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Optimization of Pectinase and Protease Produced from *Bacillus subtilis* Isolated from Market Waste

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Authors' contributions

This work was carried out in collaboration between both authors. Author EN designed the study and wrote the protocol. Author CAA performed the laboratory analyses, statistical analysis, wrote the first draft and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of the study was to produce and optimize protease and pectinase from *Bacillus subtilis* isolated from market waste.
Place and Duration of Study: Department of Microbiology (laboratory unit), Michael Okpara University of Agriculture Umudike, Abia State Nigeria.
Methodology: The production and optimization of protease and pectinase from bacteria isolated from solid market waste was investigated. Isolated bacteria from the waste were screened for protease and pectinase production using skim milk agar and pectin agar respectively. Using morphological, biochemical and molecular technique the enzymes producing isolate was confirmed as *Bacillus subtilis*. Protease and Pectinase were produced by *Bacillus subtilis* using submerged fermentation in gelatin broth and pectin broth respectively. The enzymes were purified using ammonium sulphate precipitation, dialysis and ion-exchange chromatography. Optimization using different temperatures, pH and nutrient sources was done. Enzyme activity was measured.
Results: Purified protease exhibited maximum activity of 8.72U/ml at 40^{o}C while pectinase exhibited maximum activity of 8.98U/ml at 50^{o}C. Glucose as a carbon source and peptone as a nitrogen source gave optimum activity for both enzymes. Both pectinase and protease exhibited optimum activity at pH 9. There was significant difference (*P*=.05) in enzyme activity at different

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temperatures, pH and nitrogen sources for both protease and pectinase. There was no significant difference in pectinase activity at *P*=.05 for the different carbon sources while there was significant difference for protease activity for the different carbon sources at *P*=.05.
Conclusion: Production of microbial enzymes such as protease and pectinase from waste material is an eco-friendly process and cheaper option for large scale use of enzymes in industry.

Keywords: Bacillus subtilis; optimization; submerged fermentation; protease; pectinase.

1. INTRODUCTION

Enzymes are high molecular weight proteins which catalyse biochemical reactions. The use of microorganisms for the production of extracellular enzymes for industrial use is a preferable option to the use of plants and animal sources. Enzymes such as lipase, pectinase, amylase and protease can be produced from bacteria and fungi using different fermentation techniques such as Solid State Fermentation and Submerged fermentation under optimized conditions [1]. Proteases are enzymes which catalyse the hydrolysis of proteins and are of different kinds such as serine, alkaline, acidic, neutral and carboxyl proteases [2]. They are found in all living organisms and are produced extracellularly by bacteria and fungi [3]. Protease is of industrial importance as it is used in leather processing, food industries, textiles and pharmaceuticals. Pectinase is a high molecular weight enzyme that catalyses the breakdown of pectin, a polysaccharide found in plant tissues into smaller molecules. It is utilized in the food industry and textile production. *Bacillus subtilis* is a Gram-positive, mesophilic organism found commonly in soil and the underneath of vegetation. Culturing and growth of *Bacillus subtilis* is easy, making it a choice microbe for industrial production of both pectinase and protease [4]. Waste products generated in markets usually consist of both plant and animal material. Animal waste products from meat, poultry and fish can supply a large amount of protein rich material for bioconversion to recoverable products of importance in various industries [5]. These animal waste consists of portions of an animal that cannot be sold as meat or used in meat-products. Such waste includes tendons, bones, and the contents of the gastro-intestinal tract, blood and organs and these vary with each type of animal [6]. Remains of vegetables, cassava, yam and other plant crops make up plant related market waste. Research has been carried out on many other related sources for enzyme production such as slaughter house waste, dairy effluent and sewage waste [7]. These wastes produced in market places are inexpensive sources for bioconversion processes into products such as enzymes. The objective of the study was to produce and optimize protease and pectinase from bacteria isolated from market waste. The use of fermentation techniques to produce these enzymes of economic importance provides a cheap and eco-friendly option and illustrates the advantage of white biotechnology over the use of chemicals.

2. MATERIALS AND METHODS

2.1 Sample Collection

A 50g mixed market solid waste sample of animal and plant origin was aseptically collected from Orie Ugba market, Umuahia, Abia State Nigeria in sterile containers and labelled.

