American Journal of Current Microbiology

American Journal of Current Microbiology
http://ivyunion.org/index.php/ajcmicrob/index
Vol. 2, Article ID 201300203, 5 pages

Research Article

Partial Purification and Characterization of Amylase from a newly Isolated Bacillus Megaterium Strain KAN1 from Fermented Rice Handia

Anupam Roy[1], Kalyani Khanra[2], Anjan Mishra[3], and Nandan Bhattacharyya[4*]

Department of Biotechnology, Panskura Banamali College, West Bengal, India

Abstract

This study investigated the partial purification and characterization of a new strain of *Bacillus megaterium* strain KAN1 (GenBank accession number: BankIt1545135 seq1 JX182976). It was isolated from traditional fermented drink Handia. The enzyme was then partially purified by ammonium sulphate precipitation. The results showed that the purified enzyme has specific activity of 1.05 U mg-1. This was an increase of 2.07 times than the raw enzyme extract. The optimum pH of the purified enzyme was 11, but the enzyme can work effectively in the pH range of 5.0 – 14.0. The temperature optimum for enzyme activity was 60 ℃, although the effective activity was seen in a wide range of temperature. The Km and Vmax values for soluble starch substrate were 0.65 mg/ml and 1.568 mg min^{-1}. Electrophoresis with SDS-polyacrylamide showed that the molecular weight of the enzyme was 67 kDa.

Keywords: Amylase; purification; specific activity; kinetics

Peer Reviewer: Arunava Bandyopadhaya, Department of Surgery,Massachusetts General Hospital & Harvard Medical School, Harvard University, United States; Sidharth Chopra, Division of Microbiology, CSIR-Central Drug Research Institute, India; Marius I. Mihăşan, Faculty of Biology, Alexandru Ioan Cuza University, Romania

Received: August 13, 2013; **Accepted**: January 11, 2014; **Published:** February 7, 2014

Competing Interests: The authors have declared that no competing interests exist.

Consent: We confirm that the patient has given their informed consent for the case report to be published.

[*]**Correspondence to**: Nandan Bhattacharyya, Department of Biotechnology, Panskura Banamali College, West Bengal, India

Email: bhattacharyya_nandan@rediffmail.com

Introduction

Enzymes are biologic polymers that catalyze the chemical reactions without altering themselves after the reaction. Microbial, plant and animal origin are the source of enzymes.

Among them microbial origin are mostly preferred due to cheaper production, high predictability, controllability and reliability. Beside thermostable and alkali tolerant nature makes them suitable for variety of processing condition [1].

Amylase, an industrially important enzyme degrades starch to simple sugar. This serves about 25 to 33% of the world enzyme market [2]. This enzyme is preferred to be collected in bulk from microorganisms.

Thermostable amylases are mostly preferred in industrial applications due to the decreased risk of contamination, cost of external cooling and increased diffusion rate [3]. Amylases, themostable in nature very often shows alkali tolerant in nature. These amylases are favorable for detergent industry as working pH is between 8-11 [4].

Handia is indigenous rice based fermented beverage of eastern India, and is being used as a intoxicating drink by the ethnic tribes such as Santal, Sabar, Bhumij, Paroja, Kondh, kolh, Mundari, Juang. These tribes are scattered specially in Orissa Jharkhand and West Bengal. It is prepared from mixing previous microbial culture and growth enhancer (Bakhar) with boiled rice. Details are available in Roy et al a, b & c [5,6,7] (2012). While analyzing microbial profile of Handia and its culture (Bakhar), a novel strain of bacteria was identified. One of such strain is *Bacillus megaterium* strain KAN1. The uniqueness of this strain is that it can produce alcohol directly from rice (starch) in a single step process. Depending upon the high amylase activity and the stability in wide range of temperature and pH, the strain was taken for study.

The following data represents the partial purification of amylase from the heat and temperature stable *Bacillus megaterium* strain KAN1. The effects of different environmental factors on its properties are also studied.

Materials and methods

Strain

A new strain of KAN1, isolated from fermented rice (Handia) was used in this study [5]. Although in both fermented rice (Handia) and its culture (Bakhar) presence of strain of KAN1 is proved to exist. The culture was maintained in the nutrient agar slant at 4 ℃.

