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Production, purification, and characterization of a thermo-alkali stable and metal-tolerant carboxymethylcellulase from newly isolated *Bacillus methylotrophicus* Y37

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Abstract: A carboxymethylcellulose (CMC)-degrading bacterium was isolated from soil, identified as *Bacillus methylotrophicus* according to the physiological properties and analyses of 16S rRNA and a partial sequence of the gyrase A (*gyr*A) gene, and named as *B. methylotrophicus* Y37. The CMCase enzyme was purified to homogeneity by 20.4-fold with 21.73% recovery using single-step hydrophobic interaction chromatography and biochemically characterized. CMCase showed a molecular weight of approximately 50 kDa as determined by SDS-PAGE. The activity profile of the CMCase enzyme exhibited optimum activity at 45 °C and pH 5.0. The activity was highly stable at alkaline pH levels. More than 90% of the original CMCase activity was maintained at relatively high temperatures ranging from 55 to 65 °C. The enzyme activity was induced by Ca^{2+}, Cd^{2+}, Co^{2+}, K^{+}, Mg^{2+}, and Na^{1+}, whereas it was strongly inhibited by phenylmethanesulfonyl fluoride and iodoacetic acid. The enzyme tolerated Hg^{2+} up to 10 mM and presented hydrolytic activity towards glucan, filter paper, laminarin, and CMC but not *o*-nitrophenyl β-D-galactopyranoside. Kinetic analysis of the purified enzyme showed K_m and V_{max} values of 0.19 mg mL^{-1} and 7.46 U mL^{-1}, respectively. The biochemical properties of this CMCase make the enzyme a good candidate for many industrial applications.

Key words: *Bacillus methylotrophicus*, carboxymethylcellulase, isolation, purification, characterization, metal, thermostability

1. Introduction

The production of biofuels from renewable lignocellulosic biomass has gained great attention in the last two decades. As enormous amounts of agricultural and industrial lignocellulosic wastes have been accumulating or used inefficiently, the development of bioconversion processes would solve waste disposal problems and decrease the dependence on fossil fuels to obtain energy. Although bioethanol production from cellulose (the most abundant biopolymer in nature) represents the best alternative to fossil fuels, cellulosic bioethanol generation is not frequently used yet due to the high cost of cellulolytic enzymes. Therefore, low-cost hydrolytic enzymes should be developed.[1]

It has been established that there are three main types of cellulase enzymes found in the complete enzymatic hydrolysis of lignocellulosic materials into glucose molecule: exoglucanase or cellobiohydrolase (EC 3.2.1.91), endoglucanase or carboxymethylcellulase (EC 3.2.1.4), and cellobiase or β-glucosidase (EC 3.2.1.21).

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The endoglucanases act internally on the chain of cellulose randomly cleaving the β-1,4-glycosidic bonds, and exoglucanases specifically hydrolyze cellobiosyl units from nonreducing ends. Finally, the cellobiase enzyme releases a glucosyl unit from cellooligosaccharides.[2] Cellulases have many industrial applications including the pulp and paper industry, waste management, the textile industry, bioethanol production, formulation of laundry detergents for color brightening and softening, and animal feed manufacturing.[2,3]

A number of fungi and bacteria producing cellulolytic enzymes have been identified. Among them, filamentous fungi are the major source of cellulases and hemicellulases.[4] However, the production costs of these enzymes are relatively high due to the substrates used and also the slow growth rate of fungi.[1] On the other hand, bacteria, which have the capacity to be present in a wide variety of environmental niches, can produce highly thermostable, alkali, or acid-stable enzyme complements and may serve as highly potent sources of important enzymes used in industrial applications.[5] Moreover, bacterial cellulases are considered to be a better catalyst as they encounter less feedback inhibition.[6]

Many bacterial cellulases have been purified and characterized from different bacteria including *Thermomonospora* sp.,[7] *Cellulomonas* sp.,[8] *Melanocarpus* sp.,[9] *Pseudomonas fluorescens*,[10] *Pyrococcus horikoshi*,[11] and *Bacillus* sp.[12,13]

The aim of this study was to isolate and identify a new source of thermostable carboxymethylcellulase (CMCase) to characterize its cellulolytic enzyme. It was also intended to purify the enzyme at low cost and investigate the biochemical and catalytic properties of highly purified CMCase for its potential use in biotechnological applications.

2. Results and discussion

2.1. Isolation and identification of cellulolytic bacteria

A number of cellulase-secreting bacterial strains were isolated from soil using a spread plate technique. Among them, isolate Y37 was selected as a potent carboxymethyl cellulose hydrolyzer using a CMC agar plate forming a clear zone around the growth and by cellulase assay with cell-free culture filtrate. Soil near a paper factory was selected as a source for obtaining desirable cellulase-producing organisms. The morphological and phenotypic characteristics and carbohydrate utilization pattern of isolate Y37 are summarized in Table 1 in comparison with the reference strains *B. methylotrophicus* DSM 28326, *B. amyloliquefaciens* DSM 7, and *B. vallismortis* DSM 11031. The colony appearance of strain Y37 on agar plates was a creamy color with diameters of 1–9 mm. Isolate Y37 was found to be a gram-positive, spore-forming bacterium and it gave positive test results for catalase, urease, and starch hydrolysis, whereas it was negative for nitrate reduction and hydrogen sulfide production. The absence of a black precipitate at the base of the tube indicated that hydrogen sulfide was not produced. The color of TSI agar slant turned from red to yellow, which indicated that the bacterium was able to ferment sugars including glucose, lactose, and sucrose. A temperature tolerance test revealed that the isolate was able to grow at a wide temperature range of 30–50 °C and optimal growth temperature was observed at 37 °C. No growth was observed at 60 °C. The cells were able to grow at pH values between 5 and 9 with optimal growth at pH 7 and in the presence of 3%–10% NaCl at pH 7.0. Isolate Y37 and reference strain *B. methylotrophicus* DSM 28326 showed nearly identical phenotypes according to the tested characteristics (Table 1). Differences were observed in β-galactosidase production and nitrate reduction. Phylogenetic analysis based on a BLAST search using the 16S rRNA gene sequence exhibited the highest homology (99%) with *Bacillus methylotrophicus* strain Mo-Bm (GenBank accession no. HQ325853.1), as shown in Figure 1a. The 16S rRNA gene is commonly used as a framework for modern bacterial classification, although with limitations for members

Table 1. Phenotypic properties of isolate Y37 in comparison with *B. methylotrophicus* DSM 28236, *B. amyloliquefaciens* DSM7, and *B. vallismortis* DSM11031.

