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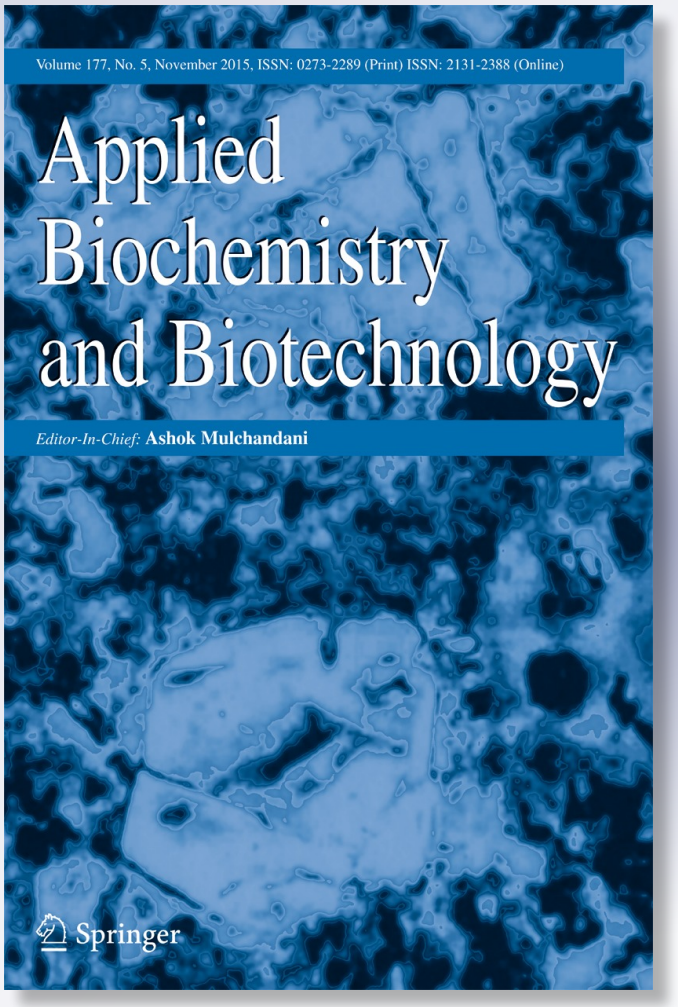
Applied Biochemistry and Biotechnology
Part A: Enzyme Engineering and Biotechnology

ISSN 0273-2289
Volume 177
Number 5

Appl Biochem Biotechnol (2015)
177:1186-1198
DOI 10.1007/s12010-015-1806-9



Springer

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Characterization of Novel Cellulase-producing Bacteria Isolated From Rotting Wood Samples

Yagya Prasad Paudel[1] · **Wensheng Qin**[1]

Received: 16 June 2015 / Accepted: 10 August 2015 /
Published online: 19 August 2015

Abstract Seventeen bacterial isolates were screened for their cellulase activity by carboxymethyl cellulose (CMC) plate assay. The bacterial strain K1 showed the largest depolymerized region in CMC plate assay and was further studied for quantitative cellulase activity. On the basis of 16S rDNA sequence analysis, the strain K1 was found to be *Bacillus* sp. This strain produced the maximum CMCase at pH 6 and 50 °C in the presence of peptone (1 %) as a source of nitrogen. The CMCase activity was stimulated by Ca^{2+} (2 mM) by 20 % over the control. The CMCase activity of this *Bacillus* sp. K1 was highly induced when lactose was used as a source of carbon during fermentation.

Keywords Cellulase · *Bacillus* · Optimization

Introduction

Due to the high rates of consumption of fossil fuel, there is an increases need for finding a new alternative source of renewable energy. Agricultural biomass is the best alternative source of biofuels [1–3]. On average, the biomass of plants contains 30–35 % cellulose, 20–35 % hemicellulose and 5–30 % lignin [4]. Cellulose is composed of glucose units joined together by β-1,4 glycosidic linkages. Hemicellulose is a heterologous polymer of five and six carbon sugars and lignin is a complex aromatic polymer. Cellulose is the major component of plant cell wall and is one of the most fascinating renewable energy sources [5, 6]. However, cellulose is not easily amenable to the fermentation which is essential for lignocellulosic biorefineries. For this, the degradation of cellulose to glucose is an important step. This can be achieved by cellulase which is produced naturally by microorganisms mainly bacteria and fungi [7].

