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PURIFICATION AND CHARACTERIZATION OF AMYLASE PRODUCED BY *HALOBACTERIUM SP. GB24* ISOLATED FROM MARINE NATIONAL PARK

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ABSTRACT
There are various enzymes used in different industries including paper, textile and food. For this propose Halophilic Bacterial strain isolated from India's 1st Marine National Park at Jamnagar. Total nine isolates were selected for amylase production on solid agar media containing 1.5% of starch as a substrate. Selected isolates were subjected to morphological, colonial and biochemical characterization. The specific activity of selected one isolate *Halobaterium sp.* GB24 is 33.62 U/mg with purification fold 2.26 and Yield-38.53% by Ammonium sulphate precipitation method and further purification by Gel Filtration Chromatography by using Sephadex G-25 shows specific activity 30.24 U/mg, Purification Fold-2.04 and 24.19% Yield. The enzyme acted optimally at pH-6, temperature-40 ºC and 20% NaCl respectively. The 16S r-RNA gene sequence analysis shows 99% similarity with *Halobaterium sp.* and the GenBank accession number is KY606985.

KEY WORDS: Amylase, Extreme halophilic bacteria, Gel filtration chromatography, *Halobaterium sp.*, Marine national park.

INTRODUCTION

The Marine National Park (Narara Island). Its area is 162.89 square km of Marine National Park and 457.95 Square Marine Sanctuary in the Gulf of Kutch. The mangroves area and creek area has high salt content due to presence of salt in the land , so some Halophilic bacteria present in to soil and water. The present work is focused on the study and isolation of Halophilic bacteria. Extremely halophilic microorganism is present in each of three domains of the life: Bacteria, Eukarya and archaea. The domain bacterium typically contains many types of halophilic bacteria that spread over a large number of Phylogenetic subgroups (Vaishali *et al.,* 2012). The Halophilic bacteria are contains a very large group of extremophiles (Ventosa *et al.,* 1998; Oren, 2002) Halophilic bacteria distinguish in to three categories on the basis of their salt requirements for their growth as slightly halophiles (1-3% w/v), moderate halophiles (3-15% w/v) and extreme halophiles (>25%w/v) (Vantosa and Nieto *et al.,1995*). These bacteria are also having physiological adaptation to highly saline environment and their ecology. Halophilic bacteria are diversified as terms of physiology *i.e*. Anaerobic, Aerobic, Photoautotroph, Chemolithotroph, as well as Photoheterotrophs. Some community inhabit the marine environment, the salt deserts and estuarine (ollivier *et al.,* 1994; Oren, 1999). The Halophilic bacteria define as a "salt loving" bacteria are grow in high salt concentration and in contrast is called halotolerant organisms required normal salt concentration (0.5-1% w/v) but they can also tolerate up to 5% w/v NaCl concentration for their growth (Garabito *et al.,* 1998).

Halophilic bacteria are normally found in Dead sea, saltern ponds, Saline lakes, desert, Salted food and other place such as saturated with salt concentrated area etc.(Kushner *et al.,*1978; Ventosa *et al.,*1998) some Halophilic archaea have been isolated form various habitats, for example, *Halorhabdus utahensis* from Great alt Lake (Wanio *et al.,* 2000); *Halobaculum gomorrense* from Dead Sea (Oren *et al.,*1995); *Halogemetricum borinquense* from solar salterns of Puerto Rico (Montalvo Rodriguez *et al.,*1998). Many strains such as *Halobacterium salinarum* have been isolated form Thai fish sauce, salted fish and hides, desert, estuaries polluted crude oil and solar salterns *etc*. (Thongthai *et al*., 1992; Raghavan *et al.,* 2000).

Extremophiles organisms that thrive in extreme environment with high stability. The life of extreme environments has been studied and which mainly focus on the diversity of bacteria, physiology, molecular and regulatory mechanisms are involved. Currently focusing on their populations and their biotechnological applications such as halophilic bacteria secrete a wide range of hydrolytic enzymes from their surrounding environments. From the several enzymes which include protease, lipase, xylanases, cellulases and amylases have polyextremophilic properties (Setati M.V., 2010). Amylases are constitute about 25% of enzyme market for sugar, paper, brewing, textile, and food processing industries (Rao *et al.,* 1998 and Sivaramakrishnan *et al.,* 2006). Theses group of organisms have stability under extreme conditions like high temperature, extreme p^H, High concentration of salt etc. and enzymes shown activity at extreme condition is called extremozymes. In the last few years, different screening programs have been carried out to study the diversity of microorganisms producing hydrolytic enzymes throughout direct planning on agar medium supplemented with specific substrates for the enzymes of interest. A limited number of halophilic bacteria showing hydrolytic activities have been isolated and characterized from Narara Island.

