SCREENING OF *XANTHOMONAS* SPECIES FOR XANTHAN GUM PRODUCTION

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ABSTRACT

Xanthomonas campestris are plant pathogens causing various disease such citrus canker, black rot disease and bacterial leave blight, xanthan gum is an extracellular heteropolysaccharides produced by *Xanthomonas* species through the fermentation of carbohydrates. This study aimed at screening *Xanthomonas* strains isolated from diseased plant leaves (tomato, pepper, mango rice and banana leaves) for potential to produce xanthan gum. Leaves with black rot spots were collected from the plants in selected farms within Niger state (Nanni village in Kaffi LGA, Badeggi in Bida LGA and Chanchaga in Bosso LGA) and transported to the research laboratory. The leaves were washed in normal saline and tenfold dilution was prepared. Aliquots (1mL) were plated on nutrient agar and incubated at 37˚C for 48h. Yellow pigmented bacteria colonies that appeared Gram negative rod after Gram staining were subjected to emulsification test. Isolates with yellow pigmentation, Gram negative rods and which exhibited stable emulsion in carbon enriched medium were regarded as potential xanthan gum producers. Eight (61.5%) of the isolates screened fulfiled these conditions. Biochemical tests on the isolates revealed that the organisms were *Xanthomonas* species. The best two xanthan gum producing isolates were further identified by molecular techniques as *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127. *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 yielded 2.10 g/L and 1.63 g/L respectively of xanthan gum after 96 h. The xanthan gum as well as the biomass yield by the two organisms were found to increase (4.51-7.26 g/L and 6.50-6.80 g/L respectively) under optimized conditions of pH: (9.0, 7.0), temperature (25℃) carbon source (0.2% pineapple peels, 0.2% sugarcane bagasse) and nitrogen source (yeast extract) respectively. The xanthan gums produced were characterized and the results showed not much variation between the xanthan gum produced and the commercial xanthan gum. However, the apparent viscosity of xanthan gum produced by *Stenotrophomonas maltophilia* IAE127 (660.6 mPas) was higher than those of *Xanthomonas campestris* CPBF 211 (526.1mPas) and commercial xanthan gum (411.3mPas). Besides, the ability of xanthan gum from *Stenotrophomonas maltophilia* IAE127 and *Xanthomonas campestris* CPBF 211 to emulsify hydrocarbon, vegetable oils, crude oil and diesel was similar to commercial xanthan gum. All the gums exhibited good thermal stability. Xanthan gum produced by the two organisms were found to recover less crude oil from the soil.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Many phytopathogenic organisms in the genus *Xanthomonas* are responsible for a wide range of diseases in monocot and dicot plants that are economically important worldwide. *Xanthomonas* species produce a range of virulence factors, such as adhesins, lipopolysaccharides, exopolysaccharides (EPSs) and extracellular degradative enzymes (Bianco *et al*., 2016). *Xanthomonas campestris* is a plant pathogen, anaerobic bacterium that is able to grow both in a complex and a defined medium. It is usually used to produce xanthan gum (Makut *et al*., 2018). The species *Xanthomonas campestris* has a vast host range of isolation (often from plants of the family Brassicaceae) such as cauliflower, spinach, cabbage, rutabaga, turnip (Ryan *et al*., 2011; Rodriguez *et al*., 2012).

Xanthomonas campestris is made up of five pathovars: incanae, armoraciae, barbareae, aberrans, and raphani, according to DNA-DNA hybridization studies. (Rombouts, 2017). The pathovars of *X. campestris* cannot be distinguished if detected on the premise of phenotypic characterization. Instead, the knowledge of the host has to be known in order to differentiate between the pathovars, Each *Xanthomonas campestris* strain or pathovar is unique to a particular plant (Vicente and Holub, 2013). Almost all *Xanthomonas* species, particularly those of the species *Xanthomonas campestris*, have the ability to produce exopolysaccharide, also known as xanthan gum (Rosalam and England, 2006).

Xanthan gum is an extracellular heteropolysaccharide that is produced by *Xanthomonas* species such as *Xanthomonas campestris* (Habibi and Khosravi-Darani, 2017),

Xanthomonas pelargonii (Niknezhad *et al*., 2015), *Xanthomonas phaseoli* and *Xanthomonas malvacearum* during aerobic fermentation (Leela and Sharma, 2000).

It is made up of repeated pentasaccharide units involving two structural molecules of mannose, glucose and one unit of glucuronic acid (Darzi *et al*., 2012). Due to its heat, acid and alkali stability, thickening property, excellent rheological properties, and pseudoplasticity, xanthan gum can be used in variety of ways which includes; in the food, agricultural, industries involve in cosmetics and pharmaceutical (Niknezhad *et al*., 2015).

Xanthan gum has also been discovered in the past few decades to be of importance in the oil industries (Hans *et al*., 1999). Xanthan gum is used extensively in the oil industry, mostly to thicken drilling mud. Due to its resistance to high temperature and salt, xanthan gum is being applied in the rheological control of tertiary oil recovery, and can help in improving the recovery factor. About 30% to 40% of xanthan gum is implored in drilling mud and tertiary oil recovery in advanced countries (Abidin *et al*., 2012).

1.2 Statement of the Research Problem

Considering the total volume of xanthan gum produced worldwide, 65% is used in the food industry, 15% is used in the oil industry, and approximately 20% is used in other applications. The use of xanthan gum is increasing for many applications, and its estimated annual growth is approximately 5–10% (Rosalam and England, 2006; Lopes *et al*., 2015). The Fufeng Group, one of the largest xanthan gum manufacturers, estimated that approximately 59,000 metric tons of xanthan gum were manufactured annually in 2012 (US International Trade Commission Report, 2013)

The popularity of this gum has grown dramatically over the years as a result of its expanding range of applications (Li *et al*., 2019). Commercial xanthan gum is currently developed using sucrose and glucose as carbon sources in fermentation. The cost of

produced xanthan gum is high due to rising costs of sucrose and glucose (Ozcan and Oner, 2015; Li *et al*., 2019; Sharmila *et al*., 2020).

Fruits are typically transformed into various fruit drinks and other products, which results in the generation of solid waste. These wastes are often disposed improperly, especially in developing countries, resulting in serious pollution issues. These waste materials are generally thought to be of little or no value since they are only used for specific purposes including animal feed (Faria *et al*., 2011) Since these waste materials are high in carbohydrate and other nutrients, they can be used to make high-value products such as xanthan gum.

Enhanced oil recovery (EOR) methods used in the oil industry are expensive and some of the methods adopted are not environmentally friendly. Thermal, miscible, and chemical processes are commonly used in EOR, with chemical treatment being one of the most common methods for reducing water production. This has been operated by building up flow resistance, which was accomplished by increasing the shear viscosity of the injected water and/or decreasing its permeability by polymer adsorption (Ali *et al*., 2020). Water-soluble polyacrylamide and polysaccharides are used in oil fields to increase oil recovery.

1.3 Justification for the Study

Xanthan gum has a special rheological property that allows it to be used as a viscosifying, stabilizing, thickening, emulsifying, and suspending agent in a variety of applications (Mohammadi *et al*., 2014). Due to its large applications and widespread usage in several processing industries that includes; food, cosmetics, toiletries, water-based paints, oil recovery, and so on, there is a fair desire to increase industrial xanthan gum production (Ryan *et al*., 2011). It has been discovered that xanthan gum aids oil

movement by increasing oil volatilization (Pu *et al*., 2018). Because of the pseudoplasticity of xanthan gum, products containing it have fluidity, adhesion, and a pleasant mouth feel (Mohammadi *et al*., 2014).

The high cost of substrate for xanthan gum production has led to finding alternative low-cost and easily accessible fermentation substrates and factors that could favor optimum production of the gum. Chestnut extract, waste sugar beet pulp, cocoa bark residue, residue of apple juice, cane juice, cheese whey, cassava whey, date extract, and sugar beet molasses have all been used to substitute sucrose and glucose in the xanthan gum manufacturing process (Moosavi-nasab *et al*., 2009; Brandao *et al*., 2010; Mabrouk *et al*., 2013; De souse costa *et al*., 2014) among others. These substitutions would lower the overall cost of xanthan gum production while also adding value to the environmentally friendly waste produced by agricultural processes, as most of these wastes are not properly disposed of, causing environmental issues.

Xanthan gum is the most widely used FDA-approved microbial polysaccharide, with a global demand of USD 987.7 million expected by 2020 (Barua *et al*., 2016). In this context, identifying a local strain of *Xanthomonas* and optimizing fermentation conditions for increased xanthan gum output would be extremely beneficial.

1.4 Aim and Objectives of the Study

The aim of this study was to screen *Xanthomonas* strains for potential to produce xanthan gum.

The objectives of the study were to:

i. isolate, identify and characterize *Xanthomonas* species from plant leaves.

ii. produce and characterize the xanthan gum.

iii. determine the optimal conditions for production of the xanthan gum.

iv. determine the potential of the produced xanthan gum for enhanced oil recovery.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of the Genus *Xanthomonas*

Walker was the first to describe the phytopathogenic bacterium that is now known as *Xanthomonas* in 1881. In the case of a Yellow Hyacinth disease, Walker initially reported a number of significant characteristics of the causal agent in his brief report which now warns any informed phytobacteriolist about the possibility that a *Xanthomonas* species was involved in a hyacinth disease. Many related bacteria were isolated from different plant diseases around the world in the century following Walker's discovery (Greenwood *et al*., 2012).

2.2 Habitat of the Genus *Xanthomonas*

The *Xanthomonas* genus is thought to originate from habitats that involve plant diseases. Most reports about the habitats of the Xanthomonads has to do with their occurrence with initial lesions on infected plants (Beattie, 2007). Xanthomonad ecosystems are extremely rare but probably authentic in soil, plant waste and asymptomatic plants. *Xanthomonas* strains are used to produce *Xanthomonas* slime a polysaccharide gum called the "xanthan," a significant commercial article in large-scale industrial fermentation processes and form a class of artificial and human habitats. Plant infection by *Xanthomonas* and disease growth are aided by warm and wet conditions (Beattie, 2007). Since hydathode is thought to be the natural route of infection, free moisture is needed to create host invasion.

2.3 Classification of X*anthomonas campestris*

The most commonly studied species of the genus *Xanthomonas* is the species *campestris*, the organism classified based on host plant it attacks (Vicente and Holub, 2013). Some of the most commonly studied *Xanthomonas campestris* include;

2.3.1 *Xanthomonas campestris* Pv. *Campestris*

Black rot is caused by the bacterium *Xanthomonas campestris.* The main destructive cruciferous disease, infecting all cultivated varieties of brassicas worldwide (Monteiro *et al*., 2005; Qian *et al*., 2005; Tonu *et al.*, 2013). This disease was first described by botanist and entomologist Harrison Garman in Lexington, Kentucky, USA in 1889. Since then, it has been found in nearly every country in which vegetable brassicas are commercially cultivated (Charles, 2006). Host infection by Xcc can occur at any stage of the plant life cycle. Blackening of vascular tissue and foliar lesions ranging from chlorotic to necrotic are recognized symptoms of the disease, causing the vegetable to rot and become unfit for human consumption (Alvarez, 2000). In wet, humid conditions, the pathogen thrives and spreads rapidly in the field. Disease prevention can be supported by crop rotation, clean seed, and other cultural practices. Due to unreliable "clean" seed, annual multiple croppings, and the high susceptibility of conventional local cultivars to disease, disease remains the greatest When Xcc infects a plant, it causes wilting, stunted growth, vascular blackening, stem rot, and V-shaped chlorotic to necrotic foliar lesions, as well as wilting, stunted growth, vascular blackening, and stem rot symptoms (Radunovi and Bala, 2012). As it spreads, the pathogen travels from the leaf margins to the veins, bacterial exopolysaccharides and components of damaged plant cell walls occluding water-conducting vessels, water stress and chlorotic symptoms increase. The term "black rot disease" comes from the

vascular tissues blackening caused by pathogen invasion. Soft-rot pathogens Pectobacterium carotovorum (formerly *Erwinia carotovora*) and *Pseudomonas marginalis* may penetrate Xcc-caused lesions. *Fusarium oxysporum* is a fungus that causes fusarium wilt of cabbage, which has similar symptoms (Radunovi and Bala, 2012). Unlike black rot, which occurs when the pathogen enters the leaf margins, Symptoms of Fusarium wilt appear first in the lower parts of the plant and then progress upward, resulting in chlorotic to necrotic symptoms that progress down the plant. In addition, Among Xcc pathogen strains, the seriousness of symptoms and aggressiveness of black rot disease vary (Alverez, 2000).

Infection and disease growth of Xcc plants are favored by warm and wet environments (Fleites *et al*., 2012). Since hydathodes are the natural route of infection, unrestricted moisture is needed invasion of the host (leave pore). Ideal temperature ranges for bacterial growth and the development of the host symptoms is between 25° and 30°C. At temperatures as low as 5°C and as high as 35°C, the rate of growth is slower. Infected hosts, on the other hand, are symptomless below 18 °C (Mikulic-Petkovsek *et al*., 2011).

Application of disease-free seeds and transplants that have been approved, chemical treatments of sodium hypochlorite, as well as hot water treatment of non-licensed seeds, insect control, crop rotation (3-4 years), Crop debris removal after harvest, cruciferous weed control to prevent the pathogen from becoming a reservoir, as well as hygiene (e.g. sterilized equipment, ignoring working in wet fields, etc.) are all examples of cultural practices (Singh *et al*., 2018). The development and application of black rot resistant cultivars has also been described as an important method of control, though it has had limited practical success (Ghazalibiglar *et al*., 2015).

2.3.2 *Xanthomonas campestris* pv. *Vesicatoria*

Xanthomonas campestris pv. Vesicatoria is a gram-negative, rod-shaped microorganism which causes bacterial leaf spot (BLS) on plants such as peppers and tomatoes. Symptoms include leaf spots, stem cankers, and fruit spots above the ground level of the plant (Abbasi and Weselowski, 2015). The disease can be managed by extracting plant content that has died and applying chemical treatments to plants that are alive because the pathogen cannot survive few week or more in the soil as dead plant inoculum. Tomatoes and peppers are the primary hosts of BLS, but other incidental hosts, primarily weeds, have been observed. Bacterial colonization of intracellular spaces causes macroscopic Water-soaked lesions on the leaves that turn necrotic on the host are among the symptoms (Thieme *et al*., 2005). Even though the spots are tiny at first (about 24 inches in diameter), they gradually increase in size and number, shading the leaves. Plant stems can also be infected with BLS, which results in elongated, elevated, light-brown cankers that are less than 25 inches long (Thieme *et al*., 2005).

Xanthomonas campestris pv. *Vesicatoria* since Vesicatoria can only last a few weeks in the soil, it feeds on seed of pepper and tomato plants, and also debris of plant (Sharma, 2018). Wheat roots and certain weeds, both of which are inoculum sources, are also present in a symbiotic relationship with the bacterium (Sharma, 2018). As it emerges from the seed layer, if it thrives on seeds, it can spread to the growing plant's cotyledons. Internally infected seeds can grow diseased plants from the moment they germinate. Plants that are infected at the germination stage do not exhibit systemic symptoms like wilting, yellowing, or dwarfing. When localized signs on leaves become apparent, however, leaf loss may occur (Peitl *et al*., 2017). When localized signs on leaves become apparent, however, leaf loss may occur (Peitl *et al*., 2017). It is spread by plants coming into direct contact with debris. Plants that have been infected grow lesions on

their leaves and fruit, which serve as inoculum sources for further infection (Sharma, 2018). The disease's distribution is heavily influenced by the environment. High humidity is needed by the bacterium (Potnis *et al*., 2015).

Xanthomonas campestris pv. *Vesicatoria* has proven to be a major concern in nurseries and greenhouses, where the bacteria thrive in the warm temperatures and high humidity. Propagation facilities are overburdened raising the risk of disease transmission by allowing contaminated plants to come into contact with healthy plants (Potnis *et al*., 2015).

Since decayed plant material is detrimental to the organism's survival, removing debris and rotating crops are two effective ways to prevent the bacterium from spreading. Eliminating plants that flourish without being intentionally planted is another successful control measure in warmer climates (volunteer plants), as they can harbor the disease (Potnis *et al*., 2015).

2.3.3 *Xanthomonas campestris pv. Graminis*

This organism causes turf grass bacterial wilt, which is the only known bacterial turf disease. The disease was previously known as C-15 decline until the causative agent was discovered in 1984. It was discovered in the mid-western United States in a creeping bent grass cultivar known as Toronto or C-15 (Hersemann *et al*., 2016).

Climbing bentgrass (Agrostis stolonifera) and annual bluegrasses are the preferred hosts for this bacterium (Poaannua). The hybrids of creeping bent grass most commonly affected are Toronto (C-15), Nemisilla, and Seaside (Singh, 2017). The pathogen infects the host plant and tampers with nutrient and water supply, causing the plant to appear drought-stressed and bluish-purple in color. Additionally, tan or brown patches, yellow leaf spots, elongated yellow leaves, water soaked lesions, and the dropping down of

blue or dark green leaves are all signs of this disease. During the winter, the bacterium is spread through water by splashes of rain or systematically via golf shoes, horses, and other equipment for gardening. This bacterium can also be found in the host during the planting process. Plant pathogens of bacterial origin unlike fungi are incapable of injuring or systematically on their own penetrate plant hosts Instead, these bacteria invade plants via injuries caused by cultivation, sand, verticutting, or natural openings like hydathodes and stomata. They take charge of the water and nutrient movements of plant cells to facilitate their own reproduction when they've made it into and settled within the plant host (Jones *et al*., 2004; Potnis *et al*., 2015).

2.4 Cell structure and metabolism of *Xanthomonas campestris*

Xanthomonas campestris (Xc) is a single straight rod-shaped Gram-negative, motile with a unipolar flagellum bacterium (García-Ochoa *et al*., 1992) yellow pigmentation and double cell walls that help to identify it. Hrp (hypersensitive response and pathogenicity) pili are a threadlike structure which are connected to type III system for protein secretion enhancing the potential to move water as well as bacterial proteins into the plant (Nino *et* al., 2006). This aerobic organism uses a variety of metabolic pathways which are highly reliant on the pathovar (Thieme, 2005). The whole genome sequencing of *Xc* shows the breakdown of carbohydrate which include: Gluconeogenesis (Glycolysis), Tricarboxylic acid (TCA cycle), Pentose phosphate pathway (PPP) e.t.c. *Xc* obtains its source of energy through phosphorylation by oxidation, fixation of carbon, nitrogen, metabolism of sulfur and methane (Thieme, 2005). It obtains glucose through gluconeogenesis from the carbon within the host. Further research shows that Xc has only the malic enzyme-PpsA pathway in gluconeogenesis, which is needed for its virulence (Leng *et al*., 2019). Additionally, this plant bacterium contains a type III secretion system (TTSS) that is essential for

attacking the host. TTSS is relevant in pathogenesis because it conveys effector proteins in order to decrease the host's defense, forming biofilm on the surfaces of plant. *Xc* demonstrate cell-cell communication using diffusible signal factor (DSF). Having the tendency to ferment, Xanthan is an extracellular polysaccharide produced by *X. campestris* that is commercially manufactured in factories and used as a stabilizing agent in a number of everyday items (Leng *et al*., 2019).

2.5 Ecology of *Xanthomonas campestris*

X. campestris produces a great loss in agriculture because they inhabit cruciferous vegetables, some of which include, cauliflower, cabbage, Brussels sprouts and also some plants such as tomato, pepper (Fig. 1), banana, rice, mango, orange. It can survive within the soil to more than one year and be dispersed by above the ground level irrigation and surface water movement (Rombouts, 2017). This pathogen thrives in hot, humid conditions with maximum temperatures of 25-30˚C (77-85℉). Water is necessary for X. campestris' survival and movement to its next host. As a result of contamination at the course of cultural operations, affected plants mostly are seen on the same row when farmed (Rombouts, 2017).

Figure 2.1: Pepper farm infected with *Xanthomonas campestris*
Source: Amanda *et al*. (2019).

2.6 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia, was first isolated in 1943 as *Bacterium booker,* previously known as *Xanthomonas maltophilia* (Swing *et al*., 1983) and *Pseudomonas maltophilia* (Hugh, 1981). It is a gram-negative bacillus, aerobic organism that is prevalent in several of environments. It has been discovered in the environment as an agent for promoting growth in the rhizospheres of plants, water, soil, sediment, sewage, frozen foods, as well as other habitats (Hauben *et al* 1999). Significantly, *S. maltophilia* is increasingly spreading wide as an opportunistic human pathogen in hospitals causing nosocomial infections on individuals that are immunocompromised and associated with postoperative infections, respiratory tracts and the infections of urinary tracts as well as other disease syndromes. This pathogen was found to be the second most often isolated

nosocomial organism after *Pseudomonas aeruginosa*, and its infections has a significant growth of clinical importance (Hauben *et al* 1999; Gherardi *et al*., 2015).

