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Isolation and identification of a novel thermo-alkaline, thermostable, SDS and chelator resistant amylase producing *Anoxybacillus* sp. IB-A from hot spring of Bakreswar, West Bengal (India): First report

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ABSTRACT

Amylases are one of the most important enzymes in present-day biotechnology. The objective of this study was to isolate and identify a potential thermophilic amylase producing bacterial strain from hot spring of Bakreswar village, Suri, West Bengal (India). Among the few isolated amylolytic strains on starch agar medium, the strain IB-A showed best amylase activity. Phylogenetic analysis based on 16S rDNA sequence homology shows that the isolated strain belongs to the genus Anoxybacillus, and named as Anoxybacillus sp. IB-A. The optimum temperature and pH for amylase production was found to be 60°C at pH 9.0. Characterization of crude amylase showed its best activity at pH 9.0 and temperature 70°C, with a thermostability of 100% for 10 hours. Enzyme activity was considerably enhanced in the presence of Mo^{+2}, K^{+}, Mn^{+2}, Cu^{+2} and Co^{+2}. The most significant observation is that the enzyme not only retained cent percent activity in presence of 10 mM Hg^{+2} and Pb^{+2} but also increased activity over the control. Among the tested metal ions Zn^{+2} and Ni^{+2} showed slight inhibitory effect. The enzyme also showed its high stability and activity in presence of surfactants and chelators, such as SDS, EDTA. So our result showed that the enzyme is highly thermostable, thermo-alkaline and chelator resistant which makes it a suitable candidate for liquefaction of starch at high temperature, in detergent and textile industries. This one is the first report of isolating a potential amylase producing thermophilic Anoxybacillus sp. from hot spring of Bakreswar, West Bengal (India).

Keywords: Thermophilic *Anoxybacillus* sp., Thermo-alkaline amylase, Thermostable, SDS and chelator resistant, Hg+2 resistant.

INTRODUCTION

Starch consists of two types of polysaccharide: amylose and amylopectin. Amylases [α-amylase, β-amylase and glucoamylase (GA)] that catalyze hydrolysis of starch are among the most important enzymes in present day biotechnology [1]. α - Amylases (EC 3.2.1.1,1,4-α-D-glucan glucanohydrolase) were classified in family 13 of glycosyl hydrolases and hydrolyzes starch, glycogen and related polysaccharides by randomly cleaving internal α-1, 4-glucosidic linkages to produce different sizes of oligosaccharides. They have diverse applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries [2]. The amylases can be derived from several sources such as plants, animals and microbes [3]. The major advantages of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzyme of desired characteristics [4], for industrial applications. Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence [5]. Thermostable α-amylases have extensive commercial applications in starch processing, brewing and sugar production, desizing and textile industries and in detergent manufacturing process [6]. On the other hand,

maltotriose and maltotetrose produced by the enzymatic activity of amylase have a wide use in food and pharmaceutical industry [7]. Thermophilic microorganisms are believed to be potential sources of industrially important thermostable enzymes [8, 9, 10]. Thermostability is a feature of most of the enzymes sold for bulk industrial usage and thermophilic organisms are therefore of special interest as a source of novel thermostable enzymes. Recent research with thermostable α-amylases has concentrated on the enzymes of thermophiles and extreme thermophiles [11, 12, 13, 10, 14, 15, 16], specially alkaline amylase for the detergent industry [17]. Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs [18]. The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination and cost of external cooling, a better solubility of substrates, a lower viscosity allowing accelerated mixing and pumping [16].

The objective of this work was to isolate and identify a thermophilic bacterial strain, which can produce thremostable α-amylase with desired characters that can be used in industrial sectors.

MATERIALS AND METHODS

Collection of water sample:

Water sample from the Bakreswar hot spring, West Bengal, India, was collected during September 2010 in a sterile glass bottle (500ml). The temperature of the water was around 60 ºC, and pH was around 9.0.

Isolation & screening of amylase producing microorganisms

The collected water sample (100µl) from a hot spring of Bakreswar was spread on nutrient agar plate with 0.1% yeast extract and 2% agar (w/v) and grown at 60^{o} C for 48 hrs. To screen a potent amylolytic bacteria the selected colonies, appeared on the plates after incubation, were patched on starch agar plate consisting of 0.5% soluble starch (w/v) and agar 2% (pH-9.0) and incubated at 60^{0}C. After incubation the plate was flooded with 5% Lugol's iodine (solution of 5 % w/v I_2 and 10% w/v KI) and kept for 5 minutes at room temperature. Colonies with a surrounding clear zone in a blue colour background were selected as amylase positive and streaked in a new starch agar plate for further study.

Morphology and biochemical Characterization:

Colony morphology of the isolates was recorded after growing them on starch mineral salt media at 60^{0}C for 24 hours, depending on growth. Overall shape, size, pigmentation, opacity, elevation, margin; whether smooth, rough, mucoid or glossy were recorded as per Smibert & Krieg [19].