MATERIALS & METHODS

Study Site

Samples of the sediment soil and water were collected from India's 1st Marine National Park and its area 457.95 squares Marine Sanctuary in the Gulf of Kutch. The Mangroves area and creek area has high salt content due to presence of salt in the land so, Some Halophilic bacteria present in to soil and water.

The present work is focused on the study and isolation of Extreme halophilic bacteria producing amylase. Sampling was carried out in duration of November. Soil sample was collected in sterile zip plastic bag and water sample was collected sterile screw cap bottle than stored under refrigeration at 4ºC.

Isolation and Screening of Halophilic bacteria

Halophiles were isolated by using nutrient agar medium containing salt concentration. The isolation medium containing (g/L^{-1})

Ingredients	Gms/Litre
Peptone	5.000
HM peptone B	1.500
Yeast Extract	1.500
Agar Powder	15.000
pH	7.2-7.5

Sodium chloride concentration were used such as 5%,10%,15%,20%,25% and 30% than incubate all the plates at 37ºC for 24-48hr well isolated colonies were selected and maintained in pure culture.

Morphological and Biochemical Characterization

Morphological characterization of Moderate and Extreme halophiles was performed by Gram's staining method. Biochemically isolates were analyzed on the basis of Bergey's Manual of Systematic Bacteriology.

Screening of Amylase hydrolysis activity

Extracellular enzyme producing bacteria among the isolates were screened by plate assay method. Amylolytic activity of the cultures were screened on starch nutrient agar plates containing g/l^{-1} starch 10.0; Peptone 5.0; Yeast extract 3.0; Agar 30.0; NaCl 200.0. The pH was adjusted by NaOH and HCl from 6.5 to 7.0 depending on experimental conditions. After incubation at 37ºC for 24-48hr, the zone of hydrolysis was determined by flooding the plates with iodine solution. The potential amylase producers were selected based on ration of zone of clearance diameter to colony diameter.

Production of Halophilic Amylase

Enzyme activity of the isolates exhibiting larger zone of clearance on the plates were further conformed by assaying enzyme activity in broth. The isolates were seeded in to the nutrient broth (Himedia) (gL^{-1}): supplements with starch, 20% NaCl and pH-6.5. The incubation was carried out at 150rpm, 37ºC for 72-96 hr. The cells were harvested by centrifugation at 10,000rpm for 10min at 4ºC and cell free supernatant was assayed by enzyme assay method (Amoozegar *et al;* 2008).

EFFECT OF P^H & TEMPERATURE ON AMYLASE ACTIVITY

1. Effect of pH on Amylase activity

The effect of pH on Amylase activity was determined by using the Nutrient agar media (Himedia) supplemented with 1% Starch and 15% NaCl. The enzyme activity was checked at different pH; pH-4, pH-5, pH-6, pH-7, pH-8 and pH-9. All the plates were incubated at 37ºC in Incubator and zone of hydrolysis of starch was measured after 24-48hr of incubation by addition of iodine.

2. Effect of Temperature on Amylase activity

The effect of temperature on the Amylase activity was observed by using starch agar containing plats (Himedia). All the plates were incubate at different temperatures such as 20ºC,30ºC,40 ºC,50 ºC,60 ºC, and 70 ºC. After 24-48 hrs measured by zone of hydrolysis by addition of Iodine.

Partial Purification of Amylase by Ammonium Sulphate Precipitation Method

The Purification of enzyme was done by ammonium sulphate precipitation method. The various fractions were collected i.e. 0%, 10%, 30%, 60%, 90%and 100% by addition of particular amount of ammonium sulphate. Precipitated fractions were collected by using cooling centrifugation (Remi) at 8,000 rpm at 4ºC for 15 min then the fractions were dissolved in less amount of phosphate buffer and dialyzed by using dialysis membrane (Himedia) at 4ºC for 24hrs.Dialyzed enzymes were used for enzyme assay by using DNSA method.

