Enzyme Engineering
ISSN: 2329-6674

Sahoo, et al., Enz Eng 2016, 5:3
DOI: 10.4172/2329-6674.1000152

Research Article Open Access

A High Salt Stable α-Amylase by *Bacillus Sp.* MRS6 Isolated from Municipal Solid Waste; Purification, Characterization and Solid State Fermentation

Sumit Sahoo[2], **Sudipta Roy**[1#] **and Smarajit Maiti**[1,2*]

[1]*Agricure Biotech Research Society, Epidemiology and Human Health Division, Midnapore-721101,India*

[2]*Department of Biochemistry and Biotechnology, Cell and Molecular Therapeutics Laboratory, Oriental Institute of Science and Technology (OIST), Vidyasagar University, Midnapore-721102, West Bengal, India*

[#]*Department of Biotechnology, Oriental Institute of Science and Technology(OIST), Vidyasagar University, Midnapore-721102, West Bengal, India*

[*]**Corresponding author:** Smarajit Maiti, Associate Professor and Head, Post Graduate Department of Biochemistry and Biotechnology, Cell & Molecular Therapeutics Laboratory, OIST, Vidyasagar University, Midnapore -721101, India, Tel: 9474504269; E-mail: maitism@rediffmail.com

Rec date: Jul 25, 2016; **Acc date:** Sep 09, 2016; **Pub date:** Sep 14, 2016

Abstract

Salt-tolerant alkaline amylase producing *Bacillus sp.* MRS6 was isolated from three municipal waste disposal site of Medinipur town in the Paschim Medinipur district of southern West Bengal, India. The strain had been characterized from a polyphasic approach. Extracellular enzyme production was carried out in mineral salt media at pH-7, 35°C with 2% soluble starch as sole carbon source. It was found that the amylase, produced by the salt-tolerant strain possessed high activity in a range of alkaline pH (6-9), with pH-8 as optimum. An incubation period of 30 min at 35°C was found as optimum temperature condition of its activity. This alkaliphilic, salt-tolerant enzyme was found to stay stable upto 80°C, 4M NaCl. $Mn^{2+,}$ Ca^{2+} plays critical role in >80% increasing of this enzyme activity. EDTA, β-merkeptoethanol and detergents strongly inhibit enzyme activity. A solid state fermentation for production of this enzyme was performed with 85% moisture content where wheat bran was found as most effective and cheap carbon source. This enzyme was purified partially by cold acetone precipitation followed by gel filtration chromatography (seralose 6B) and the molecular mass was found to be 55 kDa by SDS PAGE. The present findings suggested the enzyme to be halophilic alkaline amylase. The present enzyme is of great significance in present day biotechnology with applications ranging from food, fermentation, textile, detergent to paper industries.

Keywords: Halophilic bacteria; Salt tolerant; Alkaline amylase; *Bacillus* sp. MRS6

Introduction

Amylase is one of the important enzymes used worldwide in various industrial sectors. Microorganisms offer significant contribution in production of this enzyme. Amylases are enzyme which hydrolyses starch to yield diverse products, including dextrin and progressively smaller polymers composed of glucose units. Generally most of the amylases produce glucose and maltose as main products during starch hydrolysis. These mono and oligosaccharides have marked pharmaceutically importance as for example those are low calorigenic, less sweetening, less viscous, high moisture-retainer and those inhibit the growth of harmful intestinal microflora [1]. Unfortunately, the function of enzyme-catalysed reactions has been restricted due to the poor stabilities of enzyme proteins under industrial conditions like high temperature, salt-concentration, acid or base and in the presence of non-aqueous solvents. The diversity of nature provides various extreme environments of temperature, salinity, pH, acidity, alkalinity and pressure which can be exploited to discover novel and potentially robust enzymes that are better suited for use in industrial applications.

The searching of microbial amylase with novel activities and enhanced stability is a challenging work which can commercially support several related industries. Municipal solid waste consists of everyday items that are discarded by the public. Organic wastes are consumed by existing microbes and are no longer present to produce odours, sludge, pollution or unsightly mess. When bacteria consume waste, they convert the waste into simple compounds by releasing various enzymes. Hence there is an immense possibility to screen effective bacterial strains from waste dump sites with valuable applications. Osmotolerant and osmoactive enzyme have gained considerable attention in sugar, leather, paper/pulp, textile, detergents, baking, brewing, and distilling in animal feed industries. These enzymes are also useful in the production of cakes, starch-syrups, digestive-aids and pharmaceutical products [2,3]. The halophilic enzymes are active at salt concentrations ranging from 0.2 M to . and have been documented in waste treatment too wherein normal enzymes may not function optimally or may even get denatured [4,5].

The present study was aimed to investigate bacterial strains from municipal waste-dump sites with the ultimate objective of solid waste degradation and discovering novel enzymes with unusual properties for industrial submission.

