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Isolation and Characterization of Thermostable Amylase Producing Bacteria from Hot Water Springs of Gir National Forest

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The present study is aimed at assessing the ability of the microbial community of hot water spring to produce different industrial important enzymes. Identification was based on biochemical, morphological measures and response of different growth parameters.15 potential isolates of different thermostable enzyme producer were obtained during primary screening. Secondary screening of these bacterial isolates yielded a high thermostable α- amylase producing isolate. Among all the 6 isolates, isolate no. 2 gave remarkable response to various growth parameters.The optimum temperature and initial medium pH for amylase synthesis by the organism were 70°C and 7.0 respectively. Phylogenetic analysis on the basis of 16S rRNA gene sequence of isolate no. 2 revealed that it was new and next closest homologue was found to be *Bacillus thermodenitrificans* str.T4

Key words: Thermostable α- amylase, *Bacillus thermodenitrificans* str.T4.

One of the important characteristics of thermostable organisms is their ability to secrete enzymes of industrial use which work at higher temperature for longer period of time (Sonnleitner and Fiechter, 1983; Becker *et al.,* 1997; Lee *et al.,* 1999; Beg *et al.,* 2000). Most of the Liquid sugar industry, leather and food processing industries need thermostable á-amylase that can maintain its activity at high temperature.Amylases are the enzymes capable of degrading starch and universally distributed throughout plant, animal and bacterial kingdom. Hot spring or hot water sources are a water source that is formed as a result of ground water comes out from the earth crust after undergoing geothermal heating (Akhmaloka *et al.,* 2006) These could be the place where one can find organism with diversify genus.

A number of thermophilic bacteria have been isolated from hot water sources in india, but no one has yet reported thermostable bacillus of Gir national forest. Gram positive bacteria and particularly of genus Bacillus are major enzymes producer beside some of the gram negative organisms. More recently some amylolytic yeast and fungi of genus *Aspergillus, Penicillium, Cephalosporium, Mucor* and *Candida* had been isolated by various scientists (Nahas E. *et al.,* 2002; Xioali Z. *et al.,* 2002).

The aim of the study was to isolate, identify and characterize thermophilic bacteria from hot water spring of Gir national forest producing industrial important enzymes.

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MATERIALS AND METHODS

Sampling of thermophilic microorganisms from hot spring

The present work was started with the collection of water samples from the TulsiShyam thermal spring at Gir National Park in Junagadh district. The water samples were collected both from surface and deep waters. The samples were collected in sterilized water flasks at 5 feet depth. These samples were tested for their chemical composition.

Screening and Isolation of microorganisms

One milliliter sample was added to nutrient broth (100 ml) and incubated at 55°C for 24-48 hours. Growth was followed by measuring the increase in turbidity at 600 nm. Then, the culture was streaked onto a nutrient agar plate. Isolation of pure culture was done by using spread plate method and streak plate method recommended by Rath and Subramanyam (1998).The organism was enriched on a screening agar plate containing (grams per liter): 10.0 g Peptone, 3.0 g Meat/beef extract, 5.0 g Sodium chloride,3.0 g Agar, pH (7.4). in distilled water. Incubation at 55°C was carried out for 24-48 h, after which the plates were stained with Gram's iodine solution of 0.1% I_2 and 1% KI (Thippeswamy *et al.,* 2006). The colonies with the largest halo-forming zone were isolated for further investigation. To obtain pure culture, Subculture was prepared by a full loop of the suspension of vegetative cells streaked aseptically onto a nutrient agar (NA) several times. The pure isolates were maintained on NA slants and routinely sub-cultured.

Determination of optimum growth conditions

Study of growth kinetics of selected organisms in presence of various growth promoting factors like yeast extract, inorganic salts, vitamins and amino acids was carried out. For optimum growth of the bacterial isolate, two parameters that are temperature and pH were considered. For determination of optimum temperature, 5o ml LB broth was inoculated in four flask and each one kept at different temperature, The four flasks incubated at 40°C, 55°C,60°C and 70°C. After an incubation period of 24 h, their absorbance was taken at 600 nm. For determination of optimum pH, 6 flasks containing 50 ml of Broth was inoculated with isolates. pH of the LB broth was adjusted at 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. These flasks were incubated at 55^0C for 24 hours and their absorbance was taken at 600 nm.

