Vol.1, No.4, 112-120 (2013)
http://dx.doi.org/10.4236/aer.2013.14012

Optimized production and properties of thermostable alkaline protease from *Bacillus subtilis* SHS-04 grown on groundnut (*Arachis hypogaea*) meal

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Received 10 July 2013; revised 19 August 2013; accepted 5 September 2013

ABSTRACT

Production of alkaline protease from *Bacillus subtilis* SHS-04 was investigated under different fermentation conditions involving low-cost substrates with the aim of optimizing yield of enzyme. Maximum enzyme production (1616.21 U/mL) was achieved using groundnut meal (0.75%) as nitrogen source and 0.5% glucose as carbon source at 48 h cultivation period, pH 9, 45˚C and 200 rpm. The yield was 348% increase over comparable control samples. The alkaline protease had optimum temperature of 60˚C and remarkably exhibited 80% relative activity at 70˚C. It was highly thermostable showing 98.7% residual activity at 60˚C after 60 minutes of incubation at pH 9.0 and was stable in the presence of organic solvents studied. These properties indicate the viability of the protease for biotechnological and industrial applications. The optimized yield of enzyme achieved in this study establishes groundnut meal as potential low-cost substrate for alkaline protease production by *B. subtilis* SHS-04.

Keywords: Alkaline Protease; *Bacillus subtilis* SHS-04; Groundnut (*Arachis hypogaea*) Meal; Low-Cost Substrate; Thermostable

1. INTRODUCTION

Proteases constitute one of the most important groups of global industrial enzymes accounting for about 60% of the total enzyme sales [1]. Among the different types of proteases, alkaline proteases have wide applications in the detergent, leather and pharmaceutical industries [2]. Bacterial proteases are the most significant, compared with plants, animal and fungal proteases because they are mostly extracellular and easily produced in large amount, but their usefulness is limited by various factors, such as instability at high temperatures, extreme pH, the presence of organic solvents and the need for co-factors [3]. The present known proteases are not sufficient to meet industrial demands hence, there is a continuous search for new proteases with novel characteristics for industrial applications from diverse bacteria isolates.

Enzyme production by any organism is influenced by many factors such as pH, incubation temperature, incubation time, growth rate of culture and medium composition [4]. Currently, the overall cost of enzyme production is high and the growth medium accounts for about 30% - 40% of the production cost [5]. Reducing the cost of enzyme production by optimization of fermentation medium and process parameters is the major goal of basic research for industrial applications [6]. To this extent, the search for inexpensive carbon and nitrogen sources such as agricultural residues and marine by-products for use in media composition for protease production has been continuous.

Maximum protease synthesis by alkalophilic *Bacillus sp*. I-312 was obtained when the bacterium was grown in a medium containing wheat flour and soybean meal as carbon and nitrogen sources [7]. *Bacillus licheniformis* RP1 was shown to produce proteases when grown in media containing shrimp wastes powder as a sole carbon and nitrogen source [8]. *Mirabilis jalapa* tuber powder (MJTP) was used as organic substrate for the growth and production of fibrinolytic serine protease by *Bacillus amyloliquefaciens* An6 [9].

Groundnut (*Arachis hypogaea*) is a species in the legume family, *Fabaceae*, native to South America and cheaply cultivated in African, Asian and Arab countries [10]. Groundnut provides an inexpensive source of high quality dietary protein and oil. Groundnut seed contains

25% to 28% protein and 46% to 51% oil on a dry seed basis and it is a rich source of minerals (phosphorus, calcium, magnesium, and potassium) and vitamins (E, K, and B group) [11-13]. The proximate analysis and nutriational value of groundnut suggest groundnut meal as potential low-cost substrate in media composition for microbial growth and enzyme production. In this study, effects of low-cost substrates including groundnut meal and process parameters were investigated on protease production and properties of the crude enzyme were determined.

