

Isolation, Optimization and Characterization of α -Amylase from *Bacillus alcalophilus*

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Abstract: Amylase is one of the most important enzymes widely used in paper, textile, pharmaceutical industries etc. In the present investigation a thermostable, alkalophilic amylase was isolated from underlying soil samples of domestic wastes, and was screened on starch agar medium. The amylase activity was measured using colorimetric method at 540nm. Among forty five isolates, thirteen demonstrated clear zone of hydrolysis around the colonies when flooded with Gram's iodine solution. Maximum zone of hydrolysis was 8 mm, and tentatively identified as *Bacillus alcalophilus*. Its maximum activity was 9.210 ± 0.14 U/ml at pH 9 and 80°C temperature. It retains 70% activity after 1 h at 80°C and 60% activity after 1 hour at 90°C. With these promising features *B. alcalophilus* may be utilized in starch processing, detergent manufacturing brewing, and sugar production.

Keywords: Amylase, *Bacillus alcalophilus*, thermophilic, thermostable, domestic waste

1. Introduction

Amylases are enzymes which breakdown starch molecule to give diverse products such as dextrans and some small polymer composed of glucose [1]. They are produced by plants, animals and microorganisms but, those produced by microorganisms is still most preferred. Various fungal and bacterial species have been explored and many are still in this process to be explored [2, 3]. Amylase isolated from the bacteria has found more applications in food, textile, brewing, detergent and distilling industries [1]. Mainly two types of amylases are produced by the bacteria: α -amylase and glucoamylase. α -amylase has found much commercial importance than glucoamylases. It degrades α -1-4 glucosidic linkage of starch and related species [4]. Amylases (E.C. 3.2.1.1.) have approximately 25% of the enzyme market and have completely replaced chemical hydrolysis of starch in starch processing industries. It offers various advantages to industries i.e. risk of contamination, cost of external cooling, increase diffusion rate, resistant to denaturing agents and proteolytic enzymes [4-9]. Thermostable alkaline amylases have extensive commercial applications with pH values higher than 8.0 and in starch saccharification, industries, as an ingredient in detergent for automatic dish washers and laundries. α -amylase requires unique properties with respect to specificity, stability, temperature and pH dependencies [10-13].

In this present investigation, thermostable and alkalophilic amylase producing *Bacillus alcalophilus* was isolated and identified as potent organism for enzyme production.

2. Material and Methods

2.1 Sample Collection

Various underlying soil samples were collected from dumping sites/domestic wastes from local area of Sagar. Samples were collected in pre-sterilized bottles and brought to the lab for investigation.

2.2 Isolation and Screening

Isolation of microorganisms was done by adding sample to Starch broth medium (HIMEDIA) containing: Meat extract 0.3%, Peptic digest of animal tissue 0.5% and Starch soluble 0.2%. After 24 hours of growth medium was serial dilution method and spread on Starch Agar plates. After 48 hours of incubation at 37°C, plates were flooded with Gram's Iodine solution. Organism showing zone of clearance around the colony represents degradation of starch i.e. presence of amylase activity. All isolates were preserved in 15% glycerol in at 4°C. Identification of isolates was done by performing staining techniques, biochemical tests, advanced bacterial identification software (ABIS) and Bergey's manual.

2.3 Optimization of Culture Conditions

Screened strains of bacteria were subjected to various culture conditions to derive optimum culture condition for amylase production. Growth and amylase production was estimated; incubation period (6, 12, 18, 24, 30, 36, 42 and 48 hours), temperature (30, 40, 50, 60, 70, 80, 90, 100°C), pH (3, 4, 5, 6, 7, 8, 9, 10 and 11), Carbon source (Glucose, starch and sucrose) and Nitrogen source (peptone, yeast extract, tryptone). All experiments were carried out in 250ml Erlenmeyer flask containing 100ml medium containing peptone 0.5%, meat extract 1%, starch 1%, CaCl₂ 0.05% and NaCl 0.1%. Sterile medium was inoculated with 1ml (10⁵ CFU). Inoculated flasks were incubated at 37°C at 120 rpm for 48 hours.

2.4 Extraction and Purification

After 48 hours of incubation culture broth was centrifuged at 6000 rpm for 15 minutes. Supernatant was divided into two parts. One part of supernatant was used as crude enzyme whereas the other part was 60% saturated with ammonium sulphate. The precipitate obtained was dialysed with 20mM potassium phosphate buffer and used as purified enzyme. Protein content of both crude and purified form of amylase was estimated [14,15].

