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Production optimization and purification of halophilic amylase from bacterium mb.a.2.1 newly isolated from the coastal region of mahuva

Pal D. Dave, Happy V. Kodiya, Riddhin T. Modi, Sanjay R. Muriyasa, Dr. Rushit J. Shukla*

Department of Microbiology, Christ College, Rajkot
shuklarj@gmail.com

Abstract

Halophilic enzyme in last few decades have gained the significant attention due to its increased commercial applications. Here, the bacterium newly isolated from the coastal region of Mahuva was studied for its halophilic amylase. The bacterium was studied for different biochemical parameters wherein isolate showed MR test and utilization of various sugar test positive. Thereafter, the bacterium was optimized for halophilic amylase production. The optimum production of amylase was achieved at pH 7, 2M salt concentration and 48 hours of incubation. The partial purification of the enzyme was achieved by acetone precipitation method. The enzyme was purified by size exclusion chromatography. The molecular weight and purity of the enzyme was judged from the SDS PAGE. The studied halophilic enzyme requires detail characterization to learn more about its biochemical characteristics and may find its applications in varied field.

Key words: Halophiles, Amylase, Size exclusion chromatography, SDS PAGE

1. Introduction

Extremophiles are a type of organisms that live well in severe environments, either physically or geochemically, which are unfavorable to most other forms of life on Earth. In 1947, McElroy coined the term "extremophile" [1]. Few extremophiles are found in eukarya, while the majority are found in the archaea and bacterial domains [2]. The considerable biotechnological potential associated with microbes and their biological products is a driving force behind research on extremophiles. These microbes' peculiar characteristics are mediated by proteins and enzymes that are involved in metabolic processes and certain biological functions. Extremophiles that thrive ideally in a saline environment up to 5.2 M concentration of salt are known as halophiles. These bacteria are extensively investigated for their commercially

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valuable products, which primarily consist of enzymes and other secondary metabolites. For their distinct industrial uses, such as food coloring pigments in the food business, stress-relieving metabolites, additives in the cosmetics sector, etc., a wide range of halophiles are being employed [3]. Numerous exoenzymes, such as lipases, amylases, and proteases, have been commercially generated from halophilic Archaea and bacteria with success [4]. Proteases and amylases are thought to be the most prominent of the industrially significant enzymes because of their widespread use in the food, beverage, and detergent industries. Amylases are bio-catalysts that control particular biochemical processes. In the starch processing industry, amylases are used to hydrolyze polysaccharides like starch into their constituent simple sugars. Over 80% of the enzyme market worldwide is driven by industrial applications [5]. The most popular and still holding the lion's share of the enzyme market are food enzymes. The primary distinction made between endoamylases and exoamylases among amylase-related enzymes is based on where on starch they operate. The way the glycosidic bond is addressed determines another classification for the enzyme. The enzymes that break down starch belong to the α-, β-, and γ-amylase glycoside hydrolase families. Halophilic amylases are a great way to find new enzymes that are naturally able to work in saline and hypersaline environments. In general, they are stable to salt. Applications of halophilic α-amylase and prospects for future study are also examined. Their enzymes have special qualities that allow them to function well and remain stable in a salty environment [6].

Current study focuses on the production optimization and purification of halophilic amylase from the coastal region of Mahuva, Gujarat. The study is also focused on the biochemical behaviour of newly isolated bacterium.

2. Materials and methods

2.1 Materials

The medium components, soluble starch and Bio Gel P60 were procured from Hi-Media, Mumbai, India. The chemicals and solvents used in the experiments were of Analytical Grade and purchased from the Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India.

2.2 Methods

2.2.1 Microorganism

The bacterium was isolated in the lab by the seniors. The bacterium was revived on modified N-agar plate. The purity of the

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strain was checked by gram staining and spore staining.

2.2.2 Biochemical characterization of the bacterium

The biochemical behaviour of our organism was studied by performing various biochemical tests. The following biochemical tests were performed - Sugar utilization test, Methyl Red test, Voges Proskauer test, Citrate Utilization, Indole test, Phenylalanine deamination test, Urea hydrolysis test, Nitrate test, Ammonia production, Starch hydrolysis test, Gelatine hydrolysis test, Frazier's plate test, Lipid hydrolysis, Catalase test, Dehydrogenase test and TSI test.

2.2.3 Production optimization

Effect of various physicochemical parameters was studied for the optimization of production of the amylase.

