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Screening, Isolation, and Enzyme Kinetics of Bacterial Amylase collected from Rhizosphere soil

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ABSTRACT

Rhizosphere is a region where microbial communities are in complex association with the roots of plants where the activity of microbes and their enzymes are greatly influenced by root exudates. Amylase enzyme has great importance in biotechnology, with enormous utilization in food, fermentation, textile, and paper industries. They are produced intracellularly and extracellularly by different life forms including microorganisms. Microbial amylases are preferred over other sources because of their vast availability and it also meets the growing needs of industry. The present investigation deals with the isolation, screening, and enzyme kinetics of bacterial amylase from the rhizosphere soil samples collected from a fertile field. Soil samples were collected from the rhizosphere and amylase-producing bacteria screening was carried out by using a starch agar plate. Extracellular amylase was extracted from fermentation broth followed by quantification by starch-iodine assay. Bacterial amylase enzyme kinetics were determined by changing enzyme/substrate concentrations and incubation time. We successfully screened and isolated out starch hydrolyzing colonies from the rhizosphere soil samples. Studies on enzyme kinetics indicate that the activity of amylase increased initially as substrate and enzyme concentrations increased. If we kept enzyme concentration constant, to a certain point, there was no change in enzyme activity as the enzyme was saturated and no more enzyme was available to react with the excess substrate. Initially, enzyme activity increased as enzyme volume increased, but since substrate concentration was kept constant, higher volumes of the enzyme could not speed up the reaction. Further, under a prolonged incubation period, less amount of substrate was available at the end of a reaction. Therefore, it is concluded that the reaction velocity increases.

Keywords: Bacterial amylase, Enzyme kinetics, Rhizosphere soil.

1. INTRODUCTION

Rhizosphere, is a region where microbial communities are in complex association with roots of plant, where the activity of its microbial partners and their enzymes are greatly enhanced due to root exudates (Raaijmakers et al., 2009). Microorganisms are the most important sources for exo-enzyme production. An exoenzyme function outside of that cell, and breaks down large macromolecules for allowing their constituents to get pass through the cell membrane. Amylase-producing bacteria and fungi can be isolated from the soil and foods. Although many microorganisms produce this enzyme, the following are acknowledged and recognized as amylase producers: *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus stearothermophilus, Bacillus cereus, Bacillus polymyxa, Bacillus coagulans*, *Lactobacillus plantarum*, *Pseudomonas spp, Proteus*, *Aspergillus oryzea*, *Aspergillus. niger*, *Penicillium expansum* (El-Fallal et al., 2012), (Parmar D and Pandey A 2012), (Dash BK et al., 2015).

Amylase is an enzyme that breaks down glycogen or starch into simple sugars by targeting on α- 1,4-glycosidic bonds (Aiyer, P. V., 2005). They are produced intracellularly and extracellularly by different life forms, extending from tiny microorganisms to higher plants and humans (Ravan et al., 2009). It has significant importance in the biotechnology industries with enormous utilization in food, fermentation, textile and paper industries (Pandey et al., 2000). Amylases are classified depending on how they break down starch molecules into three basic categories: α–amylase, β-amylase, and γ-amylase.

The microbial source of amylase is preferred over other sources because of its vast availability and it also meets the growing needs of industry (Lonsane BK & Ramesh MV 1990). Many microbial amylases are usually a mixture of these amylases. Microbial amylases are utilized in many industries such as in high Fructose Corn syrup preparation, additives to detergents for removing stains, saccharification of starch for alcohol production, brewing etc. (Khan JA & Priya R 2011).

Because of increasing interest for microbial amylase, biotechnologists are attempting to elevate the production of amylase through different perspectives including a selection of better strain and utilization of cheap substrate

(Bahadure et al., 2010). Therefore, the present investigation deals with the isolation, screening, and enzyme kinetics of bacterial amylase from the rhizosphere soil collected from different fertile grounds.

2. MATERIALS AND METHODS

2.1 Collection of Rhizosphere soil samples

Rhizosphere samples were collected from 3-5 cm depth from the ground surface from the fertile fields. Soil samples were collected into sterilized plastic bags from different locations of Karachi, Pakistan during spring season and were taken to the lab for screening and isolation of amylase producers.

2.2 Physio-chemical Analysis

Equipment for on-site measurements were calibrated and checked according to the instruction manual. Electrical conductivity and pH were measured using PASCO Wireless Conductivity Sensor PS-3210 and PASCO Wireless pH Sensor PS-3204 respectively. The total moisture content of rhizosphere soil was also measured.

2.3 Screening and Isolation of Amylase Producers from *Rhizosphere*

Soil samples were serially diluted and plated on nutrient agar plate supplemented with 1% (w/v) soluble starch and incubated at ambient temperature for 24 hours. Following incubation, plates were exposed to iodine crystals to isolate amylase-producing colonies that appeared on plates with cleared zone around them. The colonies were isolated and preserved on liquid media for further analysis (Atlas et al., 1995).