Characteristic/ biochemical test	Observation			
	Y37	*B. methylotrophicus* DSM 28236	*B. amyloliquefaciens* DSM 7	*B. vallismortis* DSM11031
Colony morphology on nutrient agar plate	Large, circular, undulate, raised, viscous, translucent, creamy white color pigmented colonies	Small, circular, undulate, raised, viscous, translucent, creamy white color pigmented colonies	Large, circular, erose, raised, buttery, opaque, creamy white color pigmented colonies	Large, circular, entire, flat, dry, translucent, creamy white color pigmented colonies
Gram reaction	+	+	+	+
Endospore formation	+ (central to paracentral)	+ (central to paracentral)	+ (central to paracentral)	+ (central to paracentral)
Catalase test	+	+	+	+
β-Galactosidase	+	- [(a)]	- [(b)]	+ [(c)]
Oxidase test	+	+ [(a)]	+ [(b)]	+ [(c)]
Urease test	+	+	+	- [(c)]
Tryptophan deaminase test	+	+ [(a)]	- [(b)]	nd
NO_3 reduction to NO_2	-	+ [(a)]	+ [(b)]	+ [(c)]
Acid production from				
Glucose	+	+	+	+
Lactose	+	+	-	-
Sucrose	+	+	+	+
H_2S production	-	-	-	-
Starch hydrolysis	+	+	-	+
Gelatin hydrolysis	+	+ [(a)]	+ [(b)]	+ [(c)]
Decarboxylation of:				
Arginine	+	+ [(a)]	- [(b)]	nd
Lysine	+	nd	- [(b)]	nd
Ornithine	+	nd	- [(b)]	nd
Citrate utilization	+	+	+	+
Indole formation	+	+	+	+
Acetoin production	+	+	+	+
Growth at:				
30 °C	+	+	+	+
37 °C	+	+	+	+
50 °C	-	-	-	-
60 °C	-	-	-	-
pH 5.0	+	+	+	+
pH 5.5	+	+	+	+
pH 6.0	+	+	+	+
pH 6.5	+	+	+	+
pH 7.0	+	+	+	+
pH 7.5	+	+	+	+
pH 8.5	+	+	+	+
pH 9.0	+	+	+	+
pH 10.0	-	-	-	-
pH 11.0	-	-	-	-
pH 12.0	-	-	-	-
Growth in:				
3% NaCl	+	+	+	+
5% NaCl	+	+	+	+
7% NaCl	+	+	+	+
10% NaCl	+	+	+	+

[(a)]Results of Madhaiyan et al.[43], [(b)]Results of Borriss et al.[44], [(c)]Results of Roberts et al.[45]

of closely related taxa.[14] On the other hand, some protein-coding genes such as the *gyr*A gene sequences, coding for the DNA gyrase subunit, have been shown to exhibit much higher genetic variation and are presented as an alternative method for accurate identification of closely related taxa including *B. methylotrophicus*, *B. amyloliquefaciens*, *B. vallismortis*, *B. mojavensis*, *B. atrophaeus*, and *B. licheniformis*.[15] Therefore, in this study a partial *gyr*A gene sequence has been used for the confirmation of the results obtained from 16S rRNA sequence analysis. The phylogenetic analysis using the partial *gyr*A gene sequence also revealed that isolate Y37 has the highest homology with *Bacillus methylotrophicus* strain Mo-Bm (GenBank accession no. HQ325853.1), as shown in Figure 1b. Numbers at nodes of the tree are indications of the levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets. Isolate Y37 was identified as *Bacillus methylotrophicus* and designated as *Bacillus methylotrophicus* Y37.

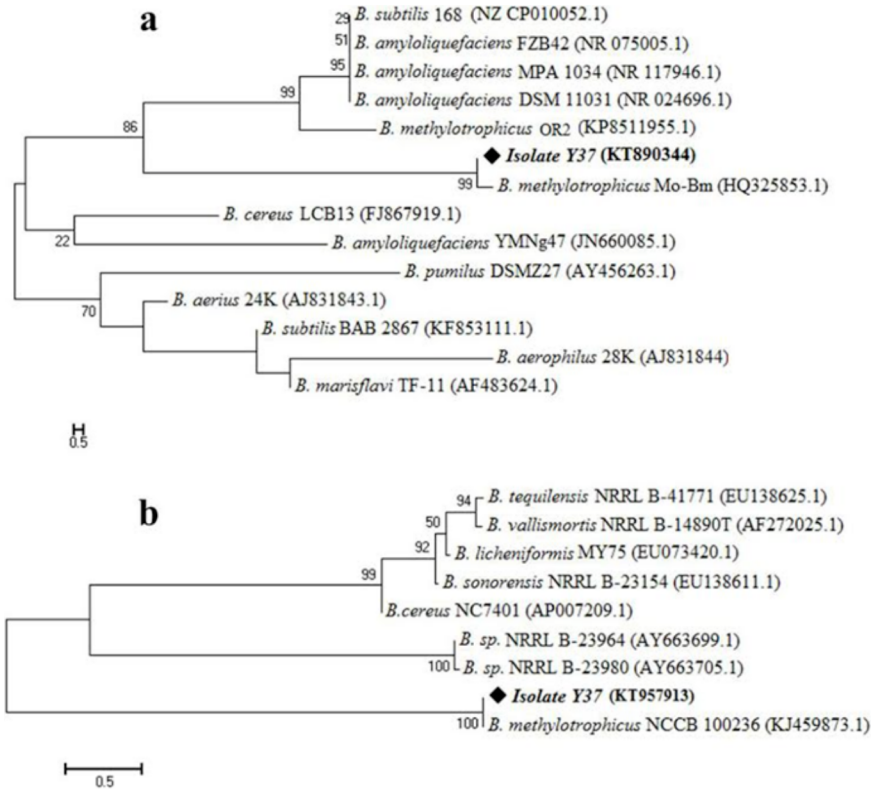

Figure 1. A phylogenetic tree of B. *methylotrophicus* Y37 associated with other members of the genus *Bacillus* using (a) the 16S rRNA sequence and (b) the *gyrA* gene sequence retrieved from the database using the neighbor-joining method.

