the cell wall of Gram-positive targets, leading to death and lysis of the target cell (Cotter et al. 2005).

Bacteriocins are generally not active against Gram-negative bacteria (Chen and Hoover 2003). Because Gram-negative bacteria possess an additional layer, the outer membrane which is composed of phospholipids, proteins, and lipopolysaccharides (LPS). This membrane is impermeable to most molecules. The presence of porins in this layer will allow the free diffusion of molecules with a molecular mass below 600 Da. The smallest bacteriocins produced by LAB are approximately 3 kDa and are too large to reach their target, the cytoplasmic membrane (Abee et al. 1995). To overcome these limitations, the use of bacteriocins has to be combined with hurdle approaches. The use of nonthermal processing technologies (high hydrostatic pressure and pulsed electric field) or using chelating agents (EDTA) is able to disrupt the outer membrane of Gram-negative bacteria. Furthermore, bacteriocins could be combined with other antimicrobial compounds such as potassium sorbate, sodium acetate, and sodium lactate resulting in enhanced inactivation of bacteria (Chen and Hoover 2003; Zacharof and Lovitt 2012). It is well documented that Gram-negative bacteria become sensitive to bacteriocins if the permeability barrier properties of their outer membrane are impaired, thus allowing bacteriocins to gain access to the cytoplasmic membrane by interfering with DNA, RNA, and protein metabolism. For example, microcin B17 (MccB17) inhibits DNA gyrase, MccJ25 inhibits RNA

polymerase, and MccC7-C51 inhibits aspartyl-tRNA synthetase. There are also exceptions, such as MccE492, that function through pore formation (Fig. 1b) (Chen and Hoover 2003; Cotter et al. 2012). The combination of nisin Z and lauric arginate caused severe changes in the cytoplasmic membrane and cell lysis of both Gram-positive and Gram-negative bacteria (Pattanayaiying et al. 2014).

4 Food-Borne Pathogenic Bacteria in Seafood

Pathogenic bacteria are usually found at fairly low levels in seafood products (Feldhusen 2000). From harvest to consumption, seafood is easily contaminated by these bacteria (Yin et al. 2007). Ghanbari et al. (2013) did the survey of bacterial hazards in fish and seafood products and found *Aeromonas* spp., *Clostridium botulinum*, *Cl. perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *V. cholera*. Huss (1994) divided seafood-borne pathogenic bacteria into two groups including indigenous and nonindigenous bacteria.

4.1 Indigenous Seafood-Borne Pathogenic Bacteria

The bacteria belonging to this group are common and widely distributed in the aquatic environments in various parts of the world. The water temperature is naturally having a selective effect. The more psychrotrophic organisms (*Clostridium botulinum* and *Listeria*) are common in Arctic and colder climate. While, the more mesophilic types (*Vibrio cholerae* and *V. parahaemolyticus*) are natural flora on fish from coastal and estuarine environments of temperate and tropical zones (Huss 1994).

4.1.1 *Clostridium botulinum*

Cl. botulinum is a Gram-positive, anaerobic spore-forming bacteria. It is widely distributed in the natural environments such as soil (Huss 1980), aquatic sediments (Hayes 1966) and fish (Hielm et al. 1996). *Cl. botulinum* can grow in food such as crab (Cockey and Tatro 1974; Harrison et al. 1996; Kautter et al. 1974), fish (Bott et al. 1966; Christiansen et al. 1968), and shrimp (Garren et al. 1994; Lalitha and Gopakumar 2001). It can produce an extremely potent neurotoxin known as "botulin" or "botulinum toxin". One of these organisms (Type E) naturally occurs in the marine environment and can grow and produce toxins at refrigerated temperatures (Fletcher et al. 2008).

4.1.2 *Vibrio* sp.

The genus contains a number of species, which are pathogenic to human. Some vibrios are marine in origin and they require Na^+ for growth. The pathogenic species are mostly mesophilic, i.e., generally occurring (ubiquitous) in tropical water and in the highest numbers in temperate water during late summer or early fall (Huss 1994).

Su and Liu (2007) review that *V. parahaemolyticus* is a human pathogen that is widely distributed in the marine environments. This organism is frequently isolated from a variety of raw seafoods, particularly shellfish. This pathogen is a common cause of food borne illnesses in many Asian countries, including China, Japan, and Taiwan. It is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the USA. Khouadja et al. (2013) studied the presence of pathogenic *V. parahaemolyticus* in waters and seafood from the Mediterranean sea. Eight strains were confirmed as *V. parahaemolyticus* by *toxR*-based polymerase chain reaction and only one was positive for *tdh* and *trh* genes. Toxigenic *V. parahaemolyticus* isolates are present in Tunisian coastal areas and they may also be present in Tunisian exported seafood products.

4.1.3 *Aeromonas* sp.

