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Application of Polyurethane Foam Matrix as Immobilizer in the Production of Thermostable Alkaline Protease by *Bacillus subtilis* Isolated from Barbecue Spots

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Abstract: Polyurethane foam (PUFoam) was used as an immobilization platform for the production of alkaline protease by *Bacillus subtilis* isolated from barbecue spots. Five *B. subtilis* isolates were subjected to alkaline protease assay. Among the isolates, *B. subtilis* IK17 exhibited highest alkaline protease production in a 48 h single batch (peak value, 20.3 AU mL^{-1}) at 40 h incubation time and was selected for immobilization studies. Effects of PUFoam volume, density and treatment in gluteraldehyde concentrations on enzyme yield were assessed. Increase in volume resulted in increased enzyme yield as volume of 3.375 cm^3 yielded a peak enzyme value of 27.2 AU mL^{-1}; 1 cm^3 foam volume led to peak enzyme value of 24.1 AU mL^{-1} while 0.125 cm^3 gave a peak value of 23.8 AU mL^{-1} at 48 h. Higher foam density led to a reduced enzyme activity as peak values obtained at 48 h incubation showed 2 g cm^{-3} = 23.8 AU mL^{-1}; 1 g m^{-3} = 24.2 AU mL^{-1} and 0.5 g cm^{-3} = 28.22 AU mL^{-1}. Effects of concentrations of gluteraldehyde exhibited maximum activity of 26 AU mL^{-1} for 5% gluteraldehyde followed by 25.4 AU mL^{-1} for 1% gluteraldehyde and 21.6 AU mL^{-1} for 10% gluteraldehyde. Up to 10 repeat cycles were conducted for enzyme production using immobilized and free cells of *B. subtilis* IK17 with free cells performing in only 2 cycles with peak total titre value of 100 AU mL^{-1} during the 1st cycle while immobilized cells produced enzyme for up to 9 cycles with peak total titer value of 200 AU mL^{-1} during the 4th cycle. The PUFoam cell leakage assessments showed that as concentration of gluteraldehyde was reduced, it resulted in higher cell leakage. Higher cell leakage was also observed in higher densities of foam while lower foam volumes resulted in higher cell leakage.

Key words: Protease, polyurethane foam matrix, *Bacillus subtilis,* immobilization

INTRODUCTION

Proteases are enzymes involved in the catalytic cleavage of peptide bonds in proteins (Godfrey and West, 1996) and are a major class of enzymes applied in various industrial processes. Their commercial viability is basically because of the physiological, degradative and biosynthetic roles and they play in the production of certain important materials in human demand. Proteases account for a huge share of the global enzyme market (Rao *et al.*, 1998) and their applications in leather processing, detergent production, meat processing, pharmaceutical formulations, peptide synthesis for infant formulas, preparation of organic fertilizers, silk industry and bioremediation processes among others have made them highly valuable (Gupta *et al.*, 2005; Naidu and Devi, 2010).

Proteases are ubiquitously occurring and can be of plant, animal or microbial origin. This is due to the abundance of protein based substrates in nature. Based on the industrial demand of proteases, there is gross inability of animal and plant protease sources to meet such requirements, thus microbial proteases are preferred (Gupta *et al.*, 2002). Microorganisms and their enzymes are also easy to manipulate with faster production rate leading to reduced cost of production, thus making them ideal candidates for biotechnological enhancements (Rao *et al.*, 1998).

Alkaline proteases are a unique class of proteases because of their characteristic high hydrogen ion concentration signature and tolerability. This property also intertwines with their broad substrate specificity and high optimal temperature, making them ideal protease candidates for application in the detergent industry (Rao *et al.*, 1998). In present times, the utilization of alkaline protease has markedly increased with large quantities derived from *Bacillus* strains (El Enshasy *et al.*, 2008; Naidu *et al.*, 2011). *Bacillus* species are industrially identified as ideal protease and alkaline protease producers due to factors such as their lack of

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pathogenicity, ease of growth, adaptability and manipulation in simple media (Maurer, 2004; Joshi, 2010).