2.7 Chemistry of Xanthan gum

Xanthan gum is a complex microbial exo-polysaccharide produced by the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* via glucose fermentation. Xanthan gum has a molecular weight of about 2 million, but it may be as heavy as 13–50 million (Silva *et al*, 2009; Lopes *et al*., 2015). It is made up of 2:2:1 molar ratios of d-glucosyl, dmannosyl, and d-glucuronyl acid residues, as well as variable amounts of O-acetyl and pyruvyl residues. Xanthan gum is an acidic polymer made up of pentasaccharide subunits that form a cellulose backbone with trisaccharide side chains consisting of mannose (1,4) and glucuronic acid (1,2) mannose linked to alternate glucose residues in the backbone by -1,3 linkages. A ketal connection is connected to half of the mannose residues terminally via a pyruvic acid moiety. On the internal mannose residues, acetyl groups are mainly found as 6-O substituents. In certain external mannoses, a second 6-O-acetyl substituent may be used (Petri, 2015). Exopolysaccharide formed by Gram-negative bacteria is thought to be similar to xanthan gum production (Miranda *et al*., 2018). There are three sections to the synthetic route:

(i) Simple sugars are absorbed and transformed into nucleotide derivatives.

(ii) Packaging of pent saccharide subunits joined to an isopentyl pyrophosphate carrier.

(iii) Polymerization of repeated pentasaccharide units and their secretion.

The backbone of xanthan is mold by consecutive additions of d-glucose-1-phosphate and d-glucose from 2 mol of uridine diphosphate-d glucose as shown in Figure 2. Then, in that order, guanosine diphosphate-mannose and UDP-glucoronic acid are included, D-mannose and d-glucoronic acid come next. The completion of each of the following steps necessitates the use of particular enzymes and substrates. The move would be obstructed if one of the two is absent (Rosalam *et al*., 2006). In *X. campestris*, Entner–Doudoroff, and tricarboxylic acid cycle pathways are the most common mechanisms for glucose breakdown, with the pentose phosphate pathway accounting for a small portion of glucose.

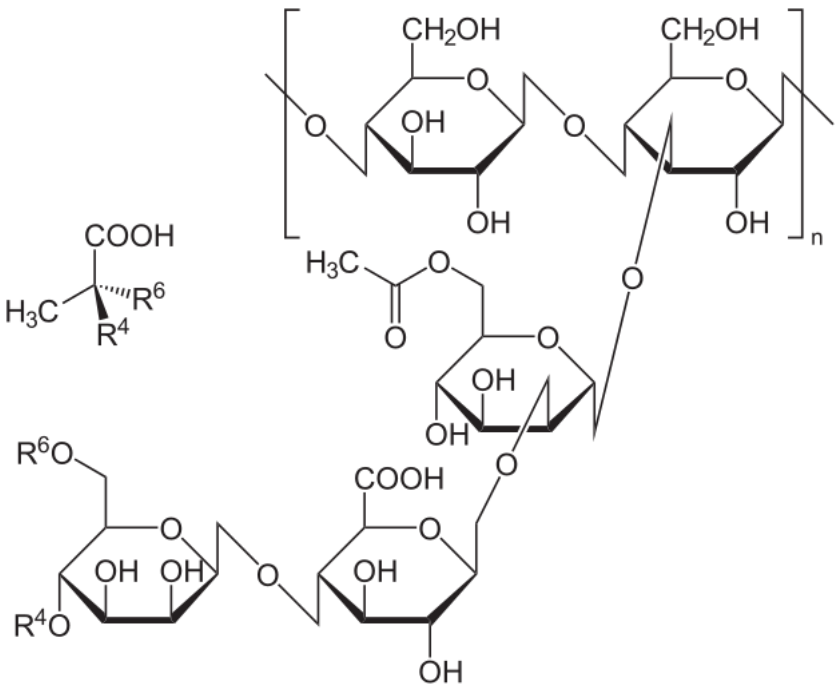


Figure 2.2 Structure of xanthan gum

Source: Rosalam *et al*. (2006)

Xanthan biosynthesis produces the polymer on a receiver molecule by combining multiple activated donors of carbohydrate similar to many polysaccharide producing bacteria. A series of monosaccharaides from nucleotide sugar di phosphates to

isoprenoid lipid receiver molecules are used to model the oligosaccharide repeated units of xanthan. Also, acyl replacements are integrated from suitable donors that are activated. It has been proposed that the building of the exo polysaccharide succeed a "tail-to-head" polymerization (Lopes *et al*., 2015). The long oligosaccharide sequence is normally added to the isoprenoid lipid diphosphate during oligomerization. Dephosphorylation of the inactive lipid carrier yields isoprenyl phosphate, which can then be reintroduced into the biosynthetic chain. Moving through the periplasm and outer membrane before being expelled into the extracellular system are the last stages for the secretion of exopolysaccharide from the cytoplasmic linning. Until transporting polymer to its final destination, all bacteria that contain polysaccharide must have this mechanism for releasing polymer from the isoprenoid lipid. The treatment requires an energy source, and it could be compared to the export of lipopolysaccharide to an external membrane, which uses ATP as an energy source (Brunchi *et al*., 2016).

2.8 Properties of Xanthan Gum

2.8.1 Physical

Xanthan gum is a powdery material that ranges in color from white to pale white. Its particle size is primarily 80-100 mesh, but it can range from agglomerate product to fine 200 mesh powder depending on the requirements needed by the consumer (Brunchi *et al*., 2016).

2.8.2 Solubility

Xanthan gum dissolves readily in water and is soluble in both hot and cold temperatures. Solubility can be determined using a wide range of salt concentrations and pH values. To ensure effective processing, when using xanthan gum, proceed with

caution. Both dry ingredients should be blended together before being poured into the liquid with high-speed agitation (Lopes *et al*., 2015).

2.8.3 Stability

When working with a wide variety of pH values, xanthan gum is very stable (2-12). It's also resistant to salt, enzymes, and heat. The heat stability of xanthan gum in a 1.1 percent citrate solution at a pH of 3.4 at 90°C for 24 hours, for example, is excellent (Brunchi *et al*., 2016).

2.8.4 Viscosity

Changes in pH, salt addition, and thermal changes do not normally affect viscosity values over long periods of time, despite the fact that other hydrocolloids are affected under the same conditions (Lopes *et al*., 2015). Xanthan gum and galactomannans including locust bean gum and guar gum are known to work well together (Verma *et al*., 2019).

2.8.5 Chemical composition

Xanthan gum is really a large molecular weight heteropolysaccharide that breaks down into individual D-mannose, D-glucose, and D-glucuronic acid monomer units when hydrolyzed. Xanthan gum's main chain, like cellulose's, is made up of b-D-glucose units connected at the 1-and 4-positions. Any alternate glucose residue has a side chain that includes a D-mannose, b-D-glucuronic acid, and a terminal b-D-mannose unit (Petri, 2015; Miranda *et al*., 2018).

2.9 Factors Influencing Production of Xanthan Gum

2.9.1 Effect of carbon sources

To be able to expand and become more effective, cells have to consume nutrients needed to produce proteins, membranes, chromosomes, cell walls and other components. Many cells use various energy and carbon sources, indicating they don't have equal internal chemical machinery. Using various limiting nutrients and substrate, for example, has no effect on the primary backbone structure, but has an effect on the side-chain structure, molecular mass, and xanthan gum yield. As a result, a mixture of xanthan gum made through a batch culture method will reflect a combination of xanthan gum produced at different growth phases, which may vary depending on the cultural conditions (Davidson, 1978). The most common carbon sources are glucose and sucrose. Since different cultures need various media and ideal conditions, several studies on the nutrients needed for side chain of product differences and optimization in xanthan gum production were conducted (Davidson, 1978; Garcia-Ochoa *et al*., 1992; Letisse *et al*., 2001; Makut *et al*., 2018). Carbon source concentration has a significant impact on xanthan yield; 2–4% xanthan concentration is suggested (Palaniraj and Jayaraman, 2011). Development is hampered by these substrates in higher concentrations Zhang and Chen (2010) used a xylose/glucose mixture media to make xanthan gum finding glucose to be a better source of carbon for xanthan production than xylose, which has a low rate of consumption.

2.9.2 Effect of pH on production conditions

Neutral pH is generally assumed to be highest for the synthesis of polysaccharide and microbial growth during fermentation process. For xanthan gum, neutral pH is optimal

for the growth of *X. campestris*, although, the pH decreases during fermentation to around 5.0 as a result of the presence of acid groups in the biopolymer. Better results are obtained with pH between the range 6.0 and 7.0. A study revealed that the control of pH enhances the growth of the microorganism without an effect on the xanthan gum produced (Qiu *et al*., 2015). Even though most authors agreed that control of pH in the range 6.0 - 8.0, using alkali such as KOH, NaOH or $(NH)_4OH$, was beneficial for the production of xanthan gum (Gumus *et al*., 2010; Niknezhad *et al*., 2015).

2.9.3 Effect of temperature

The effect of temperature on the development of xanthan gum has been extensively researched and the most desirable temperature range was discovered to be between 25 and 34ºC, while the cultured organism's temperature ranges between 28 and 30ºC. Various studies have shown that the ideal temperature for producing xanthan gum is 28ºC (Gumus *et al*., 2010; Lopes *et al*., 2015; Silva *et al*., 20017; Makut *et al*., 2018). It has been reported that lowering the temperature from 30 to 25 ºC yields the highest xanthan gum yield; however, the most favorable xanthan gum temperature for production is dependent on the medium for production (Psomas *et al*., 2007).

2.10 Applications of Xanthan Gum

Since xanthan is a water-soluble microbial polymer with unique attributes, therefore the gum has a variety of industrial applications which include;

2.10.1 Food industry

Xanthan gum is primarily used in the food industry as a thickening and emulsifying agent in several products such as fruit pulp, juice and powder beverages, yoghurt, chocolates, jellies, dairy products, desserts, margarine, bakery products, frozen foods,

sauces and gravies (Habibi and Khosravi-Darani, 2017). With its stabilizing and thickening properties, xanthan gum is a commonly used product in the food industry. Its binding and thickening properties ensured that it became an essential cooking aid food additive in dairy and bakery products. Some ordinary application of the xanthan gum are in salad dressings, gluten free breads and ice creams. The basic uses of xanthan gum across the food includes in milk based sauces and dairy products like salad dressings, as substitute for wheat and other cereals used for bread making that is gluten free, it is a simple way of thickening soya-milk rice, and non-dairy soups and ice creams (Barak and Mudgil, 2014; Habibi and Khosravi-Darani, 2017; Singh *et al*., 2018).

Xanthan gum is used in synergy with other gums like guar or locust bean gum, to decrease the cost of production (Barak and Mudgil, 2014). Xanthan gum provides current food products with the needed texture, flavour, viscosity, appearance and water-control properties. Aside these properties, its pseudo plastic behavior in solutions also helps to improve the formation of the final products (Lopes *et al*., 2015).

2.10.2 Chemical industry

The shear thinning properties of Xanthan can be used in the paint industry. Paints containing xanthan gum are extremely viscous at low shear rates and therefore do not drip when applied with a brush. Brushing, on the other hand, causes shear tension, which thins the paint and makes it easier to use (Patel *et al*., 2011). Because of its ability to spread and hydrate rapidly, xanthan is non-polluting and produces a vivid color; it has also been used in jet injection printing. Xanthan gum was revealed to settle the demands for producing environmentally safe goods in the formulation of existing generations of thermo-set coatings (Rosalam and England, 2006). Other important

applications of xanthan gel are in the removal of rust, wet slag, welding rods, and cleaning other litter from gas pipeline (Patel *et al*., 2011).

2.10.3 Agriculture industry

Xanthan gum in agriculture has been researched to be an elicitor in conjunction with fungicides in prevention of *Bipolaris sorokiniana*, which attacks cultivars of barley (Lopes *et al*, 2015). By uniformly intercepting the solid portion, it has been used to boost the flow ability of herbicides, fungicides, and insecticide formulations. Drift is reduced, and pesticide stick and permanence are improved, thanks to the specific rheological properties for solution of xanthan gum (Habibi and Khosravi-Darani, 2017).

2.10.3 Oil industry

Xanthan gum is used in the petroleum industry for oil exploration, pipeline washing, fracking, work-over, and completion. Because of its fantastic contact with salt as well as thermal resistance, xanthan gum is also used as a drilling fluid additive (Jain *et* al., 2016). Low viscosity at the drill bit, where shear rates are high, and high viscosity in the annulus, where shear rates are low, would result due to the pseudoplasticity of its solutions. As a result, xanthan will serve a dual purpose, allowing for faster penetration at the bit while also suspending cuttings in the annulus (Jha *et al*., 2014). Approximately two barrel are left in the ground for every barrel of oil produced. Xanthan gum has been found to be of great important in enhanced oil recovery (EOR). The fundamental principle used is to enhance the separation of oil and water thereby increasing recovered

oil (Abidin *et al*., 2012). However, the consistency of the xanthan gum is crucial, as a rise in impurities will make processing the oil extremely difficult. As a tertiary oil recovery technique, xanthan gum may be applied in micellar-polymer flooding. During the application, a polymer-thickened brine is used to force the surfactant slug through porous reservoir rock to collect residual oil; the polymer prevents the pushed water from bypassing the surfactant band, ensuring good area sweeping (Abidin *et al*., 2012). The purpose of polymers in both applications has to do with causing a reduction in the movement of injected water through raising its viscosity (Palaniraj and Jayaraman, 2011).

2.11 Side Effects of Xanthan Gum

i. The side effects of xanthan gum are not much, although those allergic to xanthan, are advised to stay away from products incorporated with xanthan gum. The dust particles xanthan is known to induce respiratory problems (Park *et al*., 2019).

ii. Xanthan gum is harmless if not up to 15 grams is taken in a day. It can induce some side effects like; bloating and intestinal gas (flatulence).

iii. People who are exposed to xanthan gum powder can experience flu-like symptoms, lung problems, and irritation of the nose and throat. There isn't much information about the use of xanthan gum during pregnancy and breast-feeding. Therefore, in other to be on the safer side, avoid making use of large amounts other than the optimal range giving for foods.

iv. Vomiting, nausea, appendicitis, blockage or narrowing of the intestine, difficult to expel stools that are hard (fecal impaction) or undiagnosed stomach pain: when having conditions such as the above mention, do not use xanthan gum. It is a bulk-forming laxative that can be harmful in these circumstances.

v. Xanthan gum has the potential to reduce blood sugar levels. It's possible that it'll mess with blood sugar regulation during and after surgery. Stop using xanthan gum at least two weeks before your surgical date (Webmed, 2019).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study areas from where the samples were collected were Bida, Bosso, Paikoro Local Government Area of Niger State (Fig 3.1). Niger State was created on 3rd February, 1976 by the regime of General Murtala Mohammed. It has Minna as it's capital. It has 25 Local Government Areas (LGA) out of which Bida, Kontagora, and Suleja are major cities in the state. Niger State is located in the middle belt of Nigeria, it covers 76,363 square kilometres. It is the largest Nigerian state by land area. Niger State has a population of 5,556,247 (National population Census, NPC 2006). The major tribes in the state includes; Nupe, Gbagyi, Dukawa, Kamuku, Kambari and Koro. Nupe's are dominant in Bida LGA were the constitute up to 90 % of the population, their occupations includes Farming and fishing, Badeggi in Bida LGA is basically known for rice cultivation, Paikoro LGA is located along the eastern part of the state, it is dominated by the Koro's and Gbagyi's. Nani village in Paikoro LGA is known for the cultivation of Yam, Tomato and pepper. Chanchaga settlement in Bosso LGA is known to have a mixed population consisting of both Christian and Muslim, it is a few kilometer before the entrance to the state capital, the state water works is located in

Chanchaga as such there is a river flowing through the area with mango trees along the river bank where people go to plug the fruits to eat and also sell.

Figure 3.1: Map of Niger State showing Bida (A), Paikoro (B) and Bosso (C) Local Government Council

Source: https://www.nigeriagalleria.com/Nigeria/States_Nigeria/Niger/

3.2 Collection of Samples

Plant leaves showing black rot were collected at different locations in Niger State, Nigeria. They were leaves of tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) collected from Nani village in Paikoro Local Government Area (LGA), mango (*Mangifera indica*) and banana (*Musa acuminata*) collected from Chanchaga, while infected rice seedlings (*Oryza sativa*) were collected from Bida, Niger State, Nigeria. Diseased leaf samples were collected by plucking the leaves of tomato, pepper, rice, banana and mango (Akhtar *et al*., 2008). All leaf samples were placed in clean polyethylene bags and transported to the laboratory for the isolation of bacteria.

3.3 Isolation and Screening of *Xanthomonas* species

3.3.1 Isolation of *Xanthomonas* from infected leaves

Isolation of *Xanthomonas* species was carried out following the method described by Singh *et al*. (2006). One gram (1g) of each leaf sample from infected plants showing a dark rot spot was submerged in sterile distilled water and a ten-fold serial dilution was done by transferring 1mL of the water into a test tube containing 9 mL of sterile distilled water. This step was repeated six times to obtain a dilution factor of 10^{-6}. 1mL from the dilutions 10^{-5} and 10^{-6} of each sample was withdrawn and plated in duplicates on Nutrient agar (NA) using the pour plate method and incubated at 35ºC (*Xanthomonas* optimal growth temperature) for 48 h (Singh *et al*. 2006).

3.3.2 Cultural and morphological characteristics of isolates

The incubated plates were observed for characteristic yellow mucoid colonies after 48 h which were then subcultured repeatedly on NA to obtain pure isolates. The pure isolates were gram stained and observed under the microscope for isolates showing pink colouration and were rodlike in shape. These were the presumptive *Xanthomonas* colonies that were preserved on slants for further characterization.

3.3.3 Emulsification of hydrocarbon by bacterial isolates

Isolates were screened for potential to emulsify hydrocarbon, this was done using xanthan gum production medium of Chavan and Baig (2016) with the following composition: 4g D-Glucose, 0.6g yeast extract, 0.4g K_2HP0_4, 0.01g $MgS0_4.7H_20$, 200ml distilled water. Nine millilitres (9ml) of the production medium was dispensed into test tubes inundated with 1ml of crude oil (Bonny light crude, BLC) and sterilized by autoclaving at 121ºC for 15 minutes. A 24 h culture of the isolates in buffered peptone water was introduced into the medium in test tubes and incubated at 37º C for 24 h. Different types of emulsion formed by the organisms were grouped into stable emulsion

(oil transformed remained in the emulsified form for 2 h), less stable emulsion (oil was separated out and make a layer on top of the culture broth), unstable emulsion (oil and medium were separated immediately) (Ijah and Ndana, 2003).

3.4 Characterization and Identification of Isolates

The isolates were characterized using the microbiological standard procedure as described by Singh *et al.* (2016). Biochemical tests were carried out. The isolates were identified by comparing their characteristics with those of known taxa using the scheme of Bergey's Manual of Determinative Bacteriology (George *et al*., 2004).

3.4.1 Gram staining

A pure culture was smeared on a clean grease free glass slide using wire loop and allowed to air dry, then heat fixed and placed on a staining rack. Crystal violet dye was added to the slide and rinsed with distilled water after 1minute. Gram's iodine was also added and rinsed after 1minute followed by addition of acetone which was rinsed after 30 seconds. Then safranin was added and allowed for 30 seconds and rinsed after the time has elapsed. The slide was allowed to dry after which it was examined under the microscope using an oil immersion objective (x100) to observe the Gram's reaction, and arrangement of the cells (Cheesebrough, 2006).

3.4.2 Motility test

Motility test was carried out by preparing a semi-solid nutrient broth (Nutrient broth to which 0.4g of agar was added) and dispensing 10mL into test tubes. The medium was sterilized by autoclaving at 121º C for 15 minutes. The medium was inoculated with the isolates by stabbing the medium using straight wire and incubated at 37º C for 24 h. Motile strains would move from the stab line and diffused into the medium causing the medium to become turbid. If the organism was non-motile, the growth was sharply defined and the rest of the medium was clear (Cheesebrough, 2006).

3.4.3 Catalase test

This test was carried out by placing a drop of 6% hydrogen peroxide (H_2O_2) on a clean grease free glass slide. This was emulsified with a loop full of bacterial isolate from the solid medium. A positive test was indicated by gas bubbling on the glass slide. This test was carried out to determine if a bacterial isolate was capable of producing the enzyme catalase (Gumus *et al.*, 2010).