Materials and Methods

Study location and sample collection

Solid wastes were collected from three municipal waste disposal site of Medinipur town (10°57′37″S, 19°18′56″E) in the Paschim Medinipur district of southern West Bengal, India. The study sites were near Gate Bazar, Raja Bazar and Mirza Bazar, which are about 3 Km away from each other. Sub surface semi decomposed materials were collected in sterile zip locked plastic bags. A total of 9 samples (3 from each

location) were collected and immediately placed 4°C till further experiment conducted.

Isolation of amylase producing bacteria

Serial dilution technique was employed for isolation of bacteria from collected sample. For this purpose 1 g waste was transferred in 10 ml distilled water added with Tween 80 (0.01%, v/v). Suspensions were shaken vigorously and diluted serially up to 10^5 times. From each dilution tube 0.1 ml of diluted suspension was transferred into Nutrient Agar media supplemented with 2% insoluble starch and incubated at 37°C for 24 hrs. Working on amylase producing microbes from diverse habitat, bacteria were isolated from dump garbage of municipality West Bengal, India. Isolation and enrichment was done on starch agar media (2% soluble starch). Amylase producing bacteria were identified by observing clear zone surrounding bacterial growth. Active isolates were cultured in pure form after successive passages and preserved at -20°C with glycerol (30% v/v) and evaluated for their amylase activity in DNS method by growing in starch broth [6-8].

Molecular identification and phylogeny construction

Cells were grown in LB media for 24 hrs and genomic DNA was isolated from the cell pellet after lysozyme (20 $mg.ml^{-1}$) and SDS (10%) treatment in Tris buffer pH-8 [9]. Phenol-chloroform extraction was performed followed by precipitation of DNA with isopropanol. The 16S rRNA gene was amplified using a primer 27F 5/-AGTTTGATCCTGGCTCAG-3/and 1492R 5/-GGCTTACCTTGTTACGACTT-3/ [10]. PCR conditions were programmed as follows: initial denaturing temperature of 94°C for 5 min and then 35 cycles were followed each cycle of 1 min at 95°C, primer annealing for 1 min at 55°C and primer elongation 5 min at 72°C. A final extension was done for 10 min at 72°C. Amplified products were purified with PCR column purification kit (Himedia, Mumbai, India) and sequencing was carried out with same primers.

Consensus sequence of 1306 bp was carried for BLAST with the NR database of NCBI. Based on maximum identity sequences were carried for multiple alignments by Clustal W. A distance matrix was generated using RDP (Ribosomal Database Project) and the phylogenetic tree was constructed using MEGA5 (Molecular Evolutionary Genetics Analysis) [11]. The evolutionary history was inferred using the Neighbor-Joining method [12]. The evolutionary distances were computed using the Kimura 2-parameter method [13].

Characterization of the strain MRS6

The strain MRS6, one of the isolated strains was characterized after an established study with a polyphasic approach due to its high amylase activity. The standard microbiological procedures were followed to determine its colony and cell morphology. For determining the cellular morphology, 24 hrs young culture (MRS6) was strained and observed under compound microscope at 100x magnification. A physical measurement and morphological facts were exposed after scanning electron microscopic study of the strain (Vega © Tescan, Czech Republic). Sample preparation for SEM observation was followed by graded ethanol dehydration and taken up to critical dry point [7]. Several biochemical properties like sugar utilization, extracellular enzymatic summary and antibiotic susceptibility pattern were also determined after growing it on different media. Growth features of the strain (initial media pH, incubation temperature, and salt tolerance) were also studied [8].

Enzyme production in solid state fermentation (SSF)

The SSF process was carried out in 250 ml Erlenmeyer flasks containing 5 g of Wheat Bran (WB) and Rice Husk (RH). Distilled water was added in such a way that final substrate moisture content was 85% (w/v). After sterilization by autoclaving, flasks were cooled and inoculated with a 10% (v/w) inoculum level [14]. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and incubated at 35°C for 7 days. Crude enzyme was extracted from the fermented matter by mixing the fermented matter with distilled water, 1:5 ratio (w/v) and placed on a rotary shaker (220 rpm) for one hour. Finally it was homogenized in 3 cycles of 15s. [15]. The obtained mixture was centrifuged at 10000 rpm for 15 min and the supernatant was used for enzyme assay. The activity of the enzyme was assayed at pH-8 by the DNS using 1% (w/v) soluble starch as substrate at 35°C for 30 min.

Optimization for amylase production

Optimum amylase production from strain MRS6 was carefully monitored by growing in mineral salt media formulated with soluble starch 2%, $MgSO_4$, $7H_2O$ 0.05%, K_2HPO_4 0.1%, $NaNO_3$ 0.2%, KCl 0.05%, $FeSO_4$, $7H_2O$ 0.001%, Peptone 0.1%, Yeast Extract 0.1% (w/v). Maximum production of amylase was determined by growing the strain separately at different initial media pH (2-14) and incubation temperature (10-50°C) separately. Culture was inoculated with predetermined optimum conditions and grown up to 72 hrs. Samples were drawn at each 6 h interval to determine amylase activity and parallel growth curve was also determined.