Aeromonas is ubiquitous in freshwater environments, but may also be isolated from saline and estuarine waters (Matyar et al. 2007; Pianetti et al. 2005). *Aeromonas* has been described as an emergent food borne pathogen of increasing importance. This organism may also be readily isolated from meat, fish and seafood, ice-cream and many other foods. Species of *Aeromonas* produce a wide range of toxins such as cytotoxic enterotoxin, hemolysins, and a tetrodotoxin-like sodium channel inhibitor. *Aeromonas* is very sensitive to acid and salt, and growth is unlikely to be a problem in foods where pH is less than 6.5 and the NaCl content greater than 3.0 % (Huss 1994).

Illanchezian et al. (2010) studied virulence and cytotoxicity of seafood borne *A. hydrophila*. Among 73 *A. hydrophila* strains isolated from fish and shrimp samples, 86.3 % exhibited hemolysis, 78.1 % produced slime, 98.63 % produced protease and also demonstrated cytotoxicity on Vero cells. Positive correlation was observed between proteolytic activity and cytotoxicity irrespective of hemolytic activity of the strains. Rall et al. (1998) reported that 48 % of 50 Pintado fish samples collected at the retail market of São Paulo city (Brazil) were positive for *Aeromonas* sp.

4.1.4 *Plesiomonas* sp.

Plesiomonas is widespread in nature but mostly associated with water, both freshwater and seawater. It is normally found in the environment, freshwater, fish and birds, particularly in tropical and subtropical regions (Hernández and Rodríguez de García 1997; Kain and Kelly 1989). The presence of this organism in animals as well as fish is common. It is likely that fish and shellfish is the primary reservoir of *P. shigelloides* (Huss 1994). Gonzalez-Rey et al. (2003) found *P. shigelloides* in lake water north of the Polar Circle in the northern part of Sweden. This could have ecological and clinical importance for the aquatic and terrestrial fauna at these latitudes.

4.1.5 *Listeria* sp.

L. monocytogenes is the major human pathogen among the eight species of *Listeria*. This organism causes abortion, encephalitis, gastroenteritis, arthritis, conjunctivitis, etc. in human. Apart from *L. monocytogenes*, *L. ivanovii* has also been reported to cause human infection in some cases. *L. monocytogenes* also causes clinical diseases in animals (Das et al. 2013). *L. monocytogenes* causes listeriosis—an infection that can occur when a person eats food that has been contaminated with this bacterium especially RTE foods (Miya et al. 2010).

L. monocytogenes is found in wild animals, domesticated animals, soil, and water. These bacteria make many animals sick, leading to miscarriage and stillbirth in domestic animals (Board 2011; Das et al. 2013). Vegetables, meats, and other foods can get infected with the bacteria if they come in contact with contaminated soil or manure. It is one of very few pathogenic organisms, which can grow at refrigerated temperatures. Thus, the storage of food at low temperatures cannot prevent the growth of this pathogen (Das et al. 2013).

Huss et al. (2000) reported that contamination or recontamination of seafood may also take place during processing. Low levels (<100 CFU/g) of *L. monocytogenes* are frequently found in seafood including RTE products. Apart from heat treatment, which is very effective, there are few options for eliminating *L. monocytogenes* from foods and equipment. It is essential, therefore, that growth of *L. monocytogenes* in the final product be inhibited. The preventive measures include the formulation of a cleaning and sanitizing program specifically designed to reduce the presence of *L. monocytogenes* in the factory environment. Gambarin et al. (2012) studied *L. monocytogenes* in RTE seafood and potential hazards for the consumers in the Veneto Region (Italy). Thirty-eight samples analyzed by PCR test yielded 12 positive samples. In a similar study, González et al. (2013) studied refrigerated RTE seafood products at retail in Spain and reported that low prevalence of *L. monocytogenes* was observed in surimi products, while 4.8 % of smoked salmon samples were positive for *Listeria*.

4.2 Nonindigenous Seafood-Borne Pathogenic Bacteria

Seafood has the potential to pose a wide spectrum of public health problems from common yet harmful bacteria through contamination during production and distribution from the point of harvest to final preparation (Kvenberg 1991). Nonindigenous bacteria originated from the animal/human reservoir. The bacteria belonging to this group include *Salmonella* sp., *E. coli*, *Shigella* sp., and *Staphylococcus aureus*.

4.2.1 *Salmonella* sp.

Salmonella is a Gram-negative bacillus and inhabits in the human and animal gastrointestinal tracts. It is associated with gastrointestinal problems, septicaemic disease, and abortion, due to its cellular invasion capacity and its intraphagocytic survival (Figueroa Ochoa and Verdugo Rodríguez 2005). The presence of *Salmonella* in seafood may derive from contamination occurring in the natural aquatic environment, aquaculture, or during processing (Amagliani et al. 2012).