2.2. Time course of carboxymethylcellulase (CMCase) production

The production of cellulase was carried out in a shake flask containing growth medium with 1% (w/v) CMC as the sole carbon source. The growth curve of Y37 along with the CMCase production profile (Figure 2) revealed that the enzyme production was associated with cell growth and reached a maximum at 13 h. CMCase production was simultaneous with microbial growth, indicating growth-associated production of the enzyme rather than a secondary metabolic activity.

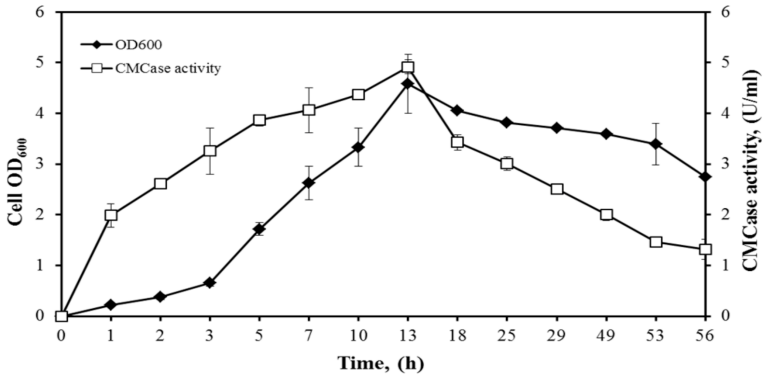

Figure 2. Time course of *B. methylotrophicus* Y37 CMCase activity with respect to cell growth in 1 L of CMC growth medium containing 1% (w/v) CMC as the sole carbon source.

2.3. CMCase purification

The CMCase enzyme was purified from the culture broth of *B. methylotrophicus* strain Y37 using single-step hydrophobic interaction chromatography. Utilization of pH 7.0 and 3 M NaCl resulted in the best purification, providing selectively passage of CMCase through the column without binding while the majority of the contaminating proteins were stacked in the column (Figure 3). A 20.4-fold purification with a recovery yield of 21.73% in comparison to the original crude extract was achieved. The molecular weight of the purified enzyme was estimated to be about 50 kDa as confirmed by the presence of a single protein band in denatured gel. The result of activity staining also showed the active band of the CMCase enzyme corresponding to the size of about 50 kDa (Figure 4). This molecular mass was much larger than the 30–42 kDa of the cellulases from *B. subtilis* AS3[16] and *B. licheniformis*,[17] but close to that of other cellulases from *B. amyloliquefaciens* DL-3,[18] *B. cereus*,[19] *Bacillus* sp. KSM-N252,[20] and *Bacillus* strain M-9.[21]

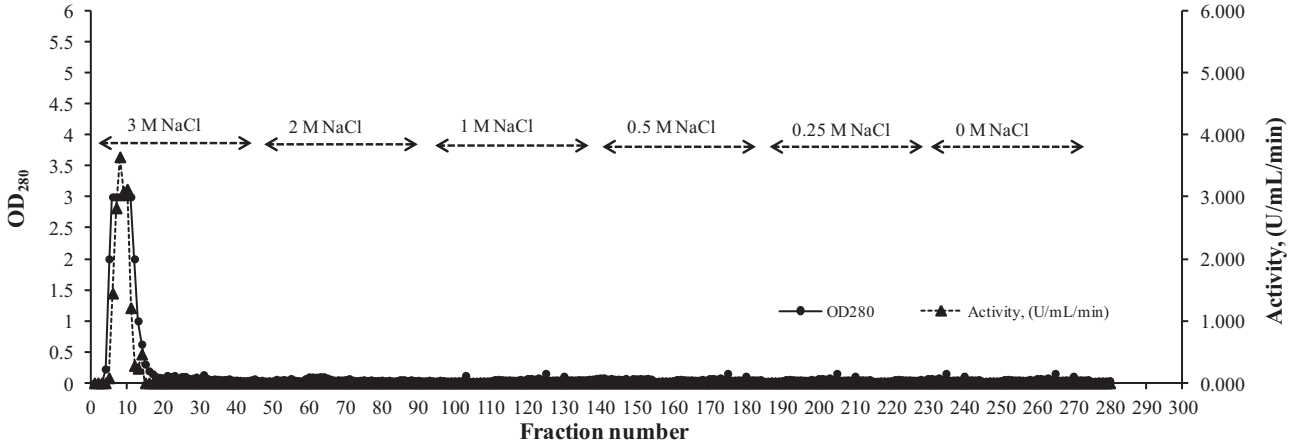

Figure 3. *B. methylotrophicus* Y37 CMCase purification by using Phenyl Sepharose high performance column at pH 7.0 and 3 M NaCl.

2.4. Effect of temperature and pH on enzyme activity and stability

The effect of temperature on the CMCase activity of the purified enzyme was examined at various temperatures ranging from 25 to 85 °C at pH 7.0. Among the seven different temperatures tested, 45 °C is the optimum

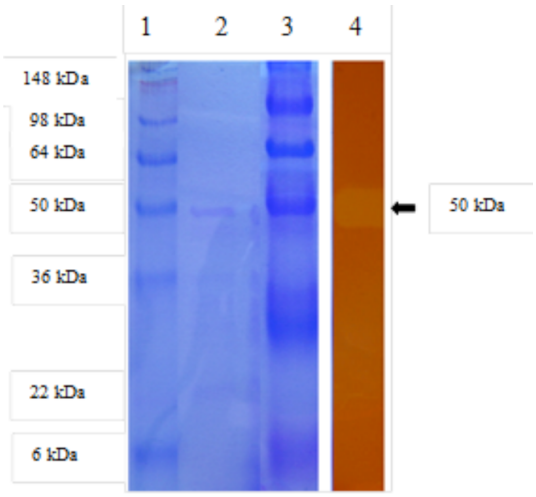

Figure 4. Electrophoretic analysis of CMCase produced by *B. methylotrophicus* Y37. Lane 1: Molecular weight marker (SeeBlue®Plus2 Pre-stained Protein Standard, LC5925), Lane 2: SDS-PAGE analysis of Phenyl Sepharose chromatography, Lane 3: SDS-PAGE analysis of crude extract, Lane 4: activity staining of CMCase with Congo red.

