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EFFECT OF SURFACTANT INCLUSIONS ON THE YIELD AND CHARACTERISTICS OF PROTEASE FROM *BACILLUS SUBTILIS*

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In this study, the effect of surfactants (Tween-80 and Acetonitrile) inclusions in the culture medium of *Bacillus subtilis* on the yield and characteristics of extracellular protease was investigated. The results showed that addition of Tween-80 increased the enzyme yield by 5 folds and thermal stability from 50^{o}C to 60^{o}C. Tween-80 did not affect the optimum pH, KM and VMax values as well as the enzyme efficiency. Acetonitrile shifts the optimum pH from 8 to 7 and reduced the affinity of the enzyme for casein. The selection of appropriate surfactants for *Bacillus subtilis* culture is required for optimum protease production.

Key words: surfactant, protease, yield, characteristics, *Bacillus subtilis*.

INTRODUCTION

Proteases are among the hydrolytic enzymes and are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. Microorganisms have been extensively used as producers of different substances with economic interest such as enzymes, antibiotics, vitamins, amino acids and steroids (Godfrey and West, 2000). Microbes have undermined plants and animals sources of proteases due to their shorter generation time, ease of bulk production, genetic and environmental manipulation to generate new enzyme with altered properties (Rahman *et al.*, 2005, Wang and Yeh, 2006).

Proteases are one of the most vital enzymes, accounting for nearly two-third of the total worldwide industrial enzyme market (Joo *et al.*, 2005, Abidi *et al.*, 2008). They have extensive applications in a range of industrial products and processes including detergent, food, pharma-ceuticals, tannery, waste treatments, resolution of amino acid mixtures, silk and silver extraction from used X-ray films (Cowan, 1996, Rao *et al.*, 1998). Proteases from *Bacillus* sp. origin possess considerable industrial potentials due to their biochemical diversity and wide applications in different industries (Agrawal *et al.*, 2004).

Surfactant are soluble amphiphiles that are surface acting and capable of reducing surface tension or free energy of the reaction medium. They solubilize membrane through disruption of lipid bilayer and through formation of mixed micelles (a close packed complex) with membrane component (Goto and Saxena, 1997) thereby increasing protein extraction from cell into medium. They are classified on the basis of charge of their hydrophilic head portion and the flexibility or chemical nature of the hydrophobic portion. Head groups may be anionic, zwitterionic, nonionic or cationic.

Various reports are available on the simulative effect of nonionic and ionic surfactants in fermentation broth of microorganism, this resulted in many fold increases in the production and secretion of enzyme such as cellulase (Pardo, 1996), phytase (Ebun *et al.*, 1995), amylase (Rahman *et al.*, 2005, Goes *et al.*, 1999), lignase (Jager *et al.*, 1985) and lipase (Mahadike *et al.*, 2002). The present study reports the effect of Tween 80 and Acetonitril inclusion on the yield and properties of protease from *Bacillus subtilis*.

MATERIALS AND METHODS

Microorganism and growth condition

A pure culture of *Bacillus subtilis* was obtained from National Cereal Research Institute (NCRI) Badeggi, Bida, Niger State, Nigeria. The organism was grown on a nutrient agar medium containing 10 g of beef extract, 10 g of peptone, 5 g of sodium chloride for 24 h and sub-cultured in a nutrient broth. Cell growth was monitored at interval of 4hours at wavelength of 420 nm. After 24 h of incubation, the nutrient broth was centrifuged at 4000 rpm for 10 min, crude enzyme activity was assayed in the supernatant.

Assay for enzyme activity

Protease activity was assayed according to method described by Rahman *et al.*, (2005) using casein as substrate. The reaction medium (2.0 ml) containing 100 mM phosphate buffer pH 7.5, casein (15 mg/ml) and 0.5 ml of crude enzyme solution. After incubation at 37^{o} C for 30 min, the reaction was terminated by addition of 5% Trichloroacetic acid (TCA) and the resultant precipitate was removed by centrifugation at 4000 rpm for 20 min. Blanks were prepared for each sample in similar manner except for the addition of TCA to enzyme solution before mixing it with casein. After centrifugation the supernatant was developed with Bradford reagent and read at 580 nm.