Purification of enzymes by Gel filtration chromatography method

Gel filtration chromatography was done by using sephadex G-25 column (Himedia). Sephadex G-25, a polymer of dextran, is a very common gel filtration material. It has the capacity to separate protein molecules with molecular weights from 1000 to 5000Da. Protein molecules with molecular weights more than 5000Da will be excluded from the beads and those with molecular weight less than 1000Da will be completely included. Therefore, a mixture containing molecules of varying size can be fractionated on a sephadex G-25 column by gel filtration chromatography.

Amylase Assay

Enzyme assay for amylase was done by Dinitrosalicylic acid (DNSA) method using starch as a substrate. The enzyme (0.5ml) was added to 0.5 ml of 1% starch solution. The reaction was incubated at 30ºC for 10min and then the enzyme reaction was stopped by addition of 1.0ml of Dinitrosalicylic acid reagent composition (gm/100ml): Dinitrosalicylic acid - 1.0 gm, Sodium Hydroxide - 1.6 gm, Sodium potassium tarterate - 30gm, D/W - 100ml.

The reaction tube was kept in to boiling water bath for 10min. and D/W was added to make final volume 10 ml.

The absorbance was measured at 540nm by using spectrophotometer (systronic).one unit of amylase was defined as the amount of enzyme liberating 1µg of maltose per minute under the same conditions. Enzyme units were measured by using maltose (100-1000µg) as a standard.

RESULTS & DISCUSSION

Isolation and Characterization of Halophiles

Soil and water samples were collected from different locations such as Mangroves area, near creek area during month of November from Narara Island, Jamnagar, Gujarat, India. Map of area shows in figure-3.1.

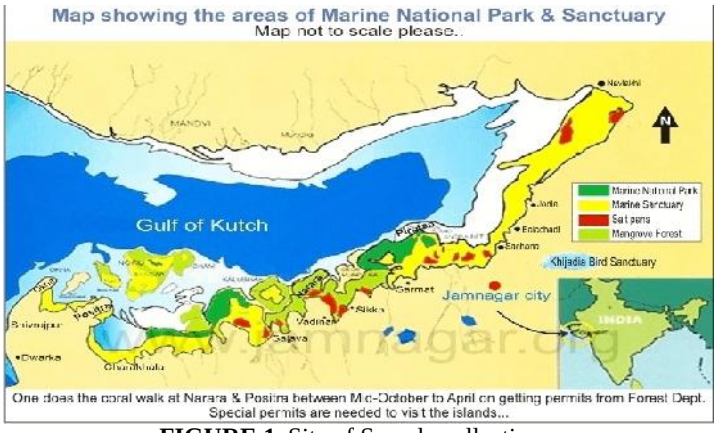

FIGURE 1. Site of Sample collections

All together 25 Moderate and extreme halophiles able to grow at the extreme level of NaCl (10-30% were isolated from the samples. *Halobaterium sp.* GB24 isolated from mangrove was able to grow 10% to 30% NaCl. *Halobaterium sp.* capacity to produce Amylase.

Morphological Characterization

Morphological data revealed that organisms were Gram's Positive or Gram's Negative. Till now majority of studies were performed gram negative halophiles there was versatility among size shape and arrangement of all the isolates.

TABLE 1: Morphological Characterization

Bacteria	Size	Shape	Arrangement	Gram reaction
GB5	Small	Coccobacili	Single, double	Gram positive
GB6	Small	Cocci	Single, pair and in group	Gram positive
GB10	Small	Cocci	Single	Gram positive
GB14	Small	Cocci	Single, pair ,tetrad form	Gram negative
GB15	Small	Cocci	Single, pair and in group	Gram positive
GB18	Small	Cocci	Single, pair and in group	Gram positive
GB22	Small	Short rod	Single, pair and in cluster	Gram negative
GB24	Small	Short rod	Single, pair and in cluster	Gram negative
GB25	Small	Short rod	Single, pair and in cluster	Gram negative

Biochemical Characterization

Bacteria were characterized with reference to Bergey's Manual of Systematic Bacteriology Show in Table 3.2. Majority of halophiles were negative Methyl red teat and Vogus –proskauer test. Majority of our isolates were able to give starch hydrolysis test positive. Halophilic bacteria were also diversified with sugar utilization like glucose, sucrose, fructose, maltose, Mannitol, xylose etc. out of total 25 isolates nine identified as a starch hydrolysis bacteria From this GB 24 give high zone of hydrolysis and hence indicate similarity with *Halobacterium sp.*