Production, extraction and purification of enzyme from bacteria (recovery of amylase)

Production of amylase for the mentioned strain was carried out on the optimized conditioned [5]. The medium used in the study was composed of 100 ml medium composed of (g/l): Peptone 0.9, Rice powder 0.1, $(NH_4)_2HPO_4$ 0.4, KCL 0.1, $MgSO_4$, $7H_2O$ 0.1, $NaH_2PO_4.2H_2O$ 0.5 (pH-7).

Particular strain was cultured at 37 ℃ for 30 h in mentioned medium (100 ml taken in 250 ml Erlenmeyer flask) and placed in a shaker incubator operated at 120 rpm at 30 ℃.The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 min using a high speed centrifuge. The supernatant obtained was collected and treated as crude enzyme source. The crude enzyme was purified by ammonium sulphate precipitation up to 80 % saturation. The precipitates from different concentrations of ammonium sulphate were re-suspended in 10 ml of 100 mM Tris buffer, pH 10. The enzyme was then dialyzed with a 30kDa molecular weight cutoff membrane in the same buffer for 24 hour, with changes in buffer made every 2 hour. The dialyzed sample was treated as purified extracellular alkaline amylase and assayed for the calculation of enzyme activity varying the environmental factor several.

Molecular weight determination by SDS-PAGE

SDS-PAGE was carried out in 12.5 % resolving gel and 4.5 % stacking gel for determination of molecular mass as per the method of Laemmli, 1970. Protein bands were detected by destaining the gel in a methanol-acetic

acid-water solution (4:1:5) after a staining process with 0.2% Coomassie brilliant Blue R250.

Protein estimation

Protein concentration in crude and purified enzyme was determined by Lowry's method of protein estimation [8] (Lowry *et al.*, 1951). The absorbance at 660nm was recorded after the reaction was compared with the standard graph plotted reacting a standard protein (BSA)

Enzyme assay

Amylase activity in crude and purified enzyme was assayed by DNS method of Miller.,1959 [11], where 0.5 ml of enzyme was reacted with 0.5 ml soluble starch (1% v/v dissolved in 100mM Tris Buffer pH 10) by incubating the reaction mixture at 37 ℃ for 5 min. The reaction was stopped by the addition of 1 mL of 3,5-dinitrosalicylic acid reagent. The tubes were placed in boiling water bath for 15 minutes and absorbance was read at 540nm. The absorbance was compared with a standard graph of maltose. Absorbance obtained after reaction of maltose with DNS reagent. Mg/ml maltose released after reaction of enzyme and substrate was calculated. This concentration was used to get the enzyme activity in U/ml. one enzyme unit is the amount of enzyme able to release one micromole (or milimole) of maltose per min.

Characterization of the purified enzyme

The effect of pH (optimum), temperature (optimum) and substrate concentration, on partially purified a-amylase was determined. pH of the buffer system was varied between 3.0 and 14.0, at temperature between 10-80 ℃. Substrate concentration (0.2 to 1.4 mg/ml) was taken into account under standard assay conditions [12].

For pH stability the enzyme preparations in different buffer solutions (between 3.0 and 14.0) and were kept at 4 ℃ for 24 h. For thermal stability, the enzyme preparations were exposed at various temperatures (10, 20, 30, 40, 50, 60, 70 and 80 ℃) for 15 min. Residual enzyme activities were determined under standard assay conditions [12]. The effect of activators (Calcium, Magnesium and Sodium of 0.2 mg/ml) on the purified enzyme was also studied. Activity of enzyme was calculated as mentioned in enzyme assay portion.

Results and Discussion

Homogeneity test of purified enzyme by electrophoresis SDS-gel polyacrylamide

The result of electrophoresis SDS-gel polyacrylamide of partially purified enzyme can be seen in electrophoregram (Figure 1). This figure showed that the molecular weight of purified enzyme was 67 kDa.