2. MATERIALS AND METHODS

2.1. Materials

Media components were products of Sigma-Aldrich (St Louis, MO, USA) except groundnut seeds, soybean meal, yam (*Dioscorea alata*) flour and locust bean meal which were purchased from the local market and processed using standard sieve. Groundnut meal was prepared by sun drying fresh groundnut seeds for two weeks. The dried groundnut seeds were pulverized and stored in glass bottles at room temperature. All other chemicals used were of analytical grade and obtained from Fisher Scientific.

2.2. Bacterial Strain

The microorganism used in this study was an alkaliphilic bacterium isolated from the soil of a slaughterhouse. Samples collected were plated onto skim-milk agar plates and were incubated 24 h at 37˚C. A clear zone of skim-milk hydrolysis gave an indication of protease-producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Depending upon the zone of clearance, isolate SHS-04 was selected for further experimental studies. This isolate was identified as *B. subtilis* in the Biotechnology Unit of the Federal Institute of Industrial Research, Lagos, Nigeria based on methods described in Bergey's Manual of Systematic Bacteriology [14] and maintained on nutrient agar slants stored at 4˚C.

2.3. Submerged Fermentation

Seed inoculum was prepared by growing a loopful of slant culture of *B. subtilis* SHS-04 in 20 mL of seed medium containing 0.75% (w/v) peptone, 0.5% (w/v) glucose, 0.05% (w/v) NaCl and 0.01% (w/v) $MgSO_4.7H_2O$ in a 200 mL conical flask with pH adjusted to 8.0. The inoculated seed medium was incubated at 37˚C for 24 h at 180 rpm in a shaking incubator (Stuart, UK). The 24 h old seed culture was used as inoculum for the production media. 2.5 mL of seed inoculum (constituting 5% v/v) was transferred into 50 mL of production media which had same composition as the seed medium. At the end of 48 h incubation period, cultures were harvested by centrifugation at 10,000 rpm for 15 min at 4˚C. The cell free supernatant was recovered as crude enzyme preparation and used for further studies.

2.4. Assay of Protease Activity

Protease activity was determined by a modified procedure based on Fujiwara *et al*. [15] using 1.0% casein in 50 mM Glycine-NaOH buffer (pH 9.0) as substrate. The assay mixture consisted of 2.0 mL of substrate and 0.5 mL of enzyme solution in 50 mM Glycine-NaOH buffer (pH 9.0). The reaction mixture was incubated at 40˚C for 30 min and reaction was terminated by the addition of 2.5 mL of 10% (w/v) trichloroacetic acid. The mixture was allowed to stand for 15 min and then centrifuged at 10,000 rpm for 10 min at 4˚C to remove the resulting precipitate. Protease activity was determined by estimating the amount of tyrosine in the supernatant which was done by measuring the absorbance at 280 nm. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per minute under the mentioned assay conditions.

2.5. Determination of Growth Kinetics and Protease Production of *B. subtilis* SHS-04

Growth kinetics over the cultivation period and production of protease were studied by cultivating the bacteria for 12, 24, 36, 48, 60, 72 and 84 h at pH 9.0 and 45˚C at 200 rpm. The growth of the microorganism was determined by measuring the absorbance of culture at 600 nm. The cultures were centrifuged at the end of each cultivation period and the supernatants were used for determination of protease activity which was the measure of protease production.

2.6. Effect of Temperature on Growth and Protease Production

Optimal temperature for growth and protease production by *B. subtilis* SHS-04 was determined by investigating growth kinetics and protease production at fixed media concentration and pH with varying temperatures. Cultures were grown at 20˚C, 30˚C, 37˚C, 40˚C, 45˚C, 50˚C, and 60˚C at 200 rpm, pH 9.0 for 48 h which was the cultivation period for optimal growth of microorganism and protease production obtained earlier in this study.

2.7. Effect of pH on Growth and Protease Production

Cultures of *B. subtilis* SHS-04 were grown at fixed media concentration and temperature, 45˚C and varying

pH of 6.0 to 11.0 at 200 rpm for 48 h using 50 mM of the following buffer solutions: sodium citrate (pH 6.0), Tris-HCl (pH 7.0 to 8.0) and glycine-NaOH (pH 9.0 to 11.0) for media preparation. Optimal pH for growth and protease production by *B. subtilis* SHS-04 was determined at the end of the cultivation period by measuring the absorbance of culture at 600 nm and determining protease activity in the cell free supernatant obtained after centrifugation at 10,000 rpm and 4˚C for 15 min.

