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The evaluation of Isolated KH1-29 properties for producing α-amylase from a novel native highly thermostable, thermophilic and alkaline Bacillus sp

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ABSTRACT

Alkaliphilic and thermophilic *bacillus sp.* KH1-29 which was isolated from Caspian Lake of Iran, produced thermostable α-Amylase at 55°C, pH 8.0. The molecular weight of the enzyme was estimated 45 kilodallton using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The enzyme showed working pH range of 7-8 with an optimum pH of 8.0. The temperature optimum of the enzyme was found at 90°C. α-Amylase production by Thermophilic *bacillus sp.* strain reached to maximum quantity at 36h after cultivation analyzed according to the Lowry method. According to Lowry method with levels of 0.63 mmol protein/min, the a-amylase production in the mentioned strain reached to maximum levels at 36 h after cultivation with levels of 0.63 mmol protein/min. As a result, according to information obtained by the enzyme it is qualified for use in biotechnological applications and all its properties make it a useful tool for bio-bleaching in pulp and paper industry.

Key words: Isolation, *Bacillus sp.*, Thermophile, Alkaline, α-Amylase, Molecular weight

INTRODUCTION

The main advantages of using microorganisms for amylases production are theirs high economical production capacity and their easy manipulation to obtain enzymes whit desired characteristics (14).

Thermophilic microorganisms are adapted to thrive at temperatures above 60°C. They are a source of interesting enzymes such as thermoactive and thermostable (22). Among the microorganisms, Bacillus species are good secretors of extracellular enzymes such as amylase, arabinase, cellulase, lipase, protease, and xylanase which play important roles in many biotechnological processes (10).The use of microbial enzymes in industrial areas has been increasing because of its economical production and immobilization of unsolvable materials in water and durable use in respect to biotechnological activities (7).

Amylases are the most important enzymes and also, nowadays, they have a great significance in biotechnology. They are used in the sugar, baking, brewing, paper, textile, distilling industries (9). Amylases include a class of industrial enzymes having approximately 25% of the enzyme market (24).

α-amylase(endo-1,4-a-D-glycan glucanohydrolase EC 3.2.1.1) are one of the most important groups of industrial enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (23,4). Amylases can be categorize into two groups such as, endoamylases and exoamylases. Endoamylases catalyse hydrolysis the interior of the starch molecule randomly. This action lead to the formation of linear and branched oligosaccharides of

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various chain lengths. Exoamylases hydrolyse from the non-reducing end, successively resulting in short end products. Today, a large number of enzymes are known which hydrolyse starch molecule into different products and a combined action of various enzymes is required to hydrolyse starch completely.

The key advantages of using microorganisms for production of amylases are theirs economical huge production capacity. Also, these microbes easily manipulate to obtain enzymes of desired characteristics (23). α-Amylases have been developed from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have important applications in industrial areas (4). Nevertheless, bacterial α-amylases particularly Bacillus amylases are more desirable according to fungal α-amylases because of their heat stability (29).

The most used bacterial α-amylases were derived from B. amyloliquefaciens, B. licheniformis and B. stearothermophilus (14, 30, and 31). It is desirable that a-amylases should be active at the high temperatures of gelatinization (100–110 -C) and liquefaction (80–90 -C) to economize processes; therefore, there has been a need and continual search for more thermophile and thermostable a-amylases. For applications in industrial processes, the enzymes should be stable at high temperature, pH, presence of salts, solvents, toxicants etc. (12).

The aim of present study was identification and isolation of new Bacillus sp. and also evaluation of the produced amylase properties by thermophile and alkaliphilic Bacillus sp. isolate KH1-29.

MATERIAL AND METHODS

Microorganisms and culture conditions:

Bacillus sp. KH1-29 was isolated from coast sediment samples collected from Caspian Lake, Iran. To select the Gram-positive spore-forming bacteria Bacillus sp., soil sample was incubated at 80 °C for 10 min (8). The isolates were cultivated in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 9.0) for 24 h at 55 °C with shaking at 200 rpm. The isolates screened for α-amylase activity on LB-agar-starch plates containing (g L-1) tryptone 10, yeast extract 5, NaCl 10, starch 5, agar 15 (pH 9.0) at 55°C (13). α -Amylase positive colonies were selected with iodine staining.

