Journal of Applied Biology & Biotechnology Vol. 10(5), pp. 193-197, Sep-Oct, 2022
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2022.100524

Isolation and characterization of starch degrading bacteria from disparate soil samples

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ARTICLE INFO

Article history:
Received on: December 05, 2021
Accepted on: May 17, 2022
Available online: July 20, 2022

Key words:
Amylase,
16srRNA,
Enzymes kinetics,
Optimum temperature and pH.

ABSTRACT

Amylases are starch degrading enzymes. These enzymes play a pivotal role in the biotechnology industries in food, fermentation, textiles, and paper production. Many industries lack local amylase supply, so the demand for amylase is often high. The study focuses on the screening and isolation of amylase producing bacteria from disparate samples of soil. The promising hydrolytic strains of bacteria from the collected samples were characterized using enzyme kinetics and further investigation is carried out to characterize the amylase enzyme produced by optimization of pH and temperature. The shortlisted isolate was identified using 16s rRNA sequencing. This study is an initial result of an exploration where soil is a source of industrially important amylase producing bacterial strains.

1. INTRODUCTION

Microorganisms play a crucial role in soil fertility due to their ability to perform biochemical transformations, which have rendered them effective sources of industrial enzymes [1,2]. The production of microbial amylase from microscopic organisms depends on the type of strain, medium arrangement, method of cultivation, cell growth, nutrient requirements, incubation time, pH, temperature, metal particles, and thermostability. Thermostability is an ideal trait of the majority of the industrial enzyme. The vast majority of strains used for the production of bacterial amylase are also with an optimum pH between 6.0 and 7.0 for growth and enzyme production [3].

Amylases are hydrolyzing catalysts that cause hydrolysis of starch molecules. Amylase is also a digestive enzyme and preferred because of its vast availability and plasticity [4]. They are categorized as exo-acting, endo-acting, and debranching compounds. Surprisingly, bacterial amylase is present in acidophilic, alkalophilic, and thermoacidophilic bacteria. Among them, β-amylase is exo-acting, while α-amylase is an endo-acting enzyme [5].

Exo-amylase or exo-hyrolases - are delivered from the cell and follow-up on the substrates. These are essentially hydrolytic compounds that debase by the expansion of high atomic weight substrates (e.g., polysaccharides, lipid, and proteins) into little parts (e.g., glucose) that can go into the cell and are later acclimatized. Enzymes required for the hydrolysis of cellulose, starch, lipid, casein and gelatin have a place with the classification of exoenzymes [6].

Endo–acting or endo–hydrolases - are utilized by the cell for further metabolic degradation of carbohydrates and are mainly responsible for synthesis. New protoplasmic necessities and creation of cell vitality from absorbed materials and these proteins work inside a cell [6]. Alpha amylase has broad application in starch preparing, fermenting and sugar creation, in detergent manufacturing processes [7]. The Pancreas likewise makes amylase (alpha amylase) to hydrolyze dietary starch into disaccharides and tri-saccharides which are converted by other enzymes to glucose to supply body with energy [8].

Some halophilic microorganisms produce enzymes that have optimal activity at high salinities and could thus be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions. Furthermore, most halobacterial enzymes are extremely thermotolerant and can be kept at room temperature for extended periods of time. *Chromohalobacter sp., Halobacillus sp., Haloarculahispanica, Halomonasmeridiana, and Bacillus dipsosauri*have all been found to produce halophilicamylases [9].

Due to the ever-increasing demand for this enzyme, industries are trying to increase the efficiency of amylase utilizing several approaches, such as the selection of high enzyme strains, process optimization, and the use of a cheap substrate [10]. The purpose of this study is therefore to isolate and characterize a bacterial strain that can produce amylase with the desired characteristics that can be used in the industry.

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2. MATERIALS AND METHODS

2.1. Sample Collection

Three variant soil samples: Rhizosphere root nodules, Lake soil sample, and Garbage soil sample.