Enzyme activity (µg/ml/min) is defined as the amount of enzyme that produced 1.5 µg tyrosine per ml per minutes under the reaction condition.

Determination of pH and Temperature Optima for enzyme activity

The enzyme solution was equilibrated in 0.5 ml of buffer at pH ranges of 6-8 for 15 min and added to substrates at same pH. Enzyme activity was assayed as described above. Enzyme activity *versus* pH plot was used to obtain optimum pH.

The enzyme was incubated at temperatures of 25^{o}–70^{o} C for 15 min while maintaining a constant pH 7.5, an aliquot of the reaction mixture was used for the enzyme activity assay.

Effect of substrate concentration on enzyme activity

The enzyme activity was assayed in reaction medium containing various substrate concentrations ranging from 0.0–0.8 mg/ml of casein substrate. The kinetic constants (K_M and V_{Max}) were estimated by the double reciprocal plots.

Effect of surfactant on growth and enzyme parameters

To measure the effect of surfactants on cell growth and enzyme characteristics, 0.8 ml of Tween-80 or acetonitrile were place into different set of nutrient broths before introduction of *Bacillus subtilis.* The culture was incubated for 24h at 37^{o}C. Microbial cell growth was determined by measuring the absorbance of culture broth at 420 nm. The amount of enzyme yield, effect on temperature and pH optima as well as K_M and V_{max} were determined as described above.

RESULTS AND DISCUSSION

The growth curve of *Bacillus subtilis* grown with or without surfactant is shown in Figure 1. The growth of *Bacillus subtilis* without surfactant followed the expected four phase of growth, showing a lag phase of growth of between 4–8 h, lag phase of 8–16 hours, stationary between 16–20 hours and decline phase 20–24 hours. The inclusion of surfactant increased cell growth and eliminated the lag phase but maintain the peak growth within 10-16 h. Tween-80 increased cell growth by 2.5 fold while acetonitrile increased cell growth by 2.1 fold. Reddy *et al.*, (1999) reported a similar increase in biomass of *Clostridium thermosulfurogenes* SV2 by Tween-80 and Zeng *et al.*, (2006) also observed increase in fungal biomass of *Penicillium simplicissimum* by the same surfactant while Kaczorek *et al*. (2008) have shown that *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida maltose* and *Yarrowia lipolytica can grow well* in natural surfactants. Tween-80, used in this study, is a well-known industrial surfactant; therefore it was thought that its presence in the culture media would affect the homogeneity of the broth and facilitate the nutrient and oxygen up take by the organism. The membrane flux of the organism may also have been increased by the surfactants, further increasing the up take of nutrients and oxygen which may explain the reason for the elimination of lag phase when Tween-80 and acetonitrile were included in the growth medium. The implication of this is that, inclusion of surfactants can shorten the growth period, increase cell growth and hence increasing the yield of useful secondary metabolites.

The enzyme yield in relation to cell growth is shown in Figure 2. Enzyme yield in the presence or absence of surfactants peaked between 12–16 hours, which corresponds to the peak of cell growth (Fig. 1). This observation suggests that inclusion of surfactants may not have affected the biochemistry of enzyme synthesis. Tween-80 and acetonitril increased the yield of enzyme by 5.0 and 4.3 folds respectively. This result agrees with the findings of Kamande *et al.*, (1999), who reported increase in protease activity by Tween-60 and Tween-80 on ruminant microorganism. Mabrouk *et al.*, (1999) and Reddy *et al*., 2008) reported increased stability and yield of protease by nonionic surfactant Tween-20 and Triton X-100 on *Bacillus* sp.

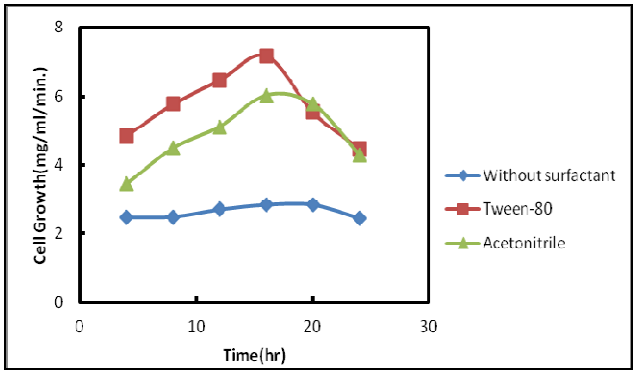

Fig. 1. Cell growth with or without surfactants.

