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ORIGINAL ARTICLE

Characterization of Cellulose-Degrading Bacteria Isolated from Soil and the Optimization of Their Culture Conditions for Cellulase Production

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Abstract

The characterization of bacteria with hydrolytic potential significantly contributes to the industries. Six cellulose-degrading bacteria were isolated from mixture soil samples collected at Kingfisher Lake and the University of Manitoba campus by Congo red method using carboxymethyl cellulose agar medium and identified as *Paenarthrobacter* sp. MKAL1, *Hymenobacter* sp. MKAL2, *Mycobacterium* sp. MKAL3, *Stenotrophomonas* sp. MKAL4, *Chryseobacterium* sp. MKAL5, and *Bacillus* sp. MKAL6. Their cellulase production was optimized by controlling different environmental and nutritional factors such as pH, temperature, incubation period, substrate concentration, nitrogen, and carbon sources using the dinitrosalicylic acid and response surface methods. Except for *Paenarthrobacter* sp. MKAL1, all strains are motile. Only *Bacillus* sp. MKAL6 was non-salt-tolerant and showed gelatinase activity. Sucrose enhanced higher cellulase activity of 78.87 ± 4.71 to 190.30 ± 6.42 U/mL in these strains at their optimum pH (5–6) and temperature (35–40 °C). The molecular weights of these cellulases were about 25 kDa. These bacterial strains could be promising biocatalysts for converting cellulose into glucose for industrial purposes.

Keywords Cellulolytic bacteria · Soil · Carboxymethyl cellulose · Cellulase · SDS-PAGE

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Introduction

In the past, humans used cellulosic materials as fertilizers, fodder, and firewood. Nowadays, it has become a cost-effective raw material and its industrial applications have become more complex. These applications have created a vast platform based on cellulose research in multidisciplinary projects. Cellulose hydrolysis is one of the approaches catalyzed by cellulases. Cellulose is a linear polymer made up of D-glucopyranose units linked by β-(1–4) glycosidic linkage and constitutes practically inexhaustible carbon and renewable energy resource [1]. Cellulose offers the best prospects for reducing the production costs of many products due to its abundance and potentially lower price than other substrates, despite the complexity of the transformation processes [2]. It constitutes a significant challenge in research, particularly in the field of bioproducts, biofuels, and chemicals. Cellulose (crystalline and amorphous) forms with hemicellulose and lignin, a water-insoluble compact network structure that limits its degradation [3]. Therefore, pretreatment (physical, chemical, and biological) is required to facilitate fermentable sugar release. Biological pretreatment (enzymes and cellulolytic microorganisms) remains the best approach to address this issue because it is eco-friendly [4].

Cellulase is a whole enzyme system composed of endoglucanase and exoglucanases including cellobiohydrolases and β-glucosidase [5], which breaks down β-1,4-linkages in cellulose polymer to release glucose units. Many investigators have reported that aerobic and anaerobic bacteria [6–9], fungi, and actinomycetes [6, 10, 11] are good cellulase enzyme producers. These microbes secrete free or cell surface-bound cellulases and exhibit an efficient enzyme decomposition. Among different types of microbes, bacteria are the most efficient cellulose degraders because they grow fast and have high cellulase synergistic activity [12]. Cellulases are very successful in the industrial exploitation of the degradation of lignocellulosic biomass. Cellulases have a wide range of applications in several sectors such as chemicals, food and feed, pulp and paper, textiles, beverages, automobiles, electronics, and, most importantly, energy [13, 14].

Recent data shows that the market demand for cellulase is 29.71% in animal feed, 26.37% in food and beverages, and 13.77% in the textile industry [15]. Also, cellulase applications are drastically rising annually. They will reach 2300 million USD by the end of 2025, with a 5.5% of annual growth rate for the 2018–2025 period according to the Global Cellulase (CAS 9012–54-8) market growth 2021–2026 report [16]. However, few cellulases perform well on an industrial scale, and their production cost remains very high. Therefore, it is essential to search for new cellulases with interesting properties from an industrial point of view. In recent years, much work has been devoted to selecting cellulolytic microorganisms, genetic mutations for obtaining hyperproductive strains, and the culture conditions of the microorganisms involved [17]. Their cellulase yields depend on a combination of various factors such as pH, temperature, inoculum size, cellulose type, aeration, incubation time, and inducers [18]. In the present study, we have characterized six cellulose-degrading bacteria isolated from the soil samples collected at Kingfisher Lake (Thunder Bay, Canada) and the University of Manitoba campus (Winnipeg, Canada). The culture conditions for these bacterial strains were optimized to achieve maximum cellulase production.

Materials and Methods

Culture Media

Different culture media were used for bacterial growth and cellulase production. These culture media include (a) Reasoner's 2A (R2A) agar, (b) Luria–Bertani (LB) broth, (c) carboxymethyl cellulose (CMC) agar, and (d) CMC broth. Their compositions were as follows:

a) R2A agar: 0.5 g yeast extract, 0.5 g peptone, 0.5 g starch, 0.5 g $MgSO_4$, 0.5 g casein hydrolysate, 0.5 g glucose, 0.3 g K_2HPO_4, 15 g agar, and distilled water up to 1 L
b) LB broth: 10 g peptone, 5 g yeast extract, 5 g NaCl, and distilled water up to 1 L
c) CMC agar: 5 g CMC, 1 g $NaNO_3$, 1 g K_2HPO_4, 1 g KCl, 0.5 g $MgSO_4$, 0.5 g yeast extract, 15 g agar, and distilled water up to 1 L
d) CMC broth: 5 g CMC, 1 g $NaNO_3$, 1 g K_2HPO_4, 1 g KCl, 0.5 g $MgSO_4$, 0.5 g yeast extract, and distilled water up to 1 L

Screening of Cellulose-Degrading Bacteria

The soil samples were collected from Kingfisher Lake and the University of Manitoba campus. The topsoil was dug by a sterile spatula, kept in a clean zip lock bag, and transported to the laboratory. The samples were mixed for bacterial isolation by dilution method [19]. The samples (0.5 g) were suspended in distilled water (50 mL) by vortexing for 2 min. A 10×dilution series was made and each dilution (5 µL) was plated onto R2A agar. All plates were incubated for 72 h at 28 °C. Based on their morphological features (size, shape, and color), forty-one bacterial colonies were selected. These colonies were streaked out in R2A agar Petri dishes. After incubation at 30 °C for 48 h, these colonies were screened for their ability to produce cellulase using Congo red method [20]. For this purpose, the isolates (bacterial colonies, negative and positive controls) were grown in LB broth (10 mL) for 24-h shaking at 30 °C. *Bacillus* sp. IM7 and *Escherichia coli* JM109 from Dr. Qin's lab were used as positive and negative controls respectively. All broth cultures (5 µL) were singly dropped onto CMC agar plates and then incubated at 30 °C for 48 h. After incubation, plates were stained with aqueous Congo red solution (0.1% w/v) as an indicator to visualize the cellulase activity. The appearance of a clear halo around the isolate confirms cellulase activity by the isolate. Halo diameters were measured using a ruler for a semi-qualitative comparison of cellulase activity among isolates. Plates were photographed, and six cellulose-degrading bacterial isolates (CDBs) were selected and stored for subsequent uses (Fig. 1). These bacteria were identified as *Paenarthrobacter* sp. MKAL1, *Hymenobacter* sp. MKAL2, *Mycobacterium* sp. MKAL3, *Stenotrophomonas* sp. MKAL4, *Chryseobacterium* sp. MKAL5, and *Bacillus* sp. MKAL6 with the NCBI accession numbers ON442553, ON442554, ON442555, ON442556, ON442557, and ON442558 respectively [21].

