British Biotechnology Journal
5(4): 196-205, 2015, Article no.BBJ.2015.019
ISSN: 2231–2927

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Production and Partial Characterization of Pectinase from Snail (*Archachatina marginata*) Gut Isolates

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Authors' contributions

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

Article Information

DOI: 10.9734/BBJ/2015/11449
Editor(s):
(1) Anil Kumar, School of Biotechnology Devi Ahilya University, Madhya Pradesh, India.
(2) Kuo-Kau Lee, Department of Aquaculture, National Taiwan Ocean University, Taiwan.
Reviewers:
(1) Anonymous, Nigeria.
(2) Anonymous, Brazil.
(3) Anonymous, South Africa.
(4) Anonymous, Slovakia.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=804&id=11&aid=7570

Original Research Article

Received 17th May 2014
Accepted 7th October 2014
Published 31st December 2014

ABSTRACT

Aim: To identify isolates for industrial production of pectinase.
Methodology: The isolates were screened for pectinolytic activity using pectin as substrate. Enzyme activity was expressed as mg of glucose equivalent released per ml of crude enzyme solution per second. The kinetic parameters of the enzyme were determined to obtain the optimum pH, temperature, K_m and V_{max}. Pectinase produced from *Bacillus subtillis* was immobilized on chitosan and its activity was compared with that of free enzyme.
Results: Pectinase from *Bacillus subtilis* was screened to have the highest enzymatic activity among bacterial isolates while pectinase from *Aspergillus niger* had the highest enzymatic activity among fungal isolates. High yield of pectinase enzyme was obtained from *B. subtilis* after 24hrs with activity of 4.01×10^{-4} mg/ml/sec while high yield of pectinase was obtained from *A. niger* on the 5th day with activity of 2.07×10^{-4} mg/ml/sec. The optimum pH for pectinase produced from *B. subtilis* and *A. niger* were 8 and 6, respectively. The optimum temperature for pectinase from *B. subtilis* and *A. niger* were at 50ºC and 40ºC, respectively. The V_{max} and K_m of pectinase from *B. subtilis* and

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A. niger were 16.88×10^{-4} (mg/ml/sec) and 10 (mg/ml); 9.29×10^{-4} (mg/ml/sec) and 30 (mg/ml), respectively. Optimum temperature and pH of immobilized pectinase were 70ºC and 4.0, respectively. Residual activity of immobilized enzyme was 92% after storage at 4ºC for 14 days.
Conclusion: This study revealed that *Bacillus subtilis* from snail gut may be considered as a good candidate for industrial production of pectinase.

Keywords: Snail gut isolates; pectinase; enzyme activity; Km and Vmax; immobilized pectinase.

1. INTRODUCTION

Terrestrial gastropods feed on fresh plants with high protein, carbohydrate and calcium content [1] and may also participate with other soil invertebrate to decompose leaf litters [2]. Consequently, they need a large set of polysaccharide depolymerase such as pectinases for digestion of plant materials. The dependence of snail on microbial activity within their gut would explain their extra ordinary efficiency in digestion of up to 60-80% plant fiber [3]. Pectic substances are complex high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. They are largely responsible for the structural integrity and cohesion of plant tissues [4]. Three major pectic polyssacharide groups are referred to as, homogalacturonan (HG) [5], rhamnogalacturonan I (RGI) [6], rhamnogalacturonan II (RGII) [7], all containing D-galacturonic acid to a greater or a lesser extent. Each of these pectic polyssacharides is digested by a particular pectinase enzyme which could be one of the following; protopectinase, pectin methyl esterase [8], pectin acetyl esterase, polymethylgalacturonase, polygalacturonase (PG), polygalacturonan acetyl esterase, pectatelyase and xylogalacturonan hydrolase [9].