Characterization of enzyme activity

Optimum conditions for amylase activity was determined, where culture supernatant (100 µl) of MRS6 was taken as enzyme source. Enzyme substrate reactions were carried out for 30 min at different pH (2–14) and temperature regions (10-70°C). Effect of glucose, metal-ions, chelating agents, reducing agents, oxidizing agents, detergents and various solvents on enzyme activity was also determined by standard methods [16]. Enzyme stability at extreme pH, temperature and salt concentrations were also checked. The enzyme was incubated in a widespread range of temperature (0-100°C) for 2 hrs. Samples were then taken at room temperature, residual was dissolved in 500 µl phosphate buffer and activity was tested against 1% soluble starch. Similarly, pH stability was also estimated by incubating at a pH range (2-14) and salt stability was determined by incubating it in graded concentration of NaCl (0-5 M) [17].

Chromatographic purification of amylase

The strain MRS6 was fermented in mineral salt starch broth under pre-optimized conditions. A total 0f 250 ml culture was centrifuged (10,000 rpm – 12 min) to separate cell mass. The culture filtrate was then mixed well with equal volume of acetone and placed at 4°C for overnight. Precipitates were obtained after centrifugation at 12,000 rpm for 15 min. It was then dissolved in 1 ml phosphate buffer (0.1 M, pH-7.2) and loaded on the top of a seralose 6B column. The crude enzyme solution was eluted with same buffer keeping the flow rate 0.5 ml/min. A total of 50 fractions were collected consisting of 2 ml each. All fractions were determined for enzyme activity. Active fractions were pooled and lyophilized, that was then dissolved in minimum amount of phosphate buffer and analysed through poly acrylamide gel electrophoresis.

Gel Electrophoresis and in-gel localization of active protein

Thus purified amylase was analysed through denaturing polyacrylamide gel electrophoresis [18]. Protein bands were visualized after comassie brilliant blue staining of the gel [19]. The molecular weight was determined comparing standard protein markers. Zymogram study was conducted in SDS-Polyacrylamide gel containing 0.1% soluble starch [20]. Protein sample was prepared with non-reducing sample loading buffer and electrophoresed at 50 mA. After electrophoresis, the gel was washed with 25% isopropanol for 1 h followed by washing in distilled water. Gel was dipped in Phosphate buffer (pH-8) saline for 1 hr at 35°C. Subsequently, the in-gel amylase band was visualized after treating the gel with Gram's iodine solution. A transparent zone on dark blue background indicates location of active amylase.

Results

Starch hydrolyzing bacteria in wastes

The isolation of bacterial strains from Medinipur Municipality waste dump site was undertaken in this study. Bacterial type of any habitat strictly depends upon local nutrient availability and environmental conditions. However, in this study, a total of 9 bacteria were isolated with starch hydrolysable ability. They produced different degree insoluble starch hydrolyzing zone, represented in (Table 1). The strains were represented as MRS1- MRS9, among which strain MRS6 was found to produce maximum starch hydrolyzed zone. This strain also indicated very high amylase activity when grown in starch broth.

Strains	Clear zone diameter (mm)
MRS1	4
MRS2	3.25
MRS3	1.5
MRS4	3.2
MRS5	2.75
MRS6	8.4
MRS7	4.1
MRS8	2.2
MRS9	5.75

Table 1: Clear zone diameter of isolated strain.

Characterization and identification of MRS6

The strain MRS6 was selected due to its comparatively higher (Table 1) amylase activity among the isolated strain from decomposed solid wastes. The detailed microbiological, biochemical and physiological characterizations were summarized in (Table 2). This gram positive, endospore forming strain appeared similar to the genus, *Bacillus*. Electron microscopic study revealed the strain as thin and long rod shaped about 1-1.5 µm long with 0.2 µm diameter (Figure 1). Morphological depiction shows that it produces milky white, large smooth colony (2-3 mm diameter) of irregular margin. However other biochemical characteristics were presented in (Table 2). The strain was found highly adaptable in different environment by producing various extracellular enzymes and utilizing different sugars as sole carbon sources.

16S rDNA analysis and molecular characterization of MRS6

From the genomic DNA of the strain about 1.5 kb DNA product had been amplified and revealed after agarose gel electrophoresis. The raw sequences of amplicon were assembled and consensus sequences were carried out for BLAST. The BLAST analysis revealed that the strain MRS6 possess 16s rRNA gene sequences which is similar (99%) to many strains of *Bacillus cereus*. It was found that the strain forms cluster with various strains of *B. cereus* and maximum similarity with *B. cereus* LN831884 with high boot strap values and hence the strain is designed as *Bacillus cereus* MRS6 (Figure 1).