2.2.3.1 Effect of salt concentration

To study the effect of different salt concentration on the production of amylase the production media was supplemented with 1.5M and 2M salt concentration. The isolate was inoculated in the production medium containing different salt. The broth was incubated for 72 hours and crude enzyme was assayed at regular interval of 24 hours of incubation.

2.2.3.2 Effect of pH

To study the effect of pH on the amylase production the pH of the production media was adjusted between 7 to 9. The isolate was inoculated in the production medium with varying pH. The broth was incubated for 72 hours and crude enzyme was assayed at regular interval of 24 hours of incubation.

2.2.3.3 Effect of incubation time

To study the effect of incubation time on amylase production medium was inoculated with isolate and incubated up to 72 hours. Crude enzyme was assayed at regular interval of 24 hours of incubation.

2.2.4 Enzyme assay

α-amylase activity was analysed by quantifying liberated maltose in the reaction mixture. For the enzyme assay, 0.5 ml of the sample (crude enzyme, partially purified enzyme) was mixed with 0.5 ml of 1 % starch prepared in 20 mM Phosphate buffer with 2M NaCl, pH 7. The reaction mixture was incubated at 37°C for 10 minutes followed by the estimation of the liberated maltose by the Dinitro Salicylic acid (DNSA) method [7]. One unit of α-amylase liberates 1 µg of the maltose per minute under our assay conditions, with maltose as the standard (100µg/ml – 1000µg/ml).

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2.2.5 Partial purification

The 48-hour culture grown under the optimized conditions was centrifuged at 5000 rpm for 20 min at 4 °C and cell-free extracts (crude enzyme) were prepared. Chilled acetone (double volume of crude enzyme) was added slowly with constant stirring for 24 h at 4 °C. The precipitate so formed was recovered (5000rpm, 20min) and dissolved in a minimum volume of 20mM phosphate buffer, pH 7, 2M NaCl. Enzyme activity and protein concentration were determined after every step using DNSA and Folin's method.

2.2.6 Purification of enzyme

The partially purified enzyme was loaded onto a Bio gel P60 column equilibrated with 20 mM Phosphate buffer with 2M NaCl, pH 7. Fractions were collected and the flow rate was maintained at 5 ml/h. Enzyme activity and protein concentration of all the fractions were determined by DNSA method and Folin Lowry's method respectively.

2.2.7 Determination of molecular weight

Purity and molecular weight of the amylase was determined by SDS-PAGE [8].

3. Results and discussion

3.1 Microorganism

3.1.1 Revival of sample organism

Reactivation of halophilic bacteria (sample organism) from glycerol stock was conducted on modified N agar. The bacterium MB.A.2.1 gave number of colonies on modified N agar plate.

3.1.2 Gram's staining

Halophilic bacterium gave gram +ve (Violet coloured) morphology after staining the microbial culture. The shape of the bacteria was coccus and arrangement were in diplococci form.

3.1.3 Spore staining

The results of spore staining showed red vegetative cells while no green spores indicating the bacterium as non-sporing bacterium.

3.2 Biochemical characterization of the bacterium

Several biochemical tests were performed to check the biochemical nature of an organism. The results of the biochemical tests are summarized in table 1.

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Sr. no.	Test	Observation	Conclusion
1	Sugar utilization test	Positive acid production, no bubbles were formed.	Acid producer
2	Methyl Red test	Development of stable red colour	Mixed acid fermenter
3	Voges Proskauer test	No colour change	Absence of acetyl methyl carbinol
4	Citrate utilization test	No colour change	Absence of citrate permease
5	Indole test	No colour change	Absence of tryptophanase
6	Phenylalanine deamination test	No colour change	Absence of deaminase
7	Urea hydrolysis test	No colour change	Absence of urease
8	Nitrate test	No colour change	Absence of nitrate reductase
9	Ammonia production test	No colour changes in litmus paper	Absence of nitrate and nitrite reductase
10	Starch hydrolysis test	Clear colourless zone was seen	Presence of amylase
11	Gelatine hydrolysis test	Medium was solidified	Absence of gelatinase
12	Frazier's Plate test	Clear zone was not observed	Absence of gelatinase
13	Lipid hydrolysis test	Clear zone was not observed	Absence of lipase
14	Catalase test	No effervescences were seen	Absence of catalase
15	Dehydrogenase test	No colour change	Absence of dehydrogenase
16	TSI test	No colour change	TSI negative

Table 1- Result of Biochemical tests

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The result indicates the organisms is acid producers with mixed acid fermentation pattern. Also, the bacterium is having ability to produce amylase enzyme.