Pectinases constitute a unique group of enzymes which catalyze the degradation of pecticpolymers present in plant cell wall [10]. It digests a galacturonic acid polymer by breaking the α-1,4glycosidic linkages between the giant molecules [11]. Through this process, it softens the cell wall and increase the yield of juice extract from the fruits. The major sources of enzyme include; plant and microorganism. From both technical and economical point of view, microbial sources of pectinase have become increasingly important. Of the hundred or so enzymes being used industrially, over a half is from fungi and yeast and over a third is from bacteria with the remainder divided between animal (8%) and plant (4%) sources [12]. Pectinolytic enzymes have been reported to be produced by a large number of bacteria and fungi such as *Bacillus subtilis*, *Clostridium* spp., *Pseudomonas* spp., *Aspergillus* spp., *Penicillium spp., Thermomyces lanuginosus.*

Pectinase has a tremendous industrial applications especially, in food industry, for extraction and clarification of both sparkling clear juice (apple, pear, grapes and wine) and cloudy (lemon, orange, pineapple and mango) juice and maceration of plant tissue [13,14].

Despite the usefulness of pectinase, the enzyme is not produced at commercial quantities in Nigeria. However, snails are natural degrader of plants, it is expected that microorganisms inhabiting their gut should be efficient in hydrolysing plants component like pectin. Therefore, the present study was undertaken to identify snail gut isolates which may become good candidates for industrial production of pectinase.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Five snails (5) were obtained from Kure Ultra-Modern market in Minna, Niger State, Nigeria. They were placed in a clean, covered bowl, containing sand, some vegetables and water. They were kept safe in the laboratory, prior to use.

2.2 Isolation of Microorganisms from the Gut of Snail

The snails were washed with tap water and outer shell rinsed with 96% ethanol for sterilization. The shells were broken (aseptically, by using flame and hand gloves) to remove the flesh and then dissected to reveal the gut. A portion of the gut was streaked onto nutrient agar plates, using inoculating loop and incubated for 18-24 hours at 37ºC for bacterial growth, another portion was

streaked in Saubourand Dextrose Agar (SDA) plates and incubated at 25ºC for 3-5days for fungal growth. Individual colonies observed were sub-cultured on Nutrient Agar and Saubourand Dextrose Agar plates for another 18-24 hours and 3-5 days respectively and finally grown on agar slants to preserve the pure cultures. Identification of bacterial isolates was based on morphological (growth pattern) and biochemical test (Gram staining, catalase and urease test). The suspected organisms were then grown on selective media. Different fungi colonies were identified morphologically and by its microbiological appearance including the pigmentation, shape, presence of special structures and characteristics of spores. A small portion of each mycelia growth was carefully picked with the aid of a sterile needle and placed on a drop of lactophenol cotton blue on a slide and covered with cover slip. The slide was then observed under the microscope with magnification of x 10 and x 40 to detect the spores and some special structures of the fungi. This was followed by catalase test and Gram's staining technique to identify the bacterial isolated [15].

2.3 Screening of Isolates for Pectinase Activity

The isolates were screened for pectinase activity. One gram (1 g) of pectin was added in to SDA and Nutrient agar. These media were sterilized and distributed aseptically in petri dishes and the isolates were inoculated on the plates. The plates were observed for a zone of clearance after 3-5 days for fungi and 18-24 hours for bacteria. Isolates showing clear zone were considered as pectinase producers [16].

2.4 Quantification of Pectinolytic Potential of the Isolates

The isolates that showed zone of inhibition on plate were subculture in a nutrient broth to quantify the isolate potential for pectinase production. They were incubated at 37ºC for 24 hours for bacteria isolates and for 5days at room temperature for fungi isolates. After the stipulated period of growth, the pectinolytic activity was determined by measuring the absorbance of reducing sugar using dinitrosalicylic acid (DNSA) reagent [17]. This was done by centrifuging the culture broth at 8,000 RPM for 5 and 15 minutes for bacteria and fungi, respectively. The supernatant was filtered using Whatman No. 1 filter paper and Pectinase enzyme was assayed by measuring half milliliter of 1% pectin in 0.1M citrate buffer (pH 5.8) in a test tube and 1.0 ml of culture filtrate was added. The reaction mixture was incubated at 50ºC for 30 minutes and the reaction terminated by adding 3ml DNSA reagent. The tubes were heated in boiling water bath for 15 minutes and then cooled to room temperature. Then, the absorbance was read at 540 nm. The absorbance of the reducing sugar was extrapolated from glucose standard curve and the enzyme activity was expressed as mg of glucose equivalent released per ml/sec [17].

