Int.J.Curr.Microbiol.App.Sci (2017) *6*(5): 2981-2987

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 5 (2017) pp. 2981-2987**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.605.338

Isolation and Partial Characterization of Amylase Producing *Bacillus flexus* PR1

P. M. Patel[1], R. G. Chaudhari[2]* and S. A. Bhatt[3]

[1]M.G. Science Institute, Ahmedabad, Gujarat, India
[2]Shri PJPN Science College, Maktupur, Unjha, Gujarat, India
[3]Department of Life Sciences, HNGU, Patan, India
**Corresponding author*

Keywords

Bacillus flexus, alkaline amylase, (G+C) content, 16S r-DNA, enzymes

Article Info

Accepted:
04 April 2017
Available Online:
10 May 2017

A B S T R A C T

Bacillus strains were isolated from highly alkaline soil from Himatnagar city of North Gujarat. The best amylase producing bacteria were screened on starch agar plate. Among the screened isolates highest amylase producer strain PR-I was selected, characterized and identified on the basis of 16S r -RNA sequence homology. The major cellular fatty acids of the strain were also identified. The strain was gram positive, spore forming and aerobic, motile bacillus species. 16S r- RNA sequence homology shows 99% similarity with *Bacillus flexus*. This strain grows in the pH range of 4.0 to 12. The optimum growth was obtained at pH 10 and temperature 37^0 C. PR-1 produced extracellular alkaline amylase, and its maximal enzyme activity was observed at 45^0C and10.0 pH.70% enzyme activity was retained at pH 12. The strain showed salt tolerance up to 4% of NaCl. The enzyme activity was found to be increased in the presence of Co^{2+} and Mg^{2+} cations. The results of the growth and enzyme activity were discussed.

Introduction

Bacteria of the genus Bacillus are potentially rich source of various valuable enzymes. These bacteria can produce extracellular enzymes with major applications in processes operated under extreme conditions due to their adaptability to environmental limits Alkaliphilic *Bacillus* species are considered to be of major interest due to their ability to produce extracellular enzymes that are active and stable at high pH, including alkaline amylase, protease and cellulase. The unusual properties of these enzymes propose a potential opportunity for their utilization in processes demanding such extreme conditions (Karnwal and Nigam, 2013). Amylases (E.C.3.2.1.1) are enzymes which hydrolyze starch molecules to give diverse products including dextrins and even smaller polymers composed of glucose units.

Amylases constitute a class of industrial enzymes, which contribute approximately 25% of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals (Das *et al.*, 2011). Thus, the potency of alkaline amylases for industrial applications have encouraged the search for microbial strains expressing activities with enzyme with desired properties (Alkando *et*

al., 2011). The strain in study PR-1 was originally isolated from alkaline soil in the Himatnagar city, it showed very high alkali-tolerance and was identified as a new member of *Bacillus flexus*. This work deals with the isolation and characterization of PR-1 and some properties of its extracellular alkaline amylase. The above features could favour current industrial demand of commercially utilizable saccharifying enzymes.

Materials and Methods

Isolation and screening of the amylase producing bacteria

Twenty *Bacillus* spp. capable of growing highly alkaline conditions were isolated from alkaline soil (pH 11.0) in Himatnagar city. Screening for starch hydrolysis activity among the isolated colonies was performed by plating them on the agar medium containing (W/V%): soluble starch 1.0, peptone 1.0, yeast extract 0.5, K_2HPO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.02, NaCl 1.0, agar 2, pH 10.0 and incubating for 24 h at 37°C. The plates were flooded with iodine reagent to reveal the zone of starch hydrolysis (Van der Maarel *et al.,* 2002).

The diameter of the halo zone against the diameter of the colony was used as a semi quantitative method for the selection of the starch hydrolyzing strains. Depending on the zone diameter and clearance isolate PR-I was selected as a good alkaline amylase producer.

