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Isolation, Purification and Characterization of Pedogenic Bacterial Amylase from Food Waste Decomposing Soil

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ABSTRACT
The aim of this work was to study the biodegradation of waste employing thermostable α-amylase enzymes producing bacteria. Three potential isolates were identified which were capable of producing maximum amylase. Amylolytic strains, *Bacillus megaterium* and *Bacillus subtilis*; *Bacillus polymyxa* respectively. The Bacillus species isolated were screened for amylolytic activities. The isolate with the widest zone of clearance (R7) was selected for further analysis. The highest activity was observed in B. *subtilis* (3.50±0.01 mm) followed by B. *megaterium* (2.5±0.10 mm) and B. *polymyxa* (2.40±0.18 mm) had the least activity. Amylase activity was determined using DNS method. The optimum temperature for the activity of the amylase produced was obtained at 70 °C. Optimum pH activity was obtained at 6.0 with a concentration of 0.376 mg/ml. Bacillus *subtilis* has the greatest potential for producing amylase than the other isolates and vegetable waste can be exploited for amylase production. The B. *subtilis* strain produced thermostable amylase with characteristics suitable for application in starch processing and other food industries.
These strains were selected based on its higher cell density, enzymatic activities and stability at a wide range of pH and temperature compared to other strains

I. INTRODUCTION

Food waste and vegetable waste is a global issue. Food waste is organic waste discharged from various sources including food processing plants, domestic and commercial kitchens, and restaurants. Approximately 1.3 billion tons of food around the world is lost or wasted each year FAO (2015). The disposal of food wastes has become a major concern and burden in India because of environmental law which prohibited the sanitary land filling of food waste since 2005. Therefore, alternative methods bioremediation of food wastes without causing secondary environmental problems became an important issue for many years (Kwon et al 2014). As food wastes contain various organic compounds, microorganisms can consume and reduce the amounts of food wastes. There is extensive research on food waste treatments using various microorganisms, such as aerobic and anaerobic fermentation Shudharshan et.al (2007). Among the various microorganisms, bacteria are widely considered for food waste treatment because various bacteria are found in food wastes. During fermentation of food wastes, mesophilic and thermophilic bacteria, including Pseudomonas spp., Xanthomonas spp., Bacillus spp., and Stearothermophilus spp., are often found in food wastes J. Jeong et.al (1999) and Fujio *et al.*1991. (Yi *et al.* 2006) reported that thermophilic bacteria exhibit good activities to grow and decompose food wastes effectively because temperature increased during fermentation to 50^0C during fermentation. Kim *et al.* 2011 isolated thermophilic Bacillus *subtilis* (B. *subtilis*) from various organic materials for food waste treatment.

This research attempted to isolate pedogenic amylase producing bacteria. The specific objectives of the research were to isolate and identify amylase enzyme producing bacteria responsible for food waste treatment and to characterize enzyme for optimum condition of activity..

II. MATERIALS AND METHODS

Soil samples were collected from different sites of wastes dump sites near potato and food

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waste dumpsites like potato fields, cold storage vegetable markets etc. . in lucknow regions. . Serial dilution was made by One gram of soil sample was serially diluted in sterilized distilled water to get a dilution factor from 10^{-1} to 10^{-6} Which is followed by aseptic transfer of 0.1 ml of each dilution to nutrient agar medium supplemented with 1 % starch. The plates were incubated in incubator adjusted at temperature 37°C for 24 hours. Pure culture of the isolates was obtained by further sub culturing on starch nutrient agar medium. Isolated pure bacterial isolates was preserved on starch nutrient agar slants were stored at 4ºC.

Screening of potent amylase producing bacteria

Screening of bacterial isolates was made for amylolytic activity by starch hydrolysis test on starch agar plate supplemented with 1% starch. The culture microbial isolates were poured in wells on the starch agar plate and incubated at 37°C for 48 hrs. After incubation iodine solution was flooded with dropper for 30 seconds on the starch agar plate. Presence of violet colour around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The isolates produced clear zones of hydrolysis were considered as amylase producers and there approximate amylase producing ability was measured by measuring clear zones diameter. (Table no.1).

Morphological and Biochemical Characteristics

The bacterial isolate with maximum diameter and frequency of occurrence was subjected for further identification. Bacterial isolates were subjected for various morphological and biochemical tests like Gram staining, motility, endospore staining, Methyl red-Vogues Proskauer's Test, citrate utilization, catalase, and amlohydrolysis out for possible identification of bacteria.