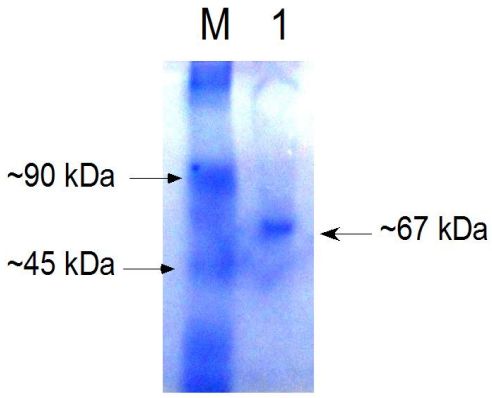

Figure 1 SDS-PAGE analysis of semi-purified α-amylase. M denotes marker, 1 denotes partially purified enzyme.

Purification profile of the enzyme

The crude enzyme was purified by ammonium sulphate precipitation up to 70 % saturation. Table 1 shows the phase of purification of amylase with purification fold. This table showed that there was increase in specific activity after purification. The total activity of the enzyme in ammonium sulphate precipitation decreases. This loss of activity might be due to coagulation process with ammonium sulphate, since the enzyme solution used was very dilute.

Table 1 PURIFICATION PROFILE OF THE ENZYME

Phase	Vol.	Total Activity (U)	Protein (mg/mL)	Protein Total (mg)	Specific activity	Yield (%)	Purification
Crude enzyme	200	552	5.45	1090	0.50	100	1
Purified enzyme	17	122.51	6.825	116.02	1.05	22.19	2.07

The optimum pH of partial purified a-amylase is shown in Fig. 2. The highest activity was found at pH 11.0. This partially purified enzyme exhibits activity between wide ranges of Ph. At pH 12, the decrease of enzyme activity was seen to start.

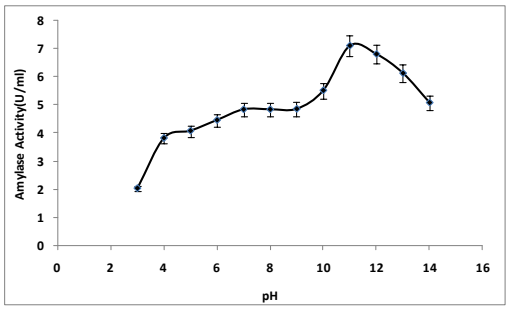

Figure 2 Effect of pH on enzyme activity.

Amylase activity was determined at different temperatures. Maximum activity was observed at a temperature of 60 ℃ (Fig.3). Activity of the enzyme decreases with increasing temperature. At 70 ℃ it offers 84% activity compared to activity at optimum temperature.

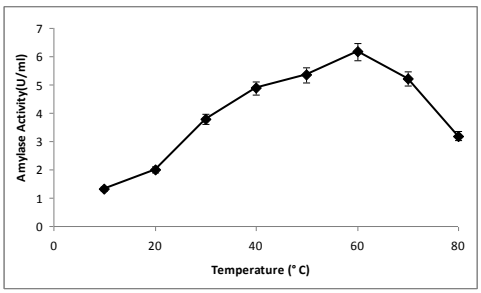

Figure 3 Effect of temperature on enzyme activity.

Upon three different tested activator at a concentration of 0.2 (mg/ml), Magnesium offers the maximum activity. Calcium exhibit similar activity compared to control (without activator). Effect of sodium is comparatively less (Fig.4).

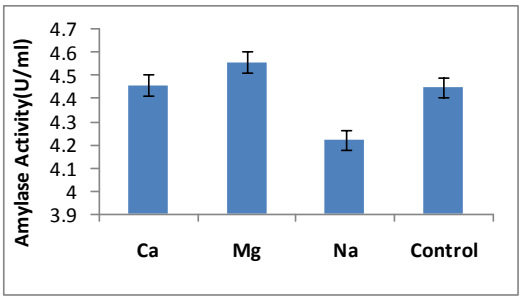

Figure 4 Effect of activator on enzyme activity.

Kinetic properties of amylase

The α-amylase activity with different substrate concentrations was estimated. The K_M and $V_{ma!}$ values of the a-amylase were determined by using Lineweaver-Burk double-reciprocal plot. The reciprocal of reaction velocity, 1/V, is plotted against the reciprocal of the substrate concentration, 1/[S]. Plotting the results gives a straight line; the best fit to the experimental points is 1/V= .394 (1 /[S]) + 0.603. The y-intercept is $1/V_{max}$ and the slope of the line is K_M/ V_{maK}. The x-intercept is -1 / K_M. Hence the V_{max} and K_M values of the -amylase from isolated bacillus species were calculated from Lineweaver-Burk double-reciprocal plot as 1.568 mg min^{-1} and 0.65 mg/ml, respectively (Fig. 4). Low values of Km indicate high affinity of the enzyme for the substrate [13].

