



BRAZILIAN JOURNAL OF MICROBIOLOGY

<http://www.bjmicrobiol.com.br/>



Fungal and Bacterial Physiology

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

Lebin Thomas, Hari Ram, Ved Pal Singh*

University of Delhi, Department of Botany, Delhi, India

ARTICLE INFO

Article history:

Received 16 October 2016

Accepted 6 May 2017

Available online 6 November 2017

Associate Editor: Lucy Seldin

Keywords:

Bacillus

Cellulases

Conserved signature indels

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 korensis*). 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 a convergent and divergent evolution from other enzymes of different substrate [(1,3)-linked β -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.

© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 β -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,

* Corresponding author.

E-mail: vp Singh_biology@rediffmail.com (V.P. Singh).

<https://doi.org/10.1016/j.bjm.2017.05.010>

1517-8382/© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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): NaNO₃ – 1; K₂HPO₄ – 1; KCl – 1; MgSO₄ – 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): NaNO₃, 1; K₂HPO₄, 1; KCl, 1; MgSO₄, 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 (1M) 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'-GGTTACCTGTACGACTT-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

the bootstrap test of 1000 replicates).²⁰ The non-uniformity of evolutionary rates among sites were modeled using a discrete gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). All evolutionary analyses were conducted in MEGA6.²¹

The 16S rRNA gene sequence was submitted to the GenBank database using the BankIt submission tool, and the obtained accession number is KJ024372.

Antibiotic susceptibility pattern

The susceptibility of the isolate SV1 to various antibiotics was tested using the standard disc diffusion method.¹² Bacterial culture (0.1 mL of 1.5 OD₆₀₀) in nutrient broth (NB) (g/L: peptone, 5; sodium chloride, 5; beef extract, 1.5; yeast extract, 1.5) was spread on NA plates, and then single antibiotic discs were placed on the surface, followed by incubation at 37 °C for 24 h. The antibiotics used were (μg/disc or units/disc) were: Streptomycin (10), Ciprofloxacin (5), Erythromycin (10), Ofloxacin (5), Norfloxacin (10), Ampicillin (10), Carbenicillin (100), Penicillin-G (2 units/disc), Tetracycline (10), Cefazolin (30), Cefaclor (30), Cefotaxime/Cephalexime (30), Amikacin (30), Cefuroxime (30), Chloramphenicol (30), Rifampicin (30), Ceftriaxone (30), Gentamicin (10), Kanamycin (5) and Cefazidime (30). The diameters of growth inhibition zones inclusive of the diameter of discs were measured, and categorized as susceptible (≥21 mm), intermediate (16–20 mm) or resistant (≤15 mm).¹²

Tolerance against environmental pollutants

Heavy metal tolerance of the isolate SV1 by measurement of minimum inhibitory concentration (MIC), was determined by growing bacteria on NA plates incorporated with different heavy metals (μg/mL), followed by incubation at 37 °C for 24 h.¹² The heavy metal ions (salts, μg/mL) used were: Pb²⁺ (lead nitrate, 0–500), Cu²⁺ (cupric sulphate, 0–200), Hg²⁺ (mercuric chloride, 0–100), Cr³⁺ (chromium chloride hexahydrate, 0–600), Zn²⁺ (zinc chloride, 0–200), Co²⁺ (cobaltous chloride hexahydrate, 0–200) and Ni²⁺ (nickel (II) chloride, hexahydrate, 0–300).

The tolerance of the isolate SV1 to organic solvents of different log *P*_{ow} was also examined. Bacterial growth was monitored in NB containing different solvents (10%, v/v) at 37 °C, 180 rpm for 48 h.²² The organic solvents used were methanol, ethanol, acetone, *n*-propanol, isopropanol, ethyl acetate, butanol, benzene, chloroform, toluene, *p*-xylene, *n*-hexane, 1-decanol and isooctane. NB without any solvent was used as the control.

Preparation of inoculum and extracellular crude cellulase

Isolate SV1 was sub-cultured in NB at 30 °C, 200 rpm for 48 h, and the obtained broth culture (OD₆₀₀ about 1.5) was used as inoculum at 1% (v/v) in all fermentation runs, carried out in 250-mL Erlenmeyer flasks. After fermentation, culture aliquots (1–2 mL) were centrifuged at 5000 × *g* for 15 min at 4 °C (Thermo Scientific, Heraeus FRESKO 21), and the obtained supernatant was used as crude cellulase.

Different media and physiological parameters effecting growth and cellulase production

The effects of seven different media on cell growth and cellulase production at 37 °C and 180 rpm for 48 h were estimated. The media (g/L) with initial pH 7.0 used were:

- I (NH₄)₂SO₄ – 1; K₂HPO₄ – 1; MgSO₄ – 0.5; NaCl – 0.1; CMC – 10.²³
- II KH₂PO₄ – 4; Na₂HPO₄ – 4; tryptone – 2; MgSO₄ – 0.2; CaCl₂ – 0.001; FeSO₄·7H₂O – 0.001; CMC – 10.²⁴
- III Yeast extract – 2.5; K₂HPO₄, NaCl – 1; MgSO₄ – 0.2; (NH₄)₂SO₄ – 0.6; CMC – 10.²⁵
- IV NaNO₃ – 1; K₂HPO₄ – 1; KCl – 1; MgSO₄ – 0.5; yeast extract – 0.5; CMC – 10.¹⁵
- V MgSO₄ – 0.2; K₂HPO₄ – 1; KH₂PO₄ – 1; NH₄NO₃ – 1; FeCl₃·7H₂O – 0.05; CaCl₂ – 0.02; CMC – 10.²⁶
- VI (NH₄)₂SO₄ – 1; KH₂PO₄ – 3; MgSO₄ – 0.1; K₂HPO₄ – 7; trisodium citrate – 0.5; yeast extract – 0.2; CMC – 10.²⁷
- VII Yeast extract – 7; KH₂PO₄ – 4; Na₂HPO₄ – 4; MgSO₄ – 0.2; CaCl₂ – 0.001; FeSO₄·7H₂O – 0.004; CMC – 10.²⁸

The optimization of physiological conditions, including pH (6.0–9.5), temperature (25–40 °C) and agitation rate (0–250 rpm) on bacterial growth and production of cellulases was then investigated through the one-factor-at-a-time (OFAT) approach in medium VII.

Estimation of inducible nature of cellulase synthesis

Inducible nature of cellulase synthesis was investigated in medium VII (initial pH 7.5, at 30 °C, 200 rpm, 48 h): (a) in the absence/presence of CMC, (b) by replacing CMC with other carbon sources (monosaccharides, disaccharides, polysaccharides and sugar alcohols), and (c) by supplementing CMC with other carbon sources (monosaccharides, disaccharides, polysaccharides and sugar alcohols) as co-substrates.¹⁵

Various nutritional factors effecting bacterial growth and cellulase production

Effects of different organic nitrogen (casein hydrolysate broth, corn steep solid, beef extract, tryptone, peptone, glycine and bacto casamino acids) sources at 0.7% (w/v) in place of yeast extract, and of different concentrations of CaCl₂ (fused, 0–5 g/L) and FeSO₄·7H₂O (0–2.9 g/L), on bacterial growth and cellulase production in medium VII were studied at initial pH 7.5, 30 °C and 200 rpm for 48 h.

Analytical methods and statistical analysis

Cellulases (CELs) were assayed using the following substrates, (a) for CMCase/endoglucanase activity: 1.8 mL of 1% (w/v) Na-CMC prepared in phosphate buffer (0.05 M, pH 7), (b) for FPase/total cellulase activity: Whatman grade 1 filter paper strip (1 × 3 cm) in 1.8 mL phosphate buffer (0.05 M, pH 7), and (c) for β-glucosidase activity: 2 mL of 0.15 g/L pNPG (*p*-nitrophenyl-β-D-glucopyranoside) in phosphate buffer (0.05 M, pH 7). For reaction, 0.2 mL of crude cellulase (replaced by phosphate buffer in control) was mixed

with substrates and incubated at 40 °C for 1 h. The reducing sugars (glucose equivalent) for CMCase and FPase activities were estimated at 575 nm, using 3 mL of freshly prepared dinitrosalicylic acid reagent (g/100 mL: sodium hydroxide – 1; dinitrosalicylic acid – 1; crystalline phenol – 0.2; sodium sulfite – 0.05), followed by keeping in a boiling water bath for 15 min, and then adding 1 mL of 40% (w/v) solution of potassium sodium tartarate (Rochelle salt) prior to cooling.²⁹ For β -glucosidase activity, the reaction was terminated by 1 M Na₂CO₃ solution (2 mL) and *p*-nitrophenol released was estimated at 400 nm in a UV-visible spectrophotometer (Shimadzu 250 1 PC, version 2.33).³⁰ One unit of CELs activity was expressed as 1 μ M of glucose (for CMCase and FPase) and *p*-nitrophenol (for β -glucosidase) liberated per mL crude cellulase per minute. The formula used for calculating the activities of CELs is

$$\frac{(\mu\text{M of glucose}/p\text{-nitrophenol released}) \times (\text{total assay volume}) \times (\text{dilution factor})}{(\text{volume of crude cellulase used}) \times (\text{volume in cuvette}) \times (\text{incubation time})}$$

where μ M of glucose/*p*-nitrophenol released is obtained from the standard curve, total assay volume is 2 mL (CMCase and FPase activities)/2.2 mL (β -glucosidase activity), volume of crude cellulase used is 0.2 mL, volume in cuvette is 1 mL and incubation time is 60 min.