2.2 Isolation of Microorganisms

The waste was degraded by grinding under aseptic conditions and then mixed with distilled water. Ten-fold serial dilution was carried out and 1ml of the diluted suspension inoculated on Nutrient agar aseptically and incubated at 35^{o}C for 48 hours [8].

2.3 Screening for Protease Activity

The isolates were streaked on skim milk agar plates and incubated at 37^{0}C for 24 hours. Colonies producing zones of hydrolysis were selected as protease producing bacteria. Zones of hydrolysis were analysed by flooding the skim milk agar plates with 0.25% tannic acid solution for 15 minutes [9].

2.4 Screening for Pectinase Activity

The isolates were screened for pectinase production using Pectin Agar Medium. The isolates were streaked on pectin agar plates and incubated at 37^{o}C for 24 hours. Colonies producing zones of hydrolysis were selected as pectinase producing bacteria. Zones of hydrolysis were analysed by overlaying the medium with 3.3% Cetyltrimethyl Ammonium Bromide solution and incubating for 10 minutes

[10]. The positive strain that produced maximum pectinase enzyme was selected.

2.5 Identification of Isolate

The isolated enzymes producing bacteria were identified based on cellular morphology, Gram's staining, endospore staining, capsule staining and other biochemical tests [11]. The isolated bacteria was confirmed using 16S rRNA method.

2.6 Storage of Isolates

The isolates was inoculated on nutrient agar slants and stored at 4°C in a refrigerator.

2.7 Protease Production

For production of protease enzyme, 100 ml of nutrient medium containing 1% gelatin w/v (as protein source), 0.5% yeast extract and 100 ml distilled water at pH 7 was inoculated with 1ml inoculums of 24 hour old *B. subtilis* culture (1 × 10^8 CFU/ ml). The flasks were incubated at 40ºC for 24 hours on a rotary shaker maintained at 2.23 x *g*. After 24 hours, the broth was centrifuged at 1957 x *g* at 4ºC for 20 min and the cell free supernatant collected. The supernatant was used as crude enzyme extract [9].

2.8 Pectinase Production

A basic liquid medium was used for the production of pectinase having a compostion of (g/ml), Pectin (1.0), Ammonium dihydrogen sulphate (0.14), Potassium dihydrogen phosphate (0.2), Potassium hydrogen phosphate (0.6)/Magnesium sulphate (0.02) at pH 7. It was inoculated with 1ml inoculum of 24 h old *B. subtilis* culture (1 × 10^8 CFU/ ml). The flasks were incubated at 40ºC for 24 hours on a rotary shaker to be maintained at 2.23 x *g*. After 24 hours, the broth was centrifuged at 1957 x *g* at 4ºC for 20 min and the cell free supernatant was collected. The supernatant was used as crude enzyme extract [12].

2.9 Purification of Crude Enzymes (protease and pectinase)

Purification of the crude extracts were carried out in three steps;

2.9.1 Ammonium sulphate precipitation

The first step was ammonium sulphate precipitation. Exactly 0.6g per ml of ammonium sulphate was added to the crude enzyme sample to get 30% saturation and kept overnight at 4ºC. It was then centrifuged at 2817 x *g* for 20 min [8].

2.9.2 Dialysis

Dialysis was then performed with the precipitate suspended in phosphate buffer, pH 7.0 and dialyzed against the same buffer to carry out desalting process. A knotted dialysis bag was immersed in dialysis buffer and the crude enzyme extract loaded using a Pasteur pipette. The dialysis bag was then clipped ensuring no leakage. The dialysis bag was then placed in a flask filled with dialysis buffer. The flask was then placed in a refrigerator at 4°C. After the dialysis had proceeded for 2 hours, fresh dialysis buffer was used to replace the buffer. It was then left overnight after which the knot was untied and the dialyzed enzyme removed using a sterlie Pasteur pipette [9].

2.9.3 Diethylaminoethyl (DEAE) column chromatography

Further purification was done by column chromatography (Normax) using Dieth ylaminoethyl (DEAE) column. A column of DEAE-cellulose was prepared by placing a filter paper at the bottom of a column to serve as a plug. DEAE-cellulose equilibrated in 0.05M buffer was added. The final height of DEAE-cellulose in the column was 8 cm. 10 ml solutions of eluting buffer from the stock 0.25M solution was added. The column was drained to just above the top of the resin. Exactly 1 ml of the crude enzyme extract was run into the column and capped. The effluent was collected in a test tube 98].