2.5 Enzyme Assay

Amylase enzyme activity was assayed by using 3,5 – dinitrosalicylic acid (DNS) method as described by of Miller (1959) [16]. By reacting 0.5 ml of enzyme to 0.5 ml of soluble starch and incubating the enzyme mixture at 37°C for 15 minutes. The reaction was stopped by addition of 1ml of DNS reagent and boiled for 15 minutes. The Absorbance was read at 540 nm and results obtained were compared with standard of maltose. One unit of enzyme was defined as one micromoles of maltose released per ml of enzyme.

2.6 Effect of Temperature and Metals on Enzyme Activity

The effect of temperature on enzyme activity was measured by incubating the enzyme at different time interval of 10, 20, 30, 40, 50 and 60 minutes in 50mM Tris buffer. Effect of different metals such as FeCl₂, CuCl₂, NaCl, MgCl₂, CaCl₂, EDTA and SDS at 1mM concentration was used to check its effect on enzyme activity. The activity of enzyme without adding any metals was taken as control.

2.7 Molecular weight determination by SDS-PAGE

Molecular weight of amylase was determined by performing SDS-PAGE with 10% polyacrylamide gel following the method described by Laemmli. The purified enzyme was loaded on wells parallel with standard protein markers of known molecular weights. Protein bands were stained by using coomassie brilliant blue and was destained. Molecular weight was calculated using Total lab ver. 10.

3. Results

Optimum growth of *Bacillus alcalophilus* and enzyme production was at stationary phase (fig. 1). Maximum growth and enzyme production was also obtained at pH – 9 (fig. 2) which signifies towards alkaline nature. Thermotolerant nature was proved when cell growth and enzyme production were highest at 60-80°C (fig. 3).

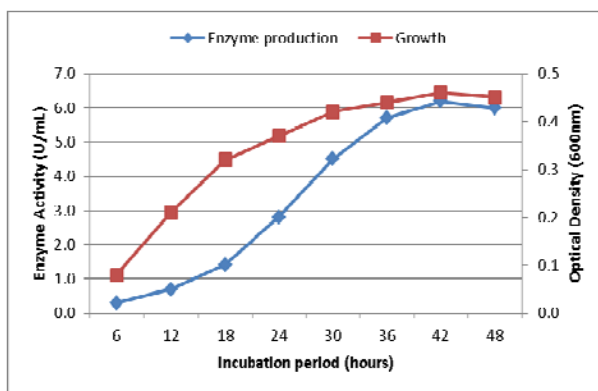


Figure 1: Effect of incubation period on growth and enzyme activity

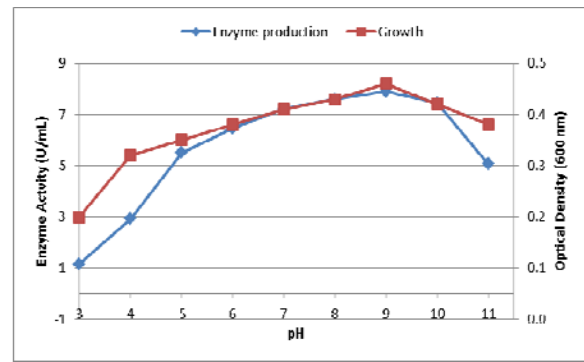


Figure 2: Effect of pH on growth and enzyme activity

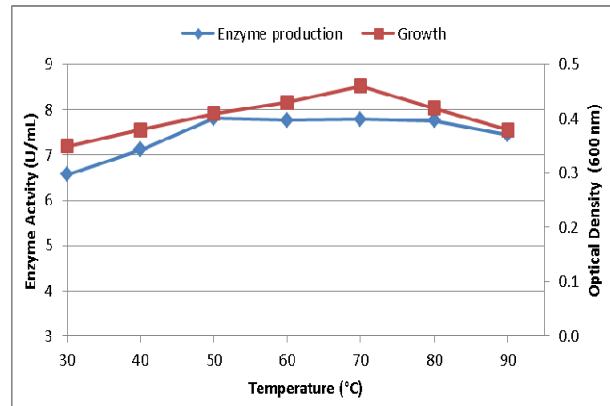


Figure 3: Effect of temperature on growth and enzyme activity

Media supplemented with lactose, starch, maltose and fructose as sole carbon source, increase the enzyme activity to 6.38 U/mL (4.42 %), 6.32 U/mL (3.44 %) 6.29 U/mL (2.95 %) and 6.25 U/mL (2.29 %) respectively. Glucose and sucrose exhibited slight increase in the activity from (0.05 to 0.12 %). In another case where the medium was supplemented with peptone and tryptone as nitrogen source, maximum enzymatic activity was 6.28 U/mL (2.78 %) and 6.24 U/mL (2.13 %) as compared from the control (Fig. 4).