3.4.4 Potassium hydroxide (KOH) solubility test

A loopful of bacteria was aseptically removed from culture plates with sterile inoculating pin, placed on glass slide in a drop of KOH (3 %) solution and stirred for ten seconds then the sterile inoculating pin was raised a few centimeters above the slide and observed for the formation of viscid strand representing the bacterium as Gram-negative (Cheesebrough, 2006).

3.4.5 Methyl red test

Methyl red indicator (0.1g methyl red dissolved in 300 mL of 95% ethanol and made up to 500 mL with distilled water) was added to test culture; change in color from yellow to red indicated the positive reaction (Cheesebrough, 2006).

3.4.6 Starch hydrolysis

Starch agar medium was prepared by adding 2 g of soluble starch into 100 mL of nutrient agar. This was sterilized by autoclaving at 121 º C for 15 min, and then 20 mL was poured into sterile Petri plates each. After solidifying, the starch agar plates were spot inoculated with loopful culture of the bacterium and incubated at 37º C for 24 h. After incubation, the plates were flooded with Lugol's iodine solution and observed for zone of clearance which indicated starch hydrolysis (Cheesebrough, 2006).

3.4.7 Oxidase test

Three drops of freshly prepared oxidase reagent (tetraethyl-p-phenylenediamine dihydrochloride) was placed on a piece of filter paper placed on a clean Petri dish, and a sterilized wire loop was used to collect the test organism and smeared on the filter paper. The appearance of blue-purple colour within ten seconds was recorded as positive result while the absence of blue-purple colour after fifteen seconds was recorded as negative (Cheesbrough, 2006).

3.4.8 H_2S production

A loopful culture of 48 h old test bacterium was inoculated into the test tubes containing peptone broth. Filter paper discs impregnated with 10% percent solution of neutral lead acetate was air dried and then inoculated. The sterilized stripes were placed into the inoculated test tubes. Which were incubated at 28º C for 72 h. Observations were drawn for the H_2S production. Blackening of the stripes indicated positive reaction (Cheesebrough, 2006).

3.4.9 Indole test

Test organisms were grown in 5 mL of 1% peptone water at 37º C for 48 h. This was followed by the addition of 0.5 mL of Kovac's reagent (prepared by dissolving 5g dimethylamino-benzaldehyde in 75 mL amyl alcohol and 25 mL concentrated HCl) and shaken gently. Appearance of red colour ring at the reagent layer was recorded as positive result while absence of coloured ring indicated negative result (Cheesbrough, 2006).

3.4.10 Triple sugar iron test (TSI)

With a sterilized straight inoculation needle, the top of a well isolated bacterial colony was touched. and inoculated on TSI agar (a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, as well as sodium thiosulfate and ferrous sulfate) by first stabbing through the center of the medium to the bottom of the tube and then streaked on the surface of the agar slant. The test tube cap was kept loosely and incubated the tube at 35° C in ambient air for 18 to 24 h. The inoculated medium was observed for colour change and gas production (Cheesbrough, 2006).

3.5 Molecular Identification of Bacterial Isolates

3.5.1 DNA extraction

Deoxyribonucleic acid (DNA) was extracted using the protocol stated by Trindade *et al*. (2007). Single bacterial colonies grown on nutrient agar medium were transferred to 1.5 mL of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600x g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% Sodium deodecyl sulfate (SDS) and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 h at 37º C, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated at 65 ºC for 10 min and kept on ice for 15 min. A volume of chloroform to iso amyl alcohol was added in the ratio of 24:1, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol was added in the ratio 1: 0.6 and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three h and finally dissolved in 50 µl of TE buffer.

3.5.2 Polymerase chain reaction

Polymerase chain reaction (PCR) sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water, 8µl DNA template. PCR was carried out in a Gene Amp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94˚C for 5 min; followed by a 30 cycles consisting of 94˚C for 30 s, 50˚C for 60s and 72°C for 1 min 30 s; and a final termination at 72°C for 10 mins, and chill at 4˚C.GEL (Frank *et al*., 2008).

3.5.3 Gel electrophoresis

The integrity of the amplified DNA, about 1.5Mb gene fragment was checked on a 1% Agarose gel run to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 min. The molten agarose was allowed to cool to 60˚C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 min to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2µl) of 10X blue gel loading dye (which gives colour and density to the sample to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the

100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 min visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was run alongside experimental samples in the gel (Frank *et al*., 2008).

3.5.4 Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mixed thoroughly by vortexing and kept at -20°C for at least 30 min. Centrifugation was carried out at 13000g for 10 min and 4°C followed by removal of supernatant (inversion of tube on trash once) after which the pellets were washed by adding 150 µl of 70% ethanol and mixed, then centrifuged at 7500 g for 15 min and 4˚C. Again all supernatant were removed, tube was inverted on tissue paper and left to dry in the fume hood at room temperature for 10-15 min. Then it was resuspended with 20 µl of sterile distilled water and kept in -20˚C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel run on a voltage of 110V for about 1h as previous to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from Thermo scientific (Frank *et al*., 2008).

3.5.5 Sequencing of DNA fragment

The amplified fragments were sequenced using a Genetic Analyzer (3130xl sequencer from Applied Biosystems, USA) according to manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

3.6 Xanthan Gum Production

3.6.1 Inoculum preparation

The media used for subculturing the isolates was yeast dextrose calcium carbonate agar (YDCC). The composition of the media was as follows: Yeast extract 10g, Calcium carbonate 20g, Dextrose 20g, Agar 20g, Distilled water 1000 mL. The YDCC agar slants were prepared and incubated at 37˚ C for 24 h to check contamination. The slants without contamination were selected and inoculated with the isolates and incubated at 37˚ C for 3 days. After 3 days, the agar slants were observed for orange colour growth of *X. campestris*. This culture was then used as the inoculum for xanthan gum production.

The inoculum was prepared by transferring cells from 72 h YDCC agar slants incubated at 37° C to 250 mL Erlenmeyer flasks containing 50 mL of YDCC broth (pH 7.0) and incubated at 37˚ C for 48 h (Kumara *et al*., 2012).

3.6.2. Xanthan estimation

One millilitre of the cultures (inoculum) was transferred to 49 mL of production medium (g/l; Glucose 20.0, Yeast extract 3.0, MgSO4 0.2, K_2HPO_4 5.0, pH 7.2) in 100 mL Erlenmeyer flask. The cultures were incubated at 37˚ C for 96 h (Kumara *et al*., 2012). The polymer was recovered from the fermentation medium by centrifugation of 5 mL broth at 10,000 rpm for 15 min. The pellet was decanted and the supernatant was precipitated with 2:3 volumes of isopropyl alcohol with shaking to precipitate out the polysaccharide. The precipitate was separated by centrifugation at 6000 rpm for 15 min. The residue was transferred to pre-weighed micro-centrifuge tube and dried for 18 h in

hot air oven at 60˚ C. The micro-centrifuge tube was cooled to 30˚ C for 1 h and the dry weight gave the xanthan concentration of the fermented broth. The concentration of xanthan gum was determined as the dry weight of xanthan gum per liter of culture medium (Kumura *et al*., 2012).

3.6.3. Biomass estimation

Growth in the medium was estimated by measuring the dry weight of washed cell mass, these was done by separating 5 mL of the broth in a centrifuge at 10,000 rpm for 15 min. After centrifugation, two fractions were formed, supernatant containing xanthan gum, and biomass deposited as a pellet.

The biomass pellet was resuspended in deionized water for washing and then recentrifuged to precipitate the biomass. The biomass deposited at the bottom of the tubes was dried in the oven at 60 ° C for 2 h and weighed to get the dry mass (Kumura *et al*., 2012).

3.7 Characterization of Xanthan Gum Produced

3.7.1 Determination of apparent viscosity

After incubation at 12º C for 72 h, the apparent viscosity of fermentation broth was measured at room temperature using a Brookfield system viscometer (Anton Paar, DV1, USA) and spindle number 3 at 40 rpm (Nejadmansouri *et al*., 2020).

3.7.2 Determination of emulsification index (IE_{24})

Five different oils (*Jatropha* oil, Castor oil, Crude oil, Diesel, Vegetable oil) were used to determine the emulsification index (IE_{24}) (Plate I). The gum suspension (0.1% w/v) and each oil sample were added to tubes at a ratio of 2:3 (v/v) and vortexed for 2 min. After 24 h of rest, the height of the emulsified layer and the total height of the liquid

layer were measured (Da Silva *et al.,* 2018). The IE_{24} was calculated using Equation 3.1.

$$IE_{24} = \frac{\text{height of the emulsified layer}}{\text{Total height}} \times 100 \quad \textbf{(3.1)}$$

3.7.3 Scanning Electron Microscopy (SEM)

Morphological analysis of the xanthan gum produced by the strains was performed using a SEM (Tescan, Mod. Vega-3 LMU). The samples were fixed in a metal support with double-sided carbon tape and covered with a thin layer of gold and viewed at a 1000-fold increase, with a 3-kV excitation voltage (Ahuja *et al*., 2012).

3.7.4 Fourier Transform Infrared Spectroscopy (FTIR)

In order to determine the functional groups in the xanthan gum, FTIR analysis was carried out according to the method used by National Research Institute for Chemical Technology, Zaria, Nigeria (NARICT). One milligram of the produced xanthan gum was ground with 100 mg of potassium bromide (KBr) and pressed with a silver coated hand presser at 7,500kg of pressure for 30 seconds to obtain translucent pellets. The pellet obtained was inserted into Fourier Transform Infrared Spectrophotometer (FTIR-8400S, Shimadzu, Japan) where the infrared spectra were recorded within the

range of 600–4000 waves cm^{-1} with a 32-scan/sample resolution of 4 waves cm^{-1} and a detector scanning speed equal to 10 kHz (Gilani *et al*., 2011).

3.7.5 Thermal Gravimetric Analysis (TGA/dTGA)

Thermal analysis of the xanthan gum was made by thermogravimetry (TGA-Perkin Elmer Mod. Pyris 1 TGA). Approximately 10.0 mg of the initial weight of each sample was subjected to a temperature change between 25 and 70º C at a heating rate of 10º C min^{-1} and an N_2 atmosphere at a flow rate of 20 mL min^{-1}. The results were compared with commercially prepared xanthan gum (Soleymanpour *et al*., 2018).

3.7.6 Differential Scanning Calorimetry (DSC)

The DSC curves were obtained in the temperature range of 25 - 400º C, with a heating rate of 10º C min^{-1} and a dynamic atmosphere of synthetic air with a flow rate of 50 mL min^{-1}. An aluminum crucible was used as the sample holder, and the mass of the analyzed samples ranged from 4.0 to 10.0 mg. The results were compared with commercial xanthan gum (Gomes *et al*., 2015).

3.8 Effect of Cultural Parameters on Biomass and Xanthan Gum Production

3.8.1 Effect of incubation time on biomass and xanthan gum production

The time for biomass and xanthan gum production was determined using three time intervals of 48 h, 96 h and 144 h. The experiments were conducted using conical flasks (250 mL) containing 50 ml xanthan gum production medium. The flasks were sterilized by autoclaving at 15 lbs for 20 min, and then inoculated with the culture of the organism in each flask. All the flasks were incubated at 30º C and flasks were withdrawn at different time of incubation keeping all other processes and conditions constant for carrying out surface fermentation (Makut *et al*., 2018). Biomass and xanthan gum were determined as described in Section 3.5 of the present study.

3.8.2 Effect of temperature on biomass and xanthan gum production

The determination of optimum temperature for biomass and xanthan gum production was done using conical flasks (250 mL) containing 50 mL of xanthan gum production medium sterilized by autoclaving at 15 lb for 20 min, then inoculated with the culture of

the organism and incubated at three different temperatures: 25º C, 30º C, 35º C, keeping all other process parameters constant (Makut *et al*., 2018). Biomass and xanthan gum were determined as described in Section 3.5 of the present study.

3.8.3 Effect of pH on biomass and xanthan gum production

The optimum pH for biomass and xanthan gum production was determine using three pH levels of 5, 7 and 9. Conical flasks of 250 mL capacity containing 50 mL xanthan gum production medium was adjusted to pH of the medium using 1 N HCL or 1 N NaOH. The flasks were sterilized by autoclaving at 15 lb for 20 min and were inoculated with the culture of the organism in each flask. The flasks were incubated at 30º C for 96 h surface fermentation, keeping other conditions constant. Thus, the effect of different pH levels on production of biomass and xanthan was determined. Biomass and xanthan gum were determined as described in Section 3.5 of the present study.

3.8.4 Effect of carbon sources on biomass and xanthan gum production

The effect of different carbon sources on biomass and xanthan production was determine using 0.2 % of carbon sources such as sugar cane bagasse, pineapple peels and glucose in the production medium keeping all other process parameters constant (Makut *et al*., 2018). Biomass and xanthan gum were determined as described in Section 3.5 of the present study.

3.8.5 Effect of Nitrogen sources on biomass and xanthan gum production

The effect of different nitrogen sources on biomass and xanthan production was determine using 0.3 % of nitrogen sources such as beef extract, ammonium sulphate, peptone, yeast extract in the production medium keeping all other process parameters

constant (Makut *et al*., 2018). Biomass and xanthan gum were determined as described in Section 3.5 of the present study.

3.8.6 Production of Biomass and Xanthan Gum at Optimized Conditions

In determining biomass and xanthan gum yield by the isolates, the cultural conditions that gave the highest biomass and xanthan gum yield for both *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 were used. For *Xanthomonas campestris* CPBF 211, the conditions were temperature 25º C, pH 9, carbon source: pineapple peels extract 0.3 %, Nitrogen source: yeast extract 0.2 % while temperature 25º C, pH 5, carbon source: sugarcane bagasse 0.3 % and Nitrogen source: yeast extract 0.2 % were used for *Stenotrophomonas maltophilia* IAE127. Production of biomass and xanthan gum was done as described in Section 3.5 of the present study.

3.9 Determination of Potential of Xanthan Gum in Enhanced Oil Recovery (EOR)

The potential application of the xanthan gum in EOR was evaluated in duplicates using the 'sand pack column' technique described by Robert *et al*. (1989). Two hundred grams (200 g) of soil was contaminated with 20 mL of Bonny light crude oil in a conical flask and was allowed to soak for 7days. Distilled water (200 mL) and a mixture of sand (200 g) and crude oil (20 g) served as control. A glass column (61 cm×3.5 cm in diameter) was vertically fixed on a retort stand and filled with the mixture (soil + crude oil) (Plate II). The surface inundated with 200 mL of xanthan gum supernatant and percolation of xanthan gum solution under the action of gravity was observed after 10 h. After percolation of xanthan gum solution through the column, the soil samples were then transferred into 250 mL conical flask and washed with 20 mL of n-hexane for the removal of the residual oil. The solvent was recovered using rotary evaporator and the

amount of oil removed was determined gravimetrically (Dahrazma and Mulligan, 2007).

3.10 Data Analysis

Data generated from this study were analyzed using the computer package SPSS (Version 23). Data were expressed as mean ± standard error while two-way analysis of variance (ANOVA) was done to determine significant differences at 5 % probability level between xanthan gum and its production conditions. Correlation analysis was also carried out to establish relationship between xanthan gum and the biomass produced at optimal production conditions (Da Silva *et al*., 2018).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Preliminary screening of bacterial isolates from plant leaves for *Xanthomonas* characteristics

Thirteen bacterial isolates were obtained from plant leaves (Banana, mango, rice, tomato, pepper) and were screened for *Xanthomonas* characteristics based on pigmentation on Nutrient agar, morphological characteristics through Gram's staining, and emulsification ability.

4.1.1.1 Cultural characteristics of bacterial isolates

Out of the thirteen bacterial isolates, eight appeared pale yellow to orange in colour with a shiny surface appearance that was slightly mucoid when incubated on Nutrient agar at 37˚C for 48h with no distinct odour.

4.1.1.2 Morphological and microscopic characteristics of bacterial isolates

The eight bacterial isolates, after gram staining, appeared rodlike in shape with a pinkish coloration when viewed under the microscope (Gram negative).

4.1.1.3 Emulsification of hydrocarbon by bacterial isolates

The bacterial isolates from the leaf of Banana (X_2, X_3, X_{12}), Rice (X_1, X_6, X_{10}), Mango (X_5, X_8, X_{13}), Tomato (X_9, X_{11}) and Pepper (X_4, X_7) were subjected to emulsification test in a carbon enriched medium, the results are shown in Table 4.1. The results revealed that, eight isolates (61.53%) caused stable emulsion of the oil medium while two (15.38%) caused less stable emulsion and three (23.07%) caused unstable emulsion (Table 4.1).

Table 4.1: Emulsification of crude oil by bacterial isolates (*Xanthomonas*) from plant leaves

Isolate code	Emulsion stability
RX_6	III
BX_2	III
BX_3	III
PX_4	III
PX_7	III
MX_8	III
TX_9	III
TX_{11}	III
RX_{10}	II
RX_1	II

BX_{12}	I
MX_{13}	I
MX_5	I

Key: III: stable emulsion, II: less stable emulsion, I: unstable emulsion,

Banana= (BX), Rice= (RX), Mango= (MX), Tomato= (TX) Pepper= (PX).

4.1.2 Biochemical characterization and identification of isolates

Table 4.2 shows the biochemical characteristics of the bacterial isolates. The bacterial isolates were identified as *Xanthomonas campestris*, *Stenotrophomonas maltophilia*, *Xanthomonas vasicola*, *Xanthomonas perforans*, *Xanthomonas gardneri*, *Xanthomonas vesicatoria*, *Xanthomonas axonopodis* and *Xanthomonas citri*.

Table 4.2: Morphology, biochemical characteristics and identification of *Xanthomonas* species

Code	Gram reaction	Shape	Motility	Catalase	KOH solubility	Methyl Red	Starch hydrolysis	Oxidase	H_2S	Indole	TSI	Isolate
TX_9	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas campestris*
BX_3	-	Rod	+	+	+	+	-	-	-	+	+	*Stenotrophomonas maltophilia*
PX_4	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas perforans*
BX_2	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas vasicola*
PX_7	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas gardneri*
MX_8	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas axonopodis*
TX_{11}	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas vesicatoria*

MX_6	-	Rod	+	+	+	-	+	-	+	+	+	*Xanthomonas citri*

(+): Positive, (-): Negative result, TSI: Triple sugar iron agar

4.1.3 Molecular identification and confirmation of *Xanthomonas campestris* and *Stenotrophomonas maltophilia*

The isolates (BX3 and TX9) with efficient xanthan gum production potentials were further identified using molecular analysis. Plate 4.1 is the image of agarose gel electrophoresis to PCR amplified DNA of the isolates.

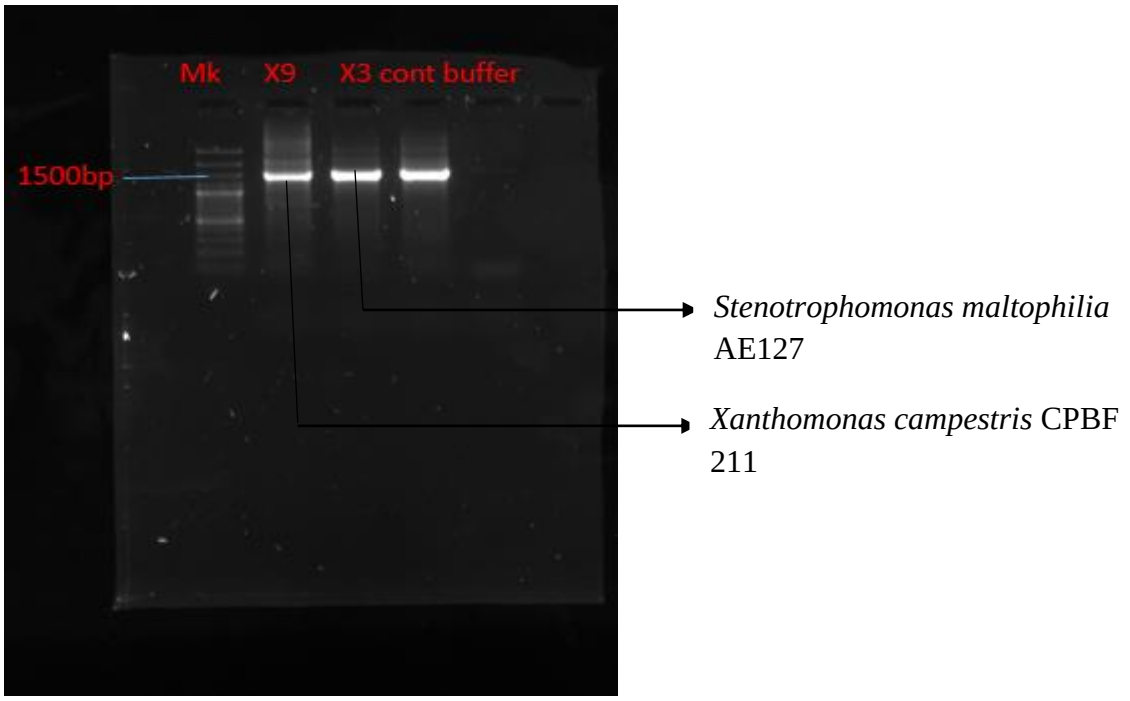


Plate 4.1: Image of Agarose gel electrophoresis to PCR amplified DNA

Key: MK=DNA ladder 1500bp, X9 = sample (*Xanthomonas campestris* CPBF 211), X3 = sample (*Stenotrophomonas maltophilia* IAE127), Buffer, Approximate size of the organism = 1500.