All experiments were performed in triplicate, and values are presented as the mean $\pm$ standard error of the mean (SEM). The statistical analysis of data by one-way ANOVA, followed by Duncan's multiple-comparison test ($p < 0.05$) was done using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Detection of cellulase gene

Two pair of primers were used, pair 1: BsFw (forward primer: 5'-GGAATTCATATGAAACGGTCAATCTC-3') and BsRv (reverse primer: 5'-TGCGGCCGCCTAATTTGGTTCTGTTC-3'),³¹ and pair 2: CelBF (forward primer: 5'-CCATGGATCATGAGGATGTGAAACTC-3') and CelBR (reverse primer: 5'-CTCGAGTGAATTTGGTTGTCTGAGCTG-3').³² The PCR reactions were carried out in a final volume of 25 μ L containing genomic DNA (2 μ L), 10 $\times$ NH₄ buffer (2.5 μ L), 50 mM MgCl₂ (2 μ L), 10 mM dNTPs (1 μ L), 10 mM of each forward/reverse primer (1 μ L each), 10 U taq DNA polymerase (0.3 μ L) and sterile double distilled water (15.2 μ L). PCR amplifications were performed with the following parameters: initial denaturation at 94 °C (3 min), followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 52.7 °C (30 s) and extension at 72 °C (2 min), with the final extension at 72 °C (7 min). The PCR products were loaded and run on 1.0% agarose gels.

In silico investigation of the evolutionary divergence in endoglucanase of *Bacillus* spp.

The protein sequence of endoglucanase component (EC 3.2.1.4) of cellulases from three species of *Bacillus*: *Bacillus agaradhaerens* (UniProt accession – O85465, 400 aa), *Bacillus cellulolyticus* (UniProt accession – E6TWN7, 488 aa) and *Bacillus subtilis* (UniProt accession – P07983, 499 aa) was taken as query. Each sequence was used to blast in the UniProtKB database, with default parameters of E-Threshold value (0.01), matrix (auto), filtering (none), gapped (yes) and hits (500).³³

The obtained 500 protein reference sequences from each blast were retrieved in FASTA format, from which fragmented, hypothetical, redundant and uncharacterized sequences were deleted, while the remaining reference sequences were then aligned by MUSCLE using MEGA6. Within the alignment, presence of conserved signature indels (CSIs) that were surrounded by conserved regions were studied. The neighbor-joining phylogenetic trees with 1000 bootstrap replications were constructed by computing the evolutionary distances using the Poisson correction method,³⁴ corroborated by the Jones–Taylor–Thornton (JTT) matrix-based model, and were in the units of number of amino acid substitutions per site.³⁵ The rate variation among sites was modeled with a gamma distribution (shape parameter=1) for the JTT model. Further, complete deletion of gaps, homogeneous pattern among

lineages and uniform rates among sites were used. The propensity (total frequencies of occurrence) of amino acids was calculated among the references in MEGA6.

Results

Cellulolytic bacterial characterization and phylogenetic analysis

The isolated strain SV1 showed a very high cellulolytic index (CI) of 7.89 on the plate assay (Supplementary Fig. 1). It also showed non-hemolytic pattern on the sheep blood agar plate (Supplementary Fig. 2). The morphological, physiological and biochemical characters were studied for the strain SV1, which have been given in Supplementary Table 1.

The obtained 16S rRNA gene sequence (1375 bp) of strain SV1 in the EzTaxon-e blast gave maximum pairwise similarities with *Bacillus* spp., including *Bacillus korlensis* (99.27%), *Bacillus beringensis* (98.61%), *Bacillus kyonggiensis* (97.58%), *Bacillus circulans* (96.85%), *Bacillus nealsonii* (96.78%), *Bacillus firmus* (96.72%) and *Bacillus eiseniae* (96.63%). In the maximum likelihood phylogenetic tree, strain SV1 formed a monophyletic group with *B. korlensis* and *B. beringensis* (Supplementary Fig. 3). The estimated major fatty acid components of strain SV1 were iso-C_{15:0} (34.02%), C_{16:1} ω 11c (18.11%) and anteiso-C_{15:0} (11.80%), which was close to its nearest neighbor *B. korlensis* (Supplementary Table 2). Hence, based on these results, strain SV1 was designated as *Bacillus* sp. SV1.

From the two primer pair of cellulase gene, pair 1 (BsFw and BsRv) was able to detect the presence of cellulase gene of about 1000 bp in *Bacillus* sp. SV1 (Supplementary Fig. 4).

Antibiotic susceptibility pattern and tolerances to heavy metals and organic solvents

Strain SV1 was susceptible to all the antibiotics tested and showed an intermediate resistance against ceftazidime (Supplementary Table 1). It showed tolerance to multiple heavy

Table 1 – The tolerance of the cellulolytic strain SV1 to organic solvents of different log P_{ow} values.

Organic solvent	Log P_{ow} ^a	Growth (O.D. at 600 nm) ^b
Control	–	0.31 ± 0.11
Methanol	–0.77	0.01 ± 0.00
Ethanol	–0.58	0.01 ± 0.00
Acetone	–0.23	0.01 ± 0.00
n-Propanol	0.25	0.01 ± 0.00
Isopropanol	0.26	0.01 ± 0.00
Ethyl acetate	0.73	0.01 ± 0.00
Butanol	0.88	0.02 ± 0.00
Benzene	2.13	0.39 ± 0.00
Chloroform	2.24	0.02 ± 0.00
Toluene	2.73	0.01 ± 0.00
p-Xylene	3.1	0.18 ± 0.01
n-Hexane	3.5	0.76 ± 0.11
1-Decanol	4.1	0.05 ± 0.01
Isooctane	4.7	0.61 ± 0.03

SEM, Standard error of the mean.

^a Log P_{ow} is the logarithm of octanol–water partition coefficient, P , of the solvent.^b Optical density (OD) at 600 nm was measured after 48 h of growth at 37 °C, 180 rpm in nutrient broth containing 10% (v/v) solvents. The values are represented as mean ± SEM.

metal ions, as inferred from bacterial growth. The minimum inhibitory concentration (MIC) of heavy metals ($\mu\text{g/mL}$) was: Pb^{2+} (100), Cu^{2+} (100), Hg^{2+} (50), Cr^{3+} (400), Zn^{2+} (50), Co^{2+} (150) and Ni^{2+} (200). It also showed high tolerance against the organic solvents which had log P_{ow} >2.0 including isooctane, n-hexane, benzene and p-xylene (Table 1).

Estimation of different media and physiological parameters on growth and cellulase production

The varied effects of different media on bacterial growth and cellulase production are shown in Supplementary Fig. 5. It was observed that medium VII supported maximum growth (of 3.6 times) and cellulase production (of 1.5 times CMCase, 1.1 times FPase and 1.6 times β -glucosidase) than the enrichment medium IV, respectively. However, in other media, both growth and cellulase production were very low. Maximum growth occurred at pH 7.0, 30 °C and 100 rpm, while maximum cellulase production occurred at pH 7.5, 30 °C and 200 rpm in medium VII (Supplementary Fig. 5b–d).

Inducible nature of cellulase synthesis

Cellulase production was higher in presence of CMC than in its absence (3.3 times CMCase, 2.9 times FPase and 2.1 times β -glucosidase) or replacement (Fig. 1). The observed pattern of cellulase production was different in conditions when CMC was replaced (Fig. 1) or used as co-substrate (Fig. 2) with different carbon sources. When CMC was replaced, raffinose, avicel and xylan promoted higher β -glucosidase production than CMCase; galactose completely suppressed CMCase and FPase productions (Fig. 1). Among co-substrates, xylose, xylan and pectin enhanced CMCase (1.7 times, 1.6 times and 1.2 times) and FPase (3.1 times, 1.9 times and 3.8 times) productions respectively, over the control containing only CMC

(Fig. 2). However, nicotinic acid, citric acid, guaiacol, glucose, fructose and cellobiose as co-substrates were inhibitory. The presence of the co-substrates sodium acetate, chitin, starch (potato), glycerol and sorbitol did not significantly affect the CMCase production, while sodium acetate, galactose, lactose and chitin did not significantly affect the β -glucosidase production.