Morphological, biochemical and molecular characterization

Microbiological properties of the isolated strain were determined according to the methods described in Bergey's Manual of Systematic Bacteriology (Sneath *et al.,* 1986; Buchanan and Gibbons, 1974). The colony characteristics of the isolates were studied and they were subcultured and screened for their ability to grow at higher temperatures. Gram's staining; Capsule Staining and Spore Staining of the selected isolates were performed. Different biochemical tests were conducted for characterization of the isolates. (Salt tolerance, Catalase test, Phenyl alanine test, Starch hydrolysis test, Casein hydrolysis test, Ammonia production test) For molecular characterization, genomic DNA was extracted as described by Carozzi *et al.,* (1991) and DNA was send to Bangalore Genei for Phylogenetic analysis on the basis of 16S rRNA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

RESULTS

Determination of optimum growth conditions

The first step was to isolate the desired microorganism that produces α- amylase. There are 6 isolate was initially selected on the basis of zone of hydrolysis on agar plate. Among the six selected *Bacillus* strains (1, 2, 3, 4, 5 and 6) that showed the highest amylase synthesis, Strain 2 was selected because it gave a larger diameter zone of clearance (Fig. 1) and the highest relative amylolytic activity compared to the other species. There-fore Strain 2 was chosen to fulfil the aim of this research which is isolation of thermostable amylase producing organism. All six isolates showed optimum growth at pH 7 (Fig. 2) and the most suitable temperature for the growth of bacterium was found to be 70°C. The growth curve pattern was studied by growing the bacterium in 1% starch and comparing with the control culture in which no starch was added.

The growth pattern of all the isolates was significantly different from each other with respect

to growth parameters. Among all the six isolates, isolates 2 respond well in the presence of yeast extract and MgSo4 up to certain concentration (Fig. 3). Moreover, Growth rate of bacterial isolate was higher in the presence of various amino acids (Fig. 4) and vitamins (Fig. 5) as compared to growth in the presence of LB medium. Isolate 1 , 2 and 4 used Alanine and arginine as nitrogen sources and shown higher growth in terms of absorbance maximum compare to control one. Pyridoxal-HCl and thymine were excellent inducer of growth. The maximum growth (O.D) was determined after 24 and 48 h of growth in (Fig. 1).

Morphological, biochemical and molecular characterization

The morphological and biochemical characteristics of the isolated amylase producing isolate No.2 are shown in Table 1. On the basis of standard method, the strain was assigned a code and identified as *Bacillus TULH. Bacillus TULH* was further used for other analytical procedure.

Phylogenetic analysis on the basis of 16 S rDNA sequence

1. The Microbe was detected to be *Unidentified* low G+C gram-positive bacterium str. HTA1418 (GenBank entry: AB002647)
2. The next closest homologue was found to be *Bacillus thermodenitrificans str. T4* (GenBank entry: AF114426) and
3. Information about other close homologue for the microbe can be found from the Alignment View table.

Alignment view table

Alignment View	Ref ID	Sequence description
☑		*B.thcatenuBacillus thermocatenulatus DSM 730 (T)*
☑		*AB034836Bacillus thermoleovorans str. B23*
☑		*B.thleovorBacillus thermoleovorans DSM 5366 (T)*
☑		*B.caldotenBacillus caldotenax DSM 406*
☑		*AF114426Bacillus thermodenitrificans str. T4*
☑		*B.caldovelBacillus caldovelox DSM 411*
☑		*AB002647str. HTA1418*
☑		*AF114424Bacillus thermodenitrificans str. NS*
☑		*B.denitrif Bacillus denitrificans DSM 466*
☑		*B.caldolyt Bacillus caldolyticus DSM 405*

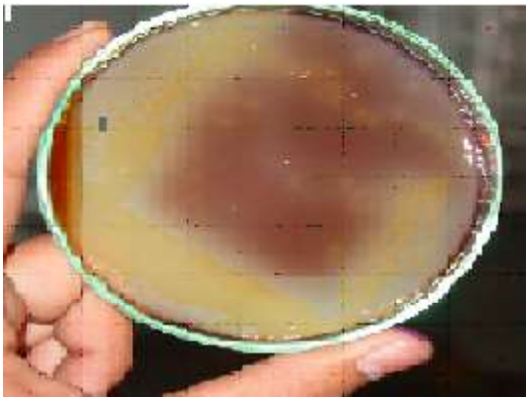

Fig. 1. Starch plate:- Zone of hydrolysis was observed when plate was flooded with iodine