The bacterial isolates, BX3 and TX9 were 99% identical to *Stenotrophomonas maltophilia* strain IAE127 and *Xanthomonas campestris* strain CPBF 211 16S ribosomal RNA gene after molecular characterization respectively (Table 4.3). Plate V shows culture of the bacterial isolates on nutrient agar, and the microscopic view of the isolates after gram staining.

Table 4.3 Molecular characterization of bacterial isolates

samples ID	Description	Max Score	Total score	Query cover	E value	% identity	Accession number
X9	*Xanthomonas campestris* CPBF 211 (XC) 16S ribosomal RNA gene, partial sequence	2400	2400	99%	0	99.56	GU596425.1
X3	*Stenotrophomonas maltophilia* (SM) IAE127 16S ribosomal RNA gene, partial sequence	1982	1982	99%	0	99.64	MK414820.1

4.1.4 Production of xanthan gum by bacterial isolates

Eight isolates which caused stable emulsion of hydrocarbon medium were subjected to xanthan gum production. The results revealed that the yield of xanthan gum ranged from 0.02 g/L to 2.10 g/L (Table 4.4) after 96 h. Isolate TX_9 showed a considerably higher ability in producing the xanthan gum than the rest of the isolates. This was followed by BX_3 with 1.63 g/L over the same period (Table 4.4).

Table 4.4: Yield of xanthan gum by *Xanthomonas* and *Stenotrophomonas* species from plant leaves

Isolate	Xanthan gum yield (g/L)
Xanthomonas vasicola BX_2	1.15 ± 0.058
Stenotrophomonas maltophilia BX_3	1.63 ± 0.054
Xanthomonas perforansz PX_4	0.95 ± 0.012
Xanthomonas citri MX_6	0.55 ± 0.105
Xanthomonas gardneri PX_7	0.02 ± 0.000
Xanthomonas axonopodis MX_8	1.30 ± 0.050
Xanthomonas campestris TX_9	2.10 ± 0.297
Xanthomonas vesicatoria TX_{11}	1.19 ± 0.040

*Values are presented as mean ± standard error

4.1.5 Characterization of Xanthan Gum Produced by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE 127

4.1.5.1 Apparent viscosity (AV) of the xanthan gum

Viscosity measurement for xanthan gum produced from *Xanthomonas campestris* CPBF 211, *Stenotrophomonas maltophilia* IAE127 and commercial xanthan gum revealed that the AV of xanthan gum produced by *Xanthomonas campestris* CPBF 211 ranged from 526.1 to 597.4 mPa.S at a maximum concentrations of 49 % while the AV of XG by *Stenotrophomonas maltophilia* IAE127 ranged from 617.7 to 660.6 mP.aS at a maximum concentration of 44.3 % (Table 4.5) Commercial XG had AV of 408.3-411.3 mPa.S at a maximum concentration of 17.2 %.

Table 4.5: Apparent viscosity of xanthan gum produced

Xanthan gum by:	**Apparent viscosity Value (mPa. S)**	**RPM**	**ºC**	**% concentration of Xanthan gum**
Xanthomonas campestris CPBF 211	597.4	12	40	23
	564.1	12	40	26
	526.1	12	40	49
Stenotrophomonas maltophilia IAE127	660.6	12	40	26.4
	617.7	12	40	36.7
	642.8	12	40	44.3
Commercial xanthan gum	408.3	12	40	16.5
	408.3	12	40	16.8
	411.3	12	40	17.2

Key: mPa.S: Millipascal per seconds, **RPM:** Revolution per minutes

4.1.5.2 *Emulsification index (EI_{24}) of xanthan gum produced*

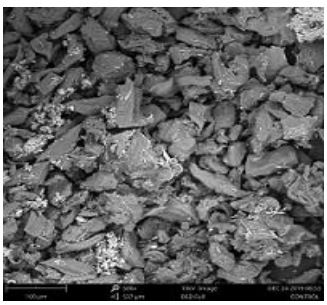
The results of the emulsification capacity of the xanthan gum after 24 h in different oils (Diesel, Crude oil, Castor oil, *Jatropha* oil, and Vegetable oil) are shown in Table 4.6. The results revealed that castor oil supported more emulsification (36.45-57.5 %) by xanthan gum produced by the two bacteria. The commercial xanthan gum caused 63.75 % emulsification on castor oil. This was followed by vegetable oil (47.5 %), *Jatropha* oil (37.5 %), crude oil (10 %) and diesel (2.65 %) in that order (Table 4.6).

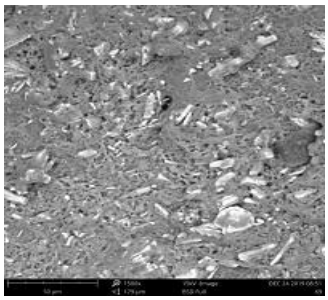
Table 4.6: Emulsification index (IE_{24}) of various oils by xanthan gum produced by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127

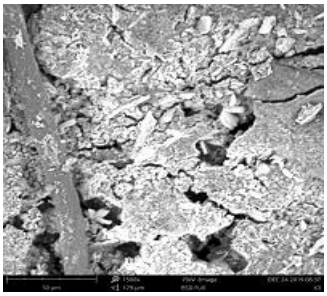
	Emulsification index (%)				
Xanthan gum by:	**Diesel**	**Crude oil**	**Castor oil**	**Jatropha oil**	**Vegetable oil**
***Xanthomonas campestris* CPBF 211**	8.75	17.5	36.45	10	33.75
***Stenotrophomonas maltophilia* IAE127**	2.50	6.5	57.5	20	36.25
Commercial xanthan gum (Control)	2.65	10	63.75	37.5	47.5

4.1.5.3 Scanning Electron Microscopy (SEM) of xanthan gum produced

Scanning electron microscopy (SEM) was used to examine the morphology and surface characteristics of xanthan gum produced by *Xanthomonas campestris* CPBF 211, *Stenotrophomonas maltophilia* IAE127 and a commercial xanthan gum at a magnification of ×1000. The results revealed commercial xanthan gum to present a rough and a more compact structural surface, xanthan gum from *Xanthomonas campestris* CPBF 211 had a more disperse heterogenous whitish structure with less surface porosity while xanthan gum produced by *Stenotrophomonas maltophilia* IAE127 presented a flat whitish surface with a pronounced fiber like structure (Plate 4.2). For xanthan gum commercial (control), the abundant elements detected were potassium, carbon, phosphorus and iodine with a weight concentration of 28.13, 17.03, 14.55, and 10.95 respectively (Table 4.7a). Potassium, carbon, calcium and phosphorus with a weight concentration of 22.55, 21.81, 17.99 and 15.88 respectively were also found to be abundant in xanthan gum produced by *Xanthomonas campestris* CPBF 211 (Table 4.7b) while xanthan gum produced by *Stenotrophomonas maltophilia* IAE127 had calcium, carbon, zirconium, phosphorus and potassium as abundant elements with weight concentration of 28.95, 15.43, 13.07, 11.95 and 10.92 respectively (Table 4.7c).







(a) Xanthan commercial (Control) (b) *Xanthomonas campestris* CPBF 211 (c) *Stenotrophomonas maltophilia* IAE127

Plate 4.2: Scanning electron micrograph of xanthan gum produced

Table 4.7a: SEM for xanthan commercial (control)

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
19	K	Potassium	19.96	28.13
6	C	Carbon	39.33	17.03
15	P	Phosphorus	13.04	14.55
53	I	Iodine	2.39	10.95
11	Na	Sodium	9.39	7.78
47	Ag	Silver	1.71	6.63
20	Ca	Calcium	2.48	3.58
17	Cl	Chlorine	1.98	2.53
26	Fe	Iron	1.08	2.18
16	S	Sulfur	1.48	1.71
7	N	Nitrogen	3.15	1.59
14	Si	Silicon	1.22	1.24
13	Al	Aluminium	0.86	0.83
8	O	Oxygen	1.42	0.82
12	Mg	Magnesium	0.51	0.45
30	Zn	Zinc	0.00	0.00

Table 4.7b: SEM for xanthan gum by *Xanthomonas campestris* strain CPBF 211

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
19	K	Potassium	14.64	22.55
6	C	Carbon	46.12	21.81
20	Ca	Calcium	11.40	17.99
15	P	Phosphorus	13.02	15.88
40	Zr	Zirconium	1.93	6.95
17	Cl	Chlorine	2.33	3.25
47	Ag	Silver	0.65	2.77
16	S	Sulfur	1.72	2.17
13	Al	Aluminium	1.45	1.55
14	Si	Silicon	1.25	1.38
7	N	Nitrogen	2.29	1.26
8	O	Oxygen	1.90	1.20
12	Mg	Magnesium	1.02	0.98
11	Na	Sodium	0.27	0.25
30	Zn	Zinc	0.00	0.00
22	Ti	Titanium	0.00	0.00

Table 4.7c: SEM for xanthan gum by *Stenotrophomonas maltophilia* IAE127

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
20	Ca	Calcium	21.63	28.95
6	C	Carbon	38.47	15.43
40	Zr	Zirconium	4.29	13.07
15	P	Phosphorus	11.56	11.95
19	K	Potassium	8.36	10.92
47	Ag	Silver	1.35	4.88
17	Cl	Chlorine	2.46	2.91
16	S	Sulfur	1.97	2.11
30	Zn	Zinc	0.80	1.75
14	Si	Silicon	1.66	1.56
22	Ti	Titanium	0.95	1.52
11	Na	Sodium	1.78	1.37
13	Al	Aluminium	1.50	1.35
26	Fe	Iron	0.41	0.76
7	N	Nitrogen	1.63	0.76
8	O	Oxygen	0.94	0.50
12	Mg	Magnesium	0.24	0.20

4.1.5.4 *Fourier Transform Infrared Spectroscopy (FTIR) of xanthan gum produced*

The chemical nature of the xanthan gum produced by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 were elucidated using FTIR. The spectra for xanthan gum produced from the FTIR analysis are shown in Figure 4.2a and 4.2b. The results revealed peaks of different shapes (weak, asymmetric, strong, broad, stretching, bending), each representing specific functional groups that are present on the molecular chain in the xanthan gums studied. Similarly, the infrared absorption pattern revealed the presence of carboxyl, carbonyl, hydroxyl and acetyl groups (Fig. 4.2a and 4.2b).

4.1.5.5 *Thermogravimetric analysis (TGA/ dTGA)*

The xanthan gum produced was also subjected to thermal gravimetric analysis/ derivative of thermal gravimetric analysis (TGA/dTGA). This analysis gives valuable information for a substance subjected to heating, indicating initial degradation temperature (T_{onset}), maximum decomposition temperature (T_{max}) and mass losses (Wl^3). It was observed that all three samples had two (2) thermal events each (Table 4.8). *Xanthomonas campestris* CPBF 211 had Tmax of 108.05˚C and 401.00˚C with mass loss of 99.88% and 95.68% respectively. *Stenotrophomonas maltophilia* IAE127 had Tmax of 200.36˚C and 515.61˚C with mass loss (WI^3) of 100.5% and 66.10% respectively while commercial xanthan gum (control) had Tmax of 120.88˚C and 411.92˚C with mass loss of 99.96% and 90.09% respectively (Table 4.8). The results (Table 4.8) revealed that xanthan gum produced by *Stenotrophomonas maltophilia* IAE127 had higher loss in weight than the xanthan gum produced by *Xanthomonas campestris* CPBF 211 and the commercial xanthan gum.

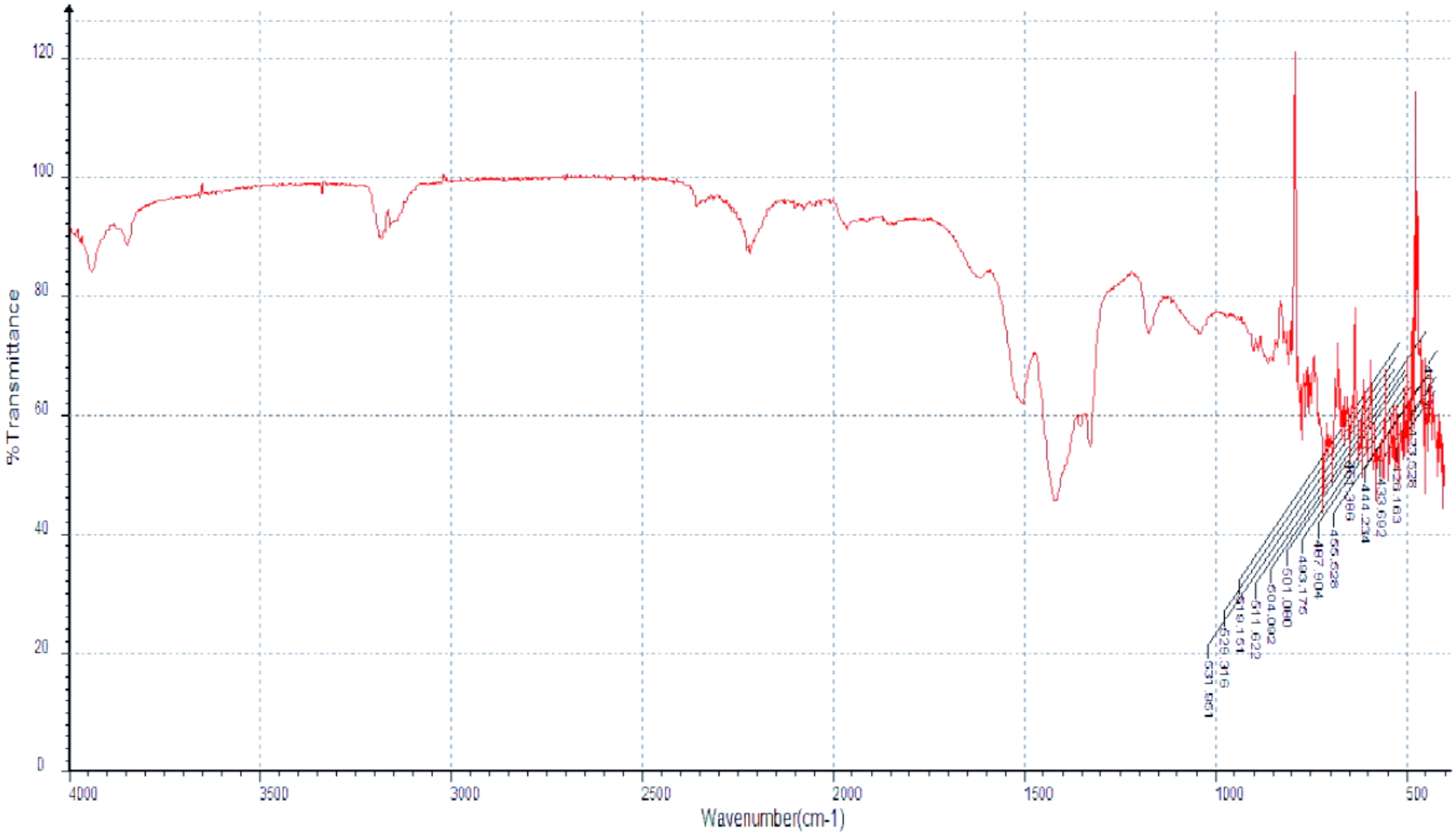


Figure 4.2a: FTIR of xanthan gum produced by *Xanthomonas campestris* CPBF 211

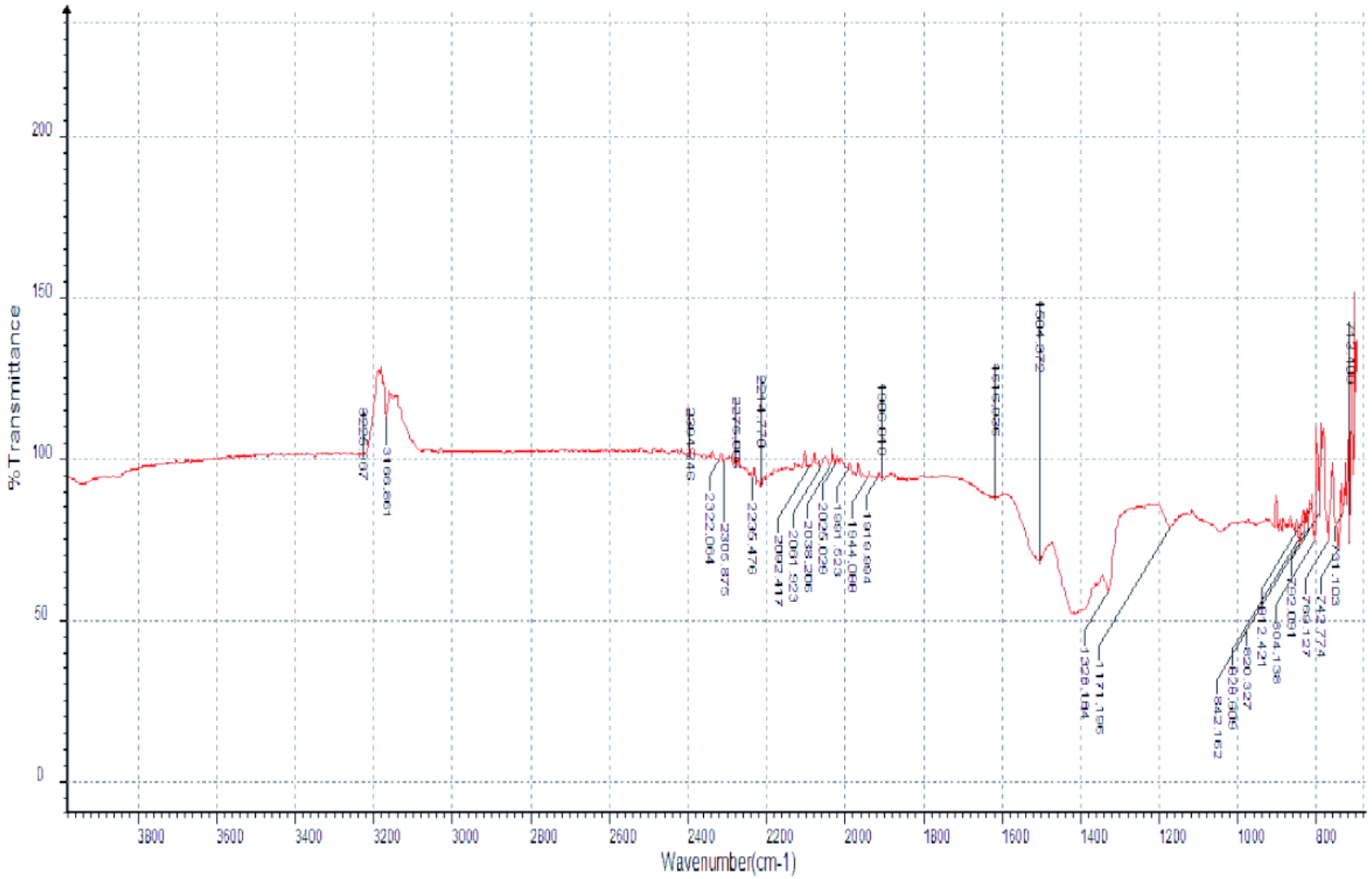

%Transmittance
Wavenumber(cm-1)
200
150
100
50
0
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
3199.861
2322.064
2305.875
2235.476
2092.417
2061.523
2038.206
2025.029
1991.523
1944.088
1919.694
1504.972
1326.184
1171.195
842.162
820.327
812.421
804.136
792.091
769.127
743.774
731.103
713.103

Figure 4.2b: FTIR of xanthan gum produced by *Stenotrophomonas maltophilia* IAE127

Table 4.8: Thermogravimetric data (TGA) of xanthan gum produced by *Xanthomonas campestris* CPBF 211, *Stenotrophomonas maltophilia* IAE12

Sample	Events	T_{onset} (°C)	T_{max} (°C)	Wl^3 (%)
XC	1	23.74	108.05	99.88
	2	309.88	401.00	95.68
SM	1	25.56	200.36	100.5
	2	400.48	515.61	66.10
CX	1	23.19	120.88	99.96
	2	309.84	411.92	90.09

Key: XC= *Xanthomonas campestris* CPBF 211
SM= *Stenotrophomonas maltophilia* IAE12
CX= Commercial xanthan gum (control)

4.1.5.6 *Differential Scanning Calorimetry (DSC)*

The xanthan gum produced was also subjected to differential scanning calorimetry (DSC). It was revealed that the xanthan gum produced by the two (2) organisms had two similar events which can be identified, one endothermic peak, and one exothermic peak. Xanthan gum produced by *Xanthomonas campestris* CPBF 211 exhibited event which varied between 25 and 160° C, showing an endothermic peak at approximately 80° C, while for xanthan gum from *Stenotrophomonas maltophilia* IAE12, the event varied between 25 and 140° C, showing an endothermic peak at approximately 80° C, The second event in xanthan gum by both *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE12 started at approximately 240° C, showing an exothermic peak related to polysaccharide degradation. These are presented in Figure 4.4 a and b.