TABLE 2: Biochemical Characterization of Isolates

Sr. No	Name of Test	GB5	GB6	GB10	GB14	GB15	GB18	GB22	GB24	GB25
1	Methyl red test	-	-	-	-	-	-	-	-	-
2	Vogus –proskauer test	-	-	-	-	-	-	-	-	-
3	Indole Production test	-	-	-	-	-	-	-	-	-
4	H_2S production test	-	-	-	-	-	-	-	-	-
5	Nitrate Reduction test	-	-	-	-	-	-	-	-	-
6	Urea hydrolysis test	-	-	-	-	-	-	-	-	-
7	Phenyl alanine deaminase	-	-	-	-	-	-	-	-	-
8	Starch hydrolysis test	+	+	+	+	+	+	+	+	+
9	Sugar Fermentation test									
	Glucose	+	+	+	+	+	+	+	+	+
	Mannitol	-	-	-	-	-	-	-	-	-
	Fructose	-	-	-	+	-	-	-	-	-
	Maltose	-	-	-	+	-	+	-	-	-
	Sucrose	-	-	+	+	+	-	+	-	+
	Xylose	+	-	+	-	-	+	-	+	-

Screening of Amylase producing Halophiles

On the basis of zone of substrate utilization on starch agar medium, it was concluded that extreme halophilic have diversity for production of Amylase. Halophiles known to produce haloenzymes like amylases, proteases, lipases, and cellulases. 9 isolates produce amylase efficiency of enzyme secretion by isolates was analyzed on the basis of zone ratio shown in Figure 1.

TABLE 3: Screening of Amylase producing Bacteria

Bacterial Isolates	Amylase producing Bacteria
GB1	-
GB2	-
GB3	-
GB4	-
GB5	+
GB6	+
GB7	-
GB8	-
GB9	-
GB10	+
GB11	-
GB12	-
GB13	-
GB14	+
GB15	+
GB16	-
GB17	-
GB18	+
GB19	-
GB20	-
GB21	-
GB22	+
GB23	-
GB24	+
GB25	+

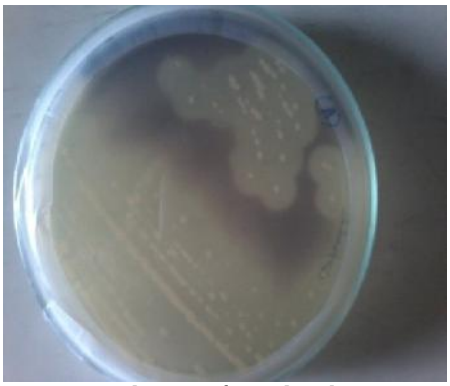

FIGURE 2: Production of Amylase by GB 24

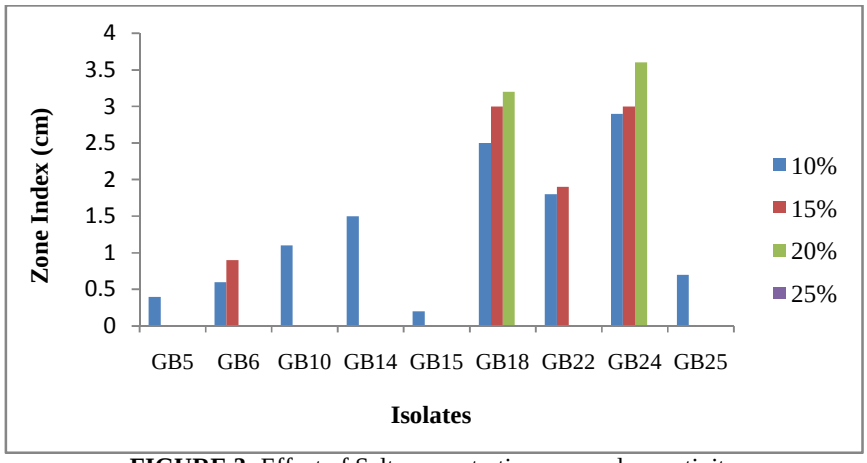

FIGURE 3: Effect of Salt concentration on amylase activity

Effect of Salt concentration on Production of Amylase

The amylase enzyme producing extremely halophilic isolates were GB-18 and GB-24 that can grown at 20% w/v of NaCl shown higher zone of hydrolysis. GB-6 and GB-22 was shown zone of clearance at 15%w/v of NaCl

concentration while other five moderately halophiles were grown at 10%w/v of NaCl shown in figure 3.2.