Field laboratories of the U.S. Food and Drug Administration collected and tested 11,312 import and 768 domestic seafood samples over a 9-year period (1990–1998) for the presence of *Salmonella*. The overall incidence of *Salmonella* was 7.2 % for import and 1.3 % for domestic seafood. The incidence in the 2734 RTE import seafood was 2.6 % cooked shrimp, shellfish or fish paste, smoked fish, salted/dried fish, and caviar. While the most frequent serotypes in import seafood were *S. Weltevreden*, *S. Senftenberg*, *S. Lexington* and *S. Paratyphi-B* (Heinitz et al. 2000). In a similar study, different *Salmonella* serovars, namely *S. Typhi*, *S. Typhimurium*, *S. Enteritidis*, *S. Mbandka*, *S. Bareilly*, and *S. Weltevreden* were detected in seafood (fish, shrimps, mussels, crabs, edible oysters, and clams) collected from the local fish markets in Cochin (India) by using PCR-based technique (Kumar et al. 2008).

4.2.2 *Shigella* sp.

Shigella sp. are Gram-negative pathogenic bacteria that may cause devastating diarrhea upon ingestion (Schroeder and Hilbi 2008). Species of the genus *Shigella* are among the bacterial pathogens most frequently isolated from patients with diarrhea. It has been reported *Shigella* sp. were isolated from seafood. Okonko et al. (2008) reported that *Shigella* sp. was found in 15 % frozen shrimp samples processed in Ibadan and Lagos, Nigeria. The comparative study regarding prevalence of microbial flora in the muscle of locally available tiger shrimp (*Penaeus monodon*) and giant water prawn (*Macrobrachium rosenbergii*) collected from Bangladesh found that *Salmonella* and *Shigella* count ranged from 2.0×10 to

1.1×10^2 cells for the shrimp and between 2.6×10 and 9.6×10^3 cells for the prawn (Yousuf et al. 2008).

4.2.3 *Escherichia coli*

E. coli are the predominant nonpathogenic facultative flora of the human intestine. Some *E. coli* strains have developed the ability to cause disease of the gastrointestinal, urinary, or central nervous system in even the most robust human hosts. Several distinct clinical syndromes accompany infection with diarrheagenic *E. coli* categories, including traveler's diarrhea (enterotoxigenic *E. coli*), hemorrhagic colitis and hemolytic uremic syndrome (enterohemorrhagic *E. coli*), persistent diarrhea (enteroaggregative *E. coli*), and watery diarrhea of infants (enteropathogenic *E. coli*) (Nataro and Kaper 1998).

The contaminations of *E. coli* in seafood have been extensively studied. Teophilo et al. (2002) reported that 32 *E. coli* strains were isolated from red snapper (*Lutjanus purpureus*) and from seabob shrimp (*Xiphopenaeus kroyeri*). A total of 14 strains produced exotoxins, of which seven were thermolabile and seven were thermostable. Thampuran et al. (2005) reported that seafood samples contained *E. coli* count ranging from 2 to 5.5 log CFU/g. The majority of the *E. coli* isolates showed a multiple antibiotic resistance. While Kumar et al. (2005) studied the prevalence of *E. coli* in tropical seafood. They found that 155 samples were positive for fecal coliforms and *E. coli* was isolated from 47 % of the positive samples.

4.2.4 *Staphylococcus aureus*

Staph. aureus is a virulent pathogen which is the most common cause of infections in hospitalized patients. The increase in the resistance of this virulent pathogen to antibacterial agents, coupled with its increasing prevalence as a nosocomial pathogen, is of major concern (Archer 1998).

Ayulo et al. (1994) studied enterotoxigenic *Staph. aureus* in fish and seafood from the southern region of Brazil. *Staph. aureus* was isolated from 20 % of the 175 samples examined, including 60 % of samples of shellfish-meat. Only 9 of 109 *Staph. aureus* strains produced enterotoxins, including enterotoxin A (4), D (1), and AB (4). It is concluded that greater care must be taken to reduce contamination of fish and seafood during harvesting and postharvest handling. In a similar study, Simon and Sanjeev (2007) reported that 168 fishery products and 87 samples from fish processing factory workers were found positive for enterotoxigenic *Staph. aureus*. Among the products, incidence was high in frozen peeled prawns and frozen fish cutlets (33 % each) compared to frozen peeled and deveined prawns and frozen squids (25 % and 20 %, respectively).

5 Food Spoilage Bacteria in Seafood

Fish and seafood products are the most important protein sources in human nutrition. At the same time, these products are perishable and could be spoiled rapidly. Microorganisms are the major cause of spoilage of most seafood products. However, only a few members of the spoilage organisms give rise to the offensive off-flavors associated with seafood spoilage (Gram and Dalgaard 2002).

Some fish products are heavily cured (salted, dried) and shelf stable at ambient temperature. An increasing number of fish products are preserved by low levels of salt, cooling, packaging in modified atmosphere, and/or addition of low levels of preservatives. The microflora of these products is often complex; however, spoilage is mostly caused by microbial action (Gram 2010). Jaffrès et al. (2009) highlighted the main bacterial genera and species related to the spoilage microbiota of cooked and peeled tropical shrimps, packaged in a modified atmosphere (MAP). LAB, mainly represented by the genera *Carnobacterium* and *Vagococcus*, were found to dominate the microbiota in shrimp, followed by *Brochothrix thermosphacta* and *Serratia liquefaciens*. Several of these bacteria were already known to be involved in the production of off-odors, characteristic of spoiled seafood products (Jaffrès et al. 2011).