temperature for maximum enzyme activity; on either side of this temperature there was a slight decline in activity. Our findings are in agreement with those of Sadhu et al.[5], Lee et al.,[18] and Lin et al.,[22] who also found either 45 or 50 °C as the most favorable temperature for CMCase activity. A closer look at Figure 5 shows that enzyme activity displays about 93% of its maximal activity at temperatures between 65 and 85 °C. Thermostability of the CMCase activity was also tested over a temperature range of 25–85 °C after 45 min of incubation (Figure 5). Almost 90% of the initial CMCase activity of the purified enzyme was maintained at temperatures ranging from 55 to 65 °C. At temperatures above 65 °C, enzyme activity was moderately lost with only 65% remaining. These results suggest that the enzyme is highly stable up to 65 °C and then a gradual decrease in stability takes place after 45 min of incubation. On the other hand, cellulases from different

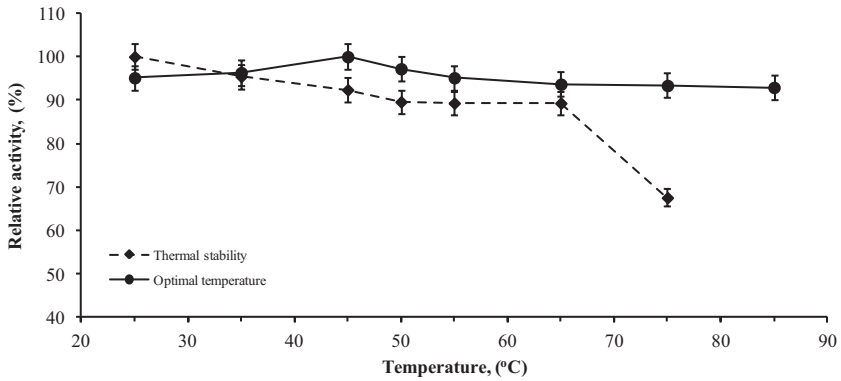

Figure 5. Effect of temperature on the enzyme activity and stability of purified CMCase produced by *B. methylotrophicus* Y37. The enzyme activity was measured at temperatures ranging from 25 to 85 °C using Tris-HCl buffer (pH 7.0). For the thermal stability of CMCase, the enzyme was incubated at indicated temperatures for 45 min. Percent activity was calculated relative to enzyme activity at different temperatures divided by the maximum enzyme activity multiplied by 100.

Bacillus species were reported to be stable up to 50 °C.[6,16,23] For industrial applications, highly thermotolerant enzymes are required. Therefore, the prolonged stability of CMCase from *B. methylotrophicus* Y37 under high temperatures would be a great advantage for its applications.

The influence of pH on CMCase activity was determined over the pH range of 3.0–10.0 at 50 °C (Figure 6). The activity profile of the purified enzyme showed its highest activity at pH 5.0 and more than 80% of the activity still retained even as the pH increased to 10.0. These results indicate that the enzyme is highly active over a broad range of pH levels. Similar findings were also reported previously by George et al.,[7] Bischoff et al.,[17] and Bajaj et al.[21] The pH stability of the purified CMCase was also evaluated at different pH values indicated above after incubations for 60 min at 50 °C and 3 h at 4 °C (Figure 6). When compared to the incubation at 4 °C, incubations at 50 °C caused more reduction in enzyme activity. CMCase samples incubated at 4 °C and pH 10.0 retained 82% of the original activity, but this was 74% for incubation at 50 °C and pH 10.0. The enzyme was active over the pH range of 3.0–10.0 and most stable at pH 6.0. An optimum pH of 4.5–9.0 has been reported for different microbial cellulases.[21,24,25] Similar to the present observations, cellulase from *Streptomyces* sp. was found to be optimally active at acidic pH levels but stable over a broad range of pH (5–10).[25,26] Since many industrial processes are operated at either acidic or alkaline pH, enzymes with a broad range of stability become significant for industrial applications.

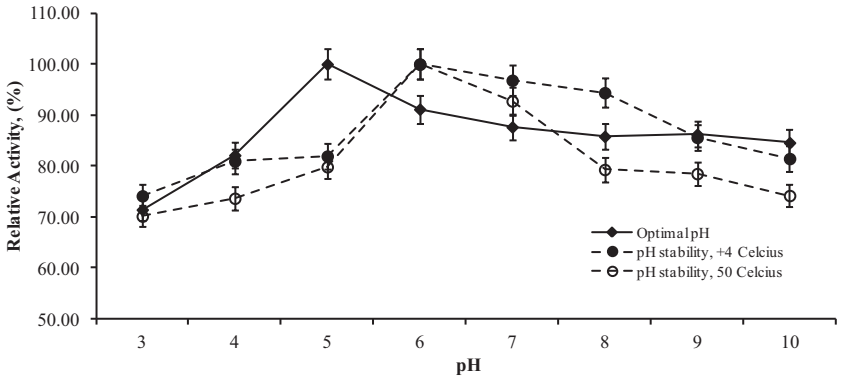

Figure 6. Effect of pH on the enzyme activity and stability of purified CMCase produced by *B. methylotrophicus* Y37. For optimal enzyme activity, the enzyme was incubated at 50 °C for 3 min with 1% (w/v) CMC dissolved in different buffers (50 mM): acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0), and glycine NaOH (pH 9.0–10.0). For pH stability, the enzyme was incubated for both 60 min at 50 °C and 3 h at 4 °C using different buffers as indicated above. Percent activity was calculated relative to enzyme activity at different pH values divided by the maximum enzyme activity multiplied by 100.