Effect of pH and Temperature on Amylase producing GB 24

GB 24 was shown enzyme activity up to 50ºC temperature and isolate found at slightly acid to neutral pH on solid agar medium it is determined by zone of hydrolysis after incubation of specific cultural condition.

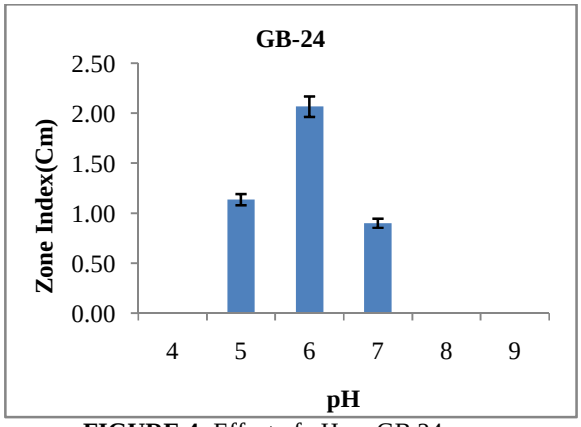

FIGURE 4: Effect of pH on GB 24

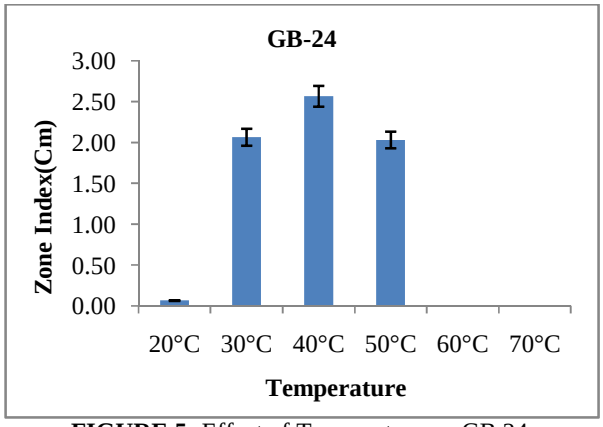

FIGURE 5: Effect of Temperature on GB 24

Purification of Amylase from GB 24 Isolate

Amylase was purified by using ammonium sulphate precipitation method. GB24 was purified in 30% to 60% ammonium sulphate fraction with 33.62 U/mg specific activity and 38.53% Yield observed in Table 3.6. After fine purification was done by gel filtration chromatography method by using sephadex G-25 column results was found in Table 3.7. It was purified in 30-60% with 30.24 U/mg specific activity and 24.19% Yield.

TABLE 4: Purification of Amylase from GB 24 by Ammonium Precipitation method

Step	Activity (Unit/ml)	Protein (mg/ml)	Specific Activity	Purification Fold	Yield (%)
Crude Enzyme	2600	175	14.85	-	100
0-30%	308	31.2	9.87	0.66	11.84
30%-60%	1002	29.8	33.62	2.26	38.53
60%-90%	303	6.00	50.50	3.40	11.65
100%	-	-	-	-	-

TABLE 5: Purification of amylase by Gel Filtration Chromatography from GB 24

Step	Activity (Unit/ml)	Protein (mg/ml)	Specific Activity	Purification fold	Yield
0-30%	141.16	49	2.88	0.19	5.4
30%-60%	629	20.8	30.24	2.04	24.19
60%-90%	164.76	11.0	14.97	1.00	6.33
100%	-	-	-	-	-

Molecular Identification of Halophilic Isolate

Molecular identification of the halophilic isolate GB24 was carried out by 16S r-RNA sequencing. It was sent for sequencing PCR amplified 16S r-RNA gene at Chromous Biotech, Bangalore analyzed by Sanger's method and the sequence obtained was submitted to NCBI under the accession number KY606985. The sequence was subjected to BLAST analysis. The BLAST result showed maximum identity of 99% towards genera *Halobaterium sp.*

CONCLUSION

The isolate GB24 was found to be interesting because of its site of sample collection is novel no work found on halophilic bacteria. Halophilic bacteria is extreme halophilic nature, activity shows at temperature 50º C thermophilic in nature, and its growth wide range of pH. Its amylolytic activity and enzyme productivity at high salt concentration and pH confirmed that much industrial potential can be expected from the isolate. The molecular identification showed 99% similarity with Halobaterium s

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