Cell morphology was observed under oil immersion lens of microscope after Gram staining. Cell shape, arrangement and size (in µm) were recorded. Cellular morphology was also determined from photomicrograph (using binocular Phase contrast Microscope, Leica DM1000).

Physico-biochemical characters were checked following standard methods [18].

Determination of optimum growth conditions

For optimum growth of the bacterial isolate, two parameters (temperature and pH) were considered. For determination of optimum temperature, 50 ml NB broth was added in 5 sets, autoclaved and inoculated with 100 µl of freshly prepared culture of bacterial isolate by growing overnight at 60°C in NB broth. The five sets of flask were incubated at 40°C, 50°C, 60°C, 70°C and 80°C. After an incubation period of 24 hr, their absorbance was taken at 600 nm using a VARIAN UV/Vis Spectrophotometer. For determination of optimum pH, 7 conical flasks each containing 20 ml of nutrient broth were prepared and their pH was adjusted at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, then autoclaved. These flasks were inoculated with 100 µl of fresh 24 hr old culture. After an incubation period of 24 hr, their absorbance was taken at 600 nm.

Utilization of different carbohydrates

Carbohydrate utilization pattern of the strain was tested using kit available from HiMedia, India. 10 µl of culture suspension was inoculated in each of the tested carbohydrate well and incubated at 60^{0}C to see the colour change.

Salt tolerance & antibiotic sensitivity.
Three sets of nutrient broth were prepared containing 1, 2, 3, 4, 5, 6, 7 and 8 % NaCl. Growth of the isolate at different salt concentration was tested using nutrient broth as the organic substrate and using a control broth without any NaCl supplementation.

The antibiotic sensitivity test was done by disc diffusion method [20]. Zone of inhibition was recorded after growing the culture at 60^0C for 24-48 hrs. The antibiotics used were streptomycin (100), tetracycline (30), vancomycin (30), erythromycin (15), trimethoprim (5), nalidixic acid (30) and kanamycin (30).

Enzyme assay
From 50 ml of 48 hr old mineral salt minimal broth (KH_2PO_4 0.3%, Na_2HPO_4 0.6 %, NH_4Cl 0.2%, NaCl 0.5%, $MgSO_4.7H_2O$ 0.01%) supplemented with 1% soluble starch, 5 ml was taken and centrifuged at 10,000 rpm for 5 minutes. The supernatant was used as crude enzyme source. Amylase activity was determined according to DNS method [21], using 1% soluble starch as the substrate at 70°C (pH 9.0). One unit of enzyme activity was defined as the amount of enzyme required produced 1 μmol of glucose per minute under the standard assay condition.

Identification of the strain
For further identification, genomic DNA of strain IB-A was extracted from fresh culture following protocol of Ausubel *et al*., 1994 [22]. The 16S rRNA gene of the strain was amplified from the genomic DNA, using the universal primers (8F-5'AGAGTTTGATCCTGGCTCAG3' & 1492R 5'TACGGTTACCTTGTTACGACTT3'). 16S rRNA gene is amplified from genomic DNA and sequenced from Chromus Biotech Pvt. Ltd., Bangalore, using Big Dye Terminator version 3.1" Cycle sequencing kit and ABI 3500xL Genetic Analyzer DNA sequencing machine. The 16S rRNA gene sequence of the isolate was aligned with reference 16S rRNA gene sequences of the European Microbiological Laboratory (EMBL), GenBank (gb, Germany) and the database of Japan (dbj) using the BLAST algorithm [23] available online in NCBI (National Centre for Biotechnology Information). Phylogenetic analysis was carried out and the phylogenetic tree was constructed by the neighbour-joining method [24].

Time course growth and amylase production
The time course of growth and the production of extracellular amylase were studied using minimal medium with soluble starch (1%). Culture samples ware collected at different time intervals over the period of cultivation, analyzed for amylase activity using the procedure described above. The culture growth was monitored my measuring the turbidity at 600 nm.

The effect of pH on amylase activity
The effect of pH was determined by standard assay protocol after incubating 0.25 ml of enzyme and 0.75 ml of the following buffers at the indicated pH (between pH 4.0-10.0) containing 1% soluble starch: citrate phosphate buffer (pH 4.0-6.0), phosphate buffer (pH 7.0-8.0), glycine-NaOH buffer (pH 9.0, 10.0).

The effect of temperature on amylase activity and stability
The effect of temperature on the enzyme activity was determined under standard assay condition mention earlier at various temperatures ranging from 30 to 80°C for 10 min at pH 9.0. The temperature stability was determined by measuring the residual activity at 70 °C, after incubation of the enzyme in70 and 80°C temperature for 1 to 10 hours at optimum pH.

