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Characterization and phylogenetic analysis of alkaline α-amylase producing *Brevibacillus laterosporus* from mountain climatic zone of India

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ABSTRACT

α-amylases (EC3.2.1.1) are glycoside hydrolases that breakdown complex starch and maltodextrins into glucose and maltose by acting upon 1,4-glycosidiclinkages. Several amylases have been isolated and purified from members of *Bacillus* community, which find extensive application in starch processing, textile and pharmaceutical industry. Keeping this in mind we isolated α-amylase producing gram positive bacterium from soils collected from mountain climatic zone of India and identified it as *Brevibacillus laterosporus*. We further studied the effect of temperature and pH on the amylase activity of this strain and found a very stable activity at alkaline pH of 10 and temperature of 45 ºC. To our knowledge this a first report on characterization and evolutionary analysis of alkaline α-amylase producing *Brevibacillus laterosporus* isolated from unexplored sites of mountain climatic zone of India.

Keywords: Climatic zone, *Brevibacillus*, Amylase, 16S rRNA gene sequencing, Phylogenetic analysis

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INTRODUCTION

The köppen system of climate classification divides the Indian climate into six zones viz., tropical wet climatic zone, tropical wet and dry climatic zone, arid climatic zone, semi arid climatic zone, humid subtropical climatic zone and mountain climatic zone[1]. This extreme climatic diversity makes India a perfect hotspot for surveying bacterial enzymes, which has extensive industrial and pharmaceutical application. One such extreme climatic zone is the mountain climatic zone, also known as alpine climate or highland climate. This zone is characterized by a temperature drop of 0.6 ºC for every 100m rise in altitude. There is also a sharp contrast in the climatic characteristics of foothills of mountain and top parts above snowline. Most of this constitutes the Himalayas which has sunny and shady slopes, inversion of temperature and variability of rainfall based on altitude and carries a great scope for studies on soil microbiota and their metabolites[2]. Reports from the literature suggest the role of climate in modulating the microbiota of soil in various terrestrial environments dictating their enzyme production capacity[3-5]. Therefore it is interesting to explore enzyme producing bacteria in terrestrial soils experiencing different climatic conditions.

Brevibacillus belongs to the phylum *Firmicutes* and stains Gram positive. It was formerly known as *Bacillus brevis* and was then reclassified in 1996. The Genus *Brevibacillus* has 23 species till date[6-8]. Many members of this genus have shown ability to produce spores that confer them resistance to survive in extreme temperatures, in presence antibiotics and chemical disinfectants[9-10]. A few reports suggest their role as human pathogens apart from being a biocontrol agent against insects[11-12]. They have been also used as human probiotics because of their ability to tolerate high temperature and low pH[13]. Many of them are also known to produce enzymes like Aldehyde oxidase and are exploited as host for expressing many biotechnologically important enzymes like alpha-amylase, Sphingomyelinase, xylanase, CGTase, and chitosanase and heterologous proteins including cytokines (EGF, IL-2, NGF, IFN-c, TNF-a, and GM-CSF), that find variety of applications in pharmaceutical, cosmetic and food industry[14].

Amylase enzymes catalyze the hydrolysis of complex starch into simple sugars such maltose and glucose[15]. The amylases produced from the microorganisms have successfully replaced the chemical hydrolysis of starch in starch processing industries. Thermostable amylase from bacteria are able to act at higher temperatures and are extensively used in starch processing, brewing and sugar production, designing in textile industries, and in detergent manufacturing processes[16]. Most class of amylases produced by bacteria are α-amylases, which randomly breaksdown long-chain saccarrides ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. The other two are the β- amylase which breaksdown second α-1,4 glycosidic bond, cleaving off

two glucose units (maltose) at a time and γ-amylases that cleaves α(1–6) glycosidic linkages and α(1–4)glycosidic linkages simultaneously at the nonreducing end of amylose and amylopectin, producing glucose units[17].

