# Novel Halotolerant Cellulolytic *Bacillus methylotrophicus* RYC01101 Isolated from Ruminant Feces in Thailand and its Application for Bioethanol Production

***Aiya Chantarasiri****
*Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok (Rayong Campus), Rayong, Thailand*

* Corresponding author. E-mail: aiyac@kmutnb.ac.th
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***Abstract***
*One impediment of large-scale biofuel production from lignocellulosic biomass is the insufficiency of cellulolytic microorganisms that can overcome extreme conditions during the industrial process. This study emphasized the isolation of a novel efficient cellulolytic bacterium. A new Bacillus methylotrophicus RYC01101, isolated from ruminant feces in Thailand, produced a hydrolysis capacity greater than that of known cellulolytic bacteria (Cellulomonas sp.). Cellulase activities were investigated on CMCase activity and FPase activity by 0.230±0.004 and 0.080±0.007 U/mL, respectively. B. methylotrophicus RYC01101 was co-cultured with Saccharomyces cerevisiae TISTR 5111 for bioethanol production. The productivity of the bioethanol was 1.38±0.40 g/L after 120 hours of fermentation. Moreover, B. methylotrophicus RYC01101 could be grown in the presence of 10% (w/v) NaCl, which could be applied in the pretreatment step of biofuel production. This study was the first report on cellulolytic activity and the halotolerant ability of B. methylotrophicus.*

***Keywords***: *Bacillus methylotrophicus, Cellulolytic bacteria, Ruminant feces, Cellulase, Biofuel*

## 1 Introduction

Synergistic increases in fossil fuel demands, its rising costs and national concern for global climate change have shifted efforts to utilize renewable and more sustainable energy as a substitution for fossil fuels [1-3]. Lignocellulosic biomass or plant biomass is a renewable material comprised of mainly cellulose, hemicellulose and lignin. Cellulose, the main component of lignocellulosic biomass, consists of between several hundred to over ten thousand D-glucose molecules connected by β-1, 4 linkages [3,4]. The cellulase complex (i.e., exoglucanase; EC 3.2.1.91, endoglucanase; EC 3.2.1.4 and β-D-glucosidase; EC 3.2.1.21) from cellulolytic microorganisms has been suggested as a sustainable strategy in biotechnological conversion of cellulose into glucose and reducing sugar for the production of biofuels [5] such as bioethanol and biobutanol. The bottleneck of this approach is the lack of efficient cellulase enzymes and cellulolytic microorganisms that can resist extreme environments during the industrial process. The chance to overcome this challenge will depend on the development of novel cellulase-producing microorganisms using a traditional microbiological isolation technique in combination with a molecular engineering technique.

In this study, cellulolytic bacteria were isolated from Thai ruminant feces to indicate a novel bacterium for use in biotechnological conversion applications.

## 2 Materials and Methods

### 2.1 *Chemicals, culture media and microorganisms*

The chemicals used in this study were of analytical or microbiological grade, purchased from Sigma-Aldrich (MO, USA), Calbiochem (Darmstadt, Germany), Merck (Darmstadt, Germany) and Univar (NSW, Australia). Culture media and constituents were purchased from Becton, Dickinson and Company (MD, USA) and HiMedia (Mumbai, India). *Cellulomonas* sp. TISTR 784, *Escherichia coli* TISTR 780 and *Saccharomyces cerevisiae* TISTR 5111 were procured from the TISTR Culture Collection, Thailand Institute

of Scientific and Technological Research (TISTR) (Pathum Thani, Thailand).

### 2.2 *Sample collection and isolation of bacteria*

Fecal samples of ruminant organisms were obtained from buffaloes, cows, horses, goats and sheep. Samples were aseptically collected from Rayong Province, on the east coast of Thailand. The samples were suspended and serially diluted with sterile water within a few hours of collection to obtain $1:10^6$ dilutions. An aliquot of each diluted sample was spread on the surface of nutrient agar (HiMedia) and incubated at 38°C, the average normal rectal temperature of ruminants [6], for 48 hours. Morphologically dissimilar colonies of bacteria were picked and streaked onto the surface of nutrient agar to obtain pure cultures.

### 2.3 *Screening of cellulolytic bacteria*

The screening of cellulolytic bacteria on agar plates was determined by Gram's iodine staining method, as previously described [7]. Overnight growth culture in the nutrient broth (HiMedia) of each isolate was spot plated on carboxymethylcellulose (CMC) agar (0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose sodium salt, 0.02% peptone and 1.7% agar) and incubated at 38°C for 48 hours. Then, all plates were flooded with Gram's iodine solution. The cellulolytic isolates were detected by the cellulolytic zone around the colonies after staining. The hydrolysis capacity of each isolate was estimated using the ratio between the diameter of the cellulolytic zone and the colony [8]. The negative control used was *E. coli* TISTR 780 and the positive control used was *Cellulomonas* sp. TISTR 784 under the same growth conditions and staining procedures. The positive control was incubated for 60 hours due to its slow growth rate [3].