5.1 Carnobacterium

The genus *Carnobacterium* contains nine species, but only *C. divergens* and *C. maltaromaticum* are frequently isolated from natural environments and foods. They are tolerant to freezing/thawing and high pressure and able to grow anaerobically at low temperatures. They metabolize arginine and various carbohydrates, including chitin, and this may improve their survival in the environment. *C. divergens* and *C. maltaromaticum* have been extensively studied as protective cultures in order to inhibit growth of *L. monocytogenes* in fish and meat products. Several carnobacterial bacteriocins are known, and parameters that affect their production have been described. However, no isolates are commercially applied as protective cultures. Carnobacteria can spoil chilled foods. Their production of tyramine in foods is critical for susceptible individuals. *C. maltaromaticum* can be a fish pathogen, although there is a suggestion to use carnobacteria as probiotic cultures for use in aquaculture (Leisner et al. 2007).

Franzetti et al. (2003) investigated LAB population in five kinds of seafood (salmon, tuna, shrimps, swordfish, and cuttlefish) packaged in modified atmospheres at 4 °C for 6 days. They found the heterofermentative rods belonging to *Carnobacterium*, *Lactobacillus*, and cocci of the *Leuconostoc* genus. The microorganisms found varied with the kind of seafood and the gas composition of the modified atmospheres: in MAP1 ($80O_2/20N_2$) richer in oxygen than MAP2 ($40CO_2/60N_2$), *Carnobacterium* represented the prevalent microbial group, especially in

tuna, shrimps, and swordfish, whereas MAP2 favored *Lactobacillus*. *Leuconostoc* was dominant in salmon and cuttlefish independently of gas composition.

5.2 Vagococcus

The genus *Vagococcus* is Gram-positive, catalase-negative, and coccus-shaped. *V. salmoninarum* and *V. fluvialis/carniphilus* have been isolated from diseased salmonid fish (Michel et al. 2007; Schmidtke and Carson 1994) and rainbow trout (Ruiz-Zarzuela et al. 2005), whereas *V. penaei* sp. *nov.* was isolated from spoilage microbiota of cooked shrimp (*Penaeus vannamei*) (Jaffrès et al. 2010).

5.3 Brochothrix thermosphacta

Br. thermosphacta, a Gram-positive bacterium, is considered as the predominant spoilage microbiota of MAP shrimp and fish (Mamlouk et al. 2012). Fall et al. (2012) studied sensory and physicochemical evolution of tropical cooked peeled shrimp inoculated by *Br. thermosphacta* and *Lactococcus piscium* CNCM I-4031 during storage at 8 °C. They reported that *Br. thermosphacta* spoiled the product after 11 days, with the emission of strong butter/caramel off-odors.

5.4 Serratia liquefaciens

Serratia liquefaciens is an organism rarely encountered in clinical practice. It also belongs to the family *Enterobacteriaceae*. It is widely distributed in nature, including river water, mineral, spring and table water, domestic sewage, fish, minced meat, and pasteurized milk or cream. It has been reported as a cause of mastitis in a dairy herd (Mossad 2000). It was found to be one of the most spoiling microorganisms in cold-smoked salmon, producing trimethylamine, dimethylsulfide, and thiobarbituric acid (Jaffrès et al. 2011).

6 Application of Bacteriocins and LAB for Seafood Biopreservation

6.1 Criteria for Seafood Biopreservation

Biopreservation is an innovative way of extending the shelf life of food products and reducing microbial risks. Biopreservation of fish and seafood products is an

alternative to meet safety standards and to control microbial deterioration without negative impact on the sensory quality of the product. Biopreservation consists of the inoculation of food products with selected bacterial strains and/or their antibacterial compounds to inhibit the growth of undesirable bacteria. LAB are particularly interesting candidates for this technique. Although, biopreservation is currently applied in many kinds of fermented food such as dry-fermented sausages (Sidira et al. 2014), charqui, a Brazilian fermented, salted and dried meat product (Biscola et al. 2013), and Fior di Latte cheese (Angiolillo et al. 2014). There are few examples in nonfermented products such as fresh and semi-cooked seafood. Some selected microorganisms that have given good results in a model medium are not efficient in seafood because they do not grow in this low-sugar content matrix and are not adapted to a chilled temperature or spoil the product. Most of the successful studies in marine products have been obtained on the inhibition of *Listeria* spp. by different species of LAB, mainly from the *Carnobacterium* genus, which is due to either bacteriocin production or competition mechanisms. However, other endogenous pathogenic bacteria, such as *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, *Cl. botulinum*, histamine-producing bacteria, and post-contaminating bacteria, such as *Staph. aureus* or *Salmonella* spp., can be associated with seafood safety and require special attention. Moreover, due to their high content of low-molecular weight nitrogenous compounds, their neutral pH and high water activity value, seafood products are also extremely sensitive to microbial spoilage. *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Aeromonas* spp., and *Pseudomonas* spp. are the main spoilers of fresh fish products stored in air or under vacuum or modified atmosphere packaging (MAP). In lightly preserved fish products (NaCl < 6 % in water phase, pH > 5) like cold-smoked fish, other microorganisms such as LAB, Enterobacteriaceae, *Br. thermosphacta*, and *Vibrio* spp. can contribute to spoilage. In order to develop the biopreservation technology, improve quality, and extend the shelf-life of seafood, the selection of LAB that show inhibitory properties against both pathogenic and spoilage bacteria at chilled temperatures is necessary (Matamoros et al. 2009).