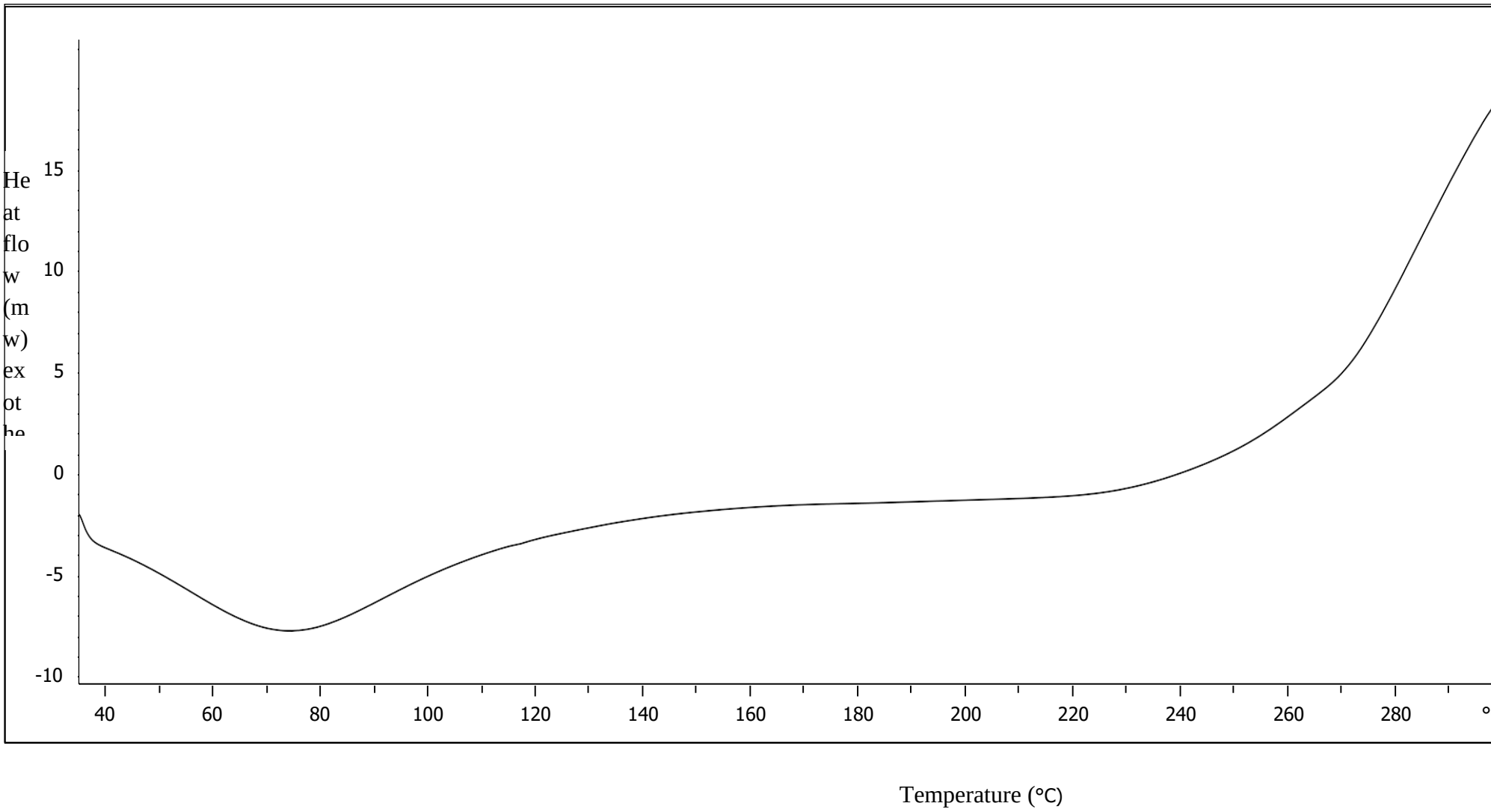


Figure 4.3a: Differential scanning calorimetry of xanthan gum produced by *Stenotrophomonas maltophilia* IAE12

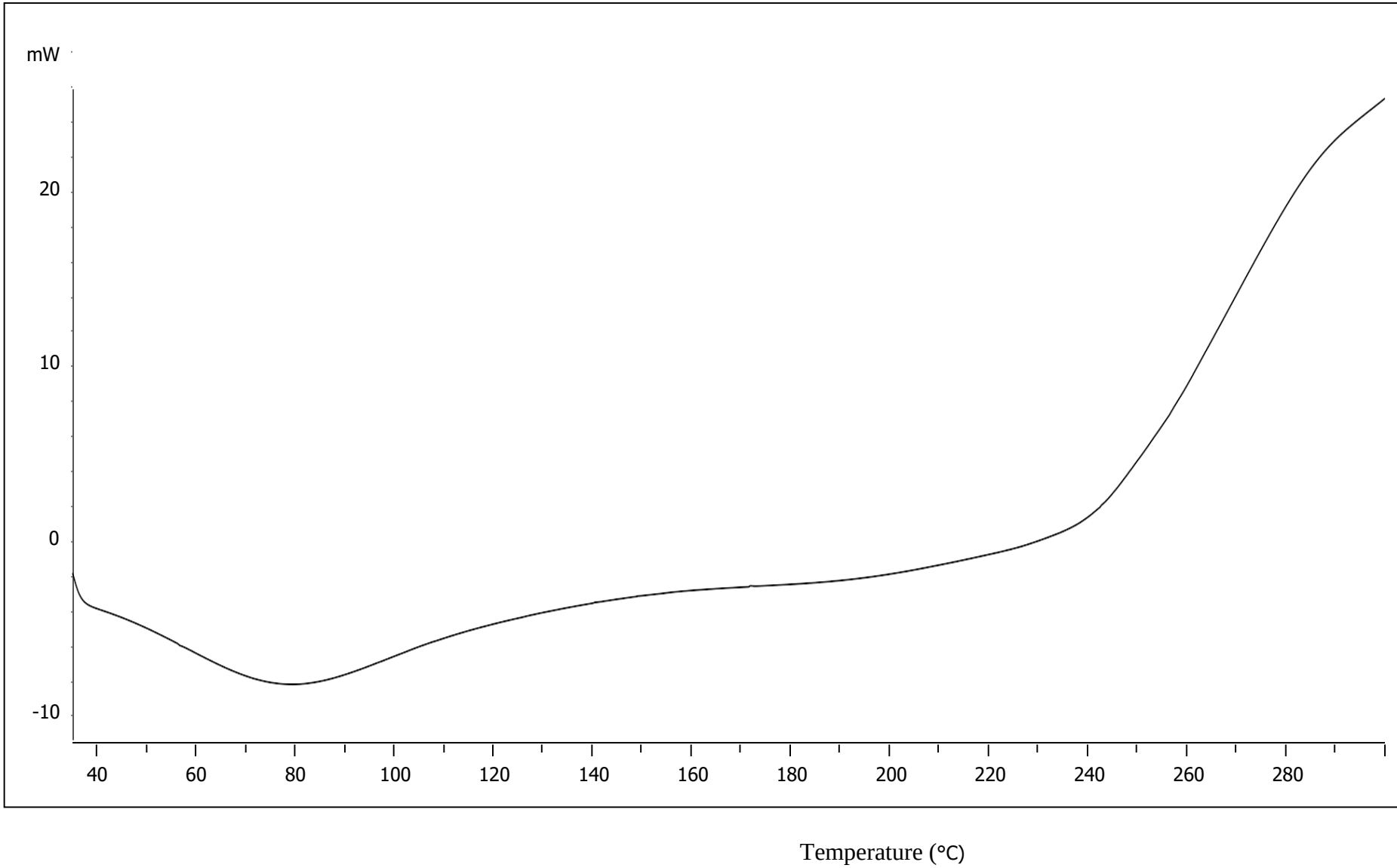


Figure 4.3b: Differential scanning calorimetry of xanthan gum produced by *Xanthomonas campestris* CPBF 211

4.1.6 Effect of cultural parameters on biomass and xanthan gum production

The effect of incubation time, temperature, pH, carbon and nitrogen sources was studied on biomass and xanthan gum production.

4.1.6.1 *Effect of incubation time on biomass and xanthan gum production*

Table 4.9 shows the effect of incubation time on biomass and xanthan gum production after 48 h, 96 h, and 144 h of incubation. The results revealed that, as the biomass increased, the xanthan gum production by the two organisms also increased. Maximum xanthan gum (1.36 & 3.64 g/L) and biomass production (4.06 & 6.74 g/L) from the organism was obtained after 144 h. It was observed that, *X campestris* CPBF211 produced more xanthan gum and biomass than *S. maltophilia* IAE127. Xanthan gum produced by the two organisms after 48h was significantly ($p>0.05$) different from the amount produced after 96 h and 144 h. No significant ($p>0.05$) differences existed among the periods of the production of biomass by the two bacteria (Table 4.9).

Table 4.9: Biomass and xanthan gum produced by bacterial by isolates at various incubation time

	Biomass (g/L)		Xanthan gum (g/L)	
Incubation time (H)	**XC**	**SM**	**XC**	**SM**
48	5.91 ± 0.043 [a]	3.83 ± 0.098 [a]	1.24 ± 0.054 [b]	1.20 ± 0.236 [a]
96	6.73 ± 0.574 [a]	4.04 ± 0.450 [a]	3.63 ± 0.012 [a]	1.36 ± 0.063[a]
144	6.74 ± 0.580 [a]	4.06 ± 0.445 [a]	3.64 ± 0.012 [a]	1.36 ± 0.066 [a]

Values are presented as mean ± standard error. Values with the same superscript in the same column are not significantly different at $p \geq 0.05$.

Key: XC= *Xanthomonas campestris* CPBF 211, **SM**= *Stenotrophomonas maltophilia* IAE12

4.1.6.2 Effect of temperature on biomass and xanthan gum production

Table 4.10 shows the effect of temperature on biomass and xanthan gum production at 25º C, 30º C and 35º C. Maximum xanthan gum and biomass production by *Xanthomonas campestris* CPBF 211 was at 25º C and 30º C respectively while maximum xanthan gum and biomass production by *Stenotrophomonas maltophilia* IAE127 was at 25º C and 35º C respectively (Table 4.10).

Table 4.10: Biomass and xanthan produced by bacterial isolates at different temperature levels

	Biomass (g/L)		Xanthan gum (g/L)	
Incubation temperature (˚C)	**XC**	**SM**	**XC**	**SM**
25	4.58 ± 0.040 [a]	3.75 ± 0.387 [a]	6.43 ± 0.395 [a]	3.75 ± 0.387 [a]
30	5.64 ± 0.367 [a]	3.76 ± 0.364 [a]	1.71 ± 0.008 [b]	1.71 ± 0.067 [a]
35	4.20 ± 0.003 [a]	4.76 ± 0.502 [a]	1.56 ± 0.020 [a]	1.54 ± 0.502 [a]

Values are presented as mean ± standard error. Values with the same superscript in the same column are not significantly different at $p>0.05$.

Key: **XC**= *Xanthomonas campestris* CPBF 211, **SM**= *Stenotrophomonas maltophilia* IAE12

4.1.6.3 *Effect of pH on biomass and xanthan gum production*

Table 4.11 shows the effect of pH on biomass and xanthan gum production. The results revealed that, as the biomass decreased the xanthan gum produced by *Xanthomonas campestris* CPBF 211 increased while the xanthan gum produced by S*tenotrophomonas maltophilia* IAE127 decreased as the biomass increased (Table 4.11) Maximum xanthan gum (4.29 g/L) and biomass production (5.25 g/L) by *Xanthomonas campestris* CPBF 211 was obtained at pH 9 and pH5 respectively while maximum xanthan gum (4.00 g/L) and biomass production (3.73 g/L) by S*tenotrophomonas maltophilia* IAE127 was obtained at pH 5 and pH 7 respectively. It was observed that, *Xanthomonas campestris* CPBF 211 produced more biomass as well as xathan gum than S*tenotrophomonas maltophilia* IAE127. The amount of xanthan gum and biomass produced by the two organisms were not significantly different ($P<0.05$) at pH 5-9 (Table 4.11).

Table 4.11: Biomass and xanthan gum produced by bacterial isolates at different pH levels

	Biomass (g/L)		Xanthan gum (g/L)	
pH	**XC**	**SM**	**XC**	**SM**
5.0	5.25 ± 0.378 [a]	2.74 ± 0.049 [a]	2.37 ± 0.038 [a]	4.00 ± 0.087 [a]
7.0	3.97 ± 0.072 [a]	3.73 ± 0.115 [a]	3.70 ± 0.170 [a]	3.06 ± 0.081 [a]
9.0	2.56 ± 0.032 [a]	3.60 ± 0.087 [a]	4.29 ± 0.430 [a]	3.02 ± 0.026 [a]

Values are presented as mean ± standard error. Values with the same superscript in the same column are not significantly different at $p>0.05$.

Key: **XC** = *Xanthomonas campestris* CPBF 211, **SM** = *Stenotrophomonas maltophilia* IAE127

4.1.6.4 *Effect of carbon sources on biomass and xanthan gum production*

The production of biomass and xanthan gum was studied using three carbon sources; glucose, pineapple peels and sugarcane bagasse (Figure 4.4). The results revealed that pineapple peels supported the highest biomass (6.99 g/L) in S*tenotrophomonas maltophilia* IAE127 while it supported the highest xanthan gum (4.82 g/L) production in *Xanthomonas campestris* CPBF 211. Similarly, sugar cane baggasse supported the highest xanthan gum (3.00 g/L) in S*tenotrophomonas maltophilia* IAE127 while it gave the highest biomass (3.84 g/L) yield in *Xanthomonas campestris* CPBF 211 (Figure 4.4). It was observed that *Xanthomonas campestris* CPBF 211 produced more xanthan gum than S*tenotrophomonas maltophilia* IAE127.

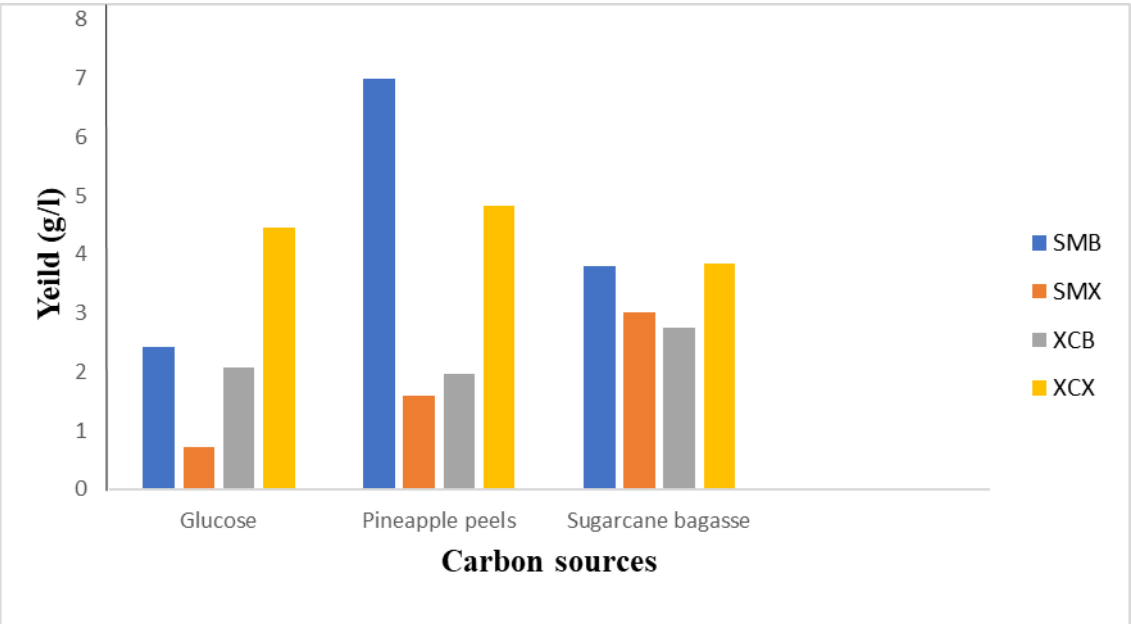


Figure 4.4: Yield of biomass and xanthan gum by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 grown on different carbon sources

Key: **XCX=** Xanthan gum from *Xanthomonas campestris* CPBF 211
XCB=Biomass of *Xanthomonas campestris* CPBF 211
SMX= Xanthan gum from *Stenotrophomonas maltophilia* IAE127
SMB= Biomass of *Stenotrophomonas maltophilia* IAE127

4.1.6.5 *Effect of nitrogen sources on biomass and xanthan gum production*

Figure 4.6 shows the results of the organism cultivated in production medium containing various nitrogen sources such as peptone, yeast extract, beef extract and ammonium sulphate. The results revealed that yeast extract supported the highest biomass and xanthan gum production in S*tenotrophomonas maltophilia* IAE127 while yeast extract and ammonium sulphate gave the highest xanthan gum and biomass respectively in *Xanthomonas campestris* CPBF 211. It was observed that S*tenotrophomonas maltophilia* IAE127 produced more xathan gum and biomass than *Xanthomonas campestris* CPBF 211 (Figure 4.6).

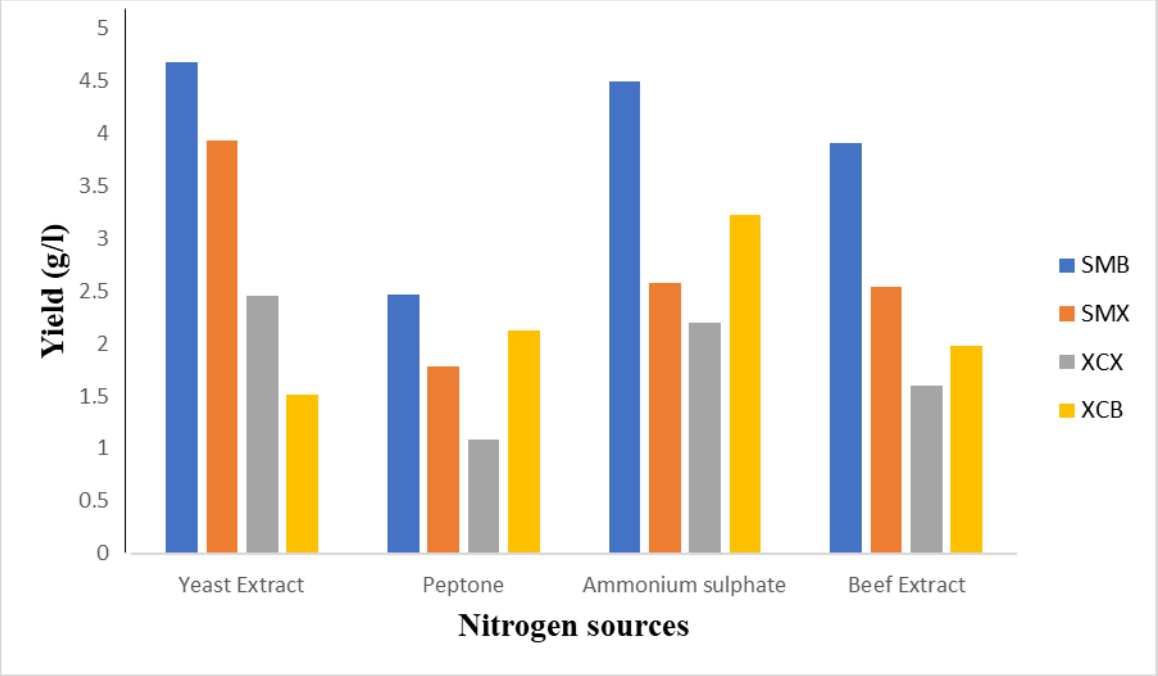


Figure 4.5: Yield of biomass and xanthan gum by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 on nitrogen sources

Key: **XCX=** Xanthan gum from *Xanthomonas campestris* CPBF 211
XCB =Biomass of *Xanthomonas campestris* CPBF 211
SMX= Xanthan gum from *Stenotrophomonas maltophilia* IAE127
SMB= Biomass of *Stenotrophomonas maltophilia* IAE127

4.1.6.6 *Production of biomass and xanthan gum under optimized culture conditions*

Xanthan gum and biomass production under optimal conditions (pH: 9.0, temperature: 25ºC; carbon source: 0.2% pineapple peels, nitrogen source: yeast extract) for *Xanthomonas campestris* CPBF211 and (pH: 7.0, temperature: 25ºC, carbon source: 0.2% sugarcane bagasse, nitrogen source: yeast extract) for *Stenotrophomonas maltophilia* IAE 127 respectively were observed. It was also observed that there was an increase in both biomass and xanthan gum production as the incubation time extended from 24 to 96 h, maximum yield was observed at 96 h (Table 4.12). Statistical analysis revealed that there were no significant differences ($P > 0.05$) in biomass production by the two organisms. However, significant difference ($p<0.05$) existed in the xanthan gum production by the two organisms, particularly after 72-96 h (Table 4.12).