2.8. Effect of Nitrogen Sources on Growth Kinetics and Protease Production

Protease production from *B. subtilis* SHS-04 was investigated using various nitrogen sources which included inexpensive and readily available groundnut meal, locust bean meal and soybean meal. Other nitrogen sources tested were gelatin, beef extract and peptone. Effects of various nitrogen sources (0.75%, w/v) on growth and protease production were studied at pH 9.0 and temperature, 45˚C. Groundnut meal, beef extract, locust bean meal, gelatin, yeast extract and soybean meal were used to substitute peptone in different production media. Cultures were grown for 48 h at 200 rpm; growth and protease production were measured at the end of the cultivation period to determine the best nitrogen sources for optimal enzyme production.

2.9. Effect of Carbon Sources on Growth Kinetics and Protease Production

Various carbon sources were investigated for their effects on protease production by *B. subtilis* SHS-04 at pH 9.0 and temperature, 45˚C. The carbon sources (0.5%, w/v) tested are fructose, maltose, lactose, yam flour, sucrose and soluble starch. These were used to replace glucose in different production media. Cultures were grown for 48 h at 200 rpm. Growth and protease production were measured at the end of the cultivation period to determine the best carbon sources for optimal enzyme production.

2.10. Effect of Temperature on Activity and Stability of Protease

Effect of temperature on activity of crude enzyme was determined by incubating the reaction mixture at temperatures ranging from 30˚C to 80˚C for 30 min in the presence and absence of 5 mM $CaCl_2$ then the activity of the protease was measured. The thermal stability was determined by incubating the crude protease at temperatures ranging from 50˚C to 80˚C in the presence and absence of 5 mM $CaCl_2$ for 30, 60, 90 and 120 min, respectively and the residual protease activity was determined according to the standard assay procedure.

2.11. Effect of pH on Activity and Stability of Protease

The effect of pH on activity of protease was determined by assaying for enzyme activity at different pH values ranging from 4.0 to 12.0. The pH was adjusted using 50 mM of the following buffer solutions: sodium acetate (pH 4.0 to 5.0), sodium citrate (6.0), Tris-HCl (pH 7.0 to 8.0) and glycine-NaOH (pH 9.0 to 12.0). Reaction mixtures were incubated at 40˚C for 30 min and the activity of the protease was measured. To determine the effect of pH on stability of protease, the protease was incubated in relevant buffers of varying pH (4.0 to 12.0) without substrate for 60 min at 40˚C. The residual protease activity was determined as described earlier.

2.12. Effects of Organic Solvents on Activity and Stability of Protease

Effects of glycerol, methanol, isopropanol, dimethyl sulfoxide (DMSO), benzene, and acetone on crude protease activity were studied by introducing the selected organic solvent into the reaction mixture at a final concentration of 25% v/v and protease activity was determined according to the standard assay procedure. Organic solvent stability of protease was investigated by pre-incubating 0.75 mL of crude protease with 0.25 mL of organic solvent at 40˚C for 30 min with shaking. The residual protease activity was determined according to the standard assay procedure and compared with the control. Distilled water was used to replace organic solvent in the control.

3. RESULTS AND DISCUSSION

3.1. Growth Kinetics of *B. subtilis* SHS-04 and Protease Production

Growth kinetics of *B. subtilis* SHS-04 and protease production were studied to determine the cultivation period that favours maximum yield of enzyme and evaluate the effect of growth of organism on protease production. Growth of organism was exponential up to 48 h, followed by a stationary phase (**Figure 1**). Protease production started in the exponential phase corresponding with growth of organism and optimum production of 464.5 U/mL was evident at 48 hour which was the late exponential phase of *B. subtilis* SHS-04. This suggests that production of protease by this *Bacillus* sp. is dependent on cell growth. Incubation time has been reported to play substantial role in enzyme production [4,16,17]. There was decline in protease production after 48 h (**Figure 1**) which could be due to autolysis of the protease which was consequent upon accumulation of the enzyme in the production media.

