Evaluation of Cellulase Production by *Zymomonas mobilis*

Tatsaporn Todhanakasem * and Siripa Jittjang

Z. mobilis has been widely studied as a potential microbe for consolidated bioprocessing to convert lignocellulosic biomass to fermentable sugars while at the same time producing ethanol. To achieve this goal, *Z. mobilis* must be evaluated for the production of cellulolytic enzyme. This work reports on the potential of intracellular and extracellular crude extracts from *Z. mobilis* ZM4 and TISTR 551 to hydrolyze various cellulosic materials including carboxymethylcellulose (CMC), delignified rice bran, microcrystalline cellulose, and filter paper. Crude intracellular extracts from ZM4 and TISTR 551 showed high endoglucanase activity with CMC substrates at an optimal pH of 6 to 7 and temperature range of 30 to 40 °C. The endoglucanase activity from the crude extracts was significantly higher than the exoglucanase activity. Of the high crystalline celluloses substrates tested, the best results were obtained for the hydrolysis of delignified rice bran by crude intracellular enzyme extracts of *Z. mobilis* TISTR 551.

Keywords: Consolidated bioprocess; Zymomonas mobilis; Cellulase; Lignocellulosic material

*Contact information: Department of Agro Industry, Faculty of Biotechnology, Assumption University, 10240, Thailand; *Corresponding author: tatsaporntdh@au.edu*

INTRODUCTION

Lignocellulosic biomass is an abundant, renewable feedstock for the production of biofuels and bio-based chemicals, if an efficient and affordable conversion technology can be established to overcome its recalcitrance. Cellulose is one of most important components of lignocellulosic material; it is composed of glucose units linked by β-1,4,D-glucosidic bonds. Cellulolytic microorganisms degrade crystalline cellulose by producing a cellulase system consisting of 3 classes of enzymes: endoglucanases, cellobiohydrolases, and β-glucosidase for the production of fermentable sugar as a final product. The bioconversion of lignocellulosic material to bioethanol represents a potential energy source to replace fossil fuels, but the production cost is not competitive with the existing technologies. Ethanol production from lignocellulosic materials includes four processes: chemical and physicochemical pretreatment to swell the biomass, hydrolysis of cellulose and hemicelluloses to fermentable sugars by cellulases and hemicellulases, microbial fermentation for the production of ethanol, and lastly, distillation to recover ethanol (Sanchez and Cardona 2008). Ethanol can be produced from lignocellulosic materials *via* separation hydrolysis and fermentation (SHF), saccharification and fermentation (SSF), or simultaneous saccharification and cofermentation (SSCF) using a variety of microorganisms, including *Z. mobilis* (Lynd *et al*. 2002, 2005; Antoni *et al*. 2007; Carere *et al*. 2008). *Z. mobilis* has some advantages over other microorganisms in ethanol production including a higher specific rate of sugar uptake, higher ethanol yield, less

production of byproducts, lower biomass production, no requirement for controlled addition of oxygen during fermentation, and tolerance to toxic inhibitors and substrates (Rogers *et al.* 2007; Yang *et al.* 2014; Gu *et al.* 2015;Yi *et al*, 2015). These characteristics make *Z. mobilis* a promising ethanologenic microorganism for large-scale production of bioethanol from sugar rich substrates or lignocellulosic materials; the technology can be further developed to provide a CBP microbe in the ethanol production using lignocellulosic material (Sarks *et al.* 2016).

CBP, which combines enzyme production, saccharification, and fermentation in a single step by one microbe, is an alternative approach with outstanding potential for the large-scale production of ethanol from lignocellulosic hydrolysate. This is considered as a promising strategy for reducing the production costs (Lynd *et al.* 2005; Cardona and Sanchez 2007; Xu *et al.* 2009; He *et al.* 2014). CBP microbes for ethanol production from lignocellulosic materials can be developed using native cellulolytic microbes that are engineered to improve ethanol production, *e.g.*, *Trichoderma reesei*, *Aspergillus*, and *Fusarium* (Ruiz *et al.* 2007; Xu *et al.* 2009, Piriya *et al.* 2012), or engineered non-cellulolytic organisms that exhibit high native ethanol production, *e.g.*, *Z. mobilis* and *Saccharomyces cerevisiae* (Van Zyl *et al.* 2007; Linger *et al.* 2010; Apiwatanapiwat *et al.* 2011; Vasan *et al.* 2011; Den *et al.* 2015; Gonçalves *et al.* 2016).