Production and partial purification of Amylolytic enzyme:

The bacterial isolate with maximum diameter and frequency of occurrence was was subjected further characterization. The selected Bacillus isolate was propagated at 37°C for 22 h in 50 ml of 8% (w/v) of starch medium in a 250 ml flask. The flask was incubated in a shaker incubator, operated at 120 rpm and at 30°C. After the incubation period, the resultant broth was centrifuged at 10,000 rpm for 15 min and the supernatant was collected as the source of crude enzyme (amylase). To partially purify the enzyme extract, a solution of 65% (w/v) of sodium sulphate was added to the clarified supernatant, centrifuged at 10,000 rpm and the pellet was suspended in 5mM Na_2HPO_4 (pH 7.0). The purified enzyme extract was used in subsequent assays.

Production medium (Minimal Agar Medium as described by (K. R. Aneja) were used with slight modification which contained (g/l) Trypticase 10gm, peptone 5gm, $(NH_4)_2SO_4$ 3gm, K_2HPO_4 2gm, $MgSO_4$ 0.2gm. Starch 5gm. ph of media was adjusted at 7.0. The culture medium was sterilized in autoclave at 121^0 C for 25 min. Medium was inoculated with bacterium showing maximum screening activity and placed in incubator shaker overnight. During incubation incubator was maintained at at 37^oC for 24 hour for growth of bacterial culture. After fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Purification of Enzyme

The crude enxyme was purified using ammonium sulphate precipitation method. The crude extract is treated with different concentration of Ammonium sulphate. In this 30% and 50% saturation of Ammonium sulphate was used for purification. Ammonium sulphate is slowly added to the extract with continuous stirring to precipitate out the enzyme. The mixture is the centrifuged at 4000 rpm for 10 mins. Both supernatant and pellet was taken in separate microfuge tube and checked for enzyme activity.

Amylase Activity and protein content measurement: The activity of the partially purified amylase enzyme was determined by using soluble starch as substrate as per the method described by (Miller et.al) using Dinitrosalicylic acid (DNSA) reagent. The DNSA reagent was prepared by adding 1.8 g of 3, 5 - DNSA to 20 ml of 1.0 N NaOH and 60 ml of distilled water. Potassium sodium tartrate (60 g) was added and the mixture was diluted to 200 ml with distilled water. The reducing sugar content from hydrolyzed starch by amylase enzyme was analysed by adding 2 ml of 3.5 DNSA reagents to 1 ml of the sample. The mixture was heated in boiling water for 5 min to stop the reaction and then cooled at room temperature. The absorbance at 540 nm of the resulting coloured solution (redish brown) was taken in a spectrophotometer against a blank; Blank was prepared by substituting the hydrolyzed sample (Maltose) with distilled water. The reducing sugar content was subsequently determined by using reference to a standard curve of known maltose concentrations.

International Journal of Engineering, Management and Humanities (IJEMH)
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Enzyme activity was defined as the amount of maltose produced from starch hydrolysis by 1 ml of enzyme in 1 min.

Effect of temperature on enzyme activity:
The optimum temperature for enzyme activity was determined by measuring enzyme activity, as described above, between 0^0C -100^0C. Thermo stability of the enzyme was determined by maintaining the enzyme extract in refrigerator and water bath at different temperatures (20 - 100°C) for 30 min.

Effect of pH on enzyme activity:
The optimum pH for enzyme activity was determined over a pH range of 2.0 - 10.5 on 1% starch medium, using 0.05 M sodium phosphate as buffer solution. The optimum pH was determined by incubating the enzyme reaction mixture in a water bath at 37^0C and the enzyme activity was then assayed as described above. The pH was adjusted by the help of 0.1 N HCl and 0.1 N NaOH. for maintaining acidity and basicity of solution respectively.

Effect of substrate concentration on enzyme activity:
The substrate concentration for enzyme activity was determined over a substrate concentration range of 0.2 – 2.5% starch, using 0.05 M sodium phosphate as buffer solution. The optimum substrate was determined by incubating the enzyme reaction mixture in a water bath at 37^0C and the enzyme activity was then assayed as described above.