Cellulases are responsible for breaking down the glycosidic linkage in a polysaccharide cellulose [8] and hydrolyse cellulose into glucose units. There are three types

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of cellulases which act synergistically. These enzymes are exoglucanase (cleaving β-1,4 glycosidic bonds from chain ends), endoglucanase (randomly cleaving β-1,4 internal linkages) and β-glucosidase (cleaving final β-1,4 linkage of cellobiose or small polysaccharides) [9]. Most of the cellulases currently used in industrial scale are produced by fungi because of their ability for high enzyme secretion. Bacteria may be considered more ideal candidates for cellulase production as they are fast growing and culturable [10]. Cellulases have several industrial applications including biofuel production, cotton softening, denim finishing, adding to detergents and washing powders [11, 12]. The cellulase-producing bacteria have been isolated from different sources over the past decades. These sources include soil, decaying wood samples, faeces of ruminants and insect guts [13]. The present study concentrates the isolation and characterization of efficient cellulase-producing bacteria from rotting wood samples which are one of the abundantly available lignocellulosic sources with the possible presence of the cellulase-producing bacteria and optimization of the enzyme activity for the possible use in industrial scale.

Materials and Methods

Bacterial Strains Isolation and Identification

The samples were collected from the premises of Lakehead University, Thunder Bay, ON, Canada. One gramme sample of the rotting wood was suspended in 100 ml of distilled water and was homogenized by vortexing. Serial dilutions of 10× were made by adding autoclaved distilled water. One hundred microlitres of each dilution was spread by using standard spread plate method over LB agar plates containing peptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 5 g l^{-1} and agar 15 g l^{-1}. The plates were incubated for 24 h before sampling. From the plates, different colonies of bacteria were selected based on their morphological features like size and colour. The pure cultures were streaked out in carboxymethyl cellulose (CMC) agar plates containing CMC 0.5 g, $NaNO_3$ 0.1 g, K_2HPO_4 0.1 g, KCl 0.1 g, $MgSO_4$ 0.05 g, yeast extract 0.05 g and agar 1.5 g in 100 ml.

Screening for Carboxymethyl Cellulase Activity

The pure bacterial strains were cultured overnight in 7 ml of LB liquid media at 30 °C along with *Cellulomonas xylanilytica* and *Escherichia coli* JM109 which were used as positive and negative controls, respectively. Five microlitres of each isolate was dropped in a petri plate containing CMC agar medium and then incubated at 30 °C for 48 h. Then, the CMC plates of all the isolates including controls were stained using Gram's iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH_2O) for qualitative cellulase assay. The iodine solution stains the agar containing CMC, forming clear zones in the areas without CMC. These clear zones are known as halo regions which indicate the cellulase activity by the bacteria.

DNA Extraction and Amplification of 16S rDNA

The genomic DNA of the cellulase-positive isolates was isolated by using ultraclean microbial DNA extraction kit. The extracted DNA was amplified using primers HAD-1 (5′-

GACTCCTACGGGAGGCAGCAGT-3′) and E1115R (5′-AGGGTTGCGCTCGTTGCGGG-3′). The reaction mixture (25 μl) composed of each primer 1 μl, PCR master mixture 12.5 μl, ddH_2O 8.5 μl and DNA template 2 μl. The PCR was used as follows: primary denaturation 3 min at 95 °C, followed by 35 amplification cycles consisting of denaturing at 95 °C for 1 min, annealing for 1 min at 63 °C, and extension at 72 °C for 1 min, upon completion of 35 amplification cycles; a final extension step was done at 72 °C for 10 min. The PCR products were visualized in 1 % gel electrophoresis. The DNA from gel was purified by using Geneaid PCR/Gel purification kit (FroggaBio, Canada) by following the manufacturer's protocol. Then, the purified samples were sent for sequencing to Euroffins Genomics (USA).

Isolates Identification and Phylogenetic Relationship

The sequencing results were inputted to NCBI database (http://blast.ncbi.nlm.nih.gov/) for possible identification of bacterial genera using Basic Local Alignment Sequencing Tool (BLAST). The phylogenetic relationship was analysed by using sequence alignment programs ClustalX and TreeView.