Enzyme production:

The organisms was propagated at 55 °C for 24 h in 100 ml of a LB medium, containing 1% soluble starch (Merck), placed in 1000-ml flasks, with shaking on a shaker (200 rpm/min). The initial pH of the medium was about 9.0. After removal of cells by centrifugation (10 000_/g, 20 min) at 4 8C, the supernatant was used for partial purification.

Enzyme assay

The relative amylase activity was assayed by adding 1 ml of enzyme to 1 ml soluble starch (1% v/v) in 50 mM Tris buffer pH 9.0, and incubating at 55 °C for 30 min. The reaction was stopped by the addition of 3 ml of 3, 5-dinitrosalicylic acid reagents. A550nm was measured in a Cecil 5500 spectrophotometer. One unit of amylase activity was defined as the amount of enzyme that released one micromole of reducing sugar equivalent to glucose per minute under the assay condition (22).

Protein determination

Proteins of wild type and mutant variants were estimated as described by Lowry et al. (17) using bovine serum albumin as the standard.

Effect of incubation period

The effect of incubation period was determined by assaying the enzyme activity in different incubation periods (12, 24, 36, 48, 60, and 72 h).

Effect of pH and temperature on activity and stability:

Temperature and pH effects on enzyme activity were assayed at various temperatures ranging from 30-100 C and pH values ranging from 6-12 for 30 min. following buffers were used in the reactions: 100mM Na-phosphate (pH 6-7) and 100 mM Tris (pH7-12) (3).

SDS–PAGE and zymogram analysis

SDS- Starch-PAGE (0.2% Starch) were done as described by laemmli (13) with slab gels (12% w/v acrylamide). For visualizing of total proteins, SDS-PAGE was stained for 1 h with the solution of 0.1% Coomassie blue R250-40% methanol-10% glacial acetic acid and then destained overnight in the same solution without dye. For activity staining (zymogram) of Starch by SDS- Starch-PAGE, SDS was removed by washing the gel at room temperature in solution-A (50 mM Na_2HPO4, 50 mM NaH_2PO4 (pH 7.2), isopropanol) for 1 h and solution-B (50 mM Na2HPO4, 50 mM NaH2PO4 (pH 7.2) for 1 h, respectively.

The gel was kept overnight in solution-C (50 mM Na2HPO4, 50 mM NaH2PO4 (pH 7.2), 5 mM β-mercaptoethanol, 1 mM EDTA) at 4°C for renaturation of the enzyme. It was then sealed with film and incubated at 55°C for 4 h. Gel was stained in a solution of iodine (iodine 5 g/l, KI 50 g/l), for 30 min, clear band indicate the presence of amylase activity (8, 3). The molecular mass of the enzyme was finally estimated from the position of standard proteins.

RESULTS

The isolated alkaline, halophilic and thermophilic strain *Bacillus sp*. KH1-29 from Caspian Lake in Iran was gram positive, rod shaped, aerobic, catalase positive and spore forming. According to the basis of various morphological and biochemical characteristic, it was identified as *Bacillus sp*. Enzyme synthesis of *Bacillus sp*. KH1-29 occurred at temperatures between 30 and 100 °C with an optimum of 90 °C. There was a variation in amylase synthesis within the pH range 6.0 and 12.0 with an optimum pH 8.0, while KH1-29 *Bacillus sp*. grew well at between 7.0 and 8 pH on starch agar medium in presence of NaCl (5% wt/v) and occurred up to 55 °C.

Enzyme properties

Productions of α-amylases at various time courses were investigated. The *Bacillus* sp. KH1-29 culture was incubated at 55 °C for 12, 24, 36, 48 and 60 hours. Maximum enzyme production was recorded after 36 h at 55 °C (Figure-1).

