HAYATI
Journal of Biosciences

Vol. 31 No. 1, January 2024 182-191
DOI:10.4308/hjb.31.1.182-191
ISSN: 1978-3019
EISSN: 2086-4094

Isolation, Identification and Assessment of Efficient Cellulase Producing Bacteria from the Termite Guts

Sirikhwan Tinrat[1*], Lueacha Tabtimmai[1], Saranya Sedtananun[1], Chakrit Tachaapaikoon[2,3], Kiattawee Choowongkomon[4]

[1]*Department of Biotechnology, Faculty of Applied science, King Mongkut's University of Technology, North Bangkok, Bangkok 10800, Thailand*
[2]*Division of Biochemical Technology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (KMUTT), Bangkok 10150, Thailand*
[3]*Excellent Center of Enzyme Technology and Microbial Utilization, Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi (KMUTT), Bangkok 10150, Thailand*
[4]*Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10400, Thailand*

ARTICLE INFO

Article history:
Received January 24, 2023
Received in revised form October 12, 2023
Accepted October 19, 2023

KEYWORDS:
cellulase,
cellulolytic bacteria,
termite gut,
carboxy methyl cellulose,
Bacillus

ABSTRACT

The present study is concerned with the screening the cellulase-producing bacteria from termite gut, assessed potential cellulase-producing bacteria and partial characterization (optimum parameters) of cellulase from isolated bacteria. The result showed that 15 out of 48 isolated strains was positive for degrading the carboxy methyl cellulose (CMC) in agar by congo-red method. After screening by DNS assay, three selected bacteria exhibited high cellulase activity that were identified as *Citrobacter amalonaticus* CM 1-3, *Bacillus cereus* CM 5-1 and *Streptococcus salivarius* CE 5-1 using 16S rRNA sequence analysis. All bacterial strains utilized CMC and showed the highest cellulase activity. Cellulase characterization of *C. amalonaticus* CM 1-3 and *S. salivarius* CE 5-1 was revealed optimum activity at 35°C, pH 7.0 and for 48 h. *Bacillus cereus* CM 5-1 represented its potential use in industrial processes due to thermostable cellulase production. The crude cellulase of this strain was purified by $(NH_4)_2SO_4$ precipitation with 1.58 purification fold and 74.38% overall recovery. The optimal temperature and pH for cellulase activity of *B. cereus* CM 5-1 were at 40°C and pH 7.0. Thus, this study provided additional information about the diversity and partial characteristic cellulase of cellulolytic bacteria from termite gut for future industrial applications.

1. Introduction

Cellulose is a linear polysaccharide of glucose units that are linked together by β-1, 4 glycosidic bonds and it is the most abundant biopolymer in the world. These solid wastes consist of 40–50% cellulose, 9–12% hemicellulose, and 10–15% lignin by dry weight (Natamihardja and Herlini 2018). The accumulation of agricultural waste produces environmental problems because it is difficult to eradicate. However, it is now known that cellulose is a potential precursor for the production of renewable fuels in the current global fuel crisis. (Aditiya *et al.* 2016; Song *et al.* 2018). Bioethanol is a renewable clean-biofuel produced by biomass fermentation (Rastogi and Shrivastava 2017). Microorganisms play an important role in cellulase production and sugar fermentation (Zhao *et al.* 2016). Therefore, the selection of suitable microbial strains with high cellulase production for ethanol production is an important (Azhar *et al.* 2017).

Symbiotic microorganisms in the organism's guts (e.g. termites and cattles) are one of the best sources for cellulose-degrading enzymes. Their life depends on the metabolism of cellulose material (Sexana *et al.* 1993). Termites are insects that use lignocellulose precursors as a source of energy, making them ideal targets for a novel cellulose-degrading enzymes (Khademi *et al.* 2002; Scharf and Tartar 2008). Among termites, these insects were more efficient in lignocellulose hydrolysis due to their diverse gut adaptations (Brune 2014). König *et al.* (2013) reported that the Total bacterial counts in the termite

* Corresponding Author
E-mail Address: sirikhwan.t@sci.kmutnb.ac.th

guts ranged from 10^7 to 10^{11} per milliliter (König *et al.* 2013). It has been previously reported that the genus *Citrobacter, Enterobacter, Cellulomonas, Clostridium* and *Bacillus* were isolated from the gut of termites which are considered as cellulolytic bacteria (Varma *et al.* 2021).

Currently, demand for cellulase enzymes accounts for up to 8% of the global enzyme industry (Shweta 2014). For decades, this enzyme have shown their potential in industrial processes such as textiles, food and feed and agriculture industry (Menendez *et al.* 2015). Industrial and laboratory cellulase is commonly obtained from fungi due to its high enzymatic activity. But there are several factors that suggest that the bacteria may have a higher potential. Bacteria have a higher growth rate than fungi resulting in higher rate of enzyme production as well. Therefore, the aims of the present study were to screen cellulase-producing bacteria from termite gut, study the characteristic enzyme (optimum temperature, pH and incubation time) and determine partial purification of crude cellulase from isolated bacteria before identification by 16s rRNA sequencing that could be a possible cellulase source for hydrolysis of cellulosic biomass and bioconversion biofuels.

2. Materials and Methods

2.1. Termite Collection

The soldier caste termites (*Coptotermes* sp.) were collected from Khao Krapak Subdistrict, Tha Yang District, Phetchaburi Province (12°43'05.6"N, 99°45'17.6"E), Thailand on December 2021. Termites were identified and sorted to species level according to the termite identification keys (Ahmad 1965; Kirton and Brown 2003; Bourguignon and Roisin 2011). After that, these termites were surface sterilized in 70% ethanol for 15 min. Then, the termites washed in sterile normal saline solution in triplicate, rinsed with distilled water and air-dried for approximately 1–2 min, respectively. Carboxymethyl cellulose (CMC) and cellulose were used for cellulolytic bacteria isolation from termite guts.