Optimum catalytic conditions and stability

It was found that the amylase production in mineral salt media was highly interconnected with optimum growth conditions (Figure 2). Highest cell density was achieved at 30 hrs of incubation whereas enzyme activity was noticed maximum at 42 hrs at its stationary phase of growth. The amylase production was evaluated in terms of measuring the extracellular enzyme activity showed highest at 35°C, pH-7 (Figure 2). This enzyme showed maximum activity within 30 min incubation with soluble starch (1%, w/v) at 35°C though it showed maximum stability at 40°C for 2 hrs. The enzyme was found to retain its activity after incubating it for 2 hrs at up to 80°C. Slightly acidic to high alkaline conditions were most suitable for catalysis by this enzyme. It shows sharp activity at pH (6-9) but falls dramatically beyond of this range. The enzyme was found to remain functionally active after incubation for 24 hrs at wide range of pH (5-9). However, enzyme activity was totally lost when incubation was done at pH (12-14) for 24 hrs (Figure 3). Surprisingly the amylase produced by this strain remains highly stable at 2.5-4 M NaCl and retained its full catalytic property at such salt concentrations. Enzyme stability at even incubating at 5 M NaCl for 24 hrs was also noted (Figure 3).

Morphological	Colony	Short colony (1.5-2 mm diameter), Regular margin, Milky white
	Cellular	Short slender rod shaped bacteria exist mostly in chain. Length 1 -1.5 µm and 0.2 µm diameter. Gram positive, Endospore forming
Biochemical	Methyl Red,	+
	Indole, Voges Prouskuer, Citrate utilization, H_2S Production	-
Sugar utilization	Mannose, Sucrose, Dextrose, Maltose, Galactose, Rhamnose,	Huge growth

	Raffinose, Fructose, Lactose, Xylose	
		Moderate growth
NaCl tolerance	0, 1, 3 M	++, +, + respectively
Growth	pH (2-14)	Huge growth (4-12)
	Temperature (20-50°C)	Huge growth (20-40°C)
Extracellular enzyme	Catalase, Amylase, Lipase, Cellulase, Nitrate Reductase	+
	Gelatinase, Caseinase, Urease, Phenylalanine Deaminase	-
	Penicillin,Amplicillin, Methicillin,Rifampcin, Streptomycin,	Resistant
Antibiotic susceptibility	CoTrimoxazole	
	Tetracycline, kanamycin, Neomycin, Norfloxacin, Chloramphenicol, Nalidixic Acid,	sensitive
"+" denotes positive and "-"denotes negative		

Table 2: Characterization of MRS6 from various aspects.

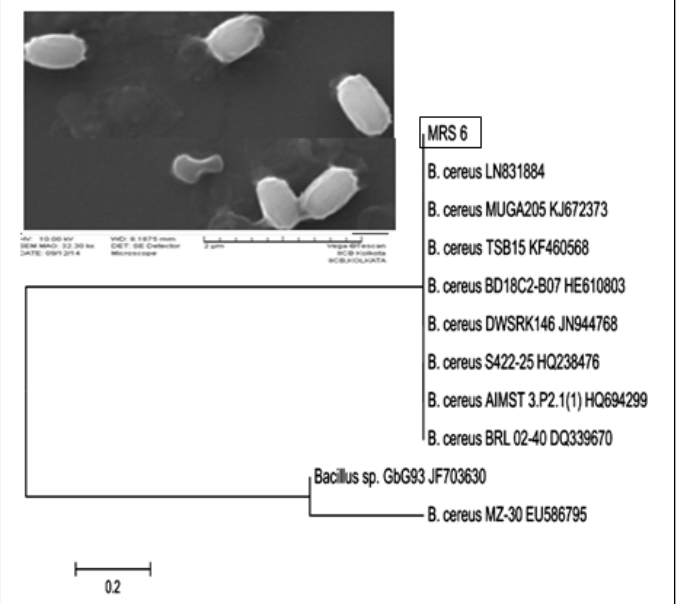

Figure 1: Scanning electron microscopic view and position in phylogenetic tree of strain MRS6.

Effect of various effectors on enzyme activity

Table 3 represents several influencing effectors that may enhance or depress the enzyme activity. Ca^{+2} played crucial role as metal ion in enzyme activity. Enzyme activity was enhanced about 84% after incubating it in 1 mm $CaCO_3$. However, Mn^{+2} also showed significant increase in enzyme activity whereas Hg^{+2} depressed the enzyme activity by 20%. Lactose and glycerol have lowered enzyme activity among which glycerol almost repressed 53% of its activity. The detergents like SDS and Tween 80 also were able to depress enzyme activity.