Figure-1. Productions of α-amylases at different time by *Bacillus sp.* isolate KH1-29

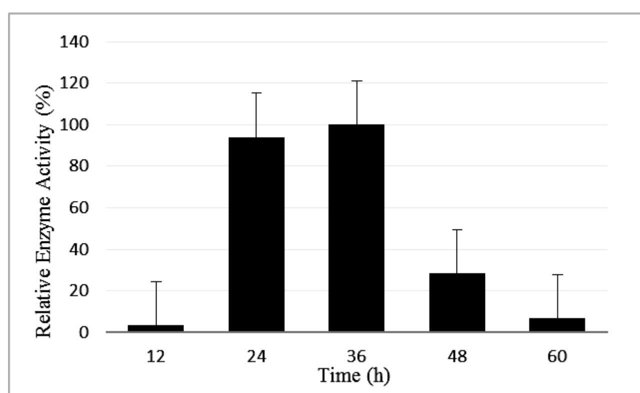
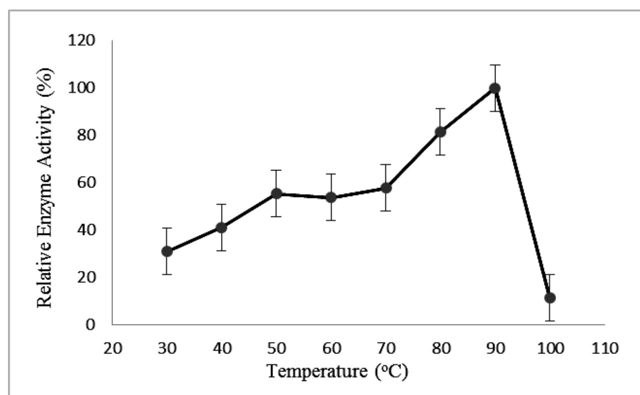
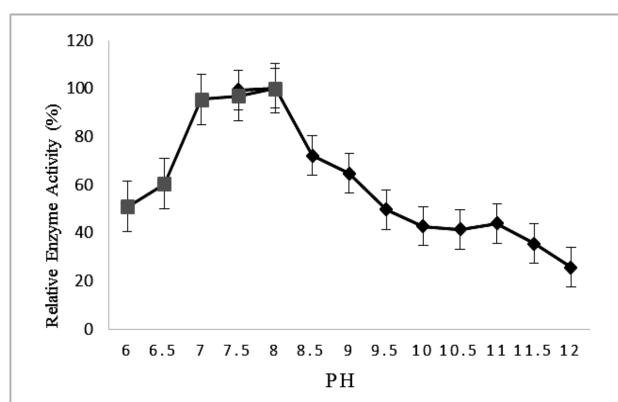


Figure-2. The effect of temperature on α-amylases activity



Out of 1865 persons, 51 persons were found suffering from disease. The disease rate was 2.73%. The enzyme had a broad temperature range between 30-100 °C and the optimum activity was observed at 90 °C. The relative enzyme activities were 31, 41, 55, 54, 58, 82 and 100 % at 30, 40, 50, 60, 70, 80 and 90 respectively, whereas only 12% activity was retained at 100°C for 30 min (Figure-2). The enzyme also showed a significant relative activity between 7 and 8.5 pH. Effects on enzyme activity was assayed at values ranging from 6-12 for 30 min. optimum activity was observed at pH 8. Following buffers were used in the reactions: 100mM Na-phosphate (pH 6-7) and 100 mM Tris (pH7-12) (Figure-3).

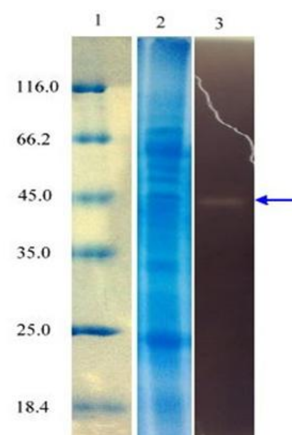
Figure-3. The effect of pH on α-amylases activity



Determination of molecular weight

Molecular weights of wild type and mutant α-amylases determined by SDS-Starch-PAGE electrophoresis revealed single bands showing α-amylase activity in gel using BioCapt MW software. The molecular mass of bands was 45kDa (Figure-4).

Figure-4. Zymogram analysis of α-amylases on SDS-PAGE. The gel was cut into two pieces, the marker and total proteins were visualized with Coomassiebrillant blue staining and the activity of enzyme revealed by iodine (1: Marker, 2: SDS-PAGE, 3: Zymogram).



Specific Activities

Total protein from Isolate *Bacillus sp*. KH1-29 was analyzed according to the Lowry (1951) method. One mg of protein in a minute was to break 0.63 mmol of substrate.