Effect of chemicals and metal ions on the amylase activity:
Effects of several metal ions and chemical agents on the enzyme activity were determined by pre incubating the crude enzyme for 1 hour at 60 °C temperature with different metal ions (10 mM solution) such as Mg^{+2}, Fe^{+2}, Pb^{+2}, Co^{+2}, Cr^{+2}, Ca^{+2}, Ni^{+2}, K^{+}, Na^{+}, Zn^{+2}, Cu^{+2}, Li^{+2}, Ag^{+2}, Hg^{+2},$Mo^{+2,}$ Cd^{+2}, Mn^{+2} and chemicals (the chelating agent Ethylenediamine tetra acetic acid(EDTA); reducing agents dithiothreitol (DTT) and *β*-mercaptoethanol; detergents SDS, Triton X100, Tween 20, Cetyltrimethyl Ammonium Bromide (CTAB)) and then performing the assay in the presence of the same inhibitor concentration at optimum temperature for 10 min. The activity of the enzyme without any metal treatment was taken as control and the activity was considered as 100 % [10, 25].

RESULTS AND DISCUSSION

Isolation of the amylolytic bacterial strain

Water sample from a hot spring of Bakreswar village, Suri, West Bengal (India) was spread on nutrient agar plate with 0.1% yeast extract and 2% agar (w/v) and grown at 60^{o} C for 48 hrs. To screen a potent amylolytic bacteria the selected colonies, appeared on the plates after incubation, were patched on starch agar plate consisting of 0.5% soluble starch (w/v) and agar 2% (pH-9.0) and incubated at 60^{0}C. Among them strain IB-A (Figure. 1) showed maximum zone of clearance after flooded with 5% Lugol's iodine (solution of 5 % w/v I_2 and 10% w/v KI) and kept for 5 minutes at room temperature, and thus selected for further studies.

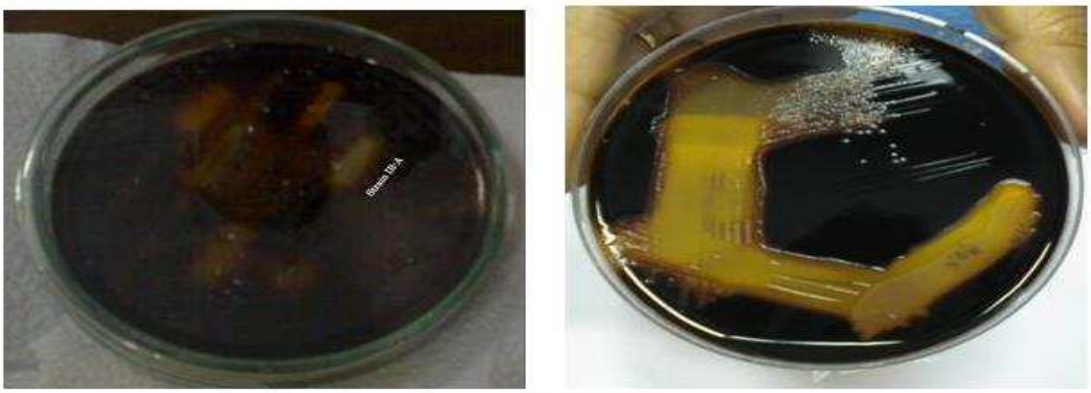

Figure 1. (a) Screening of thermophilic amylase producing bacterial strains on the basis of zone clearance on starch agar plate. (b) Strain IB-A showing zone of clearance after 12 hours of incubation on starch plate

Morphological and biochemical characterization

The purity of the isolate was assessed by colony morphology and microscopy after 24 hours growth on nutrient agar plate. Colonies of strain IB-A was small, creamish, irregular shaped with smooth edges (Figure.2, a). Phase contrast photomicrograph after gram staining revealed that cells of IB-A were rod-shaped, gram positive, motile, containing terminal spherical endospore (Figure. 2, b).

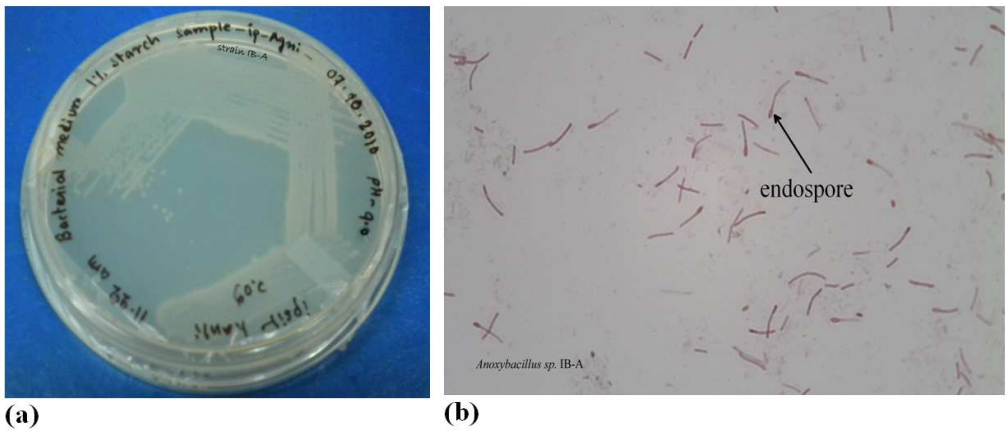

Figure 2. (a) Pure colony of strain IB-A, streaked on Nutrient agar plate supplemented with starch (1%). (b) Gram stain photomicrograph of stain IB-A under 100X (Leica DM1000)

The isolated strain IB-A is positive for catalase, amylase, oxidase, gelatinase, caseinase activity. A comparison of the phenotypic characteristics of *Anoxybacillus* sp. IB-A, with other reported *Anoxybacillus* species, showed in Table 1.