### 2.4 *Production of extracellular cellulase enzyme*

The selected cellulolytic isolate was cultured in CMC broth at 38°C for 48 hours with a shaking speed of 200 rpm. The crude extracellular cellulase produced in the culture medium was collected by centrifugation at 4,500 ×g for 30 minutes to obtain the crude cellulase as a cell-free supernatant that was immediately used for the cellulase activity assay.

### 2.5 *Cellulase activity assay*

The cellulase activity was determined by incubating the crude cellulase with the substrate solution and estimating the amount of reducing sugars liberated from the substrate. Endoglucanase activity was analyzed by CMCase activity assay [9] carried out in a 1 mL reaction mixture containing 0.5 mL of crude cellulase and 0.5 mL of 2% CMC sodium salt in 0.05 M sodium citrate buffer at pH 4.8 as a substrate. The reaction mixture was incubated at 50°C for 30 minutes. Total cellulase activity was measured by FPase activity assay [10] carried out in a 1.5 mL reaction mixture containing 0.5 mL of crude cellulase, 1.0 mL of 0.05 M sodium citrate buffer at pH 4.8 and 50 mg of Whatman No.1 filter paper (FP) strip as a substrate. The reaction mixture was incubated at 50°C for an hour. The reactions were terminated by adding 3.0 mL of 3, 5-dinitrosalicylic acid (DNS) and immediately boiled for 5 minutes. The reducing sugars released from the substrates were estimated by the DNS method [11] using glucose as the standard. The absorbance of the reaction mixtures was monitored using a GENESYS 10S UV-Vis Spectrophotometer (Thermo Scientific, USA) at 540 nm. One unit (U) of cellulase activity is defined as the amount of enzyme required to release 1 μmol of reducing sugars as glucose equivalent to that released under assay conditions.

### 2.6 *Identification of cellulolytic bacteria*

The characteristics of the selected cellulolytic isolate was identified by standard morphological and biochemical methods such as Gram staining, endospore staining, motility, catalase and oxidase. Identification of facultative anaerobic bacteria was determined on Difco anaerobic agar (Becton, Dickinson and Company) at 38°C for 48 hours in an Anaero*Jar* (Oxoid, England) assembled with Anaerocult A (Merck) for generating anaerobic conditions. Growth under the saline condition was determined by supplementing nutrient agar with 0-15% (w/v) NaCl at 38°C for 48 hours. The 16S rDNA sequencing analysis was analyzed by the Bioscience Department, Thailand Institute of Scientific and Technological Research (TISTR) (Pathum Thani, Thailand).

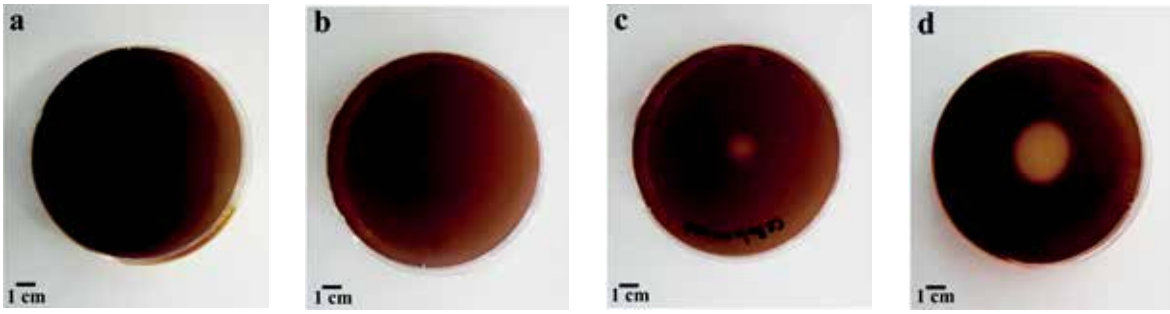


**Figure 1**: The cellulolytic zone on CMC agar plates stained with Gram's iodine solution. (a) Uninoculated. (b) Inoculated with *E. coli* TISTR 780. (c) Inoculated with *Cellulomonas* sp. TISTR 784. (d) Inoculated with bacterium strain RYC01101.