Bacterial Growth and Carboxymethyl Cellulase Assay

The isolate showing the highest activity in plate assay was further screened for quantitative cellulase assay by growing it LB liquid medium then in minimal salt medium containing 0.1 g l^{-1} $NaNO_3$, K_2HPO_4 0.1 g l^{-1}, KCl 0.1 g l^{-1}, $MgSO_4$ 0.05 g l^{-1} and 1 % CMC as a source of carbon. Its growth was observed at different time intervals. Carboxymethyl cellulase (CMCase) activity was determined by measuring the release of reducing sugars from CMC. A modified microplate-based assay using 3, 5-dinitro salicylic acid (DNS) method was used to measure the reducing sugar [14]. For this, 20 μl of cell-free enzyme supernatant was prepared and mixed with 80 μl solution of 0.5 % CMC and 0.5 M citrate buffer of pH 6 and was incubated for 30 min at 50 °C. The reaction mixture was terminated by adding 200 μl DNS, and the mixture was boiled for 5 min. The absorbance was determined at 540 nm.

Optimization of Cellulase

For the optimization of cellulase activity, in most of the experiments, 20 μl of cell-free supernatant was mixed with 80 μl solution of 0.5 % CMC and 0.5 M citrate buffer and the mixture was incubated for 30 min at 50 °C.

Effect of Incubation Period in Cellulase Production

The culture tubes containing minimal salt medium ($NaNO_3$ 0.1 g l^{-1}, K_2HPO_4 0.1 g l^{-1}, KCl 0.1 g l^{-1} and $MgSO_4$·7H2O 0.05 g l^{-1}) and 1 % CMC were cultured, and 1 ml of sample was harvested on each day starting from the first day of inoculation. The cell-free supernatant was used for enzyme assay. During CMCase assay, the reaction mixture contained 20 μl enzyme supernatant, 80 μl substrate buffer (0.5 M citrate buffer (pH 6) and 0.5 % CMC).

Effect of pH and Temperature on Cellulase Activity

The CMCase activity was measured at different acidic, neutral and basic pH. Similarly, the effect of temperature on cellulase activity was carried out at different temperatures from 30 to 70 °C.

Effect of Metal Ions and Surfactants

The effect of different metal ions, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} in their chloride salts, on the activity of cellulase was determined by performing the CMCase assay in the presence of these metal ions (2 mM) at 50 °C for 30 min. For this assay, the reaction mixture contained 20 μl enzyme supernatant, 10 μl metal ion, 70 μl 0.5 M citrate buffer (pH 6) and 1 % substrate (CMC). Further, different concentrations of the most effective metal ion were used. The effects of detergents sodium dodecyl sulphate (SDS, 10 mM) and Triton X-100 (10 %) were observed on the CMCase activity. For this, the amount of the detergents was same as that of metal ions under the similar conditions.

Effect of Different Nitrogen Sources on Cellulase Production

Nitrogen sources (0.5 %*w/v*) used were yeast extract (YE), peptone, urea and ammonium sulphate [$(NH_4)_2$ SO_4] in the enzyme production medium to determine their effects in enzyme production. For determining the best concentration of the most effective nitrogen source, the activity was tested under the same optimal pH and temperature.

Effect of Carbon Sources on Cellulase Production

Various carbon sources (1 %*w/v*) were used to determine the effect of carbon source on cellulase production medium. The carbon sources used were CMC, glucose, sucrose, sorbitol, lactose, mannose and galactose.

SDS-Polyacrylamide Gel Electrophoresis

For the determination of molecular weight of the cellulase from the isolated bacterial strain K1, the crude enzyme was first incubated at 50 °C for 5 min and was run along with standard protein markers in 10 % SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli [15]. For this, the electrophoresis was carried out with the constant supply of 200 V current. The gel was stained with Coomassie Brilliant Blue R-250 solution for 1 h and destained with decolor buffer for proteins and marker bands. The SDS gel containing 0.25 % CMC was used for the detection of cellulase activity and was washed with Triton X-100 for 15 min then it was incubated at pH 6 buffer at 50 °C for 30 min. Following this, the gel was washed and stained with 0.1 % Congo red for 30 min and destained with 1 M sodium chloride solution for zymogram analysis.