Table 1. Comparison of the phenotypic characteristics of *Anoxybacillus* sp. IB-A with related species

Characteristics	1	2	3	4	5	6	7	8	9	10	11
1. Cell morphology	Rod	Curved rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
2. Gram-stain	+	v	+	+	+	+	+	+	+	+	+
3. Relation to O2	A / F	A / F	A / F	A / F	O	A / F	F	F	F	A	A / F
4. Temperature range(°C)	45-65	50-60	30-64	30-72	39-67	40-70	30-72	30-70	40-70	35-67	40-80
5. Optimum Temperature (°C)	61	50	54	60-65	55	55-60	60-65	50	50-55	55	60
6. pH range	5.0-6.5	4.5-10.0	7.0-8.0	5.5-9.0	6.0-9.0	6.0-10.0	5.5-9.0	6.0-11.0	6.0-10.5	6.5-6.6	4.0-10.0
7. Optimum pH	5.6	7	7.0-8.0	7	7	7.5-8.0	7	7.5-8.5	7.5-8.5	6.0-6.5	9
8. Motility	+	+/-	-	+	+	+	+	+	+	+	+
9. NaCl (3%, w/v)	-	+/-	-	-	-	+	-	+	+	+	+
10.Nitrate reduction	-	+	+	+	+	+	-	+	+	-	-
11. Hydrolysis											
(a) Gelatin hydrolysis	-	+	-	-	-	+	+	-	-	ND	+
(b) Starch Hydrolysis	+	+	-	+	w	ND	ND	+	+	+	+
(c) Oxidase	-	-	+	-	ND	+	ND	+	+	ND	+
12. Utilization of											
(a) Glucose	+	+	+	-	+	+	ND	+	+	+	+
(b) Xylose	-	+	+	-	+	+	-	ND	ND	+	+
(c) Trehalose	+	+	-	ND	+	+	ND	ND	ND	ND	+

A/F = Aerobe/facultative anaerobe; F= Facultative anaerobe; O= Obligate aerobe; ND= not determined ; v= Variable. + positive; - negative; w= weak response;

***1**, Anoxybacillus amylolyticus MR3C^T(DSM 15939^T); **2**, Anoxybacillus contaminans LGM 21881^T(DSM 15866T) ; **3**, Anoxybacillus voinovskiensis TH13^T (NCIMB 13956T) ; **4**, Anoxybacillus flavithermus DSM 2641^T ; **5**, Geobacillus tepidamans GS5-97^T (DSM 16325T); **6**, Anoxybacillus gonensis G2^T(NCIMB 13933T); **7**, Anoxybacillus flavithermus (DSM 2641^T) ; **8**, Anoxybacillus ayderensis AB04T (NCIMB 13972T) ; **9**, Anoxybacillus kestanbolensis K4T (NCIMB 13971T) , **10**, Anoxybacillus rupiensis R270^T(DSM 17127^T); **11**, Anoxybacillus sp. IB-A (JF968626)*

The data presented in the above table about *Anoxybacillus amylolyticus*, *Anoxybacillus contaminans*, *Anoxybacillus voinovskiensis*, *Anoxybacillus flavithermus*, *Geobacillus tepidamans*, *Anoxybacillus gonensis*, *Anoxybacillus flavithermus*, *Anoxybacillus ayderensis*, *Anoxybacillus kestanbolensis* and *Anoxybacillus rupiensis*, were obtained from early reports [26, 27, 28, 29, 30, 31, 32, 33].

Utilization of different carbohydrates :

Utilization of organic compound as sole carbon sources by the strain IB-A, was tested by RAPID carbohydrate utilization kit of HIMEDIA. The carbohydrate utilization result showed that strain IB-A can utilize Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Inulin, Dulcitol, Mannitol, Adonitol, Arabitol, Erythritol, Alpha methyl D glucoside, Rhamnose, Cellobiose, Melezitose, Alpha methyl D mannoside, Xylitol, ONPG, D-Arabinose , Citrate, Malonate and Sorbose. This study reveals that the strain IB-A can use a wide variety of carbon source for their growth.

Determination of optimum growth conditions:

Strain IB-A showed maximum growth at pH 9.0 (Figure. 3), indicates that the strain is alkaliphilic.