Characterization of Cellulose-Degrading Bacteria

Morphological and Biochemical Characterization

CDBs were differentiated based on mobility, cell wall composition (Gram stain), vegetative cells and endospores (endospore stain), carbon source utilization, and enzymatic activities

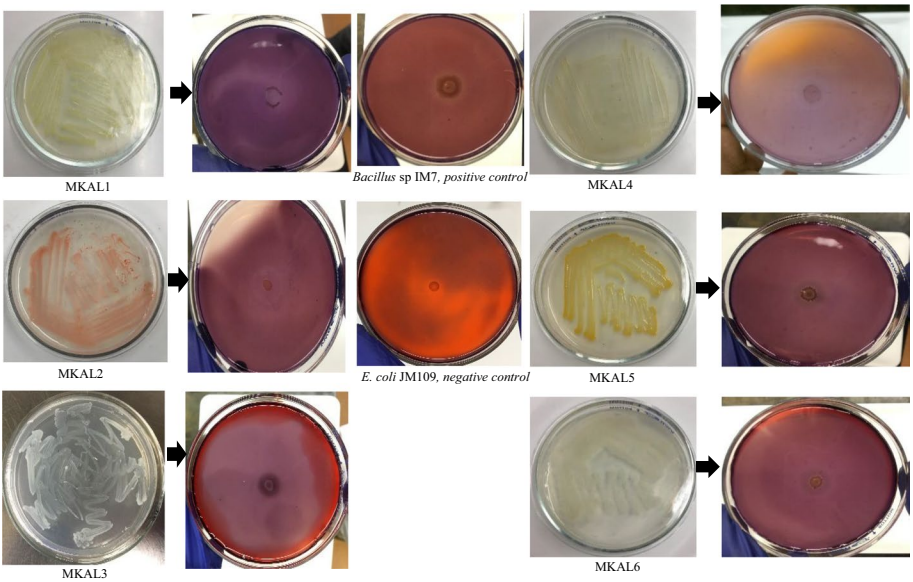

Fig. 1 Cellulase activity characterized by the appearance of clear halos around bacterial strains

by standard methods such as catalase production, gas production, starch hydrolysis, gelatin hydrolysis, DNA hydrolysis, urease test, bile esculin test, oxidase test, nitrate reduction, salt tolerance, and sugar fermentation [22].

Quantification of Cellulase Activity

Quantitative cellulase activities of CDBs were determined by measuring the release of reducing sugars from CMC using the 3,5-dinitrosalicylic acid (DNS) method [5]. CDBs were grown in 5 mL of LB broth (24 h, 30 °C, and 200 rpm). Five hundred microliters of each cultured isolate was centrifuged at 12,000 × g for 5 min and the cells were suspended in 0.05 M citrate buffer (pH 6). These bacterial samples were inoculated separately into a 250-mL Erlenmeyer flask containing CMC broth (50 mL, 1% CMC) prepared with citrate buffer (0.05 M, pH 6). Then, the flasks were incubated at 35 °C and 200 rpm for 5 days. Cellulase assay was performed using the cell-free culture supernatant as an extracellular crude enzyme. Each crude enzyme was obtained by centrifugation of 500 µL of culture at 12,000 × g for 5 min. The reaction mixture containing crude enzyme (10 µL), 0.05 M citrate buffer pH 6 (20 µL), and 1% CMC (20 µL) was transferred into a 1-mL microcentrifuge tube and incubated in the water bath at 50 °C for 15 min. The DNS solution (60 µL) was added to the reaction mixture and the tube was heated for 5 min to stop the reaction. The release of reducing sugars in reaction mixture was estimated using glucose (1.1–2 mg/mL) as a standard for the calibration curve ($y = 0.6419x - 0.1021$; $r^2 = 0.9975$). Every 24 h for 120 h, reaction mixture and bacterial growth were respectively measured at 540 and 600 nm by using a microplate reader spectrophotometer (BioTek, USA). The bacterial growth was expressed in terms of biomass, whereas the cellulase activity was measured in

U/mL (one unit of cellulase enzyme corresponds to the release of 1 μM of reducing sugar equivalent per minute from CMC) [23].

Optimization of Cellulase Production

Cellulase production was optimized by varying some parameters such as incubation time, pH, temperature, CMC concentration, salts, surfactants, carbon, and nitrogen sources. CDBs were grown in LB broth (24 h, 30 °C, and 200 rpm). The culture medium was inoculated and incubated for 5 days and the cellulase amount produced was determined from supernatant using DNS method [5].

Effect of Temperature and Incubation Period on Cellulase Production

The CMC broth (50 mL) containing overnight cultured bacterial strain (500 μL) was incubated in a shaking incubator (200 rpm) at 30, 35, 40, 45, and 50 °C for 5 days. The effect of temperature and incubation time on enzyme production was quantified by collecting culture solution (500 μL) every day.

Effect of pH on Cellulase Production

The CMC broth (50 mL) containing overnight cultured bacterial strain (500 μL) was incubated in a shaking incubator (200 rpm) in the pH ranges from 4 to 9. The effect of pH on enzyme production was investigated at the optimum temperature of each bacterial isolate.

Effect of CMC Concentration on Cellulase Production

Bacterial strain was inoculated in the culture medium with CMC (0.5–2.5% w/v) at optimum pH and temperature and shaking at 200 rpm for 120 h.

Effect of Carbon Sources on Cellulase Production

The effect of carbon sources on enzyme production was performed by replacing CMC with other carbon sources such as pure cellulose, cellulose acetate, poly(ethylene terephthalate) (PET), D-sucrose, D-glucose, D-fructose, D-sorbitol, D-mannitol, and D-xylose. Bacterial strain was inoculated with a carbon source (0.5–2.5% w/v) in the production medium at the optimum temperature and pH and shaking at 200 rpm for 120 h.

Effect of Nitrogen Sources on Cellulase Production

Effect of various nitrogen sources such as yeast extract, malt extract, tryptone, casein hydrolysate, peptone, urea, ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4)_2SO_4$), and ammonium nitrate (NH_4NO_3) was examined. Bacterial strain was inoculated with a nitrogen source (0.05–2% w/v) in the culture medium at the optimum temperature and pH and shaking at 200 rpm for 120 h.

Effect of Salts on Cellulase Production

The influence of salt supplementation was investigated by adding various salts such as potassium chloride, sodium chloride, calcium chloride, aluminum chloride, magnesium chloride, manganese chloride, cobalt chloride, nickel chloride, zinc chloride, chromium (III) chloride, lead chloride, and barium chloride. Bacterial strain was inoculated with salt (0.5–5 mM) in the production medium at the optimum temperature and pH and shaking at 200 rpm for 120 h.

Effect of Surfactants and EDTA on Cellulase Production

Effect of surfactant supplementation was performed by adding different surfactants such as tween 20, sodium dodecyl sulfate (SDS), triton X-100 (0.1–2.5% w/v), and a chelating agent, ethylenediaminetetraacetic acid (EDTA, 0.5–2.5 mM). The bacterial strain was inoculated with surfactant in the culture medium at the optimum temperature and pH and shaking at 200 rpm for 120 h.

Optimization of Cellulase Production Using Response Surface Methodology (RSM)

Response surface methodology (RSM) was used to optimize the fermentation conditions to produce cellulase. The experiment was performed by Box–Behnken design (BBD) using the SYSTAT 12 software (SYSTAT Software Inc., San Jose, USA). The temperature (X_1), initial pH (X_2), and fermentation period (X_3) were determined as independent variables based on the results of the preliminary single-factor experiments. Cellulase activity was used as a response value. The ranges and levels of these independent variables are presented in Table 2. BBD was used to generate the second-order response surface. The F test at the 0.05 significance level, coefficient of determination (R^2), and the lack of fit were used to measure the goodness of fit of the second-order polynomial model. The fitted contour plots were obtained with the response surface methods-contour/surface program in SYSTAT 12 software.

SDS–Polyacrylamide Gel Electrophoresis and Zymogram

The cellulase molecular weights were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The crude enzymes from CDBs (20 µL) were mixed with loading buffer (5 µL) and boiled (100 °C) before electrophoresis. Enzyme samples and protein ladder were run in 15% acrylamide gel. A constant supply of 120 V was maintained throughout the experiment. After gel running, the gel was divided into two parts. One part was stained overnight in Coomassie Brilliant Blue R-250, and then de-stained with a de-stain solution. Protein bands present in the gel were compared with the protein ladder (BioRad, Canada) to estimate their molecular weights. Another part of the gel was soaked in Triton X-100 (1% v/v) for 30 min to remove SDS and allow activity. Then, the gel was submerged in Congo red solution (0.1% w/v) for 30 min and de-stained with NaCl solution (1 M) until the halos appeared. The reaction was stopped by dipping the gel in acetic acid solution (4% v/v).