The selection of LAB possessing the GRAS status (US Food and Drug Administration) as protective cultures is generally agreed as beneficial for extending the shelf life of seafood products. Likewise, they also fulfill the QPS (qualified presumption of safety) requirements. Seafood-borne LAB are often able to grow even at refrigerated temperatures and are compatible to the seafood environment (MAP, low pH, high salt concentrations, the presence of additives like lactic acid or acetic acid). Importantly, their growth can also suppress spoilage bacteria and pathogenic bacteria by means of antagonistic and inhibitory activities (Ghanbari et al. 2013).

Many parameters are required for the successful application of live antagonistic bacteria to preserve seafood (Ghanbari, et al. 2013). The consumer safety is the most important aspect. The antagonistic bacteria or their metabolites may affect the quality of the seafood products. The ability of the selected bacteria to produce sufficient antimicrobial compounds against a broad spectrum of spoilage bacteria and food-borne pathogens is very important. The capability of the selected bacteria to survive adverse conditions during processing and storage must be also taken into

account. In these aspects, LAB usually meet the necessary requirements for biopreservation of seafood products.

Pilet and Leroi (2011) reported that microbial seafood-borne disease represents 10–20 % of the total food-borne outbreaks. Most seafood products have been contaminated by pathogenic *Vibrio*, *L. monocytogenes*, and histamine producing bacteria. In addition, seafood products are very sensitive to the development of spoiling bacteria producing off-odors. Pathogenic and spoiling microorganisms are not always reduced or limited by the processing steps that are currently used in these foodstuffs, and the interest in alternative techniques such as bioprotection to improve the quality and safety of seafood has increased in recent years. Among the microbiota of lightly preserved seafood products, LAB usually become dominant during storage under vacuum or modified atmosphere. In some cases these bacteria are responsible for spoilage but some of them have demonstrated potential for pathogenic or spoiling microorganisms' inhibition, mainly for the control of *L. monocytogenes* in cold-smoked salmon and to a lesser extent in other products to enhance sensory shelf life.

Seafood biopreservation strategies reinforce the need for having robust LAB since they have to survive steps of food processing, resist the food environment, and express specific functions under unfavorable conditions. The ability to quickly respond to stress is essential for survival and it is well established that LAB, like other bacteria, have evolved defense mechanisms against stress that allow them to withstand harsh conditions and sudden environmental changes. While genes implicated in stress responses are numerous, in LAB the characterization of their actual role and regulation differs widely between species. Indeed, the functional conservation of several stress proteins (e.g., heat shock proteins, Csp, etc.) and some of their regulators (e.g., HrcA and CtsR) render even more striking the potentials of LAB for use in biopreservation (Ghanbari et al. 2013).

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: (1) are generally recognized as safe (GRAS) substances, (2) are not active and nontoxic on eukaryotic cells, (3) become inactivated by digestive proteases, having little influence on the gut microbiota, (4) are usually pH and heat tolerant, (5) have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (6) show a bactericidal mode of action, usually acting on the bacterial cytoplasm membrane: no cross resistance with antibiotics, and (7) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Gálvez et al. 2007). Application of bacteriocins may help to reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, RTE, and lightly preserved. In recent years, considerable effort has been made to develop food applications for many different bacteriocins and bacteriocinogenic strains. Depending on the raw materials, processing conditions, distribution, and consumption, the different types of foods offer a great variety of scenarios where food poisoning, pathogenic, or spoilage bacteria may proliferate. Therefore, the effectiveness of bacteriocins requires

Table 1 Bacteriocins and/or bacteriocinogenic lactic acid bacteria strains used as biopreservative in seafoods