In present study we isolated and characterized amylase producing bacteria from soils collected at pine forests of Himanchal Pradesh India, which falls in the mountain climatic zone. The soil was collected at a depth of 20cm from the surface in order to obtain the native bacterial community. The bacterial isolates were then screened for amylase activity and potent isolate was further characterized and identified using 16S rRNA gene sequencing.

MATERIALS & METHODS

Soil Sampling

Soil samples were collected from 20 different spots in the pre–monsoon season from February 2017 to March 2017 from pine forest located at hills of kasauli from Himanchal Pradesh, India. The soil samples were collected from a mean elevation of 1800 m and at a depth of 15–20cm using a sterile shovel into sterile sampling bags (Nasco: Hi–media, India). The sample were then transported in ice to laboratory and stored at 4 ˚C until further processing[18].

Isolation and Pure Culture

The 20 samples were pooled together to make a composite soil sample which was serially diluted using normal saline (0.45%) and plated on to General purpose media like NA (Nutrient Agar; Hi–Media, India), followed by incubation at 30ºC for a week subsequently monitoring bacterial growth at 24hrs, 48hrs, 72 hrs etc. Quantitative analysis of bacterial growth in media plates was performed by colony counting and CFU (colony forming units) determination. The phenotypic variant was selected on the basis of colony characteristics like shape, size and color for further purification and enzyme screening[19].

Enzyme Screening and Amylase Activity

In primary screening, the amylase activity was detected by plating the isolates in corn starch agar (2% soluble corn starch; 0.5g/100ml peptone; 0.25g/100ml yeast extract; 0.1g/100ml NaCl; 2.5g/100ml agar) followed by incubation at 30°C and staining with Grams iodine to observe clear zone around the colonies. The strain showing maximum zone was further selected for secondary screening and enzyme production assay. The fermentation for enzyme production was carried out in orbital shaker with 250ml Erlenmeyer flask containing 100ml starch broth media (2% soluble corn starch; 0.5g/100ml peptone; 0.25g/100ml yeast extract; 0.1g/100ml NaCl; 0.1g/100ml KCl; 0.1g/100ml $MgSO_4.7H_2O$; 0.1g/100ml K_2HPO_4; 0.1g/100ml $CaCl_2$) at 120rpm and 35°C for 48hrs with subsequent monitoring of enzymatic activity[20]. Detection of amylase activity at different pH and temperature was carried out using crude enzyme, obtained after centrifuging the culture broth at 10,000 rpm and removing cell pellet. The amylase activity was calculated at different pH and temperature using colorimetric assay with disappearance of starch substrate upon addition of acidic iodine, which reacts with starch to produce blue color compound with a maximum absorbance of 620nm[21].

Strain Identification

The amylase producing isolate was subjected to Gram staining, microscopic examination and biochemical characterization as described in Bergey's Manual of Determinative Bacteriology. Further, for molecular characterization the isolate was purified on TSA medium and identified using 16S rRNA gene sequencing. The Genomic DNA was extracted using Nucleospin nucleic acid kit (Machery and Nigel, Germany) and subjected to PCR amplification in a thermal cycler (Eppendorf, Germany) with universal eubacterial primers 27F (5′– AGA GTT TGA TCM TGG CTC AG–3′) and 1492 R (5′– CGG TTA CCT TGT TAC GAC TT–3′). Further the purified PCR product was amplified again using 1100R (5'– GGG TTG CGC TCG TTG–3') primer and sequenced using Sanger dideoxy methodology[22]. The raw reads containing 16S rDNA sequences were searched for similar sequences from 16S rRNA gene database of EzTaxon server using BLAST[23].

Phylogenetic Analysis

The phylogenetic analysis of the identified isolate was performed using MEGA software version 7.0 and phylogenetic trees were constructed using the neighbour joining algorithm[24]. Bootstrap analysis of 1,000 bootstrap replications was performed to assess the confidence limits of the branching[25].