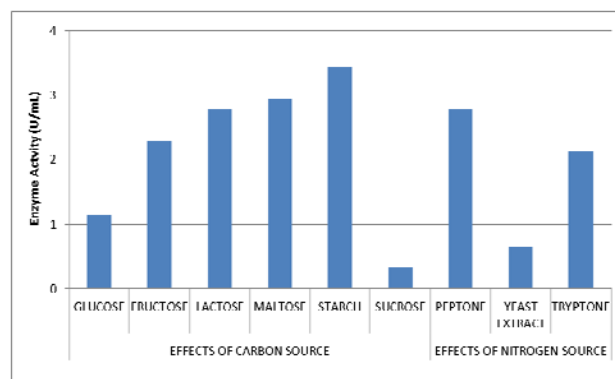


Figure 4. Effect on Carbon/Nitrogen source on enzyme activity

When MnCl₂, CaCl₂ and NaCl metal ions were used 3.60 %, 2.95 % and 2.29 % increase in the amylase activity was reported whereas with SDS, EDTA and Urea, 1.31 %, 3.27 % and 2.29 % decrease in the enzymatic activity was observed (Fig. 5). Subsequently decrease up to 90°C and beyond 90°C no enzyme activity were found. With purified

enzyme high specific activity of 24.95 U/mg was found. Molecular weight determination of the purified amylase on SDS-PAGE showed a single band with molecular weight of 52 kDa indicating the purity of amylase.

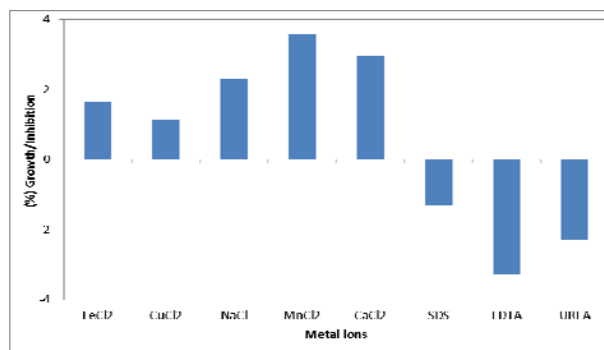


Figure 5: Effect of metal ions/inhibitors on enzyme production

4. Discussion

Enzyme production and Genus *Bacillus* has a very long relationship and is still followed. *Bacillus* is the most exploited genus for production of variety of extracellular enzymes and they have a wide range of industrial applications. Of these enzymes, amylases are particularly significant to industry as it contributes 25% of enzyme market. This present study has reported optimization of culture conditions for amylase production by *B. alcalophilus*, which shows optimum growth and enzyme production at pH 9, 70-80°C. Igarashi *et al.*, (1998) reported that *Bacillus* sp. is having optimum growth at 30°C and the enzyme maxima at 55°C [17]. Similar finding were also reported by Sodhi *et al.*, (2005) for another *Bacillus* spp. PS-7 [18]. *Bacillus* sp. TS-23 exhibits optimum growth at 55°C and enzyme activity at 70°C Lin *et al.*, (1998) [8]. The growth and the enzyme production by *B. alcalophilus* are more or less same. Further, soluble starch serves as the best carbon source for maximum growth and enzyme production. This phenomenon is also shown by many workers [19].

Many of the amylase reported are not alkaline where as some of them are alkaline activity but not thermostable. The amylase isolated in this study solves this purpose of being alkaline in nature and thermostable. Mn²⁺ and Ca²⁺ ions catalyse the enzyme activity as well as stability. These metal ions must be acting as a cofactor required to increase the enzyme activity. Inhibitory activity of SDS, EDTA and Urea may be due to the competition between the exogenous cations resulting in the decrease in metalloenzyme activity. The molecular weight of the amylase enzyme found in the present study as 52 kDa.

The result obtained suggests that *Bacillus alcalophilus* with the activity of (9.08 ± 0.14 U/ml) can be considered as a good candidate for thermostable amylase production and could be used as a potential microorganism. This bacterium plays an important role in degrading starch from the domestic waste. Many researchers have studied *Bacillus alcalophilus* isolated from various extremes condition and have checked for various enzyme productions.

Acknowledgement

This work was supported by a grant from the University Grant Commission, New Delhi and Department of Botany, Dr. H. S. Gour University, Sagar (M.P.).