Phenotypic and genotypic identification

Identification of selected isolate was carried out on the basis of their morphological and biochemical tests were performed according to Bergey's manual of systematic bacteriology and the methods described in the Genus *Bacillus* (Das *et al.,* 2004). It was then confirmed with 16S r RNA sequencing, pure cultures of the target bacteria were grown in nutrient broth medium on a rotary shaker (150 rpm) at 30°C for 24 h for the isolation of genomic DNA (Yadav *et al.,* 2009).

Characteristics of amylase

Enzyme production medium

The medium for enzyme production used having (W/V%): starch 2.0, peptone 1.0, yeast extract 1.0, K_2HPO_4 0.1, and $MgSO_4 \cdot 7H_2O$ 0.02 (Kaur *et al.,* 2012). The medium (50 mL) was inoculated with 0.5 mL of the inoculum with an optical density of 0.6 at 600 nm and incubated at 37°C (El-Tayeb *et al.,* 2007). Samples were harvested till 72 h by centrifugation at 6000×g for 10 min and the cell free supernatant was analysed for amylase activity as described below.

Enzyme assay

The amylase activity was estimated on the basis of the reduction in blue color intensity resulting from enzymatic hydrolysis of starch. The reaction mixture containing 1% starch (0.1 mL), 0.3 mL of 0.05 mol/L glycine NaOH buffer (pH 10.0) and 0.1 mL of culture filtrate was incubated at 37°C for 15 min in a water bath. The reaction was terminated by adding 1 mL chilled 1 mol/L HCl. To this mixture, 2 mL of diluted iodine was added and the volume was made to 10 mL with double distilled water.

A substrate control-lacking enzyme was also kept with each set of reaction. The amylase activity was estimated after appropriate dilution and absorbance was read at 620 nm against a substrate blank. One dextrinizing unit of amylase activity is defined as the amount of enzyme that results in 10% decline in the optical density of the starch iodine complex at 620 nm when compared to substrate blank. All the experiments were carried out in triplicates and results presented are the mean of three values.

Partial purification

The cell free supernatant fluid was precipitated using ammonium sulphate to85% saturation. The precipitate was dissolved in 0.2 M glycine NaOH buffer (pH 10.0) and dialysed overnight against the same buffer. The dialysate was used for all enzyme characterization studies.

Effect of pH on enzyme activity and stability

The pH optimum of the enzyme was determined by varying the pH of the assay reaction mixture using the following buffers (0.2 mol/L): Na_2HPO_4/citrate phosphate buffer (pH 3.0−8.0), glycine/NaOH buffer (pH 9.0−10.0), Na_2HPO_4/NaOH buffer (pH 11.0) and KCl/NaOH buffer (pH12.0−13.0). To determine the stability of amylase, the enzyme was pre-incubated in different buffers (pH 7.0−13.0) for 30 and 60 min. The residual enzyme activity was determined as described earlier.

Effect of temperature on enzyme activity and stability

The temperature optimum of the enzyme was estimated by measuring the amylase activity at different temperatures (40°C −80°C) in 0.2 mol/L glycine/NaOH buffer (pH 10.0).

The effect of temperature on amylase stability was determined by measuring the residual activity after 20, 40 and 60 min of preincubation in 0.2 mol/L glycine/NaOH buffer (pH10.0), at temperatures ranging from 40°C to 70°C (Vaseekaran *et al.,* 2010)

Effect of metal ions on enzyme activity

For determining the effect of metal ions on amylase activity, enzyme assay was performed after pre-incubation, at 40°C (optimum) for 60 min, of the enzyme with various metal ions each at a concentration of 50 mmol/L. The enzyme assay was carried out in the presence of $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $FeSO_4$, $CoCl_2$, $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4$ and EDTA.

Results and Discussion

Morphological, physiological and biochemical properties

Twenty *Bacillus* spp. capable of growing highly alkaline conditions were isolated from alkaline soil (pH 11.0) in Himatnagar city, among these we have screened a potent alkaline amylase-degrading microorganism **(**Alkando *et al.,* 2011).