Solid State Fermentation (SSF)

The strain *B. cereus* MRS6 is able to produce amylase by fermenting solid untreated substrate. It could utilize wheat bran and rice husk with 85% moisture content. Amylase production was found better with wheat bran than rice husk (Figure 4). About two-fold increased amylase activity was observed with wheat bran employed as natural starch source.

Molecular weight and in gel enzyme activity in SDS-PAGE

After gel filtration chromatography the active fractions (25-31) were pooled and lyophilied. The lyophilized sample was dissolved in phosphate buffer (0.1M, pH 7) and analysed through polyacrylamide gel electrophoresis. Molecular mass of the enzyme was studied after comparing the protein band position with marker proteins on polyacrylamide gel. Presence of active protein was determined comparing its in-gel substrate hydrolysing ability and the most probable mass was estimated about 55 kDa. Figure 5 represent the electrophoretic pattern and in gel position of active protein.

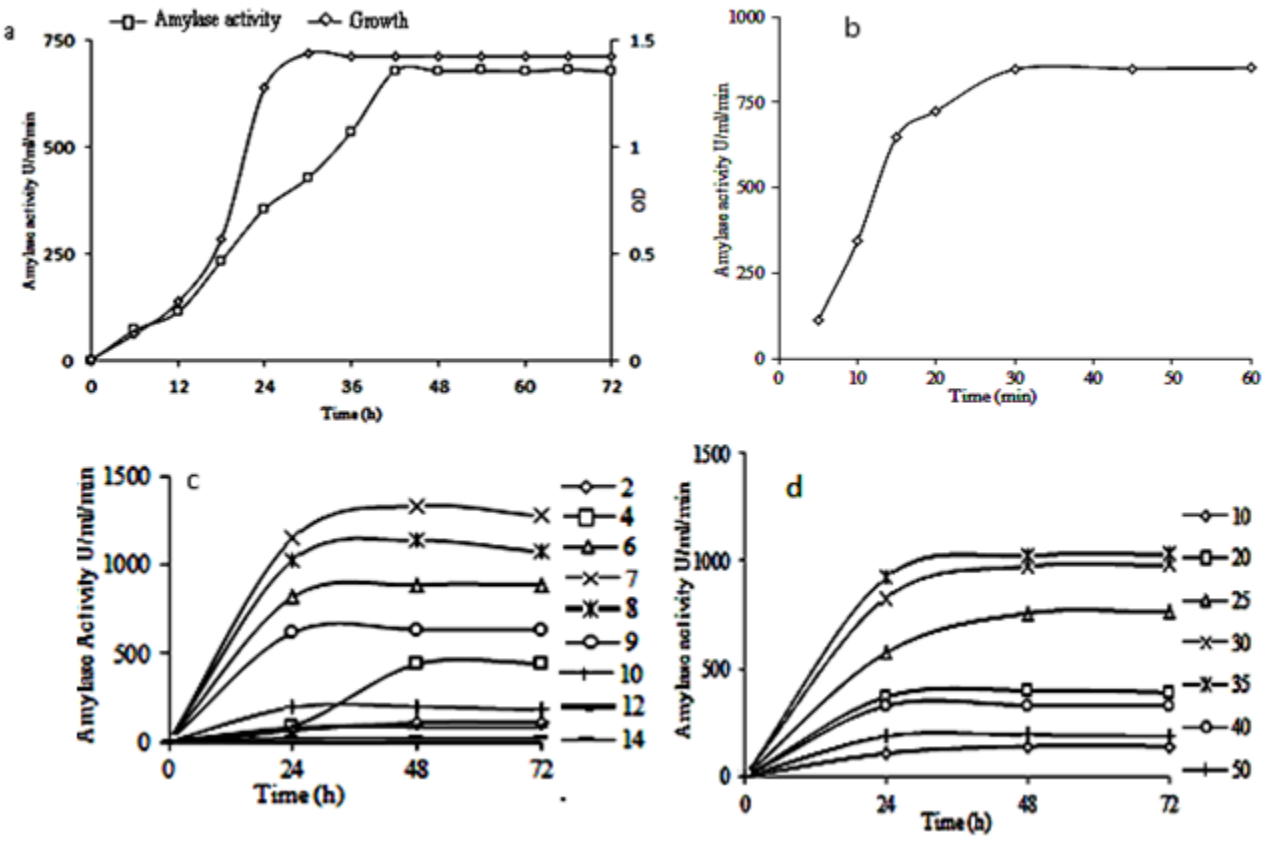

Figure 2: Optimization of bacterial growth and amylase production; a - bacterial growth and amylase production in different time, b- optimization of enzyme activity in different time c- amylase production in different pH, d- amylase production in different temperature.