Statistical Analysis

All the experiments were performed in triplicates, and the results are expressed in terms of mean±SD (standard deviation). The statistical analysis of data was performed to test the significant difference by one-way analysis of variance (ANOVA) followed by Tukey's HSD test ($p<0.05$) using system.

Results and Discussion

Isolation and Identification

Sixty bacterial samples were collected from different locations around Thunder bay, ON, Canada. Seventeen strains showed cellulase activity in CMC agar plate assay (Fig. 1). This method of isolation was found easy for preliminary screening of cellulolytic bacteria. The strains were compared with a cellulase-producing positive control (*C. xylanilytica*) and negative control (*E. coli* JM109) with no cellulase activity [16]. The bacterial strain K1 showed the largest diameter of halo region and was selected for further enzyme assay (Fig. 1). The morphological examination showed the colonies of the strain K1 as a rough opaque and grey. The other bacterial colonies also exhibited similar morphological features. As there are wide varieties of cellulase-producing bacteria in the environment, their morphological features make the isolation of bacteria easier from different sources.

DNA Extraction and Amplification of 16S rDNA

The genomic DNA of all the 17 isolates was successfully extracted. The PCR primers successfully amplified 16S rDNA fragments. 1 % agarose gel showed the clear bands of about 800 bp.

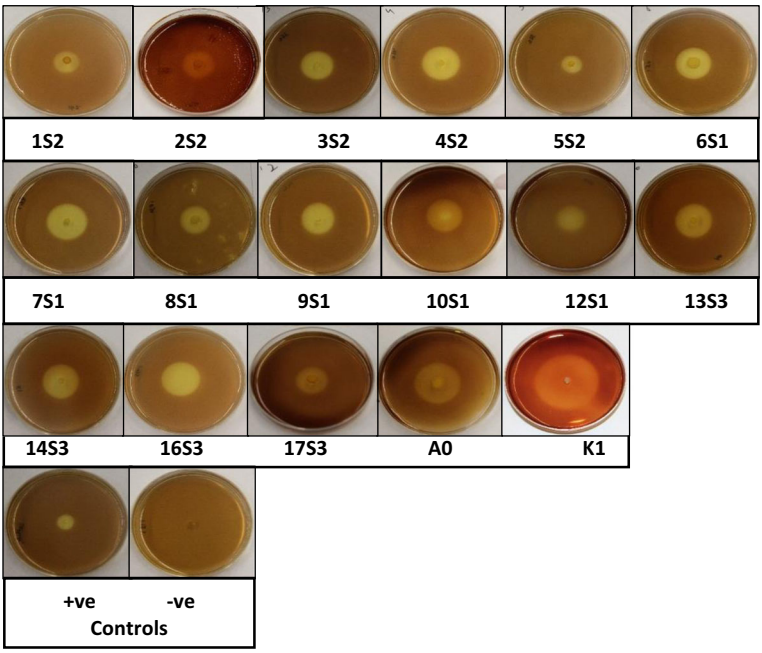

Fig. 1 Seventeen cellulase-producing isolates and positive and negative controls, *C. xylanilytica* and *E. coli* JM109

Isolate Identification and Phylogenetic Analysis of 16S rDNA Sequences

The sequences of all the 17 isolates were analysed by using nucleotide blast of NCBI database. The genera of 17 isolates were identified on the basis of DNA sequences homology. The isolates are related to *Bacillus* (12), *Pseudomonas* (3), *Rahnella* (1) and *Buttiauxella* (1). The sequence for K1 was successfully uploaded to NCBI gene bank database (Accession no. KP987117).

For the phylogenetic analysis, the sequencing results of all the 17 cellulase-producing bacterial isolates were aligned using ClustalX UPGMA algorithm. The sequences were uploaded into TreeView for phylogenetic relationship analysis (Fig. 2). The phylogenetic analysis revealed that the isolates belong to two groups *Firmicutes* and *Proteobacteria*. The *Bacillus* strains are related to Gram-positive *Firmicutes* and the strains *Pseudomonas*, *Rahnella* and *Buttiauxella* are related to Gram-negative *Proteobacteria*. Both the groups of bacteria can degrade the cellulosic materials.