III. RESULTS AND DISCUSSION

Three *Bacillus* species were isolated and identified from the collected soil samples from potato dumpsites near vegetable markets and cold storage. The morphological and biochemical examination showed the presence of *Bacillus subtilis* (R2, R3, A5 and R7). *B. Polymyxa* (R6), *B. megaterium* (R1, R4 and R8). These isolates were analyzed by gram-staining, morphology, endospore staining and found that all were Gram-positive, rod-shaped, spore formers and Starch hydrolyzers. The amylolytic activity of Bacillus isolates according to their clear zones produced due to starch hydrolysis is presented in Table 1. R7 (*Bacillus subtilis* had the highest clear zone diameter (3.5 mm), and *B. Megaterium* (R4) had the smallest (1.2 mm). Since the difference between the isolates resides in the soil origin and taxonomical features of isolates, it is not easy to define whether amylolytic variation between the strains was the consequence of genetic makeup or because of environmental effects on the same micro organisms as environmental factors affects the biochemical characters. Since the distinction between the strains lies in the soil origin and taxonomic characteristics of isolates, it was not possible to define whether amylolytic variation between the strains was the consequence of species variability or environmental effect on the same microorganisms (Oyeleke and Oduwole, 2009). Similarly Bertrand *et al.* (2004) reported difference in amylolytic zones produced by different yeast strains isolated from starchy soil. The strain R7 identified as *Bacillus subtilis*, which showed the highest amylolytic halo, was selected for further study. The isolated *B. subtilis* had the highest frequency (50%), while *B. polymyxa* had the lowest frequency (13%) (Table 2 and Figure 1).

The growth and amylase activity of A7 (*B. subtilis*) in 1 % starch fermented broth is shown in Figures 1 and 2. There was a gradual increase in growth of *Bacillus subtilis* in the starchy medium, probably as a result of its ability to utilize the starch following its amylolytic activity to maltose. These results shows similar pattern of findings with the report of Oguntimehin (1998) regarding the use of *Bacillus licheniformis* isolated from waste cassava sites for the production of amylase. The activity of amylase produced increased from 0 to 48 h with a amylase activity of 0.7 mg/ml (Figure 2). These results found a comparable trend in amylase production by *Shwanniomyces occidentalis*, which was isolated from potato fermentation Kocher and Katgal (2008). The temperature stability result of amylase obtained from *Bacillus subtilis* (R7) is shown in (Figure 4). The figure illustrate that the enzyme was stable between 20 and 70^0C with maximum activity at 35^0C.

Table 1. Amylase Activity of Bacterial isolates

Codes fo isolates	Possible identification	Clear zone shown by amylase activity (mm)
R1	*Bacilus megaterium*	2.3
R2	*Bacilus subtilis*	2.1
R3	*Bacillus subtilis*	1.8
R4	*Bacillus megaterium*	1.2
R5	*Bacilus subtilis*	1.4

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R6	*Bacilus polymyxa*	2.4
R7	*Bacilus subtilis*	3.5
R8	*Bacilus megaterium*	2.5

The enzyme stability decreases at temperatures above 70°C. The stability of enzyme pattern in present study agrees with the behaviour of amylases from *Bacillus sps.* investigated by Cordeiro *et al.* (2002), in which a soluble starch medium was used. The amylase in our study seems more thermostable than those reported by other researchers who reported 70 oC (Oyeleke and Odunwole, 2009) and 37 oC (Dhanya *et al.*, 2006). A pH 6.0 was recorded as the optimum for the production of this amylase. This result is similar to that of other researchers. Oyeleke and Odunwole (2009) reported optimum pH 6.5 and 7.5 for *Bacillus sp* from cassava waste while Anturin *et al.* (1990) reported a pH range of 5.5 – 6.0 for *Bacillus licheniformis*. The results suggest that there is a stimulation of enzyme at pH towards neutrality. Sudharhsan *et al.* (2007) also made similar observation. Starch and food Industries that exploit amylase for operations in acid environment will find the enzyme in this study worthy harnessing.