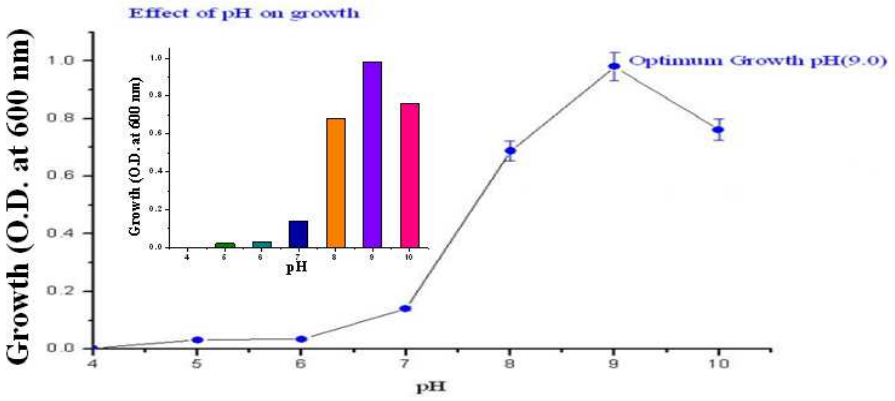

Figure 3. Effect of pH on growth

After incubation at different temperature for 24 hours, strain IB-A showed maximum growth at 60 ºC. So, the optimum growth temperature of the strain is 60 ºC (Figure. 4).

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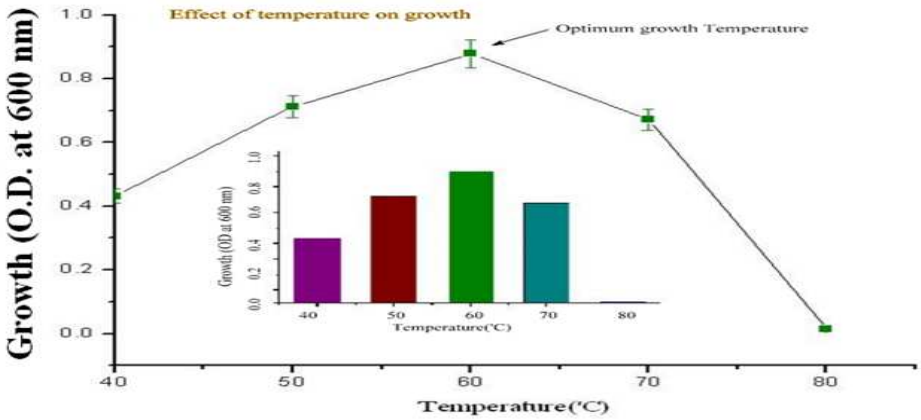

Figure 4. Effect of temperature on growth

Salt tolerance & antibiotic sensitivity:

The growth of the strain, IB-A was inhibited with a salt concentration greater than 3%; where as maximum growth was found at 2% salt concentration. Hence this strain can be categorized as slight halophilie (1.2-3% salt tolerance) [34].

The inhibition zone against antibiotics suggested that the isolate IB-A was susceptible to all the tested antibiotics. Hence, this strain may be exploited industrially without any health hazards.

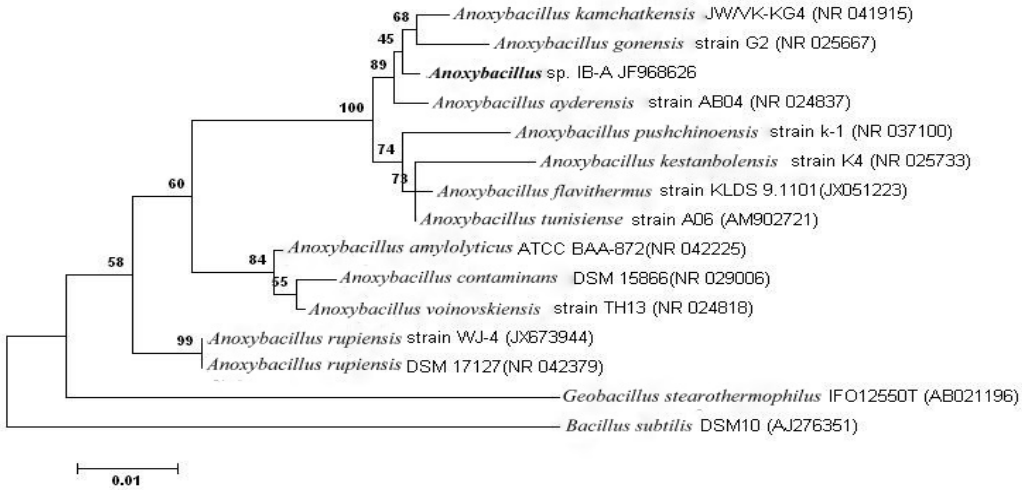

Figure 5. Phylogenetic tree of Anoxybacillus sp. IB-A with the closely related species:16s rDNA sequence based phylogenetic neighbour joining tree, using MEGA5, showing the phylogenetic relationship of strain IB-A relative to the closely related species in the genera *Anoxybacillus*. 0.01 base substitutions per site