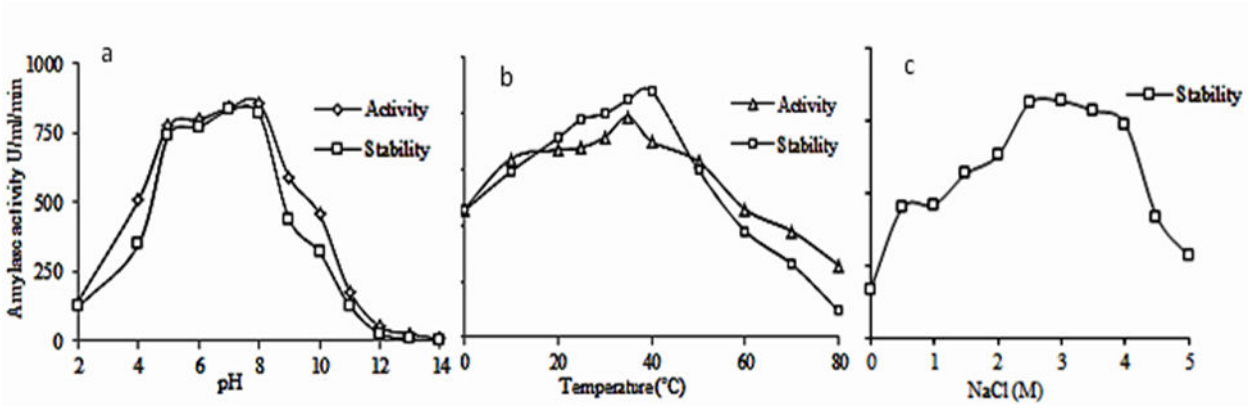

Figure 3: Characterization of amylase activity and stability; a- different pH, b- temperature, c- NaCl.

	Effectors	Concentration	Residual activity	Induction	Inhibition
		(1mM)	(%)	(%)	(%)
Crude(Untreated)		0	100	-	-

Sugars	Glucose	1	123	23	-
	Lactose	1	100	-	-
Metal ion	$MnSO_4$	1	173	73	-
	$CaCO_3$	1	184	84	-
	$K_2Cr_2O_7$	1	109	9	-
	$NaASO_2$	1	110	10	-
	NaN_3	1	114	14	-
	$HgCl_2$	1	80	-	20
	$KMnO_4$	1	76	-	24
	MnO_2	1	139	39	-
	$AgNO_3$	1	104	4	-
	$CuCl_2$	1	78	-	22
Surfectant	SDS	1	99	-	1
	Tween 80	1	89	-	11
	Triton x	1	76	-	24
Denaturing agent	β-mercepto ethanol	1	78	-	22
Organic Compound	Urea	6000	89	-	11
	Glycine	1	102	2	-
Chelating agent	EDTA	1	100	-	-
Alcohol	Glycerol	1	47	-	53
	Methanol	1	113	13	-

Table 3: Various effectors on enzyme activity.

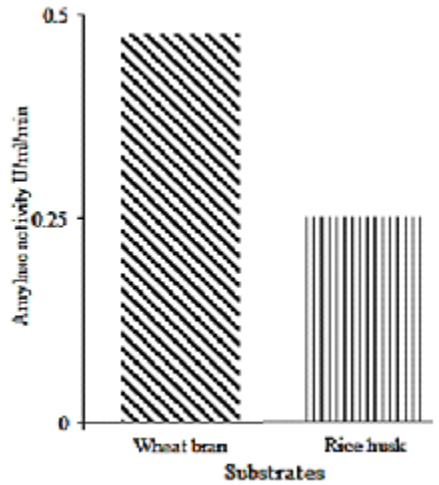

Figure 4: Determination of solid state fermentation (SSF).

Discussion

In this present research, several bacterial strains were isolated, but few strains showed amylolytic activity. According to the 16S rDNA sequences the strains belonged to the genus *Bacillus* after studying with different characterization approaches of MRS6 in phylogenetic tree, which was able to produce amylase with high level. The strain can be able to grow at alkaline pH (8-12) and adequate saline environment (0-3M) (Table 2). Therefore, according to the salt tolerance in the presence of various NaCl concentrations (up to 3M) which indicate its high biotechnological prospective. Based on the present findings the enzyme was stated to be alkaline amylase. The stability of the amylase at higher pH, could be explained that the enzyme in the present study may contains more acidic amino acid that are exposed to or excreted to the external medium and a Na+ cycle that facilitates the solute uptake. The strain has the capability to utilize various sugars efficiently, so it can be cultivated using various cheap carbon sources. Several *Bacillus* sp. are known to produce variety of extracellular enzymes and they have a varied range of industrial applications. Amylases are of explicit significance to the industry. Amylase production is maximum around 42 hrs while production started at 12 hrs. The amylase activity appeared to be doubled with the growth. The enzyme production was found to be maximum when the cell population entered into the