In a bid to harness, the inherent qualities of *Bacillus* species in alkaline protease production, various bioprocessing mechanisms that are both engineering and microbiology based have been designed. Biotechnological studies, assessments and improvement methods that have veered into the genetics of the organism have also been gaining indepth interests globally (Rao *et al.*, 1998; Naidu *et al.*, 2011). The commercial viability of many of these new methods, however remains to be evaluated by quantitative and qualitative assessments of yield. Some models of obtaining increased enzyme yields, however have employed the improvement and alteration of fermentation states, cell immobilization techniques, repeated batch and continuous culture systems and improvement of nutrient formulation. Cell immobilization techniques have been easily adopted for enzyme production and production of various other metabolic products of microorganisms (Adinarayana *et al.*, 2004; Martins *et al.*, 2013). Cell immobilization is important due to its operational stability, ease of separation from bulk liquid for onward reduced biomass loss, recycling and continuity in operation (Helmo *et al.*, 1985). Modifications and increases in chemical, thermal and mechanical resistances relative to the method and material used have also been observed in cells that were immobilized (Naidu *et al.*, 2011), thus increasing their activity level and operational functionality in industrial enzyme production. Immobilization methods include adsorption unto substances, entrapment in matrices and covalent bonding with desired material. Gel-like matrices have been employed in immobilizing cells; examples of such include alginate, gelatin, polyacrilamide and agar (De Araujo and Santana, 1996; Kumar and Vats, 2010). Based on chemical bonding mechanisms, simple covalent bonds can be formed between whole cells and external inorganic solid platform as a method of cell immobilization. Solid carriers like wool and stainless steel have been utilized in alkaline protease production by *Bacillus* spp. (Ahmed and Abdel-Fattah, 2010). The use of polyurethane which is a synthetic polymer in solid form for the production of alkaline protease by *Bacillus subtilis* is to be assessed in this study.

MATERIALS AND METHODS

Bacterial isolation and characterization: Soil samples from 25 different local barbecue spots within the city of Lagos, Nigeria were collected. The areas in Lagos where the samples were collected were Lekki, Ikeja, Agege, Mushin and Victoria Island. The collection was done by scraping the earth in and around the barbecue fireplaces. Serial dilution was carried out and the samples were plated

on Nutrient Agar. Pure cultures were obtained and colonies were examined for *Bacillus* with standard descriptions of Bergey's Manual of Determinative Bacteriology (Bergey *et al.*, 1974) and Cowan and Steel's Manual for identification of medical bacteria (Barrow and Feltham, 1993). Identification and characterization was based on morphology, spore shape and formation, growth in 10% sodium chloride, oxygen requirement, carbohydrate and sugar fermentation tests as well as biochemical characteristics. Species identified as *Bacillus subtilis* were maintained on nutrient agar slants at 4°C.

Preliminary screening for protease production: The *Bacillus subtilis* isolates were screened for extracellular protease production by inoculating in skim milk agar (pH 10) at 37°C for 48 h. A clear zone of hydrolysis around colonies due to proteolytic digestion indicated protease production by identified isolates. The isolates were stored in slants and promising proteolytic bacilli were selected for further assay and alkaline protease production.

Inoculum preparation: Each isolate (stock culture) was aseptically transferred in two loopfuls into 5 mL of freshly prepared nutrient broth. This was kept at room temperature for 10-12 h before being transferred into 250 mL Erlenmeyer flasks containing 50 mL of inoculum medium. The inoculum medium was prepared with slight modifications according to Kumar and Vats (2010) and was composed (g L^{-1}) of: glucose 2.0; casein, 0.5; peptone, 0.5; yeast extract, 0.5 and 50 m L^{-1} salt solution (containing, g L^{-1}-KH_2PO_4, 5.0; $MgSO_4.7H_2O$, 5.0 and $FeSO_4.7H_2O$, 0.1). The pH was maintained at 7.0 for the inoculum medium and the flask was placed in a shaker at 100 rpm at 37°C for 20 min. The contents of the flask were centrifuged at 4000 rpm for 7 min and the supernatant was decanted. Thereafter, the cell mass (pellets) was subjected to thorough washing using sterile double distilled water. The resulting cell mass was then suspended in sterile saline solution (10 g L^{-1}) and was used as the source of inoculum for all the experiments.