3.3 Production optimization

3.3.1 Effect of salt concentration

The salt concentration is an important parameter that determines the growth rate of halophilic bacteria and significantly affects the level of enzyme production. Different organisms have different optimal salt concentration and any change in salt concentration on either side of optimum value result in poor microbial growth and enzyme production. In current study, the optimum salt concentration is found to be 2M with maximum enzyme activity of 1215 µg/ml/min along. The optimum salt concentration of *Halobacillus* sp. strain MA-2 was at 5% salt concentration. Maximum amylase activity was exhibited in the medium containing 5% (w/v) NaCl (2.4 U ml^{-1}) [9]

3.3.2 Effect of pH

pH is an important parameter that determines the growth rate of microorganism and significantly affects the level of enzyme production. Different organisms have different pH optima and decrease or increase in pH on either side of optimum value result in enzyme production. In current study, the optimum pH is found to be 7 with highest enzyme activity of 1215 µg/ml/min. Rodriguez-Valera and his group reported pH 7.2 to be the usual pH of culture media for growing *Haloferax mediterranei* [10]. Isolate MB.A.2.1 showed maximum amylase production at pH 7 indicating its neutral nature.

Table 2- Effect of salt and pH at different incubation time

Sr no.	24 hours		48 hours		72 hours	
	pH 7	pH 9	pH 7	pH 9	pH 7	pH 9
1.5 M	638.5	423.5	727	496.5	444	343
2 M	688.5	391	1215	999	424.5	370

3.3.3 Effect of incubation time

The enzyme productions of microorganisms are totally dependent on culture condition and incubation time required for utilization of nutrients. Increase in incubation period increase enzyme production but in case of this

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bacterial strain there is no further increase in enzyme production rather it decreased after 48 hrs incubation. The optimum time for enzyme production by the studied bacterium is 48 hrs. Incubation time of *Aspergillus Niger* for enzyme production was 120 hrs that was 5th day [11]. Similar results were observed in case of Bacillus sp. strain TSCVKK [12].

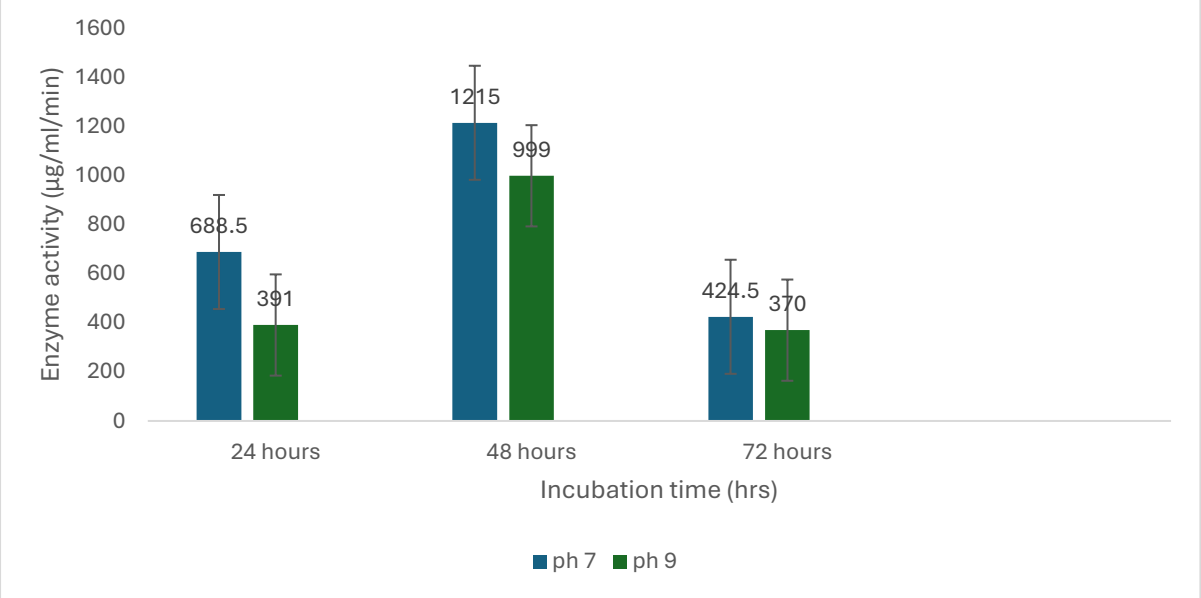

Figure 1- Production of amylase at different incubation time under different pH and 2 M salt concentration