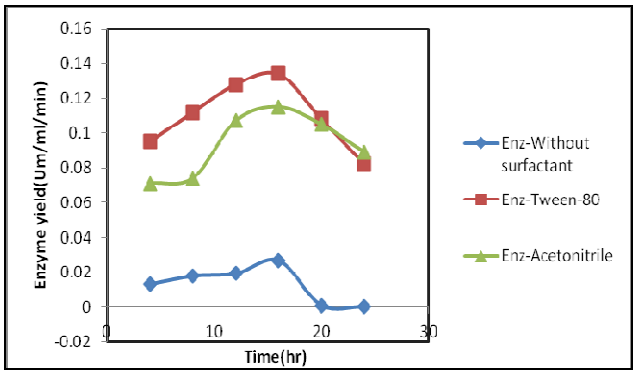

Fig. 2. Enzime yield with period of cell growth.

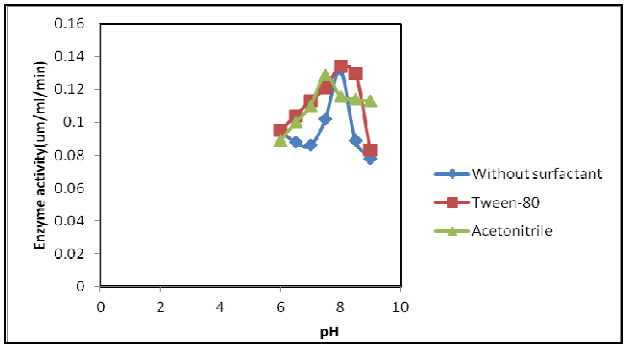

Fig. 3. pH profile of protease *Bacillus* spp grown with or without surfactants.

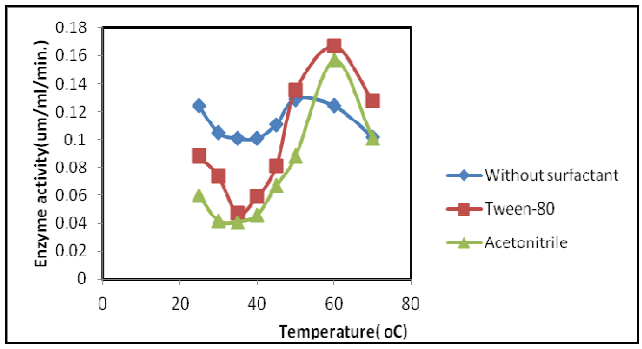

Fig. 4. Temperature profile of *Bacillus* spp grown with or without surfactants.

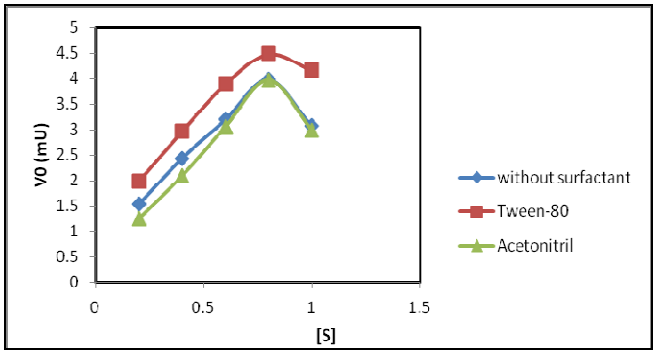

Fig. 5. Substrate-Activity for protease with or without surfactants.

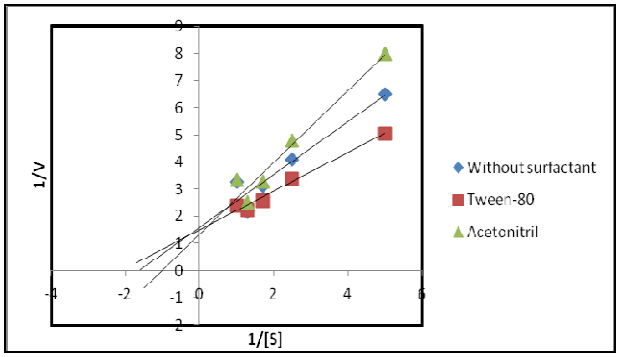

Fig. 6. Lineweaver-Burk Plot for Km amd Vmax.