2.10 Protease Assay

Protease activity was measured by Caseinolytic method. One unit of enzyme is defined as the amount of enzyme that liberates 1µml of tyrosine per minute. Protease activity was determined according to the modified Anson's method. Exactly 1.0 ml of the enzyme was taken in a 100 ml flask and 1.0 ml of pH 7.0 phosphate buffer added to it. One ml of the substrate (2% casein) was added to the buffer-enzyme solution and incubated at 37°C for 10 minutes in a water bath. At the end of 10 minutes, 10 ml of 5N TCA (Trichloroacetic acid) was added to stop the reaction. The precipitate was then filtered off and 5.0 ml of the filtrate were taken in a test tube. To this 10.0 ml of 0.5N NaOH solution and then 3.0 ml of the Folin- ciocalteu reagent were added. Final readings were taken in a spectrophotometer (Jenway SP/25402) at 750

nm. Standard curve using Tyrosine as standard was prepared [13].

2.11 Pectinase Assay

Pectinase activity was measured by estimation of D-galacturonic acid by Dinitrosalicylic acid (DNS) method using pectin as substrate. One unit of Pectinase activity is defined as the amount of enzyme which liberated 1µm of D-galacturonic acid per min. 1ml of the enzyme was mixed with an equal volume of 1% (w/v) pectin in 0.2M Tris-HCl buffer (pH 8) as the substrate. The mixture was incubated at 40°C for 15 min. Dinitrosalicylic acid reagent (3 mL) was then added and the reaction mixture was boiled in water bath for 15 min. Immediately after boiling, 1mL sodium potassium tartrate (40% w/v) was added into the mixture for colour stability. The mixture was cooled in a water bath to room temperature and the absorbance of cooled reaction mixture was measured at 540 nm. Standard curve was prepared using D-galacturonic acid as standard [14].

2.12 Optimization of Enzymes (Protease and Pectinase)

The activity of enzyme was evaluated at different pH values. The purified enzyme was incubated using sodium phosphate buffer between pH 3-11. Optimum temperature for enzyme activity was determined by incubating the reaction mixture at temperature ranging from 30^{o}c to 70^{o}c in 28 Mm of sodium phosphate buffer (pH 7.0). For optimization of carbon source, glucose, sucrose, lactose and mannitose were used in the media for enzyme production. For optimization of nitrogen source, yeast extract, beef extract, tryptone and peptone were used in enzyme production [15].

2.13 Statistical Analysis

One- way Analysis of Variance (ANOVA) was used to analyse the data generated using R.

3. RESULTS AND DISCUSSION

3.1 Morphological and Biochemical Properties of Isolates

Morphological, physiological and biochemical characteristics of the enzyme producing bacterial isolate were investigated according to the method described by Sneath et al. [11]. It was identified as a member of the genus *Bacillus.* Taxanomic identification using 16S rRNA method confirmed the isolate to be *Bacillus subtilis*.

3.2 Protease Activity

Purified protease exhibited maximum activity of 8.72U/ml at 40^{o}C. Enzyme activity was tested at varying temperatures to determine optimum activity. Protease activity at different temperatures (30^{o}C, 40^{o}C, 50^{o}C, 60^{o}C and 70^{o}C) was statistically different (*P*=.05). From Fig. 1, we can see that protease activity increased until it peaked at 40^{o}C. Decline in protease activity between 40^{o}C and 50^{o}C was minimal. Thereafter there was a steady decline and beyond 60^{o}C protease activity dipped rapidly to 4.32 U/ml at 70^{o}C. Enzyme activity was tested at varying pH to determine optimum activity. From Fig. 2, it can be seen that protease exhibited more activity under alkaline conditions in comparison to acidic conditions. Maximum activity of 5.5 U/ml was observed at pH 9. Though enzyme activity declined at pH 11, it was still higher than that observed at pH 3 and pH 5. The protease produced can be deduced to be alkaline in nature due to optimum activity observed in alkaline conditions.