Effects of different nutritional factors on growth and cellulase production

Presence of an appropriate organic nitrogen source and of metals (CaCl_2 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was highly preferred for cellulase production, as was observed among different media. Hence, absence or presence of different organic nitrogen sources was investigated for their effects on cellulase production. Absence of organic nitrogen source (growth – 9.8%, CMCase – 10.8%, FPase – 18.8%, β -glucosidase – 9.8%) and presence of glycine (growth – 6.0%, CMCase – 11.0%, FPase – 0.0%, β -glucosidase – 9.7%) and bacto casamino acids (growth – 9.4%, CMCase – 5.9%, FPase – 38.1%, β -glucosidase – 10.5%) caused drastic decrease in both growth and cellulase production, than in the control containing yeast extract (Fig. 3A). In the presence of corn steep solids, growth (145.6%) and CMCase production (115.1%) was maximum, compared to the control. Presence of CaCl_2 (up to 5 mg/L) caused enhancement in β -glucosidase production (1.5 times) compared to its absence, while higher concentrations (>100 mg/L) were inhibitory (Fig. 3B). The presence of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (up to 36 mg/L) was found to be essential for increasing productions of CMCase (of 1.5 times) and β -glucosidase (of 1.1 times) than its absence, whereas higher concentrations (>108 mg/L) were inhibitory for both growth and cellulase production (Fig. 3C).

In silico investigation of evolutionary divergence in endoglucanase component of cellulases of *Bacillus* spp.

The protein sequence blast of each *Bacillus* spp. cellulase query in the UniProtKB database included references that belonged to various taxa within the domain Bacteria and Eukaryota. Bacterial references belonged to phyla Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Spirochaete, Fibrobacteres, Ignavibacteriae, Planctomycetes and Verrucomicrobia. Eukaryota references included members of Arthropoda (*Psacotha*, *Apriona*, *Anoplophora*) and Nematoda (*Ditylenchus*, *Heterodera*, *Radopholus*, *Aphelenchoides*, *Pratylenchus*). Among the references, different enzymes including endo-1,3-beta-glucanase, endo-1,4-beta-xylanase and chitosanase were also present. The phylogenetic relationship of these references is indicated in the neighbor-joining trees for each query (Fig. 4; Supplementary Figs. 6 and 7). In all the three trees, similar taxa were found to be grouped together, and eukaryota references had a common ancestor with the members of the phylum Bacteroidetes.

During alignment of references obtained from blast of the three *Bacillus* spp. cellulase queries (*B. agaradhaerens*, *B. cellulolyticus* and *B. subtilis*), characteristic taxon-specific conserved signature indels (CSIs) were observed. Among *B. cellulolyticus* references, a 7 aa long insert, specific to Actinobacteria and to one Bacteroidetes member *Niastella koreensis* was found

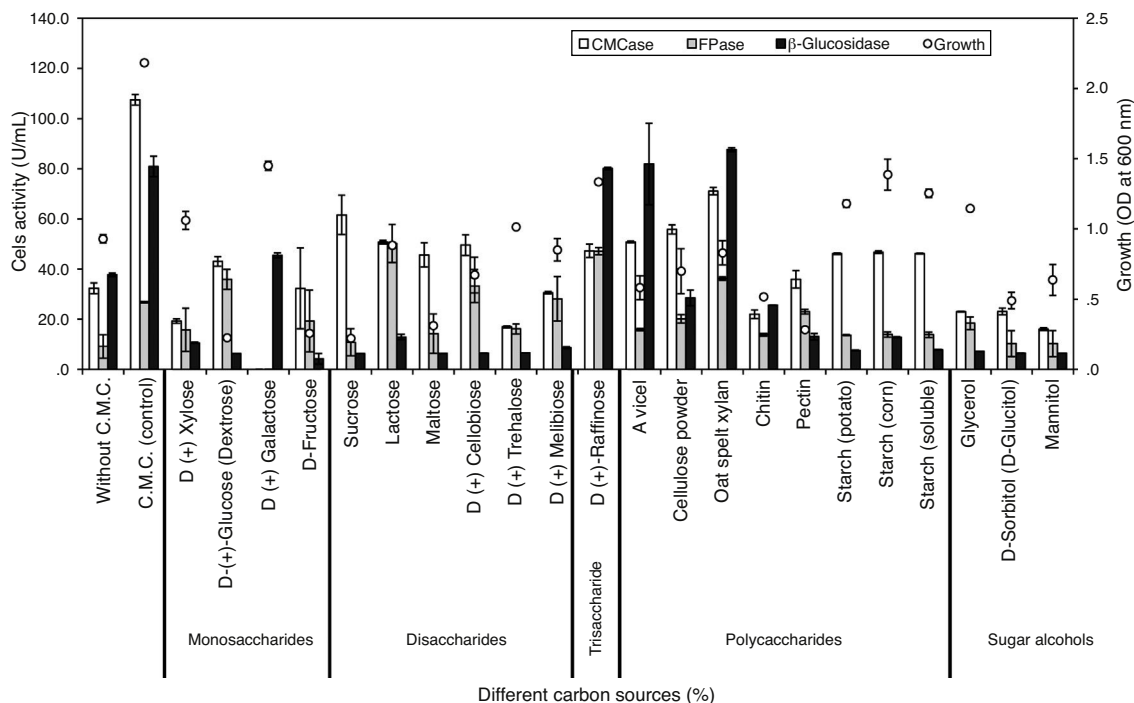


Fig. 1 – Effects of different carbon sources on cellulase production and bacterial growth of the cellulolytic *Bacillus* sp. SV1. All values are represented as mean of triplicate experiments $\pm$ SEM, taken after 48 h of bacterial culturing at 30 °C, 200 rpm in medium VII (initial pH 7.5).

(Fig. 5A). *N. koreensis* also branched within the monophyletic group of Actinobacteria members (Fig. 4). Further, among *B. subtilis* references, an 8 aa long insert specific only to Actinobacteria members (Fig. 5B) and an 8 aa (Fig. 6A) and a 9

aa (Fig. 6B) long inserts specific to certain members of Firmicutes (species of *Caldicellulosiruptor*, *Caldanaerobacter* and *Clostridium*) were observed. A similar 9 aa long insert shared among these three members of Firmicutes was also observed

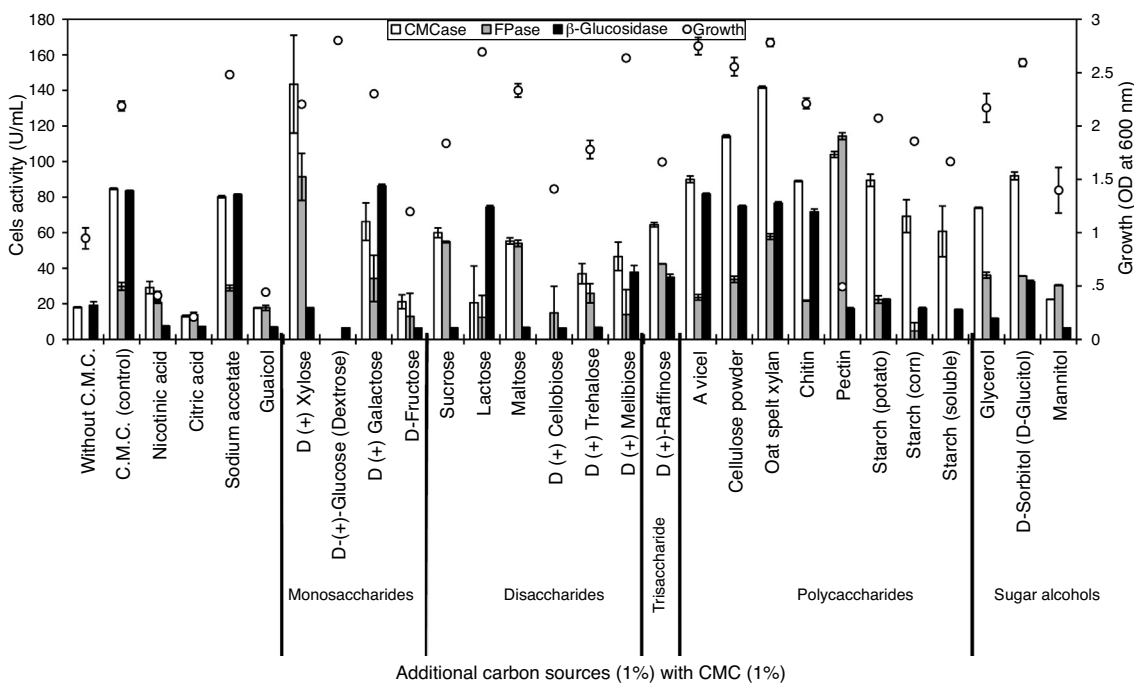


Fig. 2 – Effects of different additional carbon sources as co-substrates to CMC on cellulase production and bacterial growth of the cellulolytic *Bacillus* sp. SV1. All values are represented as mean of triplicate experiments $\pm$ SEM, taken after 48 h of bacterial culturing at 30 °C, 200 rpm in medium VII (initial pH 7.5).

