Identification and Biochemical Characterization of a Thermophilic Amylase from the Microbes in the Soil of Pathankot Region

Najuruddin, Daljeet Singh Dhanjal, Reena Singh Chopra, Chirag Chopra*

School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India.

Abstract

Amylases are highly prevalent in environment and perform an imperative role in the field of biotechnology, as they share around 25% of global enzyme market. This enzyme induces the hydrolysis of internal alpha 1,4-glycosidic bonds of starch to produce glucose. The wide range of the applications of amylases in the various processes of industries like fermentation, textile, food, paper, detergent and in pharmaceutical industries has impelled the exploration of the unique and extreme regions to isolate the amylase-producing bacteria. The current study focusses on isolating the extremophilic amylase-producing bacteria from the crop fields of Pathankot region. After isolation, the isolated amylase producing strain was optimized, for which optimal activity at 90^{o}C and pH 5.0. Additionally, the metal ion like $MnCl_2$ and $CoCl_2$ were found to be positive co-factor for enhancing enzyme activity, whereas, $ZnCl_2$ and $CaCl_2$ showed the inhibiting effect on the enzyme. Further, the bacteria showing the optimum activity was found to be gram negative and rod-shaped (phenotype similar with *Bacillus*).

Keywords: Amylase, Culture Dependent, Extremophile, Optimization, Pathankot

Introduction

Amylase is an important enzyme of the class hydrolases used for hydrolysis of starch to produce discrete products comprising dextrin and also the glucose units. Based on their mode of action, amylase is subdivided into debranching amylases, endo-amylases and exo-amylases **(Souza, 2010)**. It has been comprehended that amylase holds the 25-33% share in the world enzyme market and plays vital position in biotechnological industries **(Singh et al., 2016)**. Amylase has broad spectrum application in various industries like baking, brewing, paper, sugar and textile industry. Mesophilic amylase producing microbes have wide applications in industries **(Mehta and Satyanarayana, 2016)**. But, the application of mesophilic amylase gets limited because of other factors like extreme pH, high temperature, salt concentration, heavy metals and organic solvent which suppress the enzyme activity. In biotechnology field, microbes are believed to be important source of enzyme worthy for industrial process, particularly the enzymes obtained from extremophilic bacteria as they synthesize stable and significant enzymes **(Gopinath et al., 2017)**.

Among extremophiles, thermophiles are microbes which possess the ability to multiple and survive even at high temperature. Generally, these microbes inhabit geothermal site and hot spring, where the temperature is above 70°C **(Panda et al., 2013)**. Due to extensive cautions, time-consuming process and difficult in isolation and maintenance

process for isolating these extremophiles have restricted their expedition. Hence, their diversity and biotechnology ability have remained unexplored from diverse thermal habitats **(Azhar et al., 2014)**. Due to the growth of microbes at high temperature, these microbes might possess unique properties that enable them to synthesize chemically and physically stable enzyme. Recently, thermophilic microbes have gained significant attention worldwide because of their ability to synthesize thermostable enzyme possess broad spectrum application in food and pharmaceutical industries **(Di Donato et al., 2019)**. Therefore, exploring of microbes residing in these extreme environments and having the ability to produce amylase will enable us to isolate novel amylases matching industrial requirements.

This work focusses on isolating and characterizing the amylase producing thermophilic bacterial strain from crop field of Pathankot Region. Additionally, characterization of optimum culture parameter for optimum enzyme production will also be evaluated for isolated strains.

Material and Methods

Sample Collection

The soil sample was collected from crop fields of Pathankot region (Latitude-32.3143° N and Longitude-75.5975° E) using sterilized scrapper in clean polythene zip-lock bags and stored at 4°C. On transferring the soil sample to the lab, the soil was dried in the oven at 45^{o}C and was stored for further use.

Isolation of Amylase Producing Bacteria

About 0.2 gram of dried soil collected from crop fields of Pathankot region was added in 50ml of 2x-YT broth supplemented with 0.5% Starch and was incubated at 70^{o}C for 72 hrs. The prepared inoculum was later spread on Luria Agar plates and incubated at 37^{o}C for 96 hrs. After that, the morphologically different colonies were re-streaked onto Starch Agar plates in duplicates and grown for 48 hrs.