2.5. Effect of metal ions and inhibitors on enzyme activity

The influence of metal ions on the purified CMCase was determined by performing the assay with the addition of metal ions at final concentrations of 1, 10, and 100 mM. The presence of Co^{2+} metal ions in the reaction mixture enhanced the enzyme activity to 319% of the original level, while metal ions of K^{+} and Mg^{2+} increased the activity at moderate levels (148% and 141%, respectively). It is clear from Table 2 that the enzyme activity was strongly inhibited by Hg^{2+}, while no significant inhibition was observed in the case of Ca^{2+} and Cd^{2+}. The strong inhibition by Hg^{2+} of the cellulase activity was also reported in *B. amyloliquefaciens*[18] and *B. subtilis*.[27] It has been suggested previously that the inhibitory effect of Hg^{2+} results from its binding to either

the thiol groups or tryptophan residue in the enzyme.[28] According to the studies reported previously, Co^{2+} activated cellulases from *B. subtilis*,[23,29] *B. mycoides*,[30] and *Cellulomonas* sp.[31] The effect of a number of cellulase inhibitors on CMCase was analyzed with CMC as the substrate. Inhibitors were selected according to the information in the literature.[8,31,32] Results presented in Table 3 show that there is inhibition in the presence of phenylmethanesulfonyl fluoride (PMSF) and iodoacetic acid (IAA), both indicated as inhibitors of cellulases.[8,22,29] Significant inhibition by PMSF and IAA revealed that serine and cysteine residues would be essential for the enzyme catalysis.

Table 2. CMCase enzyme activity affected by the presence of various metal ions with the final concentrations of 1, 10, and 100 mM dissolved in Tris-HCl buffer (pH 7.0).

	Relative activity, %		
Metal salt	1 mM	10 mM	100 mM
Control	100 ± 0.12	100 ± 0.12	100 ± 0.12
KCl	119.4 ± 1.98	148.4 ± 1.98	148.4 ± 0.84
NaCl	91.1 ± 3.49	96.1 ± 3.37	103.1 ± 3.46
$CoCl_2$	121.2 ± 2.74	175.9 ± 2.93	319.1 ± 3.09
$HgCl_2$	92.7 ± 3.65	82.5 ± 1.56	25.7 ± 0.92
$CdCl_2$	91.5 ± 2.77	96.9 ± 2.55	114.2 ± 0.77
$CaCl_2$	97.2 ± 2.74	110.7 ± 2.92	114.2 ± 3.43
$MgCl_2$	109.6 ± 2.92	109.6 ± 3.19	141.2 ± 3.42

Table 3. CMCase enzyme activity affected by the presence of various inhibitors with the final concentrations of 1, 5, and 10 mM dissolved in Tris-HCl buffer (pH 7.0).

	Residual activity, %		
Inhibitor	1 mM	5 mM	10 mM
Control (no inhibitor)	100 ± 0.17	100 ± 0.17	100 ± 0.17
PMSF	73.53 ± 0.02	61.58 ± 0.94	35.54 ± 0.05
N-Bromosuccinimide	106.92 ± 0.05	116.11 ± 0.14	114.27 ± 0.07
Iodoacetic acid	66.63 ± 0.05	58.82 ± 0.05	30.33 ± 0.07
Woodward K	86.55 ± 0.12	80.42 ± 0.14	56.98 ± 0.05
Phenyl glyoxylate	70.46 ± 0.14	77.97 ± 0.09	75.06 ± 0.10
TLCK	88.08 ± 0.06	74.14 ± 0.05	72.15 ± 0.14
TPCK	93.13 ± 0.07	106.92 ± 0.04	121.32 ± 0.05

2.6. Substrate specificity

CMC is a soluble cellulosic substrate with β-1,3-1,4 linkage. The synergistic action of the hydrolyzing effect of cellulolytic enzymes (β-1,3 and β-1,4 glycosidic bonds; β-1,3 glycosidic bonds and β-1,4 glycosidic bonds) is required for effective cellulose hydrolysis. If not, large amounts of cellulases are still required for efficient decomposition of biomass, and this increases the cost of the industrial application of cellulase.[33] In our study, the substrate specificity of the purified CMCase was determined by assays with different substrates. As shown in Table 4, the purified enzyme degraded β-glucan (including β-1,4 endoglucanase), laminarin (including β-1,3 endoglucanase), filter paperm and CMC (including β-1,3 and β-1,4 glycosidic bonds), but there was no detectable activity on *o*-nitrophenyl-D-glucopyranoside (ONPG). The rate of β-glucan and laminarin degradation was higher than that of any other substrates tested.

Table 4. Substrate specificity of the CMCase produced by *B. methylotrophicus* Y37.

Substrate	Activity, U/mL
Glucan	14.89 ± 0.85
Laminarin	14.19 ± 0.44
Filter paper	13.13 ± 0.56
CMC	10.39 ± 0.32
ONPG	n.d.

n.d., Activity was not detectable.

The enzyme showed the capacity to hydrolyze β-1,3, β-1,4, and β-1,6 glycosidic linkages. From these results, it seems that the nature of this enzyme resembles an important endo type of cellulase.

2.7. Kinetic analysis

K_m and V_{max} values of purified extracellular CMCase were determined by a Lineweaver–Burk double reciprocal plot of the initial reaction against substrate (CMC) concentration and were found as 0.19 mg mL^{-1} and 7.46 U mL^{-1}, respectively (Figure 7). The K_m value observed was quite lower than that found in the range of 0.6–7.2 mg mL^{-1} for some other cellulases produced from different *Bacillus* strains.[23,26,27] Lower K_m values reflect a higher affinity between the substrate and enzyme, indicating that CMCase from *B. methylotrophicus* Y37 has the highest affinity for CMC among the other cellulases reported earlier.

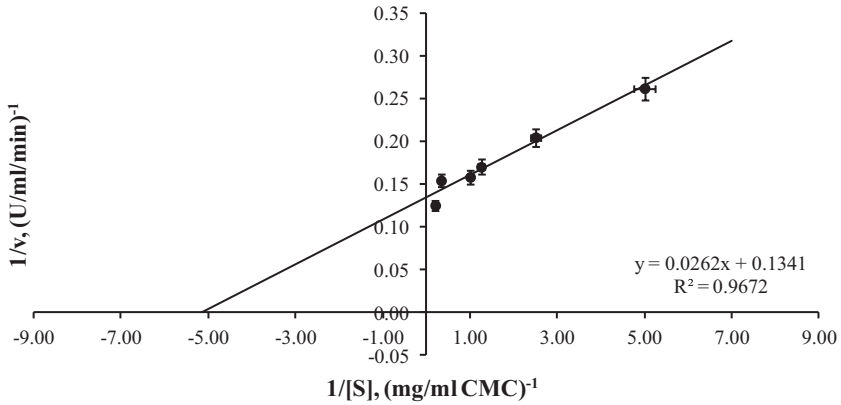

Figure 7. Lineweaver–Burk double reciprocal plots of purified CMCase produced by *B. methylotrophicus* Y37. Data are means of two different triplicate experiments.