Identification on the basis of 16S rRNA sequence analysis

Depending on phylogenetic analysis based on 16S rDNA sequence homology, the strain IB-A from hot spring of Bakreswar village, Suri, West Bengal (India) was identified as a member of the genus *Anoxybacillus* and named as *Anoxybacillus* sp. Ip-C. The neighbour-joining phylogenetic tree (Figure.5) clearly indicates that it forms a clade within the cluster represented by *Anoxybacillus gonensis* G2^T(NR 04195), *Anoxybacillus kanchatkensis* JW/VK-KG

(NR 041915). The strain showed closest sequence similarity with *Anoxybacillus gonensis* G2^T(NR 04195), 98 %; *Anoxybacillus kanchatkensis* JW/VK-KG (NR 041915), 99% and *Anoxybacillus ayderensis* AB04 (NR 024837), 99%.

The 16S ribosomal RNA gene of *Anoxybacillus* sp. IB-A, was submitted to GenBank and the following accession numbers was assigned for isolates IB-A is JF968626.

Time course growth and amylase production

The time course of growth and the production of extracellular amylase were studied using minimal medium supplemented with starch (1%). Soon after inoculation the culture started growing and reached a highest biomass at around 48 hours. In comparison with growth, the amylase production increased gradually and reached a highest value at around 48 hours of the incubation and remained same thereafter (Figure. 6). So, the enzyme production by the strain IB-A, is in a linier relationship with growth.

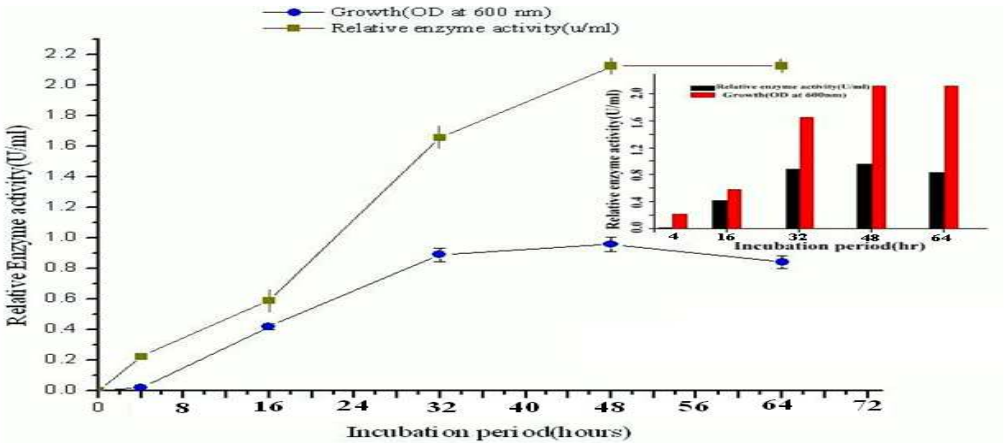

Figure 6. Time course for growth and amylase production

Effect of pH and temperature on crude amylase and its thermostability

Amylase assays were performed under different conditions for enzyme activity. Optimum pH and temperature of the amylase from *Anoxybacillus* sp. IB-A, was found to pH 9.0 (Figure 7) and 70°C (Figure. 8), hence it can be characterized as a thermo-alkaline amylase as reported in early studies [27, 28, 30, 31, 33, 35, 36].

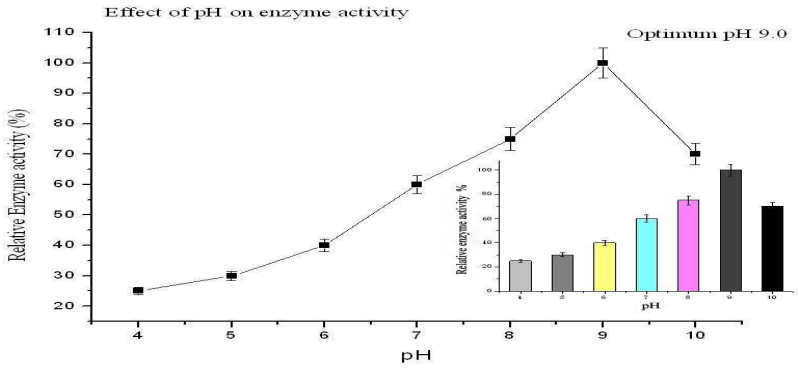

Figure 7. Effect of pH on amylase activity

The thermal stability of crude amylase produced by this strain IB-A showed its 100 % stability for 10 hours at 70°C and retain 90 % of its original activity at 80°C for 3 hours.(Figure. 9). Though many of the amylases reported earlier were thermostable but not active in alkaline condition whereas some of them were alkaliphilic but not thermostable [37, 38, 39, 40, 41], our strain can produce alkaline thermostable amylase.