2.2. Enrichment and Isolation Cellulase Producing Bacteria

Ten surface sterilized termites were ground and aseptically transferred into a conical flask containing 25 ml sterile CMC or Cellulose (CMC or Cellulose 10 g/l, yeast extract 3.0 g/l, peptone 1.5 g/l, NaCl 3.5 g/l, $NaNO_3$ 1.0 g/l, KH_2PO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.3 g/l; pH = 7.0) broth. After incubation at 37°C for 3 days, the bacterial strains were isolated on CMC or Cellulose agar medium by 10-fold dilution and spread plate technique. Then, all plates were incubated at 37°C for 24–48 hr with both aerobic and anaerobic condition. All isolated bacteria were purified by re-streaking on nutrient agar containing CMC or cellulose.

2.3. Qualitative Screening of Cellulolytic Bacteria

The overnight culture of all the pure isolates were spotted on CMC or cellulose agar plates. After incubation at 37°C for 1–2 day, all plates were flooded with 1% congo red solution for 15–20 min and then de-stained with 1M NaCl solution for 10–15 min. Colonies showing a clear area in the otherwise congo red stained background on CMC or cellulose agar plates were considered as cellulase positive. For evaluating cellulose hydrolysis, the hydrolysis capacity of CMC/cellulose (HC value) was calculated from the ratio of the clear zone diameter to the colony diameter. The ratios considered to have high activity ranged from 3.1–4.0, while moderate activity ratios ranged from 2.1–3.0 (Kanokkanjana and Garivait 2013). Pure cultures of these microorganisms were maintained at 4°C.

2.4. Morphological and Biochemical Characterization

Morphology in macroscopy on agar plates was observed. The morphology and color of the cells were observed under a 100x magnification microscope after gram staining technique. For biochemical characterization, the selected strains were characterized using API 20E Kit (Biomerieux, France) and then studied oxidase and catalase tests.

2.5. 16S rRNA Sequencing and Sequence Analysis

Only three effective isolates were selected for genomic DNA extraction and 16S rRNA gene analysis. Genomic DNA was isolated using GF-1 Bacterial DNA Extraction Kit (Vivantis) following manufacturer's instructions. The universal primers used were 27F (5′-AGA GTTTGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTTGTT ACG ACT T T-3′). The condition for amplification reactions was as follows: 95°C for 3 min, 50 cycles of 45 sec at 95°C for 30 sec, 62°C and 72°C and final extension at 72°C for 7 min. The amplicons

were purified using a QIAquick PCR purification kit (QIAGEN, USA) after amplification. The nucleotide sequencing of 16S rDNA was determined with automated sequencing. Sequence analysis were aligned by the BLAST program at http://www.ncbi.nlm.nih.gov/genbank. Finally, phylogenetic tree was built by neighbor-joining method at 1,000X bootstraps with MEGA X programme (Thompson *et al.* 1997; Kumar *et al.* 2018).

2.6. Cellulase Activity Assay

For quantitative assay, the isolated strains were inoculated into 50 ml of CMC or cellulose broth and incubated under different incubation conditions. The culture broth of isolated strains (incubation for 24 hr at 37°C) was centrifuged at 8,000×g for 15–20 min to separate the bacterial cells. The culture supernatants were collected for quantitative assay. Cellulase activity was calculated by measuring the reducing sugar content by dinitrosalicylic acid (DNS) method (Miller 1959) using glucose as the standard. A mixture of 0.5 ml of a crude cellulase solution, 0.5 ml of 50 mM sodium phosphate buffer (pH 7.0) or 50 mM sodium citrate buffer (pH 5.0) and 0.5 ml of 1% CMC or cellulose was incubated at 37°C for 30–45 min. After that, the mixture is shaken with vortex for 10 sec after adding 1.5 ml of DNS reagent. Then, the solution is heated in boiling water for 15 min and cooled in ice water, respectively. The absorbance of the mixture reaction were read at 540 nm wavelength. Concentration of glucose is calculated by standard curve. Cellulase activity was calculated by using the formula; Enzyme Activity (Unit/ml) = [(X × D)/(M × t × V), X = Glucose content compared to the standard curve (µg), D = dilution factor of enzyme, M = 180.156 µg/µmol, V = volume of used enzyme (ml) and t = incubation time (minutes). The obtained enzyme activity was expressed in Units/ml, one unit of cellulase activity is a number of enzymes that produce 1 µmol of reducing sugar (glucose) per minute under certain conditions. The total protein concentration was determined by the Bradford assay using bovine serum albumin as standard. The enzyme-specific activities were calculated by using the formula; Specific Activity (Unit/mg protein) = Enzyme Activity/Protein Level.

2.7. Optimization of Various Parameters for Determination of Maximum Cellulase Activity

Various parameters were optimized for cellulase production. For the optimization of temperature, experiment was performed at 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C. To study the effect of pH on cellulase enzyme activity, 50 mM sodium citrate (pH 5.0–6.0), 50 mM potassium phosphate (pH 7.0), 50 mM Tris HCl (pH 8.0–9.0) were prepared. To determine appropriate incubation period, bacterial cultures were grown in nutrient broth supplemented with 1% CMC at the optimum temperature and pH were taken out at various time intervals. The enzymatic activity was determined.

2.8. Purification of Cellulases by Ammonium Sulfate

The crude enzyme (supernatant) of selected strain was precipitated overnight with 80% ammonium sulphate ($(NH_4)_2SO_4$) at 8,000×g for 20 min at 4°C. The precipitated cellulase was resuspended in 0.1M phosphate buffer (pH 7.0) and dialyzed using dialysis tube (pore size <10 kDa) in same buffer. The recovered cellulase was dissolved in fresh buffer and then evaluated the enzyme activity and protein content.