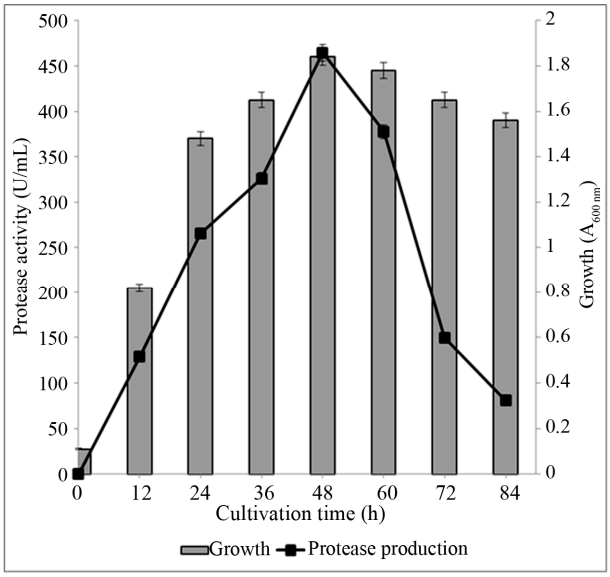

Figure 1. Growth kinetics of *B. subtilis* SHS-04 and protease production over 84 h cultivation period. Protease activity (U/mL) was used to measure protease production. Symbols and bars represent mean values and standard deviations of triplicate determinations.

3.2. Effects of Temperature on Growth and Production of Protease

In bioprocesses, specific temperature requirement and its regulation is one of the most critical parameters [18]. *B. subtilis* SHS-04 exhibited cell growth and protease production at all temperatures studied (20˚C to 60˚C). Optimum temperature for cell growth was 37˚C but optimum protease production was recorded at 45˚C (**Figure 2**). Sepahy and Jabalameli [19] reported a similar finding on a *Bacillus sp*. with maximum protease production at 45˚C. Gouda [20] reported a lower optimum temperature of 30˚C for protease production by *Bacillus sp*. MIG and Nascimento [21] reported a higher optimum temperature of 60˚C for *Bacillus sp.* SMIA-2, respectively. Cultivation temperature affects protein synthesis by influencing rate of biochemical reactions within the cell and consequently inducing or repressing enzyme production [22].

3.3. Effects of pH on Growth and Production of Protease

B. subtilis SHS-04 grew over the entire pH 6.0 - 11.0 studied with optimum growth recorded at pH 9.0. Protease production followed same trend as cell growth with optimum enzyme production of 462.6 U/mL at pH 9.0 (**Figure 3**). Cell growth and protease production declined after pH 9.0. *Bacillus* sp. strain APP1 also grew well in pH range 5.0 - 12.0 but demonstrated optimum protease production at pH 9.0 [23]. Similar finding was reported by Joshi *et al*. [24] on *Bacillus cereus* which showed

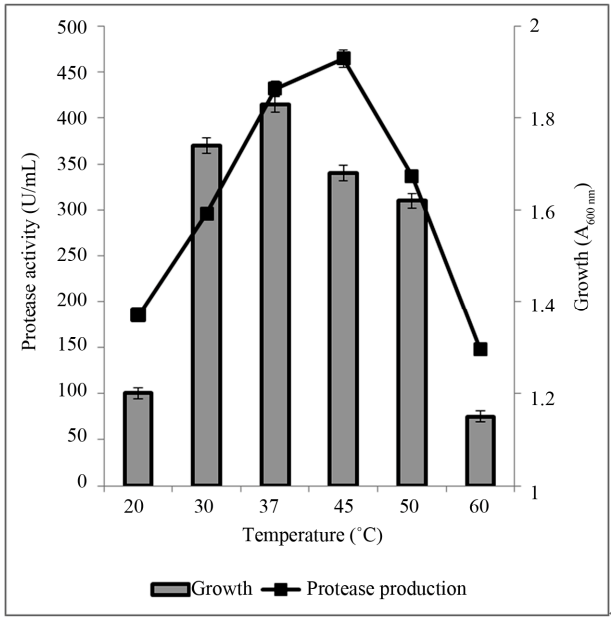

Figure 2. Effects of temperature on growth and protease production over 48 h cultivation period. Symbols and bars represent mean values and standard deviations of triplicate determinations.