Incorporation of Tween-80 into various fermentation medium have shown to enhanced enzyme production and secretion (Guang *et al.*, 2006). Therefore the surfactants used in this study may have improved the permeability of the cell membrane through disruption of lipid bilayer (Goto and Saxena, 1997), thereby increasing the uptake of nutrient into the organism and the secretion of enzyme into the culture medium. The amphipathicity of the surfactant may also play a role in exposing the active sites available for enzyme substrate hydrophobic interaction (Triggle, 1970).

The pH profile of protease activity with or without surfactants is shown in Figure 3. The optimum pH of protease activity without surfactant was found to be at 8.0, while that of Tween-80 and acetonitrile were at 8.0 and 7.0 respectively. It has been reported that proteases secreted by *Bacillus* sp. presented activity at a wide range of pH (7.0 to 11.0) (Gupta *et al.*, 2002, Joo *et al.*, 2003).The pH optima observed in the present study agrees with the expected range and specifically, with the findings of Fikret (2011) who reported a pH of 8 for *Bacillus cereus* grown in different surfactants. Tween-80 is a non-ionic surfactant, therefore, it is not surprising that it did not alter the pH of the protease compared to the pH of the protease from the medium without surfactant. However,

acetonotrile, an ionic surfactant, with dielectric constant of 3.92D, reduced the pH for optimum enzyme activity to 7. The pH ranges observed in this study agrees with earlier reports of alkaline protease from *Bacillus* spp (Rahman, *et al.*, 2005; Abidi *et al.*, 2008; Oyeleke *et al*., 2011). The observation in the present study suggests that inclusion of Tween-80 into a culture medium can increase extracellular enzyme yield without altering the enzyme property.

The temperature profile of protease activity with or without surfactant is shown in Figure 4. The optimum temperature of protease activity without surfactant was found to be 50^{o}C. This finding agrees with the finding of Udandi and, Rajendran (2009) and Oyeleke *et at.*, (2011) who have reported optimum temperature of 50^{o} C for proteases produced from *Bacillus* spp. Growing organism in culture containing Tween-80 and acetonitrile shifted the temperature for optimum protease activity to 60^{o}C. This observation suggests that surfactant inclusion could be a means of achieving enzyme thermostability at higher temperatures. Sanjay *et al*., (2011) have shown that alkaline protease stable in Tween-80 and other surfactants is an indication that the protease could be used in commercial laundry detergent. It therefore suggests that growing *Bacillus* spp. in a culture containing surfactant may confer to the resulting protease a better industrial usefulness like application in commercial laundry detergent.

A plot of enzyme activity against substrate concentration (Fig. 5) showed the expected Michealis-Menten's curve. The Line -Weaverbulk's plot in Figure 6, reveals that the K_M and V_{Max} values and enzyme efficiency of protease without surfactant (0.575 µgml^{-1}, 0.618 µgmin^{-1} and 2.82) is similar to that with Tween-80 (0.502 µgml^{-1}, 0.675 µgmin^{-1} and 2.95). This finding further supports the fact that Tween-80 does not affect the biochemistry of the enzyme but may increase the yield by increasing nutrient and membrane flux. The kinetic parameters observed in the present study agree with the report of Shubba *et al.*, (2009) showing K_M and V_{Max} values of *0.597* mgmil^{-1} *and 13825* µmolmin^{-1} respectively for protease from *Bacillus circulans*, Acetonitrile reduced enzyme affinity for casein with increased K_M and V_{Max} values of 1.161 µgml^{-1}and 0.743 µgmin^{-1} respectively. The deviation observed with acetonitrile further suggests the protonation of the enzyme thereby changing the biochemistry of the enzyme. Therefore the selection of surfactant type may be important in the use of surfactant to increase the yield of enzyme from microbial culture without altering the native enzyme properties.

CONCLUSIONS AND FUTURE PROSPECTS

The inclusion of Tween-80 into *Bacillus subtilis* culture increases the yield of protease enzyme without changing the basic characteristics of the enzyme. Tween-80 may also improve the thermal stability of the enzyme thereby improving the industrial usefulness.

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