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Inducible cellulase production from an organic solvent tolerant *Bacillus* sp. SV1 and evolutionary divergence of endoglucanase in different species of the genus *Bacillus*

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ABSTRACT

Bacteria are important sources of cellulases with various industrial and biotechnological applications. In view of this, a non-hemolytic bacterial strain, tolerant to various environmental pollutants (heavy metals and organic solvents), showing high cellulolytic index (7.89) was isolated from cattle shed soil and identified as *Bacillus* sp. SV1 (99.27% pairwise similarity with *Bacillus korlensis*). Extracellular cellulases showed the presence of endoglucanase, total cellulase and β-glucosidase activities. Cellulase production was induced in presence of cellulose (3.3 times CMCase, 2.9 times FPase and 2.1 times β-glucosidase), and enhanced (115.1% CMCase) by low-cost corn steep solids. An *in silico* investigation of endoglucanase (EC 3.2.1.4) protein sequences of three *Bacillus* spp. as query, revealed their similarities with members of nine bacterial phyla and to Eukaryota (represented by Arthropoda and Nematoda), and also highlighted of a convergent and divergent evolution from other enzymes of different substrate [(1,3)-linked beta-D-glucans, xylan and chitosan] specificities. Characteristic conserved signature indels were observed among members of Actinobacteria (7 aa insert) and Firmicutes (9 aa insert) that served as a potential tool in support of their relatedness in phylogenetic trees.

Introduction

Glycoside hydrolases or glycosidases are enzymes that hydrolyze O-, N- and S-linked glycosides, which have diverse industrial applications. Cellulases are glycoside hydrolases that hydrolyze O-linked glycosides, *i.e.* the β-1,4-glycosidic bonds between two glucose residues of cellobiose units in cellulose. They form a synergistically functioning enzyme system, that chiefly consist of endoglucanase (EC 3.2.1.4), exoglucanase/cellobiohydrolase (EC 3.2.1.176/EC 3.2.1.91) and beta-glucosidase (EC 3.2.1.21). Cellulases have important industrial applications in textile processing, as detergent components, as part of macerating enzymes in food processing,

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in production of animal feed, for removing bacterial biofilms, and more recently in biorefinery for production of value-added chemicals and biofuels from renewable biomass feedstocks.[1,2] Majority of the cellulases are commonly obtained from fungi, but bacteria are considered important because of their high growth rate, production of effective complex enzymes and ease of genetic engineering for enhancing enzyme production.[3] Among various sources, an excellent cellulolytic environment is the cattle rumen which contains a very dense and complex mixture of cellulolytic bacteria (in majority), fungi and protozoa.[4] The bacterial species able to utilize crystalline cellulose are known frequently from the phyla Firmicutes, Actinobacteria and Proteobacteria.[5] Within Firmicutes, most species of *Bacillus* are known to be endoglucanase producers, in that, they are able to hydrolyze amorphous forms of cellulose like carboxymethyl cellulose. However, relatively only few strains of *Bacillus* are known to utilize crystalline forms of cellulose and produce multiple cellulase enzymes.[3,6,7]

In addition to the use in industrial fermentations, the prefatory potential of such microorganisms in other areas like bioremediation could also be studied for their multiple applications. Organic solvents are often used in the manufacture of pharmaceutical products, paints, varnishes and adhesives, and they cause significant air and water pollution, and land contamination.[8] Microorganisms that are tolerant to the destructive effects of solvents have been explored for their potential in industrial and environmental biotechnology to carry out bioremediation and biocatalytic processes.[9] Moreover, pollution due to the heavy metals from mining and industrial wastes, vehicle emissions and fertilizers have negative consequences on the hydrosphere, and one of the best procedures considered in removing the toxic metals from the environment is using metal tolerant bacteria.[10,11] Thus, it is required to find microorganisms that apart from being representatives for production of a number of economic products *i.e.* enzymes, are also capable of tolerating environmental pollutants.[12]