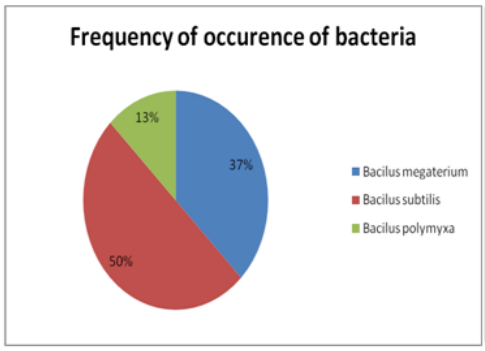

Figure 1 Frequency of occurrence of bacteria

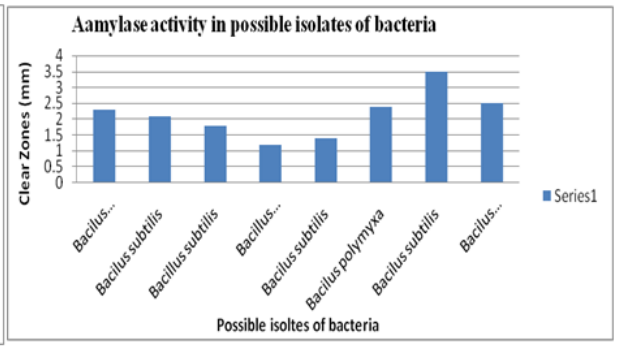

Figure 2. Amylase Activity of Bacterial isolates

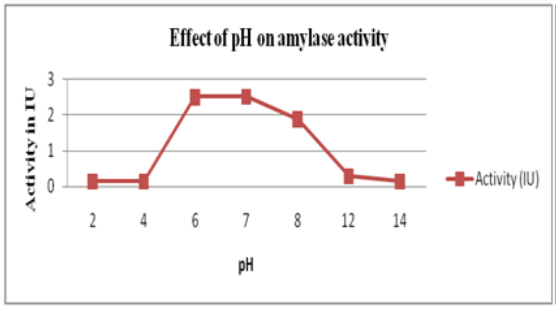

Figure 3: Effect of pH on amylase activity

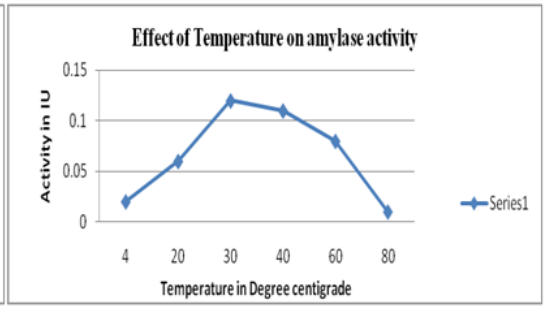

Figure 4: Effect of Temperature on amylase activity

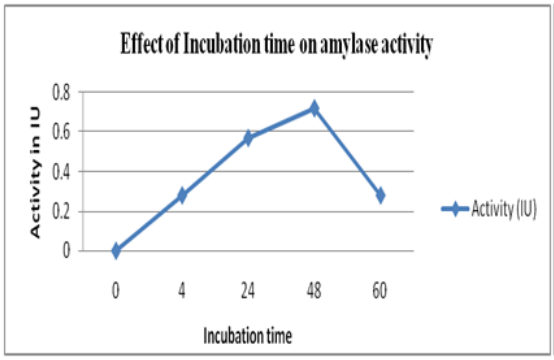

Figure 5: Effect of Temperature on amylase activity

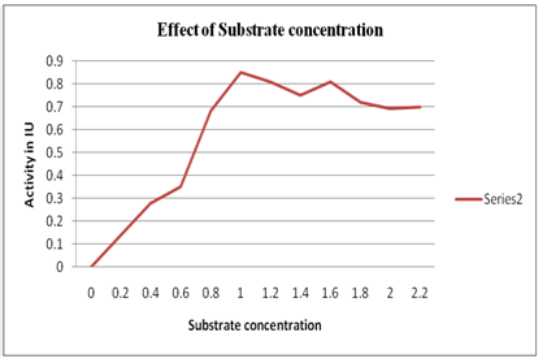

Figure 6: Effect of substrate concentration on amylase activity

The enzyme stability decreases at temperatures above 70°C. The stability of enzyme pattern in present study agrees with the behaviour of amylases from *Bacillus sps.* investigated by Cordeiro *et al.* (2002), in which a soluble starch medium was used. The amylase in our study seems more thermostable than those reported by other researchers who reported 70 oC (Oyeleke and Odunwole, 2009) and 37 oC (Dhanya *et al*., 2006). A pH 6.0 was recorded as the optimum for the production of this amylase. This result is similar to that of other researchers. Oyeleke and Odunwole (2009) reported optimum pH 6.5 and 7.5 for *Bacillus sp* from cassava waste while Anturin *et al.* (1990) reported a pH range of 5.5 – 6.0 for *Bacillus licheniformis*. The results suggest that there is a stimulation of enzyme at pH towards neutrality. Sudharhsan *et al.* (2007) also made similar observation. Starch and food Industries that exploit amylase for operations in acid environment will find the enzyme in this study worthy harnessing.

IV. CONCLUSION:

Bacteria isolated from food, wastes and soils were identified as three different *Bacillus* species. The highest potential nd thermostability were achieved by *B. subtilis* R7 and *B. megaterium* R1. They were found a good source of amylase enzyme and may have great potential to degrade food and vegetable wastes as well as source of industrially important enzymes.

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