2.5 Pectinase Production Profile from Growing Culture of Snail Gut Isolates

The isolates showing pectinase activity were placed in a basal medium, the medium consist of $NaNO_3$, 2%; K_2HPO4, 1%; MgSO4, 5%; KCl 5%; FeSO4, 0.001%; Pectin 15%. The culture was grown for 7days at 25ºC for fungi and 30 hours for bacteria. The culture broth was sampled and assayed every 24 hours for fungi and 6 hours for bacteria.

2.6 Microbial Biomass Determination

The bacterial growth was determined by spectrophotometric method. This was carried out by growing the isolates in nutrient broth and sampled to determine the optical density (OD) at 575 nm; it was carried out simultaneously with enzyme production profile. This measurement of turbidity or optical density is an indirect measurement of cell biomass that includes both living and dead cell [18].

2.7 Effect of Temperature on Pectinase Activity

The optimum temperature for pectinase was determined by incubating 1 ml of the crude enzyme with 2 ml 3% pectin in citrate buffer, pH (5.8) at different temperature (30-90ºC) for 30 minutes. Reducing sugars were estimated by the dinitrosalcyclic acid reagent method [19].

2.8 Effect of pH on Pectinase Activity

The optimum pH was determined by incubating 1 ml crude enzyme with 2 ml of 3% pectin in 1 ml citrate buffer at different pH (3-8) for 30 minutes at 50ºC. Reducing sugars thus released were estimated spectrophotometrically by dinitrosalicylic acid reagent method.

2.9 Effect of Substrate Concentration on Pectinase Activity

Crude enzyme (1ml) was added to 2ml of different substrate concentrations, 1%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% (W/V) and incubated at appropriate temperature and pH obtained above. The reducing sugar was estimated spectrophotometrically, Vmax and Km were then determined from Line Weaver-Burk plot.

2.10 Immobilization of Pectinase on Chitosan

Pectinase produced from *Bacillus subtilis* was immobilized by physical adsorption on Chitosan bead as described by [20]. Chitosan powder (2 g) was soaked in hexane under agitation conditions (100 rpm) for 1 hour. Excess hexane was removed by decantation followed by the addition of 10ml of pectinase crude enzyme. The pectinase was on the support under agitation for 3 hours at room temperature followed by additional period of 18 hours under static conditions at 4ºC. The derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with hexane. The optimum enzyme loading capacity of the chitosan and the loading time were determined. The effects of other factors, such as optimum pH, temperature and enzyme storage stability of immobilized enzyme activity were investigated as described in reference [20]. Optimum pectinase loading time was determined by sampling the bead-enzyme mixture at an hour time interval for 6 hours. The enzyme activity was assayed after centrifuging the sample at 1500 rpm for 30 minutes to remove the carrier. Storage stability of immobilized pectinase was determined from its decay constant and half-life [21].

3. RESULTS AND DISCUSSION

The bacteria isolated from the gut of snail include *B. subtilis, streptococcus sp, and S. aureus* while fungal isolates were *A. niger, P. notatum, Trychophyton violacium and Torulopsis datilla*.

3.1 Screening and Quantification of Pectinolytic Potential of the Isolates from Snail Gut

All the bacteria and only two of the fungal isolates showed zone of clearance on pectin agar plates, thus indicating the ability of these organisms to produce pectinase. From the bacteria screened for pectinase production, *Bacillus subtilis* showed the highest enzymatic activity ($3.0x10^{-4}$ mg/ml/sec). The pectinase activity of *A. niger* ($2.6x10^{-4}$ mg/ml/sec) was greater than that of *P. notatum* after secondary screening as shown in Table 1.