### 2.7 *Bioethanol production*

Cassava stalk, an agricultural byproduct in Rayong Province, was used as the carbon source for bioethanol production. The cassava stalk was pretreated with an alkaline solution following the method described by Cheng et al [12]. The selected cellulolytic isolate was cultured in a basal medium (0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4$, 0.05% KCl and 0.02% peptone) supplemented with 1% pretreated cassava stalk at 38°C with a shaking speed of 200 rpm. After 72 hours of incubation time, *S. cerevisiae* TISTR 5111 was added to the culture medium for co-culturing. Saccharification of lignocellulosic biomass and fermentation of bioethanol was completed at 30°C for 120 hours under stationary conditions. Bioethanol production (g/L) was analyzed using the $K_2Cr_2O_7$ reagent test [13].

## 3 Results and Discussion

Forty fecal samples of ruminant organisms were aseptically collected and diluted. All samples were spread on nutrient agar and incubated under experimental conditions. Two hundred and thirty morphologically dissimilar bacteria were isolated and subsequently streaked onto the surface of nutrient agar for single colony purification.

The purified bacteria were screened using a plate staining method. Twenty-two strains exhibited a cellulolytic zone around the colonies after staining the plates with Gram's iodine. The hydrolysis capacity values of the cellulolytic isolates were estimated. The cellulolytic bacterium strain RYC01101 was isolated from cow feces and had elite hydrolysis capacity 3.09±0.39 that was greater than the positive control (*Cellulomonas* sp. TISTR 784) by 2.41-fold (see Table 1 and Figure 1).

**Table 1**: Hydrolysis capacity of bacteria after being cultured on CMC agar at 38°C for 48 hours

| No. | Source of feces | Bacterial strain | Hydrolysis capacity |
|---|---|---|---|
| 1 | Buffalo | RYB01106 | 2.74±0.06 |
| 2 | Buffalo | RYB01107 | 1.02±0.03 |
| 3 | Buffalo | RYB02101 | 1.00±0.00 |
| 4 | Buffalo | RYB02102 | 2.08±0.21 |
| 5 | Buffalo | RYB03102 | 1.03±0.04 |
| 6 | Buffalo | RYB05104 | 1.79±0.29 |
| 7 | Cow | RYC01101 | 3.09±0.39 |
| 8 | Cow | RYC03102 | 1.01±0.01 |
| 9 | Cow | RYC03104 | 1.02±0.02 |
| 10 | Cow | RYC03105 | 2.14±0.20 |
| 11 | Cow | RYC04101 | 1.01±0.01 |
| 12 | Cow | RYC08101 | 2.06±0.12 |
| 13 | Cow | RYC09105 | 2.37±0.30 |
| 14 | Cow | RYC12102 | 1.96±0.08 |
| 15 | Horse | RYH01104 | 1.83±0.07 |
| 16 | Horse | RYH01106 | 2.24±0.35 |
| 17 | Goat | RYG01103 | 2.79±0.04 |
| 18 | Goat | RYG02103 | 2.34±0.02 |
| 19 | Goat | RYG02104 | 2.25±0.33 |
| 20 | Goat | RYG07105 | 2.81±0.09 |
| 21 | Sheep | RYS02104 | 2.65±0.62 |
| 22 | Sheep | RYS04103 | 2.07±0.08 |
| 23 | TISTR* | *Cellulomonas* TISTR 784** | 1.28±0.46 |

* TISTR Culture Collection (Pathum Thani, Thailand)
** Positive control

The bacterium strain RYC01101 was selected for cellulase production. The extracellular cellulase was collected and enzyme activity determined. The activity assays confirmed that the bacterium strain RYC01101 yielded 0.230±0.004 U/mL of CMCase activity and 0.080±0.007 U/mL of FPase activity. This cellulase activity was compared to other bacteria isolated from herbivores such as cows, goats and rhinoceros (see Table 2). Comparisons showed that the bacterium strain RYC01101 produced active cellulase with higher activity, particularly FPase activity.

**Table 2**: Cellulase activity of bacterium strain RYC01101 and related species

| Bacteria | Source | CMCase activity (U/mL) | FPase activity (U/mL) | Ref. |
|---|---|---|---|---|
| *Bacillus subtilis* AS3 | Cow feces | 0.07 | 0.02 | [14] |
| *B. licheniformis* JK7 | Ruminal fluid of goat | 0.75 | ND | [15] |
| *B. amyloliquefacien* SS35 | Rhinoceros feces | 0.08 | ND | [16] |
| Bacterium strain RYC01101 | Cow feces | 0.23 | 0.08 | This study |

ND denotes "not determined".