2.2. Reagents

Distilled water, Conical flask, measuring cylinder, test tubes, Petri plates, Starch agar media, Iodine stain, Crystal violet reagent, Grams iodine reagent, Decolorizing agent: ethanol, Counter stain: safranin, Slide, microscope 100X, oil. Slide, wire gauge, bunsen burner, Malachite green, Adsorbent paper, Safranin. LB Broth, magnetic stirrer. Glucose stock solution (10 mg/ml of distilled water), Dinitrosalicylic Acid Reagent Solution, Potassium sodium tartrate solution(40%), Starch Solution (1%), DNS reagent solution, Rochelle salt (40%), α-amylase solution (1%), and pH buffers ranging from 4 to 9.

2.3. Isolation and Screening of Amylase Producing Organisms

Fifteen grams of nutrient agar were suspended in 100 cm^3 distilled water. The solution was boiled to dissolve agar completely. 40 g of soluble starch was heated in 100 cm^3 of distilled water to form a suspension. The solutions were allowed to cool and then mixed. The starch agar media was prepared and sterilized. The sterilized starch agar media was poured into Petri plates and allowed the media to solidify. 1 g of soil sample was collected from the rhizosphere of root nodules, which was further dissolved into 99 ml of sterile distilled water (1/100 dilution). Series of 5 test tubes were labeled as T1, T2, T3, T4, and T5. Test tubes containing 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions were prepared. 0.1 ml of concentration sample from 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilution was transferred onto the starch agar plate. Using the sterile spreader, the sample was evenly spread and the plates were incubated in 37°C incubator for 48 h. Same procedure was repeated for two other samples which include lake soil sample and garbage soil sample collected from different locations.

2.4. Sub Culturing and Screening of Amylase Microorganism

Autoclaved starch was poured in petri plates and allowed to solidify. Pure culture was obtained by streaking the isolated colonies on petri plates. As per the zone of clearance, dilutions showing maximum zone of clearance were considered for sub culturing. Regular sub culturing was performed in a span of every 3 days to obtain pure culture. Sub culturing was performed for all samples. The plates were flooded with iodine solution to screen and isolate starch hydrolyzing colonies showing clear zone around it. This protocol was repeated for two other soil samples, garbage waste sample, and lake waste sample.

2.5. Gram Staining Method and Endospore Staining

The cells were obtained from various samples and then Gram stained with crystal violet staining reagent. Using crystal violet stain, the cells were colored, followed by the addition of mordant: Gram's iodine. It was then flooded with decolorizing agent, ethanol. The slide was finally flooded with counter stain, safranin. The results of the staining procedure were observed under oil immersion using a 100× microscope. On completion of the Gram Stain, Gram-negative bacteria were stained pink/red, and Gram-positive bacteria were stained blue/purple. The procedure was performed for all the three samples [11].

Smears of organisms were prepared to be tested for presence of endospores on a clean microscopic slide which was allowed to air dry. Blotting paper was heated and a drop or two of malachite green was added. The blotting paper was removed from the burner and the slide was allowed to cool to room temperature for 2 min. The slide was rinsed thoroughly with distilled water. The smear was stained with safranin, which acts as a counter stain for 2 min. It was observed under 100× microscope using oil emulsion. The experiment was performed for all the three samples [11].

2.6. Isolation of Amylase Enzyme

LB broth was measured and prepared in conical flasks with space for the organisms to grow. They were inoculated with the cells which were sub cultured and allowed to grow for 24 h in magnetic stirrer at 37°C. After 24 h, LB broth was centrifuged. Supernatant was stored in fridge until further use [12].

2.7. Estimation of Glucose by Dinitro Salicylic Acid (DNS) Assay Method

The glucose standards were prepared ranging from 0.1 ml to 1 ml using dry clean and labeled test tubes (for all the three samples). 3 ml of Dinitro Salicylic Acid reagent was added to all the test tubes and incubated till brick red color appeared, followed by addition of 1 ml of 40% potassium sodium tartrate solution. Optical density was measured at 540 nm [12].