Table 4.12: Yield of biomass and xanthan gum under optimized culture conditions

	Biomass (g/L)		Xanthan gum (g/L)	
Incubation time (Hours)	**XC**	**SM**	**XC**	**SM**
24	0.96 ± 0.021 [a] *	2.52 ± 0.028 [a]	0.92 ± 0.113 [a]	0.99 ± 0.014 [a]
48	1.32 ± 0.247 [a]	3.02 ± 0.028 [a]	1.22 ± 0.282 [a]	1.17 ± 0.035 [a]
72	3.69 ± 0.438 [a]	4.05 ± 0.028 [a]	4.40 ± 0.000 [b]	3.39 ± 0.014 [a]
96	6.50 ± 2.828 [a]	6.80 ± 0.056 [a]	7.26 ± 0.197 [b]	4.51 ± 0.014 [a]

*Values are presented as mean ± standard error. Values with the same superscript in the same column are not significantly different at $p \geq 0.05$

Key: **XC**= *Xanthomonas campestris* CPBF 211, **SM**= *Stenotrophomonas maltophilia* IAE127

4.1.7 Enhanced Oil Recovery from Soil using Xanthan Gum

Figure 4.6 revealed that the rates of enhanced oil recovery (EOR) using commercial xanthan gum and xanthan gum produced from *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 were not high. However, more oil was recovered using the commercial xanthan gum (8.25 %) than xanthan gum produced by either of the two organisms (2.7 - 5.0 %).

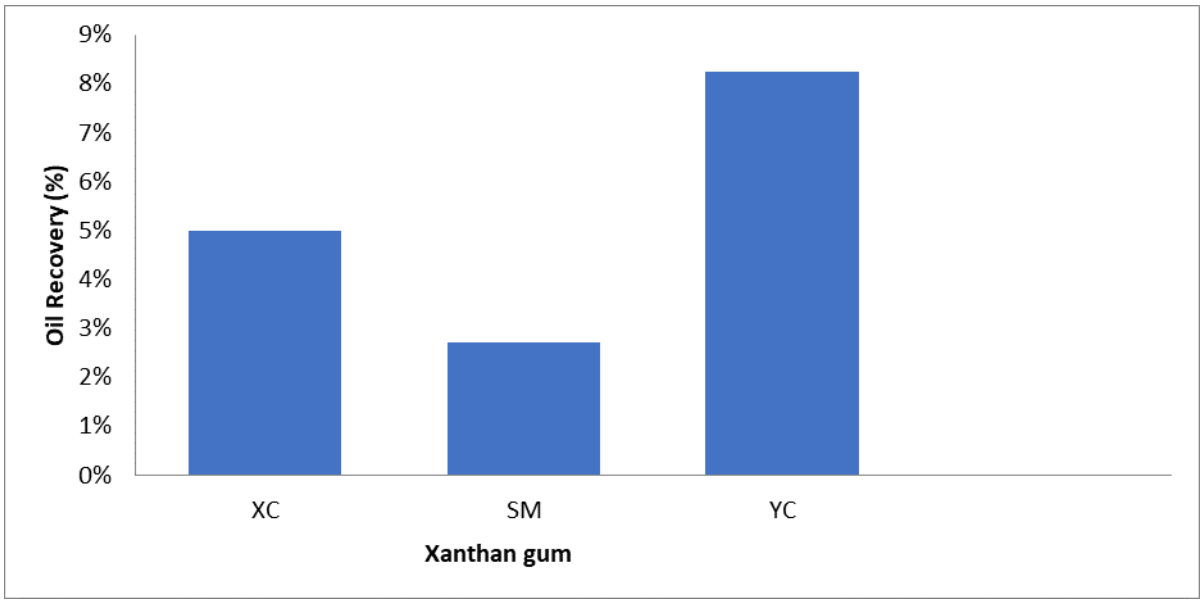


Figure 4.6: Crude oil recovery from contaminated soil by xanthan gum from *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127

Key: Xc = Xanthan gum from *Xanthomonas campestris* CPBF 211, Sm = Xanthan gum from *Stenotrophomonas maltophilia* IAE127, Yc xanthan commercial

4.2 Discussion

Xanthomonas species were initially selected based on pigmentation on nutrient agar, morphological characteristics and emulsification ability on carbon source. The organisms exhibited pale yellow to orange pigmentation on nutrient agar, gram negative rod and emulsified the carbon source. These qualities helped in the initial selection of X*anthomonas* species. Other investigators (Roumagnac *et al*., 2004; Li *et al*., 2019; Ogolla and Neema, 2019; Izadiyan and Taghavi, 2020) have used these parameters in the primary isolation and selection of *Xanthomonas* species. The yellow/orange colour appearance on nutrient agar is due to a membrane bound pigment "xanthomonadin" which may protect bacteria from photobiological damage (Habibi and Khogravi-Darani, 2017). The isolated *Xanthomonas* species emulsified hydrocarbon to varying degrees, forming stable, less stable of unstable emulsions. This means that, the organisms produced surface active agents such as xanthan gum which are useful in various industries including food and petroleum industries (Paraniraj and Jayaraman, 2011; Ijah and Olarinoye, 2012; Habibi and Khogravi- Darani, 2017).

The *Xanthomonas* species were identified based on biochemical test as *X. vasicola, X. citri, X. campestris, S. maltophilia, X. axonopodis, X. vesicatoria, X. gardneri* and *X. perforans.* The two best xanthan gum producing strains were subjected to molecular identification, it was observed that the two bacterial strains (BX3 and TX9) were 99% identical to *Stenotrophomonas maltophilia* strain IAE127 and *Xanthomonas campestris* strain CPBF 211 respectively according to 16S ribosomal RNA gene after molecular characterization. Other investigators have isolated these organisms from different plant parts where they occur as pathogens (Beattie, 2007; Tonu, 2013; Abbasi and Weselowski, 2015; Sharma, 2018, Newberry *et al*., 2019; Izadiyan and Taghavi, 2020).

The viscosity of xanthan gum is important for its thickening properties, as well as its rheological and pseudoplastic effects in aqueous solutions; these properties are important for its industrial application and are therefore, the main indicators of xanthan quality (Li *et al*., 2016; Wang *et al*., 2017; Xu *et al*., 2019). The rheological properties of the xanthan gum produced and commercial xanthan gum (control) assessed by apparent viscosity (AV) analysis exhibited considerable differences at varying concentrations for both the commercial xanthan gum and the xanthan gum produced in the laboratory at 12rpm and temperature of 40˚ C. Generally, the AV of solutions increased with increasing xanthan gum concentration for commercial xanthan gum while increase in gum concentration for *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 led to a decrease in the AV value. The commercial xanthan gum showed a maximum viscosity value of 411.3 followed by that of *Xanthomonas campestris* CPBF 211 with a maximum viscosity of 526.1. Higher viscosity values were obtained from *Stenotrophomonas maltophilia* IAE127 with a maximum of 660.6, indicating that the strain had a greater effect on the variation in maximum apparent viscosity.

Previous studies stated that xanthan gum with various viscosities had different applications. Xanthan gum with high viscosity is usually used in petroleum industry, agriculture industry and water-based paints (Rosalam and England, 2006; Palaniraj and Jayaraman, 2011). In contrast, xanthan gum with low viscosity has higher dosage and transparency, and thus can be potentially used in foods and other fields (Li *et al*., 2016; Wang *et al*., 2017). Low viscosity makes xanthan gum have better synergistic effect with other additives and gums (Wang *et al*., 2017; Filimon *et al*., 2018). Therefore, these gums produced in the present study can find application in the food industry as emulsifiers, stabilizers, and suspension agents.

Polysaccharides are used to control the rheological properties of the emulsions, contributing to the stability of the emulsion. Xanthan gum is one of the main stabilizers in food emulsions because it is a good thickening agent for aqueous solutions. Its ability to increase both the viscosity and the stability of the emulsion depends on the concentration and structure of the polymer. The results of emulsification index (IE_{24}) revealed that xanthan gum from both *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 gave better IE_{24} with castor oil than other oils reaching values of 67.5 and 36.45 % respectively. This was followed by vegetable oil with IE_{24} of 33.75-47.5 %, Diesel gave the least emulsification index (2.5 – 8.75 %) which means that the xanthan gum poorly emulsified diesel. The IE_{24} of a commercial xanthan gum solution (1g $^{L-1}$ w/v) with oil also gave the highest emulsification in castor oil as (63.75 %) and the least emulsification with diesel (2.65 %). This implies that castor oil was the best of the five oils used. Iyer *et al*. (2006) investigated polysaccharides produced by *Enterobacter cloaceae* in paraffin, cotton, coconut, jojoba, peanut and sunflower oils at a concentration of 1 g/L. The investigators found an IE_{24} of 60 % for paraffin, coconut and cotton oils whereas that of jojoba oil and sunflower oil was 65 and 75 % respectively. However, the highest value was for peanut oil (95 %). The IE_{24} of commercial xanthan and Arabic gums was also evaluated at the same concentration, with findings of 33 and 61 %, respectively.

The emulsifying activity of an exopolysaccharides (EPS) can be attributed to certain functional groups in the biopolymer, such as 6-deoxyhexoses and acetyl moieties, which provide hydrophobicity to the EPS and subsequently, contribute to its emulsifying capacity (Iyer *et al*., 2006). The occurrence of a high percentage of hydrophobic amino acids in the protein moiety also favours the emulsification properties of a polysaccharide (Iyer *et al*., 2006). Furthermore, due to their texturing properties,

polysaccharides, such as xanthan, have been widely employed to control rheological properties of oil-in-water emulsions, thus also contributing to emulsion stability (Desplanques *et al*., 2012). Considering that production process conditions affect some structural characteristics, such as molecular weight, pyruvilation and acetylation degree of xanthan, with implications for its physico-chemical properties, different carbon sources and stress conditions could affect the molecular structure of xanthan and lead to different behaviour related to its emulsifying capacity (Trindade *et al*., 2018).

The scanning electron microscopy revealed xanthan commercial (control) to present a rough and a more compact structural surface, probably due to subjecting the gum to some special treatment such as alkali stress (addition of 2N NaOH to increase the pH thereby improving the morphology) (Luvielmo *et al*., 2016). The xanthan gum produced by *Xanthomonas campestris* CPBF 211 had a more disperse heterogeneous whitish structure with less surface porosity probably as a result of the sizes of the gum particles and the presence of a white colouration may be due to the high calcium content present in the fermentation medium. The xanthan gum produced by *Stenotrophomonas maltophilia* IAE127 presented a flat whitish surface with a pronounced fiber like structure probably suggesting part of the substrate remained in the gum during the separation process. According to Ahuja *et al*. (2012) samples that have rough surfaces causes blockage and result in less flow of irregularly shaped particles.

From xanthan gum commercial (control) the most abundant elements were found to be potassium, carbon, phosphorus and iodine with a weight concentration of 28.13, 17.03, 14.55, and 10.95, respectively. Potassium, carbon, calcium and phosphorus with a weight concentration of 22.55, 21.81, 17.99 and 15.88 respectively were also found to be abundant in xanthan gum produced by *Xanthomonas campestris* CPBF 211 while xanthan gum by *Stenotrophomonas maltophilia* IAE127 had calcium, carbon,

zirconium, phosphorus and potassium to be abundant elements with atomic weight concentration of 28.95, 15.43, 13.07, 11.95 and 10.92, respectively. It is possible that these elements contribute to the structural morphology of the xanthan gum.

The Fourier Transform-infrared spectrum (FT-IR) is used to detect similarities or differences in chemical structures of compounds. The region studied included all the spectra bands located in the window between the wave numbers 1000 and 4000 cm^{-1} and 1000 and 3800 cm^{-1} for *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 respectively. The region studied includes spectra bands between 4000–1000 cm^{-1} for *Xanthomonas campestris* CPBF 211 which were: (3950–3200 cm^{-1}: axial deformation of –OH; 2150–2350 cm^{-1}: axial deformation of C–H (may be due to absorption of symmetrical and asymmetrical stretching of CH_3 or even groups of CH_2) and CHO; 1700–1600 cm^{-1}: axial deformation of C=O ester, acid carboxylic, aldehydes and ketones; 1450–1600 cm^{-1}: axial deformation of C=O of enols (-diketones); 1350–1450 cm^{-1}). While the most important bands recorded in the range of 3800–1000 cm^{-1} for *Stenotrophomonas maltophilia* IAE127 were: (3100–3200 cm^{-1}: axial deformation of C–H and CHO; 1450–1550 cm^{-1}: axial deformation of C O ester, acid carboxylic, aldehydes and ketones (Li *et al*., 2016). Comparison of the peak values, representing a specific functional group in *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127, revealed that they have almost the same value, the cumulative stretching vibrations of all the functional groups closely resemble the structure of xanthan gum as reported by Faria *et al*. (2011) and Li *et al*. (2016).

Table 4.9 shows the thermogravimetric analysis (TGA/dTGA) of xanthan gum produced from *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127. Two thermal events were observed. The first thermal events correspond to dehydration of polysaccharides. The presence of water in the samples may be related to the rapid absorption of water during gum weighing. According to Faria *et al* (2011), xanthan gum absorbs water due to the presence of polar groups in its chemical structure. The second thermal event was associated with the degradation of the xanthan gum polymer chain (Da Silva *et al*., 2018), which began by disrupting groups belonging to the side chain, and subsequently caused the main chain to unfold. The initial degradation temperature (T_{onset}), Maximum decomposition temperature (T_{max}) and mass losses are shown in Table 4.8. According to Zohuriaan and Shokrolahi (2004), the degradation temperature range for commercial xanthan gum was between 251.15 and 330.30˚ C, with weight loss ranging from 8.7 to 41.6 %. Xanthan gum produced by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 was observed to have a similar thermal stability to the commercial xanthan gum indicating their potential for use in processes that require high temperatures. Faria *et al*. (2011) evaluated the thermal conditions of xanthan gum from cane juice and showed that thermal degradation occurred at temperature between 220 and 320˚ C with a mass loss of 40 % and maximum loss occurring at 283˚ C. According to Villetti *et al*. (2002), mass loss between 25 and 150º C was related to the water outlet of the polymer, whereas between 150 and 400º C, carbonization of the biopolymer occurred, and between 400 and 550º C, xanthan degradation occurred.

The differential scanning calorimetry (DSC) curves shown in Figure 4.4a and b revealed that the xanthan gum from *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas*

maltophilia IAE127 behaved similarly when subjected to the same temperature conditions. Two events could be identified, one endothermic peak, and one exothermic peak. The first event for *Xanthomonas campestris* CPBF 211 varied between 25 and 160 °C, showing an endothermic peak at approximately 80 °C, attributed to a loss of moisture in the sample (Zohuriaan and Shokrolahi, 2004). The second event started at approximately 240 °C, showing an exothermic peak related to polysaccharide degradation (Faria *et al*., 2011). while for *Stenotrophomonas maltophilia* IAE12 the event varied between 25 and 140 °C, showing an endothermic peak at approximately 80 °C. The second event started at approximately 270 °C. The implication is that, xanthan gum produced by the two bacteria requires similar amount of heat to cause loss of moisture in the samples thus strengthening the structure of the samples.

Biomass and xanthan gum production by both *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 was found to increase between 48 h and 96 h, but as incubation time extended from 96 h to 144 h, there was a little/no increase in biomass as well as the xanthan gum produced by the organisms. This may be due to the presence of acid groups in the biopolymer which resulted to a decrease in pH as the fermentation time extended beyond 96 h (Psomas *et al*., 2007; Borges *et al*., 2009; Lopes *et al*., 2015). Hence optimum time of xanthan gum production was 96 h, this agrees with the study by Chavan and Baig, (2016) who found a relationship between biomass and xanthan production by *Xanthomonas campestris*. The investgators found that, there was a decrease in both biomass and xanthan gum produced after 96h of incubation. However, significant differences existed between the amount of xanthan gum produced by *Xanthomonas campestris* CPBF 211 at 48 and 96 h of incubation at $p<0.05$. Earlier studies have shown that the maximum xanthan production was produced

after 48 h and increased with increase in time (Mohan *et al*., 2010: Palaniraj and Jayaraman, 2011).

It was observed that, as temperature increased from 25° C to 35° C, the production of biomass increased and xanthan gum production decreased in *Stenotrophomonas maltophilia* IAE127. In *Xanthomonas campestris* CPBF 211, increase in temperature from 25° C to 35° C led to a decrease in both biomass and xanthan gum production. This may be due to the fact that the organism is not thermotolerant and therefore, could not withstand slightly higher temperature other than the optimum (30° C ± 2) to give xanthan gum (Lopez *et al*., 2015: Da silver *et* al., 2018: Makut *et al*., 2018). There was a significant difference in the amount of xanthan gum produced by *Xanthomonas campestris* CPBF 211 at 25° C and 30° C at $p<0.05$. Therefore, it was clear that the optimum temperature for xanthan gum production in *Stenotrophomonas maltophilia* IAE127 was 30° C, whereas the optimum temperature for *Xanthomonas campestris* CPBF 211 was less than 30° C. Maximum production of biomass and xanthan gum was found to be 4.76 and 3.75 g/L, respectively for *Stenotrophomonas maltophilia* IAE127, while it was 5.64 and 6.43 g/L, respectively for *Xanthomonas campestris* strain CPBF 211. This implies that *Xanthomonas campestris* strain CPBF 211 is a better strain to use for xanthan gum production at these conditions.

The effect of pH on biomass and xanthan gum production revealed that, as pH increased from 5 to 9, biomass produced by *Xanthomonas campestris* CPBF 211 decreased while xanthan gum increased. Highest yield of biomass and xanthan gum was obtained at pH 5(5.25 g/L) and pH 9 (4.29 g/L) respectively. This agrees with most authors (Psomas *et al*., 2007; Silva *et al*., 2009; Gumus *et al*., 2010; Palaniraj and Jayaraman, 2011; Lopes *et al*., 2015) who reported that for xanthan gum production, neutral pH was ideal for the

growth of *X. campestris*. However, pH may decrease during fermentation to around 5.0 due to acid groups present in the biopolymer. Also, it was observed from the study that, as the pH increased from 5 to 9 the xanthan gum produced by *Stenotrophomonas maltophilia* IAE127 decreased while the biomass increased, implying that optimal xanthan gum production was at pH 7. There was no significant difference in the biomass and xanthan gum produced by the two organisms between pH 5 and 9 at $p<0.05$.

Effect of carbon source on biomass and xanthan gum production showed that, *Stenotrophomonas maltophilia* IAE127 produced the highest biomass (2.43 g/L) in pineapple peels and the highest xanthan gum in sugarcane bagasse medium while *Xanthomonas campestris* CPBF 211 gave the highest biomass and xanthan gum yield of 3.84 and 4.46 g/L, respectively in sugarcane bagasse containing medium, *Stenotrophomonas maltophilia* IAE127 produce the highest biomass of 3.79 g/L while *Xanthomonas campestris* CPBF 211 gave the highest xanthan yield of 3.84 g/L. *Stenotrophomonas maltophilia* IAE127 cells grown in pineapple peels gave the highest biomass yield of 6.99 g/L, while *Xanthomonas campestris* CPBF 211 grown in the same medium gave the highest xanthan gum yield of 4.82 among the carbon sources tested. The present results agree with Souw and Demain (1979) and Kawahara and Obata (1998) who reported that, maximum xanthan production was obtained when sucrose was used as a carbon source. The total soluble sugar found in pineapple is predominantly sucrose (Sangprayoon *et al*., 2019). The least xanthan gum yield was found in the medium containing glucose (0.72 g/L). Glucose concentrations of less than 2 to 5 % are not effective for maximum cell growth. Also, high concentration of glucose has no significant effect on cell growth and xanthan gum production (Amanullah *et al*., 1998; Leela and Sharma, 2000; Niknezhad *et al*., 2015).