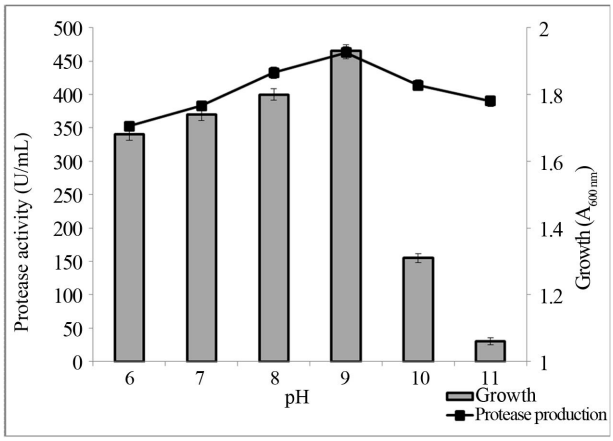

Figure 3. Effects of pH on growth and protease production over 48 h cultivation period. Symbols and bars represent mean values and standard deviations of triplicate determinations.

maximum protease production at pH 9.0. Some have reported optimum protease production at pH 7.0 and 8.0 [21,25]. Further studies to optimize protease production from *B. subtilis* SHS-04 were carried out at pH 9.0. The pH of culture medium has earlier been reported to have strong influence on metabolic processes within the cell [26].

3.4. Effects of Nitrogen Sources on Growth and Production of Protease

All the nitrogen sources studied supported cell growth and protease production from *B. subtilis* SHS-04 at pH

9.0 and temperature 45˚C. However, maximum enzyme production of 1616.2 U/mL was achieved with groundnut meal as nitrogen source (**Figure 4**). This was 348% increase in protease production over what was obtained in the basal media which contained peptone as nitrogen source. Groundnut meal served as complex organic nitrogen source and also provided vitamins and minerals [13] which in this study promoted growth and enzyme production in *B. subtilis* SHS-04. The stimulative role of oil seed cakes on alkaline protease production has been reported in some studies [27]. Beef extract and soybean meal supported 234% and 145% increase in protease yield over peptone. These findings show that organic nitrogen sources are effectively utilized for protease production [4]. Joo and Chang [7] reported optimum protease production from *Bacillus sp*. I-312 in a medium containing wheat flour and soybean meal as the carbon and nitrogen sources. Furthermore, protease production from *Bacillus cereus* MCM B-326 using media containing deoiled groundnut cakes have been reported but with enzyme yield below 200 U/mL [28]. The protease production by *B. subtilis* SHS-04 using groundnut meal as nitrogen source in the present study has greatly enhanced protease production more than other nitrogen sources studied. These results indicate that groundnut meal is an excellent low-cost and readily available substrate for protease production by *B. subtilis* SHS-04.

3.5. Effects of Carbon Sources on Growth and Production of Protease

B. subtilis SHS-04 grew and produced protease in the presence of various carbon sources tested (**Figure 5**).

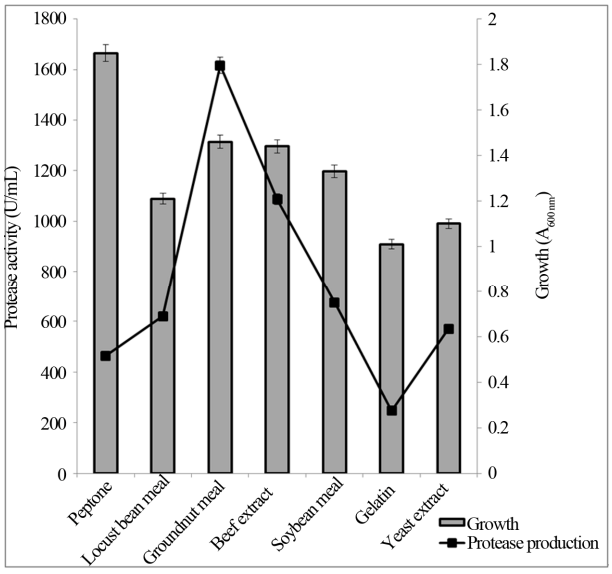

Figure 4. Effects of nitrogen sources on growth and protease production. Symbols and bars represent mean values and standard deviations of triplicate determinations.