Growth of Strain K1 and CMCase Production

A time course of the bacterial strain and enzyme production was performed over a period of 120 h. The strain K1 showed maximum growth after 3 days of incubation. Also, the cellulase yield reached a maximum at 72 h of incubation (Fig. 3) which was significantly different to the cellulase production at 24, 96 and 120 h. The fermentation period is an important factor for enzyme production by microorganisms [17]. Similar results of maximum production of cellulase at 72 h of incubation were found by other researchers. The *Bacillus pumulis* EWBCM1 and *Bacillus* sp. B21 showed maximum endoglucanase after 72 h incubation [18, 19]. However, this enzyme production time was different from other researchers who reported the maximum endoglucanase after 24 h in *Pseudomonas* sp. HP207 [20] and *Pseudomonas flourescens* NCIB [21], 96 h in *Bacillus circulans* and *Bacillus subtilis* and 142 h incubation for *Clostridium cellulolyticum* [22]. The *Bacillus* strains produce cellulase at

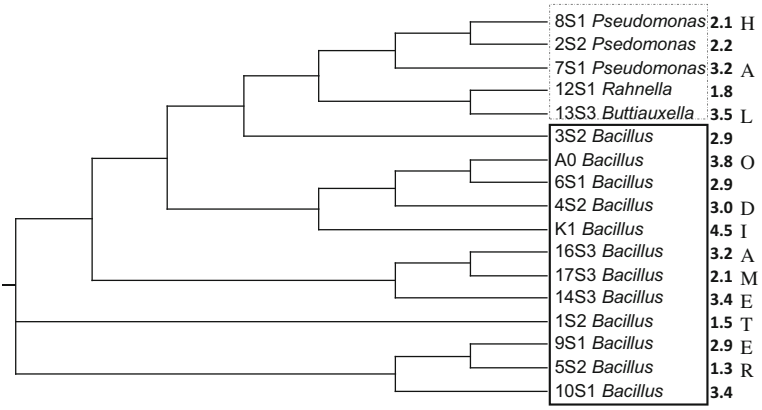

Fig. 2 Phylogenetic tree depicting the evolutionary relationships between the 17 cellulase-positive bacterial isolates (displayed using TreeView) and halo diameter (cm). The isolates outlined in *black* belong to *Firmicutes* and those *dashes* that outlined isolates belong to Proteobacteria. The *numbers* represent the halo diameters produced by the cellulase-producing bacteria in CMC agar plates

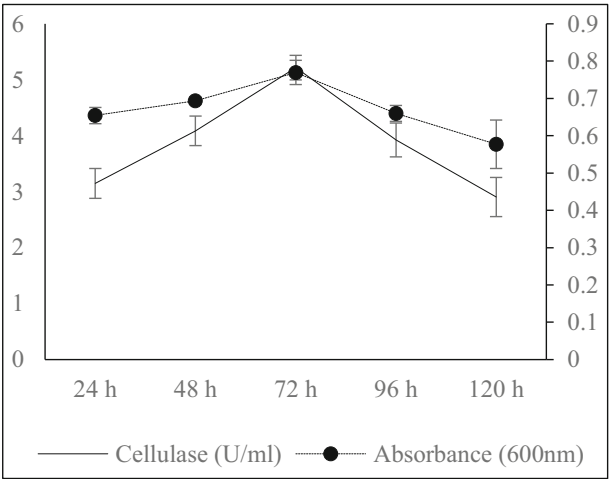

Fig. 3 Growth of *Bacillus* sp. K1 and its CMCase production

different time intervals and are regarded as the important cellulase producers in enzyme industry [23].

The CMCase activity of strain K1 was compared with positive control *C. xylanilytica* and negative control *E. coli* JM 109. The CMCase activity of strain K1 was 5.21±0.21 U/ml (Fig. 3) whereas this activity for *C. xylanilytica* was 2.28±0.51 U/ml and *E. coli* JM 109 exhibited no CMCase activity. One unit (U) of cellulase activity is defined as the amount of enzyme necessary to release 1 μmol reducing sugar per minute per millilitre. This enzyme activity of strain K1 was found higher than those of widely studied bacteria and some fungi, which have received wide attention for commercial production of cellulase [24]. Sheng et al. [20] reported endoglucanase activity by *Pseudomonas* sp. under optimized conditions to be 1.432 U ml^{-1}. Under different nutritional and environmental factors, the endoglucanase activity of *Bacillus pumilus* and *Aspergillus niger*, *and Trichoderma harzianum* Rut-C 8230 did not exceed 1.0 U ml^{-1} [25–28]. Similarly, CMCase activity was only 0.12 U/ml by *Bacillus* sp. [29] and 0.8 U/l by *Geobacillus* sp. [30]. However, this CMCase activity by the isolate K1 was less than that of other *Bacillus* species such as *B. subtilis* subsp. *subtilis* A-53 [31] and *B. subtilis* CY5 and *B. circulans* TP3 [32].