Sequence Deposition and Accession Number

The 16S rRNA gene sequence of the enzyme producing bacterial isolate was deposited in Genbank database under the accession number MF680666.

RESULTS AND DISCUSSION

Strain Isolation and Preliminary Screening

The pH of the soil was mildly acidic (5.4), although it was plated onto a neutral pH media during isolation. The colonies which showed distinct morphology were selected and examined under microscope. Isolates which stained gram positive were further selected for enzyme screening assay in corn starch agar medium. Maximum zone of clearence in starch agar medium was shown by strain irn26 (**Fig.1**). The strain irn26 stained Gram positive having dry round opaque colony morphology. Studies have shown amylase production by many gram positive bacteria isolated from terrestrial environments in starch agar media by addition of iodine, which forms a dark blue complex with starch[26-27].

Figure 1: Preliminary screening of amylase activity. A cross section of starch agar medium plate showing the maximum zone of clearence (marked by red arrow) produced by isolate G9, after addition of iodine.

Strain Identification and Phylogenetic analysis

The amylase producing strain was identified by sequencing the 16S rRNA gene sequencing, using eubacterial universal primer, adopted as a Gold Standard for bacterial identification. Strain irn26 producing amylase was 100% similar to *Brevibacillus laterosporus* (**Table 1**). Further a phylogenetic tree was constructed to confirm its identity and evolutionary relatedness, which showed that the strain irn26 was identical to *Brevibacillus laterosporus* IAM 12465^T

and also had evolutionary closeness to *Brevibacillus halotolerans* LAM0312^T. Whereas, the other members of the genus were distinctly placed in a separate branch of the phylogenetic tree, that shows greater difference in evolutionary lineage between *Brevibacillus laterosporus* and other members of that genus (**Fig.2)**. The sequencing of 16S rRNA gene and BLAST analysis gave 100% similarity match of the amylase producing strain, which enabled us to characterize the isolate with accuracy at species level. Although reports show the 16S rRNA gene based identification and phylogenetic analysis of genus *Brevibacillus*, our study is the first report on characterization and phylogenetic analysis of alkaline alpha amylase producing *Brevibacillus laterosporus*[28-29].

Table 1: Identification of amylase producer using partial 16S rRNA gene sequencing

Isolate Code	Climatic zone	Collection depth	Strain name	Closest phylogenetic affiliation	Percentage similarity	Enzyme produced
MCZ-NA-3	Mountain	20cm	irn26	*Brevibacillus laterosporus*	100	Alkaline alpha amylase

Growth and Amylase activity at different pH and temperature

The growth and amylase activity was carried out in different pH and temperature for the *Brevibacillus laterosporus* strain irn26. Growth was determined by measuring the optical density (O.D.) using a spectrophotometer at 600nm after 48hrs of incubation. Maximum growth was observed at a pH of 10 and temperature of 35^{o}C. Minimum growth was observed at a pH of 5.0, at a temperature of 25^{o}C **(Fig.3)**. The effect of pH and temperature on amylase activity of *Brevibacillus laterosporus* strain irn26 was also assessed by measuring the absorbance of starch