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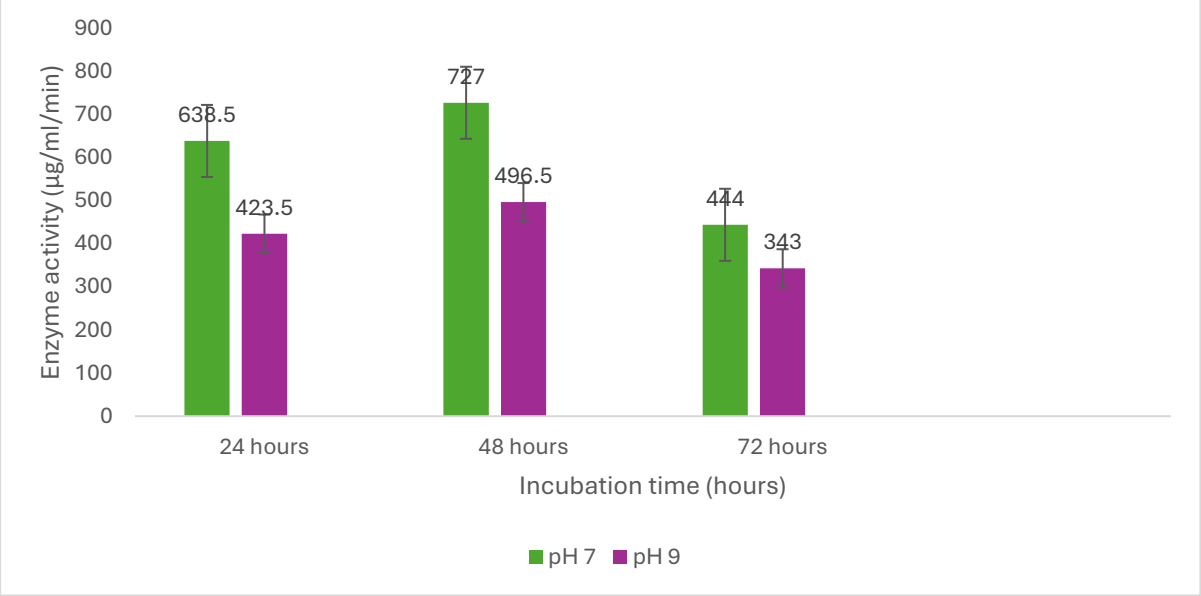

Figure 2- Production of amylase at different incubation time, different pH and 1.5M salt concentration

3.4 Partial purification

Partial purification of enzyme was done by acetone saturation method. The enzyme was partially purified 3.40-fold with 50% yield and specific activity of 1,712.39 (µg/ml/min). The purification profile in terms of specific activity, fold purification and % yield is shown in table 3. Shukla et.al, reported partial purification of α-amylase from thermophilic *Bacillus licheniformis* TSI-14, 3.23-fold with 17.6% yield [13].

3.5 Purification of enzyme

The amylase enzyme was purified by Gel permeation chromatography using a glass column. The column was packed using Bio Gel P60. The fractions were examined using DNSA method for checking the enzyme activity and Folin's method [14] was used for the determination of protein concentration of each fraction. The amylase was purified up to 7.18-fold with the yield of 0.8 %. The elution profile shows that maximum enzyme is eluted in fraction no. 21. The results are summarized in the table 3 and the elution profile is shown in figure 3.