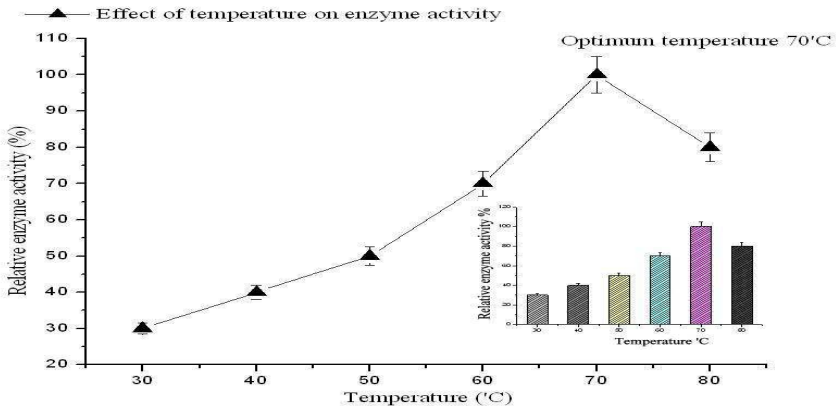

Figure 8. Effect of temperature on amylase activity

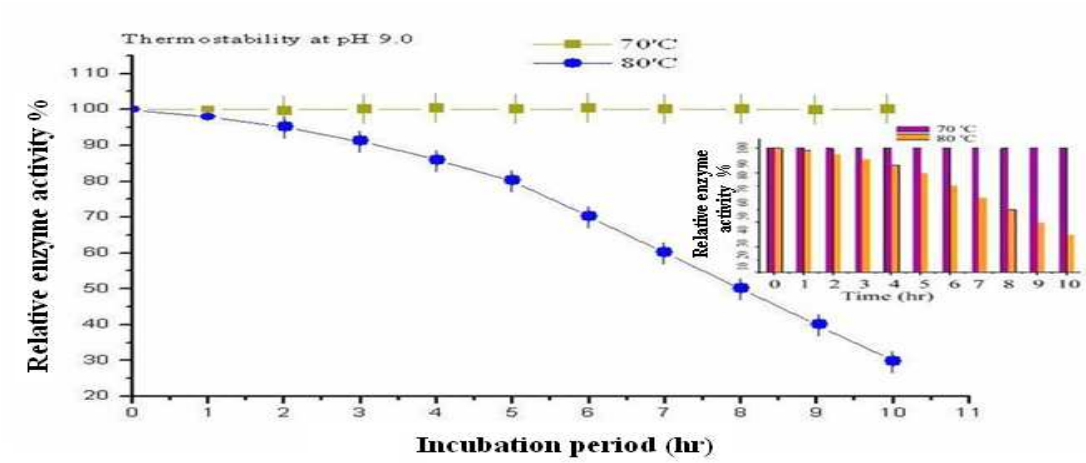

Figure 9. Thermal stability of amylase at 70 and 80 °C

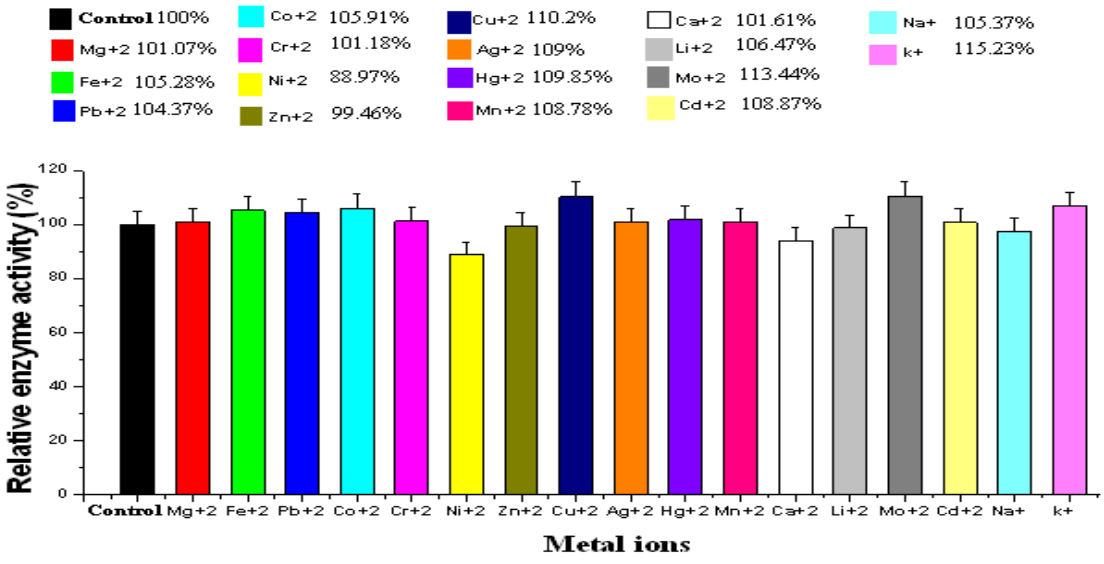

Figure 10. Effect of metal ions on crude amylase activity

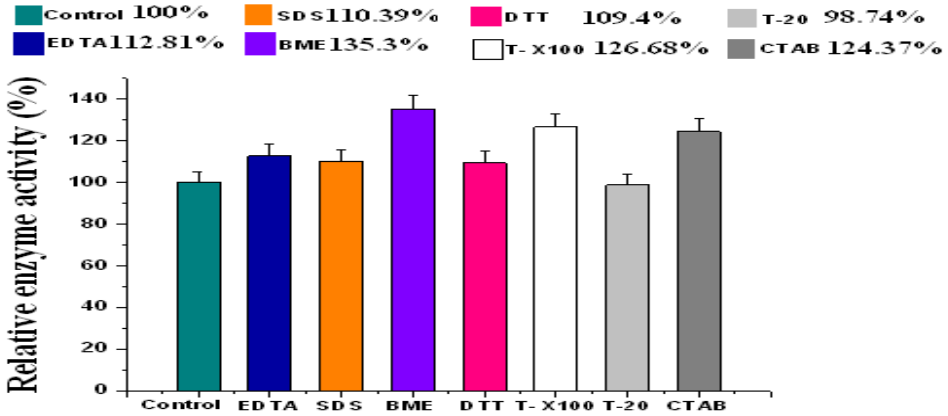

Figure 11. Effects of detergents, chelators and inhibitors on crude amylase activity. (SDS = sodium dodecyl sulphate, T-X100= Triton X 100, Ethylenediamine tetra acetic acid (EDTA), Dithiothreitol (DTT) and *β*-mercaptoethanol (BME), Cetyltrimethyl Ammonium Bromide(CTAB))

Effect of metal ions, chelators, surfactants and inhibitors on amylase activity:
Effects of different metal ions and chemicals on the activity of xylanase were determined by using soluble starch as a substrate. It was seen that Mo^{+2}, K^{+}, Mn^{+2}, Cu^{+2}, Hg^{+2}, Pb^{+2} and Co^{+2} enhance the enzyme activity to 113.4, 115.2, 108.9, 110.2, 109.9, 104.39 and 106 % respectively (Figure.10); but significant enhancement was found in presence of EDTA (112.81 %), SDS (110.39 %), Triton X100 (121.68%), beta-marcaptoethanol (135.30%) and CTAB (124.37%) (Figure.11). This result not only proves that it's a chelators and SDS resistant amylase, but also proves that this amylase is not a metallozyme, like other amylases [2]. Ni^{+2} and Zn^{+2} slightly inhibit the enzyme activity to 89 and 99 % (Figure. 10).

CONCLUSION

Bakreshwar is a pilgrimage centre in Birbhum- Suri, West Bengal, India. Bakreshwar, known for an ancient temple of Lord Shiva. The main temple is surrounded by eight hot springs of varying temperatures. The hottest one known as the Agni Kundu, is having water around 200 degrees Fahrenheit temperature. According to ancient rituals, all devotees throw raw rice to the hot springs as offering to Lord Shiva. As per our intuitions, these hot springs should be a good source of thermophilic amylase producing microorganisms.