Results

Morphological and Biochemical Characterization

Among forty-one bacterial isolates, only six were selected based on the appearance of a clear halo around confirming cellulase production by these bacteria. Morphological and biochemical characteristics of CDBs are presented in Table 1. Different shapes

Table 1 Biochemical and enzymatic characteristics of cellulose-degrading bacteria

Tests	Characteristics					
	MKAL1	MKAL2	MKAL3	MKAL4	MKAL5	MKAL6
Motility	Non-motile	Motile	Motile	Motile	Motile	Motile
Gram stain	$Gram^+$	$Gram^-$	$Gram^+$	$Gram^-$	$Gram^-$	$Gram^+$
Shape	Circular	Rod	Circular	Rod	Rod	Rod
Pigmentation	Pale	Red	White	Pale	Yellow	Creamy
Endospore stain	-	-	-	-	-	-
D-Xylose	+	+	+	+	+	+
D-Arabinose	-	-	-	-	-	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Galactose	-	-	-	-	-	+
D-Mannitol	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	+
Inositol	-	-	-	-	-	+
D-Rhamnose	+	-	-	-	-	+
Dulcitol	-	-	-	-	-	+
D-Sucrose	+	+	+	+	+	+
D-Lactose	-	-	+	+	+	+
Cellobiose	+	+	+	+	+	+
D-raffinose	-	+	-	-	-	+
Pectinase	-	+	+	-	-	-
Xylanase	+	-	-	-	+	-
Acetate	+	-	-	-	+	-
Malonate	+	+	-	+	+	+
Bile esculin	+	+	-	+	+	+
α-Amylase	+	+	-	+	+	+
DNase	+	+	-	+	+	+
Phenylalanine deaminase	-	-	-	-	-	-
Lysine deaminase	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-
Ornithine decarboxylase	+	-	-	+	-	+
Urease	+	+	+	-	-	+
Gelatinase	-	-	-	-	-	+
Nitrate reductase	-	-	+	+	+	-
Citrate permease	-	-	-	-	-	-
Catalase	+	+	-	+	+	+
Oxidase	-	-	-	-	-	-
H_2S	-	-	-	-	-	-
Gas	-	-	-	-	-	-
Indole	-	-	-	-	-	-
Salt tolerance (6.5%)	+	+	+	+	+	-

+: production/degradation/tolerant, -: no production/no degradation/no tolerant

were observed among these strains. MKAL1 and MKAL3 are circular-shaped while MKAL2, MKAL4, MKAL5, and MKAL6 are rod-shaped. Strains MKAL2, MKAL4, and MKAL5 are negative Gram bacteria. MKAL1, MKAL3, and MKAL6 are positive Gram bacteria. MKAL1 and MKAL4 are pale colored, while MKAL2, MKAL3, MKAL5, and MKAL6 are red, white, yellow, and creamy colored respectively. All strains are non-endospore-forming bacteria. Except for MKAL1, all tested strains are motile. All strains did not produce indole, hydrogen sulfide, gas, phenylalanine deaminase, citrate permease, lysine decarboxylase, and lysine deaminase. Except for MKAL3, all strains produced catalase and α-amylase and hydrolyzed malonate, DNA, and esculin. Only MKAL6 was non-salt-tolerant and showed a gelatinase activity. All strains degraded most sugars tested.

Effect of Temperature and Incubation Period on Cellulase Production

The effect of different temperatures was evaluated on the enzymatic activity and growth rate of isolates. Bacterial isolates were separately cultured in 250-mL conical flasks containing CMC broth (50 mL) for 5 days at 30, 35, 40, 45, and 50 °C. The results are presented in Fig. 2. All strains did not produce cellulase at 50 °C and no cell growth was observed at this temperature. MKAL3 showed cellulase activity only at 35 °C. Strains MKAL1, MKAL4, MKAL5, and MKAL6 exhibited maximum activity at 35 °C while the optimum temperature of MKAL2 for cellulase production occurred at 40 °C. The cell growth of isolates increased until the optimum temperatures and then declined. All strains exerted optimum cellulase production after 96 h of incubation (Fig. 3).

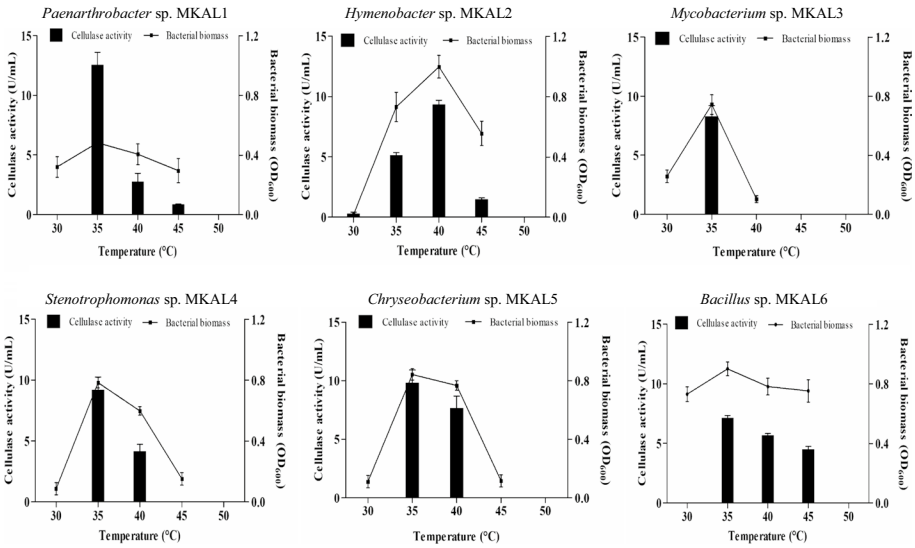

Fig. 2 Effect of temperature on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6

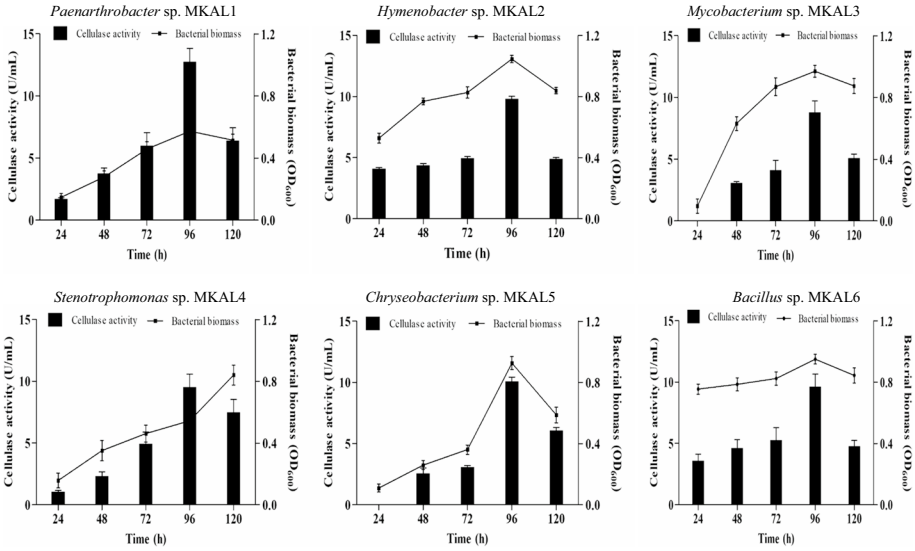

Fig. 3 Effect of incubation period on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6

Effect of pH on Cellulase Production

The effect of pH on cellulase production and bacterial growth was studied by adjusting the pH of the culture medium between 4 and 9. No cellulase activity and cell growth of strains were observed at pH 4 (Fig. 4). Only MKAL2 and MKAL6 produced cellulase at pH 5. MKAL3 exhibited cellulase activity only at pH 6. The optimum pH of