2.9. Statistical Analysis

All data were presented the mean ± SD (n = 9) and analyzed by Graphpad prism 9.0 software using one-way analysis of variance (ANOVA) and followed by Tukey's multiple comparisons ($\alpha = 0.05$)

3. Results

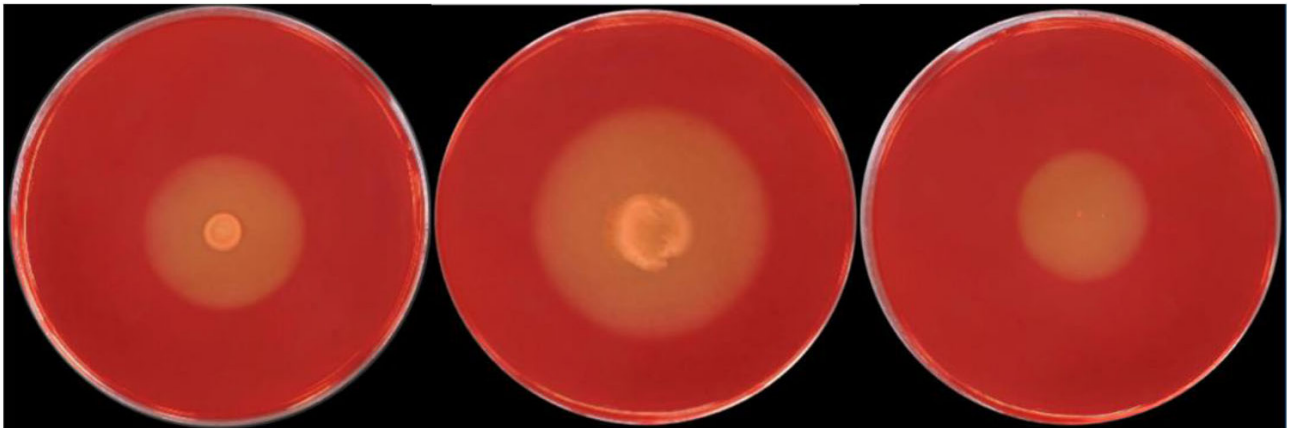
3.1. Isolation of Bacteria and Primary Screening for Cellulase

A total of 48 isolated bacteria were isolated from the termite guts. The isolates were further screened for their cellulolytic abilities. The cellulolytic index was used to test the cellulolytic potential of the positive isolates. Cellulolyic index and cellulase activity of isolated bacteria after 24 hr of incubation shown in the Table 1. The result indicated that fifteen bacterial cultures presented the growth on nutrient agar supplemented with CMC congo-red for producing clear zone. All of them were considered as active cellulase bacteria (31.25% of the isolated strains) on CMC agar. The HC values of the cellulolytic bacteria ranged from 1.25±0.00–4.75±0.20. Isolate CM 1-3 showed significantly maximum HC of 4.75±0.20, followed by isolate CE 5-1 (HC value of 4.67±0.24) and CM 5-1 (HC value of 2.51±0.60), respectively ($p \leq 0.05$) (Figure 1).

Table 1. Cellulolyic index and cellulase activity of isolated cellulolytic gut bacteria after 24 hr of incubation

Isolates	Primary screening			Secondary screening	
	Diameter of colony (mm)	Diameter of cellulolytic zone (mm)	Cellulolytic index (level)	Cellulase activity (U/ml)	Specific activity (U/mg)
CM 1-2	9.00±0.00	16.00±0.82^{e}	1.78±0.09^{g} (2)	2.798±0.011^{hi}	0.181±0.001^{f}
CM 1-3	4.00±0.00	19.00±0.82^{c}	4.75±0.20^{ab} (3)	7.198±0.028^{b}	0.522±0.002^{b}
CM 2-1	11.00±0.00	18.67±0.94^{c}	1.70±0.09^{gh} (2)	2.944±0.013^{h}	0.168±0.001^{h}
CM 2-2	12.00±0.00	20.67±0.47^{b}	1.72±0.04^{gh} (2)	2.750±0.023^{hi}	0.169±0.001^{h}
CM 2-3	6.00±0.00	12.00±0.00^{f}	2.00±0.00^{f} (2)	3.125±0.013^{g}	0.225±0.001^{e}
CM 3-1	10.00±0.00	17.67±0.94^{d}	1.77±0.09^{g} (2)	2.842±0.014^{h}	0.174±0.001^{gh}
CM 3-2	12.00±0.00	20.67±0.47^{b}	1.72±0.04^{gh} (2)	2.717±0.032^{hi}	0.167±0.002^{h}
CM 4-2	10.00±0.00	19.67±0.47^{c}	1.97±0.05^{f} (2)	3.052±0.017^{g}	0.220±0.002^{e}
CM 5-1	11.67±2.36	28.00±2.83^{a}	2.51±0.60^{d} (2)	6.880±0.021^{b}	0.462±0.002^{c}
CE 2-2	4.00±0.00	8.00±0.00^{h}	2.00±0.09^{f} (2)	3.112±0.030^{g}	0.193±0.002^{f}
CE 3-2	8.67±0.94	21.67±1.25^{b}	2.50±0.94^{d} (2)	5.354±0.017^{d}	0.323±0.001^{d}
CE 3-3	4.33±0.47	11.33±0.94^{f}	2.63±0.26^{c} (2)	5.718±0.021^{c}	0.392±0.001^{d}
CE 4-1	4.00±0.00	6.67±0.47^{i}	1.67±0.12^{hi} (2)	2.641±0.007^{i}	0.192±0.000^{f}
CE 4-2	4.00±0.00	5.00±0.00^{j}	1.25±0.00^{j} (1)	3.410±0.008^{f}	0.253±0.001^{e}
CE 5-1	4.00±0.00	18.67±0.94^{c}	4.67±0.24^{b} (3)	7.533±0.013^{a}	0.548±0.000a

^{abcdefghij}: Values on the same column with different superscripts were significantly different (p≤0.05)



A B C

Figure 1. Hydrolyzing zones of bacterial strains on agar plates containing CMC or cellulose after Congo-red staining. (A) Isolate CM 1-3, (B) Isolate CM 5-1, and (C) Isolate CE 5-1

3.2. Secondary Screening for Cellulase

The cellulase activity was determined by incubating the CMC agar plates at 37°C for 24 hr with culture medium of isolated bacteria. In the present study, The isolate CE 5-1 (7.533±0.013 U/ml) were significantly revealed the highest cellulose activity, followed by the isolate CM 1-3 (7.198±0.028 U/ml) and CM 5-1 (6.880±0.021 U/ml) with 0.548±0.000 U/mg, 0.522±0.002 U/mg and 0.462±0.002 U/mg of specific enzyme activity after 24 hr of cultivation, respectively (p≤0.05) (Table 1). Therefore, these isolates (CM 1-3, CM 5-1 and CE 5-1) were chosen for further studies of their enzymatic assay.