Many ethanologenic *Zymomonas* strains have been engineered to harbor cellulolytic enzymatic activities with the aim of producing CBP strains. For example, two cellulolytic enzymes E1 and GH12 from *Acidothermus cellulolyticus* were successfully expressed in *Z. mobilis via* its native signal secretion protein. *Z. mobilis* produces endogeneous extracellular cellulose activity against carboxymethyl cellulose (CMC) *via* both native and engineered cellulolytic expressing genes (Linger *et al.* 2010). Cellulolytic enzymes from *Cellulomonas fumi* and *Ruminococcus albus* have been introduced, co-expressed, and secreted in *Zymobacter palmae*, which is then able to successfully ferment cellulosic polysaccharide (barley β-glucan) to ethanol with 79.5% theoretical yield (Kojima *et al.* 2013). Finally, cellulase encoding genes from *Enterobacter cloace* and *Heterotermes indicola* have been isolated from the gut of a wood-feeding termite and transferred to *Z. mobilis*; this new strain performs CBP processes (Vasan *et al.* 2011). Thus, it is clear that *Z. mobilis* can express and secrete foreign microbial cellulases. However, there are some limitations to recombinant *Z. mobilis* strains containing cellulolytic enzymes, such as lower levels of protein expression, protein stability, and protein secretion when the protein are engineered from foreign microbes. This is due to the differences in the native codon usages and protein secretion system from the host organism (Gunasekaran and Raj 1999; Vasan *et al.* 2011; Davis and Olsen 2011; Luo and Bao 2015). Thus, the most obvious mean to achieve cellulolytic enzyme activity is to use native cellulolytic enzymes with a native translocation system for a protein secretion to direct contact with lignocellulosic substrate. The systematic analysis of available *Z. mobilis* strains that have native cellulolytic activities may reveal more cellulase producing strains that could be used as CBP microbes for hydrolyzing lignocellulosic material and ethanol production in the future. Genomic and enzymatic studies of *Z. mobilis* ZM4 (ATCC 31821) and *Z. mobilis* ATCC 29192 encode the ZMO 1086 (endoglucanase) and Zymop_0203 cellulases (endoglucanase) are only recently available (Luo and Bao 2015; Rajnish *et al.* 2008).

In this work, the cellulolytic activities of crude extracellular and intracellular extracts of *Z. mobilis* wild type strains ZM4 and TISTR 551 were evaluated for the ability

to hydrolyze carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), filter paper and treated rice bran. The pH and temperature optimum of the crude intracellular and extracellular cellulolytic enzymes of *Z. mobilis* strain ZM4 and TISTR 551 were analyzed. This is the first report on *Z. mobilis* endoglucanase activity and exoglucanase activity against various cellulosic materials. This raises exciting potential for this microorganism in further CBP studies.

EXPERIMENTAL

Bacterial Strains and Cultivation

Z. mobilis strain ZM4 (a type strain, ATCC 31821) and TISTR551 (from Thailand Institute of Scientific and Technological Research (TISTR)) were used in this study. *Z. mobilis* was grown in yeast peptone glucose (YPG) medium (10 g peptone, 10 g yeast extract, and 20 g glucose per liter, pH 6.4) at 30 °C for approximately 24 h until the optical density at 600 nm (OD_{600}) reached 1.0 prior to all studies.

Enzyme Activity Test on CMC Plate

Overnight cultures of *Z. mobilis* strains ZM4 and TISTR 551 (OD_{600} approximately 1.0) were streaked on YPG agar supplemented with 0.12% w/v CMC. The plates were incubated at 30 °C for 48 h. The cellulolytic abilities of all strains were detected by the Congo red overlay method. The colonies were removed from the agar, and plates were flooded with 0.2% Congo red solution for 10 min then washed with 1 M NaCl. A clear zone surrounding colonies indicates cellulose hydrolysis (Wood 1980; Linger *et al.* 2010). The diameters of the cleared zones in three replicates were measured.