2.8. Effect of pH on Amylase Enzyme Activity

0.1M pH buffer solutions were prepared ranging from pH 4 to pH 9 using different chemicals. 1 ml of 1% starch solution was taken in test tubes, followed by addition of different pH buffer solution in all test tubes. Test tubes were incubated for 30 min to which 1 ml of α-amylase enzyme solution was added. 3 ml DNS reagent was added and the test tubes were incubated at room temperature for optimum time. Next step involved addition of SPT. Optical density was measured at 540 nm using spectrophotometer [13].

2.9. Effect of Temperature on Amylase Enzyme Activity

1 ml of 1% starch solution and 1 ml of 0.1 M 7 pH buffer were taken in test tubes.1ml of α-amylase enzyme solution was added and incubated. 3 ml of DNS reagent was added and incubated followed by SPT. Optical density was measured at 540 nm using spectrophotometer [13].

2.10. Enzyme Kinetics

1 ml of 1% substrate starch solution was taken along with pH buffer. To this 1.0 ml of α-amylase enzyme solution was added and incubated. 3 ml of DNS reagent was added followed by addition of SPT. Optical density was measured at 540 nm using spectrophotometer.

2.11. PCR Analysis and *16s rRNA*

All reagents were thawed on ice. Reaction mix was prepared and buffer was added (1x). Taq polymerase 0.05 units/µL and dNTP mix 200 µM were added. $MgCl_2$ was added0.1-0.5 mM followed by forward and reverse primer 0.1−0.5 mM. Template was added about 200 pg/µL along with addition of DMSO. Reaction mix was assembled into 50 µL volume in a thin walled 0.2 mL PCR tubes. It was gently mixed by tapping tube and also centrifuged briefly. Negative control reaction was prepared without template DNA. Results of PCR reaction were observed through gel electrophoresis [14].

The trimmed and corrected 16s rRNA gene sequence of sample 1 was added to NCBI Nucleotide BLAST website and the list of 100 sequences which were most similar to query sequence were obtained [14].

3. RESULTS AND DISCUSSION

The three isolates, namely, Isolate 1- Rhizosphere root nodules, Isolate 2-, Lake soil sample and Isolate 3-Garbage soil sample were found to be Gram negative long or short rods [15]. Gram-negative bacteria are usually *E. coli*, *Klebsiella*, *Acinetobacter*, and *Pseudomonas* spp. Endospores are usually seen as oval or spherical structures within the stained cell. Spore structures are resistant to heat, radiation, chemical, and any other agent that is lethal to the organisms. The function of endospore staining is to differentiate between bacterial spores and other vegetative cells and to differentiate spore formers from non-spore. All the isolates showed formations of green spores. By spore formations, bacteria can survive in hostile conditions. The isolates may belong to groups such as *Bacillus* and *Clostridium sp.* The crude extract of isolate cultures was used for kinetics and optimization studies.