Cellulose-degrading bacteria have been isolated from soil. Among them, isolate Y37 exhibited the highest CMCase activity and has been further identified as *B. methylotrophicus* on the basis of physiological properties and 16S rRNA and partial *gyrA* gene sequence analyses. The produced enzyme was considered to be a thermostable endoglucanase with a broad range of pH tolerance and the ability to break down a wide variety of cellulosic substrates. Additional properties like increasing relative activity at increasing metal ion concentrations, especially in the presence of Co^{2+}, K^{+}, and Mg^{2+}; having high stability at alkaline pH levels; and having the highest affinity to its substrate make the CMCase from *B. methylotrophicus* Y37 a promising candidate in different fields of industrial applications like the food and baking industry, paper and pulp industry, and feed industry. Further optimization for large-scale production for CMCase using this strain is underway in our laboratory.

3. Experimental

3.1. Chemicals

CMC, inhibitors, and salts came from Sigma-Aldrich (St. Louis, MO, USA). Phenylglyoxal and $HgCl_2$ were obtained from Merck. Phenyl sepharose 6 Fast Flow was purchased from Pharmacia (Uppsala, Sweden). The other chemicals were of analytical grade and purchased from either Sigma-Aldrich or Merck.

3.2. Isolation and screening of cellulolytic bacteria

Nine different soil samples were collected from different localities of Yuvacık in northwestern Turkey. A presterilized spatula and plastic Falcon tubes were used for sample collection, and before bacterial isolation the samples were stored at 4 °C in an icebox for approximately 16 h. Aliquots (100 μL) of different dilutions of soil suspensions were heat-shocked at 80 °C for 10 min, spread on Difco powdered nutrient agar plates, and then incubated at 37 °C. According to the morphological characteristics of different colonies on agar plates, inocula from these grown colonies were transferred to replicates of slants containing the same specific media. Purified isolates were maintained on agar slants of the same medium at 4 °C.

For the screening of cellulolytic activity, the bacterial isolates were streaked on CMC agar medium (peptone, 1 g; yeast extract, 5 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 5 g; agar 15, g; CMCellulose, 10 g in 1 L of distilled water)[8] and incubated at 37 °C for 18 h. Then the bacterial colonies were flooded with 1% Congo red for 3 h. The stain was poured off, and the plates were washed with 1 M NaCl.[1] The formation of a clear zone of hydrolysis indicated cellulose degradation. The strain that showed the highest production of CMCase enzyme was selected for further studies.

3.3. Morphological and biochemical characterizations of isolate Y37

Cells grown on nutrient agar medium were examined for their morphological and cultural characteristics, including cell shape, colony appearance, endospore formation, and pigmentation, after being incubated at 37 °C for 24 h. β-Galactosidase and tryptophan deaminase, Voges–Proskauer reactions, indole production, gelatin hydrolysis, nitrate reduction, citrate utilization, and arginine, lysine, and ornithine decarboxylations were determined as described in *Bergey's Manual of Systematic Bacteriology*.[34] Gram staining and endospore staining were done as per standard protocols. The catalase activity was determined by adding few drops of 3% (v/v) H_2O_2 to 5 mL of culture grown for 18 h. The oxidase activity was tested according to the methods described by Sneath et al.[34] Triple Sugar Iron (TSI) slants (Merck 1.03915) containing three sugars, namely glucose, lactose, and sucrose, were used for acid and H_2S production. Acid production after carbohydrate fermentation was detected by the visible change in color from red to yellow. Growth at different pH levels (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0, 11.0, and 12.0), different NaCl concentrations (3, 5, 7, and 10% (w/v)), and at different temperatures (30, 37, 50, and 60 °C) were tested by using nutrient broth medium. All tests were carried out by incubating the cultures at 37 °C, except for investigations into the effect of temperature on growth.

3.4. 16S rRNA and partial gyrase A (*gyr* A) gene sequencing

Genomic DNA for molecular identification of the selected bacterial strain was extracted using a peqGOLD Bacterial DNA Kit (Peq Lab). The 16S rRNA gene was amplified by PCR with two pairs of universal primer sets (A: adenine, T: thymine, C cytosine, G: guanine) pF1 (5' - AGAGTTTGATCCTGGCTCAG - 3') / pR1

(5' - ACGGCTACCTTGTTACGACTT - 3') and pF2 (5' - AGAGTRTGATCMTYGCTWAC - 3') / pR2 (5' - CGYTAMCTTWTTACGRCT - 3') (IUPAC nucleotide base code: R stands for C or T, M for A or C, Y for A or T, and W for A or T).[27] The *gyr*A region was amplified using the primers p – gyrA - F (5' - CAGTCAGGAAATGCGTACGTCCTT - 3') and p – gyrA - R (5' - CAAGGTAATGCTCCAGGCATTGCT - 3').[35,36] The PCR reaction mixture (25 μL of final volume) contained 20.16 ng of genomic DNA, 200 μM dNTP mix, 1.5 mM $MgSO_4$, 0.3 μM primer pF1 (or primer pF2), 0.3 μM primer pR1 (or primer pR2), 0.02 U/μL KOD Hot Start DNA Polymerase (Novagen), and 1X buffer for KOD Hot Start DNA Polymerase (Novagen). The process of PCR was done under the following conditions: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s; 1 cycle of 72 °C of 10 min; and then 4 °C forever. PCR amplified products were sequenced with the ABI 3500XL (Thermo Fisher Scientific). The 16S rRNA and partial *gyr*A gene sequencing results were compared using the Basic Local Alignment Search Tool (BLAST) of the NCBI using GenBank data (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignment and calculation levels of sequence similarity were determined with the ClustalW 1.7 program.[37] A phylogenetic tree was constructed using the neighbor-joining model of the MEGA 5.1 program.[38]

3.5. Nucleotide sequence accession number

The 16S rRNA and partial *gyr*A gene sequences of the selected bacterial strain have been deposited in the NCBI nucleotide sequence database under accession numbers KT890344 and KT957913, respectively.