Production of alkaline protease: A single batch process for enzyme production was adopted. The production was carried out in a production medium (Naidu *et al.*, 2011) composed (g L^{-1}) of glucose 5, peptone 7.5 and 5% salt solution (5 g L^{-1}, $MgSO_4.7 H_2O$; 5 g L^{-1} KH_2PO_4; 0.1 g L^{-1} $FeSO_4.7 H_2O$) with an alkaline pH of 10. The set up was incubated at 37°C for 36 h and at 4 hourly intervals, sample was withdrawn for alkaline protease assay.

Alkaline protease assay: Alkaline protease activity was assessed by caseinolysis of the substrate by crude extracts of the enzyme according to the modified method of Leighton *et al.* (1973) and Tsuchida *et al.* (1986). The

2 mL of crude enzyme was obtained by centrifugation of the fermentation broth at 4000 rpm for 20 min. The crude extracts were filtered using 0.45 μm syringe filter in test tubes and added to the 10 mL of the substrate (2% w/v casein+0.1 M Tris/HCL buffer solution maintained at pH 10). The mixture was swirled and incubated at 37°C for 30 min. Subsequently, 5 mL of trichloroacetic acid (10%v/v) was added as stop solution and the absorbance was read at 420 nm. For standardization, 1.1 mM of tyrosine (100 μg mL^{-1}) was prepared as stock solution and aliquots in the ranging from 5-100 μg were read spectrophotometrically at 420 nm and subsequently interpreted as a standard tyrosine concentration curve plotted based on the absorbance (Kumar and Vats, 2010). Enzyme activity was observed and one activity unit was defined as the amount of enzyme that released 1 μg of tyrosine released from 1 mL crude enzyme in 1 min incubation.

Conditioning of polyurethane foam immobilization platform: Effects of foam density, volume and curing in gluteraldehyde concentrations: Commercially available polyurethane foam (PUF) platforms were obtained. The polymers were cut into cubes of measured volumes (3.375, 1 and 0.125 cm^3) and their densities (2, 1 and 0.5 g cm^{-3}) were determined. The cut cubes were pre-treated by washing with double distilled water, boiled for 20 min at 100°C and cured in gluteraldehyde. Varied concentrations of gluteraldehyde (10, 5 and 1%) were applied and its effects on enzyme yield were determined. To determine the effects of foam density on enzyme yield, the different density values were assessed in constant volume (1 cm^3) and gluteraldehyde concentration (5%). To determine the effects of foam volume on enzyme yield, the different volume values were assessed in constant density (1 g cm^{-3}) and gluteraldehyde concentration (5%). To determine the effects of gluteraldehyde on enzyme yield, the different concentrations of gluteraldehyde were assessed in constant density (1 g cm^{-3}) and volume (1 cm^3). All experiments to determine the effects of polyurethane conditions were assessed in single batch fermentations while the concentration of gluteraldehyde, density of foam and volume of foam with best effect on alkaline protease production was used for enzyme production in repeated batch fermentations. The volumes and densities of the polymer cubes were calculated as follows:

$$\text{Volume (cm}^3) = \text{Length (cm)} \times \text{breadth (cm)} \times \text{height (cm)}$$

$$\text{Density (g cm}^{-3}) = \frac{\text{Mass (g)}}{\text{Volume (cm}^3)}$$

Immobilisation by covalent bonding: Initially prepared cell mass suspended in sodium chloride solution (10 g L^{-1})

was used. Cell pastes were removed after centrifugation, dried and weighed under a laminar flow chamber. Specifically, 0.03 g of dry cell mass was measured and added to 10 mL sterile distilled water (Adinarayana *et al.*, 2005). The conditioned polymer cubes were added to sterile 0.1 M phosphate buffer (pH 7.0) together with the cell suspension and mixed thoroughly. The same numbers of the cubes (20 cubes) were used throughout the study.