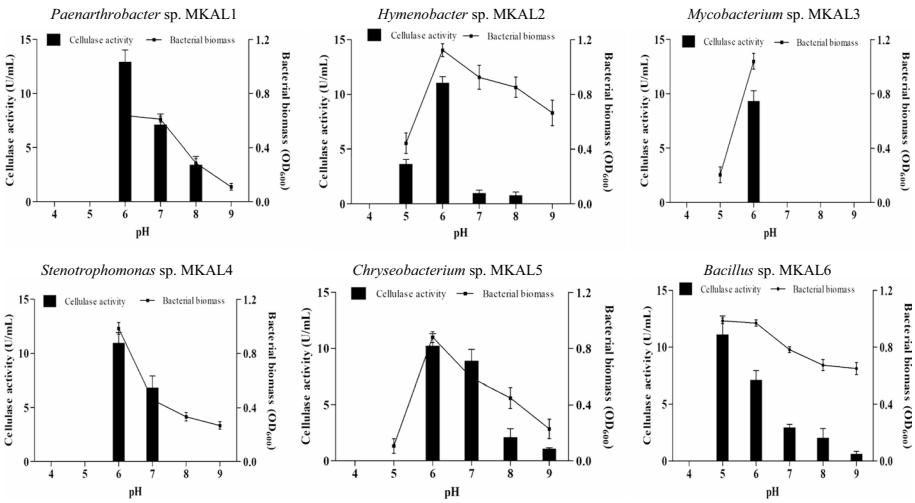

Fig. 4 Effect of pH on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6

MKAL6 for cellulase production was 5, while MKAL1, MKAL2, MKAL3, MKAL4, and MKAL5 showed maximum activity at pH 6.

Effect of CMC Concentration on Cellulase Production

The effects of CMC concentration on cell growth and cellulase production are presented in Fig. 5. No cellulase production was observed at 0.5% CMC. However, isolates showed cellulase activity at a range of CMC concentrations from 1 to 2.5% except for MKAL3 which exerted cellulase activity only at 1% CMC (9.66 ± 0.75 U/mL). MKAL1 (13.22 ± 0.53 U/mL) and MKAL5 (11.51 ± 0.95 U/mL) showed optimum cellulase production at 1.5% CMC, while MKAL2 (16.50 ± 1.36 U/mL), MKAL4 (10.93 ± 0.83 U/mL), and MKAL6 (18.06 ± 1.30 U/mL) exhibited maximum activity at 2% CMC.

Effect of Carbon Sources on Cellulase Production

The effect of carbon sources on enzyme production was determined by replacing CMC in the culture medium with various carbon sources (0–2.5%). All tested carbon sources boosted cellulase production at different concentrations except for pure cellulose, cellulose acetate, and PET (Fig. 6, Table S1). Some carbohydrates enhanced cellulase production by strains compared to CMC (9.66 ± 0.75 to 18.06 ± 1.30 U/mL). MKAL1 exhibited maximum activity at 1% sucrose (158.27 ± 10.48 U/mL), 1.5% fructose (21.16 ± 8.08 U/mL), 1.5% xylose (25.56 ± 9.51 U/mL), 2% sorbitol (33.34 ± 10.98 U/mL), and 2% mannitol (44.22 ± 7.13 U/mL). MKAL2 showed higher activity at 1.5% sorbitol (34.01 ± 4.01 U/mL), 2% sucrose (78.87 ± 4.71 U/mL), 2% mannitol (40.20 ± 4.72 U/mL), and 2% xylose (26.57 ± 5.47 U/mL). MKAL3 exhibited maximum activity at 1.5% sucrose (100.82 ± 8.93 U/mL), 1.5% glucose (15.40 ± 4.62 U/mL), 1.5% mannitol (39.72 ± 6.66 U/mL), and 2% sorbitol (44.01 ± 7.06 U/mL). MKAL4 showed higher activity at 2% of sucrose

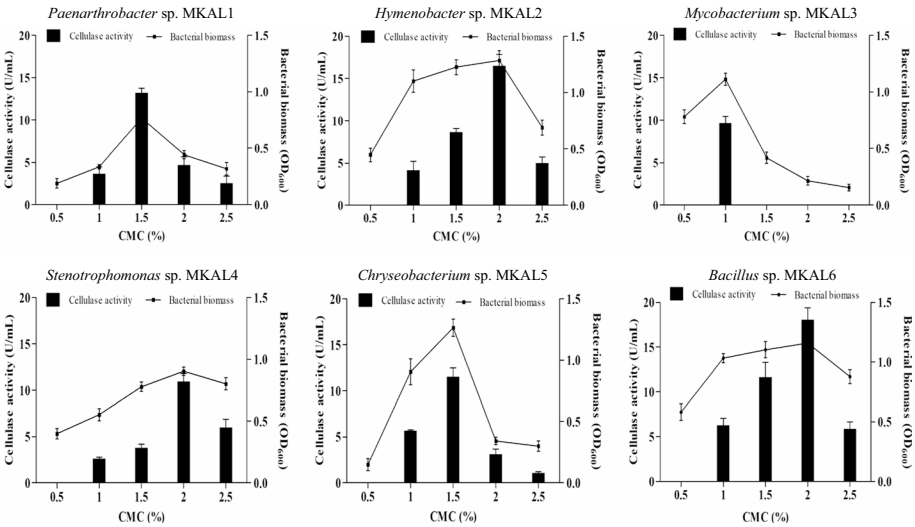

Fig. 5 Effect of CMC concentration on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6

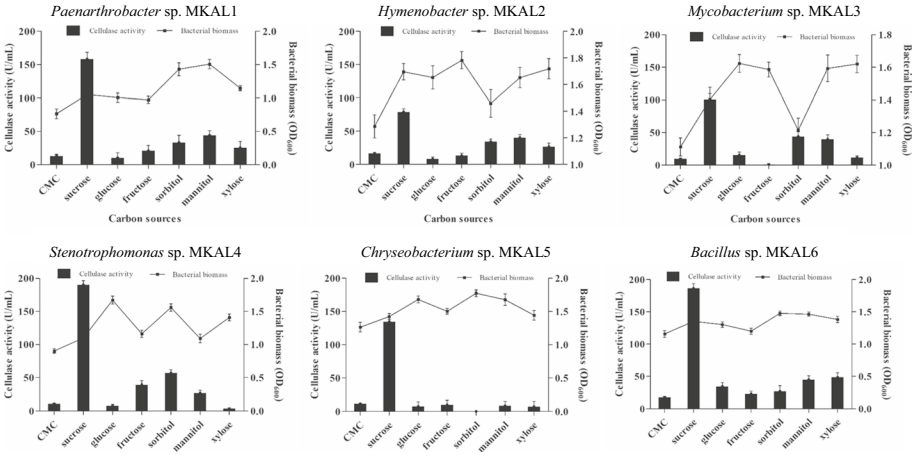

Fig. 6 Effect of carbon sources on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6. Carbon sources enhanced cellulase production at different concentrations: MKAL1 (1% sucrose, 1.5% CMC, 1.5% glucose, 1.5% fructose, 1.5% xylose, 2% sorbitol, and 2% mannitol), MKAL2 (1.5% sorbitol, 2% CMC, 2% sucrose, 2% glucose, 2% fructose, 2% mannitol, and 2% xylose), MKAL3 (1% CMC; 1.5% sucrose, 1.5% glucose, 1.5% mannitol, 2% fructose, 2% sorbitol, and 2% xylose), MKAL4 (2% of all tested carbon sources), MKAL5 (1% xylose, 1.5% CMC, sucrose, 1.5% fructose, 2% glucose, and 2% mannitol), and MKAL6 (1.5% sucrose, 1.5% sorbitol, 1.5% mannitol, 2% CMC, 2% glucose, 2% fructose, and 2% xylose). CMC, carboxymethyl cellulose

(190.30 ± 6.42 U/mL), fructose (39.44 ± 5.96 U/mL), sorbitol (56.96 ± 4.75 U/mL), and mannitol (27.25 ± 4.27 U/mL). Higher cellulase production by MKAL5 was found only at 1.5% sucrose (134.76 ± 9.11 U/mL). Maximum cellulase activity by MKAL6 occurred at 1.5% sucrose (186.54 ± 7.23 U/mL), 1.5% sorbitol (27.48 ± 8.58 U/mL), 1.5% mannitol (44.99 ± 5.99 U/mL), 2% glucose (34.90 ± 5.65 U/mL), 2% fructose (23.33 ± 4.28 U/mL), and 2% xylose (48.52 ± 6.89 U/mL). However, sucrose was the best cellulase production inducer by these bacterial strains.