stationary phase suggesting that enzyme secretion is growth associated. The activity was observed to start rapidly after beginning of incubation, and the crude enzyme extract obtained after 42 hrs of growth in the culture medium exhibited highest activity at the beginning of the stationary phase. The optimum temperature and pH for production of amylase is found to be 35°C and pH-7. The enzyme is optimally active and stable at pH-8, though it remains active at a range of basic pH (8-10). Medium pH is very important in nutrients absorption and growth of bacteria, stimulation of enzyme production via signalling pathways and release of extracellular enzymes based on amylolytic mechanism of signal peptidases [21]. Enzyme is retained activity at temperature (up to 80°C). The temperature regulates the enzyme synthesis at mRNA transcription level and probably translation levels of proteins, thus increases the stability of the proteins and also the production. However, the other possible reason for production at elevated temperature may be temperature influences their secretion; likely by changing the physical properties of the cell membrane [22]. Sugars are inhibiting to this enzyme activity probably by blocking the substrate binding site to form un-dissociated enzyme-product complex. The report on metals like Hg^{2+} inhibition of microbial amylase and these findings support present results [23,24]. The Mn^{2+} and Ca^{2+} ions augmented the enzyme activity possibly playing as the co-factors of this enzyme. The result demonstrated that Mn^{2+} and Ca^{2+} enhanced (70%) the enzyme activity. Such divalent ions stimulate the enzyme activity by forming a link bridge between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme at their proper location [25]. The amylase from MRS6 is remaining same in the presence of EDTA. It is found that mercaptoethanol that act as a reducing agent, inhibit enzyme activity. SDS, tween, and triton-X are stimulating the enzyme activity about 20% of the original activity. SDS was found to be strong inhibitor as in the case of *Bacillus* thermoleovorans causing almost complete inhibition of enzyme activity [26]. The cause of strong inhibition by SDS could be due to the local conformation changes in the active site of the enzyme molecule that results in inhibition, partial reversible unfolding, and subsequent inactivation. The polyacrylamide gel electrophoresis showing the enzyme activity in gel corresponds to the commassie stained band and the protein marker (left most, Figure 5). This enzyme is calculated to have molecular weight ~55 kDa. Beside high amylolytic activity, this strain also apparent adequate cellulolytic, lipolytic, gelatinase and nitrate reductase activities which may be exploited for various commercial purpose and environmental issues. In SSF (solid state fermentation), the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation [27]. In the present studies WB (wheat bran) and RH (rice husk) were used for growth and amylase production by the MRS6. The results were shown in Figure 4 that the substrates supported growth and enzyme formation by the culture. A high amylase activity was obtained in a medium containing WB alone as the substrate than RH. It was reported that WB was found to be the best substrate and suitable for necessary manipulation [28]. Widespread suitability of WB may be due to the presence of sufficient nutrients and its ability to remain loose even in moist conditions thus providing a large surface area [29].

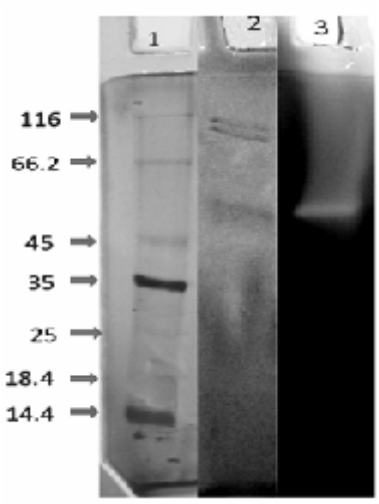

Figure 5: PAGE analysis of bacterial extra-cellular proteins and in gel localization of amylase within it. (Lane 1- Marker, lane 2- gel filtrate lyophilized protein, lane 3-substrate hydrolysis in gel).

Therefore, according to the findings it should be noted that the amylase producing MRS6 strains might be called halophilic because the halophilic enzymes are usually inactive when the NaCl concentration is less than 2 M. Mader et al. [30] and Perez-Pomares et al. [31] showed that the optimum salt concentration for amylase production of *Haloferax mediterranei* was 3 M NaCl. Therefore, this halophilic stability and adaptation of halothermophilic strains with capability to produce amylase suggested that, these strains could be a good choice for some biotechnological application.

Conclusion

It is evident from the above study that; this amylase has the requisite properties of significant industrial importance. Amylase that is stable at high alkaline conditions and high salt is rare. In the present study *Bacillus* sp. MRS6, which produces inducible, extracellular, alkalophilic amylase stable in high salt operation industry, makes it ideally suited for industrially importance. However, further investigation is required to ascertain the molecular characteristics, kinetic properties and application to access the true potential of this microbial amylase.