3.3 Pectinase Activity

Purified pectinase exhibited maximum activity of 8.98U/ml at 50^{o}C. Pectinase activity was tested at different temperatures to determine the optimum temperature of activity. From Fig. 3, it is observed that there was a steady rise in enzyme activity from 30^{o}C to 50^{o}C. After pectinase activity peaked at 50^{o}C, there was a sharp decline to 4.01 U/ml at 70^{o}C. Pectinase activity was statistically higher under alkaline conditions in comparison to acidic conditions with optimum activity of 8.62U/ml observed at pH 9. The produced pectinase can be deduced to be alkaline in nature due to optimum activity observed in alkaline conditions.

3.4 Optimization of Production Media

In the production medium for protease and pectinase, various carbon supplements such as glucose, sucrose, mannitose and lactose were added. Different nitrogen sources were also used to determine the most suitable for enzyme production. From Figs. 5 and 6, it can be seen that crude extracts of both protease and pectinase showed maximum activity of 4.30U/ml and 4.47U/ml respectively with glucose as carbon source. Protease displayed significant higher activity with glucose as carbon source in

comparison to mannitose and lactose. There was no significant difference (*P*=.05) in pectinase activity for the four different carbon sources with a *P* value of .06. From Figs. 7 and 8, it can be seen that both protease and pectinase displayed maximum activity of 7.40U/ml and 4.90U/ml respectively with peptone as nitrogen source. There was significant difference (*P*=.05) in enzyme activity exhibited with the four different nitrogen sources for both enzymes. Beef extract was a better nitrogen supplement than tryptone and yeast extract for the production of both pectinase and protease. The results indicate that the maximum enzyme production was enhanced with the usage of peptone water for both protease and pectinase.