Effect of Nitrogen Sources on Cellulase Production

The cellulase production was highly affected by various nitrogen sources. The effect of each nitrogen source (0.05–2% w/v) on enzyme production by strains was investigated at their optimum pH (5 and 6) and temperature (35 and 40 °C). Except for MKAL6, all tested bacterial isolates cannot degrade CMC without a nitrogen source in the culture medium. No cellulase production and cell growth by MKAL1, MKAL2, MKAL3, and MKAL4 were observed with urea. Also, ammonium nitrate inhibited cellulase production by MKAL6, while ammonium chloride and ammonium sulfate inhibited cellulase activity of MKAL2. Organic nitrogen sources promoted higher cellulase production than inorganic nitrogen sources (Fig. 7, Table S2). The results showed higher cellulase production when yeast extract (MKAL2 and MKAL6), casein hydrolysate (MKAL1, MKAL4, and MKAL5), and tryptone (MKAL3) were used. MKAL2 (20.50 ± 1.35 U/mL) and MKAL6 (26.60 ± 3.36 U/mL) exerted maximum activity at 0.5 and 1.5% yeast extract respectively. Maximum cellulase production by MKAL1 (19.62 ± 2.55 U/mL), MKAL5 (17.75 ± 1.49 U/mL), and

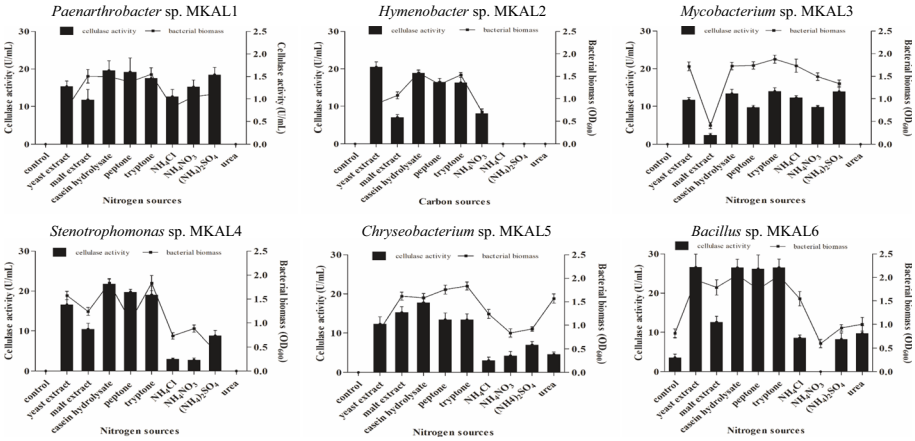

Fig. 7 Effect of nitrogen sources on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6. Nitrogen sources enhanced cellulase production at different concentrations: MKAL1 (1% of all tested nitrogen sources except for urea), MKAL2 (0.5% yeast extract, 0.5% malt extract, 0.5% NH_4NO_3, 1.5% casein hydrolysate, 1.5% peptone, and 1.5% tryptone), MKAL3 (0.5% malt extract, 1% NH_4Cl, 1% NH_4NO_3, 1.5% yeast extract, 1.5% casein hydrolysate, 1.5% peptone, 1.5% tryptone, and 1.5% $(NH_4)_2SO_4$), MKAL4 (0.5% malt extract, 0.5% NH_4Cl, 0.5% NH_4NO_3, 1% $(NH_4)_2SO_4$, 1.5% yeast extract, 1.5% casein hydrolysate, 1.5% peptone, and 1.5% tryptone), MKAL5 (0.5% NH_4Cl, 0.5% NH_4NO_3, 0.5% urea, 1% yeast extract, 1% malt extract, 1% casein hydrolysate, 1.5% peptone, 1.5% tryptone, and 1.5% $(NH_4)_2SO_4$), and MKAL6 (0.5% NH_4Cl, 0.5% $(NH_4)_2SO_4$, 0.5% urea; 1% malt extract, 1.5% yeast extract, 1.5% casein hydrolysate, 1.5% peptone, and 1.5% tryptone)

MKAL4 (21.80 ± 1.14 U/mL) occurred at 1 and 1.5% casein hydrolysate respectively. The highest cellulase activity in MKAL3 (14.00 ± 0.91 U/mL) was observed when 1.5% tryptone was used.

Effect of Salts on Cellulase Production

The effect of salts (0.5–5 mM) on enzyme production by strains was performed at their optimum pH (5 and 6) and temperature (35 and 40 °C). Except for MKAL3, all bacterial strains stimulated cellulase production in a non-salt supplemented culture medium (Fig. 8, Table S3). Some salts enhanced cellulase production by strains compared to control (0.09 ± 0.00 to 10.92 ± 2.45 U/mL). MKAL4 (24.38 ± 2.60 U/mL), MKAL6 (28.71 ± 1.22 U/mL), and MKAL2 (23.23 ± 1.90 U/mL) exhibited maximum activity at 1 and 2.5 mM $CoCl_2$ respectively. The optimum enzyme production by MKAL1 (21.15 ± 3.29 U/mL) and MKAL3 (16.39 ± 1.41 U/mL) occurred at 2.5 mM KCl, while the maximum production by MKAL5 (20.05 ± 2.29 U/mL) was at 2.5 mM $MgCl_2$.

Effect of Surfactants and EDTA on Cellulase Production

No cellulase production was observed with triton X-100, SDS, and EDTA. The presence of tween 20 in the culture medium enhanced cellulase production by MKAL6

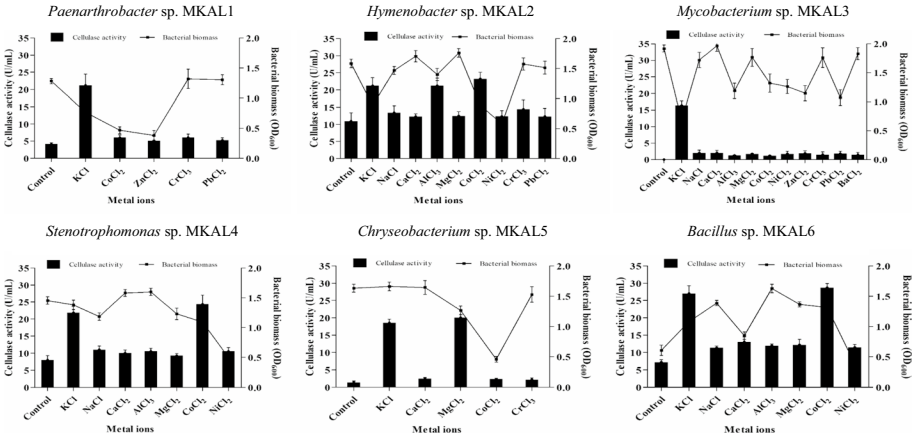

Fig. 8 Effect of salts on cellulase production by strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6. Salts enhanced cellulase production at different concentrations: MKAL1 (1 mM $CoCl_2$, 1 mM $ZnCl_2$, 1 mM $PbCl_2$, 2.5 mM KCl, and 2.5 mM $CrCl_3$), MKAL2 (1 mM NaCl, 1 mM $CaCl_2$, 1 mM $CrCl_3$, 1 mM $PbCl_2$, 2.5 mM KCl, 2.5 mM $AlCl_3$, 2.5 mM $MgCl_2$, 2.5 mM $CoCl_2$, and 2.5 mM $NiCl_2$), MKAL3 (1 mM NaCl, 1 mM $CaCl_2$, 1 mM $AlCl_3$, 1 mM $MgCl_2$, 1 mM $CoCl_2$, 1 mM $CrCl_3$, 1 mM $PbCl_2$, 1 mM $BaCl_2$, 2.5 mM KCl, 2.5 mM $NiCl_2$, and 2.5 mM $ZnCl_2$), MKAL4 (1 mM KCl, 1 mM $CaCl_2$, 1 mM $AlCl_3$, 1 mM $MgCl_2$, 1 mM $CoCl_2$, 2.5 mM NaCl, and 2.5 mM $NiCl_2$), MKAL5 (1 mM $CaCl_2$, 1 mM $CrCl_3$, 2.5 mM KCl, 2.5 mM $MgCl_2$, and 2.5 mM $CoCl_2$), and MKAL6 (1 mM NaCl, 1 mM $CaCl_2$, 1 mM $AlCl_3$, 1 mM $MgCl_2$, 1 mM $CoCl_2$, 2.5 mM KCl, and 2.5 mM $NiCl_2$)