Enzymatic Assays for Optimum pH and Temperature

Activities of crude extracellular enzymes (unpurified cellulase enzyme secreted into culture supernatant) and crude intracellular enzymes (obtained from the cell lysate) from *Z. mobilis* strain ZM4 and TISTR 551 were analyzed for optimal pH and temperature requirements. The crude extracellular enzymes were obtained by centrifugation of overnight cultures at 5000 rpm (RCF= 3588g) for 2 min, and the supernatants were saved for further testing. The crude intracellular enzymes were obtained by disrupting cells with glass beads (Sigma, G8772), centrifuging, and saving the supernatant. Standard enzymatic activity was measured by the dinitrosalicylic acid (DNS) method with carboxymethyl cellulose (CMC) as the substrate (Miller 1959). Briefly, 20 μL of the enzyme diluted to an appropriate concentration and 320 μL of 1% w/v CMC in buffer at a specific pH were incubated at the specific temperature test for 5 min prior and then the reaction was carried out at that specific temperature for 15 min. The reaction was terminated by adding 680 μL of DNS solution. The level of reducing sugars produced by the enzymatic reaction was subsequently monitored at 540 nm with a spectrophotometer (UNICO (model 1200), cuvette dimension 12.5 ×12.5 ×45 mm). The protein content of the crude extracts was analyzed according to the method of Lowry *et al*. (1951). One unit of enzyme activity (U) was defined as the amount of the enzyme that produces the equivalent of 1 μmol of glucose per min under the assay conditions. Specific activity of the crude enzyme was determined by the activity of an enzyme per milligram of total protein (U/ mg). The optimal pH and

temperature were determined by subjecting the reaction mixtures to various pH from 4 to 8 (0.2 M sodium acetate pH 4.0 to 5.0 and 0.2 M potassium phosphate pH 6.0 to 8.0) and various temperature ranges from 30 to 50 °C. The optimum pH and temperature for cellulose activity of extracellular and intracellular crude enzymes of *Z. mobilis* ZM4 and TISTR551 were analyzed based on triplicate analysis, and the results were statistically analyzed by ANOVA.

Substrate Specificity of Crude Enzymes

The crude intracellular and extracellular cellulolytic enzymes of *Z. mobilis* ZM4 and TISTR 551 were monitored for substrate specificities on CMC, microcrystalline cellulose, filter paper (Whatman no. 1), and treated rice bran under specific pH and temperature optimums according to previous analysis. To measure enzymatic activity against CMC, 20 μL of the enzyme diluted to an appropriate concentration was reacted with 320 μL of 1% CMC. Activity to hydrolyze MCC was tested by adding 20 μL of the enzyme diluted to an appropriate concentration to 1% microcrystalline cellulose in 320 μL. To measure enzymatic activity against filter paper (Whatman no. 1 (50 mg, 1 × 1.5 cm) (50 mg)), 0.5 mL of the enzyme diluted to an appropriate concentration was added to filter paper (in 1 mL of buffer). Enzyme and substrate were separately incubated at specific temperature and pH test for 5 min prior to the reaction. The reactions were carried out at optimum temperature and pH for each crude extract for 2 h, 15 min, and 1 h when microcrystalline cellulose, CMC and filter paper were used as substrates, respectively, and stopped by adding 680 μL of DNS solution. For the filter paper test, the reactions were terminated by adding 1 mL of the mixture to 3 mL of DNS solution and then the amount of reducing sugar was monitored. Delignified rice bran was used as a representative lignocellulosic material and was prepared by treating rice bran with 0.2 M potassium hydroxide (KOH) for 4 h at room temperature (10% w/v). The materials were filtered through cheese cloth and washed in tap water until the pH became neutral. The samples were then dried at 85 °C until the weight was constant. Pretreated rice bran (2 g) was added to 10 mL specific buffer containing enzymes diluted to appropriate concentrations. The reactions were performed for 4 h for intracellular crude extract and 1 h for extracellular crude extract. The reducing sugar and specific enzyme activity were elaborated. The experiments were performed in triplicate, and the results were statistically analyzed by ANOVA.