3.3. Characterization and Identification of Bacteria

After secondary screening of cellulase activity, only 3 isolated strains were selected for further studies because they had high potential the cellulase and specific activity in the first 3, including isolates CM 1-3, CM 5-1 and CE 5-1. The morphological and biochemical characteristic of the three isolates are presented in Table 2. Based on 16s rRNA sequencing, these cellulose-degrading bacterial strains were closely similar to 3 genus; *Citrobacter, Bacillus* and *Streptococcus*. Isolates CM 1-3, CM 5-1 and CE 5-1 were identified as *Citrobacter amalonaticus*

Table 2. Morphological and biochemical characteristics of cellulose-degrading bacteria

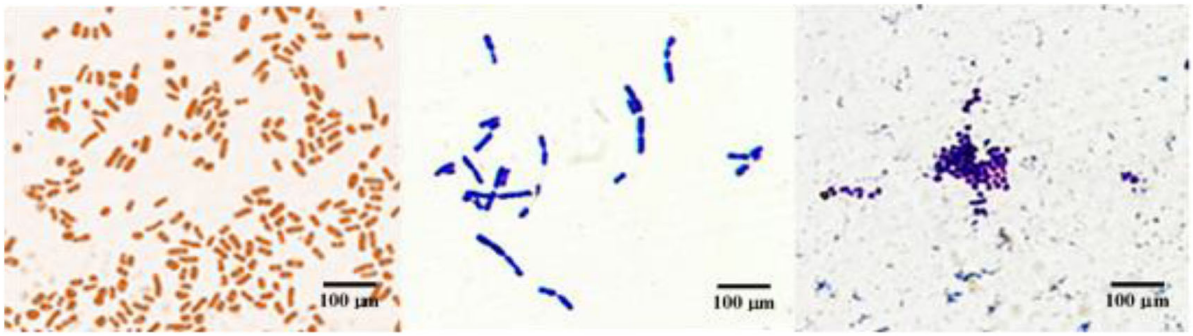
Characteristics	Isolated strains		
	CM 1-3 (M8)	CM 5-1 (M3)	CE 5-1 (E4)
Shape	Rod	Rod	Cocci
Gram staining	Negative	Positive	Positive
Oxidase	-	-	-
Catalase	+	+	-
β-galactosidase	+	+	+
Arginine dehydrolase	+	+	+
Lysine decarboxylase	+	-	+
Ornithine decarboxylase	+	+	+
Citrate utilization	+	+	+
H_2S production	-	-	-
Indole production	+	+	+
Acetoin production	-	+	+
Urease	+	-	-
Deaminase	+	+	+
Gelatinase	-	+	+
Acid fermentation:			
Glucose	-	+	+
Mannitol	-	+	+
Inositol	-	-	+
Sorbitol	-	-	-
Rhamnose	-	+	-
Sucrose	-	-	-
Melibiose	-	-	+
Amygdalin	+	+	+
Arabinose	-	+	+
Growth temperature range	30-40°C; optimum at 37°C	30-50°C; optimum at 37°C	30-40°C; optimum at 37°C
Growth pH range	5-9; optimum at 7	5-9, optimum at 7	5-8; optimum at 7

+: positive reaction, -: negative reaction

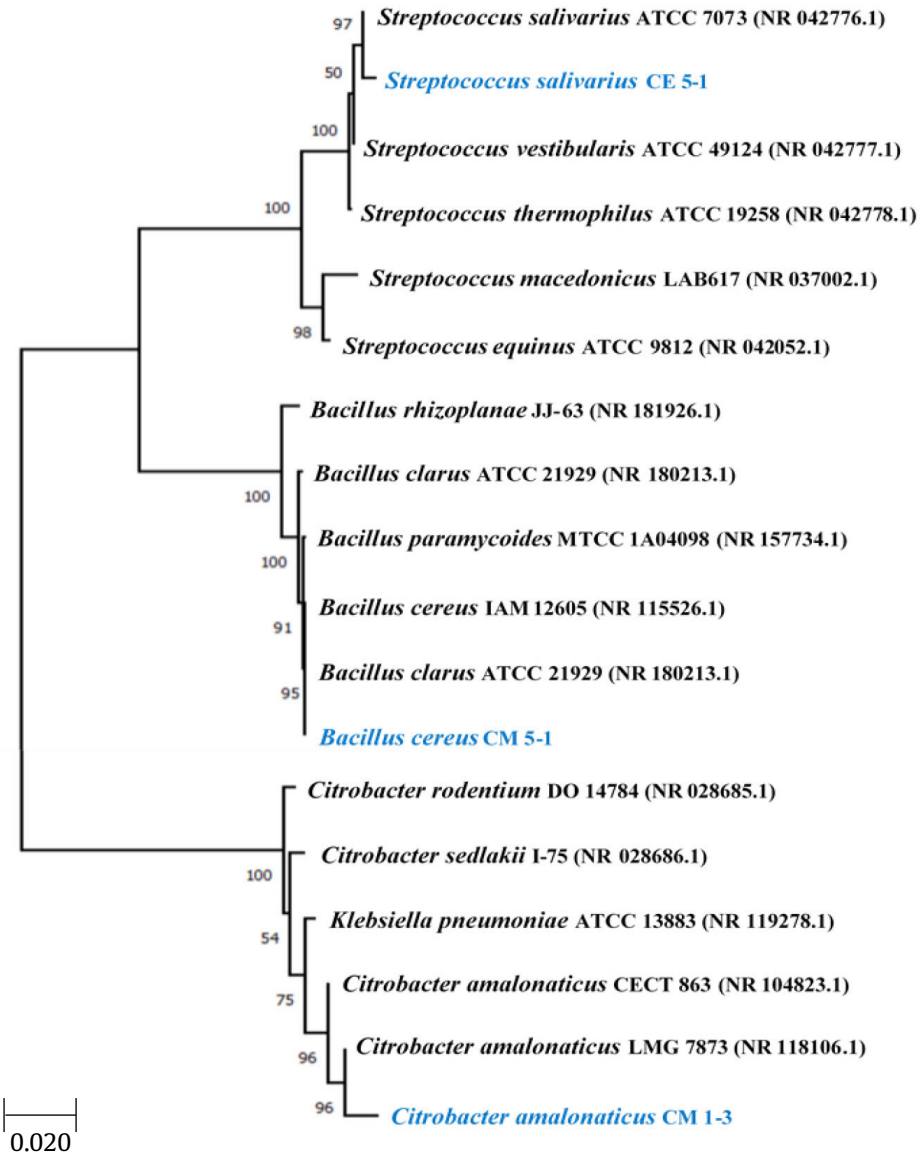
LMG 7873 (Accession No. NR_118106.1 with 97.2% identity), *Bacillus cereus* IAM 12605 (Accession No. NR_115526.1 with 98.31% identity) and *Streptococcus salivarius* ATCC 7073 (Accession No. NR_042776.1 with 98.37% identity), respectively. *Citrobacter amalonaticus* CM 1-3 was Gram-negative bacilli bacterium (Figure 2A) while, *Bacillus cereus* CM 5-1 was Gram-positive bacilli bacterium (Figure 2B). Both strains showed negative and positive reaction for oxidase and catalase. For *Streptococcus salivarius* CE 5-1 was Gram-negative cocci bacterium (Figure 2C) and negative reaction for oxidase and catalase. The phylogenetic tree of the three cellulolytic bacteria using software MEGA X with Neighbor-Joining method at 100,000 bootstrap replications is shown in Figure 2D.