3.6. Quantitative determination of extracellular CMCase production

The isolate, selected on the basis of plate staining method, was grown in 5 mL of Difco powdered nutrient broth. Overnight grown culture of selected bacteria was then inoculated into 100 mL of enzyme production medium (at pH 7.0). This medium was the same as the previously used medium during isolation, with the only difference being the exclusion of agar. The main culture inoculated with 1.5% of the preculture medium was incubated at 160 rpm and 37 °C for 56 h. Samples were collected every hour and centrifuged at 10000 rpm for 15 min. The cell-free culture broth containing crude enzyme was used for estimation of CMCase activity. The enzyme production by the isolate was monitored with cell growth at 600 nm using a UV-visible spectrophotometer (Bio-Rad).

3.7. CMCase purification and biochemical characterization

3.7.1. Purification of CMCase

Enzyme purification was performed with the Bio-Rad Fraction Collector 2110 and Econopump system. All the steps of purification were performed at 4 °C. Crude enzyme solution was fractionated at 2 mL/min flow rate by single-step hydrophobic interaction chromatography. NaCl was directly added to the crude extract enzyme solution to bring the final NaCl concentration to 3 M and 20 mL of crude extract was applied to a Phenyl Sepharose 6 Fast Flow (high sub) (HIC) (GE Healthcare, Sweden) (2.5 × 10 cm) column previously equilibrated with 50 mM Tris-HCl buffer at pH 7 containing 3 M NaCl. Later, elution of adsorbed protein was achieved by applying a decreasing (3–0 M) NaCl gradient. Each fraction was tested for purity by SDS-PAGE and the concentration of the pooled fractions was determined by the Bradford assay using bovine serum albumin as a standard.[39]

3.7.2. Enzyme assay

CMCase activity was determined by measuring the amount of reducing sugar liberated from CMC using the 3,5-dinitrosalicylic acid (DNS) method.[40] First, 200 μL of appropriately diluted enzyme was incubated with 800 μL of 1% (w/v) CMC in 50 mM Tris-HCl buffer (pH 7.0). The mixture was incubated at 50 °C for 3 min and the reaction was stopped by adding 1 mL of DNS. The reaction mixture was boiled for 5 min, then cooled in an ice bath, and optical density was determined at 540 nm. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of reducing sugar as glucose per minute.

3.7.3. SDS-PAGE and zymogram analysis

To determine the apparent molecular weight of the purified enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 4 °C and the bands were visualized by Coomassie Brilliant Blue staining. For zymogram analysis, samples were applied to 12% Native-PAGE gel containing 1% (w/v) CMC incorporated directly into the resolving gel at 4 °C. The gel was incubated at 50 °C in Tris-HCl buffer (pH 7.0) for 2 h, stained with Congo red (1%, w/v) for 90 min, and destained with 1 M NaCl until the CMCase activity was visualized as a clear band against the red background.[27]

3.7.4. Effect of temperature and pH on enzyme activity and stability

Temperature and pH profiles of purified CMCase were estimated by measuring the enzyme activity at different temperatures (25–85 °C with an interval of 10 °C) and pH levels (3.0–10.0 with an interval of 1.0) using the following buffers: 50 mM acetate buffer (pH 3.0–5.0), 50 mM phosphate buffer (pH 6.0–8.0), and 50 mM glycine-NaOH (pH 9.0–10.0). Thermal stability of the enzyme was determined at respective temperatures with the preincubation of the enzyme for 45 min and pH stability was determined at respective pH levels with preincubation of the enzyme for both 60 min at 50 °C and 3 h at 4 °C. The residual activity of each sample for hydrolysis of CMC was then quantified under the optimized conditions of the enzyme assay.

3.7.5. Effect of metal ions and inhibitors on enzyme activity

The effect of various metals and inhibitors on enzyme activity was also examined. The additives used in this study were the salts of K^+, Na^+, Co^{2+}, Hg^{2+}, Cd^{2+}, Ca^{2+}, and Mg^{2+}. Salts were dissolved in 50 mM Tris-HCl buffer (pH 7.0) at final concentrations of 1, 10, and 100 mM. Purified enzyme was diluted with these solutions and enzyme activity was determined as in Section 3.7.2 for each metal ion concentration. Inhibitors examined were PMSF, N-bromosuccinimide, IAA, Woodward's reagent K, phenylglyoxylic acid, SDS, tosyllysine chloromethyl ketone (TLCK), and tosyl phenylalanyl chloromethyl ketone (TPCK). For inhibition experiments, standard reaction mixtures were used in the presence of the inhibitor dissolved in the reaction buffers. The reaction mixtures with various additives and inhibitors were incubated for 60 min at 50 °C and the CMCase activity was assayed by DNS method.

3.7.6. Substrate specificity

The substrate specificity of the purified enzyme was determined by performing the assay with different substrates including CMC, filter paper, ONPG (*o*-nitrophenyl-D-galactopyranoside), laminarin, and β-D-glucan from barley. The filter paper cellulase and CMCase activities were determined using the IUPAC standard

procedure.[41] β-Galactosidase activity was measured by hydrolysis of the ONPG substrate at 405 nm.[42] β-Glucanase activity was determined by measuring the reducing sugar produced in the reaction; 0.1 mL of appropriately diluted enzyme was incubated with 1% (w/v) β-D-glucan (50 mM acetate buffer, pH 5.0) at 37 °C for 3 min and the reaction was stopped by adding 1 mL of DNS. The reaction mixture was boiled for 5 min then cooled in an ice bath and absorbance was measured at 540 nm. One unit of enzyme was defined as the amount of enzyme required to release 1 μg of reducing sugar per minute using glucose as a standard.[8]

3.7.7. Enzyme kinetics

The kinetics of the CMCase enzyme were characterized in terms of Michaelis–Menten kinetic constants (K_m and V_{max}) using Lineweaver–Burk plots[24] by assaying the enzyme activity at CMC concentrations ranging from 0.4 to 5 mM in 50 mM Tris-HCl buffer (pH 7.0) at 50 °C for 2 min.