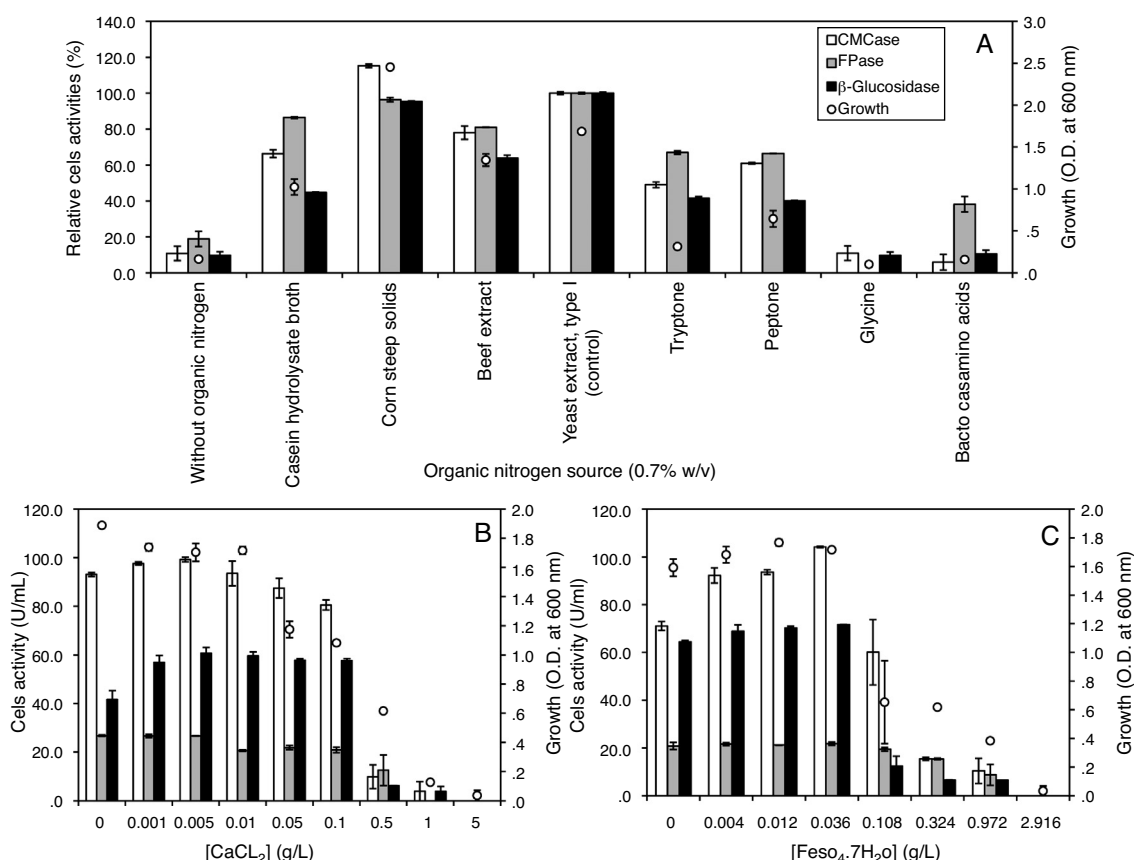


Fig. 3 – Effects of different organic nitrogen source (A), and concentrations of CaCl_2 (B) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (C) on cellulase production and growth of *Bacillus* sp. SV1. All values are represented as mean of triplicate experiments $\pm$ SEM, taken after 48 h of bacterial culturing at 30 °C, 200 rpm in medium VII (initial pH 7.5). In (A), the production of cellulases is expressed as relative CELs activity, defined as the percentage of the activities observed with respect to the control (containing yeast extract).

in *B. agaradhaerens* references (Supplementary Table 3). Even in the phylogenetic trees, species of *Caldicellulosiruptor*, *Caldanaerobacter* and *Clostridium* formed monophyletic grouping (Supplementary Figs. 6 and 7). CSIs both specific to and shared among the members of Actinobacteria and Firmicutes were found within the references of *B. agaradhaerens* and *B. subtilis* cellulase queries (Supplementary Table 3).

Among the different amino acid residues, propensity of alanine, glycine and serine was high, whereas that of cysteine, methionine and histidine was very low, though variations were also observed in distribution of residues in different references (Supplementary Table 4). For instance, the distribution of the catalytic residue aspartate was low in the *Cystobacter* (Proteobacteria), *Hymenobacter* (Bacteroidetes) and the Actinobacteria *Verrucospora* and *Actinoplanes*, but very high in Firmicutes members *Coprococcus*, *Butyrivibrio* and in the Actinobacteria *Bifidobacterium*.

Discussion

In the present study, a cellulase producing bacterium was isolated from soil collected from a cattle shed area. The soil here is usually mixed with cattle dung, due to which different cel-

lulolytic organisms from the cattle rumen might be available. It was considered to isolate cellulase producing bacteria not only based on high cellulolytic index (CI), but also on its non-hemolytic behavior which provides safer working conditions and lesser risk of therapeutic hurdles.^{15,17} Most of the bacteria that are reported for the production of cellulases, do not have information on the non-pathogenic behavior (in terms of non-hemolytic behavior) of the bacteria. Therefore, if this behavior of the bacteria is already studied, that makes the bacteria more safer to use, causing lesser obstacles for therapy and treatment if any infection is caused. Among the isolates, some had low growth with low CI, some had high CI but showed either α - or β -hemolytic behaviors on sheep blood agar plates, and some had low CI with γ (non-hemolytic) behavior on blood agar plates. Only the strain SV1 was considered as it had a very high CI (7.89) and non-hemolytic behavior. The CI known from cellulolytic bacteria (members of Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes) isolated from the gut of the larvae of *Holotrichia parallela* ranged from 1.1 to 9.0.^{36,37} The CI known for some of the cellulolytic bacteria are *Bacillus megaterium* RU4 (6.5),³⁸ *Paracoccus yeii* RA2 (3.0),³⁸ *Bacillus* sp. KC2 (6.86),³⁹ *Bacillus* sp. KC4 (4.37),³⁹ *Bacillus* sp. KC8 (3.95),³⁹ actinomycetes (1.1–2.3).⁴⁰ Strain SV1 was susceptible to all the antibiotics tested, which also suggests of its safe