Seafood products	LAB strains	Bacteriocins	References
Brined shrimp	*Lactobacillus bavaricus* MI 401	Bavaricin A	Einarsson and Lauzon (1995)
Fish sausage	*Lactococcus lactis* subsp. *lactis*	Nisin	Raju et al. (2003)
Refrigerated seafood	*Pediococcus pentosaceus* ACCEL	Pediocin ACCEL	Yin et al. (2007)
Vacuum-packaged cold-smoked salmon	*Enterococcus*, *Lactobacillus*, *Pediococcus*	Antilisterial bacteriocin	Tomé et al. (2008b)
Senegalese fish fermentation	*Lactococcus lactis* subsp. *lactis*	Nisin	Diop et al. (2009)
Fish and shellfish products	*Enterococcus* spp.	Enterocins A, B, L50 and P	Chahad et al. (2012)
Fresh salmon fillets	*Lactobacillus pentosus* 39	Bacteriocin-like substance	Anacarso et al. (2014)
Cooked, peeled, and ionized tropical shrimps	*Lactococcus lactis* subsp. *lactis* KT2W2L	Nisin Z	Hwanhlem et al. (2015)

careful testing in the food systems for which they are intended to be applied against the selected target bacteria (Galvez et al. 2008).

It has been extensively studied that bacteriocins and bacteriocinogenic LAB strains were used as biopreservative in many kinds of fish and seafood products (Ghanbari et al. 2013). Many successful results have been obtained at the laboratory scale, nevertheless, the application in the seafood industry is still limited (Pilet and Leroi 2011). Table 1 presents a survey of bacteriocins and bacteriocinogenic LAB strains used as biopreservative in several seafood products.

6.2 *Application of Bacteriocin in Seafood*

The deterioration of fresh seafood is generally caused by Gram-negative microorganisms. However, in vacuum-packed fresh fish and seafood, some pathogenic organisms can also cause problems. Application of bacteriocins in seafood has been performed as potential biopreservatives in many foods such as brined shrimp (Einarsson and Lauzon 1995), fresh fish fillets (Yin et al. 2007), and vacuum-packaged cold-smoked salmon (Tomé et al. 2008a), etc. The combination of nisin and Microgard reduced the total aerobic bacteria populations of fresh chilled salmon, increased its shelf life, and also reduced the growth of inoculated *L. monocytogenes* in frozen thawed salmon. It has been also demonstrated the synergistic effect of combination of lactic acid, sodium chloride, and/or nisin in rainbow trout, and more recently showed the effect of LAB cultures on pathogenic microorganism control in fish (Ananou et al. 2007). Einarsson and Lauzon (1995) studied the biopreservation of brined shrimp (*Pandalus borealis*) by bacteriocins

from LAB. They reported that effects of three different LAB bacteriocins (purified nisin Z, carnocin, and crude bavaricin A) on bacterial growth and shelf life were compared with those of a benzoate–sorbate solution and a control with no preservatives. The shelf life of shrimp subjected to the control treatment was found to be 10 days. Carnocin UI49 did not extend the shelf life, while crude bavaricin A (a cell-free supernatant of *Lactobacillus bavaricus* MI 401) resulted in a shelf life of 16 days, as opposed to 31 days with nisin Z for both its crude and purified forms. The benzoate–sorbate solution preserved the brined shrimp for the whole storage period (59 days). In the control, carnocin UI49, and crude bavaricin A treatments, a Gram-positive flora dominated toward the end of the storage period while in the nisin Z treatment a Gram-negative flora was more pronounced.

Inhibition of *L. monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere in in vitro model studies and in trials with cold-smoked salmon has been studied by Nilsson et al. (1997). They found that addition of nisin caused various degrees of inhibition and sometimes death of *L. monocytogenes* in model experiments performed at 10 °C. The antilisterial effect of nisin was improved in the presence of 100 % CO_2 and increasing NaCl concentrations (0.5–5.0 % w/v). Minimal bactericidal concentrations (MBC) of nisin varied from 30 to more than 500 IU/ml. Raju et al. (2003) studied the use of nisin as a preservative in fish sausage stored at ambient (28 ± 2 °C) and refrigerated (6 ± 2 °C) temperatures with nisin on the keeping quality of fish sausage in synthetic casing at ambient (28 ± 2 °C) and refrigerated (6 ± 2 °C) temperatures. Fish sausage treated with 50 ppm of nisin was acceptable after storage at ambient temperature for 20–22 days compared with the control which were acceptable only for 2 days. The keeping quality of the sausages, at refrigerated temperature, varied from 30 days in the control to 150 days in 50-ppm nisin-treated samples.

Yin et al. (2007) investigated the effectiveness of pediocin ACCEL on refrigerated sea foods. Fresh fish fillets were immersed in various concentrations of pediocin ACCEL and then stored at either 0 or 4 °C. Samples treated with nisin were used as a positive control. The aerobic plate counts (APC) of samples with bacteriocins were <2.0 log CFU/g after 2 days storage at 0 °C, except that with 1500 IU/ml of pediocin ACCEL. The APC of samples with nisin were >2.0 log CFU/g after 2 days storage, while those with pediocin ACCEL occurred after 1 day storage at 4 °C. In refrigerated sea foods, pediocin ACCEL and nisin suppressed the growth of inoculated *L. monocytogenes* during 1 and 2 weeks of storage at 4 °C, respectively. Compared with nisin, the pediocin ACCEL was considered to be more effective on the suppression of *L. monocytogenes* growth in refrigerated sea foods during 2 weeks of storage at 4 °C.