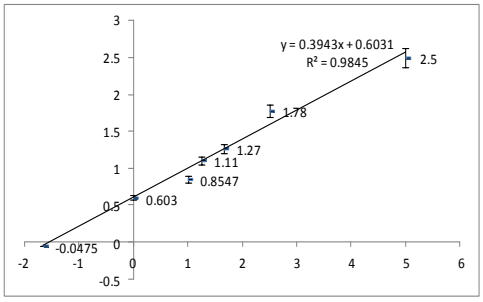

Figure 5 Lineweaver-Burk double reciprocal plot of enzyme kinetics

Conclusions

The production and characterization of amylase produced by *Bacillus megaterium* strain KAN1 (GenBank accession number: BankIt1545135 seq1 JX182976) isolated from traditional fermented drink Handia was examined. Steamed rice is the substrate for this particular alcoholic fermented beverage. The novelty of this partially purified enzyme is that the enzyme is thermostable and can withstand wide range of pH without affecting its activity. Above study concludes that this strain can be a good source for the production of a very important thermostable-alkaline amylase enzyme being used industrially.

In order to attain higher specific activity further purification needs to be done that includes chromatography techniques such as affinity chromatography, ion exchange chromatography, HPLC etc.

References

1. Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A, Osman G ,Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus Sp. Isolate ANT-6. ***Process Biochem***. 2003, 38: 1397-1403
2. Asoodeh A, Chamani J, Lagziana M ,A novel thermostable, acidophilic α-amylase from a new thermophilic "*Bacillus* sp. Ferdowsicous" isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization. ***Int J Biol Macromol***. 2010, 46:289-97
3. Saxena RK, Kakoli Dutt, Lata Agarwal and Preeti Nayyar.. A highly thermostable and alkaline amylase from a Bacillus sp. PNS. Biores. Technol. (2007) 98: 260-265.
4. Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S and Hatada Y. Alkaline detergent enzymes from alkaliphiles: Enzymatic properties, genetics, and structures. ***Extremophiles***. 1998, 2:185-190.
5. Roy A, khanra N, Saha S, Bhattacharya C, Mishra A, and Bhattacharyya N. An antioxidant-rich fermented substrate produced by a newly isolated bacterium showing antimicrobial property against human pathogen,may be a potent nutraceutical in the near future. ***ALSA***. 2012, l:36-44
6. Roy A, khanra N, Mishra A, and Bhattacharyya N. General analysis and Antioxidant study of Traditional fermented drink Handia, its concentrate and volatiles. ALSA. 2012, 1:54-57
7. Roy A, khanra N, Mishra A, Bhattacharya C and Bhattacharyya N. Bakhar-Handia Fermentation: General Analysis and a Correlation between Traditional Claims and Scientific Evidences
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurment with the Folin phenol reagent, ***J Biol Chem***. 1951, 193-265
9. Hoque MM, Akter S, Ahmed MU, Nurun N, Khan MR and Khan ZUM. a-amylase activity of Streptomyces misakiensis activity. ***Bangladeshi Microbiol.*** 2003, 32: 199-125
10. Hamilton LM, Kelly CT and Fogarty WM. Raw starch degradation by the non-raw starch-adsorbing bacterial alpha amylase of *Bacillus sp.* IMD 434. ***Carbohydrate Research***. 1998, 314: 251-257
11. Asgher M, Asad MJ, Rahman SU and Legge RL. A thermostable amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. ***J Food Eng***. 2007, 79: 950-955
12. Horikoshi K. Alkaliphiles: Some applications of their products for Biotechnology. ***Microb Mol Biol Rev***. 1999, 63: 735-750
13. Miller GL. Use of Dinitrosalicylic acid reagent for determination of reducing sugar, ***Anal Chem***. 1959, 31: 426-429