Effect of pH and Temperature on Cellulase Activity

The CMCase activity of strain K1 was found maximum at pH 6 (Fig. 4a) which was significantly different to other pH tested during the experiment ($p<0.05$). The enzyme showed significant decrease after this pH retaining 38 % of its activity at pH 8. A similar result was also reported in *Bacillus* sp. CH43 [33]. A pH of 6.5 was found to be optimal in other *Bacillus* strains [31, 33]. The *Bacillus* strains CH43 and HR68 showed stable cellulase activity in pH 6–8 [34].

Microbial cellulase activity has been influenced by temperature. The optimal temperatures are different in different bacteria. The bacterial strain K1 showed cellulase activity from 30 to 70 °C. The maximum enzyme activity was found at 50 °C, and this activity was significantly different to the CMCase activity at 30, 60 and 70 °C ($p<0.05$). At 70 °C, the enzyme showed

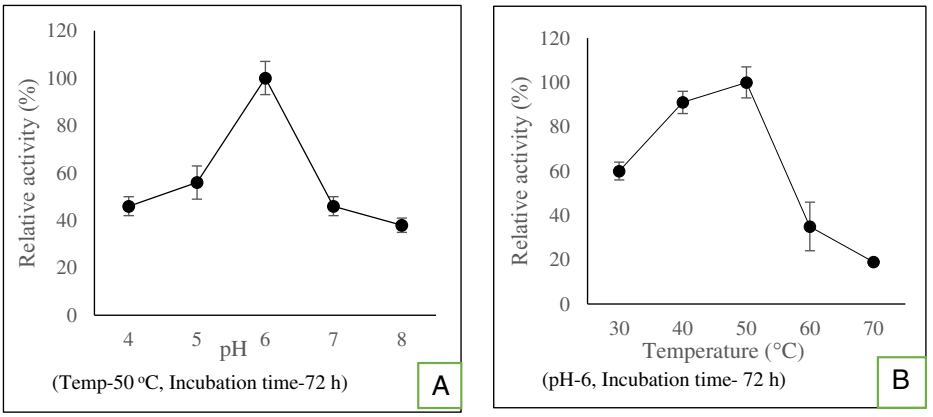

Fig. 4 Effect of pH (**a**) and temperature (**b**) on CMCase activity by *Bacillus* sp. K1

19 % of its relative enzyme activity (Fig. 4b). Similar results have been reported in other *Bacillus* spp. [31, 34–37].

Effects of Metal Ions and Surfactants on Cellulase Activity

The CMCase activity by Ca^{2+} was significantly different ($p<0.05$) to control other metal ions and detergents used in the experiment (Fig. 5a). Fu et al. [38] also reported that Ca^{2+}, Mg^{2+} and Mn^{2+} had a positive effect on endoglucanase activity of *Paenibacillus* sp. BME-14. Ca^{2+} ions have been found essential for enhancing the substrate binding affinity of the enzyme [39]. Maximum enzyme activity was observed at 2 mM Ca^{2+} (Fig. 5b).

The cellulase produced by strain K1 was not tolerant to the common detergents SDS and Triton X-100. The enzyme was reduced to about 60 % while using these surfactants (Fig. 5a) which was significantly lower than the control ($p<0.05$). It might be because of the interaction of detergents with the hydrophobic group of amino acids. The surfactant-like SDS has been found to reduce the endoglucanase activity [40].