3.4. Effect of Temperature, pH and Incubation Time on Cellulase Production by Isolated Strains

Three potential cellulose-degrading bacteria (isolates CM 1-3, CM 5-1 and CE 5-1) in this study were examined the different parameters for optimal cellulase production. The enzyme activities of potential isolates at various parameters are shown in Table 3. *Bacillus cereus* CM 5-1 significantly showed maximum cellulase activity at 40°C (3.092±0.010 U/ml and 0.478±0.002 U/mg) ($p \leq 0.05$) whereas, *Citrobacter amalonaticus* CM 1-3 (2.937±0.008 U/ml and 0.432±0.001 U/mg) and *Streptococcus salivarius* CE 5-1 (3.044±0.008 U/ml and 0.466±0.001 U/mg) showed maximum enzyme activity at 35°C. (Figure 3A). The low enzyme activity of *B. cereus* CM 5-1



A B C



D

Figure 2. Morphologies of isolated bacteria from termite gut under microscope (1,000×), CM 1-3 (A; short rod); CM 5-1 (B; rod); CE 5-1 (C; cocci) and Phylogenic tree of cellulolytic bacteria (D)

Table 3. Optimization of parameters for cellulase production of isolated strains

Parameters	Values	*Citrobacter amalonaticus* CM 1-3 (U/mg)	*Bacillus cereus* CM 5-1 (U/mg)	*Streptococcus salivarius* CE 5-1 (U/mg)
Temperature (°C)	30	0.384±0.001^{b}	0.394±0.003^{a}	0.299±0.002^{b}
	35	0.432±0.001^{a}	0.403±0.001^{a}	0.466±0.001^{a}
	40	0.290±0.001^{bc}	0.478±0.002^{a}	0.298±0.002^{b}
	45	0.236±0.003^{c}	0.392±0.002^{a}	0.208±0.003^{b}
	50	0.103±0.002^{d}	0.249±0.003^{b}	0.092±0.002^{c}
	55	0.082±0.002^{d}	0.238±0.003^{b}	0.072±0.002^{c}
	60	0.054±0.002d	0.175±0.003^{b}	0.055±0.002^{c}
pH	5	0.222±0.002^{c}	0.235±0.002^{b}	0.231±0.002^{c}
	6	0.384±0.002^{ab}	0.400±0.001^{a}	0.411±0.002^{a}
	7	0.484±0.004^{a}	0.493±0.002^{a}	0.494±0.002^{a}
	8	0.382±0.002^{b}	0.401±0.001^{a}	0.373±0.002^{ab}
	9	0.266±0.002^{c}	0.288±0.003^{b}	0.274±0.003^{bc}
Incubation period (hr)	0	0.000±0.000	0.000±0.000	0.000±0.000
	6	0.063±0.006^{c}	0.059±0.010^{d}	0.062±0.005^{d}
	12	0.427±0.006^{b}	0.425±0.003^{bc}	0.418±0.010^{bc}
	24	0.480±0.002^{ab}	0.473±0.002^{ab}	0.489±0.004^{b}
	48	0.542±0.001^{a}	0.570±0.001^{a}	0.522±0.005^{a}
	72	0.379±0.002^{b}	0.346±0.002^{c}	0.328±0.003^{c}

^{abcd}: Values on the same column with different superscripts were significantly different in each experiment (p≤0.05)