3.2 Biomass Yield-enzyme Activity Profile

Bacillus subtilis gave the highest enzyme yield after 24 hours as shown in Fig. 1, with an enzyme activity of 4.01 X 10^{-4} mg/ml/sec. *A. niger* gave the highest enzyme yield after 5 days with activity of 2.06×10^{-4} mg/ml/sec as shown in Fig. 2. *Bacillus subtilis* showed its highest enzymatic activity (4.01 x 10^{-4}mg/ml/sec) after 24 hours with highest biomass growth after 20 hours (Fig. 1). This is in correlation to findings of Kashyap et al. [22] who observed pectinolytic activity after 24 hours for *Bacillus subtilis* but the result is contrary to Abdul et al. [23] who observed maximum pectinase activity from *B. subtilis* at 48 hours with the activity of 1700 µ/ml. It was observed that, the time required by the pectinase from snail gut isolate (*B. subtilis*) to develop peak activity was significantly shorter than the time reported by Abdul et al. [23]. Therefore, the Bacillus isolate in the present study may be considered a better source of pectinase production. The difference observed in the present result could be due to difference in strain of *B. subtilis* used. Pectinase activity increased, with exponential growth of the isolates, the decrease with time of pectinase activity during incubation was regarded as the death phase of the isolates. *Aspergillus niger* exhibited its highest enzymatic activity on the 5^{th} day of incubation with an activity of 2.07×10^{-4} mg/ml/sec and then declined to the 7^{th} day of incubation. A similar result was reported by Anithara [24] who recorded maximum pectinase activity after 4 days. The slight difference observed could be due to the difference in substrate and or difference in strains of *A. niger.* Therefore, the incubation period at which pectinase had maximum activity (24 hours for *Bacillus subtilis and* 5 days for *A. niger*) is the best time at which pectinase should be harvested from these isolates which is the period of maximum production.

3.3 Effect of Temperature on Pectinase Activity

The optimum temperature of pectinase from *B. subtilis, A. niger, P. notatum* were 50ºC, 40ºC and 60ºC respectively, with activities of 0.43 ×

10^{-4}, 0.44 × 10^{-4} and 0.27 × 10^{-4} mg/ml/sec respectively as shown in Fig. 3. Optimum temperature of pectinase from *Bacillus subtilis* observed in the present study agreed with the findings of Eleni et al. [25] who recorded optimum temperature between 45ºC and 55ºC for pectinase from *B. subtilis*. Optimum temperature attained by pectinase produced from snail gut *Aspergillus niger* supports the findings of Mohammed et al. [26] who reported optimum temperature of 40ºC. Pectinase from *Penicillium notatum* showed highest activity at optimum temperature of 60ºC which agreed with the finding of Sinitsyna et al. [27]. Increased in temperature resulted to an increase in pectinase activity up to a certain point (optimum temperature), where activities decreased as the temperature increased further. Every enzyme has optimum or apparent temperature at which its activity is highest, above this range, the enzyme is denatured, therefore losses its active site for catalysis. Below this range, the enzyme is less active due to intra-molecular hydrogen bond within the enzyme for catalysis to take place.

3.4 Effect of pH on Pectinase Activity

The optimum pH of pectinase from *B. subtilis, A. niger, P. notatum* were 8, 6 and 4, respectively, with activities of 2.56×10^{-5}, 1.30×10^{-4} and 1.17×10^{-4} mg/ml/sec, respectively (Fig. 4). Pectinase from *Bacillus subtilis* has its optimum activity at pH that correlates to the finding of Kashyap et al. [22] but contrary to Soriano et al. [29] who reported optimum pH at 10.0 for pectinase from *Bacillus subtilis*. Optimum pH (6) for pectinase from *A. niger* (with activity of 1.3 x 10^{-4} mg/ml/sec) was in agreement to the finding of Oyeleke et al. [28] who reported optimum pH at 6.0 for pectinase from *A. niger*.

Optimum pH of pectinase from *Penicillium notatum* agreed with Alana et al. [29] and Sinitsyna et al. [27]. The pH above or below this range alter the conformation of the enzyme and ultimately its activity, therefore compromising the binding of the enzyme with substrate.