The effect of nitrogen sources on biomass and xanthan gum production indicated that biomass was highest in yeast extract (4.68 g/L) for *Stenotrophomonas maltophilia* IAE127 while *Xanthomonas campestris* CPBF 211 had highest biomass (2.20 g/L) in ammonium sulphate. Both *Stenotrophomonas maltophilia* IAE127 and *Xanthomonas campestris* CPBF 211 had the highest yield of xanthan gum in yeast extract medium (3.93 and 3.22 g/L), respectively. This implies that the amount of nitrogen required in the fermentation media is less than that required in the growth medium of the organism. Ammonium sulphate therefore, is a better substrate for biomass accumulation while xanthan gum yield was higher with nitrate used as nitrogen source (Muniyasamy *et al*., 2019). This is in agreement with the work of Palaniraj and Jayaraman (2011) who reported highest xanthan gum production when yeast extract was used as nitrogen source in the medium.

When the conditions were optimized (pH: 9.0, 7.0; temperature: 25º C; carbon source: 0.2 % pineapple peels, sugarcane bagasse; nitrogen source: 0.3 % yeast extract for *Xanthomonas campestris* CPBF211 *and Stenotrophomonas maltophilia* IAE 127, respectively) xanthan gum and biomass increased in yield; maximum yield of xanthan gum and biomass for both organisms was observed at 96 h of incubation. There was a strong positive correlation between biomass and xanthan gum produced by the organisms and it was highly significant at $P< 0.01$.

The application of xanthan gum produced by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 in oil recovery from soil revealed that the xanthan gum produced was not so effective to enhance oil recovery. The xanthan gum produced by *Xanthomonas campestris* CPBF 211 was able to recover only 5 % of crude from the soil while xanthan gum from *Stenotrophomonas maltophilia* IAE127 recovered 2.7 % crude oil from the soil, as compared to the commercial xanthan (served

as control) which recovered 8.25 % of crude oil from the soil after 10 h. A study by Coolman *et al*. (2020) examined the effect of xanthan gum and aquagel polymer treatment on oil recovery factor in a typical well in Trinidad and found that, due to different properties, the performance of xanthan gum and aquagel polymer was dependent on working conditions, concentration of the polymer as well as the viscosity, the results showed that aquagel gel polymer flood resulted in a higher oil recovery than xanthan gum polymer flood.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study revealed that *Xanthomonas* species isolated from diseased plant leaves had potentials for xanthan gum production. *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 were efficient xanthan gum producers, yielding 2.101 g/l and 1.63 g/l respectively of xanthan gum after 96 h. Characterization of the produced revealed that, increase in xanthan gum concentration for *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127 led to a decrease in their apparent viscosity thus can be potentially useful in the food industries. The ability of xanthan gum from *Stenotrophomonas maltophilia* IAE127 and *Xanthomonas campestris* CPBF 211 to emulsify hydrocarbon vegetable oils, crude and diesel oil was similar to that of commercial xanthan gum; the produced xanthan gums also exhibited good thermal stability. Differential scanning calorimetry revealed that xanthan gum produced by the two bacteria required similar amount of heat to cause loss of moisture in the samples thus strengthening the structure of the samples. Fourier Transform Infrared Spectrometry revealed that functional groups of the xanthan gum produced were closely related to those of commercial xanthan gum. The xanthan gum yield was found to increase under optimized conditions of pH: (pH: 9.0, 7.0; temperature: 25ºC; carbon source: 0.2% pineapple peels, sugarcane bagasse; nitrogen source: 0.3% yeast extract) with a maximum yield after 96h of incubation. There was a strong positive correlation between biomass and xanthan gum produced by the organisms and it was highly significant at $P<0.01$ meaning xanthan gum production depends the biomass

produced. Xanthan gum produced by the two organisms were found to recover less crude oil from the soil.

Recommendations

It is recommended that:

1. Local isolates of *Xanthomonas* can produce useful products such as xanthan gum which has characteristics similar to commercial xanthan gum used in food, chemical agricultural and petroleum industries.
2. The optimized production parameters enhanced xanthan gum production by the organisms.
3. The xanthan gum produced can be useful in the oil industries.
4. The *Xanthomonas* species should be screened for potential to produce other biopolymers such as biosurfactants which are useful in various industries. This is because the organisms can emulsify various carbon sources.

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APPENDIX
APPENDIX A: Partial sequence of:

(i) *Xanthomonas campestris* CPBF 211

Gene sequence obtained from the molecular characterization of the isolate is 99% identical to *Xanthomonas campestris* strain CPBF 211 16S ribosomal RNA gene, presented as:

TCTTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTC
TTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTA
CGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGATTGAATGAGCCGATGT
CGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGC
TGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCC
AGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTG
GGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGA
ATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAACGAAGGGTGCATG
CGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTGGTTTAAGTCT
GTTTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTGGGTCACT
AGAGTGTGGTAGAGGGTAGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAG
AGATCGGGAGGAACATCCGTGGCGAAGGCGGCTACCTGGACCAACACTGAC
ACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTA
TCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTT
AATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAACTTT
CCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCCTTAGTTGCCGCACGTAATGGTGGGAACTCTAAGGAGACCGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAGTCATCATGGCCCTTACG
ACCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAACCCG
CGAGGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGC
AACTCGACTCCATGAAGTCGAATCGCTAGTAATCGCAGATCAGCATTGCTG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TTTGTTGCACCAGAAGCAGGGCATT

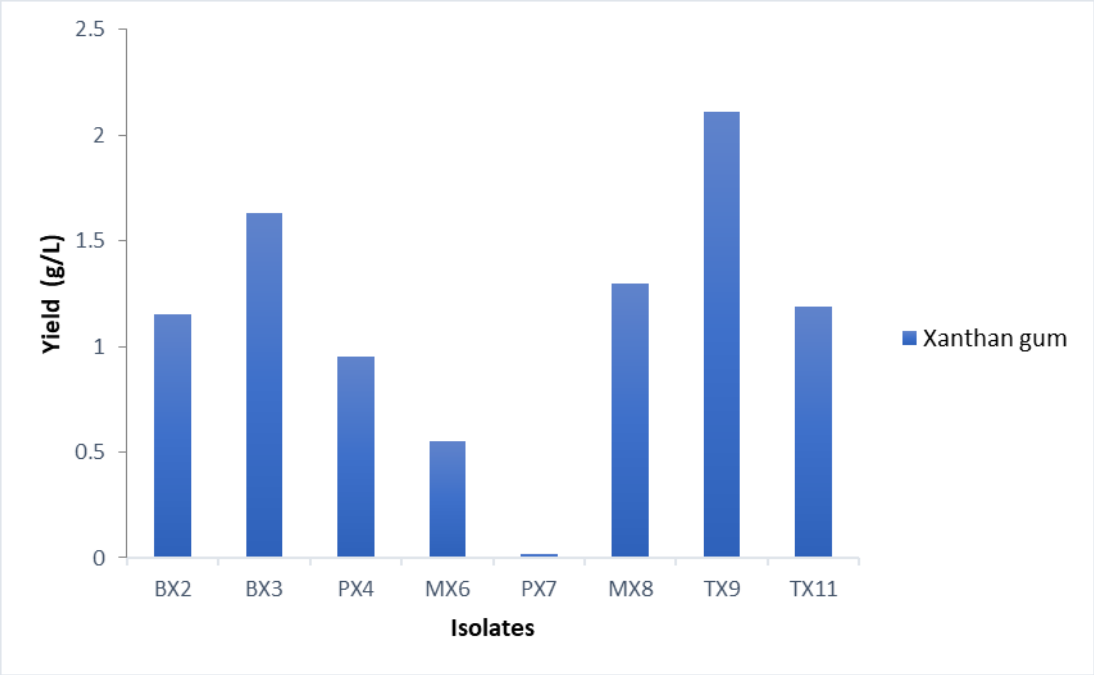
(ii) *Stenotrophomonas maltophilia* IAE127

Gene sequence obtained from the molecular characterization of the isolate is 99% identical to *Stenotrophomonas maltophilia* strain IAE127 211 16S ribosomal RNA gene, presented as:

CCTGCAGTCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGG
CGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGGGGGATAACGTA
GGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATC
TTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGG
GGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGA

AGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGG
CTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTT
CGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTAC
TGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTG
GGCTCAACCTGGGAACTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAG
GGTAGC
GGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCAT
GGCGAAGGCAGCTACCTGGACCAACATGACACTGAGGCACGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAA
CTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTT
CGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGATTGTGCCTTC
GGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGT
AATGGTGGGAACTCTAAGGAGACCGCCGGTGACCCAGCGTA

Appendix B: (i) Xanthan gum yield by *Xanthomonas* species isolated from various plant leaves



Key: Isolates from: BX= Banana, PX =Pepper, RX =Rice, MX = Mango, TX =Tomato.

(i) Setup for the production of xanthan gum by eight *Xanthomonas* isolates

Appendix C: Interaction effect between isolates and incubation time on biomass and xanthan gum

Interaction effect	Xanthan gum	Biomass
48h × Sc	1.20^{d}	3.83^{d}
48h × Xc	1.24^{c}	4.04^{c}
92h × Sc	1.36^{b}	4.06^{c}
92h × Xc	3.63^{a}	5.91^{b}
144h × Sc	1.37^{b}	6.73^{a}
144h × Xc	3.64^{a}	6.74^{a}
Partial Eta Squared	89.1	3.6
P – value	0.001	0.895

Key:

Xc= *Xanthomonas campestris* CPBF 211, Sc= *Stenotrophomonas maltophilia* IAE127

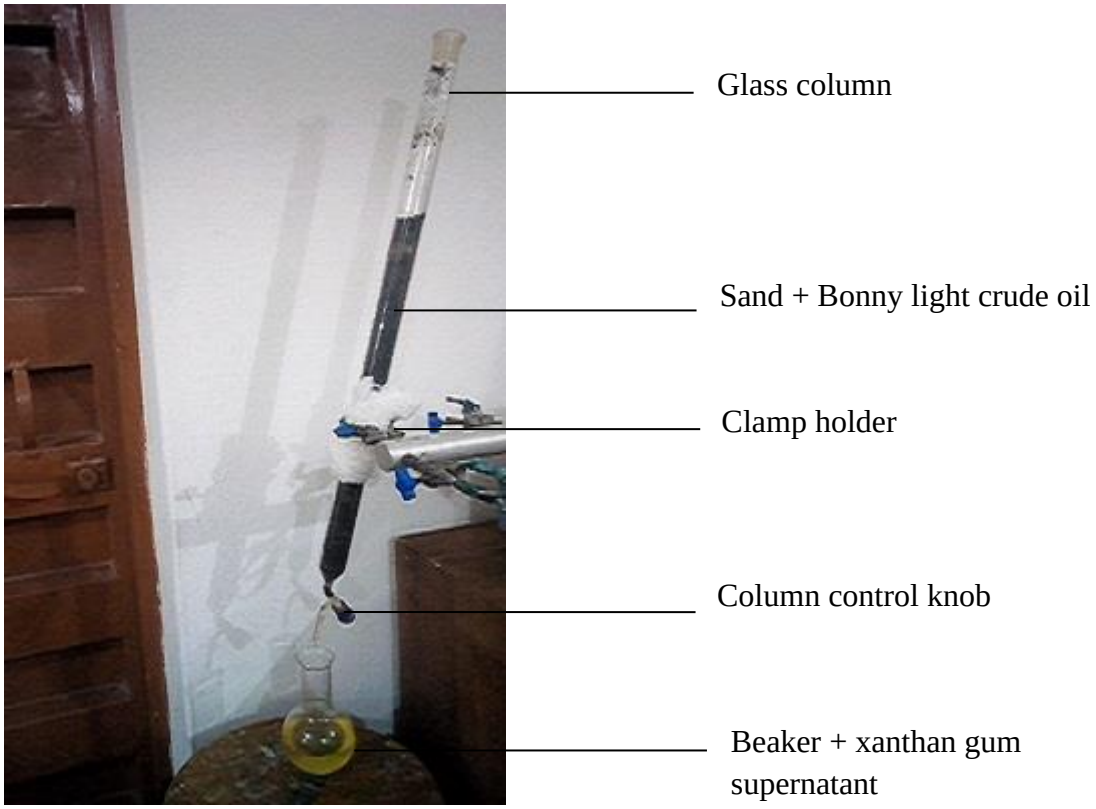
IT= incubation time, d= days

Appendix D: Determination of emulsification index of xanthan gum produced

Plate I: Experimental set up for determination of emulsification of oils

Key: C=castor oil, V= vegetable oil, D= diesel, *Jatropha* oil, CO= crude oil

Appendix E: Experimental setup for enhanced recovery of oil using xanthan gum



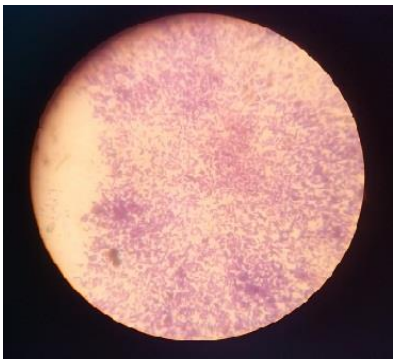
Appendix F: Cultures of some bacterial isolates showing yellow colouration on nutrient agar (a-d).

(a) **(b)**

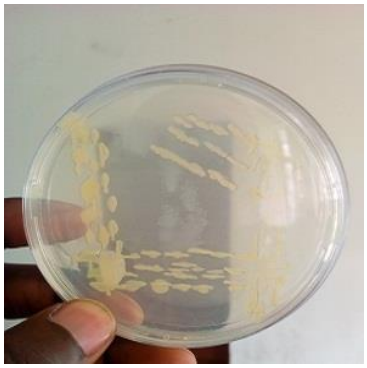
(c) **(d)**

Appendix G: (a) 48h culture of *Xanthomonas campestris* CPBF 211 (b) *Xanthomonas campestris* CPBF 211 viewed under microscope (c) 48h culture of *Stenotrophomonas maltophilia* IAE127 (d) *Stenotrophomonas maltophilia* IAE127 viewed under microscope.

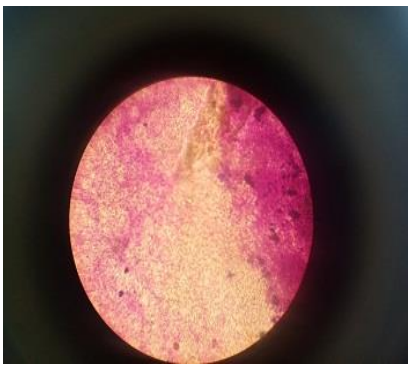
(a)



(b)

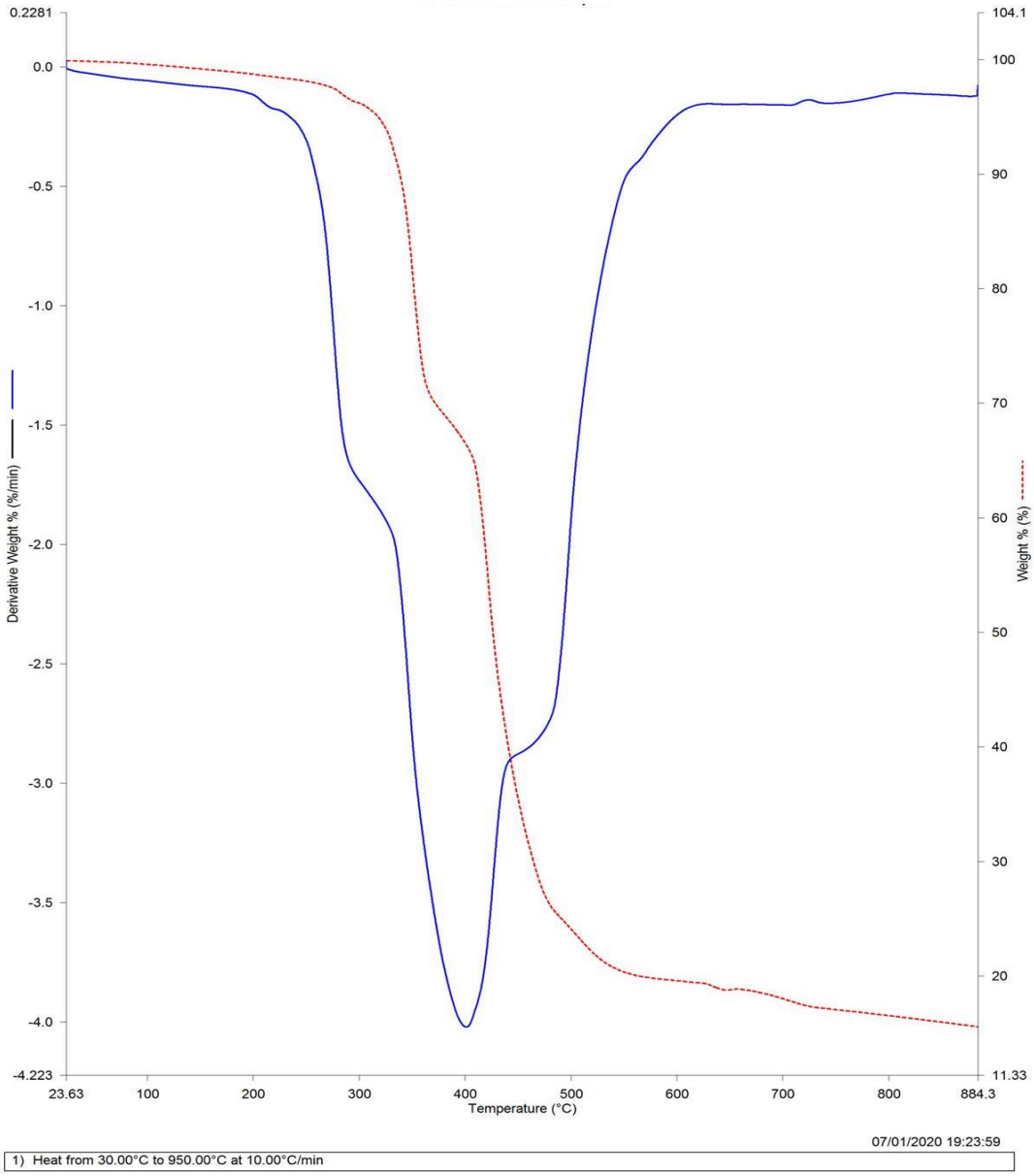


(c)



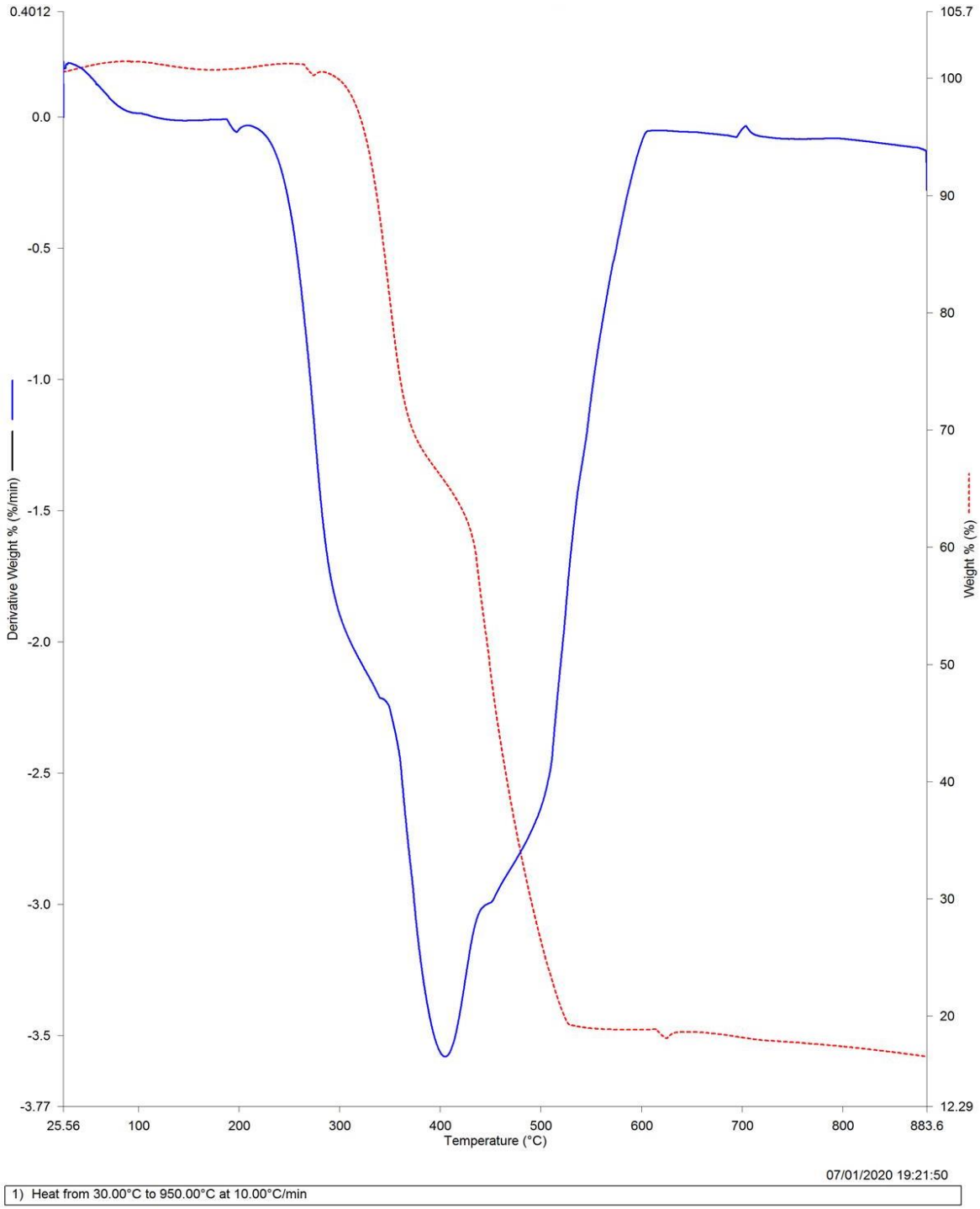
(d)