Primary Screening, Secondary Screening and Morphological Identification for Amylase-producing Bacterial Isolates

Primary screening of the amylase producing was done by observing the hydrolysis zone around the isolated colonies after staining it with I_2/KI solution. Later, Secondary screening was conducted to determine the localization of the enzyme. Colonies showing the positive result for amylase were cultured in growth media. And after growth, the media was centrifuged. Then lysis of the cell-pellet was done by using bead-beating lysis to obtain cell-free extract (CFE). Both Media and CFE were further evaluated for amylase activity via standard DNSA method **(Miller G.L., 1959)**. Furthermore, enzyme activity was determined by comparing the value of DNS assay with glucose standard curve in Figure 2a. One unit of amylase enzyme can be defined as the amount of enzyme which liberates 1µmole of reducing sugar (glucose equivalent) per minute under standard assay conditions (**Kaur *et al*., 2017**). This test was performed in triplicates and to identify whether the isolated strain is of gram +ve bacteria or gram –ve bacteria, Gram Staining was done according to the standard protocol of **Cappucino and Shermann (2008)**.

Biochemical Characterization of Amylase

Effect of temperature on amylase activity

The temperature optima were determined by assessing the amylase activity at different temperature ranging from 10-120°C. For this enzyme extract containing 500µl of 50mM of Lysis buffer, 300µl of 0.5% starch solution and 200µl of enzyme was incubated at varied temperature from 10-120°C for 30 mins. The enzyme activity was determined by following the standard DNSA protocol (Miller G.L., 1959).

Effect of pH on amylase activity

The pH optima were determined by assessing the amylase activity at different pH ranging from 3-12. For this enzyme extract containing 500µl of 50mM of Lysis buffer, 300µl of 0.5% starch solution and 200µl of enzyme was used. In this different type of buffer of varied pH were used, i.e. Acetate Buffer (pH 3-4), Citrate Buffer (pH 5-6), Phosphate Buffer (pH 7), Tris-HCl Buffer (pH 8-9) and Glycine-NaOH Buffer (pH 10-12). The reaction mixture was then incubated at 90^{o}C for 30 mins. The enzyme activity was determined by following the standard DNSA protocol (Miller G.L., 1959).

Effect of Metal ions on Amylase Activity

The metal ion effect on the amylase activity was determined in the absence and presence of metal ions (2mM or 5mM). The metal ions used for the analysis are as followed i.e. $CaCl_2$, $CoCL_2$, $CuCl_2$, $FeCL_2$, $FeCl_3$, $HgCl_2$, $MnCl_2$, and $ZnCl_2$. In order to assess the metal ion effect on the amylase effect, the reaction mixtures were incubated for 30 mins at 90^{o}C. The effect of metal ions on amylase activity was determined by following the standard DNSA protocol **(Miller G.L., 1959, Asgher *et al*., 2007; Carvalho et al., 2008**).

Result and Discussion

Isolation of Amylase Producing Bacteria

The isolation of amylase producing bacteria was done by spreading the enriched media onto the Luria Agar plates and was incubated for overnight. Later, the 7 distinct colonies on the basis of their texture and morphology were selected and sub-culture on both Luria Agar and Starch plate to obtain the pure cultures of amylase producing bacteria.

Primary Screening, Secondary Screening and Morphological Identification for Amylase-producing Bacterial Isolates

The primary (1°) screening of the amylase producing bacteria was assessed by observing the hydrolysis zone on starch plate stained with I_2/KI solution. Among the 7 pure isolates, only two isolates (1 and 2) which exhibited the measurable hydrolysis zone on starch plate in **figure 1**. After that secondary (2°) was conducted to determine the localization of the enzyme. For this the prepared media and CFE were incubated with reaction mixture (with or without 1% starch and buffer) and determined by using the standard DNSA protocol. The isolate 2 showed the

highest OD for media confirming the enzyme to be extracellular. Hence, the gram staining result revealed the isolated colony i.e. 2 to be gram negative and rod-shaped.