Depending on the zone diameter and clearance, the isolate PR-I was selected as a good alkaline amylase producer. The isolate was able to grow and produce extracellular amylase when it was cultured in amylase induction medium (Kanimozhi *et al.,* 2014). It was a Gram-positive, aerobic, motile, spore-forming, alkaliphilic bacterium, and its cells were rod-shaped. It grew at a wide range of pH values (4.0−12) in LB medium, with the optimum being at pH 10. Growth occurred at 10°C−60°C, with the optimum growth temperature being at 37°C. Its NaCl tolerance was high up to 4 %. It showed catalase-positive and oxidase negative reactions but did not reduce nitrate to nitrite Table 1.

16S rDNA sequences analysis and (G+C) content were done and to understand the phylogenetic position of our isolate, we constructed a phylogenetic tree based on comparison of 16S rDNA sequences of our isolate and correlative taxa (Fig. 2). PR-I has 99% sequence similarity with all known strains of *B. flexus* and formed a tight cluster with them. The (G+C) content of the genomic DNA of strain PR-I (39.1mol %) was almost

identical to that of *B. flexus* (37−39 mol %).

Characteristics of amylase

pH optima and stability check : Optimum pH of PR-I amylase is shown in (Fig. 1-A). Determination of enzyme activity at 40°C at pH values ranging from 3.0 to 13.0 showed the amylase found to be active in a wide range of pH values with maximum production at pH 10.0 (14.63 U/mL). More than 70% of enzyme activity was found between pH 9.0 and 13.0. A significant decline of enzyme activity was observed in the acidic pH and only 50% residual activity was retained at pH7.0, whereas 90% retained at pH 11.0. The pH stability was determined at 40°C with different pH values (pH 7.0−13.0) (Kamm and Kamm, 2004).