Table 1. Pectinase activity of the snail gut isolates

Isolates	Enzyme activity (mg/ml/sec) × 10^{-5}
S. aureus	0.10
B. subtilis	3.00
Streptococcus spp	1.10
Aspergillus niger	2.60
Penicillium notatum	2.20

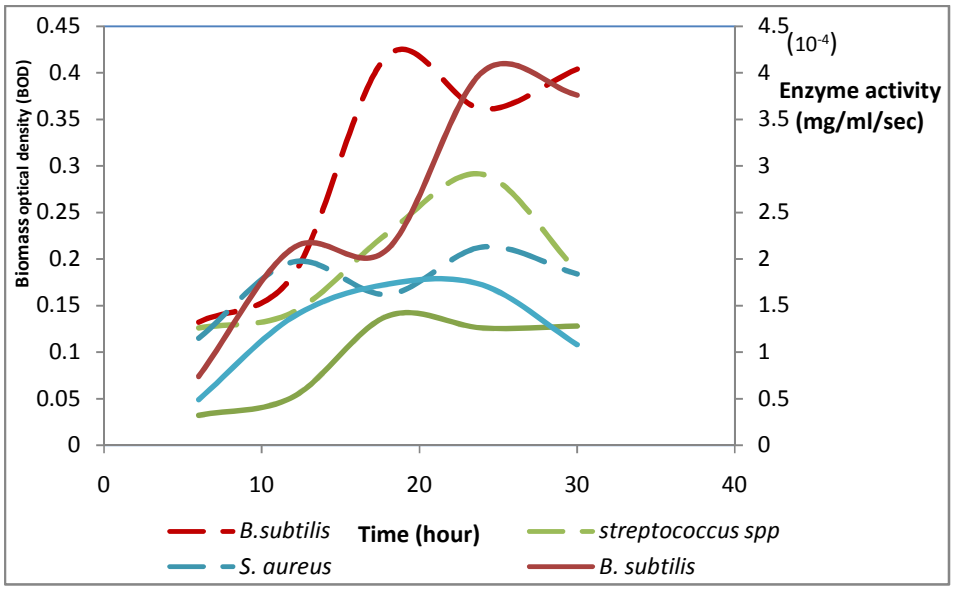

Fig. 1. Pectinase activity (mg/ml/sec) and biomass optical density of *B. subtilis, streptococcus sp*and *S. aureus*

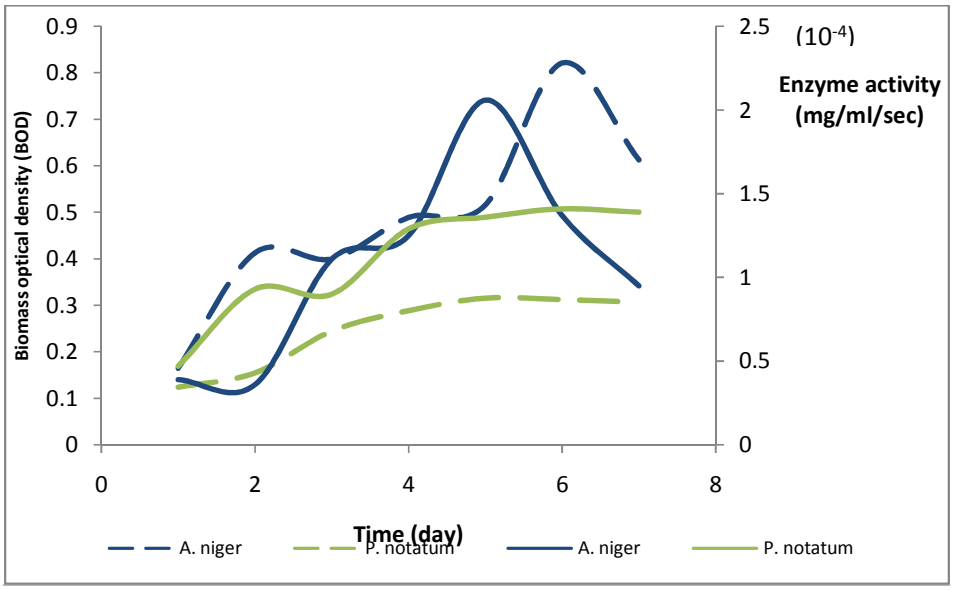

Fig. 2. Pectinase activity (mg/ml/sec) and biomass optical density of *A. niger* and *P. notatum*