DISCUSSION

In the present study, water and soil samples were collected from Caspian Lake of Iran and used for isolation of Gram (+), spore forming, and aerobic bacterial strains. About 120 strains were isolated and screened for α-amylase activity. Among these isolates, 36 bacteria showed amylolytic activity on LB-agar plate containing starch. The *Bacillus* sp. isolate KH1-29 selected for further studies because of its maximum amylolytic hollow zone around the colony. α-mylases from alkaline and thermophilic *Bacillus* species were reported previously (21, 9, 11, 15, 3, 5).

Most of the *Bacillus* strains used commercially for the production of α-amylases have an optimum pH between 6.0 and 9.0 for growth and enzyme production [3, 25]. The strain *Bacillus* sp. KH1-29 was improved for α-amylase production. *Bacillus* sp. isolate KH1-29 Maximum enzyme production was recorded after 36 h at 55 °C. The optimum pH values for native amylases were 8.0. The optimal temperature values for enzyme activity were 90°C. These pH and temperature values are similar to *Bacillus licheniformis* and *Gracilibacillus* (6), *Bacillus* sp. GUF8 (18), *Halomonas* sp. AAD21 (29), *Bacillus cereus* MS6 (20) enzymes.

CONCLUSION

The *bacillus sp*. KH1-29 strain produced high levels of thermostable α-amylases. The KH1-29 α-amylases is the thermostable (90 ºC) and slightly alkaline with wide range of pH (7-8). Hence, it is qualified for use in biotechnological applications and all its properties make it a useful tool for biobleaching in pulp and paper industry. The KH1-29 α-amylases production process can be commercialized after further optimization for enhanced enzyme production.

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Conflict of Interests:

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. **Alghabpoor, S.S., Panosyan, H. and Popov, Y. (2013).** Purification and characterization of a novel thermostable and acid stable α-amylase from Bacillus Sp. Iranian S1. Int J Eng 26(8): 815-820.
2. **Al-Quadan, F., Akel. H. and Natshi, R. (2009).** Characteristics of a novel highly thermostable and extremely thermophilic alkalitolerant amylase from hyperthermophilic Bacillus strain HUTBS51. OnLine J Biol Sci 9(3): 67- 74.
3. **Amal A. Hassan, Esam H. Mansour, Abo El-Fath A. El Bedawey and Mohamed S. Zaki** (2015). Effect of α-amylase enzyme on rheological properties and quality of betifore-type cookies. Biolife 2015; 3(1); 31-39.
4. **Arikan, B., Unaldi, N., Coral, G., Colak, O., Aygan, A. and Gulnaz, O. (2003).** Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. isolate ANT- 6. Process Biochem 38: 1397-1403.
5. **Asgher M, Asad MJ, Rahman SU, Legge RLA.(2007).** thermostable α -amylase from a moderately thermophilic Bacillus subtilis strain for starch processing. J Food Eng; 79:950-955.
6. **Aygan, A., Arikan, B., Korkmaz, H., Dinçer, S. and Çolak, Ö. (2008).** Highly thermostable and alkaline α-amylase from a halotolerant-alkaliphilic Bacillus sp. AB68. Braz J Microbiol 39(3): 547-553.
7. **Bakhtiary, N., Hosseinkhani, S., Mohajeri-Tehrani, M. R., & Hedayati, M** (2014). Critical role of plasma C-peptide on control of ATP/ADP ratio of RBC. *The Ame J Sci & Med Res,* 1(1), 7-15.
8. **Fehimeh, M., Nima, B. and Majid, B. (2013).** Isolation, characterization and identification of amylase producing halothermophilic isolates from Howz Soltan Lake, Iran. Afr J Microbiol Res 7(36): 4483-4490.
9. **Gümüşel F. 2002.** Biyoteknoloji, genetik ve sağlık sektörü.Kocaeli Sanayii İçin Teknolojik Uzgörü Ortak Projesi, 73-135.
10. **Hamilton LM, Kelly CT, Fogarty WM et al. (1999).** Production and properties of the raw starch-digesting a-amylase of Bacillus sp. IMD 435. Process Biochem 35: 27–31.
11. **Igarashi, K., Hatada, Y., Hagihara, H., Saeki, K., Takaiwa, M., Eumura, T., Ara, K., Ozaki, K., Kawai, M., Kobayashi, T. and Ito, S. (1998).** Enzymatic properties of a novel liquefying a-amylase from an alkaliphilic Bacillus isolate and entire nucleotide and amino acid sequences. Appl Environ Microbiol 64(9): 3382-3389.
12. **Joo MH, Hur SH, Han YS, Kim JY. (2007).** Isolation, identification, and characterization of Bacillus strains from the traditional Korean

soybean-fermented food, Chungkookjang. J Appl Biol Chem 2007; 50:202-210.