Fig.1 The effects of temperature and pH on the amylase activity and stability

Fig.1A The effects of pH on the amylase activity

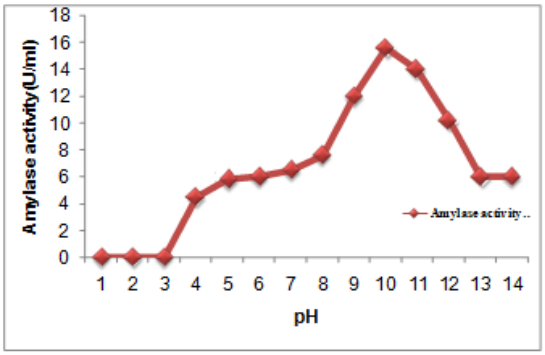

Fig.1B pH stability of the enzyme

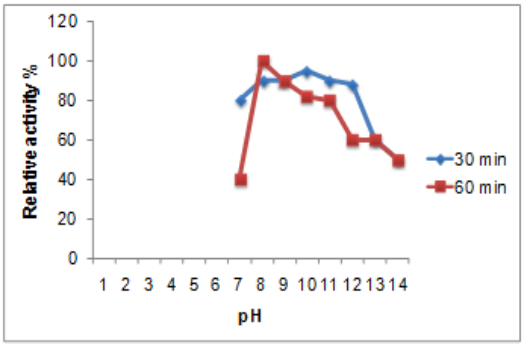

Fig.1C The effects of temperature on the amylase activity

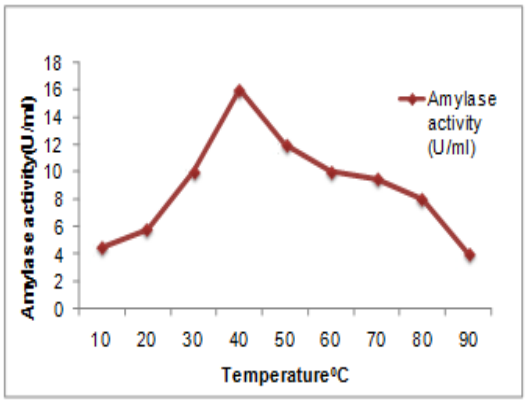

Fig.1D Thermal stability of the enzyme

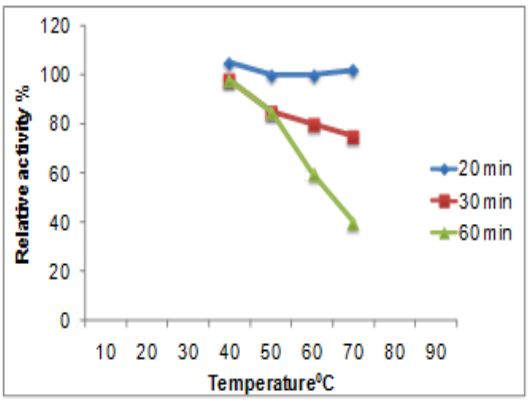

Table.1 Physiological and biochemical properties of *B flexus* PR-I

Characteristics	*B flexus* PR-1	Characteristics	*B flexus* PR-1
Colony color	Cream	Hydrolysis of	
Gram reaction	Gram positive	Casein	+
Tolerence of pH 4-12	+	Starch	+
Growth in NaCl		Xylan	+
5% (W/V)	+	Cellulose	-
8% (W/V)	+	Acid production from	
15%(W/V)	+	D- fructose	+
Growth at		D-glucose	+
40^0C	+	D-lactose	-
50^0C	+	Maltose	+
60^0C	+	D-Mannitol	+
Catalase	+	D-Raffinose	-
Oxidase	-	Sucrose	+
Nitrate reduction	-	D- Xylose	+
		D-Trehalose	+

Table.2 Effects of metal ions

Cations 50 mmol/l	Relative activity %
Control	100
Fe^{+2}	52
Co^{+2}	125
Cu^{+2}	73
Mn^{+2}	41
Zn^{+2}	88
Mg^{+2}	130
EDTA	63

Amylase was stable between pH8 and 13, and more than 60% of the activity was retained after incubated for 1h (Fig. 1-B). It is clearly evident that amylase produced by PR-I, which was designated as PR-I amylase, is very stable to extremely alkaline environment.

Temperature optimum and stability: Using starch as substrate, the optimum temperature of the amylase was 40°C (Fig.1-C). Under some constant temperature, we found that PR-I amylase was very stable at 50°C (Thippeswamy *et al.*, 2006). Even more than 60% of amylase activity still observed when it was incubated at 60°C for 1 h (Fig.1-D), (Das *et al.*, 2004).

Effect of different metal ions and chelating agents: The effects of various metal ions and chelating agents on enzyme activity were examined at pH 10 and 40°C (Table 3). Slight effects were observed in the presence of Zn^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+} and chelating agents of EDTA only inhibited activity of the enzyme to 52, 78, 39, 54 and 65%, respectively. In contrast, Mg^{2+}, Co^{2+} obviously increased the enzyme activity to 138 and 123%, respectively (Jogezai *et al.*, 2011) (Table 2).

Bacillus species are the most important industrial microorganism with versatile roles. The development of a variety of commercial enzymes products with the desired temperature, pH activity, and stability properties require to be of significant. In this study the isolate PR-I could grow and produce extracellular amylase when it was cultured in amylase agar medium. Combined with morphological, physiological and biochemical characteristics with 16S rDNA sequence analysis and the (G+C) content of the genomic DNA, the strain identified as *B.flexus*. The 16S rDNA sequences, the hydrolysis of urea, the utilization of D-Xylose, D-Lactose and D- Raffinose, and the excellent pH tolerance, which were different from those of the reference strain *B. flexus* DSM1320T and the description of the Manuals of Bergey, the strain was classified as a new member of *B. flexus*. The alkaliphiles can grow at pH 9 and the optimum pH is 10.0−12.0, but they can grow weakly or not grow at pH6.5 approximately. The alkaliphiles can grow at pH 9 and the optimum pH is 10.0−12.0, but they can grow weakly or not grow at pH6.5 approximately (Gangadharan *et al.*, 2006), while alkali-tolerant bacteria cannot grow at the pH over 10.5, but can grow at pH7.0−9.0. Our results indicate that *B. flexus* can be a potential organism for various applications including the treatment of alkaline agroindustrial wastes.