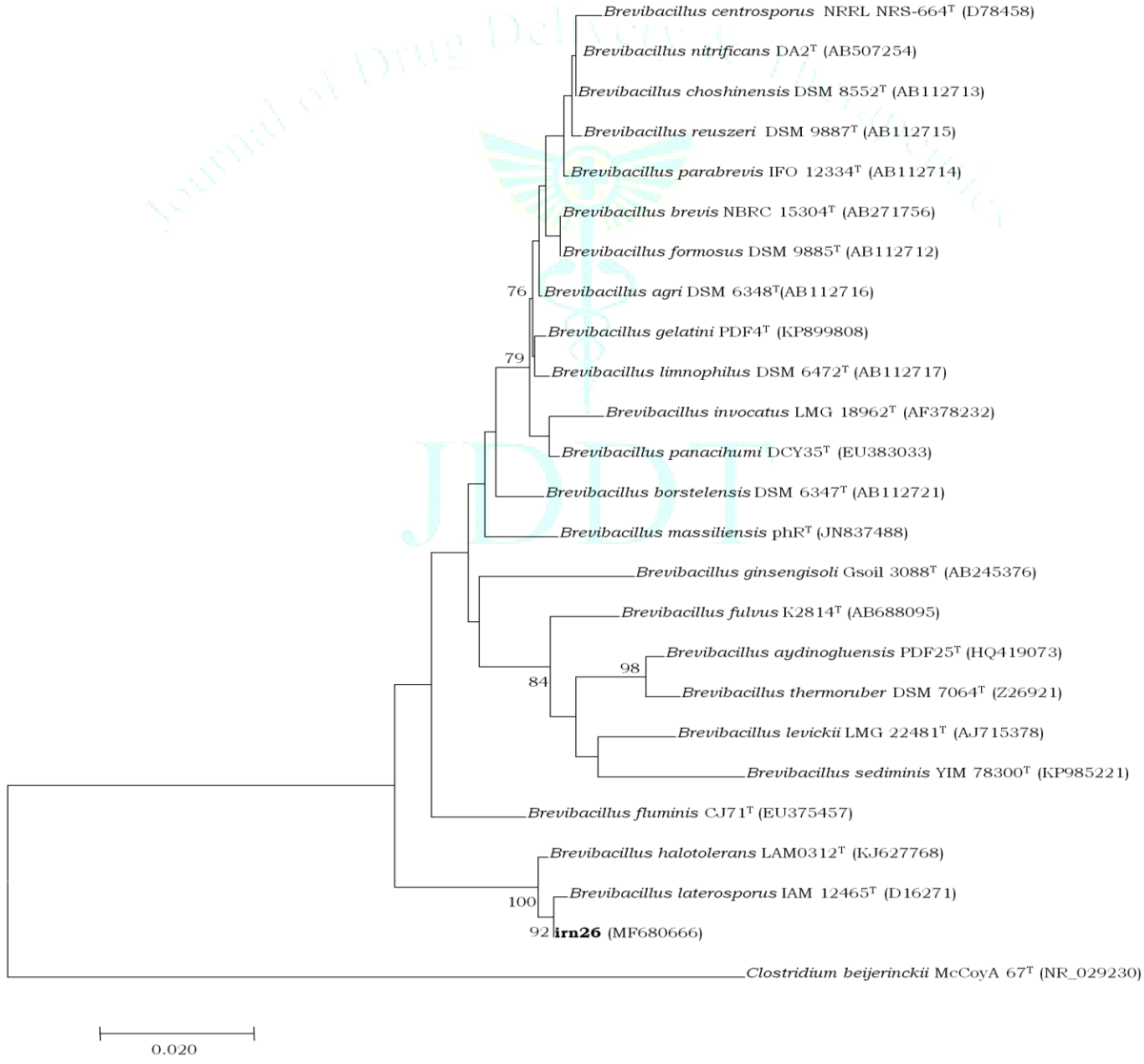

Figure 2: Phylogenetic tree based on 16S rDNA sequences constructed using Neighbor joining method showing the identity and evolutionary relatedness of amylase producing strain irn26 (in bold). Boot-Strap values (1,000 replicates) greater than 70 are given at the nodes. Clostridium beijerinckii McCoyA 67T (NR_029230) was used as outgroup for constructing the tree. Scale bar represents the number of substitutions per nucleotide position iodine complex at 620nm. The peak amylase activity was observed at a temperature of 45 ^{o}C, which started to decrease at higher temperatures. The amylase activity of *Brevibacillus laterosporus* was also stable at a temperature of 70^{o}C.

Interestingly, the amylase activity was high at an alkaline pH of 10 compared to other lower pH ranges and neutral pH and showed a sharp decrease at a pH of 11 **(Fig.4).** Amylases active at alkaline pH find a variety of applications in pharmaceutical industries, starch and textile industries and as an ingredient of detergents[30].