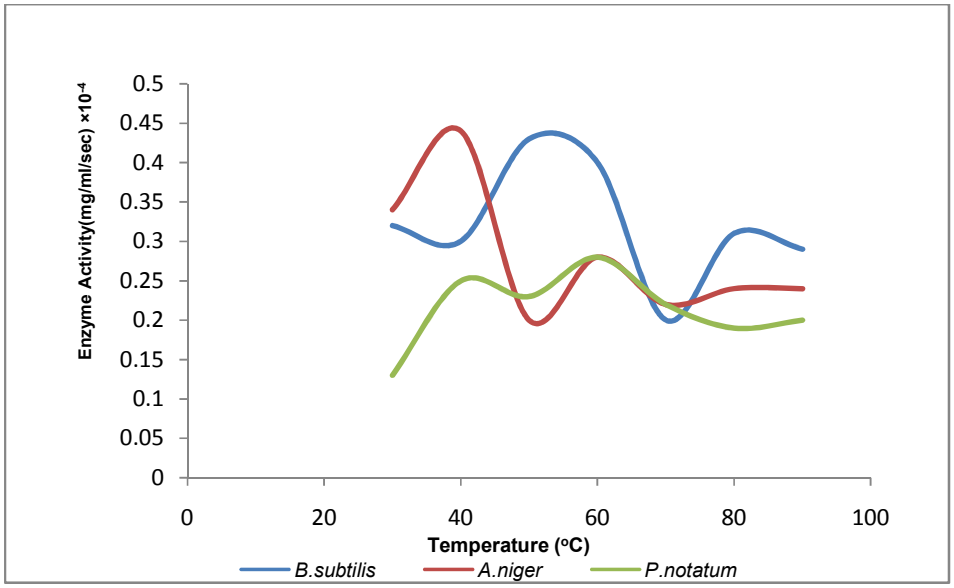

Fig. 3. Temperature profile of pectinase enzymes from *B. subtilis, A. niger* and *P. notatum* isolated from snail gut.

3.5 Effect of Substrate Concentration on Enzyme Activity

The K_m and V_{max} values of pectinase from different sources were calculated using Line weaver-Burk plot and were shown in Table 2. This result indicated that, pectinase from *Bacillus subtilis* has the highest affinity for substrate due to its lowest K_m, it also has the highest utility of pectin substrate as a result of its highest V_{max}. The present finding is similar to the finding of Anna et al. [30] who reported that pectinase from *Bacillus firmus* was better than that isolated from *Aspergillus niger.* Due to this high binding of pectinase from *B. subtilis* with pectin substrate, small quantity of the enzyme will digest a considerably high amount of substrate. This may therefore reduce the cost for the enzyme in industrial use.

Furthermore, the present results were compared with the observation of Adesina et al. [31] who reported the V_{max} of pectinase produced by *Rhodotorulla* spp and *Mucor mucorales* to be 0.023304 µ/mg/min and 0.043304 µ/mg/min, respectively. The maximum velocities (V_{max}) of pectinase from *A. niger* and *B. subtilis* isolated from the gut of snail were higher than the value recorded by Adesina et al. [31]. This suggested that pectinase produced from snail gut isolates has better utility of its substrate.

3.6 Characterization of Immobilized Pectinase

3.6.1 Effect of pectinase loading time

The effect of loading time on Chitosan support is shown in Fig. 5. The maximum enzyme loading time was obtained at 4 hours with enzyme activity of 1.18 X 10^{-4} (mg/ml/sec).At this loading time, the intermolecular spaces of the chitosan (carrier) have been completely adsorbed with the enzyme and beyond this time, the activity of immobilized pectinase remained constant.

3.6.2 Effect of temperature on immobilized pectinase

The optimum temperature of immobilized pectinase from *B. subtilis* was 70ºC while optimum temperature of free pectinase from *B. subtilis* was 50ºC. This result supported the findings of Egwim et al. [20] who showed that the optimum temperature of immobilized lipase increased from 35ºC for free enzyme to 40ºC. The result also supported Flashmy et al. [32] who reported optimum temperature of immobilized urease increased from 55ºC for free enzyme to 65ºC. The increase in temperature in the immobilized enzyme over free enzyme in the present study suggested that immobilized enzyme has a higher resistance to changes in temperature than free enzyme. The stability of the enzyme up to 70ºC could make it less susceptible to thermal inactivation during industrial processes.