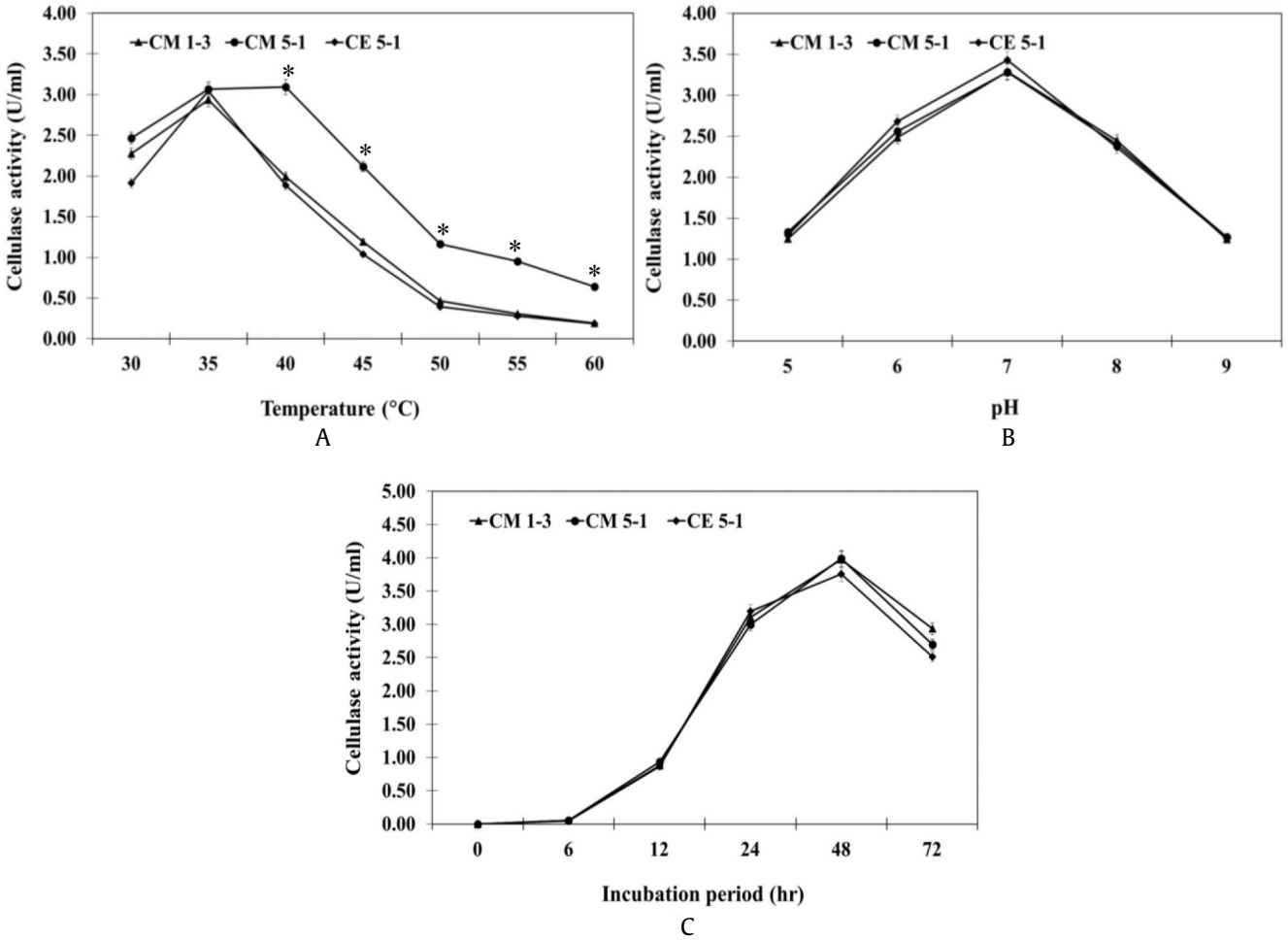


Figure 3. Effect of temperature, pH and incubation period on cellulase production by the isolated strains. *: p≤0.05 by paired t-test comparing with all isolated strains

occurs during a decrease in temperature below 35°C and an increase in temperature above 45°C. The enzyme activity of *Citrobacter amalonaticus* CM 1-3 and *Streptococcus salivarius* CE 5-1 was declined at temperatures below 35°C and above 40°C. The temperature profiles demonstrated that cellulase activity of *Bacillus cereus* CM 5-1 was retained at 35 – 40°C whereas cellulase activity of other both selected strains was stable at 35°C.

Like temperature, pH is also one of the important factors that affects enzyme yield. All isolates showed maximum cellulase activity at pH 7.0 with 3.284±0.010–3.428±0.012 U/ml. The low enzyme activity in all isolated strains occurs during the pH below or above 7.0. (Figure 3B). On increasing the pH level from 7.0 to 9.0 or decreasing the pH level 7.0 to 5.0, the enzyme activity was progressively reduced. Similarly, different incubation periods showed altering in cellulase production. The optimum incubation time for cellulase production all the isolates was observed at 48 hr with enzyme activity of 3.757±0.034–3.991±0.010 U/ml. Thereafter, the cellulase activity was significantly reduced after 48 h of cultivation ($p \leq 0.05$) (Figure 3C).

3.5. Purification of Crude Cellulase

Based on effect of temperature, pH and incubation time on cellulase production, *Bacillus cereus* CM 5-1 showed the potential cellulase production at optimum parameters. Moreover, cellulase enzyme of this strains was stable at temperature 35–40°C and exhibited the more enzyme activity than other strains at 45°C. These characteristic was the suitable for applying in bioethanol and/or industrial processes. Therefore, *B. cereus* CM 5-1 was selected to purify the crude cellulase. The result showed that crude of extracellular cellulases of *B. cereus* CM 5-1 with maximum activity against CMC was initially purified by ammonium sulfate precipitation (salting in) and dialysis (salting out). The crude enzyme and cellulase activity was recovered in a fraction of 80% $NH_4(SO_4)_2$. The fraction had the total protein of 64.907±1.40 mg/ml and 82.49±0.52 U/mg of specific activity with 74.38% recovery and 1.58 purification folds (Table 4).

4. Discussion

The intestines of termites (*Odontotermes* sp.) are potential source for isolating cellulose-degrading bacteria. This study represented the presence of cellulose-degrading bacteria in termites to degrade carboxymethyl cellulose or cellulose supplements in the agar medium by congo-red assay, indicating their potential in cellulose degradation due to cellulase production. In several studies were chosen congo-red staining to screen cellulose-degrading microorganisms. Although the relationship between the diameter of the hydrolyzed zone and the enzyme concentration was considered, But this relationship could not reveal the enzyme-producing capacity of the microorganisms. (Teather and Wood 1982; Manzum and Al Mamun 2018). In the current study, some strains show large transparent zones, the cellulase activity produced by them was not consistent with HC values in all isolated strains. Thus, cellulase activity of isolated strains was further assessed with DNS assay.

The cellulolytic bacteria have been isolated by various researchers from termite gut. In present study, the potential cellulase-producing bacteria were *Bacillus cereus* CM 5-1, *Streptococcus salivarius* CE 5-1 that were identified by 16s rRNA sequceing. In a previous study, Shinde *et al.* (2017) reported that the cellulolytic bacteria from termite guts belonged to genera *Pseudomonas, Klebsiella, Salmonella, Serratia, Enterobacter Acinetobacter, Bacillus, Citrobacter, Paenibacillus*, *Paenibacillus* and *Serratia* (Shinde *et al.* 2017; Boontanom and Chantarasir 2021). Sharma *et al.* (2015) and Afzal *et al.* (2019) have isolated *Enterobacter cloacae* and *Bacillus licheniformis* from termites gut, respectively (Sharma *et al.* 2015; Afzal *et al.* 2019).