References

Alkando, A. A., Ibrahim, H. M., (2011) A potential new isolate for the production of a thermostable extracellular a-amylase. J. Bacteriol.Res. 3, 129–137.

Das K, Doley R, Mukherjee A K, (2004) Purification and biochemical characterization of thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03, a strain isolated from the traditional food of India. *Biotechnol Appl Biochem*, 40: 291-298.

Das S, Singh S, Sharma V, Soni M L (2011) Biotechnological applications of industrially important amylase enzyme. *Inter J Phar Bio Sci*, 2(1):486-496.

El-Tayeb O, Mohammed F, Hashem A, Abdullah M (2007) Optimization of the industrial production of bacterial alpha amyalse in Egypt. IV. Fermentor production and characterization of the enzyme of two strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*. *Afr J Biotechnol*, 7(24):4521-4536

Gangadharan D, Sivaramakrishnan S, Namboothiri K M, Pandey A, (2006) Solid culturing of *Bacillus amyloliquefaciens* for -amylase production. *Food Technol Biotechnol*, 44(2):269-274

Jogezai, N., Raza, A., Abbas, F., Bajwa, M., Mohammad, D., Kakar, W., Saeed, M., Awan, A. (2011). Optimization of cultural conditions for microbial alpha amylase production. J. Microbiol. Antimicrob.3, 221–227.

Kamm, B., Kamm, M. (2004). Biorefinery – systems. Chem. Biochem.Eng. 18, 1–6.

Kanimozhi, M., Johny, M., Gayathri, N., Subashkumar, R., (2014). Optimization and production of a -amylase from halophilic bacillus species isolated from mangrove soil sources. J. Appl.Environ. Microb. 3, 70–73.

Karnwal, A. and Nigam, V., (2013). Production of amylase enzyme by isolated microorganisms and its application. IJPBS 3, 354–360.

Kaur P, Vyas A (2012) Characterization and optimal production of alkaline α-

amylase from Bacillus sp. DLB 9. *Afr J Microbiol Res*, 6(11):2674-2681

Kumarai, B. L., SaiRam, C. V. S., Kumar, T. S., Sudhakar, P., Vijetha, P., (2011). Screening and isolation of thermostable a-amylase producing bacteria and optimization of physico-chemical parameters for increasing the yield. Int. J. Pharm. Technol. 3, 1570–1583.

Thippeswamy, S., Girigowda, K., Mulimami, H. V., (2006). Isolation and identification of a- amylase producing Bacillus sp. from dhal industry waste. Ind. J. Biochem. Biophys. 43, 295–298.

Van der Maarel, M. J. E. C., van der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., Dijkhuizen, L., (2002). Properties and applications of starch converting of alpha amylase family. J. Biotechnol. 94, 137–155.

Vaseekaran, S., Balakumar, S., Arasaratnam, V., (2010). Isolation and identification of a bacterial strain producing thermostable aamylase.Trop. Agric. Res. 22, 1–11.

Yadav, V., Prakash, S., Srivastava, S., Verma, P. C., Gupta, V., Basu, V., Rawat, A. K., (2009). Identification of Comamonas species using16S rRNA gene sequence. J. Bioinform. 3, 381–383.

How to cite this article:

Patel, P. M., R. G. Chaudhari and Bhatt, S. A. 2017. Isolation and Partial Characterization of Amylase Producing *Bacillus flexus* PR1. *Int.J.Curr.Microbiol.App.Sci.* 6(5): 2981-2987.
doi: http://dx.doi.org/10.20546/ijcmas.2017.605.338