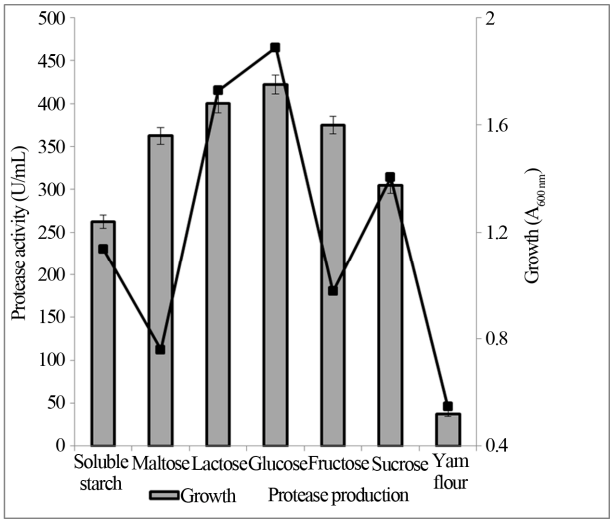

Figure 5. Effects of carbon sources on growth and protease production. Symbols and bars represent mean values and standard deviations of triplicate determinations.

However, only glucose and lactose were most effectively utilized by the organism for protease production with optimum enzyme production of 465.2 U/mL in the presence of 0.5% glucose and 415.0 U/mL with 0.5% lactose. Protease production was repressed in the presence of fructose, maltose and soluble starch (**Figure 5**). Our results agree with some earlier reports where production of extracellular protease was enhanced in the presence of glucose as carbon sources [7,29]. However, other previous studies have reported the repression of protease production by glucose [24,30].

3.6. Thermostability of Crude Protease

It has been well reported that the stability of alkaline proteases is dependent on calcium ion [31-33]. It is highly remarkable that alkaline protease from *B. subtilis* SHS-04 demonstrated unique stability both in the presence and absence of Ca^{2+} with optimum temperature of 60˚C as shown in **Figure 6**. The protease had relative activity of 80% at 70˚C in the absence of Ca^{2+} and 89 % relative activity in the presence of Ca^{2+}. The enzyme retained 76% of its original activity at 80˚C in the presence of 5 mM Ca^{2+} and exhibited 46% relative activity in the absence of calcium ion at 80˚C (**Figure 6**). Some reports on crude and purified thermo tolerant protease show considerable activity over 40˚C to 65˚C but are completely inactivated at 80˚C [25,34,35]. The protease under study showed very high thermostability retaining 99% of its original activity at 60˚C after 60 min of incubation at pH 9.0 in the presence of 5 mM Ca^{2+} as shown in **Figure 7** and 78% in the absence of Ca^{2+}. The thermostability decreased sharply on increase of temperature to 70˚C exhibiting 46% residual activity in the presence of Ca^{2+} and 35% residual activity in the absence of this