13. **Kim, T.U., Gu, B.G., Jeong, J.Y., Byun, S.M. and Shin, Y.C. (1995).** Purification and characterization of a maltotetraose-forming alkaline α-amylase from an alkaliphilic Bacillus strain, GM8901. Appl Environ Microbiol 61: 3105–3112.
14. **Kumar S, Karan R, Kapoor S, Singh SP, Khare SK. 2012**. Screening and isolation of halophilic bacteria producing industrially important enzymes. Braz J Microbiol 2012;1595-1603.
15. **Laemmli, U.K. (1970).** Cleauage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680-685.
16. **Lee, S., Morikawa, M., Takagi, M. and Imanaka, T. (1994).** Cloning of the aapt gene and characterization its product, α-amylase-pullulunase (AppT), from thermophilic and alkaliphilic Bacillus sp. KAL601. Appl Environ Microbiol 60: 3761-3773.
17. **Lin, L.L., Chyau, C.C. and Hsu, W.H. (1998).** Production and properties of a raw starch-degrading amylase from the thermophilic and alkaliphilic Bacillus sp. TS-23. Biotechnol Appl Biochem 28(1): 61-68.
18. **Lonsane BK, Ramesh MV.1990**. Production of bacterial thermostable α-amylase by solid state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis. Adv Appl Microbiol 1990; 35:1-56.
19. **Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951).** Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-271.
20. **Mahdavi, A., Sajedi, R.H., Rassa, M. and Jafarian, V. (2010)** Characterization of an α-amylase with broad temperature activity from an acid-neutralizing Bacillus cereus strain. Iran. J Biotechnol 8(2): 103-111
21. **McTigue MA, Kelly CT, Doyle EM, Fogarty WM. (1995).** The alkaline amylase of the alkalophilic Bacillus sp. IMD 370. Enzyme Microb Technol 1995;17:570_/3.
22. **Michelin, M., Silva, T.M., Benassi, V.M., Peixoto-Noqueira, S.C., Moraes, L.A., Leão, J.M., Jorge, J.A., Terenzi, H.F. and Polizeli Mde, L. (2010).** Purification and characterization of a thermostable α-amylase produced by the fungus Paecilomyces variotii. Carbohydr Res 345(16): 2348-2353.
23. **Morgan, F.J., Priest, F.G. (1981).** Characterisation of a thermostable α-amylase from Bacillus licheniformis NCIB 6346. J Appl Bacteriol 50: 107-114.
24. **Niehaus, F., Bertoldo, C., Kahler, M., Antranikian, G., (1999**). Extremophiles as a ource of novel enzymes for industrial application. Applied Microbiology and Biotechnology 50, 711–729.
25. **Nielsen JE, Borchert V. (2000).** Protein engineering of bacterial α -amylases. Biochim Biophys Acta 2000; 1543:253-274.
26. **Rao, M.B., Tanksale, A.M., Gathe, M.S., Deshpande, V.V., (1998).** Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews 62 (3), 597–635.
27. **Rasooli, I., Astaneh, S.D.A., Borna, H. and Barchini, K.A. (2008).** A thermostable α-amylase producing natural variant of Bacillus spp. Isolated from soil in Iran. Am J Agric Biol Sci 3(3): 591-596.
28. **Sambrook, J. and Russell, D.W. (2001).** Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York, USA.
29. **Saxena, R.K., Dutt, K., Agarwal, L. and Nayyar, P. (2007).** A highly thermostable and alkaline amylase from a Bacillus sp. PN5. Bioresour Technol 98: 260-265.
30. **Shen, H., Mo, X., Chen, X., Han, D. and Zhao, C. (2012).** Purification and enzymatic identification of an acid stable and thermostable α-amylase from Rhizopus microspores. J Inst Brew 118(3): 309-314..
31. **Uzyol KS, SarıyarAkbulut B, Denizci AA, Kazan D et al. (2012).** Thermostable α-amylase from moderately halophilicHalomonas sp. AAD21. Turk J Biol 36: 327-338.

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