Temperature is an important physical factor that influences enzyme production. Among the selected four temperatures [Figure 1], the optimum temperature was determined to be 45°C. Isolate 1 showed a higher rate of reaction at this temperature. At higher temperatures than 45°C, bacterial growth is suppressed and, as a result, enzyme production is also inhibited [16]. According to the previous study (Optimum temperature = 40°C), there is a decrease in enzyme activity as the incubation temperature rises. There is also a positive correlation between growth/enzyme output and incubation temperatures of up to 45°C, accompanied by a steady decrease in growth/enzyme production thereafter [17]. Another study also shows that the thermal stability of alpha-amylase is stable up to 50°C and some at 40°C after 15 min of incubation [18]. However, a range of the incubation temperature of 40°C–45°C was seen in the 3 isolates with isolate 1 having a higher rate reaction at 45°C. The three bacterial isolates were optimized for their pH by allowing growing in media of different pH ranging from 4.0 to 9.0 [Figure 2]. Isolate 3 showed maximum activity at pH 6 while the other two isolates had lesser reaction rates compared to isolate 3.There are many species with different pH optima. A decrease or increase in pH results in low microbial growth on either side of the optimum value [19]. The optimum pH was found to be 6 which results in higher amylase production and increased cell growth. Other studies have found that *Pseudomonas mendocina* developed a maximum amylase activity at pH 7.The enzyme activity can either decrease or denature if the pH is below or above 7 [19]. Similar work was noted when amylase activity was recorded at different pH ranges (5-10). The activity of amylase at base pH=10 increases and the acidic medium decreases when pH=5 [20]. Therefore, at neutral pH, there is a stimulation of enzyme synthesis, high enzyme production, and increased cell growth [19]. The enzyme activity of each bacterial species is determined by its growth temperature. Likewise, differences in pH requirements among species are dependent on the susceptibility of their individual enzyme systems due to the denaturation process at various pH levels. As a result, determining the ideal pH and temperature of a bacterial species for effective enzyme synthesis is essential. Using the incubation temperature of 45°C and pH of buffer as 6, Enzyme kinetics [Figure 3 and Table 1] was performed for the three isolates and it was found that enzyme reaction rate is highest for Isolate 1, that is, rhizosphere of root nodules. The Vmax was 12.82*10^{-4}mmol/ml.min which was at least 1.5 × 10^{-4} mmol/ml.min higher than the other two isolates. The Km also was lower than the other isolates indicating greater affinity to the substrate like amylose/ sugar. Therefore, Isolate 1 was further identified by colony PCR and 16srRNA sequencing. BLAST was performed for the 16srRNA forward and reverse sequence and the organism was found to be *Bacillus sp. B51f*. The results obtained make it possible to conclude that an isolate obtained from the rhizosphere of plants was *Bacillus sp. B51f* which has potential to produce an industrially relevant amylase enzyme. Originally found in the gut of fishes and known for probiotic effects,

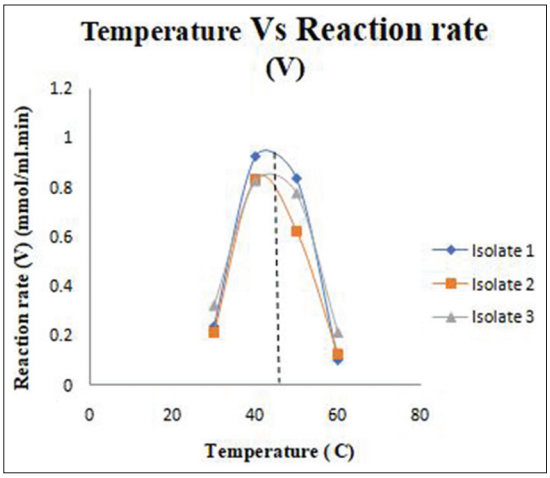

Figure 1: Amylase activity at different temperature.

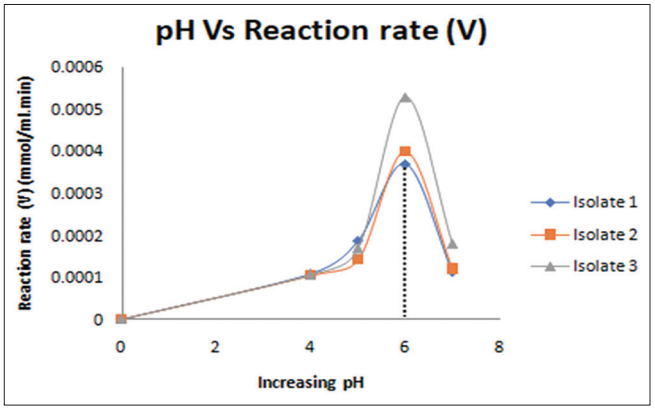

Figure 2: Amylase activity at different pH.

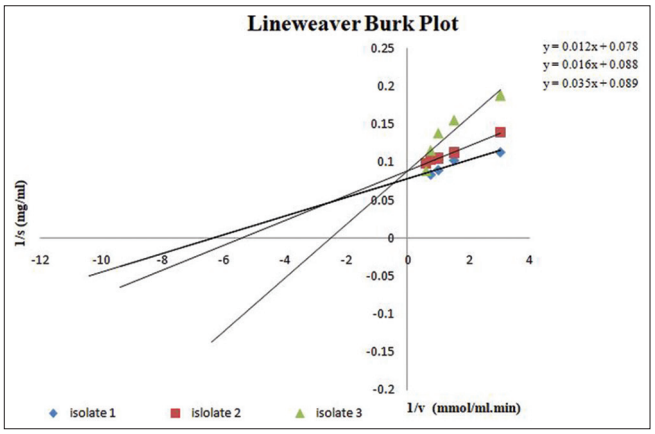

Figure 3: Enzyme kinetics activity of amylase.