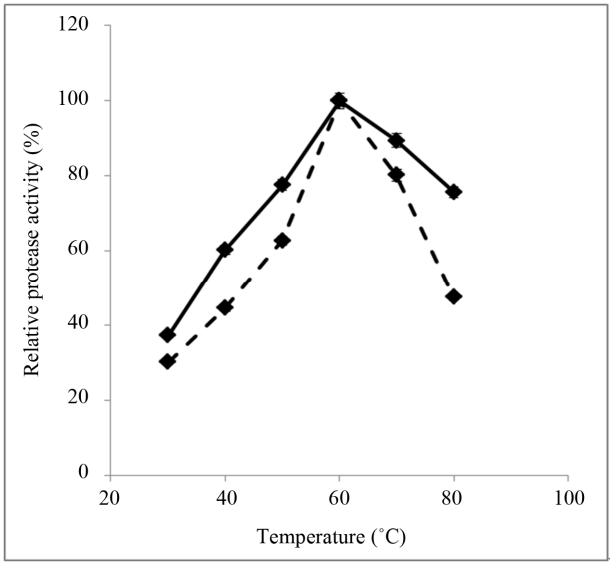

Figure 6. Effect of temperature on activity of protease from *Bacillus subtilis* SHS-04 in the presence (*bold line*) and absence of $CaCl_2$ (*dashed line*).

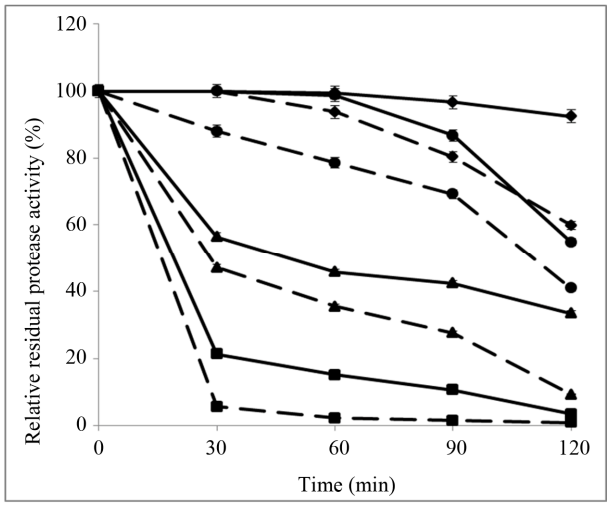

Figure 7. Thermostability of protease in the absence (*dashed lines*) and presence (*bold lines*) of 5 mM $CaCl_2$. Stability at 50˚C (♦), 60˚C (●), 70˚C (▲) and 80˚C (■) were determined by assaying for activity of enzyme after incubation at specified temperatures for 30, 60, 90 and 120 minutes, respectively.

metal ion at 70˚C after 60 min of incubation at pH 9.0 (**Figure 7**). These results are similar to earlier reports which show that calcium was required for the stability of proteases [31,36]. This is further established by Alexander *et al*. [33] who reported that there are two calcium-binding sites in the crystal structure of subtilisin which play very important role in maintaining the thermostability.

3.7. pH Stability of Crude Protease

The optimum pH for protease activity was 9.0. The protease activity rapidly declined above pH 9.0 with the enzyme having relative activity of 53% at pH 10.0 (**Figure 8**), the activity decreased significantly after pH 11.0 showing only 11% relative activity at pH 12.0. The enzyme was very stable over a broad range of pH (7.0 - 11.0). The important detergent enzymes, subtilisin Carlberg and subtilisin Novo showed maximum activity at pH values of 8.0 - 10.0 [30].

3.8. Organic Solvent Stability of Protease

Microbial proteases have been successfully applied to the synthesis of several small peptides of pharmaceutical and nutritional interests [37]. However, the use of proteases in peptide synthesis is limited by the specificity and the instability of the enzymes in the presence of organic solvents [36]. Protease from *B. subtilis* SHS-04 had shown remarkable activity and stability in the presence of the organic solvents studied (**Figure 9**). Protease showed above 80% relative activity in the presence of acetone, methanol and glycerol when compared with control. The enzyme had relative activity of 78% and 66% in the presence of isopropanol and DMSO. The activity of the protease was inhibited by almost 50% in the presence of benzene (**Figure 9**). The effect of organic solvents on stability of protease was determined by pre-incubating the enzyme with selected organic solvent (25% v/v) for 30 min with shaking. **Figure 9** shows that protease from *B. subtilis* SHS-04 retained above 90% of its original activity in the presence of DMSO, acetone