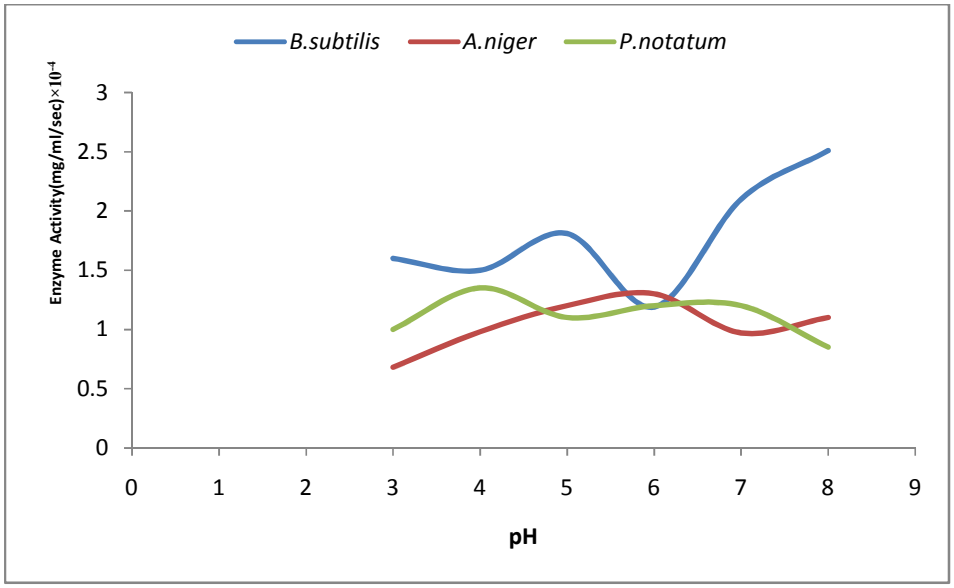

Fig. 4. pH profile of pectinase enzymes from *B. subtilis, A. niger* and *P. notatum* isolated from snail gut

Table 2. V_{max} and K_m of pectinases from pectinolytic isolates

Isolates	V_{max}(mg/ml/sec) ×10^{-5}	K_m (mg/ml)
B. subtilis	16.88	10
S. aureus	0.88	410
Streptococcus sp	3.62	60
Aspergillus niger	9.29	30
P. notatum	1.10	630