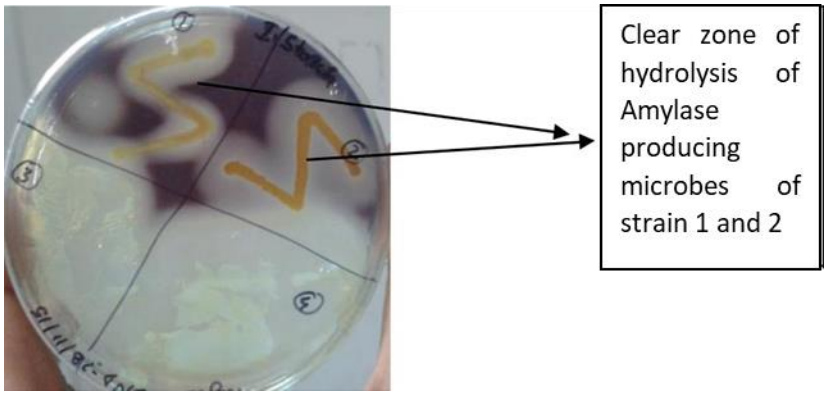

Fig 1. Iodine-stained starch agar plate showing primary screening of amylase-producing strains. The zone of hydrolysis indicated positive amylase activity

Biochemical Characterization of Amylase

Effect of temperature on amylase activity

The temperature optima was determined by conducting the standard DNSA protocol at varied temperatures ranging from 10-120°C. It was found that amylase enzyme showed the maximum value of **0.036 IU/mL** at 90°C in Figure 2b. The result obtained were found to have high activity as compared to the result obtained by **Mishra and Behera (2008)**. Hence, the isolated amylase producing bacteria was found to be thermophilic in nature.

Effect of pH on amylase activity

The pH optima was determined by conducting the standard DNSA protocol at varied pH ranging from 3-12. It was found that amylase enzyme showed the maximum value of **0.19 IU/mL** at pH 5 in Figure 2c. The result obtained for pH optima was found to be in positive correlation with the result of **Mishra and Behera (2008)**. Hence, the isolated amylase producing bacteria was found to be acidophilic in nature.

Effect of Metal ions on Amylase Activity

The metal ion effect on the amylase activity was determined in the absence and presence of metal ions (2mM or 5mM) and was determined by conducting the standard DNSA protocol. It was found that amylase enzyme showed the favorable activity in the presence of $MnCl_2$ (2mM or 5mM). The activity was found to be **0.16 IU/mL** in Figure 2d**.** Whereas, the $MgCl_2$ and $CoCl_2$ were also found to be the inducer of amylase activity, $FeCl_3$ and $ZnCl_2$ were found to be the inhibitor of amylase activity. As per the previous literature, Mg^{2+} ion was found to be positive inducer for amylase production, but our result showed that Mn^{2+} is the positive inducer for amylase production (**Sirohi & Prakash, 2015**).