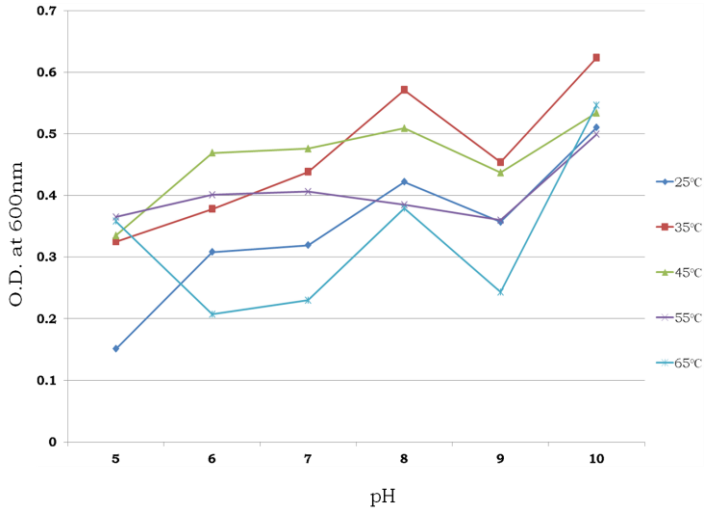

Figure 3: Effect of pH and temperature on the growth of *Brevibacillus laterosporus* strain irn26.

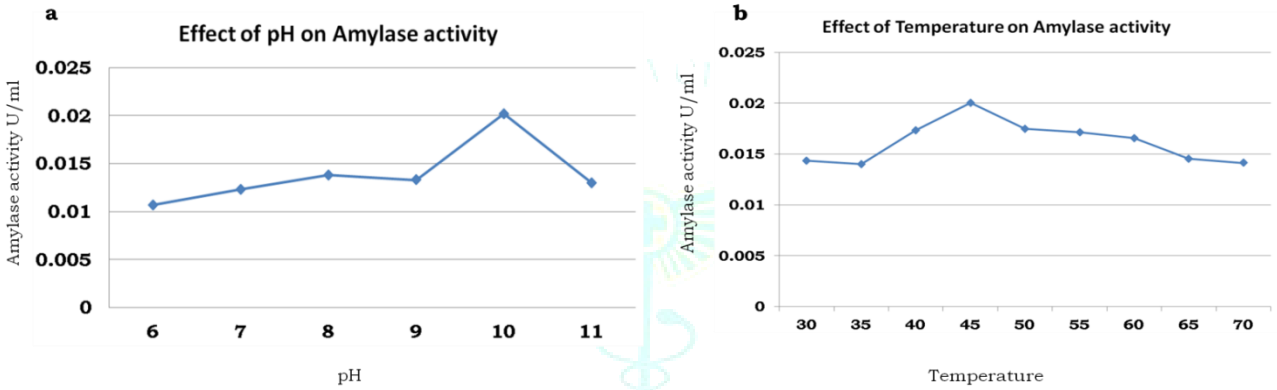

Figure 4: Effect of pH and temperature on amylase activity of *Brevibacillus laterosporus* strain irn26. Maximum activity was observed at temperature of 45°C (a). The amylase activity was highest at an alkaline pH of 10 (b).

CONCLUSION

Present study characterized alkaline alpha amylase producing bacteria *Brevibacillus laterosporus* strain irn26, isolate from the cold mountains of Himanchal pradesh falling in the mountain climatic zone according to the koppen system of climate classification. The produced amylase enzyme was highly active at an alkaline pH of 10 and could be exploited for variety of industrial and pharmaceutical applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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AUTHOR CONTRIBUTION

Dr. Suresh SS Raja (SSR): Concept and study design, Mr. Girish R Nair (RGN): Manuscript preparation, data analysis, review and editing.

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