References

- [1] W. W. Windish, N. S. Mhatre, "Microbial Amylases," in Advances in Applied Microbiology Wayne WU Academic Press 1965.
- [2] V. Ivanova, D. Yankov, L. Kabaivanova, D. Pashkoulov, "Simultaneous biosynthesis and purification of two extracellular *Bacillus* hydrolases in aqueous two-phase systems," Microbiological Research, 156 (1). pp.19-30, 2001.
- [3] B. K. Lonsane, M. V. Ramesh, "Production of bacterial thermostable alpha-amylase by solid-state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis," Adv Appl Microbiol, 35 pp.1-56, 1990.
- [4] K. R. Babu, T. Satyanarayana, "Parametric optimization of extracellular α -amylase production by thermophilic *Bacillus coagulans*," Folia Microbiol, 38 (1). pp.77-80, 1993.
- [5] R. K. Saxena, K. Dutt, L. Agarwal, P. Nayyar, "A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5," Bioresour Technol, 98 (2). pp.260-265. Epub 2006 Mar 2009., 2007.
- [6] Burhan, U. Nisa, C. Gökhan, C. Ömer, A. Ashabil, G. Osman, "Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6," Process Biochemistry, 38 (10). pp.1397-1403, 2003.
- [7] Pandey, P. Nigam, C. R. Soccol, V. T. Soccol, D. Singh, R. Mohan, "Advances in microbial amylases," Biotechnol Appl Biochem, 31 (Pt 2). pp.135-152., 2000.
- [8] L. L. Lin, C. C. Chyau, W. H. Hsu, "Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23," Biotechnol Appl Biochem, 28 (Pt 1). pp.61-68., 1998.
- [9] J. M. Bragger, R. M. Daniel, T. Coolbear, H. W. Morgan, "Very stable enzymes from extremely thermophilic archaeobacteria and eubacteria," Appl Microbiol Biotechnol, 31 (5-6). pp.556-561, 1989.
- [10] T. U. Kim, B. G. Gu, J. Y. Jeong, S. M. Byun, Y. C. Shin, "Purification and Characterization of a Maltotetraose-Forming Alkaline (alpha)-Amylase from an Alkaliphilic *Bacillus* Strain, GM8901," Appl Environ Microbiol, 61 (8). pp.3105-3112., 1995.
- [11] E. Lévêque, Š. Janeček, B. Haye, A. Belarbi, "Thermophilic archaeal amylolytic enzymes," Enzyme and Microbial Technology, 26 (1). pp.3-14, 2000.
- [12] J. Hewitt, G. L. Solomons, "The production of α -amylase (E.C.3.2.1.1.) by *Bacillus amyloliquefaciens*, in a complex and a totally defined synthetic culture medium," Journal of Industrial Microbiology, 17 (2). pp.96-99, 1996.
- [13] S. C. Peixoto, J. A. Jorge, H. F. Terenzi, L. Polizeli Mde, "Rhizopus microsporus var. rhizopodiformis: a thermotolerant fungus with potential for production of

- thermostable amylases," *Int Microbiol*, 6 (4). pp.269-273. Epub 2003 Aug 2015., 2003.
- [14] M. A. M. Tigue, C. T. Kelly, E. M. Doyle, W. M. Fogarty, "The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370," *Enzyme and Microbial Technology*, 17 (6). pp.570-573, 1995.
- [15] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, "Protein measurement with the Folin phenol reagent," *J Biol Chem*, 193 (1). pp.265-275., 1951.
- [16] G. L. Miller, "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar," *Analytical Chemistry*, 31 (3). pp.426-428, 1959.
- [17] K. Igarashi, Y. Hatada, H. Hagihara, K. Saeki, M. Takaiwa, T. Uemura, K. Ara, K. Ozaki, S. Kawai, T. Kobayashi, S. Ito, "Enzymatic properties of a novel liquefying alpha-amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequences," *Appl Environ Microbiol*, 64 (9). pp.3282-3289., 1998.
- [18] H. K. Sodhi, K. Sharma, J. K. Gupta, S. K. Soni, "Production of a thermostable α -amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production," *Process Biochemistry*, 40 (2). pp.525-534, 2005.
- [19] H. Yang, L. Liu, J. Li, G. Du, J. Chen, "Heterologous expression, biochemical characterization, and overproduction of alkaline alpha-amylase from *Bacillus alcalophilus* in *Bacillus subtilis*," *Microb Cell Fact*, 10 (77). pp.1475-2859, 2011.

Author Profile



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