Tomé et al. (2008b) studied the suitability of bacteriocin-producing lactic acid bacteria, as potential biopreservation cultures in vacuum-packaged cold-smoked salmon in vitro tests. They reported that nine bacteriocin-producing LAB strains isolated from vacuum-packaged cold-smoked salmon (CSS) were used as biopreservative cultures against *L. monocytogenes*. Only five strains were able to secrete active bacteriocins into the culture medium, at high salt concentrations and low temperatures, both in aerobic and anaerobic atmospheres. Enterococci showed

neither hemolytic activity nor vancomycin resistance. The production of histamine was not observed for any of the bacteriocin-producing strain.

Chahad et al. (2012) reported that 84 LAB strains isolated from sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) were identified and characterized for their inhibitory activities against the most relevant seafood-spoilage and pathogenic bacteria potentially present in commercial products. The bioactive strains belonged to the genus *Enterococcus* and exhibited inhibition against *Carnobacterium* sp., *Bacillus* sp., *L. monocytogenes*, *A. salmonicida*, *A. hydrophila*, and *V. anguillarum*. Molecular analysis led to the identification of the bacteriocins investigated, including enterocins A, B, L50, and P. All of these proteins demonstrated remarkable anti-Listeria activity and were found to be heat-resistant small class IIa bacteriocins. The results presented in their work open the way for potential applications of these LAB strains to the biopreservation of minimally processed seafood products.

6.3 Application of Lactic Acid Bacteria in Seafood

LAB have long been disregarded for seafood preservation because they are thought to be not present in fresh fish flesh. In the 1990s, the presence of LAB in high quantity has been highlighted in lightly preserved seafood products and studies on the selection of bacteria with antimicrobial properties have intensified. However, very few commercial applications have currently appeared in seafood products. A major hurdle is that these products are not fermented and the selected LAB strains should not change their delicate organoleptic qualities. Many bacteria that gave promising results in liquid medium proved to be ineffective in products, either because they were poorly established in the environmental conditions or because they produced unpleasant odors. A more recent strategy involves selecting LAB naturally present in seafood products in order to ensure their good growth in the marine matrix stored at chilled temperature (Leroi 2011).

LAB are able to grow at refrigeration temperatures. They tolerate atmosphere packaging, low pH, high salt concentrations, and the presence of additives such as lactic acid, ethanol, or acetic acid. LAB strains were found in cooked and brine shrimp stored under a modified atmosphere between 0 and 25 °C. The major organism found at 15–25 °C was *Ent. faecalis*, whereas *C. divergens* and *L. curvatus* were the major organisms found at 0–8 °C (Calo-Mata et al. 2007). In a similar study by Ben Embarek et al. (1994), the antibacterial potential of *Enterococcus faecium* strains isolated from sous-vide cooked fish fillets was reported. They highlighted that *E. faecium* showed the potential use as biopreservation in shrimp extracts or sous-vide cooked fish because the isolates of *E. faecium* did not produce off-odors in these products. They also reported that in the presence of 10^7 CFU/ml of *E. faecium*, *L. monocytogenes* (10^2 CFU/ml) was strongly inhibited at 3 °C and partially at 5 and 15 °C. Nilsson et al. (1999) reported that *L. sake* strain LKE5 and four strains of *C. piscicola* were evaluated as biopreservation cultures to control the

growth of *L. monocytogenes* on vacuum-packed cold-smoked salmon stored at 5 °C. All five strains were antilisterial as live cultures in an agar diffusion assay. CFS of two strains of *C. piscicola* and *L. sake* LKE5 were also antilisterial because of the production of bacteriocins. The presence of high cell numbers of strains of *C. piscicola* had no influence on the sensory quality of cold-smoked salmon stored at 5 °C, but *L. sake* LKE5 caused strong sulfurous off-flavors and was rejected as a culture for the biopreservation of cold-smoked salmon. A bacteriocin-producing strain of *C. piscicola* (A9b) initially caused a 7-day lag phase of *L. monocytogenes*, followed by a reduction in the numbers of *L. monocytogenes* from 10^3 CFU/ml to below 10 CFU/ml after 32 days of incubation, coinciding with the detection of antilisterial compounds.