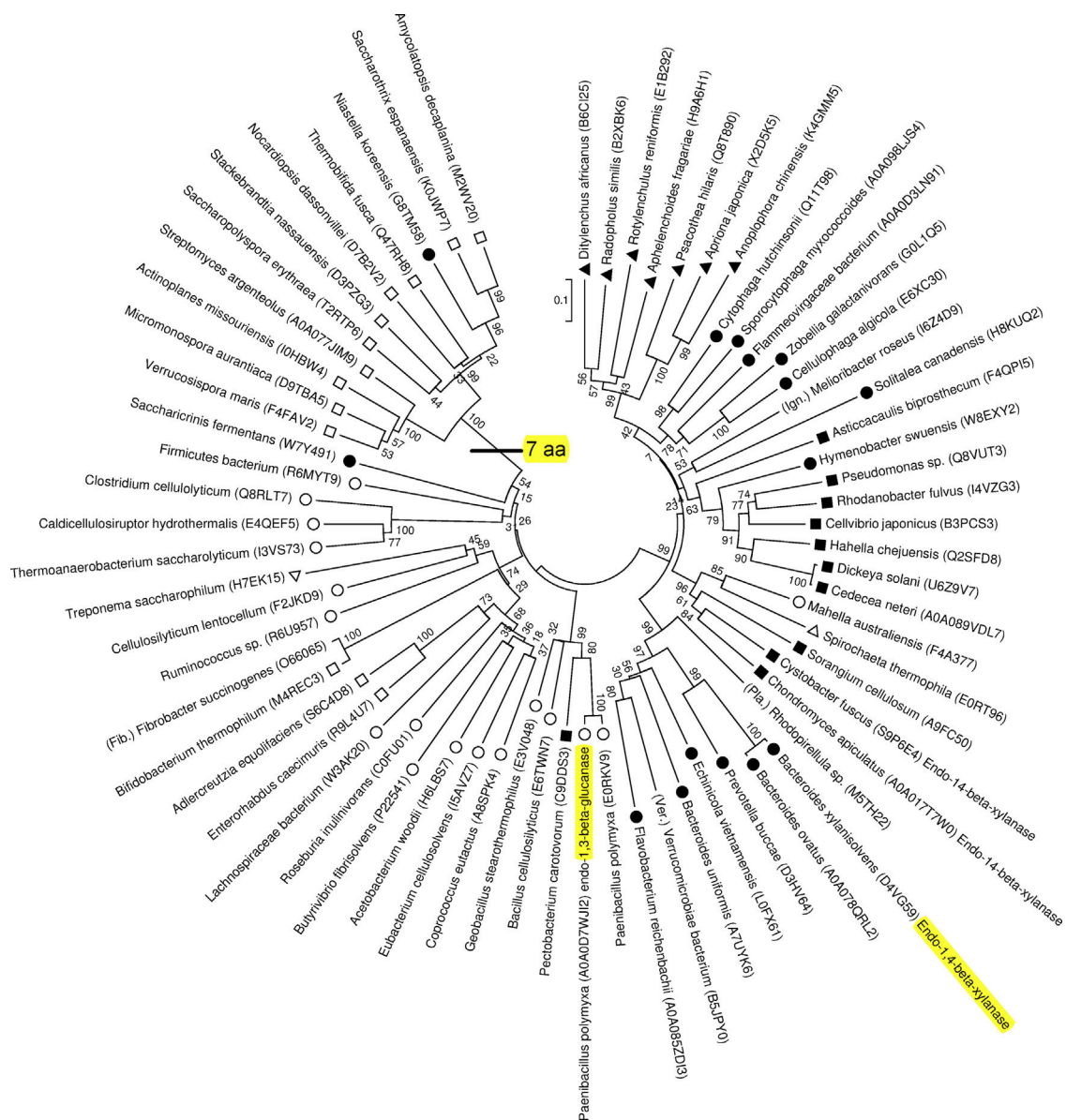


Fig. 4 – Neighbor-joining phylogenetic tree of cellulase (endoglucanase) references of *Bacillus cellulosilyticus* based on the amino acid sequences. Highlighted regions include clade between endoglucanase of *Bacteroides ovatus* and xylanase of *Bacteroides xylanisolvans*, clade between endoglucanase and 1, 3-beta glucanase of *Paenibacillus polymyxa*, and a 7 amino acid (aa) insert shared among members of Actinobacteria and *Niastella koreensis* (of Bacteroidetes). Symbols and abbreviations: Firmicutes (open circle), Proteobacteria (closed square), Spirochaete (open triangle), Ignavibacteriae (Ign.), Planctomycete (Pla.), Bacteroidetes (closed circle), Verrucomicrobiae (Ver.), Eukaryota (closed triangle), Actinobacteria (open square), Fibrobacteres (Fib.). Bar, 0.1 substitutions per nucleotide position.

utilization for application in cellulase production. However, it showed an intermediate resistance against ceftazidime. It is considered that ceftazidime resistance in bacteria arise by either the horizontal acquisition of β -lactamases or from an altered expression of the chromosomal wide-spectrum class C β -lactamase.^{41,42} Due to its tolerance against the toxic heavy metals Cr, Ni, Co and other metals, some physiological mechanism might be involved, which may include their degradation into non-toxic forms. The different mechanisms, often plasmid-associated, known are active efflux, sequestration, complexation, biosorption, ionic interaction, changing toxic

to non-toxic forms, enzymatic metal reduction, immobilization and precipitation.^{43,44} The reduction of Cr to non-toxic forms has been known from various bacteria like *Bacillus*, *Pseudomonas*, *Escherichia coli*, *Exiguobacterium*, *Desulfovibrio* and *Microbacterium*, while a Cr(VI) reducing *Bacillus* sp. FM1 was also found to be tolerant to multiple heavy metals including Cu, Co, Cd, Ni and Zn.⁴⁵ *B. circulans* EB1 has been used for the biosorption of Mn, Zn, Cu, Ni and Co, thus making it suitable for the bioremediation of metal contaminated aqueous systems.⁴⁶ A nickel tolerant *B. megaterium* SR28C, that showed tolerance to other metals (Cu, Zn, Cd, Pb, Cr), was found to have plant

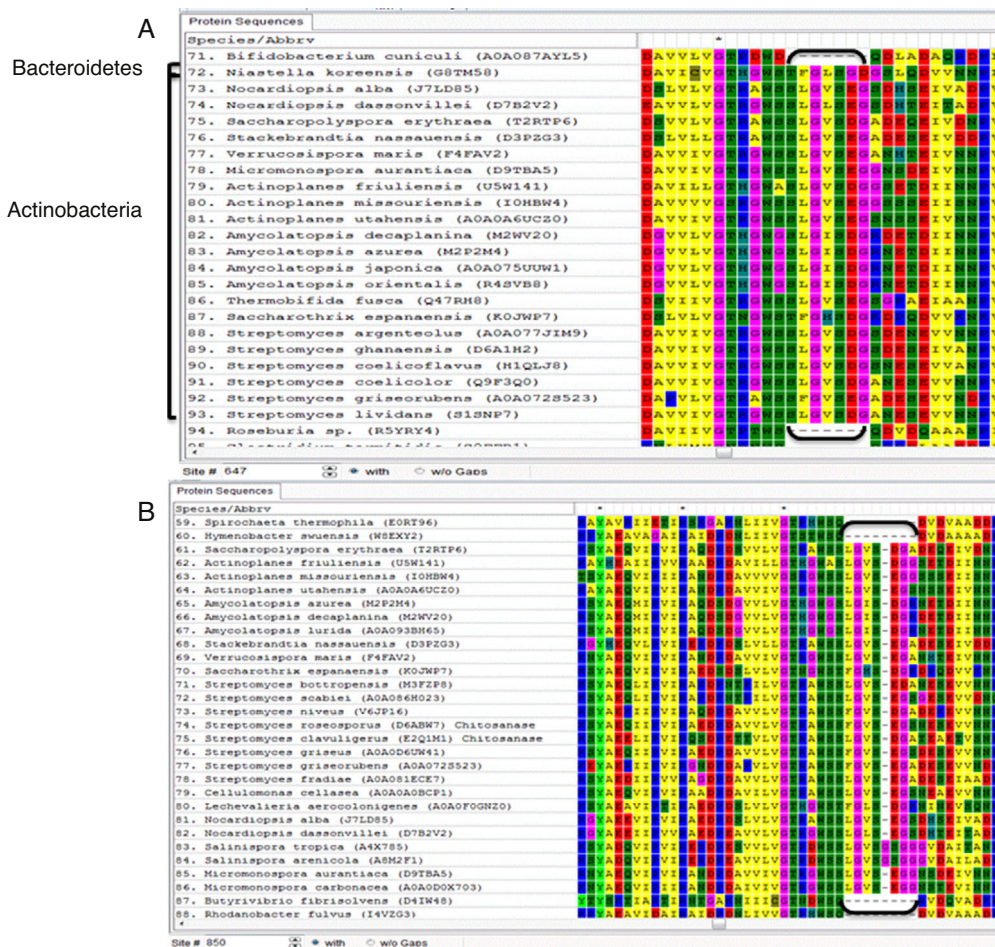


Fig. 5 – Conserved signature indels (CSIs) shared (A) among members of Actinobacteria and one Bacteroidetes member, and (B) specifically among members of Actinobacteria.

growth promoting property by decreasing Ni toxicity in soils, which is useful for phytostabilization.⁴⁷ Furthermore, all the organic solvents that the strain SV1 was tolerant to (isooctane, *n*-hexane, benzene and *p*-xylene) had a $\log P_{ow}$ value greater than 2.0, which indicates of their more hydrophobicity. Besides showing tolerance, the strain SV1 also had enhanced growth in the presence of the solvents *n*-hexane, isooctane and benzene, which suggests of its ability to either utilize or degrade these solvents. Similarly, the tolerance to organic solvents with higher $\log P_{ow}$ values has also been found in several species of *Bacillus* used for enzyme productions, some of which are *Bacillus aquimaris* (benzene, heptane),²² *Bacillus sphaericus* (*n*-benzene, toluene, ethyl benzene and *p*-xylene),⁴⁸ *Bacillus licheniformis* PAL05 (benzene and toluene),⁴⁹ *B. licheniformis* YP1A (tetradecane and decane),⁵⁰ and *Bacillus cereus* BG1 (hexane).⁵¹ Thus, the strain SV1 could be valuable for the bioremediation of multi-metal or organic solvent polluted areas.