Conserved signature indels (CSIs) in protein sequences, characterized by their defined size and being flanked on both sides by conserved regions, are rare and specific genetic changes that provide useful phylogenetic markers for understanding evolutionary relationships among different organisms.[13] Presently, relationship of cellulolytic bacteria among different phyla is not well known. One way of establishing this is by studying protein sequences among various organisms, which can indicate specific patterns occurring in a particular or several group of species. CSIs specific for different lineages can provide definitive means of differentiation of organisms of these groups, while those shared among different taxa enable the determination of phylogenetic relationships based on multiple protein sequences.[14]

Thus, in the present study a cellulase producing bacterial strain of *Bacillus* sp. SV1 was isolated from a cattle shed soil. Its tolerance to different environmental pollutants including toxic heavy metals and organic solvents was checked. Factors effecting cellulase production, different cellulose substrate hydrolyzing capacity of the extracellular cellulase and cellulase gene detection were studied. Cellulase sequences from three species of *Bacillus* were used as query to analyze phylogenetic relationships and occurrence of CSIs among different taxa.

Materials and methods

Chemicals

Chemical reagents of analytical grade were obtained from Sigma–Aldrich (St. Louis, MO, USA), Hi-Media laboratories (Mumbai, India) and Fisher Scientific (Mumbai, India).

Isolation of cellulolytic bacterium

Soil sample was collected in sterile vials from a cattle shed (located in Sonia Vihar, New Delhi – 28.70743° N and 77.25993° E) and stored at 4 °C. One gram soil was suspended in 50 mL of sterilized liquid medium IV (pH 7.0) containing (g/L): $NaNO_3$ – 1; K_2HPO_4 – 1; KCl – 1; $MgSO_4$ – 0.5; yeast extract – 0.5; sodium carboxymethylcellulose (Na-CMC, 1500–3000 cP) – 10.[15] Actidione (100 ppm) was included to prevent fungal growth. Enrichment of cellulolytic bacteria was done at 37 °C and 180 rpm in an incubator shaker (Kuhner LT-X) for 48 h. This suspension after serial dilution in 0.87% (w/v) NaCl solution was spread (100 μL) on nutrient agar (NA) (g/L: peptone, 5; sodium chloride, 5; beef extract, 1.5; yeast extract, 1.5; agar, 15) plates and incubated at 37 °C for 48 h, from which ten discreet colonies were selected and purified on NA. Each of the ten isolates were then point inoculated on solid medium IV (pH 7.0) containing (g/L): $NaNO_3$, 1; K_2HPO_4, 1; KCl, 1; $MgSO_4$, 0.5; yeast extract, 0.5; sodium carboxymethylcellulose (Na-CMC, 1500–3000 cP), 10; agar, 16, and incubated at 37 °C for 48 h, followed by Congo red (1 mg/mL) staining–NaCl (1 M) de-staining and measurement of cellulolytic index (CI) (ratio of the diameter of the clearing zone around the colony to the diameter of colony).[16] Hemolytic behaviors of the isolates were examined by streaking them on sterile sheep blood agar plates and incubating at 37 °C for 24 h.[17] Out of ten, one isolate, designated as SV1, was selected for further study based on CI and non-hemolytic nature.

Characterization and phylogeny of the cellulolytic isolate

Morphological, physiological and biochemical characters for the isolate SV1 were studied. Genomic DNA was extracted using a Fermentas GeneJET genomic DNA purification kit. The universal primer pair, 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) was used to amplify 16S rRNA gene.[18] PCR parameters adopted were: initial denaturation at 94 °C (3 min), followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (2.5 min), with the final extension at 72 °C (6 min). Amplified region was sequenced with an Applied Biosystems (ABI) Prism 310 Genetic Analyzer. The obtained sequence was used to blast in the EzTaxon-e server, followed by multiple sequence alignment of the retrieved closest neighbors reference sequences.[19] Based on lowest calculated Bayesian information criterion scores, Kimura 2-parameter model was used for constructing a maximum likelihood phylogenetic tree (with