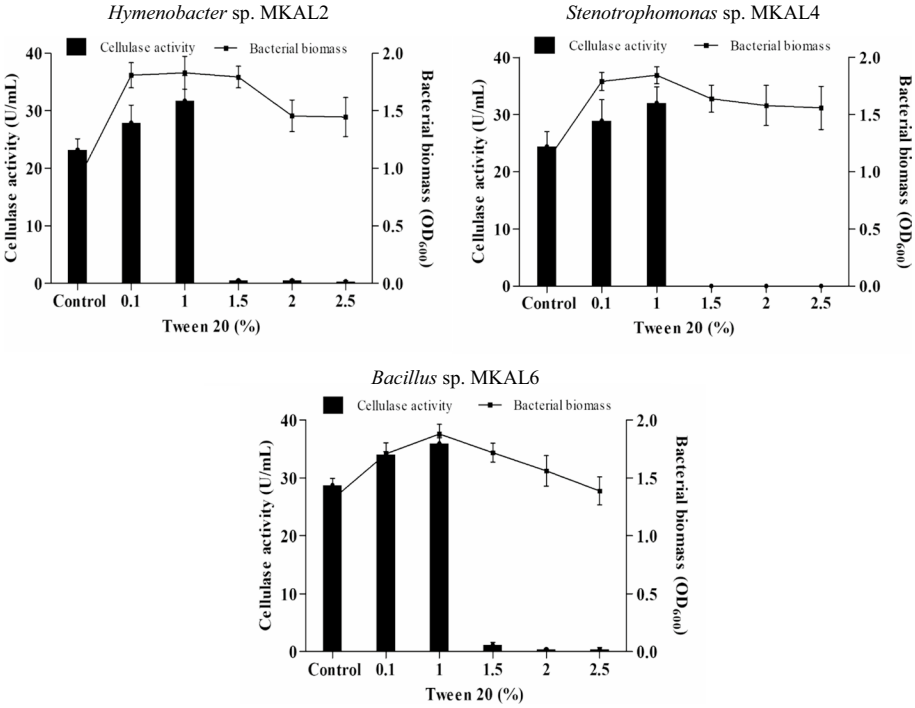

Fig. 9 Effect of tween 20 on cellulase production by strains MKAL2, MKAL4, and MKAL6

(33.99 ± 0.58 to 35.91 ± 1.03 U/mL), MKAL2 (27.87 ± 3.10 to 31.72 ± 4.40 U/mL), and MKAL4 (28.93 ± 3.64 to 32.00 ± 2.86 U/mL) compared to control (23.23 ± 1.90 to 28.71 ± 1.22 U/mL) (Fig. 9). This cellulase production gradually increased with an increase in tween 20 concentration and reached a maximum production at 1% (w/v) concentration in the medium. This trend was also observed in bacterial growth.

Optimization of Fermentation

The Box–Behnken design was used to optimize the fermentation conditions (Table 2). Results are presented in Table S4. Cellulase was the response variable, while temperature (X_1), pH (X_2), and fermentation time (X_3) were independent variables. Quadratic equations showing the linear relationship between response and independent variables were as follows:

$$\begin{aligned}\text{MKAL1}: \text{Cellulase (U/mL)} &= -6.174X_1^2 - 5.676X_2^2 - 5.764X_3^2 + 1.380X_1X_2 \\ &+ 0.345X_2X_3 + 1.123X_1X_3 + 1.336X_1 + 1.585X_2 \\ &+ 0.734X_3 + 13.230\end{aligned} \tag{1}$$

$$\begin{aligned}\text{MKAL2}: \text{Cellulase (U/mL)} = &-8.677X_1^2 - 4.814X_2^2 - 7.387X_3^2 \\ &+2.682X_1X_2 + 0.667X_2X_3 - 0.210X_1X_3 \\ &-2.469X_1 - 1.918X_2 + 0.559X_3 + 18.513\end{aligned} \tag{2}$$

$$\begin{aligned}MKAL3: Cellulase(U/mL) = &-4.757X_1^2 - 4.757X_2^2 - 4.757X_3^2 \\ &+1.208X_1X_2 + 0.335X_2X_3 + 0.675X_1X_3 + 9.513\end{aligned} \tag{3}$$

$$\begin{aligned}MKAL4: Cellulase(U/mL) = &-5.253X_1^2 - 4.700X_2^2 - 4.153X_3^2 + 0.900X_1X_2 \\ &+0.495X_2X_3 - 0.093X_1X_3 + 1.174X_1 + 1.450X_2 + 0.201X_3 + 10.853\end{aligned} \tag{4}$$

$$\begin{aligned}\text{MKAL5}: \text{Cellulase (U/mL)} = &-4.867X_1^2 - 5.112X_2^2 - 4.235X_3^2 + 1.208X_1X_2 \\ &+0.335X_2X_3 + 0.675X_1X_3 + 1.646X_1 + 1.524X_2 + 0.505X_3 + 11.187\end{aligned} \tag{5}$$

$$\begin{aligned}\text{MKAL6}: \text{Cellulase (U/mL)} = &-7.974X_1^2 - 7.651X_2^2 - 4.129X_3^2 + 3.215X_1X_2 \\ &+0.235X_2X_3 + 0.252X_1X_3 + 4.976X_1 + 5.138X_2 + 0.244X_3 + 18.840\end{aligned} \tag{6}$$

The analysis of variance revealed that p values of regression and lack of fit were 0.000–0.009 ($p < 0.05$) and 0.074–0.778 ($p > 0.05$) respectively for strains MKAL1, MKAL2, MKAL4, and MKAL5 (Table S4). This indicates that the built quadratic equation is relatively credible for the evaluation of glucose isomerase activity of these bacterial strains. However, the p value of the lack of fit was 0.001 ($p < 0.05$) respectively for MKAL3 and MKAL6. This suggests that the relationship between parameters is not significant, or the response surface quadratic model does not fit well for the assessment of enzyme activity of those two bacteria. Contour plots were produced based on the fitted model to estimate response surface shape. All contour plots appeared as ellipses, suggesting interactions