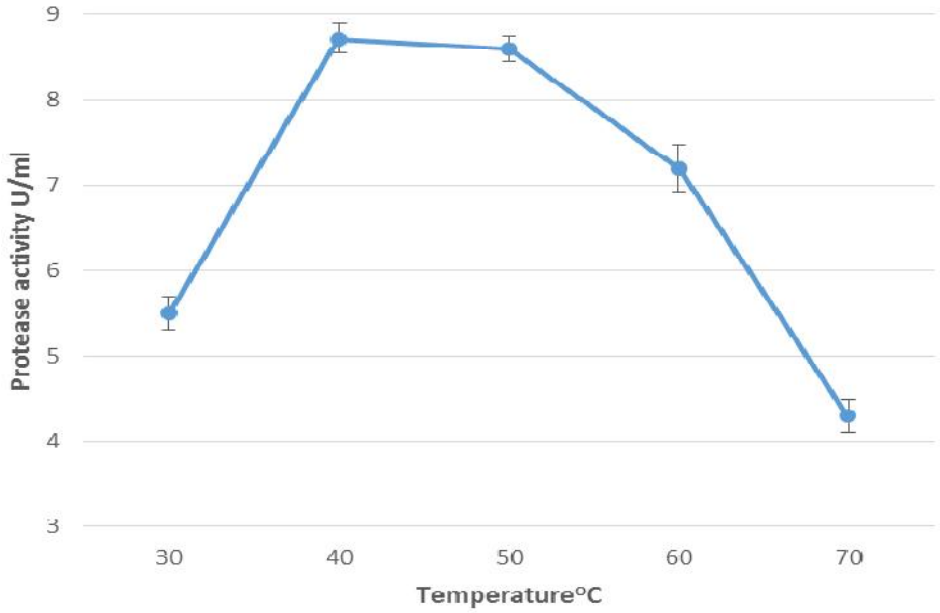

Fig. 1. Effect of temperature on protease activity

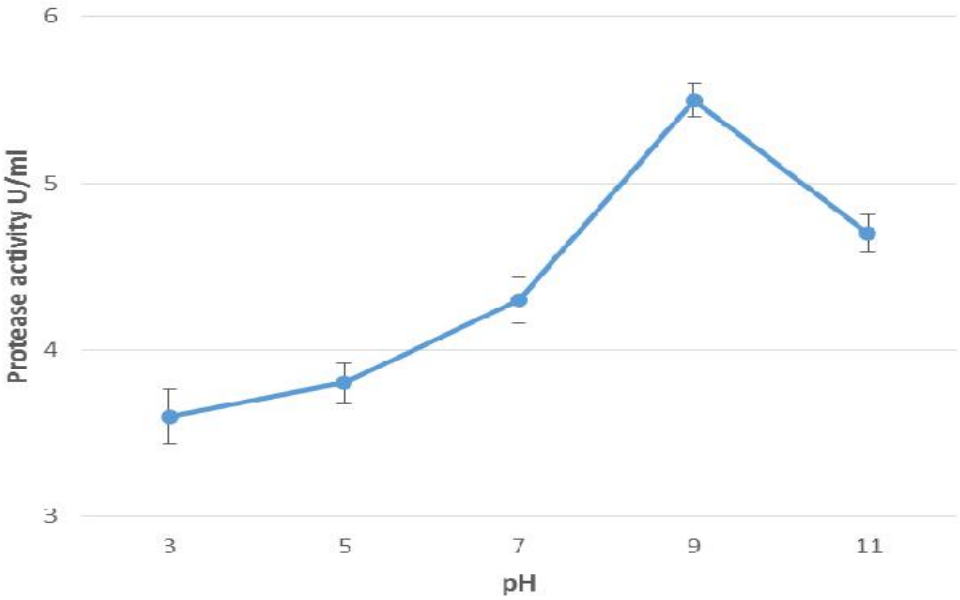

Fig. 2. Effect of pH on protease activity

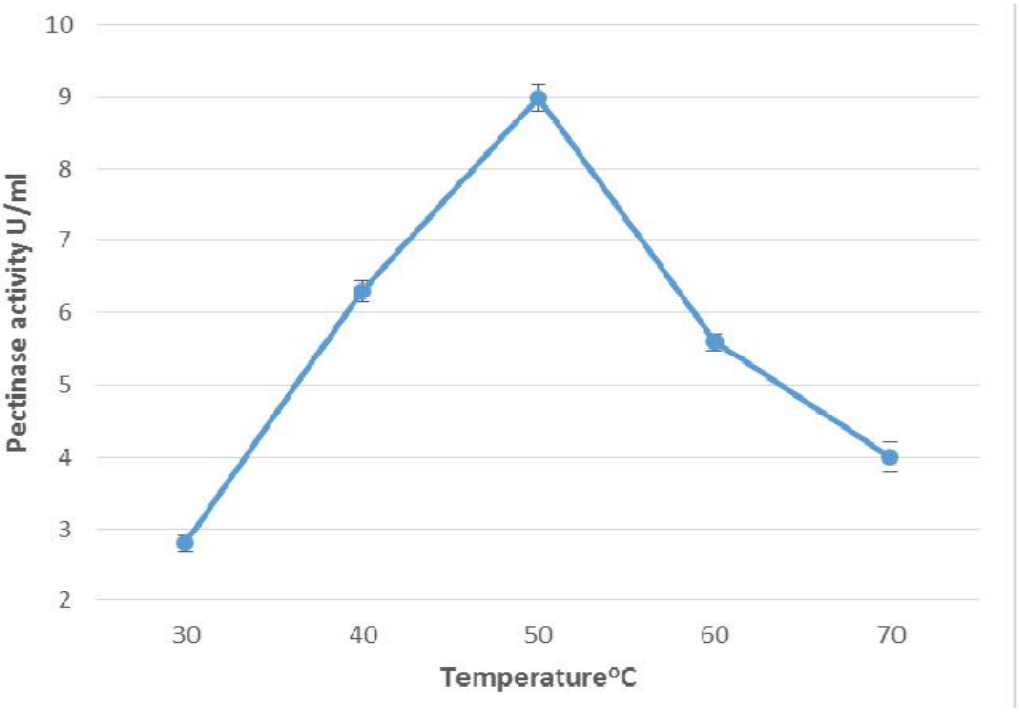

Fig. 3. Effect of temperature on pectinase activity

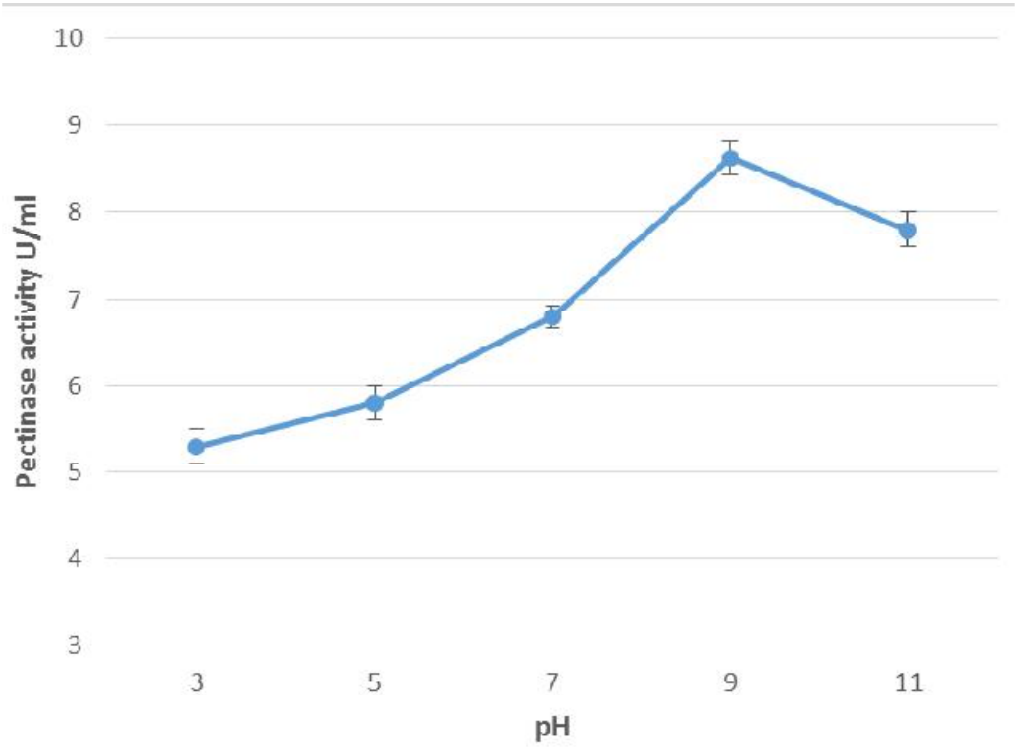

Fig. 4. Effect of pH on pectinase activity

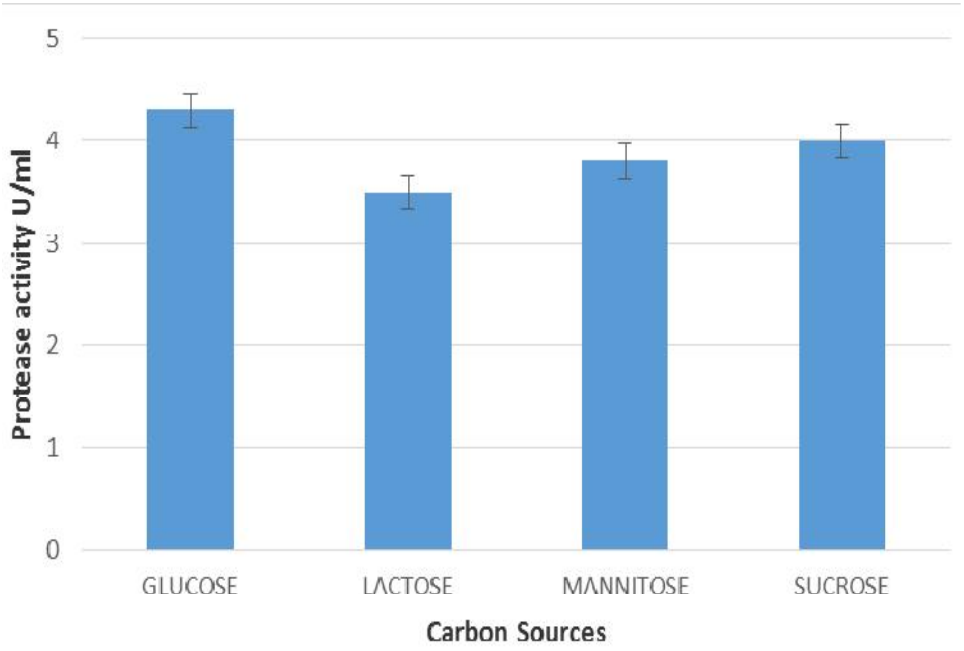

Fig. 5. Effect of different carbon sources on protease activity

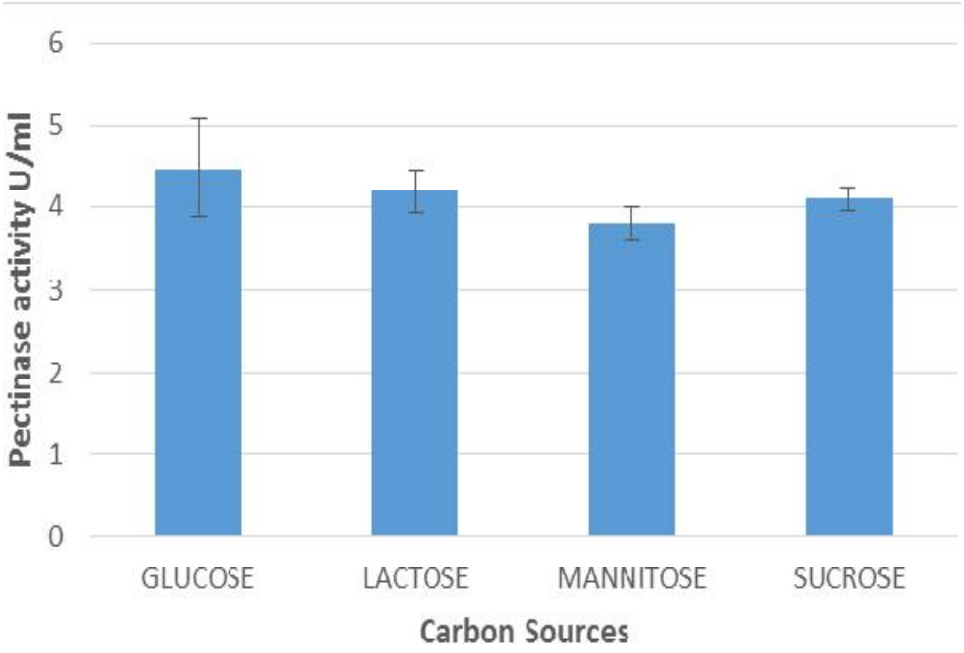

Fig. 6. Effect of different carbon sources on pectinase activity

3.5 Discussion

The use of *Bacillus subtilis* for the production of extracellular enzymes protease and pectinase was investigated. Studies have identified the ability of this bacteria to produce proteases, amylases and pectinases in a liquid fermentation medium and these enzymes have a wide range of use in industry [16]. The study focused on enzyme activity under optimized conditions while not measuring bacterial growth.

The produced pectinase and protease from *Bacillus subtilis* isolated from mixed plant and animal waste using submerged fermentation technology yielded reasonable quantities. After the crude enzymes were purified by ammonium sulphate precipitation, dialysis and ion-exchange chromatography, enzymes of high purity were obtained. Assay was done using spectrophotometric analysis of the purified enzymes. Media for production of microbial extracellular enzymes was optimized to maximize enzyme production. Supplementing different carbon and nitrogen sources and adjusting physical parameters such as pH and temperature ensured that optimal conditions for enzyme activity were obtained.

