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Original Article

Characterization of an industrially important alkalophilic bacterium, *Bacillus agaradhaerens* strain nandiniphanse5

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ABSTRACT

Aim: The phenotypic and phylogenetic characterization of an alkalophilic bacterium producing two industrially important enzymes – alkaline protease and alkaline amylase, was performed. A Gram-positive, rod-shaped, spore-forming alkalophilic bacterium (2b) isolated from alkaline dairy soil of Indore region of Madhya Pradesh, was selected in this work.
Methods: The alkalophile was subjected to a taxonomic investigation, including standard bacteriological characterization and 16S rRNA gene sequencing. Phylogenetic trees were constructed by different treeing algorithms: neighbour joining, maximum parsimony tree, maximum-likelihood and UPGMA method using MEGA5 software. The secondary structures of 16S rRNA gene sequence were predicted using GeneBee package and UNAFOLD, a Linux based software.
Results: Its overall biochemical, physiological and phylogenetic characteristics indicated that strain 2b is an alkaliphilic Bacillus belonging to the species agaradhaerens. The free energy of the secondary structure of rRNA was −171.7 and −265.13 kcal/mol respectively. The structure obtained with a Gibb's free energy, ΔG −265.13 kcal/mol by UNAFOLD seems to be more stable in the present study.
Conclusion: The taxonomic investigation identifies 2b as Bacillus *agaradhaerens*.

1. Introduction

Alkaliphilic *Bacillus* species are by far the most important source of several commercial microbial enzymes like proteases, amylases, xylanases, pullulanases, and cellulases. They can be cultivated under extreme pH conditions and these species produce extracellular enzymes that are resistant to high pH and/or high temperature conditions.[1,2] Since enzymes produced by alkalophiles are active in the alkaline pH range, they are found to be most suitable in detergent formulations. The search for new species of microbes having the ability to produce industrially important enzymes with novel properties is a continuous process. The aim of this study was to search for alkalophilic bacteria having the ability to produce two industrially important alkaline enzymes viz. alkaline protease and alkaline amylase. Looking to the increased demand of alkaline protease and alkaline amylase[3–5] in detergent industry and in treatment of alkaline wastes, studies on the cost effective

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production of these enzymes is essential. Multiple enzymes produced from a single organism can be a useful step in this direction.[6] The work undertaken deals with the concomitant production of alkaline protease and alkaline amylase by an alkalophilic bacterium viz. *Bacillus agaradhaerens*. This study focuses on phenotypic and phylogenetic analysis performed in order to establish the taxonomic position of the isolated strain of *B. agaradhaerens*.

2. Methods

2.1. Isolation of alkalophiles

Alkalophilic bacteria were screened by enrichment culture technique from diverse samples collected in and around the city of Indore of Madhya Pradesh, India. These samples included soil, sewage and industrial effluents. The samples were inoculated in Horikoshi's broth medium[7] I, pH 10.0, containing (g %) glucose; 1.0, peptone; 0.5, yeast extract; 0.5, KH_2PO_4; 0.1, $MgSO_4$; 0.02, Na_2CO_3 1.0 (separately sterilized), distilled water 100.0 ml, followed by isolation on Horikoshi's agar medium I (pH 10.0). Single colonies that developed after 48 h of incubation at 30 °C were isolated. The same medium was used for maintenance of the strains. The alkalophilic/alkalotolerant nature of isolates was determined by growing each isolate on Horikoshi's M-I (pH 7.0) agar medium and incubating at 30 °C for 24 h.