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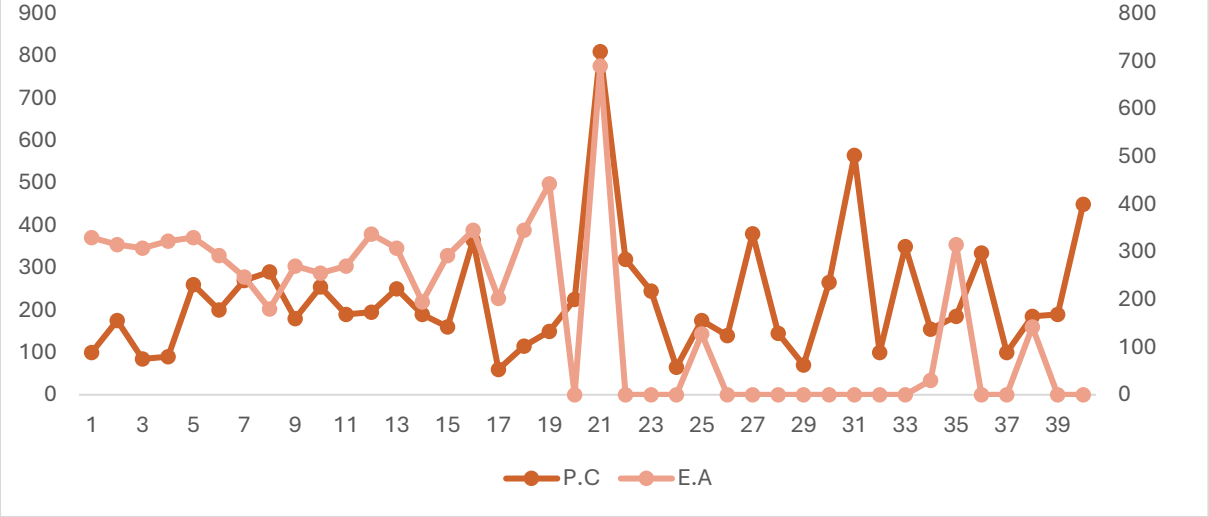

Figure 3 -Total E.A and Total P.C. of eluted sample

Table 3- Summary of partial purification of amylase from the bacterium MB.A.2.1

Fractions	Total E. A (U)	Total PC (mg)	Specific activity (µg/ml/min)	Fold purification	%Yield
Crude	34,800	69.05	503.97	1	100
Partially	17,415	10.17	1,712.39	3.40	50.04
Fraction	307.5	0.085	3617.65	7.18	0.88

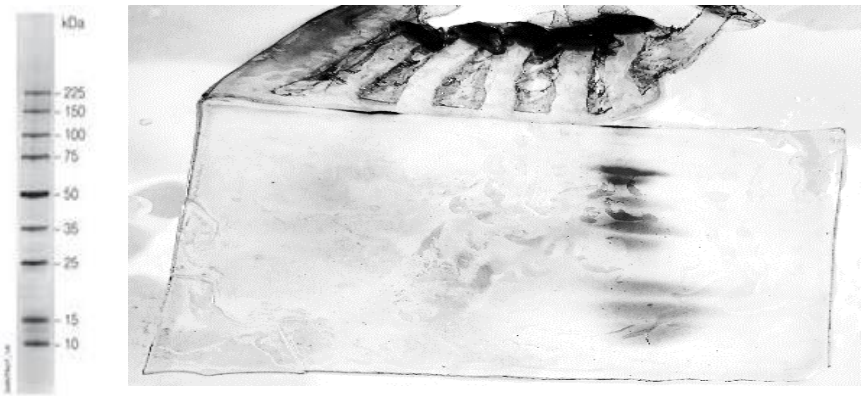

Figure 4- Figure 4- SDS PAGE: From left to right Lane 2- partially purified enzyme, Lane 4- purified enzyme and Lane 6- medium range protein marker

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3.6 Determination of molecular weight

Partially purified enzyme and the purified fractions were analysed on SDS PAGE. The faint band of the purified protein was observed on the gel. The results suggest need of concentrating the sample. The approximate molecular weight of the amylase was calculated to be 43 kDa from the above SDS PAGE. The photograph of SDS PAGE is shown in figure 4

Conclusion

Amylase is one of the most important enzymes known and is of great significance having approximately 25 % of share in enzyme market. Amylase have potential applications in food, pharmaceutical and fine chemical industries. Earlier several studies reported the isolation and purification of amylase from microorganisms. This study reports production optimization and purification of halophilic amylase from bacterium MB.A.2.1 newly isolated from the coastal region of Mahuva. The biochemical behaviour of bacterium shows it as an acid producer with mixed acid fermentation pattern of sugar utilization. The best enzyme production was found in presence of 2M NaCl concentration and at pH 7.0. The approximate molecular weight of the amylase was calculated as 43 kDa. The further characterization of purified enzyme will be useful in determining the best applicability of the enzyme.

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