Anacarso et al. (2014) studied the ability of *Lactobacillus pentosus* 39, a BLS (bacteriocin-like substance)-producing strain to control the growth of *A. hydrophila* ATCC 14715 and *L. monocytogenes* ATCC 19117 artificially added to fresh salmon fillets at refrigeration temperatures and under simulated cold-chain break conditions. They found that at refrigeration temperatures, *Lb. pentosus* 39 protective culture and its putative bacteriocin significantly reduced the number of *Aer. hydrophila* and *L. monocytogenes*. Under simulated cold-chain break conditions, an increase in temperature (30 °C for 12 h) produced an evident increase in the development of *A. hydrophila*, *L. monocytogenes*, but also of *Lb. pentosus* 39, with a consequent increase in the BLS production. This condition resulted in a greater reduction of both pathogens compared with samples stored at 4 °C throughout the experiment (2.8 log CFU/g reduction for *A. hydrophila* and 5.8 log CFU/g reduction for *L. monocytogenes*). In samples treated with the putative bacteriocin alone, a less marked decrease was observed. Their study demonstrates the capability of *Lb. pentosus* 39 to control the growth of psychrotrophic bacteria in an experimental seafood model system.

Hwanhlem et al. (2013) isolated *Lactococcus lactis* subsp. *lactis* KT2W2L which produced bacteriocin from mangrove forest and the bacteriocin was purified and characterized as nisin Z. Application of *Lactococcus lactis* subsp. *lactis* KT2W2L for biopreservation in cooked, peeled, and ionized tropical shrimps (CPITS) during storage at 8 °C under modified atmosphere packaging was studied (Hwanhlem et al. 2015). The neutralized and filtered cell-free supernatant produced by this strain exhibited a broad spectrum of inhibition against 23 strains from 24 indicator strains of food-spoilage bacteria and food-borne pathogens by agar well diffusion assay. The growth of *B. thermosphacta* CD274, *C. maltaromaticum* CD263, and *L. innocua* CIP 80.11T was inhibited by *Lact. lactis* subsp. *lactis* KT2W2L when co-cultivated at 25 °C. However, only *L. innocua* CIP 80.11T was inhibited in a co-cultivation at 8 °C. *Lact. lactis* subsp. *lactis* KT2W2L was then used for biopreservation in CPITS. Growth of bacteria group inoculated in CPITS was monitored at regular intervals during storage period under MAP at 8 °C by microbial counts and the thermal temperature gradient gel electrophoresis (TTGE) technique. *Lact. lactis* subsp. *lactis* KT2W2L inhibited the growth of *Br. thermosphacta* and *L. innocua* CIP 80.11T batches after 7 days of storage. However, it was inactive against *C. maltaromaticum* batch. In addition, TTGE revealed

to be an excellent tool to monitor the change of the microbial ecosystem in this product.

Diop et al. (2009) reported that *L. lactis* subsp. *lactis* strain CWBI B1410, which produced organic acids and nisin, was used as a starter culture to improve the traditional Senegalese fish fermentation to guedj by spontaneous fermentation for 24–48 h at an ambient temperature followed by salting (with NaCl) and sun drying. When raw fish fillets were supplemented with 1 % glucose and inoculated with *L. lactis* (10^7 CFU/g), the pH decreased to about 4.60 after 10 h at 30 °C and nisin activity was detected in juice from the fillets. These data suggest that this new fish fermentation strategy combined with salting and drying can be used to enhance the safety of guedj.

Karthik et al. (2014) studied the efficacy of bacteriocin producing *Lactobacillus* sp. strain AMET 1506 isolated from curd sample as a biopreservative for shrimp under different storage temperature conditions. Shrimp samples were divided into two groups. One group was stored directly and another group was dipped in cold distilled water containing bacteriocin of *Lactobacillus* sp. Both treatments were stored at –4 and –20 °C for 30 days and the microbial load was assessed at different time intervals. The presence of total heterotrophic bacteria, total coliforms and *E. coli*, *V. cholerae*, *V. parahaemolyticus*, *Salmonella* sp., and *Shigella* sp. were assessed in specific media. Their result indicated that the microbial load was reduced in the treatment which was preserved with bacteriocin of *Lactobacillus* sp. strain AMET 1506.

Several reports indicated that using bacteriocins and bacteriocinogenic LAB strains as biopreservatives have the potential to increase the safety and extend the storage life of seafood products. However, it has been reported that some strains of LAB influence the production of biogenic amines (BA), ammonia (AMN) and/or trimethylamine oxide (TMA-O) in seafood products (Françoise 2010; Küley et al. 2013). Therefore, the selection of suitable LAB strain to be used as biopreservative in each product is one of the most important parameter to be considered for the application of LAB in seafood products.

7 Conclusions

During the last 10 years, bacteriocins and bacteriocinogenic LAB strains were studied as biopreservatives in many kinds of food products as well as seafood. Most of the successful results have been obtained at the laboratory scale. Nevertheless, the application in the seafood industry is still limited. The production strain must be proofed as GRAS status. The selected bacteriocins or LAB should not have any deleterious effects on food quality. In addition it must survive the harsh conditions of the seafood products. The use of bacteriocins in combination with other antimicrobial compounds will broaden the inhibitory spectrum. The production of either the suitable bacteriocins or the bacteriocinogenic LAB at low cost and

the ease of application with long lasting efficiency are necessary for the successful application in seafood industries.

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