2.4 Separation of Extracellular Amylase from culture broth

Separation of extracellular amylase was performed by using 250 mL Erlenmeyer's flask containing broth media and isolated amylase producers. After incubation on a rotary shaker at 180 rpm for 24 hours, the culture broth was centrifuged at 6000 rpm and the supernatant was collected which served as the source of bacterial amylase (Asgher et al., 2007).

2.5 Quantification of Bacterial Amylase

In order to quantify bacterial amylase, the starch-iodine assay was used as described earlier (Fuwa H. 1954). Beer lambert law, Unitary method, and Gradient method were also used to quantify the enzyme produced by using the following equations:

2.5.1 Beer & lambert's law:

Used established law having formula

$$A = \text{Ɛ}\ b\ C$$

Where A = Absorbance (Optical density) (No units)

Ɛ = absorptivity of the dye (L g^{-1} cm^{-1})

b = length of the path (cm)

C = Concentration of the dye (g L-1)

2.5.2 Gradient Method:

$$m = gradient = \frac{y2 - y1}{x2 - x1} = \frac{Absorbance}{concentration}$$

2.5.3 3- Unitary Method:

$$C_T = \frac{conc.\ of\ sample * abs.\ of\ test}{abs.\ of\ sample} = \frac{0.1 * 0.00467}{0.36900} = 0.00124$$

2.6 Enzyme Kinetics

Modified method (Butré C.I & Wierenga P.A, 2014) was used to assess enzyme kinetics of bacterial amylase as follows:

2.6.1 Effect of substrate concentration

To determine the effect of substrate concentration on bacterial amylase enzyme, serial dilutions of 1 % starch as substrate was made. Then, 15 µL bacterial amylase was added to each tube containing starch. All tubes were incubated for 5 min. The reaction was stopped by adding 1 mL stop solution and optical density at 590 nm (OD_{590}) was measured.

2.6.2 Effect of enzyme concentration

Two fold dilutions of amylase were made to determine the effect of enzyme concentration. Then, each dilution

was added to the tube containing 1 % starch. All tubes were incubated for 5 min. The reaction was stopped and OD_{590} nm was measured as described above.

2.6.3 Determination of reaction velocity

For enzyme reaction velocity, 15 µL of bacterial amylase was added to the tubes containing 1 % starch as substrate. The tubes were incubated for 3 min, 6 min, 9 min, 12 min and 15 min respectively. The reaction was stopped by adding 1 mL stop solution at respective time and OD_{590} was measured. The tube without any incubation was marked as zero.

3. RESULTS AND DISCUSSION

The present investigation was aimed to isolate and screen out starch hydrolyzing bacteria and to study enzyme kinetics of bacterial amylase from the rhizosphere soil samples, whose physio-chemical parameters are mentioned in **Table 1**. Sasmita Mishra and Niranjan Behera (Sasmita Mishra & Niranjan Behera, 2008) isolated amylase producers from kitchen waste containing soil and growth conditions were evaluated. We successfully screened and isolated amylase-producing colonies from the rhizosphere soil samples as depicted in **Figure 1** and their colonial morphology was recorded as shown in **Table 2**. Quantification of extracellular amylase was carried out by using the starch iodine method. However, the production of bacterial amylase can also be magnified by using different growth media as described earlier (Ruth Rize et al., 2017). The dilutions of starch we prepared were not enough to produce data to build a standard curve that would allow us to detect very low absorbance of test runs. We should have made more dilutions of starch with lower concentrations to acquire more data and produce a better standard curve (Figure 2). However, we used the aforementioned three different methods to quantify bacterial amylase produced as exhibited in Table 3. One of the previous studies found that species of the genus *Bacillus* were capable of producing different types of amylases (Nusrat, A. & Rahman, S.R, 2007). However, this work does not focus on the identification of bacterial isolates.

Table 1: Physio-chemical Analysis of Rhizosphere Soil samples

Rhizosphere soil samples	pH	Electrical conductivity (uS/cm)	Moisture content
1	7.35	2763.7	37.25 %
2	7.12	1715.7	42.4 %
3	7.35	27.3	39.1 %

Table 2: Colony Morphology of Amylase producing Bacterial Isolates on Starch Agar Medium

Colony morphology						
Isolates	Colour	Density	Texture	Elevation	Shape	Margin
1	White	Translucent	Smooth	Flat	Irregular	Filiform
2	yellowish	Translucent	Smooth	Raised	Irregular	Entire
3	Orangish-Yellow	Translucent	Smooth	Flat	Circular	Undulate
4	White	Opaque	Smooth	Raised	Irregular	Lobate
5	Clear	Transparent	Smooth	Flat	Circular	Entire
6	Yellow	Translucent	Smooth	Flat	Irregular	Entire