The cellulase activity decrease when the initial pH and incubation temperature were not optimal. The cellulase activity of *B. cereus* CM 5-1 has optimum temperature and pH of at 35–40°C and pH for 48 hr of cultivation with enzyme activity 3.991±0.010 U/ml. Whereas, the optimum temperature, pH and incubation period of *C.*

Table 4. Purification of cellulase from isolated *Bacillus cereus* CM 5-1

Purification steps	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	7198.00±2.03	138.00±1.58	52.16±0.26	1.00	100.00
Precipitation $(NH_4)_2SO_4$	5354.00±1.70	64.907±1.40	82.49±0.52	1.58	74.38

amalonaticus CM 1-3 and *S. salivarius* CE 5-1 were at 35°C, pH 7.0 for 48 hr. Jamila *et al.* (2018) and Islam *et al.* (2019) have been reported that cellulases of *Citrobacter* and *Bacillus* sp. were stable at 25°C and 45°C, respectively (Jamila *et al.* 2018; Islam *et al.* 2019). Decreased levels of cellulase production may be possibly due to lower transport of substrates through the cells at lower temperatures, resulting in a decline in the productivity of the enzyme (Rehman and Elahi 2018). As the temperature increased, enzyme production is reduced, because the high temperature can alter membrane composition and cause protein catabolism and inhibit microbial growth (Nehad *et al.* 2019). Change in pH of medium decreased cellulase activity because the microorganism require a neutral pH to grow and produce the cellulase. The reason for reducing enzyme production at higher pH was probably due to the inhibition of cellulase proteolysis (Nehad *et al.* 2019). There have been several studies on acid stable cellulases from *Bacillus* sp. (Mawadza *et al.* 2000; Islam *et al.* 2019). However, there are only a few reports of thermo-neutral stable cellulases from *Bacillus* species (Afzal *et al.* 2019). For cellulase from *Citrobacter* sp., it have been performed on acid (pH 5.5) stable cellulases from *Citrobacter* sp. (Jamila *et al.* 2018) while, cellulase of *Citrobacter amalonaticus* CM 1-3 was stable at pH 7.0.

From this study, the maximum cellulase activity of isolate *B. cereus* CM 5-1 was observed at temperature 40°C (stability at 35°C to 40°C), pH 7.0 and after 48 hr of incubation period while, *C. amalonaticus* CM 1-3 and *S. salivarius* CE 5-1 showed the optimum temperature of cellulase activity at 35°C, pH 7.0 after 48 hr of incubation period (Figure 3). Cellulase biosynthesis of *C. amalonaticus* CM 1-3 and *S. salivarius* CE 5-1 was significantly declined with the increase in the incubation temperature above 40°C. Cellulase production of three selected strains tended to increase from the 6th hr of incubation period and decreased after the 48th hr of incubation period. However, *Bacillus cereus* CM 5-1 was the potential bacteria for application in industrial process due to thermostable cellulase producing. The optimized enzyme activity value of *B. cereus* CM 5-1 (3.991±0.010 U/ml at 40°C, pH 7.0 for 48 hr) obtained in this study is much higher than the reported values with previous study. Deka *et al.* (2011) and Boontanom *et al.* (2021) reported the highest cellulase production (0.26 U/ml and 0.43 U/ml) of *Bacillus* sp. when the culture was grown on nutrient agar containing 1% CMC with enzyme activity of 0.43 and 2.190 U/ml, respectively (Deka *et al.* 2011; Boontanom *et al.* 2021). Crude cellulase of *B. cereus* CM 5-1 were also performed on partial purification by ammonium sulphate precipitation and dialysis for evaluating the cellulase activity. However, cellulase enzyme from this bacterial strain should be purified by chromatography technique before application in food industrial and/or bioethanol production in the future.

Acknowledgements

The authors would like to thank Energy Conservation Promotion Fund (Confirmation letter No. KorThoOr. 64-03-0117), Thailand for financial support. We would like to thank to the Institute of Technological Development for Industry (ITDI), King Mongkut's University of Technology North Bangkok for coordinating and clarifying several points in this fund.

References

Aditiya, H.B., Mahlia, T.M.I., Chong, W.T., Nur, H., Sebayang, A.H., 2016. Second generation bioethanol production: a critical review. *Renew. Sustain. Energy Rev.* 66, 631–653. https://doi.org/10.1016/j.rser.2016.07.015

Afzal, M., Qureshi, M. Z., Khan, S., Khan, M. I., Ikram, H., Ashraf, A., Qureshi, N.A., 2019. Production, purification and optimization of cellulase by *Bacillus licheniformis* HI-08 isolated from the hindgut of wood-feeding termite. *Intl. J. Agric. Biol.* 21, 125-134. https://doi.org/10.17957/IJAB/15.0872

Ahmad, M., 1965. Termites (Isoptera) of Thailand. *Bulletin of the AMNH.* 131, 1–113.

Azhar, S.H.M., Abdulla, R., Jambo, S.A., Marbawi, H., Gansau, J.A., Faik, A.A.M., Rodrigues, K.F., 2017. Yeasts in sustainable bioethanol production: a review. *Biochem. Biophys. Rep.* 10, 52–61. https://doi.org/10.1016/j.bbrep.2017.03.003

Boontanom, P., chantarasir, A., 2021. Diversity and cellulolytic activity of culturable bacteria isolated from the gut of higher termites (*Odontotermes* sp.) in eastern Thailand. *Biodivers.* 22, 3349–3357. https://doi.org/10.13057/biodiv/d220831

Bourguignon, T., Roisin, Y., 2011. Revision of the termite family Rhinotermitidae (Isoptera) in New Guinea. *ZooKeys.* 148, 55–103. https://doi.org/10.3897/zookeys.148.1826

Brune, A., 2014. Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev. Microbiol.* 12, 168–180. https://doi.org/10.1038/nrmicro3182

Deka, D., Bhargavi P., Sharma, A., Goyal, D., Jawed, M., Goyal, A., 2011. Enhancement of cellulase activity from a new strain of Bacillus subtilis by medium optimization and analysis with various cellulosic substrates. *Enzyme Res.* 2011, 1–8. https://doi.org/10.4061/2011/151656