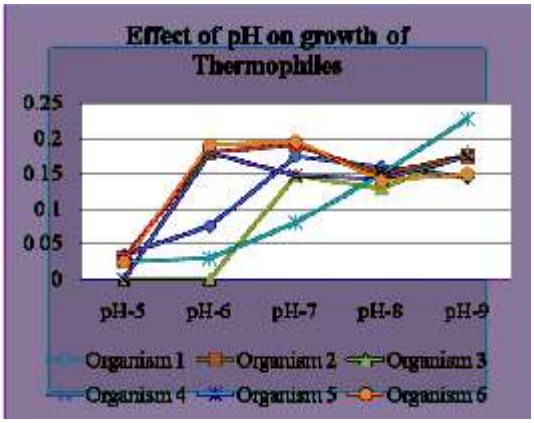

Fig. 2. Effect of pH on growth of 6 isolates

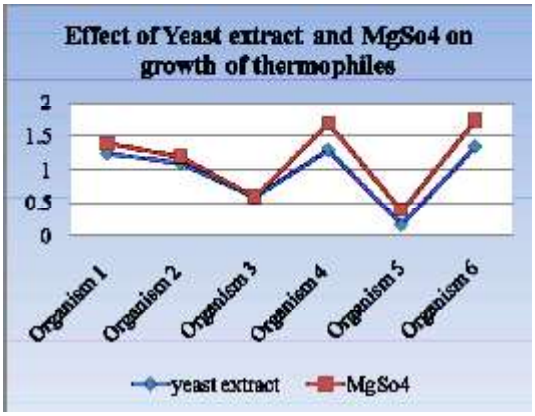

Fig. 3. Effect of yeast extract and $MgSo_4$ on growth of 6 isolates

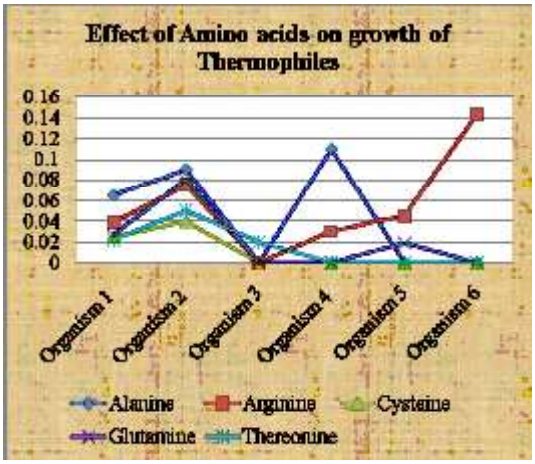

Fig. 4. Effect of various amino acids on growth of 6 isolates

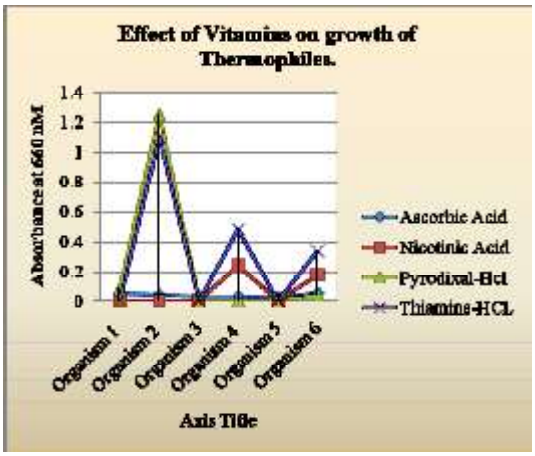

Fig. 5. Effect of various vitamins on growth of 6 isolates

Phylogenetic tree

16S r RNA Sequence analysis was performed using Applied Biosystems Microseq microbial analysis software and database. The top ten alignment matches are represented. 16S rDNA gene sequence analysis indicated that isolate no. 2 was phylogenetically related to members of the genus *Bacillus*. The phylogenetic tree (Fig. 6) also indicated that isolate no. 2 clustered with *Bacillus* species and this cluster was strongly supported (100%). *Bacillus isolate no. 2 16S r RNA sequence* was publicly deposited with accession no. EF063151 *to NCBI* .The isolate matched best with the genus *Bacillus* and showed similarity to *Bacillus thermodenitrificans str. T4* (GenBank entry: AF114426).