Table 3: Quantification of Bacterial Amylase isolated from Rhizosphere Soil

	Method	Concentration of bacterial amylase
1	Beer lambert law	0.00126 M
2	Gradient method	0.00175 M
3	Unitary method	0.00124 M

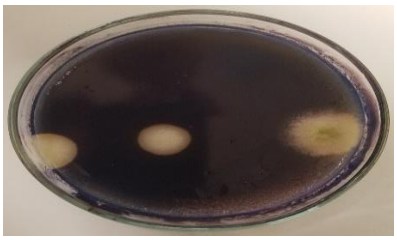

Figure 1: Screening of Amylase producers from Rhizosphere soil collected from Fertile field

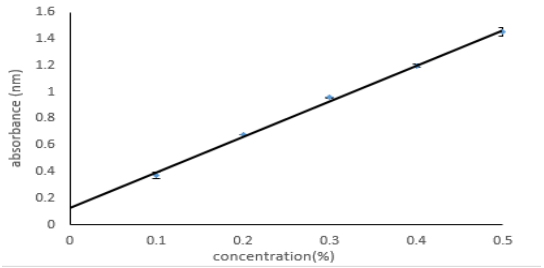

Figure 2: calibration curve of Starch

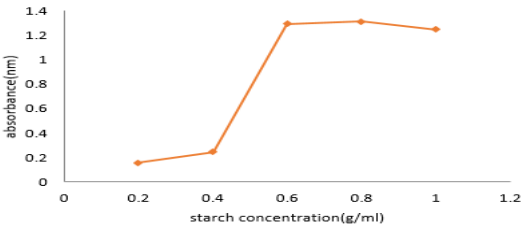

Figure 3: Effect of Substrate concentration

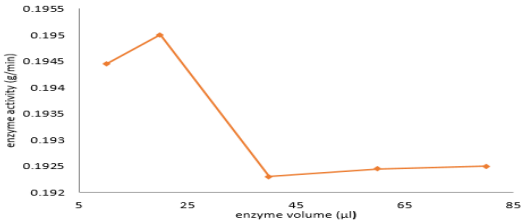

Figure 4: Effect of Enzyme concentration

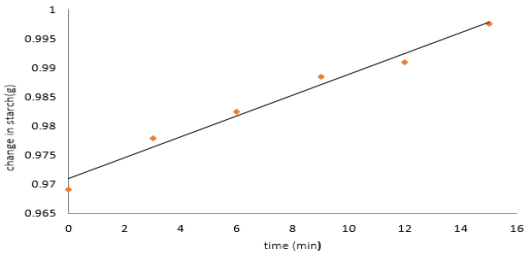

Figure 5: Enzyme activity of bacterial amylase (Time vs Reaction velocity)

Firstly, we identified the effect of starch concentration on the activity of our extracted bacterial amylase by changing substrate concentrations. Figure 3 shows that enzyme activity increases initially as substrate concentration increases. To a certain point, there was no change in enzyme activity as the enzyme was saturated and no more enzyme was available to react with the substrate. Likewise, Ruth Rize Paas Megahati *et al., 2017* showed that in the presence of excess substrate, the activity of enzymes is kept constant (Ruth Rize et al., 2017). The obtained results are somehow coinciding with the previous study which declared that the increase in substrate concentration did not affect the increase of enzyme activity (Halder et al., 2014).

Further, we determine the effect of enzyme concentration on substrate by keeping the starch concentration constant in an experiment (Figure 4). Initially enzyme activity increased as enzyme volume increased, but since substrate concentration was kept constant, higher volumes of enzyme could not speed up the reaction. This is because there was nothing for additional enzymes to bind to and all the substrate was converted to product leaving enzyme in its free state. Abdul Ghani *et al.*, 2014 showed that the concentration of the enzyme must be based on the substrate concentration used (Abdul Ghani Kumar et al., 2014).

To ascertain bacterial amylase reaction velocity, we decided to readjust the incubation time. As incubation time increased conversion of colored substrate into a colorless product was also increased due to which absorbance decreased linearly. This is because, at the end of the prolonged incubation period, less amount of substrate was available at the end of reaction. Therefore, it is estimated that the reaction velocity increases with time as shown in Figure 5.

4. CONCLUSION

Current study presents isolation and screening of starch hydrolyzing bacteria and to study enzyme kinetics of bacterial amylase from the rhizosphere soil samples collected from fertile ground. In commercially available amylase, cost, stability, and yield of amylase are the major obstacles. Thus, studies on amylase coming from microbial sources have been broadly concentrated by various researchers. Furthermore, there is a need the search for potent microbial amylases from different sources including plants, fruits, and rhizosphere soil. This work can be advanced by genetically enhancing the potential strains at a faster level, which can prove beneficial to different sectors including food processing, textile, paper, biofuel, and other biotechnological industries.

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