Table 2 Box–Behnken design matrix for optimization of cellulase activity

Bacterial strains	Run	X_1 Temperature (°C)	X_2 pH value	X_3 Time (h)	Cellulase activity (U/mL)
Paenarthrobacter sp. MKAL1	1	− 1 (30)	− 1 (5)	0 (96)	0.00 ± 0.00
	2	1 (40)	− 1	0	0.00 ± 0.00
	3	− 1	1 (7)	0	0.00 ± 0.00
	4	1	1	0	5.52 ± 0.17
	5	− 1	0 (6)	− 1 (72)	0.00 ± 0.00
	6	1	0	− 1	0.34 ± 0.01
	7	− 1	0	1 (120)	0.00 ± 0.00
	8	1	0	1	4.83 ± 0.11
	9	0 (35)	− 1	− 1	0.00 ± 0.00
	10	0	1	− 1	2.89 ± 0.06
	11	0	− 1	1	0.00 ± 0.00
	12	0	1	1	4.27 ± 0.18
	13	0	0	0	12.27 ± 1.29
	14	0	0	0	14.52 ± 1.08
	15	0	0	0	12.90 ± 1.88
Hymenobacter sp. MKAL2	1	− 1 (35)	− 1 (5)	0 (96)	14.15 ± 2.91
	2	1 (45)	− 1	0	0.00 ± 0.00
	3	− 1	1 (7)	0	4.68 ± 0.25
	4	1	1	0	1.26 ± 0.00
	5	− 1	0 (6)	− 1 (72)	2.45 ± 0.03
	6	1	0	− 1	1.78 ± 0.03
	7	− 1	0	1 (120)	3.54 ± 0.10
	8	1	0	1	2.03 ± 0.05
	9	0 (40)	− 1	− 1	7.98 ± 1.29
	10	0	1	− 1	3.08 ± 0.09
	11	0	− 1	1	8.21 ± 1.79
	12	0	1	1	5.98 ± 0.99
	13	0	0	0	17.86 ± 2.77
	14	0	0	0	18.15 ± 3.07
	15	0	0	0	19.53 ± 3.35
Mycobacterium sp. MKAL3	1	− 1 (30)	− 1 (5)	0 (96)	0.00 ± 0.00
	2	1 (40)	− 1	0	0.00 ± 0.00
	3	− 1	1 (7)	0	0.00 ± 0.00
	4	1	1	0	0.00 ± 0.00
	5	− 1	0 (6)	− 1 (72)	0.00 ± 0.00
	6	1	0	− 1	0.00 ± 0.00
	7	− 1	0	1 (120)	0.00 ± 0.00
	8	1	0	1	0.00 ± 0.00
	9	0 (35)	− 1	− 1	0.00 ± 0.00
	10	0	1	− 1	0.00 ± 0.00
	11	0	− 1	1	0.00 ± 0.00
	12	0	1	1	0.00 ± 0.00
	13	0	0	0	9.70 ± 0.49
	14	0	0	0	9.47 ± 0.80
	15	0	0	0	9.37 ± 0.97

between temperature, pH, and fermentation time. These variables affect cellulase activity and optimum conditions for maximum enzyme production yield were in the design range (Fig. S1). The optimal responses, 13.474, 18.982, 11.052, and 11.502 U/mL with a 95% confidence interval, were obtained by canonical analysis for MKAL1, MKAL2, MKAL4, and MKAL5 respectively. The coded factor values for the stationary point were as follows:

Table 2 (continued)

Bacterial strains	Run	X_1 Temperature (°C)	X_2 pH value	X_3 Time (h)	Cellulase activity (U/mL)
Stenotrophomonas sp. MKAL4	1	− 1 (30)	− 1 (5)	0 (96)	0.00 ± 0.00
	2	1 (40)	− 1	0	0.00 ± 0.00
	3	− 1	1 (7)	0	0.00 ± 0.00
	4	1	1	0	3.60 ± 0.05
	5	− 1	0 (6)	− 1 (72)	0.00 ± 0.00
	6	1	0	− 1	3.08 ± 0.01
	7	− 1	0	1 (120)	0.00 ± 0.00
	8	1	0	1	2.71 ± 0.04
	9	0 (35)	− 1	− 1	0.00 ± 0.00
	10	0	1	− 1	3.01 ± 0.15
	11	0	− 1	1	0.00 ± 0.00
	12	0	1	1	4.99 ± 0.72
	13	0	0	0	10.56 ± 1.38
	14	0	0	0	10.67 ± 1.08
	15	0	0	0	11.33 ± 0.76
Chryseobacterium sp. MKAL5	1	− 1 (30)	− 1 (5)	0 (96)	0.00 ± 0.00
	2	1 (40)	− 1	0	0.00 ± 0.00
	3	− 1	1 (7)	0	0.00 ± 0.00
	4	1	1	0	4.83 ± 0.52
	5	− 1	0 (6)	− 1 (72)	0.00 ± 0.00
	6	1	0	− 1	2.82 ± 0.05
	7	− 1	0	1 (120)	0.00 ± 0.00
	8	1	0	1	5.52 ± 0.67
	9	0 (35)	− 1	− 1	0.00 ± 0.00
	10	0	1	− 1	3.01 ± 0.07
	11	0	− 1	1	0.00 ± 0.00
	12	0	1	1	4.35 ± 0.08
	13	0	0	0	10.56 ± 1.75
	14	0	0	0	11.67 ± 1.78
	15	0	0	0	11.33 ± 1.91
Bacillus sp. MKAL6	1	− 1 (30)	− 1 (4)	0 (96)	0.00 ± 0.00
	2	1 (40)	− 1	0	0.00 ± 0.00
	3	− 1	1 (6)	0	0.00 ± 0.00
	4	1	1	0	12.86 ± 0.61
	5	− 1	0 (5)	− 1 (72)	0.00 ± 0.00
	6	1	0	− 1	12.97 ± 0.11
	7	− 1	0	1 (120)	0.00 ± 0.00
	8	1	0	1	13.98 ± 0.35
	9	0 (35)	− 1	− 1	0.00 ± 0.00
	10	0	1	− 1	13.65 ± 0.71
	11	0	− 1	1	0.00 ± 0.00
	12	0	1	1	14.59 ± 0.09
	13	0	0	0	18.73 ± 1.09
	14	0	0	0	18.78 ± 1.50
	15	0	0	0	19.01 ± 1.17

(1) MKAL1: $0.133(X_1), 0.158(X_2), 0.081(X_3)$, with corresponding experimental conditions: temperature 35.67 °C, pH 6.16, and fermentation time 97.94 h
(2) MKAL2: $-0.181(X_1), -0.248(X_2), 0.029(X_3)$, with corresponding experimental conditions: temperature 39.10 °C, pH 5.75, and fermentation time 96.70 h
(3) MKAL4: $0.126(X_1), 0.168(X_2), 0.033(X_3)$, with corresponding experimental conditions: temperature 35.63 °C, pH 6.17, and fermentation time 96.79 h

(4) MKAL5: $0.197(X_1), 0.175(X_2), 0.082(X_3)$, with corresponding experimental conditions: temperature 35.99 °C, pH 6.18, and fermentation time 97.97 h

The fitness of the model was checked by performing triplicate experiments under predicted optimum fermentation conditions. Experimental values were 13.303 ± 1.57, 18.817 ± 2.08, 10.89 ± 0.78, and 11.381 ± 1.12 U/mL for MKAL1, MKAL2, MKAL4, and MKAL5, respectively. This demonstrates reliable goodness of fit to predict cellulase production yield during the fermentation process with these bacterial strains.

Molecular Weight Determination and Zymogram

Protein bands of cellulases were observed in 15% acrylamide gel. Multiple bands were observed in the gel. However, the bands with hydrolytic zone correspond to 25 kDa (Fig. 10) confirming the presence of cellulase.

Discussion

Cellulases are demanding industrial enzymes and play a major role in the bioconversion of cellulosic biomass into fermentable sugars which are further exploited for many applications [24]. Cellulose-degrading bacteria are widely spread in nature, soils, and agricultural environments.

We identified six cellulose-degrading bacteria from soil samples belonging to genera *Bacillus*, *Hymenobacter*, *Chryseobacterium*, *Paenarthrobacter*, *Mycobacterium*, and *Stenotrophomonas*. Many investigators reported cellulase activity of the members of these bacteria isolated from various sources [25–29]. Their cellulase production was influenced by growth parameters such as temperature, incubation period, pH, carbon and nitrogen sources, metal ions, surfactants, and incubation time.