The varied effects of different media on bacterial growth and cellulase production clearly reflected on the drastic effects that different components could have. Medium IV used for isolating the bacterium supported less cellulase production than medium VII. Both of these media had several compositional differences, the major being the increased concentration

of yeast extract and presence of CaCl_2 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in medium VII. Medium II also contained CaCl_2 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, but had a different organic nitrogen source tryptone, which supported less cellulase production that was also confirmed in the study of different organic nitrogen sources that effected cellulase production. Medium I and V had no organic nitrogen source, due to which there was very less growth and cellulase production in them. Medium VI had very low concentration of yeast extract than medium III, and thus growth and FPase production observed was very low in medium VI than in medium III. The ratio of C/N, with both their type and relative concentration, can be important in the production of enzymes. Low level of nitrogen can be inadequate, while higher levels can be detrimental for enzyme production, which could also vary for different strains producing various enzymes.⁵² For strain SV1, the media I, II, IV, V and VII have a C/N ratio of 1.0, while media III and VI have the C/N ratio of 0.5. Moreover, when only the C source (Na-CMC) was provided and the organic nitrogen was removed, the production of cellulases were reduced. Similarly, the C/N ratio of 1.0 was found to give maximum production of another enzyme, amylase from *B. licheniformis* SPT 27.⁵² In contrast, for *Trichoderma reesei* ZU-02, the cellulase production was found to increase with increasing C/N ratio of up to 8.0,⁵³ while for *Penicillium occitanis*, cellulase production was very

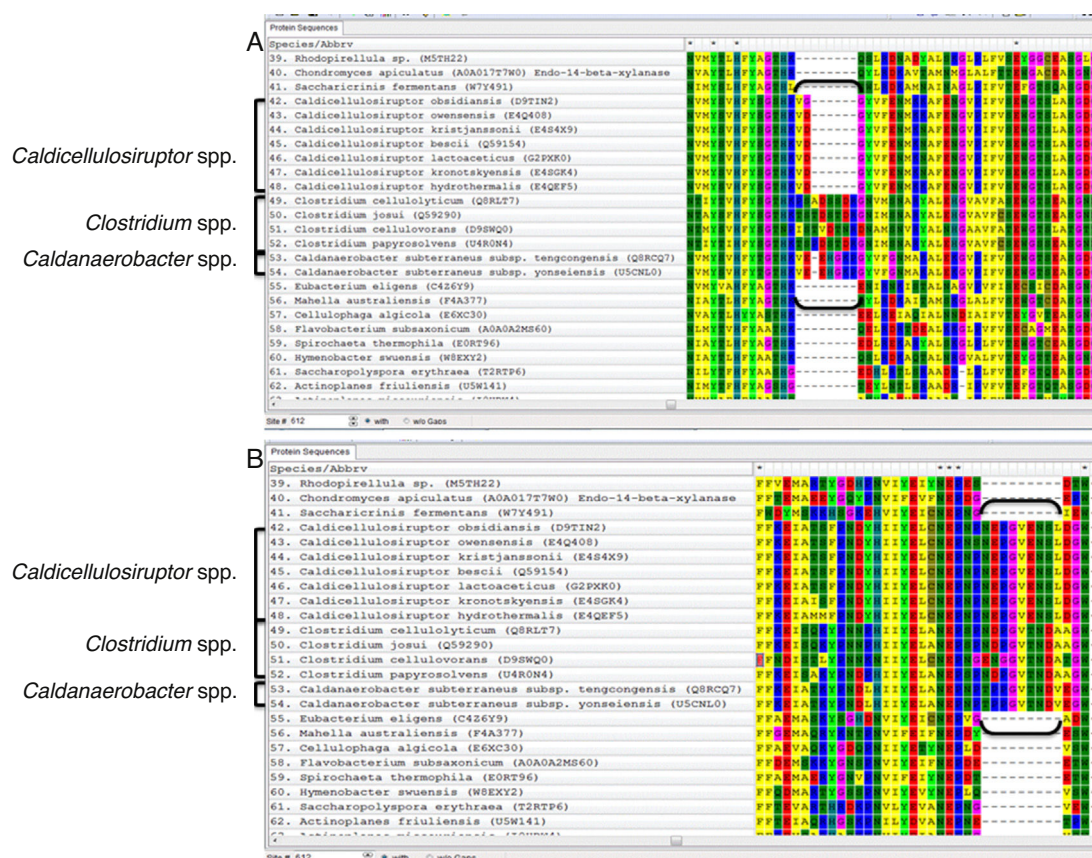


Fig. 6 – Conserved signature indels (CSIs) shared (A and B) among members of Firmicutes including the species of *Caldicellulosiruptor*, *Caldanaerobacter* and *Clostridium*.

Table 2 – The comparison of the production of cellulases of strain SV1 with different cellulolytic strains of *Bacillus*, using various cellulosic substrates.

Cellulolytic strains	Cellulosic substrate used	Production of cellulases (U/mL)	Reference
<i>Bacillus</i> sp. SV1	Na-CMC	CMCase – 107.45, FPase – 26.79 and β -glucosidase – 80.90	This study
<i>Bacillus pumilus</i> EB3	CMC	FPase – 0.05, CMCase – 0.31 and β -glucosidase – 0.18	3
<i>Bacillus</i> sp. SMIA-2	Sugarcane bagasse	CMCase – 0.29	6
<i>Bacillus subtilis</i> AS3	CMC	CMCase – 0.57	65
<i>Bacillus thuringiensis</i> var. <i>israelensis</i>	CMC	CMCase – 0.07, FPase – 0.01	66
<i>Bacillus thuringiensis</i> var. <i>thompsoni</i>	CMC	CMCase – 0.06, FPase – 0.01	66
<i>Bacillus amyloliquefaciens</i> SS35	CMC	CMCase – 0.13–0.53	67
<i>Bacillus vallismortis</i> RG-07	Sugarcane bagasse	CMCase – 4105.00	68
<i>Bacillus pumilus</i> EB3	Oil palm empty fruit bunch	CMCase – 0.06	69

Na-CMC, Sodium-carboxymethyl cellulose; CMC, Carboxymethyl cellulose; CMCase, Carboxymethyl cellulase; FPase, Filter paper cellulase.

high at a C/N ratio below 20.2.⁵⁴ Thus, presence of the organic nitrogen yeast extract and the metals CaCl_2 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were essential for both growth and cellulase production by the strain SV1. Yeast extract is a complex hydrolysate of yeasts, which provides nitrogenous compounds, carbon, sulfur, trace nutrients, vitamin B complex and other important growth factors.^{55,56} The organic nitrogen sources that promoted very low growth and cellulase production are among those that have simpler components containing either single amino acid (glycine) or mixtures of amino acid and peptides (casamino acids, tryptone, peptone and casein hydrolysate broth). However, beef extract (mixture of amino acids, peptides,

organic acids, minerals and some vitamins) caused slightly more growth and cellulase production than glycine, casamino acids, tryptone, peptone and casein hydrolysate broth. Among the organic nitrogen other than yeast extract, only corn steep solids could enhance maximum growth and CMCase production. Corn steep solids are low-cost by-products that contain amino acids, vitamins and minerals, which has also been found to cause high CMCase production from *Streptomyces malaysiensis*,⁵⁷ *Streptomyces drozdowiczii*,⁵⁸ and *T. reesei*.⁵⁹ The enhanced production of cellulases in the presence of CMC than its absence or replacement by other carbon sources, and the suppressed CMCase production in presence of glucose

and cellobiose (which are among the end-products of cellulose hydrolysis) indicated cellulase synthesis to be inducible and under catabolite repression, respectively. Thus, cellulase production was increased only for the requirement of the utilization of cellulose, but when simple sugars like glucose, fructose and cellobiose were present, bacteria utilized these easily metabolizable sugars and did not produce cellulase either due to its non-requirement (in condition when CMC was replaced) or for utilizing less complex sugars (in condition when CMC was a co-substrate with monosaccharides and disaccharides).⁶⁰ Moreover, glucose and cellobiose are among the end-products of cellulose hydrolysis which also cause a catabolite repression of cellulase synthesis. Certain co-substrates did not significantly affect the production of cellulases, which indicated that cellulases might have been co-produced with other enzymes needed to utilize different substrates for supporting bacterial growth. The strain also utilized microcrystalline cellulose (avicel) for the production of different cellulases especially higher β -glucosidase than with CMC. Raffinose as a single carbon source enhanced β -glucosidase production. This trisaccharide comprising of galactose, glucose and fructose, has been used as an inducer for maximum production of another cellulase component CMCase from only *B. subtilis* AU-1, where it was suggested that raffinose was initially hydrolyzed into its component sugars, which then induced higher CMCase production.⁶¹ A combination of cellulolytic bacteria (*Streptomyces*, *Cohnella* and *Cellulosimicrobium*) have been used to reduce the soybean meal non-starch polysaccharide containing raffinose.⁶² However, raffinose has also been found to suppress the production of CMCase from *Penicillium expansum*.⁶³ The enhanced cellulase production caused by the co-substrates xylose, xylan, pectin and cellulose powder might be attributed to the fact that plant cellulose is mostly in association with different polysaccharides of hemicellulose and pectin, due to which in order to get access to cellulose, the production of cellulolytic enzymes might be controlled not only in the presence of cellulose, but also hemicellulose (like xylan) and pectin.⁶⁴ The optimal cellulase produced by strain SV 1 was 107.45 ± 2.12 U/mL (CMCase), 26.79 ± 0.27 U/mL (FPase) and 80.90 ± 4.05 U/mL (β -glucosidase), which is comparable to that observed in different strains of *Bacillus* sp., utilizing different cellulosic substrates (Table 2).