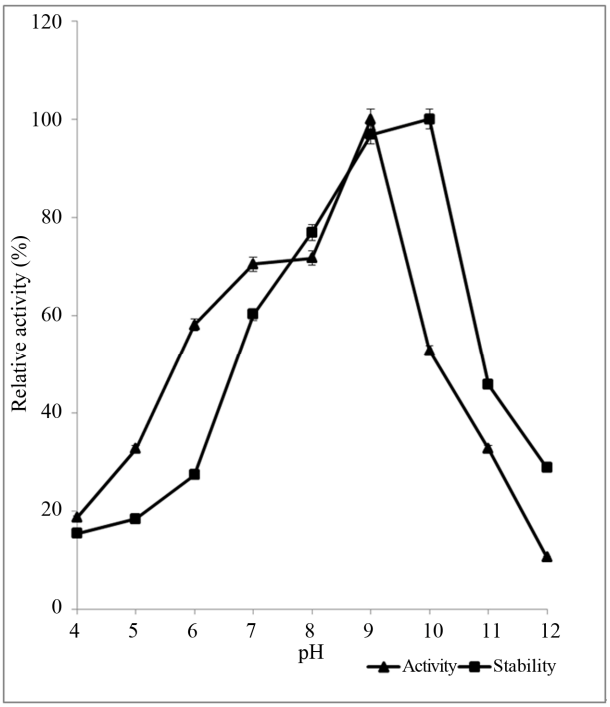

Figure 8. Effect of pH on activity and stability of protease from *Bacillus subtilis* SHS-04.

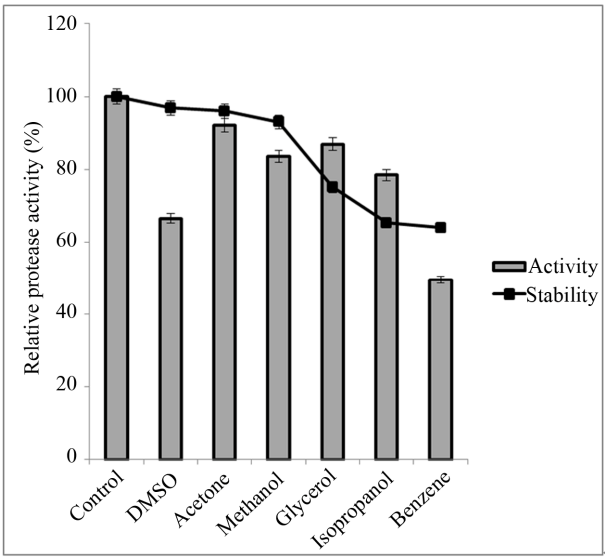

Figure 9. Effect of organic solvents (25% v/v) on activity and stability of protease from *B. subtilis* SHS-04. Symbols and bars represent mean values and standard deviations of triplicate determinations. Dimethyl sulfoxide is abbreviated DMSO.

and methanol with 75%, 65% and 64% residual activity in the presence of glycerol, isopropanol and benzene. This property is highly remarkable when compared with the alkaline protease reported by Hadj-Ali *et al.* [36] from *Bacillus licheniformis* NH1which exhibited only 12% residual activity in the presence of isopropanol. Also, a purified protease from *Pseudomonas aeruginosa* PseA strain was stable in the presence of some organic solvents but unstable in benzene [38]. However, the protease under study from *B. subtilis* SHS-04 was stable in the presence of isopropanol, benzene, DMSO, acetone, methanol and glycerol.

4. CONCLUSION

This study ascertains groundnut meal as the most effective low-cost substrate for protease production from *B. subtilis* SHS-04. The optimized condition for maximum enzyme production (1616.21 U/mL) using groundnut meal (0.75%) was achieved at 48 h cultivation period, pH 9, 45˚C and 200 rpm. The enzyme produced was stable over a wide range of pH and temperature, and in the presence of organic solvents. The use of low-cost growth medium for protease production would significantly reduce the cost of enzyme production.

5. ACKNOWLEDGEMENTS

The author is very grateful to the International Foundation for Science (IFS), Sweden for supporting this research through the awarded IFS grant (F/3775-2). Author also acknowledges with thanks the support received from Mr. Olatope and staff of the Biotechnology Unit of Federal Institute of Industrial Research, Lagos in identification of bacterial isolates.

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