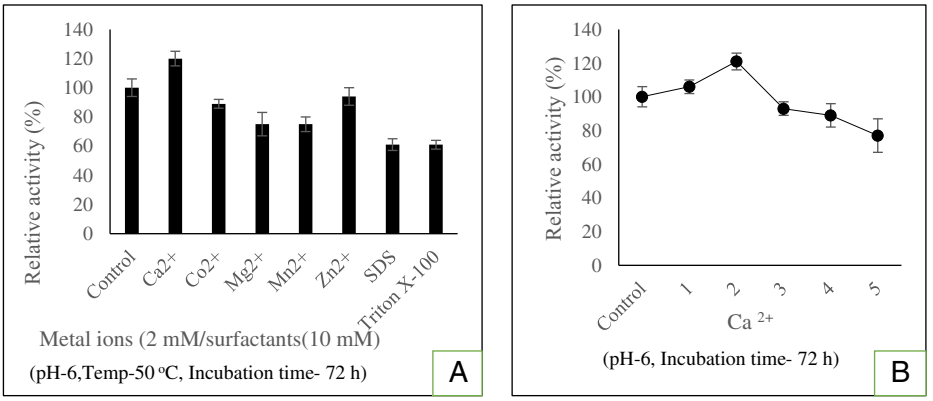

Fig. 5 Effect of metal ions and surfactants (**a**) and Ca $^{2+}$ on CMCase activity (**b**) by *Bacillus* sp. K1

Effect of Different Nitrogen Sources in Culture Medium During Cellulase Production

The production of cellulase is sensitive to the source of nitrogen. The maximum CMCase activity was found by using peptone as a source of nitrogen (Fig. 6a) which was significantly different to ammonium sulphate and urea ($p<0.05$). The *Bacillus* strain could utilize the source of organic nitrogen. The reduction in the production of inorganic nitrogen source might be due to the medium acidification which affected the cellulase production. The *Bacillus* sp. isolated by Yang et al. [41] and Bairagi et al. [42] showed similar results of organic nitrogen source for cellulase production. However, the *B. subtilis* could utilize both the inorganic and organic nitrogen sources for cellulase production [43]. The use of 1 % of peptone enhanced the production of cellulase by 12 %. On increasing the concentration of peptone after 1 %, the enzyme activity was decreased significantly (Fig. 6b).

Effect of Different Carbon Sources in Culture Medium During Cellulase Production

In this experiment, the results showed that the strain K1 could utilize various carbon sources in the production medium and the use of lactose in the culture medium showed a significantly different and higher CMCase activity ($p<0.05$) to that of another source of carbon used in the experiment (9.96±0.23 U/ml) (Fig. 7a). While using different concentrations of lactose, the maximum cellulase was produced when 1 % lactose was used in the medium (Fig. 7b). Since the cellulase is an inducible enzyme, the production of enzyme is enhanced sometimes by some sources of carbon in the medium.

Lactose in the production medium was quickly taken up by the isolated *Bacillus* strain and the CMCase was produced. It might be due to the lactose-induced enzyme activity or increased rate of penetration through the cell membrane [44]. Also, lactose enhances the cellulase yield by stimulating the secretion of various proteins with cellulase. Other researchers also reported the maximum CMCase production by using lactose as a source of carbon by *Microbacterium* sp. [45], *Aspergillus hortai* [46] and *Trichoderma reesei* [47].

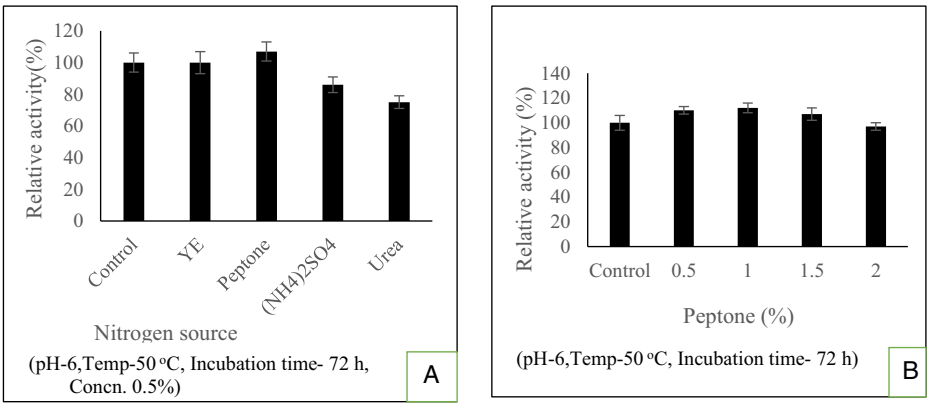

Fig. 6 Effect of nitrogen source (**a**) and peptone concentration (**b**) on CMCase production by *Bacillus* sp. K1