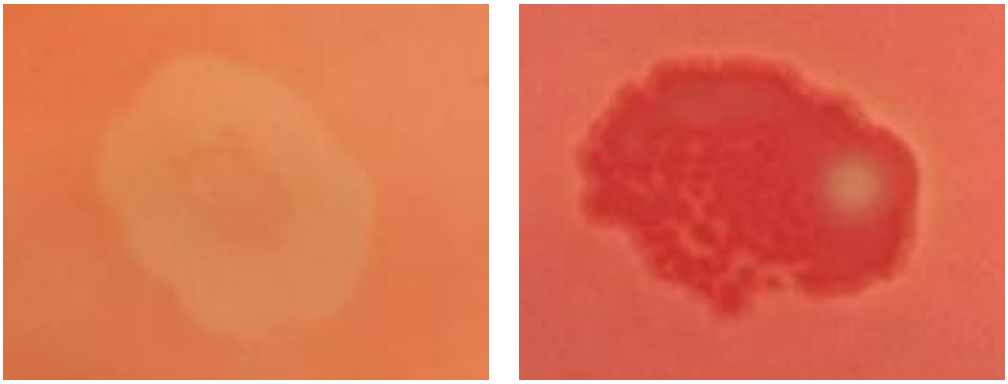
RESULTS AND DISCUSSION

The preliminary quantitative analysis on cellulase production and secretion was conducted on *Z. mobilis* strains ZM4 and TISTR551 using agar plates containing 0.12% w/v CMC. ZM4 and TISTR551 exhibited extracellular cellulolytic activities by hydrolyzing CMC with diameters of the zones of clearance 2.23 ± 0.25 cm and 1.79 ± 0.10 cm, respectively (Table 1, Fig. 1). This rapid detection demonstrated that ZM4 represented higher hydrolytic activity than TISTR551, as indicated by the higher diameter in clear zone. However, in order to ensure on the presence of cellulolytic activities of both strains, the activities of crude extracts needed to be revealed.

Table 1. Hydrolytic Activity of Microbial Cells *

Strain	Diameter of Clear Zone (cm)
Z. mobilis ZM4	2.23 ± 0.25
Z. mobilis 551	1.79 ± 0.10

* The diameter of clear zones (cm) created by *Z. mobilis* ZM4 and TISTR 551 on YPG agar supplemented with 0.12% w/v CMC indicates hydrolytic activity of microbial cells.



ZM4 TISTR 551

Fig. 1. CMC Degradation by cellulolytic enzyme activities

The effects of temperature and pH on crude extracellular and intracellular cellulases of ZM4 and TISTR 551 were determined using 1% w/v CMC in buffer at various pH (4, 5, 6, 7, and 8) and temperatures (30, 35, 40, 45, and 50 °C) for 15 min. The cellulase specific activities of crude extracts were determined through the production of reducing sugar (Konig *et al.* 2002). *Z. mobilis* ZM4 crude intracellular extracts showed the highest cellulolytic activity at pH 7 and 30 °C (837.98 ± 22.5 U/mg) (Table 2). Intracellular crude extracts of TISTR 551 demonstrated optimal cellulolytic activity at pH 6 and 40 °C (667.30 ± 43.2 U/ mg) (Table 3). At pH 8, the specific activities of the crude intracellular cellulases were dramatically reduced for both strains.

Table 2. *Z. mobilis* strain ZM4 Intracellular Cellulose Specific Activities with the Statistical Analysis using ANOVA

Specific Activity (U/ mg)					
pH	30 °C	35 °C	40 °C	45 °C	50 °C
4	144.01 ± 4.7^{i}	155.68 ± 3.8^{hi}	220.08 ± 13.8^{fg}	252.47 ± 13.9^{def}	142.98 ± 9.4^{i}
5	225.23 ± 26.4^{fg}	442.11 ± 13.3^{b}	203.30 ± 16.9^{g}	196.70 ± 2.2^{gh}	121.32 ± 2.2^{ij}
6	132.43 ± 3.5^{i}	279.57 ± 7.8^{cd}	234.02 ± 19.9^{efg}	422.52 ± 30.6^{b}	298.76 ± 11.5^{c}
7	837.98 ± 22.5^{a}	133.00 ± 3.9^{i}	272.87 ± 25.3^{cde}	122.39 ± 5.4^{ij}	267.04 ± 4.9^{cde}
8	89.41 ± 7.2^{j}	195.44 ± 13.4^{gh}	89.90 ± 4.7^{j}	42.64 ± 4.0^{k}	38.16 ± 3.1^{k}

Data represent the mean and standard deviation of three replicates. Based on ANOVA, a, b, and c had the three highest activity levels. The different letters represent a significant difference of $p < 0.05$. The data were compared in all conditions.

Table 3. *Z. mobilis* strain TISTR 551 Intracellular Cellulose Specific Activities with the Statistical Analysis using ANOVA

Specific Activity (U/ mg)					
pH	30 °C	35 °C	40 °C	45 °C	50 °C
4	271.46 ± 19.8^{d}	51.66 ± 1.5^{ghi}	81.85 ± 10.9^{fgh}	552.46 ± 17.5^{b}	346.87 ± 15.5^{c}
5	265.53 ± 23.7^{d}	126.32 ± 10.8^{f}	158.18 ± 5.5^{f}	142.39 ± 15.1^{f}	78.52 ± 6.5^{fgh}
6	262.87 ± 32.5^{d}	304.87 ± 27.5^{cd}	667.30 ± 43.2^{a}	110.26 ± 5.2^{fg}	166.70 ± 6.5^{ef}
7	260.99 ± 36.3^{d}	225.30 ± 20.9^{de}	242.53 ± 29.4^{d}	13.59 ± 0.8^{i}	510.35 ± 39.9^{b}
8	44.26 ± 2.1^{hi}	46.72 ± 3.5^{ghi}	104.34 ± 2.3^{gh}	45.47 ± 4.3^{hi}	43.96 ± 2.3^{hi}

Data represent the mean and standard deviation of three replicates. Based on ANOVA, a, b, and c had the three highest activity levels. The different letters represent a significant difference of $p < 0.05$. The data were compared in all conditions.