For characterization of the isolate RYC01101, the morphological and biochemical analyses revealed that RYC01101 colonies were creamy white, convex, translucent, entire margin and 3-4 mm in diameter after 48 hours incubation at 38°C on nutrient agar. Cells were rod-shaped, Gram-positive, endospore forming and motile. Catalase and oxidase were positive. The RYC01101 isolate was a strictly aerobic bacterium and able to grow in the medium supplemented with 0-10% (w/v) NaCl. The 16S rDNA sequencing analysis informed that the RYC01101 isolate had homology to *Bacillus methylotrophicus* [17] and *Bacillus siamensis* [18] with 99.84% and 99.77% similarity, respectively. The differential characteristics of bacterium strain RYC01101 are summarized in Table 3. In reference to morphology, biochemical characteristics, 16S rDNA sequence and growth in the presence of NaCl, the bacterium strain RYC01101 could be identified as halotolerant *B. methylotrophicus*. However, previous studies have reported that *B. methylotrophicus* was negative for cellulase activity [17,19], while growth in the presence of more than 4% (w/v) NaCl has not been reported [17].

From experiment to application, bioethanol production from agricultural waste was preliminarily investigated. Cassava stalk is a good carbon source for bioconversion due to it containing high cellulose content in a low amount of hemicelluloses [20]. Pretreated cassava stalk was saccharified by the extracellular cellulase of *B. methylotrophicus* RYC01101 under incubation conditions, yielding cassava stalk hydrolysate. After 72 hours of incubation, cassava stalk hydrolysate contained a maximum amount of glucose of 0.41±0.01 mg/mL. The hydrolysate was subsequently co-cultured with *S. cerevisiae* TISTR 5111 for bioethanol fermentation using the glucose in the hydrolysate as the carbon source. At the end of fermentation, the productivity of bioethanol was 1.38±0.40 g/L. Cassava stalks were investigated as a potential substrate for bioethanol production [20]. The pretreated cassava stalks were saccharified using a cocktail of biomass degradation enzymes and converted to bioethanol by fermentation of *S. cerevisiae* TISTR 5048. The bioethanol was 5.97±0.006 g/L after 18 hours of fermentation. To improve the production of bioethanol in further studies, a cocktail of cellulolytic bacteria producing a variety of biomass degradation enzymes such as cellulase, hemicellulase, amylase, xylanse and pectinase will be necessary in the saccharification step to yield a high glucose concentration of biomass hydrolysate.

**Table 3**: Differential characteristics of bacterium strain RYC01101 and related species

| Bacteria | Characteristics | | | | | | |
|---|---|---|---|---|---|---|---|
| | Similarity (%)* | Catalase activity | Oxidase activity | Cellulase activity | Oxygen requirement | Growth in NaCl (%) | Ref. |
| *B. methylotrophicus* | 99.84 | Positive | Positive | Negative | Aerobe | 0-4 | [17] |
| *B. siamensis* | 99.77 | Positive | Negative | Not determined | Facultative aerobe | 0-14 | [18] |
| Bacterium strain RYC01101 | NA | Positive | Positive | Positive | Aerobe | 0-10 | This study |

* Compared with 16S rDNA sequence of bacterium strain RYC01101
NA denotes "not available."

Thailand and other agricultural countries have high potential for producing biofuels from crop and agro-manufacture waste. In biofuel production, a pretreatment step is required in order to break down the lignocellulosic structure, leading to improved efficiency of cellulolytic enzymes [12], by using a strong acid, alkaline and ionic liquid (liquid forms of salt) solution. High salinity solutions possibly inactivate the cellulolytic enzymes used in the subsequent step by protein aggregation. A washing process is required to remove residual salt and other inhibitors, such as phenolic compounds, from the lignocellulosic biomass after the pretreatment step, which consumes energy and increases cost, especially in large-scale production [21]. To save costs, energy and time, *B. methylotrophicus* RYC01101 could be alternatively preferred in the pretreatment step of biofuel production and any high salinity applications due to its cellulase activity and halotolerant ability.

## 4 Conclusions

This study focused on the isolation of novel cellulolytic bacteria from Thai ruminant feces for use in biotechnological conversion applications. The bacterium strain RYC01101, isolated from cow feces, was a potential cellulolytic microorganism and identified as halotolerant *B. methylotrophicus*. The *B. methylotrophicus* RYC01101 could be used in saccharification of cellulosic biomass and applied in bioethanol production. With its halotolerant ability, *B. methylotrophicus* RYC01101 could be preferred in applications with high salinity conditions. Bioethanol production by *B. methylotrophicus* RYC01101 co-culturing with *S. cerevisiae* will be optimized in further studies.

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