The aim of this study was to isolate and identify thermophilic strains having potential amylase activity. We combined the morphological, physiological and DNA sequence analysis method [35] to identify the taxonomic position of the isolated thermophilic amylolytic strain.

In this study, we have isolated a thermostable and alkali-tolerant strain IB-A from hot spring of Bakreshwar. Depending on phylogenetic analysis based on 16S rDNA sequence homology, this strain was identified as a member of the genus *Anoxybacillus* and named as *Anoxybacillus* sp. IB-A. The genus *Anoxybacillus* was firstly separated from *Bacillus* by Pikuta *et al.*, 2000 [30] based on the phenotypic properties and 16S rDNA sequence. Characterization of the crude amylase produced by *Anoxybacillus* sp. IB-A, revels that this one is a thermo-alkaline amylase, similar to some amylases reported earlier [42, 43, 44, 45]. Study of the effect of metal ions, chelators, detergents and inhibitors on enzyme activity reveals that it's not only a chelators and SDS resistant amylase, but also proves that this amylase is not a metallozyme, like other amylases [2].

In conclusion, the newly isolated *Anoxybacillus* sp. IB-A from hot spring of Bakreswar, West Bengal (India), is a potent source of thermostable and thermoalkaline amylase. It's high activity and stability towards alkaline pH, high temperature and resistance towards metal ions, detergents and chelators, suggests that the enzyme has a potential in starch liquefaction at high temperature and detergent industry.

Acknowledgements
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