Each microorganism needs optimum temperature for enzyme production stabilization. MKAL1, MKAL3 MKAL4, MKAL5, and MKAL6 exhibited maximum activity at 35 °C, while the optimum temperature of MKAL2 for cellulase production occurred at 40 °C. *Bacillus subtilis* subsp. subtilis JJBS300 [30], *Bacillus velezensis* [31], and *Bacillus subtilis* strain MU S1 [32] were reported to produce higher cellulase yield at 35 °C. Some other bacteria such as *Bacillus pacificus*, *Pseudomonas mucidolens* [33], *Bacillus pseudomycoides* [34], and *Streptomyces thermocoprophilus* strain TC13W [35] showed higher cellulase activity at 40 °C. No cellulase production by strains was observed at 30 and 50 °C. Enzyme inactivation at these temperatures would be due to weak intermolecular interactions on the enzyme structure stability, decreasing enzyme catalytic abilities. At lower temperatures, substrate transport across the cell is suppressed, while at a higher temperature, the enzyme is unfolded and inactivated (thermal denaturation) [36]. However, some researchers revealed cellulase production by *Paenibacillus* sp. IM7, *Bacillus* sp., *Bacillus wiedmannii*, and *Chryseobacterium* sp. at 30 and 50 °C [37–41]. Optimal cellulase production was achieved at an incubation period of 96 h. Beyond, enzyme activity decreased. This occurred due to nutrient depletion in the fermentation medium, inhibition by end-products, or by-product production. Nutrient depletion causes bacterial stress leading to enzyme secretion inactivation and cell death [42]. *Micrococcus* sp. SAMRC-UFH3 [43], *Bacillus amyloliquefaciens* AK9 [44], and *Bacillus albus* [45] were reported to produce maximum cellulase after 96 h of incubation.

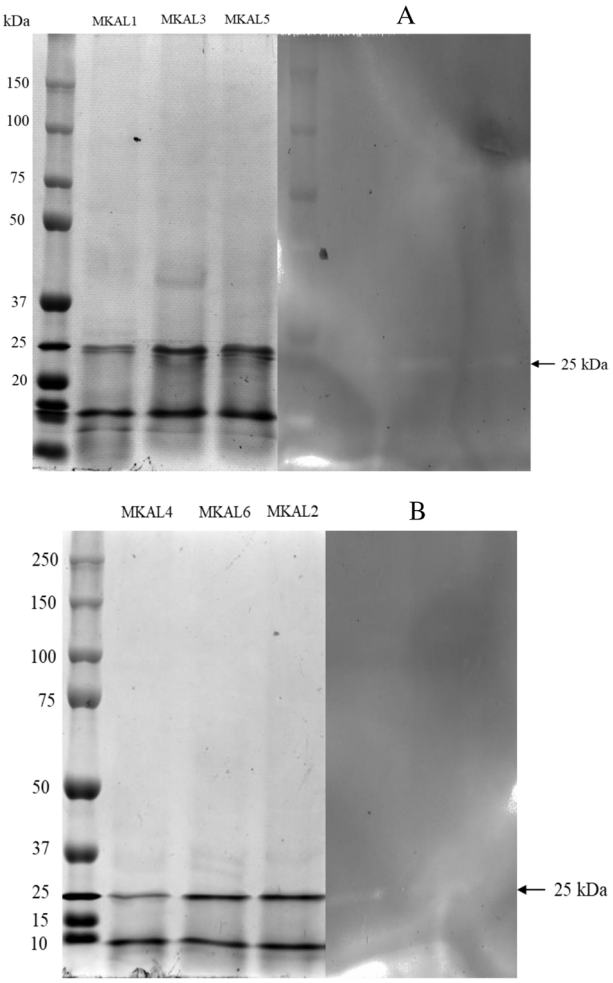

Fig. 10 SDS-PAGE of crude cellulase from strains MKAL1, MKAL2, MKAL3, MKAL4, MKAL5, and MKAL6. **A**, **B** Hydrolytic bands in zymogram

Medium pH is an essential factor for enzyme production and enzyme stability. All isolates tested showed cellulase production at optimum pH ranging from 5 to 6. Similar optimum pH of 5 to 6 was reported in *Chryseobacterium* sp. [37], *Stenotrophomonas maltophilia* [46], and *Bacillus albus* [45]. However, MKAL1, MKAL2, MKAL4, and MKAL6 also exerted cellulase production in the broader pH ranges from 6 to 8. These results were also recorded for different bacterial cellulases [34, 36, 39, 40, 47–49]. We observed a decrease in enzyme activity that may be due to ionization group change at the enzyme active site or conformational change of the enzyme slowing or preventing the enzyme–substrate complex formation [36].

CMC is widely used to produce microbial cellulase because it is a soluble cellulose derivative with a high degree of polymerization. Its concentration in the culture influences enzyme production [50]. All tested strains exhibited cellulase activity at concentrations

ranging from 1 to 2.5%. MKAL6 exhibited cellulase activity of 6.27 ± 0.79 U/mL at 1% CMC. Malik and Javed [51] reported cellulase activity of 2.4 U/mL in *Bacillus subtilis* CD001 at 1% CMC. Sugars act as inducers or repressors for enzyme production. Sucrose enhanced higher production, which suggested the negligible requirement of this sugar for appropriate enzyme induction. Hussain et al. [52] showed that *Bacillus amyloliquefaciens* SA5, *Bacillus subtilis* BTN7A, *Bacillus megaterium* BMS4, and *Anoxybacillus flavithermus* BTN7B exhibited maximum cellulase production when sucrose was used as sole carbon in the culture medium. Pure cellulose, cellulose acetate, and PET did not stimulate enzyme production because of their structural complexity and insolubility.

The fermentation medium not supplemented by nitrogen sources did not promote cellulase production by isolates. Thus, nitrogen sources are essential for cellulase production. Yeast extract, casein hydrolysate, and tryptone boosted higher cellulase activity. Other investigators recorded similar results [34, 53]. Organic nitrogen sources have stimulated higher production than inorganic nitrogen sources because their metabolism contributes to culture medium acidification, affecting cellulase production. However, other studies revealed that inorganic nitrogen sources such as urea and ammonium chloride promoted maximum cellulase production by *Bacillus licheniformis* 2D55 [54] and *Aneurinibacillus aneurinilyticus* BKT-9 [55].

Metal ions play a vital role in enzyme catalysis by binding directly or indirectly to the enzyme active site [56]. $CoCl_2$, KCl, and $MgCl_2$ promoted higher cellulase production. Other reports showed these salts enhanced higher cellulase activity in *Bacillus tequilensis* S28 [57], *Bacillus cereus* [58], and *Bacillus amyloliquefaciens* [59].

The presence of tween 20 in the culture medium boosted cellulase production in MKAL2, MKAL4, and MKAL6 at 0.1 and 1%. This cellulase production declined at higher concentrations. Bhagia et al. [60] revealed that even nonionic surfactants at high concentrations such as tween 20 could negatively affect enzymatic hydrolysis.

Hydrolytic bands of cellulases indicated cellulolytic activity in zymogram corresponding to ~ 25 kDa. Cellulase bands in the range of 24.4–185 kDa have been estimated from SDS-PAGE [61–64]. A similar molecular weight of 25 kDa has been reported in *Bacillus licheniformis* SVD1 [65], *Bacillus subtilis* MA139 [66], *Penicillium verruculosum* [67], and *Novosphingobium* sp. Cm1 [68].

Conclusions

This study aimed to characterize cellulose-degrading bacteria and optimize their cellulase production. Six CDBs were isolated from soil samples showing that soil is a vast cellulolytic bacteria untapped reservoir and identified as *Paenarthrobacter* sp. MKAL1, *Hymenobacter* sp. MKAL2, *Mycobacterium* sp. MKAL3, *Stenotrophomonas* sp. MKAL4, *Chryseobacterium* sp. MKAL5, and *Bacillus* sp. MKAL6. The higher cellulase production in these strains occurred at the culture conditions of 35–40 °C, pH 5–6, 1–2% CMC, and 96 h of incubation. The presence of yeast extract, casein hydrolysate, tryptone, sucrose, potassium chloride, cobalt chloride, magnesium chloride, and tween 20 boosted their cellulase production. Response surface quadratic model was reliable to predict cellulase production during the fermentation process with strains MKAL1, MKAL2, MKAL4, and MKAL5. The purification of these cellulases for hydrolysis and saccharification of lignocellulosic biomasses are being studied.

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Data Availability All data generated or analyzed during this study are included in this article.

Declarations

Ethics Approval Not applicable.

Consent to Participate The authors agreed to participate in this work.

Consent for Publication The authors agreed to publish this work.

Conflict of Interest The authors declare no competing interests.

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