Batch and repeated batch production of alkaline protease on immobilized polymers: For batch production, the polymer cubes carrying the cells were aseptically transferred into 50 mL of production medium in flasks (Naidu *et al.*, 2011), incubated at 37°C for 48 h. At regular time intervals of 4 h each, alkaline protease was assayed. Repeated batch culture production was also done using 50 mL production medium. In re-cycling, the cell laden cubes were filtered and washed in 30 mL, 10% saline, before re-introduction into the production medium. Samples were withdrawn at hourly intervals of 4 h from each of the flasks and enzyme activity was measured. The process was repeated until maximum cell leakage was determined.

Cell leakage: Biomass leakages from the polymer matrix were determined by measuring the optical densities of the media broth at 600 nm and correlating it with cell mass (Kumar and Vats, 2010). A biomass standard curve was plotted by introducing aliquots of weighed cells from the range of 50-300 mg mL^{-1} into a liquid medium and then spectrophotometrically determining its absorbance. Effects of various concentrations of gluteraldehyde on binding or leakage of cells were also determined. Density and volume based polyurethane foam cell leakage were also ascertained. The corresponding absorbance was converted to μg of cell leakage from matrix based on the biomass standard curve and subsequently converted to percentages with values from cell free foam matrix as the control and maxima percentage limit.

Effects of temperature on enzyme activity: To characterize the thermal stability of alkaline protease from *B. subtilis* produced by the best immobilization parameters, the effects of various temperatures (30, 40, 50, 60, 70 and 80°C) on enzyme activity was measured during the assay according to the procedure of Akcan and Uyar (2011). Relative enzyme activity recorded for the different temperatures was determined in percentage ratio to the optimum temperature measured.

RESULTS

A total of thirty six bacterial isolates were obtained from the various barbecue spots in Lagos (Agege, Lekki, Ikeja, Mushin and Victoria Island). After morphological

and physiological characterization, five *Bacillus subtilis* isolates were identified (Table 1). After characterization of isolates, *B. subtilis* AG06, *B. subtilis* LK34, *Bacillus subtilis* IK17, *B. subtilis* MS13 and *B. subtilis* VI03 isolates from barbecue sites in Agege, Lekki, Ikeja, Mushin and Victoria Island areas of Lagos, respectively were identified. *Basillus subtilis* IK17 was observed to be the highest alkaline protease producing isolate with peak value of 20.3 AU mL^{-1} at 48 h incubation time and was selected for immobilization and further enzyme production studies (Fig. 1). Results of the effects of PUFoam volume on enzyme yield (Fig. 2) revealed that enzyme titre up to 27.2 AU mL^{-1} was obtained when 3.375 cm^3 PUFoam volume was used. The PUF volume 1 cm^3 led to a peak enzyme yield of 24.1 AU mL^{-1} and a value of 23.8 AU mL^{-1} was obtained for 0.125 cm^3 volume of foam. Fig. 3 shows the effects of PUFoam density values on enzyme yield. The PUFaom density 2 g cm^{-3} led to

enzyme maxima of 23.8 AU mL^{-1}; the density of 1 g cm^{-3} resulted in maxima of 24.2 AU mL^{-1} while 0.5 g cm^{-3} yielded maxima of 28.22 AU mL^{-1}. Application of different concentrations of gluteraldehyde as a spacer/binder for cell immobilization was assessed and results (Fig. 4)

Fig. 2: Alkaline protease production by *B. subtilis* IK17 immobilised in different volumes of PUFoam cubes

Table 1: Morphological and physiological characteristics of alkaline protease producing *B. subtilis* isolates

Morphological and physiological characteristics	Results
Gram-staining	Positive
Cell shape	Bacillus
Endospore presence	Positive
Endospore shape	Ellipsoidal
Voges-Proskauer test	Negative
Growth temperature range	5-55°C
pH growth range	5.0-8.0
Anaerobic growth	Negative
Catalase test	Positive
Indole test	Negative
Urease test	Negative
Oxidase test	Negative
Motility	Positive
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Nitrate reduction test	Positive
NaCl tolerance	≤10% (w/v)
Gelatin hydrolysis	Positive
Sugar fermentation tests	
Glucose	Positive
Arabinose	Positive
Mannitol	Positive
Xylose	Positive