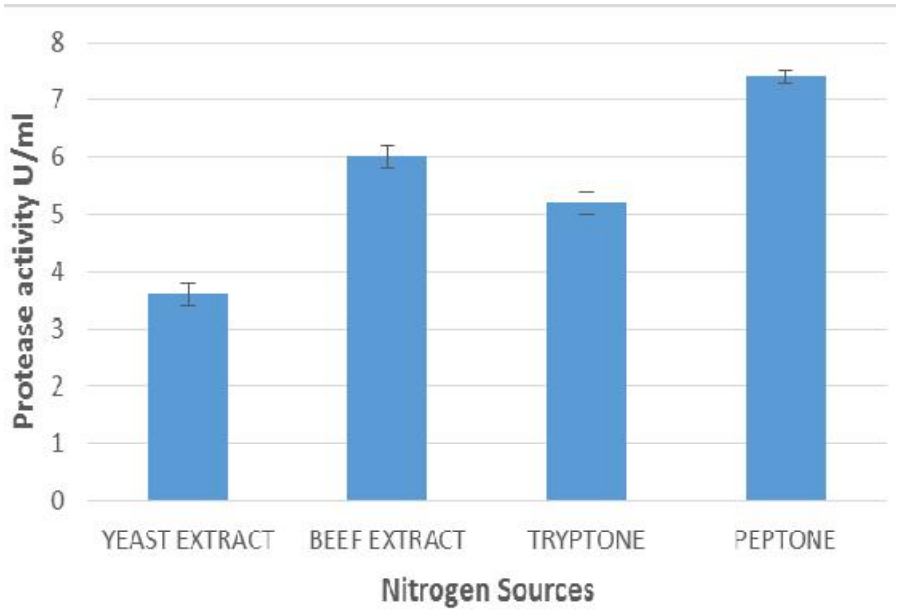

Fig. 7. Effect of different nitrogen sources on protease activity

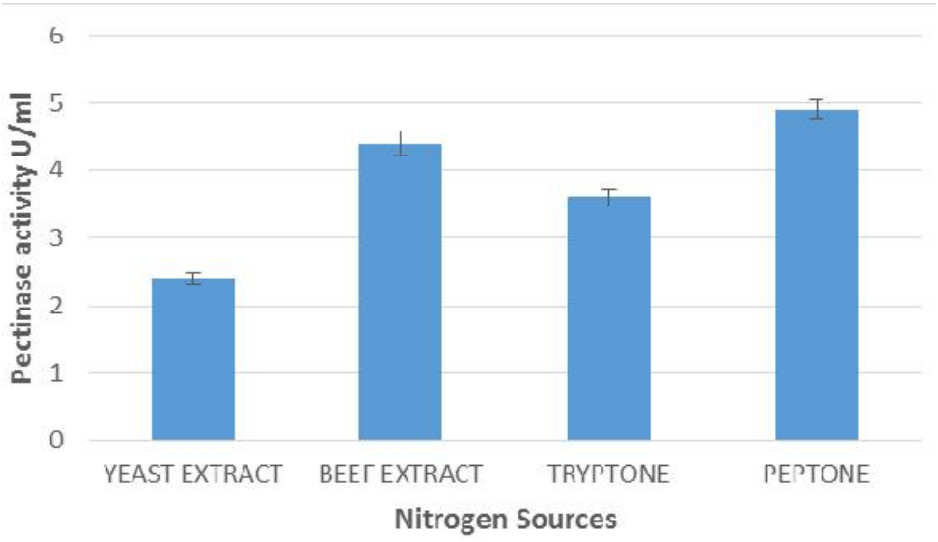

Fig. 8. Effect of different nitrogen sources on pectinase activity

Using glucose and peptone as carbon and nitrogen sources respectively in the production media gave the highest activity for both enzymes. This indicates that the enzymes producing organism metabolized glucose and peptone water faster than other nutrient sources. Glucose giving highest activity was similar to findings in other studies [17]. Optimum temperature for production of protease by *Bacillus subtilis* was 40^{o}C which is similar to findings in other studies [8] which indicated that optimum temperature for *B.subtilis* growth is 40^{o}C. At this temperature, nutrients in the growth medium are maximally utilized. Submerged fermentation media for the production of enzymes using *B.subtilis* must have an operating temperature of 40^{o}C to ensure optimal production.

The effect of different pH on the produced enzymes expectedly had varying effects on enzyme activity after they were assayed using different buffers with varying pH ranging from 3 to 11. Maximum protease activity was recorded at pH 9 similar to a study [18] which gave an optimum pH of 9 for the protease de-hairing of goat. Pectinase also showed maximum activity at pH 9 indicating the alkaline nature of the produced enzymes.

4. CONCLUSION

From the present study, it can be concluded that protease and pectinase can be produced from *B. subtilis* using submerged fermentation technique. Both enzymes displayed highest activity under alkaline conditions and with glucose and peptone as preferred nutrient sources. The use of enzymes in white biotechnology techniques is attracting wider patronage due to the obvious advantages it has over traditional methods especially in developing countries. The consumption of energy and raw-materials, as well as increased awareness of environmental concerns related to the use and disposal of chemicals into landfills, water or release into the air during chemical processing in industry are the principal reasons for the application of enzymes in different sectors. Microbial extracellular enzymes are cheaper to produce and give higher yields in comparison to animal and plant sources. The present study shows that waste derived from human activities can be utilized for the production of enzymes using isolated bacteria in liquid medium. This can be attributed to the rapid growth of bacteria under optimized conditions. The use of enzymes such as pectinase and protease are just a few applications of biotechnology with many other such potentials yet to be explored. Usage of enzymes in industry are beneficial from an ecological point of view and also from an economic angle as they reduce water and energy consumption which ultimately reduces the cost of production of materials.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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