Appendix H: Thermogravimetric data (TGA and dTGA) of xanthan gum produced by *Xanthomonas campestris* CPBF 211, *Stenotrophomonas maltophilia* IAE12



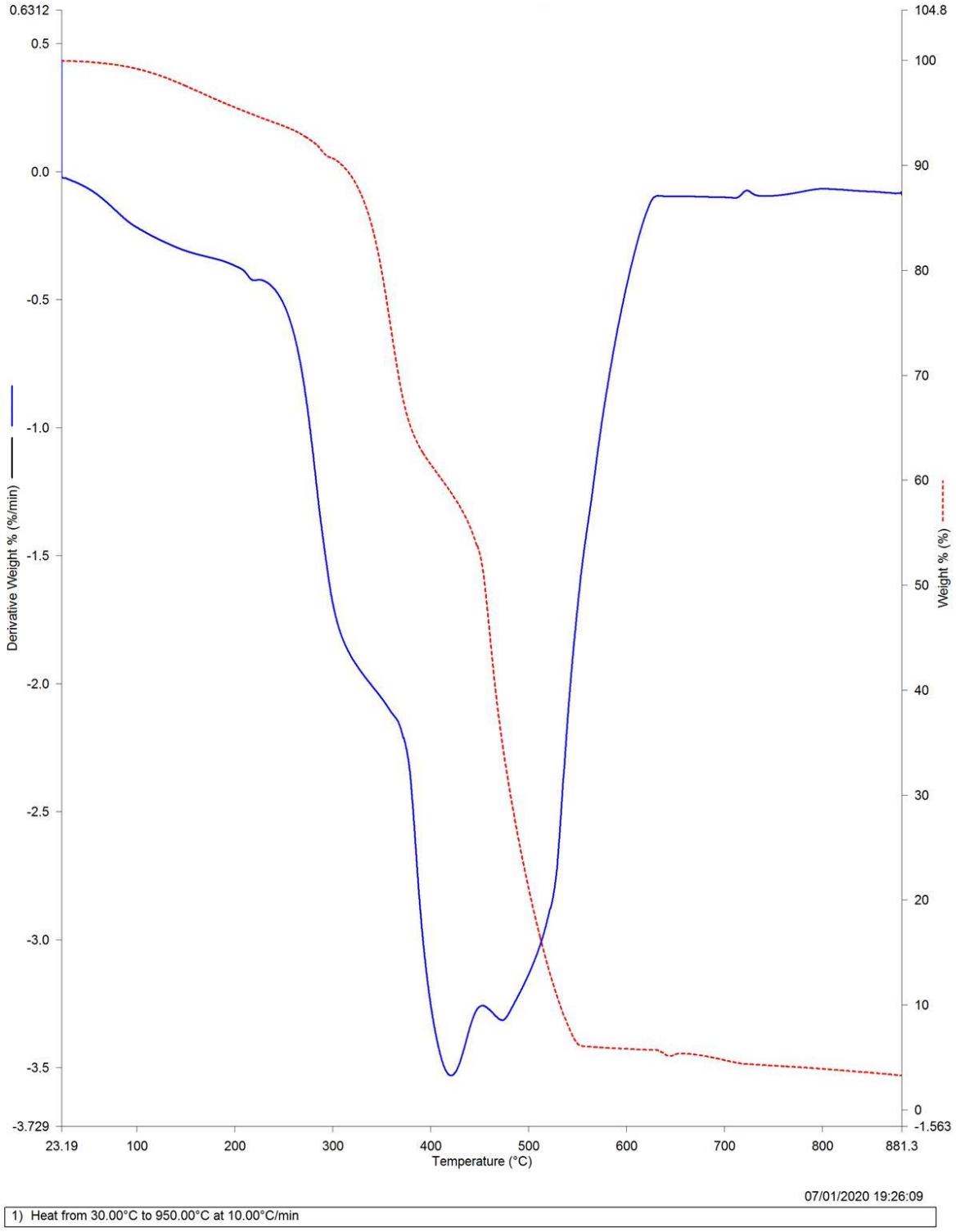
TGA =……. DTA = ———

Figure 4.3a: TGA/ dTGA for *Xanthomonas campestris* CPBF 211



TGA =……. DTA = ———

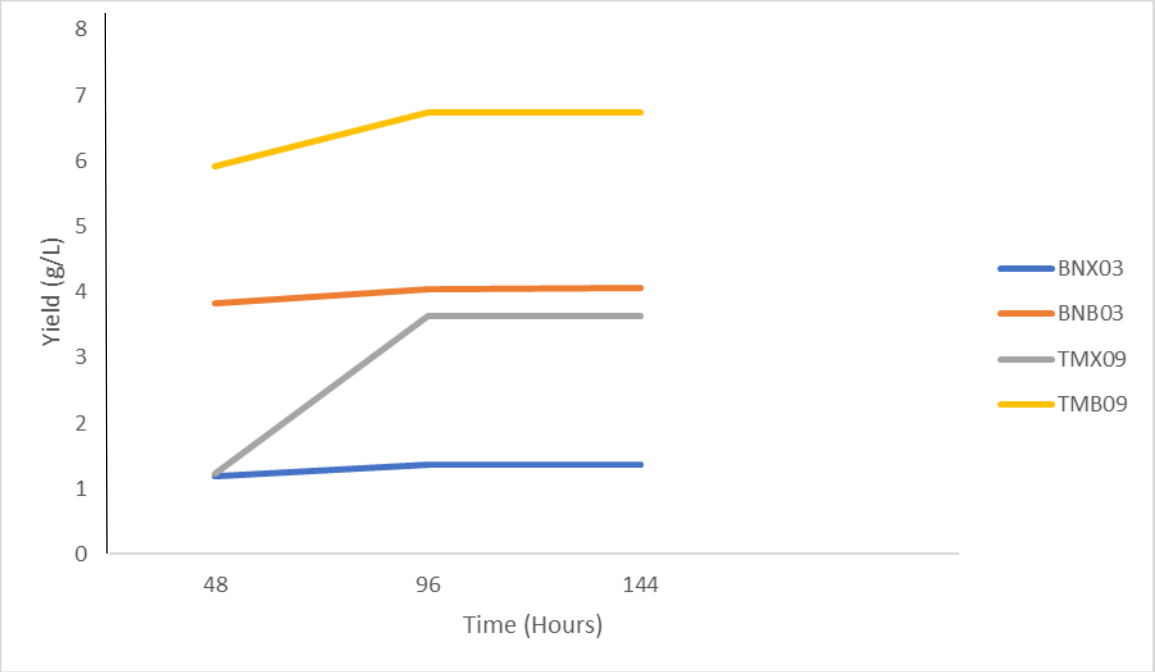
Figure 4.3b: TGA/ dTGA for *Stenotrophomonas maltophilia* IAE127



TGA =……. DTA = ———

Figure 4.3c: TGA/ dTGA for commercial xanthan gum (control)

APPENDIX J: Effect of incubation time on biomass and xanthan gum production by *Xanthomonas campestris* CPBF 211 and *Stenotrophomonas maltophilia* IAE127



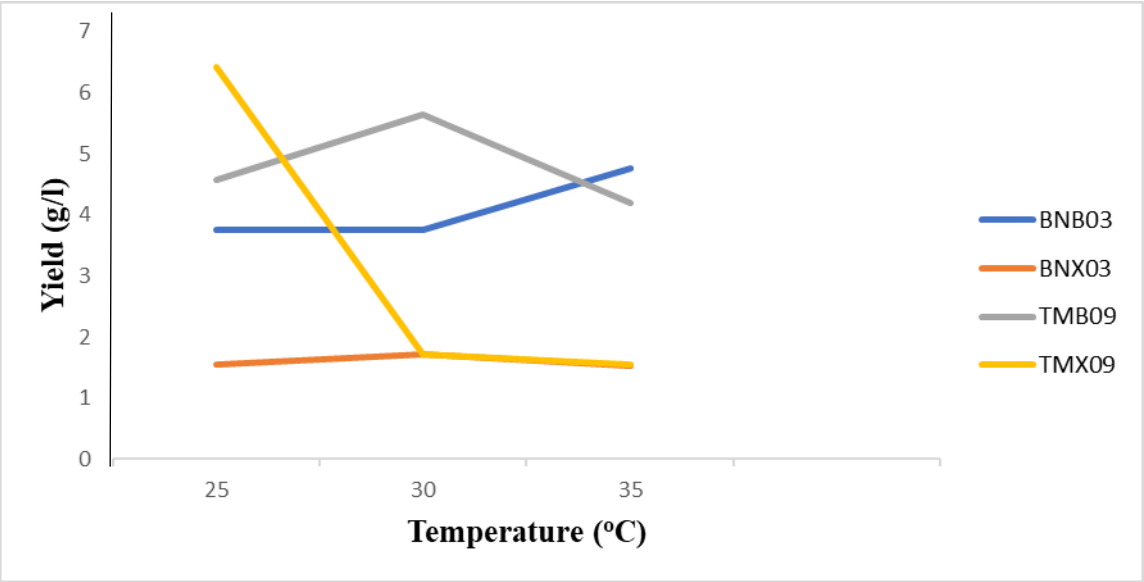
KEY: **TMX09=** Xanthan gum from *Xanthomonas campestris* CPBF 211

TMB09=Biomass of *Xanthomonas campestris* CPBF 211

BNX03= Xanthan gum from *Stenotrophomonas maltophilia* IAE127

BNB03= Biomass of *Stenotrophomonas maltophilia* IAE127

APPENDIX K: Effect of temperature on biomass and the xanthan gum production by *Xanthomonas campestris* strain CPBF 211 and *Stenotrophomonas maltophilia* strain IAE127



Appendix K: Interaction effect between isolates and temperature on biomass and xanthan gum

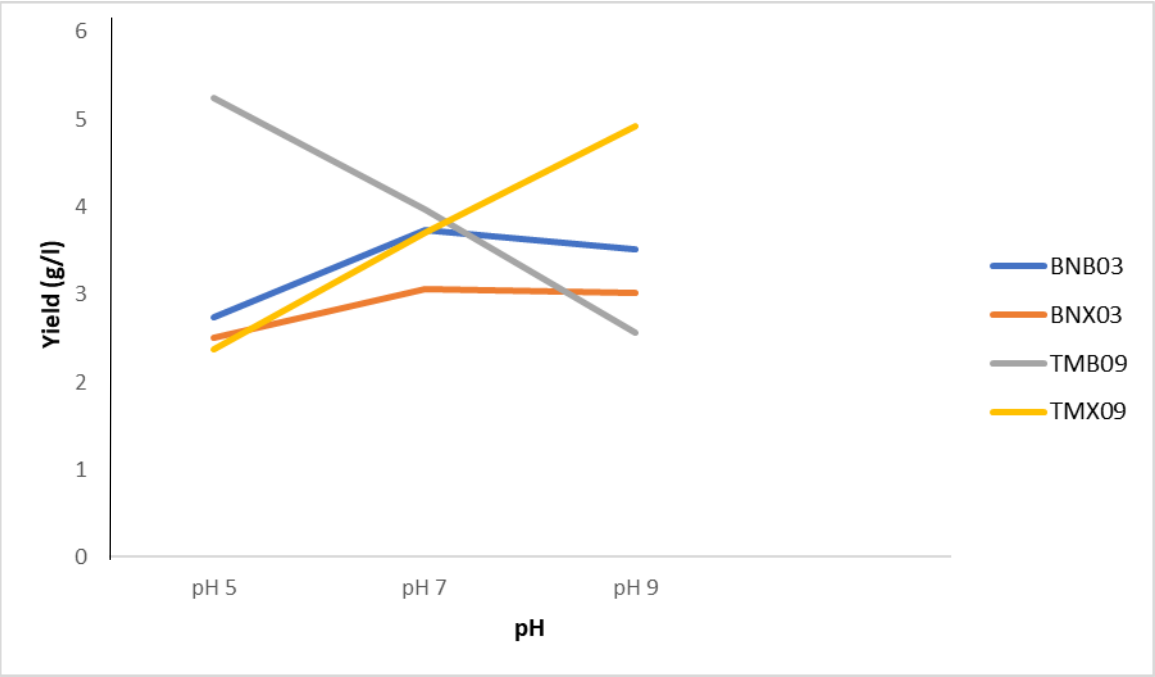
Interaction effect	Xanthan (g/L)	Biomass (g/L)
T 25 × Sc	1.55^{c}	3.75^{d}
T 25 × Xc	6.43^{a}	4.58^{b}
T 30 × Sc	1.71^{b}	3.76^{d}
T 30 × Xc	1.71^{b}	5.64^{a}
T 35 × Sc	1.54^{d}	4.76^{b}
T 35 × Xc	1.56^{e}	4.20^{c}
Partial Eta Squared	86.4	42.7
P-value	0.002	0.188

Key:

Xc= *Xanthomonas campestris* CPBF 211, Sc= *Stenotrophomonas maltophilia* IAE127

T= temperature

APPENDIX L: Effect of pH on the biomass and xanthan gum production by *Xanthomonas campestris* strain CPBF 211 and *Stenotrophomonas maltophilia* strain IAE127


6
5
4
3
2
1
0
Yield (g/l)
pH 5
pH 7
pH 9
pH
BNB03
BNX03
TMB09
TMX09

Appendix M : Interaction effect between isolates and pH on biomass and xanthan gum

Interaction effect	Xanthan (g/L)	Biomass (g/L)
pH 5 × Sc	4.00^{b}	2.74^{e}
pH 7 × Sc	3.06^{d}	3.73^{c}
pH 9 × Sc	3.02^{e}	3.60^{d}
pH 5 × Xc	2.37^{f}	5.25^{a}
pH 7 × Xc	3.70^{c}	3.97^{b}
pH 9 × Xc	4.29^{a}	2.56^{f}
Partial Eta Squared	51.2	42.2
P – value	0.116	0.193

Key:

Xc= *Xanthomonas campestris* CPBF 211, Sc= *Stenotrophomonas maltophilia* IAE127

Appendix N: Effect of carbon source on biomass and xanthan gum production by bacterial isolates

	Biomass (g/L)		Xanthan gum (g/L)	
Carbon source	**XCB**	**SMB**	**XCX**	**SMX**
Glucose	$2.06^{b} \pm 0.035$	$2.43^{c} \pm 0.629$	$4.46^{a} \pm 0.127$	$0.72^{b} \pm 0.155$
Pineapple peels	$1.97^{b} \pm 0.982$	$6.99^{b} \pm 0.600$	$4.82^{a} \pm 0.357$	$1.60^{b} \pm 0.161$
Sugarcane bagasse	$3.84^{a} \pm 0.029$	$5.70^{a} \pm 23.97$	$3.84^{b} \pm 0.028$	$3.00^{a} \pm 0.138$

Values are presented as mean ± standard error. Values with the same superscript in the same column are not significantly different at $p<0.05$.

Key:

XCB= *Xanthomonas campestris* CPBF 211 Biomass, SMB= *Stenotrophomonas maltophilia* IAE127 Biomass

XCX= *Xanthomonas campestris* CPBF 211 Xanthan gum, SMX= *Stenotrophomonas maltophilia* IAE127

Xanthan gum

Appendix O: Interaction effect between isolates and carbon sources on biomass and xanthan gum

Interaction effect	Xanthan (g/L)	Biomass (g/L)
CS gl × Sc	0.72[d]	2.43[c]
CS gl × Xc	4.46[a]	2.06[d]
CS pp × Sc	1.60[c]	6.98[a]
CS pp × Xc	4.82[a]	1.97[e]
CS sb × Sc	3.00[a]	75.70[a]
CS sb × Xc	2.74[b]	3.84[b]
Partial Eta Squared	88.0	99.8
P-value	0.002	0.000

Key:

Xc= *Xanthomonas campestris* CPBF 211, Sc= *Stenotrophomonas maltophilia* IAE127

CS= carbon source, gl= glucose, pp= pineapple peels, sb= sugarcane bargasse.

Appendix P: Yield of biomass and Xanthan gum by bacterial isolates on various Nitrogen sources

	Biomass (g/L)		**Xanthan gum (g/L)**	
Nitrogen source	**XCB**	**SMB**	**XCX**	**SMX**

Yeast extract	2.46^{a} ± 0.277	4.50^{a} ± 0.130	3.22^{a} ± 0.282	3.93^{a} ± 0.300
Peptone	1.09^{b} ± 0.040	2.47^{b} ± 0.266	2.12^{ab} ± 0.115	1.78^{b} ± 0.300
Ammonium sulphate	1.60^{ab} ± 0.115	4.68^{a} ± 0.087	1.98^{ab} ± 0.230	2.58^{ab} ± 0.329
Beef extract	2.20^{a} ± 0.057	3.91^{a} ± 0.017	1.5^{b} ± 0.150	2.54^{ab} ± 0.288

Values are presented as mean ± standard error. Values with the same superscript in the same column are not significantly different at $p<0.05$.

Key:

XCB= *Xanthomonas campestris* CPBF 211 Biomass, SMB= *Stenotrophomonas maltophilia* IAE127 Biomass

XCX= *Xanthomonas campestris* CPBF 211 Xanthan gum, SMX= *Stenotrophomonas maltophilia* IAE127

Xanthan gum

Appendix Q: Interaction effect between isolates and nitrogen sources on biomass and xanthan gum

Interaction effect	Xanthan (g/L)	Biomass (g/L)
AS × Sc	2.58^{b}	4.50^{a}
AS × Xc	3.22^{a}	2.20^{a}
BE × Sc	2.54^{b}	3.91^{a}
BE × Xc	1.98^{d}	1.60^{b}

P × Sc	1.78[e]	2.47[a]
P × Xc	2.12[c]	1.09[c]
YE × Sc	3.93[a]	4.69[a]
YE × Xc	1.51[f]	2.46[a]
Partial Eta Squared	67.4	34.8
p-value	0.024	0.306

Key:

Xc= *Xanthomonas campestris* CPBF 211, Sc= *Stenotrophomonas maltophilia* IAE127

AS= ammonium sulphate, BE= beef extract, p= peptone, YE= yeast extract

Appendix R: Correlations analysis on optimized culture conditions for xanthan gum and biomass production by *Stenotrophomonas maltophilia* IAE127

		SM_xanthan	SM_biomass
SM_xanthan	Pearson Correlation	1	.933**
	Sig. (2-tailed)		.001
	N	8	8
SM_biomass	Pearson Correlation	.933**	1
	Sig. (2-tailed)	.001	
	N	8	8

**. Correlation is highly significant at the 0.01 level.

Appendix R: Correlations analysis on optimized culture conditions for xanthan gum and biomass production by *Xanthomonas campestris* CPBF 211

		Xc_xanthan	Xc_biomass
Xc_xanthan	Pearson Correlation	1	.895**
	Sig. (2-tailed)		.003
	N	8	8
Xc_biomass	Pearson Correlation	.895**	1
	Sig. (2-tailed)	.003	
	N	8	8

**. Correlation is highly significant at the 0.01 level (2-tailed).

Appendix S: Percentage oil recovery from soil by xanthan gum produced by *Xanthomonas campestris* CPBF 211, *Stenotrophomonas maltophilia* IAE127 and commercial xanthan gum

Xanthan gum by:	**Oil recovery (%)**
Xanthomonas campestris CPBF 211	5.0 ± 1.5
Stenotrophomonas maltophilia IAE127	2.7 ± 0.7
Xanthan commercial (control)	8.25 ± 0.8