Table 1: Enzyme kinetics values for the three bacterial isolates.

Isolate	K_m (mg/ml)	V_{max} (mmol/ml.min)
Isolate 1	0.15	12.82×10^{-4}
Isolate 2	0.18	11.36×10^{-4}
Isolate 3	0.39	11.23×10^{-4}

the sample of soil obtained would have a pond rhizosphere mixed in it [21]. Bacillus is a facultative anaerobic, spore-forming bacterial species usually obtained from the soil. Probiotics mean substances that are beneficial for the human body and Bacillus species is considered one of the top three probiotic producing organisms presently being consumed [22]. *Bacillus sp. B51f* is known for its Inulin as enzyme activity recently in Food industry. There has been no published work on amylase production in *Bacillus sp. B51f* bacteria and hence this can be considered as a novel result. Thermophilic bacteria and fungi are efficient producers of thermostable amylases. Thermostable alpha amylases have been isolated from a variety of bacterial strains and produced with both SmF and SSF. However, it has been discovered that the use of SSF is more advantageous than the use of SmF and allows for the production of enzymes at a lower cost. The production of alpha amylase by SSF is limited to the genus Bacillus. *B. subtilis, B. polymyxia, B.mesentericus, B. vulgarus, B. megaterium,* and *B.licheniformis* have been used for amylase production in SSF. In the starch processing industry, thermostable amylases from *Bacillus stearothermophilus* or *Bacillus licheniformis* are currently used [23]. For an efficient large scale production of amylases, the structural and functional relationships of these enzymes have to be studied and correlated. This will be further facilitated by implementation in established fermentation processes with the isolate *Bacillus sp. B51f* and complementation with other biotechnological aspects. Further investigations like biochemical characterization, effect of carbon and nitrogen sources can be performed for a better understanding of amylase production from B*acillus sp. B51f.* Previous studies suggest that several Bacillus species have been examined for amylase production and are suitable for future use in various industries [23]. The majority of the starch-degrading bacterial strains revealed a pH range of 6.0 to 7.0 for normal growth and enzyme activity. Similarly, a study found that *Bacillus sp.* 3.5AL2 and *B.amyloliquefaciens* had optimal pH values of 7.0 and 7.024. Nevertheless, Bacillus sp. WA21 showed the optimal pH of the amylase at 6.0 which is less than that found in the other two mentioned bacterial species. This type of research is essential in the development of commercial amylase production [24]. Statistically, the organisms (*B. subtilis, B. licheniformis, A. niger,* and *A. fumigatus*) showed significant growth with moderate temperature but decreased with increasing temperature (Pearson correlation).In general, as incubation time increases, so does enzyme activity. Except for *B.licheniformis*, an increase in pH reduces the enzyme activity [25]. Thus further optimization studies in *Bacillus sp. B51f* for fermentation, enzyme purification, thermostability, influence of different metal ions, and substrates and efficiency will mostly yield positive results.

4. CONCLUSION

The essence of this work was to bridge the gap between efficient and cost effective amylase bacteria and the everlasting search for industrially useful amylase producers. This work is an initial study to discover isolates with never before studied amylase properties and we suggest that *Bacillus sp. B51f* is a hidden potent industrially sound amylase producer.

5. ACKNOWLEDGMENT

We the authors thank the Biotechnology Department of SRMIST for all its support and encouragement to do this work.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

There is no funding to report.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

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How to cite this article:
Srivathsan V, Bhandari M, Swaminathan P. Isolation and characterization of starch degrading bacteria from disparate soil samples. J App Biol Biotech. 2022;10(5):193-197. DOI: 10.7324/JABB.2022.100524