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References

1. Maki, M.; Leung, K. T.; Qin, W. *Int. J. Biol. Sci.* **2009**, *5*, 500-516.
2. Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. *Cellulose* **2015**, *22*, 2081-2093.
3. Bhat, M. K. *Biotechnol. Adv.* **2000**, *18*, 355-383.
4. Baldrian, P.; Valaskova, V. *FEMS Microbiol. Rev.* **2008**, *32*, 501-521.
5. Sadhu, S.; Saha, S.; Sen, S. K.; Mayilraj, S.; Maiti, T. K. *Springerplus* **2013**, *2*-10.
6. Acharya, S.; Chaudhary, A. *Braz. J. Microbiol.* **2012**, *43*, 844-856.
7. George, P. S.; Ahmad, A.; Rao, M. B. *Bioresour. Technol.* **2001**, *77*, 171-175.
8. Yin, L. J.; Huang, P. S.; Lin, H. H. *Agric. Food Chem.* **2010**, *58*, 9833-9837.
9. Kaur, J.; Chadha, B. S.; Kumar, B. A.; Kaur, G. S.; Saini, H. S. *Electron. J. Biotechnol.* **2007**, *10*, 260-270.
10. Hazlewood, G. P., Laurie, J. I., Ferreira, L. M.; Gilbert, H. J. *J. Appl. Bacteriol.* **1992**, *72*, 244-251.
11. Kang, H. J.; Uegaki, K.; Fukada, H.; Ishikawa, K. *Extremophiles* **2007**, *11*, 251-256.
12. Fujimoto, N.; Kosaka, T.; Nakao, T.; Yamada, M. *Open Biotechnol. J.* **2011**, *5*, 7-13.
13. Ariffin, H.; Hassan, M. A.; Umi Kalsom, M. S.; Abdullah, N.; Ghazali, F. M.; Shirai, Y. *J. Biosci. Bioeng.* **2008**, *106*, 231-236.
14. Fox, G. E.; Wisotzkey, J. D.; Jurtshuk, P. J. *Int. J. Syst. Bacteriol.* **1992**, *42*, 166-170.
15. Deka, D.; Jawed, M.; Goyal, A. *Prep. Biochem. Biotechnol.* **2013**, *43*, 256-270.
16. Bischoff, K. M.; Rooney, A. P.; Li, X. L.; Liu, S.; Hughes, S. R. *Biotechnol. Lett.* **2006**, *28*, 1761-1765.
17. Lee, Y.; Kim, B.; Lee, B.; Jo, K.; Lee, N.; Chung, C.; Lee, Y.; Lee, J. *Bioresour. Technol.* **2008**, *99*, 378-386.
18. Yan, H.; Dai, Y.; Zhang, Y.; Yan, L.; Liu, D. *Afr. J. Biotechnol.* **2011**, *10*, 16277-16285.
19. Endo, K.; Hakamada, Y.; Takizawa, S.; Kubota, H.; Sumitomo, N.; Kobayashi, T.; Ito, S. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 109-116.
20. Bajaj, B. K.; Pangotra, H.; Wani, A. M.; Sharma, P.; Sharma, A. *Indian J. Chem. Technol.* **2009**, *16*, 382-387.

21. Lin, L.; Kan, X.; Yan, H.; Wang, D. *Electron. J. Biotechnol.* **2012**, *15*, 1-7.
22. Mawadza, C.; Hatti-Kaul, R.; Zvauya, R.; Mattiasson, B. *J. Biotechnol.* **2000**, *83*, 177-187.
23. Immanuel, G.; Dhanusha, R.; Prema, P.; Palavesam, A. *Int. J. Env. Sci. Technol.* **2006**, *3*, 25-34.
24. Alani, F.; Anderson, W. A.; Young, M. M. *Biotechnol. Lett.* **2008**, *30*, 123-126.
25. Kim, C. H. *Appl. Environ. Microbiol.* **1995**, *61*, 959-965.
26. Huang, X. P.; Monk, C. *World J. Microbiol. Biotechnol.* **2004**, *20*, 85-92.
27. Rawat, R.; Tewari, L. *Extremophiles* **2012**, *16*, 637-644.
28. Lamed, R.; Tormo, J.; Chirino, A. J.; Morag, E.; Bayer, E. A. *J. Mol. Biol.* **1994**, *244*, 236-237.
29. Yin, L. J.; Lin, H. H.; Xiao, Z. R. *J. Mar. Sci. Technol.* **2010**, *18*, 466-471.
30. Balasubramanian, N.; Toubarro, D.; Teixeira, M.; Simõs, N. *Appl. Biochem. Biotechnol.* **2012**, *168*, 2191-2204.
31. Saxena, S.; Bahadur, J.; Varma, A. *BioMetals* **1992**, *5*, 209-212.
32. Tsuji, A.; Tominaga, K.; Nishiyama, N.; Yuasa, K. *PLoS One* **2013**, *8*, 1-13.
33. Sneath, P. H. A.; Mair, N. S.; Sharpe, M. E.; Holt, J. G. *Bergey's Manual of Determinative Bacteriology, Vol. 2*; Williams and Wilkins: Baltimore, MD, USA, 1986.
34. Singh, S.; Moholkar, V. S.; Goyal, A. *ISRN Microbiol.* **2013**, *2013*, 1-7.
35. Chun, J.; Bae, K. S. *Antonie van Leeuwenhoek* **2000**, *78*, 123-127.
36. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res.* **1994**, *22*, 4673-4680.
37. Tamura, K.; Battistuzzi, F. U.; Billing-Ross, P.; Murillo, O.; Filipski, A.; Kumar, S. *P. Natl. Acad. Sci. USA* **2012**, *109*, 19333-19338.
38. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-250.
39. Miller, G. L. *Anal. Chem.* **1959**, *31*, 426-428.
40. Ghose, T. K. *Pure Appl. Chem.* **1987**, *59*, 257-268.
41. Duman, Y. A.; Kaya, E. *Protein Expr. Purif.* **2013**, *91*, 155-160.
42. Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658-666.
43. Madhaiyan, M.; Poonguzhali, S.; Kwon, S. W.; Sa, T. M. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 2490-2495.
44. Borriss, R.; Chen, X. H.; Rueckert, C.; Blom, J.; Becker, A.; Baumgarth, B.; Fan, B.; Pukall, R.; Schumann, P.; Spröer, C. et al. *Int. J. Syst. Evol. Microbiol.* **2011**, *61*, 1786-1801.
45. Roberts, M. S., Nakamura, L. K.; Cohan, F. M. *Int. J. Syst. Evol. Int. J. Syst. Bacteriol.* **1996**, *46*, 470-475.