Fig. 3: Alkaline protease production by *B. subtilis* IK17 immobilised in different densities of PUFoam cubes
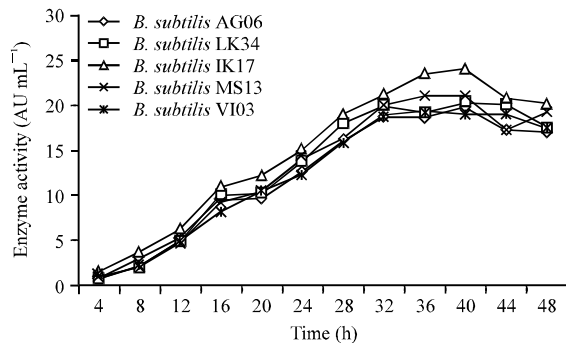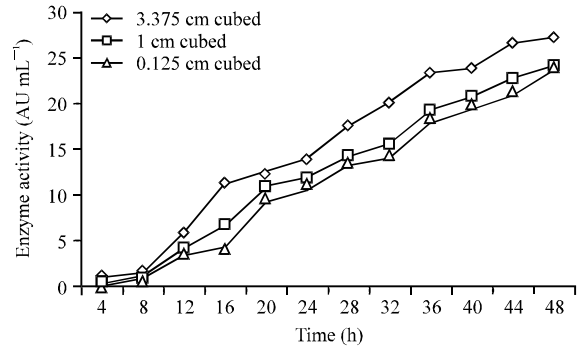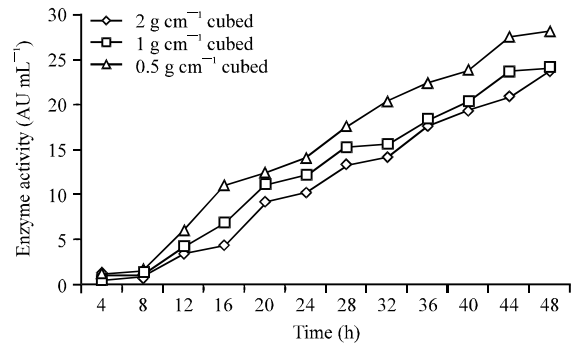

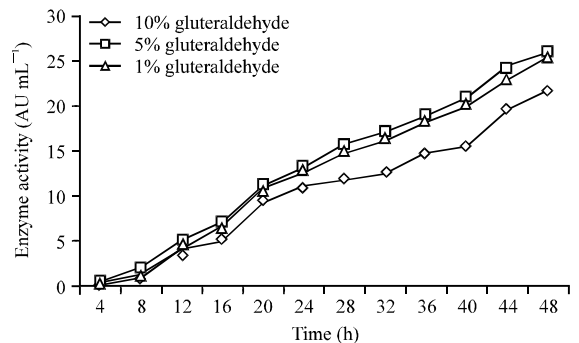Fig. 1: Alkaline protease production by different *B. subtilis* isolates from barbecue spots