References

1. Dey G, Singh B, Banerjee R (2003) Immobilization of exo-amylase production by Bacillus circulans GRS 313. Brazilian Archives of Biology and Technology 46: 167–176.
2. Kiran K, Chandra T (2008) Production of surfactant and detergent-stable, halophilic and alkali tolerant α-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK. Appl Microbiol Biotechnol 77: 1023–1031.
3. Syed D, Agasar D, Pandey A (2009) Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis*. J Ind Microbiol Biotechnol 36: 189–194.
4. Oren A (2010) Industrial and environmental applications of halophilic microorganisms. Environ Technol 31: 825–834.
5. Ryu K, Kim J, Dordick JS (1994) Catalytic properties and potential of an extracellular protease from an extreme halophile. Enzyme Microb Technol 16: 266–275.

6. Bernfeld P (1955) Amylase: α and β, in methods in enzymology. Academic Press New York 1:149-158
7. Harris JL, Howe HB, Roth IL (1975) Scanning electron microscopy of surface and internal features of developing perithecia of Neurospora crassa. Journal of bacteriology 122: 1239-1246.
8. Abdelkafi S, Chamkha M, Casalot L, Sayadi S, Labat M (2005) Isolation and characterization of a novel *Bacillus* sp., strain YAS1, capable of transforming tyrosol under hypersaline conditions. FEMS microbiology letters 252: 79-84.
9. Hatzinger PB, Palmer P, Smith RL, Penarrieta CT, Yoshinari T (2003) Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. Journal of Microbiological Methods 52: 47-58.
10. Siqueira JF, Rocas IN (2005) Uncultivated phylotypes and newly named species associated with primary and persistent endodontic infections. J Clin Microbiol 43: 3314-3319.
11. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.
12. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.
13. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120.
14. Lonsane BK, Ghildyal NP, Budiatman S, Ramakrishna SV (1985) Engineering aspects of solid state fermentation. Enzyme Microb Technol 7: 258-265.
15. Balkan B, Balkan S, Ertan F (2011) Optimization of parameters for α-amylase production under solid state fermentation by *Trichothe ciumroseum*. Romanian Biotechnological Letter. 16: 6591-6600.
16. Hajji M, Kanoun S, Nasri M, Gharsallah N (2007) Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1. Process Biochem 42: 791–797.
17. Sajedi RH, Naderi MH, Khajeh KH, Ahmadv R, Ranjbar B et al. (2005) A Ca independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. Enzyme Microb Technol 36: 666-671.
18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
19. Heukeshoven J, Dernick R (1988) Improved silver staining procedure for fast staining in phast system development unit. I. staining of sodium dodecyl sulfate gels. Electrophoresis 9: 28-32.
20. Asoodeh A, Lagzian M (2012) Purification and characterization of a new glucoamylo pullulanase from thermotolerant alkaliphilic *Bacillus subtilis* DR8806 of a hot mineral spring. Process Biochemistry 47: 806–815.
21. Palekar AA, Vasudevan PT, Yan S (2000) Purification of lipase: A review. Biocatal Biotransform 18: 177-200.
22. Paetzel M, Karla A, Strynadka NCJ, Dalbey RE (2002) Signal Peptidases. Chem Rev 102: 4549–4579.
23. Goyal N, Gupta JK, Soni SK (2005) A novel raw starch digesting thermostable α-amylase from Bacillus sp. I-3 and its use in the direct hydrolysis of raw potato starch. Enzyme Microb Technol 37: 723–734.
24. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. Process Biochem 38: 1599–1616.
25. Afifi AF, Kamel EM, Khalil AA, Fouaad E, Fazxi M et al. (2008) Purification and characterization of α-amylase from *Penicillium olsonii* under the effect some antioxidant vitamins. Global J Biotechnol Biochem 3: 14-21.
26. Tanaka A, Hoshino E (2002) Thermodynamic and activation parameters for the hydrolysis of amylose with *Bacillus* α-amylases in a diluted anionic surfactant solution. Journal of Bioscience and Bioengineering 93: 485-490.
27. Kunamneni A, Permaul K, Singh S (2005) Amylase production in solid state fermentation by the thermophilic fungus Thermomyces lanuginosus. Journal of Bioscience and Bioengineering 2: 168-171.
28. Chimata MK, Sasidhar P, Challa S (2010) Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation. African Journal of Biotechnology 9: 5162-5169.
29. Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A (2007) Alpha amylase production by *Aspergillus oryzae* employing solid-state fermentation. J Sci Ind Res 66: 621-626.
30. Mader P, Edenhofer S, Boller T, Wiemken A, Niggli U (2000) Arbuscular mycorrhizae in a long-term field trial comparing low-input (organic, biological) and high-input (conventional) farming systems in a crop rotation. Biol Fertil Soils 31: 150-156.
31. Perez-Pomares F, Bautista V, Ferrer J, Pire C, Marhuenda-Egea FC et al. (2003) α-amylase activity from the halophilic archaeon *Haloferax mediterranei*. Extremophiles 7: 299-306.