There are few cellulase producing bacterial species (though majority are fungi) that are used in the markets for the production and usage of cellulases. The species of *Bacillus* is one of them. Therefore, an evolutionary study of the cellulase amino acid sequence of different cellulolytic species of *Bacillus* was studied to know its relationship with other cellulolytic organisms. This was done by the use of phylogenetic trees and conserved signature indels. The protein sequence blast of each *Bacillus* spp. cellulase query (endoglucanase, EC 3.2.1.4) included references of almost nine bacterial phyla. Reference cellulases of Eukarya represented by Arthropoda and Nematoda indicated of their identity with the bacterial queries, which was supported by them having common ancestor with the bacterial phylum Bacteroidetes in the phylogenetic trees. Such similarity is suggested to be either due to the involvement of horizontal transfer of genes from symbiotic organisms or from a convergent evolution event which was passed on

from a now extinct ancestor.⁷⁰ The query cellulase, which causes endohydrolysis of (1,4)- β -D-glucosidic linkages in cellulose, showed similarity in blast and formed clade in phylogenetic tree (Fig. 4) with another type of cellulase, endo-1,3- β -glucanase (EC 3.2.1.6), which causes endohydrolysis of (1,3)- or (1,4)-linkages in β -D-glucans. Their similarity might be attributed with a convergent evolution that occurred from the selective pressure of the abundant availability of cellulose. Cellulase query also showed similarity in blast and formed clade in phylogenetic tree (Fig. 4, Supplementary Figs. 6 and 7) with functionally different enzymes, endo-1,4- β -xylanase (EC 3.2.1.8) and chitosanase (EC 3.2.1.132). Both are glycoside hydrolases, which either hydrolyze the β -1,4 linkages between xylan (xylanase) or between D-glucosamine residues in a partly acetylated chitosan (chitosanase). This is highly indicative of a divergent evolution among these enzymes from a basic protein fold so as to incorporate different substrates.⁶⁴

The sharing of the conserved signature insertions and deletions (CSIs) observed between *N. koreensis* (a Bacteroidetes member) and different members of Actinobacteria (Fig. 5A) was also supported by its branching within the monophyletic group of Actinobacteria (Fig. 4). This CSI of 7 aa insert must have been introduced in the cellulase of their common ancestor. The monophyletic grouping of the species of *Caldicellulosiruptor*, *Caldanaerobacter* and *Clostridium* (Supplementary Figs. 6 and 7) was supported by their sharing of an 8 aa (Fig. 6A) and 9 aa (Fig. 6B) (Supplementary Table 3) inserts, which must have been acquired and then later diverged from their ancestral species during evolution. CSIs observed to be specific to different bacteria phyla including Actinobacteria and Firmicutes (Supplementary Table 3) would be important tools for characterization and distinguishing of the members of their common evolutionary decent. Similarly, evolutionary relationships among cellulases, using CSIs are also known for various species of Archaea, Bacteria and Eukarya.⁷¹ From the different amino acid residues in cellulase, frequency of occurrence of alanine, glycine and serine were high, however the reason for very high and low variations observed in several references for certain residues is not known. Such variations were distributed among the members of Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Fibrobacteres, and in some Nematoda and one Arthropoda (*Psacotheta*).

Conclusion

A cellulolytic and non-hemolytic soil bacterium, *Bacillus* sp. SV1 showed susceptibility to various antibiotics, and tolerances to toxic heavy metals ($\text{Cr}^{3+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Pb}^{2+} > \text{Cu}^{2+} > \text{Hg}^{2+} = \text{Zn}^{2+}$) and organic solvents (*n*-hexane > isooctane > benzene > *p*-xylene). Extracellularly synthesized cellulases of the strain SV1 had endoglucanase, filter paper and β -glucosidase activities, which were increased in the presence of cellulose. CMC replaced by raffinose, avicel and xylan promoted higher β -glucosidase production, while xylose, xylan and pectin as co-substrates to CMC enhanced CMCase and FPase productions. The low-cost corn steep solids caused maximum growth and production of CMCase, which also required the

presence of CaCl_2 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Therefore, the strain SV1 has potential in the production of complete cellulases in a cheaper medium, and also for prefatory bioremediation (of multiple heavy metals and organic solvents) applications. An evolutionary relationship of the cellulase of different species of *Bacillus* was studied using their protein amino acid sequences. The protein sequence blast of three *Bacillus* spp. cellulase (EC 3.2.1.4) showed that it had similarities with members of bacterial and eukaryotic phyla, with another cellulase enzyme (endo-1,3-beta-glucanase), and with two other hydrolase (endo-1,4-beta-xylanase and chitosanase) enzymes. Within the alignment of the reference cellulases for the queries, conserved signature indels were observed for the members of Actinobacteria and Firmicutes, which were in support of their evolutionary decent in phylogenetic trees.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Fellowships from C.S.I.R., New Delhi to Lebin Thomas, from U.G.C., New Delhi to Hari Ram, and the DST-purse and R & D grants from the University of Delhi are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjm.2017.05.010](https://doi.org/10.1016/j.bjm.2017.05.010).

REFERENCES

- Juturu V, Wu JC. Microbial cellulases: engineering, production and applications. *Renew Sustain Energy Rev.* 2014;33:188–203.
- Acharya S, Chaudhary A. Bioprospecting thermophiles for cellulase production: a review. *Braz J Microbiol.* 2012;43:844–856.
- Ariffin H, Abdullah N, Kalsom MSU, Shirai Y, Hassan MA. Production and characterization of cellulase by *Bacillus pumilus* EB3. *Int J Eng Technol.* 2006;3:47–53.
- Wilson DB. Microbial diversity of cellulose hydrolysis. *Curr Opin Microbiol.* 2011;14(3):259–263.
- Koeck DE, Pechtl A, Zverlov VV, Schwarz WH. Genomics of cellulolytic bacteria. *Curr Opin Biotechnol.* 2014;29:171–183.
- Ladeira SA, Cruz E, Delatorre AB, Barbosa JB, Martins MLL. Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. *Electron J Biotechnol.* 2015;18:110–115.
- Balasubramanian N, Simões N. *Bacillus pumilus* S124A carboxymethyl cellulase; a thermo stable enzyme with a wide substrate spectrum utility. *Int J Biol Macromol.* 2014;67:132–139.
- Reichardt C. Solvents and solvent effects: an introduction. *Org Process Res Dev.* 2007;11:105–113.
- Sardesai YN, Bhosle S. Industrial potential of organic solvent tolerant bacteria. *Biotechnol Prog.* 2004;20:655–660.
- Aono R, Negishi T, Nakajima H. Cloning of organic solvent tolerance gene *ostA* that determines n-hexane tolerance level in *Escherichia coli*. *Appl Environ Microbiol.* 1994;60:4624–4626.
- Alboghobeish H, Tahmourespour A, Doudi M. The study of Nickel Resistant Bacteria (NiRB) isolated from wastewaters polluted with different industrial sources. *J Environ Health Sci Eng.* 2014;12:44.
- Bisht D, Yadav SK, Gautam P, Darmwal NS. Simultaneous production of alkaline lipase and protease by antibiotic and heavy metal tolerant *Pseudomonas aeruginosa*. *J Basic Microbiol.* 2013;53:715–722.
- Gupta RS. Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among archaeobacteria, eubacteria, and eukaryotes. *Microbiol Mol Biol Rev.* 1998;62:1435–1491.
- Naushad HS, Lee B, Gupta RS. Conserved signature indels and signature proteins as novel tools for understanding microbial phylogeny and systematics: identification of molecular signatures that are specific for the phytopathogenic genera *Dickeya*, *Pectobacterium* and *Brenneria*. *Int J Syst Evol Microbiol.* 2014;64:366–383.
- Thomas L, Ram H, Kumar A, Singh VP. Production, optimization, and characterization of organic solvent tolerant cellulases from a lignocellulosic waste-degrading actinobacterium, *Promicromonospora* sp. VP111. *Appl Biochem Biotechnol.* 2016;179:863–879.
- Teather RM, Wood PJ. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol.* 1982;43:777–780.
- Rahman Z, Singh VP. Cr(VI) reduction by *Enterobacter* sp. DU17 isolated from the tannery waste dump site and characterization of the bacterium and the Cr(VI) reductase. *Int Biodeterior Biodegrad.* 2014;91:97–103.
- Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A.* 1985;82:6955–6959.
- Kim OS, Cho YJ, Lee K, et al. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol.* 2012;62:716–721.
- Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* 1980;16:111–120.
- Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol.* 2013;30:2725–2729.
- Trivedi N, Gupta V, Kumar M, Kumari P, Reddy CRK, Jha B. Solvent tolerant marine bacterium *Bacillus aquimaris* secreting organic solvent stable alkaline cellulase. *Chemosphere.* 2011;83:706–712.
- Sadhu S, Ghosh PK, De TK, Maiti TK. Optimization of cultural condition and synergistic effect of lactose with carboxymethyl cellulose on cellulase production by *Bacillus* sp. isolated from fecal matter of elephant (*Elephas maximus*). *Adv Microbiol.* 2013;3:280–288.
- Saha S, Roy RJ, Sen SK, Ray AK. Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes). *Aquac Res.* 2006;37:380–388.
- Gao W, Lee EJ, Lee SU, Li J, Chung CH, Lee JW. Enhanced carboxymethylcellulase production by a newly isolated marine bacterium *Cellulophaga lytica* LBH-14, using wheat bran. *J Microbiol Biotechnol.* 2012;22:1412–1422.
- Lo YC, Saratale GD, Chen WM, Bai MD, Chang JS. Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for biohydrogen production. *Enzyme Microb Technol.* 2009;44:417–425.