Islam, M., Sarka, P.K., Mohiuddin, A.K.M., Suzauddula, Md., 2019. Optimization of fermentation condition for cellulase enzyme production from *Bacillus* sp. *Malays. J. halal Res.* 2, https://doi.org/19–24. 10.2478/mjhr-2019-0009

Jamila, A.D.J., Kerr, R.G., Haltli, B.A., Tinto, W.F., 2018. Temperature and pH effect on glucose production from pretreated bagasse by a novel species of Citrobacter and other bacteria. *Heliyon*. 4, e00657. https://doi.org/10.1016/j.heliyon.2018.e00657

Kanokkanjana, K., Garivait, S., 2013. Alternative rice straw management practices to reduce field open burning in Thailand. *Int. J. Environ. Sci. Technol.* 4, 119–123. https://doi.org/10.7763/IJESD.2013.V4.318

Khademi, S., Guarino, L.A., Watanabe, H., Tokuda, G., Meyer, E.F., 2002. Structure of an endoglucanase from termite, *Nasutitermes takasagoensis. Acta. Crystallogr. D Biol. Crystallogr*. 58, 653–659. https://doi.org/10.1107/s0907444902002366

Kirton, L.G., Brown, V.K., 2003. The taxonomic status of pest species of Coptotermes in Southeast Asia: resolving the paradox in the pest status of the termites, Coptotermes gestroi, *C. havilandi* and *C. travians* (Isoptera: Rhinotermitidae). *Sociobiology*. 42, 43–63.

König, H., Li, L., Fröhlich, J., 2013. The cellulolytic system of the termite gut. *Appl. Microbiol. Biotechnol.* 97, 7943–7962. https://doi.org/10.1007/s00253-013-5119-z

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Manzum, A.M., Al Mamun, Md.A., 2018. Isolation of *Bacillus* spp. bacteria from soil for production of cellulase. *Nepal J. Biotechnol.* 6, 57–61. https://doi.org/10.3126/njb.v6i1.22338

Mawadza, C., Hatti-Kaul,R., Zvauya, R., Mattiasson, B., 2000. Purification and characterization of cellulases produced by two *Bacillus strains. J. Biotechnol.* 83, 177–187. https://doi.org/10.1016/s0168-1656(00)00305-9

Menendez, E., Garcia-Fraile, P., Rivas, R., 2015. Biotechnological applications of bacterial cellulases. *AIMS Bioeng.* 2, 163–182. https://doi.org/10.3934/bioeng.2015.3.163

Miller G.L., 1959. Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, 426–428. https://doi.org/10.1021/ac60147a030

Natamihardja, Y., Herlini, H., 2018. Isolation and identification of cellulolytic bacteria from termites gut (*Cryptotermes* sp.). *J. Phys.: Conf. Ser.* 1013, 012173. https://doi.org/10.1088/1742-6596/1013/1/012173

Nehad, E.A., Yoness, M.F., Reem, A.A., 2019. Optimization and purification of cellulase produced by *Penicillium decumbens* and its application. *Egypt. Pharm. J.* 18, 391–402. https://doi.org/10.4103/epj.epj_31_19

Rastogi, M., Shrivastava, S., 2017. Recent advances in second generation bioethanol production: an insight to pretreatment, saccharification and fermentation processes. *Renewable Sustainable Energy Rev*. 80, 330–340. https://doi.org/10.1016/j.rser.2017.05.225

Rehman, A., Elahi, A., 2018. Bioconversion of hemicellulosic materials into ethanol by yeast, *Pichia kudriavzevii* 2–KLP1, isolated from industrial waste. *Rev Argent Microbiol.* 50, 417–425. https://doi.org/10.1016/j.ram.2017.07.008

Scharf, M.E., Tartar, A., 2008. Termite digestomes as sources for novel lignocellulases. *Biofuels Bioprod. Biorefining*. 2, 540–552. https://doi.org/10.1002/bbb.107

Sexana, S., Bahadur, J., Varma, A., 1993. Cellulose and hemicellulose degrading bacteria from termite gut and mound soils of India. *Ind. J. Microbiol.* 33, 55–60.

Sharma, S., Chatterjee, S., Datta, S., Prasad, R.K., Sharma, A., Dubey, D., Vairale, M.G., Veer, V., 2015. Isolation and characterization of cellulose degrading bacteria of termite gut from north eastern region of India. *South. Asian J. Exp. Biol.* 5, 283–290. https://doi.org/10.38150/sajeb.5(6).p283-290

Shinde, V.S., Agrawal, T., Kotasthane, A.S., 2017. Molecular characterization of cellulolytic bacteria derived from termite gut and optimization of cellulase production. *Int. J. Curr. Microbiol. App. Sci*. 6, 2474–2492. https://doi.org/10.20546/ijcmas.2017.610.292

Shweta, A., 2014. Cellulases of bacterial origin and their applications: a review. *Int. J. Sci. Res.* 3, 1652–1655.

Song, B., Li, B., Wang, X., Shen, W., Park, S., Collings, C., Feng, A., Smith, S. J., Walton, J.D., Ding, S.Y., 2018. Real-time imaging reveals that lytic polysaccharide monooxygenase promotes cellulase activity by increasing cellulose accessibility. *Biotechnol. Biofuels*. 11, 1–11. https://doi.org/10.1186/s13068-018-1023-1

Teather, R. M., Wood, P. J., 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* 43, 777–780. https://doi.org/10.1128/aem.43.4.777-780.1982

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic. Acids Res.* 25, 4876–4882. https://doi.org/10.1093/nar/25.24.4876

Varma, Y., Chovatiya, S., Patel, D., Ingle, S., Daxini, N., Lal, S., 2021. Media optimization and saccharification of lignocellulosic waste by cellulolytic bacteria isolated from gut of a termite. *Int. J. Biotech. Trends Technol.* 11, 8–14. https://doi.org/10.14445/22490183/IJBTT-V11I2P602

Zhao, X., Xiong, L., Zhang, M., Bai, F., 2016. Towards efficient bioethanol production from agricultural and forestry residues: exploration of unique natural microorganisms in combination with advanced strain engineering. *Bioresour. Technol.* 215, 84–91. https://doi.org/10.1016/j.biortech.2016.03.158