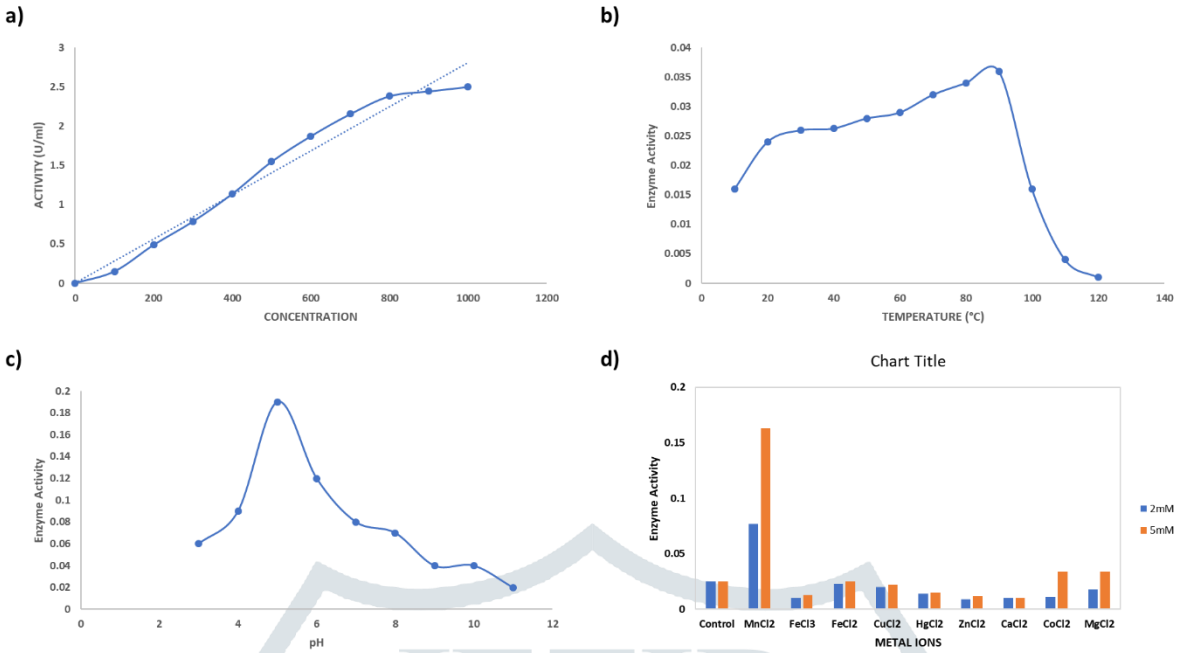

Figure 2: Biochemical Characterization of Amylase; a) Glucose Standard Curve, b) Temperature Optimum, c) pH Optimum and d) Effect of Metal ions on Amylase Activity

Conclusion

The isolated amylase bacterial strained showed the optimum amylase activity at pH 5 and temperature 90^{o}C. Moreover, Mn^{2+}metal ion was found to be the inducer of the amylase activity. This study revealed that this gram negative and rod-shaped bacteria has the ability to synthesize amylase at very high temperature and low pH. As amylase enzyme is of great importance in various industries like baking, brewing, paper, sugar and textile industry. Furthermore, the extremophilic bacterial strain can be used to obtain potent enzyme under severe conditions for wide-spectrum industrial applications.

References

Asgher, M., Asad, M. J., Rahman, S. U., & Legge, R. L. (2007). A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *Journal of food engineering*, *79*(3), 950-955.

Azhar, M., Uniyal, V., Chauhan, N., & Rawat, D. S. (2014). Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India. *International Journal of Current Microbiology and Applied Sciences*, *3*(12), 753-760.

Cappuccino, J. G., & Sherman, N. (2008). Microbiology: A Laboratory Manual, Pearson/Benjamin Cummings. *San Francisco, US*.

Carvalho, R. V. D., Côrrea, T. L. R., Silva, J. C. M. D., Mansur, L. R. C. D. O., & Martins, M. L. L. (2008). Properties of an amylase from thermophilic *Bacillus* sp. *Brazilian Journal of Microbiology*, *39*(1), 102-107.

Gopinath, S. C., Anbu, P., Arshad, M. K., Lakshmipriya, T., Voon, C. H., Hashim, U., & Chinni, S. V. (2017). Biotechnological processes in microbial amylase production. *BioMed research international*, *2017*.

Kaur, A., Kaur, M., Samyal, M. L., & Ahmed, Z. (2012). Isolation, characterization and identification of bacterial strain producing amylase. *Journal of Microbiology and Biotechnology Research*, *2*(4), 573-579.

Mehta, D., & Satyanarayana, T. (2016). Bacterial and archaeal α-amylases: diversity and amelioration of the desirable characteristics for industrial applications. *Frontiers in microbiology*, *7*, 1129.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, *31*(3), 426-428.

Mishra, S., & Behera, N. (2008). Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. *African journal of Biotechnology*, *7*(18).

Panda, M. K., Sahu, M. K., & Tayung, K. (2013). Isolation and characterization of a thermophilic *Bacillus* sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India. *Iranian journal of microbiology*, *5*(2), 159.

Panneerselvam, T., & Elavarasi, S. (2015). Isolation of-Amylase producing *Bacillus subtilis* from Soil. *International Journal of Current Microbiology and Applied Sciences*, *4*(2), 543-552.

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Microbial enzymes: industrial progress in 21st century. *3 Biotech*, *6*(2), 174.

Sirohi, R., & Prakash, V. (2015). Effect of metal ions on amylase production using *Bacillus subtilis* isolated from soil of Almora District, Uttrakhand, India. *International Journal of Pure and Applied Bioscience*, *3*, 37-41.

Souza, P. M. D. (2010). Application of microbial α-amylase in industry-A review. *Brazilian journal of microbiology*, *41*(4), 850-861.