Fig. 4: Alkaline protease production by *B. subtilis* IK17 immobilised in PUFoam matrix cured with different concentrations of gluteraldehyde
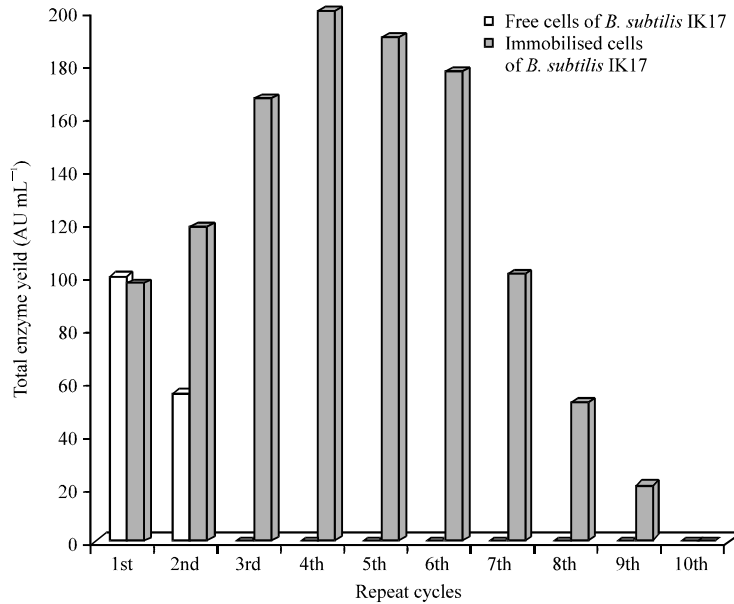
Fig. 5: Enzyme yield for repeated cycles of immobilized and free cells of *B. subtilis* IK17
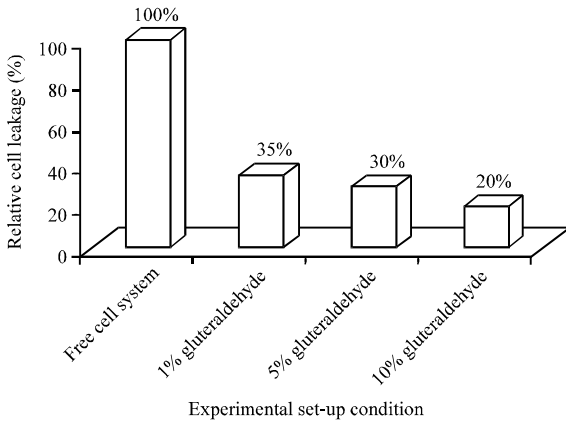

Fig. 6: Percentage cell leakage assessment of *B. subtilis* IK17 immobilised in PUFoam treated with different concentrations of gluteraldehyde
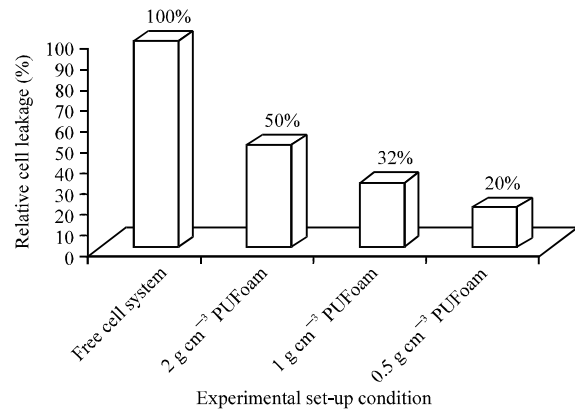

Fig. 7: Percentage cell leakage of *B. subtilis* IK17 immobilised in different densities of PUFoam

show that 10% gluteraldehyde, 5% gluteraldehyde and 1% gluteraldehyde resulted in maxima yields of 21.6, 26 and 25.4 AU mL^{-1}, respectively. The recyclability of immobilized cells in comparison with free cells was determined and the immobilized cells were recycled for up to 9 times as compared to the free cells that performed in only 2 cycles (Fig. 5). The maxima value for total enzyme titre for free cells was 100 AU mL^{-1} as obtained after the 1st cycle while maxima value for total enzyme titre for immobilized cells was 200 AU mL^{-1} as obtained after the 4th cycle. Cell leakage assessment for the various