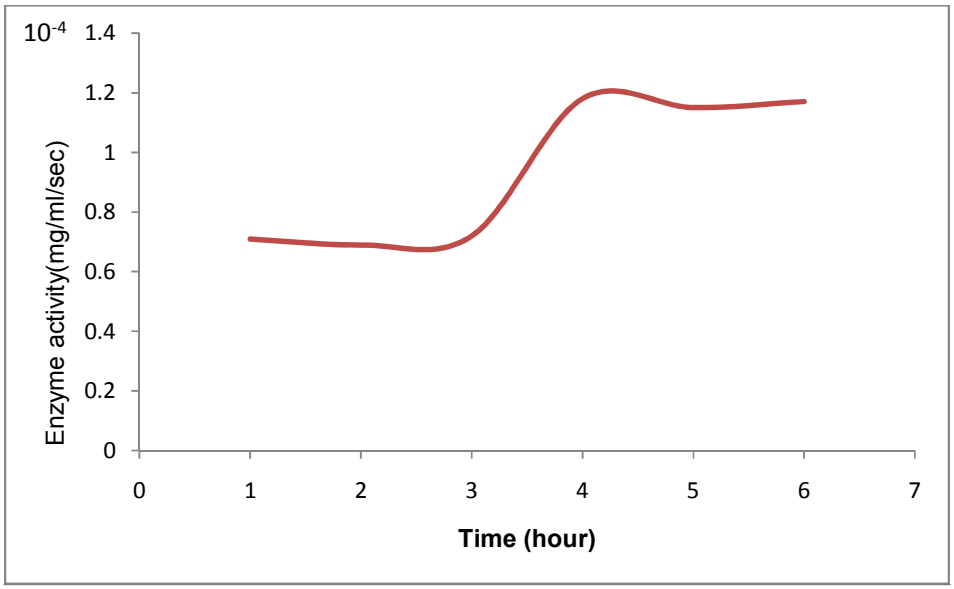

Fig. 5. Pectinase loading capacity and loading time on chitosan

3.6.3 Effect of pH on immobilized pectinase

The optimum pH of immobilized pectinase was found to be at 4.0. Optimum pH of immobilized pectinase was shifted to acidic range (pH 4.0) from pH 8.0, which was the optimum pH for free enzyme. This acidic shift in the optimum pH was in agreement with the general observation that the positively-charged supports displace pH-activity curve of the enzyme attached to them towards lower pH values [33]. The stability of pH in acidic region could be an advantage in fruit processing such as citrus fruits as a result of their acidic nature.

3.6.4 Storage stability of immobilized enzyme

The residual activity of immobilized pectinase achieved after storage for 14 days was 92%. Thus, it would take 132 days for immobilized pectinase to attain half of its activity (half-life). These results are similar to the findings of Egwim et al. [20] who reported 96% residual activity of immobilized lipase after storage for 90 days. Furthermore, the storage stability observed was higher than the previous study of Li et al. [21] who reported that half-life of immobilized pectinase was 30 days under 4ºC condition. The higher half-life of immobilized pectinase produced by *B. subtilis* isolated from snail gut could be found advantageous in industrial applications because, most enzymes are lost after first use while other loss activity after a few days of storage. This difference could be as a result of the effect of support use for immobilization on enzyme storage or the nature of the enzyme itself. The half-life indicated possible reusability of the immobilized enzyme while residual activity showed the potency of the immobilized enzyme after each cycle. The storage stability of immobilized enzyme could be due to intramolecular linkage conferred by the carriers (chitosan) and depends on the length of such linkage [34]. It could also be due to the restriction of the enzyme within the carrier. Therefore, for industrial application, pectinase immobilized on chitosan bead can be reused over a long period of time and can withstand harsh industrial conditions (high temperature and acidic medium).

3.6.5 Decay constant (κ) and half -life (T1/2)

The κ and T½ values for immobilized pectinase enzyme were computed as follow;

$$de/dt = -KE \quad (1)$$

$$\text{At } t = 0;\ E = E_o$$

Integrating

$$E = E_o\, e^{(-Kt)} \quad (2)$$

Where E_o is the initial active enzyme concentration and t is time elapsed during reaction. The residual enzyme activity A_r is

directly proportional to the concentration of the active enzyme (E):

$$A_rA_o = E/E_o \quad (3)$$

From equation 2&3, the residual enzyme activity follows as:

$$Ar = Aoe^{(-Kt)} \quad (4)$$

From equation 4;

$$K= 2.303/t \log A_r/A_o \quad (5)$$

Enzyme half-life (t1/2) is the time lapse for the enzyme activity to decrease by 50% of the initial value. From equation (5) it means that half life (t1/2) can be calculated.

$$T1/2 = \ln (0.5)/K = 0.693/K \quad (6)$$

$A_o = 0.92\times10^{-4}$(mg/ml/sec)
$_r = 0.99\times10^{-4}$(mg/ml/sec)
t = 14days
Using equation 5;
$K = 2.303/t \times \log(A_o/A_r)$
K = -0.00524
Therefore, half-life can be calculated using equation 6
$T_{1/2} = \ln (0.5)/K$
$T_{1/2}$ = 132 days.

4. CONCLUSION

It can be concluded that *Bacillus subtilis* and *Aspergillus niger* obtained from snail gut may be good candidates for industrial production of pectinase.

ACKNOWLEDGEMENTS

Our sincere gratitude to the Department of Microbiology and Department of Biochemistry of Federal University of Technology, Minna for providing the chemicals and materials used during this research.

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