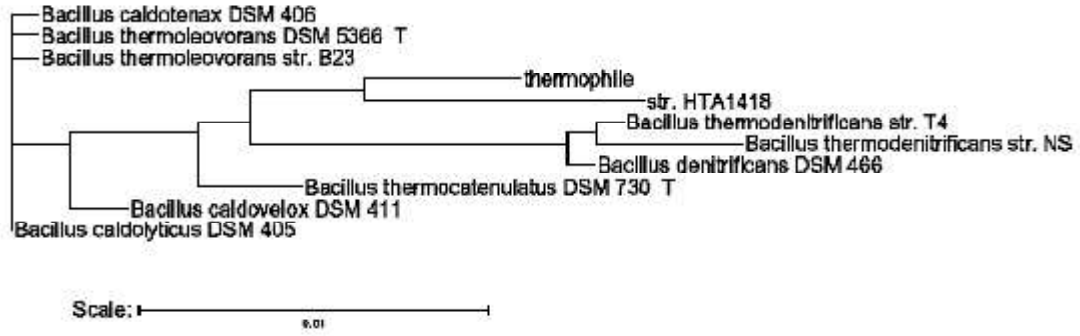

Fig. 6. Phylogenetic tree constructed with the neighbour-joining method according to 16S rDNA gene sequence evolutionary distance among *Bacillus* sp. TULH and the type strains of recognized members of the genus *Bacillus*

Table 1. Morphological and Biochemical characteristics of the isolate no. 2

Morphology and Biochemical characteristics of Isolate no. 2 (*Bacillus TULH*.)	
Morphology:	Rods occurring as single cells, non- motile,
Growth:	Agar Abundant, pale-yellow colonies
Gram's staining	Gram positive, small rods, occurring in pairs
Spore staining	Spore formers
Capsule staining	Capsule formers
pH	Optimum 6.8, range 6-9
Temperature	Optimum 70°C, range 55-70°C
Salt tolerance	Growth observed up to 7% NaCl.
Catalase test	Positive
Phenyl alanine test	Positive
Starch hydrolysis test	Positive
Casein hydrolysis test	Positive
Ammonia production test	Positive
Aerobic	Abundant growth
Anaerobic	No growth
NaCl slant	No growth in 10%
Colony characteristics	
Size:	Medium
Shape	Round
Margin	Undulate
Elevation	Umbilicus (having central pit)
Surface	Smooth
Opacity	Translucent

DISCUSSION

Microbial ecosystem is a complex with different morphological, biochemical and physiological groups. One of the most interesting and currently most worked upon organisms are Thermophiles. Thermophile is a type of extremophile, which are active at high temperature, above 45°C.

Thermophiles contain enzymes that can function at high temperature. Thermo stable enzymes isolated from some extremophiles have been proven to be greater use in Biotechnology related industries, which are even able to function under condition that would denature enzymes taken from most normal organisms.

Hot water springs have relatively higher amount of sulphate. Thermophiles required sulphate for their metabolism and this requirement is fulfilled by hot water springs which contain more amount of sulphate. Various moderate Thermophiles have been isolated from the hot spring.

In our study the analysis of water sample suggested that it contain unusual high amount of sulphur which advised that organism was evolved from its ancestor. Initially there were 15 different isolate were selected according to its colony morphology, subsequently pure culture was prepared through repeated culturing using four flame method. At last there were six isolates selected on the basis of zone of hydrolysis. However isolate no. 2 was shown excellent growth on starch plate and highest zone of hydrolysis. Growth of thermophiles normally takes place at slightly acidic to slightly alkaline pH (between pH 6 - 8) indicating their ranges of pH optima. Isolate No.2 gave good zone on the starch agar plate and within 10 hrs it showed maximum growth hence was selected for production of thermostable amylase. Before the production stage isolate was checked for various biochemical tests. It indicates that the organism is also capable for the production of caseinase and catalase. The morphology of the isolate no. 2 was rod shaped; non motile and aerobic Catalase producing, more over it was spore former, characteristics of members of family Bacillaceae. The physiological characteristics of isolates varied over wide range of conditions. Some gave good intense growth but not good producer of amylase but aim of the research is to isolate amylase producing micro organisms. Henceforth we have decided to proceed with isolate no. 2 which revealed highest zone of hydrolysis. The optimum temperature for growth of organism was 70^0C and can grow very well upto 7 % Nacl concentration which is good for industrial applications. The isolate subjected to 16S rRNA gene sequence studies, was placed in the genus Bacillus. The sequence information thus obtained was analysing through applied biosystem software but it revealed no similarity with already known sequence in the database. The sequence information was deposited to NCBI with accession No. EF063151. Hence we intended to make phylogenetic tree to find out relative organism in the database. We conclude that we have isolated new species of *Thermophilic* bacteria and advocated to name it as *Bacillus TULH.*

CONCLUSION

In conclusion, based on the results of this study, isolation of thermophiles from hot spring is highly successful if water sample collect at the bottom of the spring. For isolation of thermophiles producing industrial important enzymes, hot water springs represents one of the efficient sources. Our work was one of the stepping stone in the field of extremophiles.

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