concentrations of gluteraldehyde applied as binding agent on PUFoam (Fig. 6) showed that 10, 5 and 1 gluteraldehyde concentrations of treatment effected 20, 30 and 35% cell leakages, respectively. Figure 7 showed the effects of density values of PUFoam on cell leakage. Density values of 2, 1 and 0.5 g cm^{-3} resulted in 50, 32 and 20% cell leakages. Varied volumes of PUFoam 3.375, 1 and 0.125 cm^3 showed percentage cell leakages as 20, 35 and 65%, respectively (Fig. 8). Figure 9 shows that alkaline protease produced by *B. subtilis* IK17 was active between a temperature range of 30-80°C with optimum activity observed at 60°C.
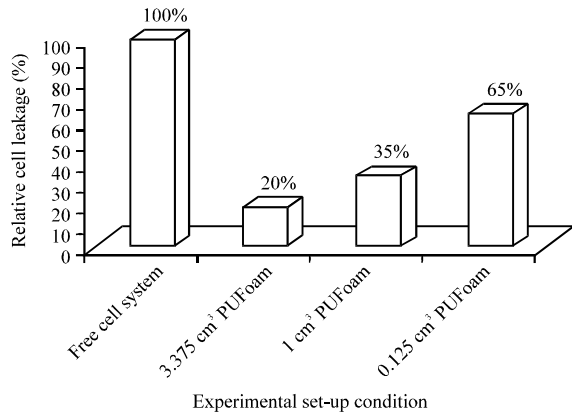
Fig. 8: Percentage cell leakage of *B. subtilis* IK17 immobilised in different volumes of PUFoam
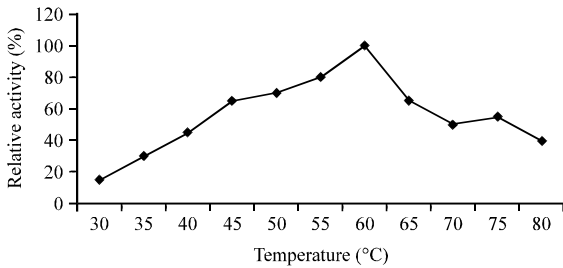

Fig. 9: Effect of temperature on activity of *B. subtilis* IK17 alkaline protease

DISCUSSION

After isolation and characterization of isolates, *B. subtilis* AG06, *B. subtilis* LK34, *B. subtilis* IK17, *B. subtilis* MS13 and *B. subtilis* VI03 isolates from barbecue sites in Agege, Lekki, Ikeja, Mushin and Victoria Island areas of Lagos, respectively were identified.

Among the five isolates, *B. subtilis* IK17 an isolate obtained from a local and very active barbecue site in the densely populated center of Ikeja, Lagos; best expressed alkaline protease production. The isolate was selected among other isolates for immobilization application. Production of alkaline protease by *B. subtilis* has been reported by authors (Sen and Satyanarayana, 1993; Singh *et al.*, 1999; Ahmed and Abdel-Fattah, 2010), thus underscoring the need for improvement in production methods and processes. However, the consideration of thermal stability of alkaline protease produced and its effect on its activity was valued based on the inductively acquired environment-based traits peculiar with the site of isolation. Thermal stability of the protease produced goes a long way in affecting its industrial relevance given the characteristic high temperature signatures of industrial

processes. Theoretically, *Bacillus* species with the ability to produce alkaline protease have to be able to respond favourably to characteristically high pH and temperature limits (Rao *et al.*, 1998). Thus, enzyme production can be induced within these physiological parameters. This informed the choice of isolating alkaline protease producing *B. subtilis* from barbecue spots in Lagos, a densely populated state in Nigeria in correlation with the ever busy barbecue production at such sites and the unique high temperature conditions applied during the meat processing.

Immobilisation of *B. subtilis* IK17 was carried out using polyurethane foam (PUFoam) matrix. Similarly, immobilization experiments for production of protease by *Bacillus* spp. have been reported (Adinarayana *et al.*, 2004) using gel-like matrices. However, these matrices can be easily dissolved over time leading to a gradual cell wash-out and also can be prone to disintegration when subjected to high temperatures. The PUFoam matrix was applied in microbial enzyme production as reviewed by Romaskevic *et al.* (2006). Specifically, Prasad *et al.* (2006) applied PUFoam matrix to obtain increased yield of laccase by *Pleurotus ostreatus* 1804. Martins *et al.* (2013) stated observed advantages of polyurethane based matrix over conventionally applied gel-like substances to include reduced biodegradability, stability and better mechanical performance. In this study, a synthetic solid polymer matrix (polyurethane foam) was used in bacterial protease production with *Bacillus* spp. with a need to obtain higher yield as well as enzyme stability at high pH and temperature values.