2.2. Enzyme profile studies for selection of isolates

Individual bacterial colonies obtained on Horikoshi's M-I (pH 10.0) agar plates were evaluated for their proteolytic ability by measuring the zone of casein hydrolysis on milk agar medium, pH 10.0, containing (g %) peptone; 1.0, meat extract; 0.5, NaCl; 0.5, Na_2CO_3; 1.0, distilled water; 100.0 ml, agar; 2.0. Separately sterilized 10% skimmed milk and Na_2CO_3 were added to the sterilized nutrient agar base, cooled up to 45 °C. Likewise the amylolytic activity of the alkalophilic isolates was evaluated by measuring the zone of starch hydrolysis on starch agar medium, pH 10.0, containing (g %) starch; 2.0, peptone; 0.5, yeast extract; 0.1, KH_2PO_4; 0.2, $MgSO_4$; 0.02, Na_2CO_3; 1.0, agar; 2.0, distilled water; 100.0 ml Na_2CO_3 was sterilized separately and mixed. The inoculated plates were incubated at 30 °C for 48 h and observed for zones of clearance. In case of detection of amylase, the starch agar medium plate was flooded with 1% iodine solution, to observe the zone of hydrolysis. The bacterium, 2b, found to produce maximum zone of hydrolysis around the colony on the casein agar medium and on starch agar medium was selected for further study. The isolate was maintained on Horikoshi medium slants (pH 10.0) and stored at 4 °C.

2.3. Morphological characteristics

The morphological characteristics of the selected isolate 2b obtained on Horikoshi's –I (pH 10.0) agar plates were studied. The shape, size and arrangement of the cells were studied in Gram-stained preparations. Endospore staining was carried out according to the method of Schaeffer and Fulton.[8] Motility of 12 and 24 h old cells was observed by phase contrast microscopy of hanging-drop preparations.

2.4. Standard bacteriological characterization

Growth experiments at pH 7–11 were performed on Horikoshi I broth adjusted to various pH values: pH 7–9 (adjusted by adding $NaHCO_3$) and pH 10–11 (adjusted by adding). Growth at various NaCl concentrations (2–10%) and at various temperatures (4–55 °C) was investigated in Horikoshi I broth (pH 10.0). Acid production from carbohydrates was determined by the method of using thymol blue instead of bromothymol blue at pH 10.0 [9,10]. Physiological and biochemical tests such as indole production from tryptophan, methyl-red and Voges–Proskauer tests, Simmons' citrate utilization test, catalase and oxidase activity, urea hydrolysis, production of H_2S from cysteine, nitrate reduction to nitrite, hydrolysis of casein, gelatin and starch were examined according to Smibert and Krieg.[11]

2.5. Identification

The taxonomic status of the selected bacterium 2b was identified following the criteria laid down by Bergey's Manual of Systematic Bacteriology.[12] The identification was further confirmed by Microbial Type Culture Collection Center and Gene Bank (MTCC), Institute of Microbial Technology, (IMTECH), Chandigarh, India.

2.6. Genotypic characterization

2.6.1. Sequencing of PCR product

The 16S RNA gene sequencing of the isolate was performed by National Center for Cell Sciences (NCCS), Pune, India. The purified PCR product of 16sr RNA was sequenced using ABI Prism. The sequence obtained was BLAST searched and compared with sequences of other closely related members of genus *Bacillus* retrieved from GenBank database. Phylogenetic tree was constructed from 16S rRNA gene sequences of members of genus *Bacillus* using neighbour-joining method.[13] The analysis involved 39 nucleotide sequences of genus *Bacillus*.

2.6.2. 16S rRNA-based phylogeny

The sequence so obtained was taken up for running NCBI BLAST against nonredundant nucleotide database using megablast algorithm for getting homologous sequences[14,15]. Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program[16] and multiple sequence alignment was performed.

Phylogenetic trees were constructed by different treeing algorithms: neighbour-joining,[13] maximum parsimony tree[17] and maximum-likelihood[18] and UPGMA method[19] using MEGA5.[20] All the phylogenetic analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 667 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.[20]

2.7. Phylogenetic analysis and elucidation of rRNA secondary structure

The 16S rRNA gene sequence was further used to predict the secondary structure of rRNA. The secondary structure was elucidated using GeneBee package[21,22] and UNAFOLD.[23] The