27. Balasubramanian N, Toubarro D, Teixeira M, Simões N. Purification and biochemical characterization of a novel thermo-stable carboxymethyl cellulase from Azorean isolate *Bacillus mycoides* S122C. *Appl Biochem Biotechnol*. 2012;168:2191–2204.
28. Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V. Industrial waste based compost as a source of novel cellulolytic strains and enzymes. *FEMS Microbiol Lett*. 2013;339:93–101.
29. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*. 1959;31:426–428.
30. Wood TM, Bhat KM. Method for measuring cellulase activities. In: Wood WA, Kellogg JA, eds. *Methods in Enzymology: Cellulose and Hemicellulose*. New York: Academic Press; 1998:87–112.
31. Cucurachi M, Busconi M, Marudelli M, Soffritti G, Fogher C. Direct amplification of new cellulase genes from woodland soil purified DNA. *Mol Biol Rep*. 2013;40:4317–4325.
32. Zafar M, Sheikh MA, Rehman S, Jamil A. Cloning of a Cellulase gene from indigenous strain of *Bacillus* species. *Pak J Life Soc Sci*. 2011;9:116–120.
33. UniProt Consortium. UniProt: a hub for protein information. *Nucleic Acids Res*. 2015;43:D204–D212.
34. Zuckerkandl E, Pauling L. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, eds. *Evolving Genes and Proteins*. New York: Academic Press; 1965:97–166.
35. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci*. 1992;8:275–282.
36. Huang S, Sheng P, Zhang H. Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *Int J Mol Sci*. 2012;13:2563–2577.
37. Sheng P, Huang S, Wang Q, Wang A, Zhang H. Isolation, screening, and optimization of the fermentation conditions of highly cellulolytic bacteria from the hindgut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *Appl Biochem Biotechnol*. 2012;167:270–284.
38. Ferbiyanto A, Rusmana I, Raffiudin R. Characterization and identification of cellulolytic bacteria from gut of worker *Macrotermes gilvus*. *HAYATI J Biosci*. 2015;22:197–200.
39. Singh S, Thavamani P, Megharaj M, Naidu R. Multifarious activities of cellulose degrading bacteria from Koala (*Phascolarctos cinereus*) faeces. *J Anim Sci Technol*. 2015;57:23.
40. Goyari S, Devi SS, Kalita MC, Talukdar NC. Population, diversity and characteristics of cellulolytic microorganisms from the Indo-Burma Biodiversity hotspot. *SpringerPlus*. 2014;3:700.
41. Du SJ, Kuo HC, Cheng CH, Fei ACY, Wei HW, Chang SK. Molecular mechanisms of ceftazidime resistance in *Pseudomonas aeruginosa* isolates from canine and human infections. *Vet Med*. 2010;55:172–182.
42. Kos VN, McLaughlin RE, Gardner HA. The elucidation of mechanisms of ceftazidime resistance among clinical isolates of *Pseudomonas aeruginosa* using genomic data. *Antimicrob Agents Chemother*. 2016. AAC-03113.
43. Gadd GM, Griffiths AJ. Microorganisms and heavy metal toxicity. *Microb Ecol*. 1977;4:303–317.
44. Valls M, De Lorenzo V. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS Microbiol Rev*. 2002;26:327–338.
45. Masood F, Malik A. Hexavalent chromium reduction by *Bacillus* sp. strain FM1 isolated from heavy-metal contaminated soil. *Bull Environ Contam Toxicol*. 2011;86:114–119.
46. Yilmaz EI. Metal tolerance and biosorption capacity of *Bacillus circulans* strain EB1. *Res Microbiol*. 2003;154:409–415.
47. Rajkumar M, Ma Y, Freitas H. Improvement of Ni phytostabilization by inoculation of Ni resistant *Bacillus megaterium* SR28C. *J Environ Manag*. 2013;128:973–980.
48. Hun CJ, Rahman RNZA, Salleh AB, Basri M. A newly isolated organic solvent tolerant *Bacillus sphaericus* 205y producing organic solvent-stable lipase. *Biochem Eng J*. 2003;15:147–151.
49. Anbu P, Hur BK. Isolation of an organic solvent-tolerant bacterium *Bacillus licheniformis* PAL05 that is able to secrete solvent-stable lipase. *Biotechnol Appl Biochem*. 2014;61:528–534.
50. Li S, He B, Bai Z, Ouyang P. A novel organic solvent-stable alkaline protease from organic solvent-tolerant *Bacillus licheniformis* YP1A. *J Mol Catal B: Enzym*. 2009;56:85–88.
51. Ghorbel B, Sellami-Kamoun A, Nasri M. Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb Technol*. 2003;32:513–518.
52. Aiyer PD. Effect of C:N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. *Afr J Biotechnol*. 2004;3:519–522.
53. Liming X, Xueliang S. High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. *Bioresour Technol*. 2004;91:259–262.
54. Chaabouni SE, Belguith H, Hassairi I, M'Rad K, Ellouz R. Optimization of cellulase production by *Penicillium occitanis*. *Appl Microbiol Biotechnol*. 1995;43:267–269.
55. Ames JM, Leod GM. Volatile components of a yeast extract composition. *J Food Sci*. 1985;50:125–131.
56. Grant CL, Pramer D. Minor element composition of yeast extract. *J Bacteriol*. 1962;84:869.
57. Nascimento RP, Junior NA, Pereira N Jr, Bon EP, Coelho RR. Brewer's spent grain and corn steep liquor as substrates for cellulolytic enzymes production by *Streptomyces malaysiensis*. *Lett Appl Microbiol*. 2009;48:529–535.
58. de Lima ALG, do Nascimento RP, da Silva Bon EP, Coelho RRR. *Streptomyces drozdowiczii* cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. *Enzyme Microb Technol*. 2005;37:272–277.
59. Farid MA, El-Shahed KY. Cellulase production on high levels of cellulose and corn steep liquor. *Zentralbl Mikrobiol*. 1993;148:277–283.
60. Béguin P, Aubert JP. The biological degradation of cellulose. *FEMS Microbiol Rev*. 1994;13:25–58.
61. Chan KY, Au KS. Studies on cellulase production by a *Bacillus subtilis*. *Antonie Van Leeuwenhoek*. 1987;53:125–136.
62. Opazo R, Ortuzar F, Navarrete P, Espejo R, Romero J. Reduction of soybean meal non-starch polysaccharides and α -galactosides by solid-state fermentation using cellulolytic bacteria obtained from different environments. *PLoS ONE*. 2012;7:e44783.
63. Spalding DH, Wells JM, Allison DW. Catabolite repression of polygalacturonase, pectin lyase, and cellulase synthesis in *Penicillium expansum*. *Phytopathology*. 1973;63:840–844.
64. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev*. 2002;66:506–577.
65. Deka D, Das SP, Sahoo N, et al. Enhanced cellulase production from *Bacillus subtilis* by optimizing physical parameters for bioethanol production. *ISRN Biotechnol*. 2013. <http://dx.doi.org/10.5402/2013/965310>.
66. Lin L, Kan X, Yan H, Wang D. Characterization of extracellular cellulose-degrading enzymes from *Bacillus thuringiensis* strains. *Electron J Biotechnol*. 2012;15:3.
67. Singh S, Moholkar VS, Goyal A. Optimization of carboxymethylcellulase production from *Bacillus amyloliquefaciens* SS35. *3 Biotech*. 2014;4:411–424.

-
68. Gaur R, Tiwari S. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnol.* 2015;15:19.
 69. Ariffin H, Hassan MA, Md Shah UK, Abdullah N, Md Ghazali F, Shirai Y. Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by *Bacillus pumilus* EB3. *J Biosci Bioeng.* 2008;106:231–236.
 70. Tanimura A, Liu W, Yamada K, Kishida T, Toyohara H. Animal cellulases with a focus on aquatic invertebrates. *Fish Sci.* 2013;79:1–13.
 71. Thomas L, Ram H, Singh VP. Evolutionary relationships and taxa-specific conserved signature indels among cellulases of Archaea, Bacteria, and Eukarya. *J Comput Biol.* 2017, <http://dx.doi.org/10.1089/cmb.2016.0161>.