Immobilisation studies revealed that PUFaom volume, density and binding chemical (gluteraldehyde) application affected enzyme values. Increased matrix volume led to an increase in production with maxima of 27.2 AU mL^{-1} for 3.375 cm^3 at 48 h of production while reduced volume (1 cm^3, 0.125 cm^3) resulted in lower yields. Theoretically, this can be attributed to the surface area ratio of the polymer matrix. Increased internal surface area can directly lead to higher cell mass binding and subsequent enzyme production. The resultant effect of density increase was however, the opposite with maxima limits of 28.22 AU mL^{-1} (Fig. 3) obtained for foam density value of 0.5 g cm^{-3} and reduced yield of 24.2 AU mL^{-1} and 23.8 AU mL^{-1} for 1 and 2 g cm^{-3}, respectively. This could similarly be as a result of reduced internal surface ratio as a higher matrix density resulted in reduced interstitial pore size within the polymer, thus resulting in the accommodation of reduced biomass. The effect of gluteraldehyde was however, not similar to gradient-like results obtained for matrix density and volume as gluteraldehyde concentration of 5% performed slightly

above 1 and 10% concentrations. A maxima value of 26.5 AU mL^{-1} was obtained for 5% concentration of gluteraldehyde and proved to be the most suitable concentration value to be adopted for immobilization studies. Naidu *et al.* (2011) also applied a gluteraldehyde value of 5% on immobilization experiments using agar-agar gel matrix.

As enzyme is being produced within an immobilized system, increased protein crowding within the interstices of the carrier material, which might hamper proper cell-substrate contact as well as conformational changes required for optimum catalysis (Kumar and Vats, 2010). Gluteraldehyde was however proposed as a binder for increased biomass activity as it led to more efficient bonding of cells to carrier platform. This occurs as gluteraldehyde acts as a spacer unit thus changing the local surface area with a resultant effect on increased biomass bonding and reduced protein crowding (Krastanov, 1997). Despite this fact, higher concentrations of gluteraldehyde can be inhibitory to microbial life subsequently affecting enzyme production (Russell, 1994; Maris, 1995). This was evident in reduced enzyme yield when increase in concentration (to 10% gluteraldehyde) was applied. From results obtained, the final combination of PUFoam parameters that was used in the recycle studies with immobilized cells was 5% gluteraldehyde, 0.5 g cm^{-3} density and 3.375 cm^3 volume.

The use of PUFoam afforded the opportunity to recycle the system in repeated batch production for up to 10 batches. In comparison with the free cells that were effective in enzyme production for just 2 batches, the immobilized cells were functional for up to 9 batches. The peak total enzyme yield with respect to cycles was obtained at the 1st cycle for free cells (100 AU mL^{-1}) and 4th cycle for immobilized cells (200 AU mL^{-1}). This proves that immobilization of cells on polyurethane foam matrix was more advantageous for continuous production and subsequent increased enzyme yield when compared with free cells. This is in correlation with the work carried out by Prasad *et al.* (2006).

Cell leakage efficiency of foam immobilization with respect to the different parameters was determined relative to the free cells. Concentrations of gluteraldehyde applied as spacing agent affected cell leakage/loss. It was observed that increased concentrations had better efficiency against cell loss, as lower percentage cell leakage was recorded when gluteraldehyde concentration was increased. In the case of density, reduced density was more effective against cell leakage than increased foam density. Foams of higher volume were more efficient as they had lower cell leakage than foams of lower volume. Results obtained from cell leakage/loss assessment proved that higher internal surface areas

evident in higher foam volume and lower density positively affected cell holding capacity of PUFoam. Increased concentration of gluteraldehyde treatment on PUFoam also aided proper binding of cells subsequently leading to reduced cell loss.

In agreement with reports by Rao *et al.* (1998), the alkaline protease produced by *B. subtilis* IK17 was thermotolerant and active between a temperature range of 30-80°C with optimum activity observed at 60°C, thus making it an effective candidate for industrial application.

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