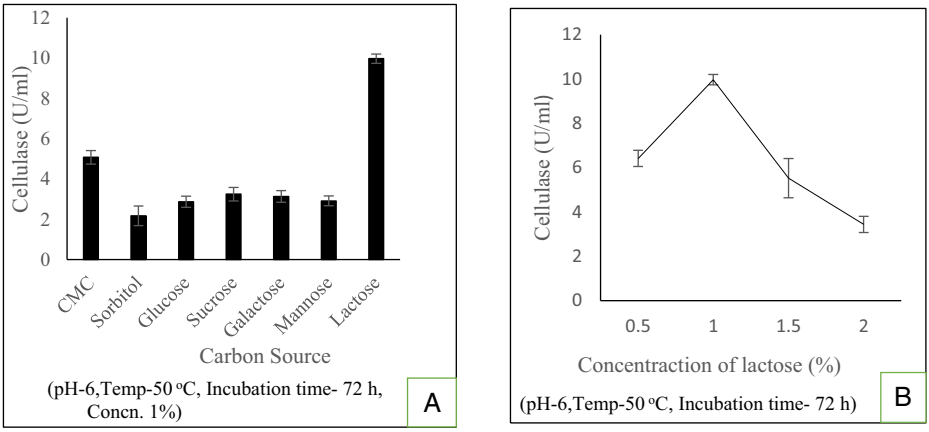

Fig. 7 Effect of carbon source (**a**) and lactose concentration (**b**) on CMCase production by *Bacillus* sp. K1

SDS-PAGE and Zymogram Analysis

Based on the zymogram which was run under the conditions of SDS-PAGE, the molecular weight of the crude cellulase was estimated ~36 kDa (single band of K1, Fig. 8). This is similar to the findings of many researchers who reported the molecular weight of cellulases from 37 to 43 kDa in *Bacillus* species [48–50]. However, this molecular weight of cellulase was lower than the other species of *Bacillus* from which cellulases had molecular weights of 53–78 kDa [51–53].

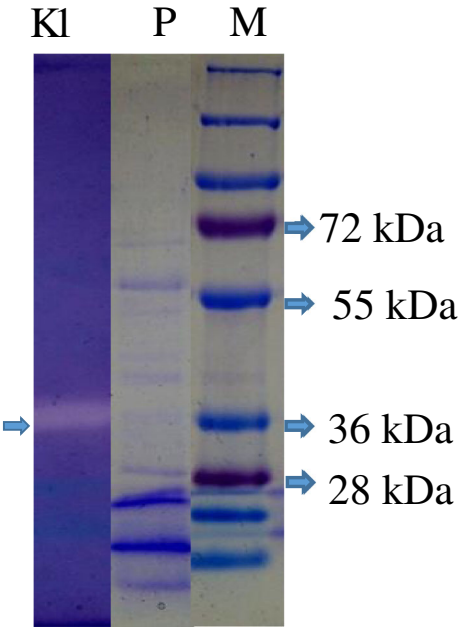

Fig. 8 SDS-PAGE and zymogram of crude cellulase enzyme (*K1* cellulase zymogram, *P* protein in supernatant and *M* marker; based on the gel, the molecular wt. of the enzyme was estimated about 36 kDa)

Conclusion

Seventeen cellulase-producing bacterial isolates were obtained from different rotting wood samples. The isolate K1 produced higher cellulase in plate assay than other isolates. On the basis of 16S rDNA sequence analysis, the strain K1 was found to be *Bacillus* sp. This strain produced maximum CMCase (5.21±0.21 U/ml) at pH 6 and 50 °C after 72 h of incubation. The cellulase produced by this strain was enhanced by Ca^{2+} ions. In the production medium, 1 % peptone enhanced the cellulase production by 12 % over the control. Similarly, lactose induced the CMCase, nearly doubling the enzyme activity (9.96±0.23 U/ml). So, this strain is of particular interest using induction for producing maximum cellulase which might be valuable for biorefining industries. Based on SDS-PAGE analysis, the molecular weight of the cellulase was found ~36 kDa.

Conflict of Interest The authors declare that they have no competing interests.

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