Specific cellulase activities of extracellular crude extracts from *Z. mobilis* ZM4 and TISTR 551 exhibited optimal enzyme activity at pH 6, 40 °C (81.80 ± 7.7 U/ mg) and pH 8, 40 °C (111.61 ± 7.4 U/ mg), respectively (Tables 4, 5). Thus, the optimal enzymatic reactivity conditions for crude extracellular and intracellular cellulases were in the ranges 30 to 40 °C and pH 6 to 7, except for the TISTR 551 extracellular cellulases, which had an optimum pH of 8.0. However, TISTR 551 extracellular cellulases also represented high enzymatic activity at pH 6.0 and 35 °C.

Crude intracellular and extracellular extracts from the individual strains also showed different pH and temperature optimums (Table 6), implying that several cellulases may be produced by one native strain, as previously suggested (Lynd *et al.* 2002). The optimum pH and temperature for intracellular and extracellular crude cellulase enzyme activity correlated well with the optimal conditions reported for growth and ethanol production for *Z. mobilis* (30 to 37 °C and pH 6 to 7.5), which are proximal to the optimum cellulase activities (King and Hossain 1982). Therefore, *Z. mobilis* ZM4 and TISTR 551 are good candidates for consolidated bioprocessing (CBP) for on-site cellulolytic activity and ethanol production by a single microbe.

Table 4. *Z. mobilis* strain ZM4 Extracellular Cellulose Specific Activities with the Statistical Analysis using ANOVA

Specific Activity (U/ mg)					
pH	30 °C	35 °C	40 °C	45 °C	50 °C
4	67.49 ± 5.1^{bcd}	15.47 ± 1.3^{hij}	61.67 ± 5.8^{cde}	48.48 ± 3.9^{f}	26.39 ± 3.3^{gh}
5	18.52 ± 1.3^{hi}	22.38 ± 1.1^{hi}	25.73 ± 1.7^{ghi}	17.80 ± 1.3^{hij}	22.70 ± 7.1^{hi}
6	23.94 ± 2.2^{ghi}	55.42±3.5^{ef}	81.80 ± 7.7^{a}	28.15 ± 0.7^{gh}	34.77 ± 1.4^{g}
7	70.90 ± 6.2^{abc}	50.50 ± 5.2^{ef}	49.98 ± 4.8^{ef}	13.85 ± 0.9^{ij}	75.72 ± 3.5^{ab}
8	55.87 ± 3.4^{def}	75.86 ± 2.7^{ab}	33.03 ± 2.6^{gh}	9.90 ± 1.2^{ij}	6.38 ± 0.8^{j}

Data represent the mean and standard deviation of three replicates. Based on ANOVA, a, b, and c had the three highest activity levels. The different letters represent a significant difference of $p < 0.05$. The data were compared in all conditions.

Table 5. *Z. mobilis* strain TISTR 551 Extracellular Cellulose Specific Activities with the Statistical Analysis using ANOVA

Specific Activity (U/ mg)					
pH	30 °C	35 °C	40 °C	45 °C	50 °C
4	21.39 ± 1.5^{g}	99.84 ± 3.5^{b}	53.78 ± 3.1^{e}	58.66 ± 1.7^{e}	87.37 ± 6.5^{c}
5	69.31 ± 3.2^{d}	18.49 ± 1.6^{gh}	11.33 ± 1.8^{gh}	57.05 ± 2.9^{e}	9.41 ± 0.8^{hi}
6	70.94 ± 2.9^{d}	104.95 ± 6.0^{ab}	58.73 ± 2.1^{e}	58.61 ± 4.1^{e}	57.75 ± 3.1^{e}
7	24.31 ± 0.7^{fg}	33.79 ± 4.2^{f}	100.46 ± 0.5^{b}	66.48 ± 1.7^{de}	51.18 ± 5.8^{e}
8	19.50 ± 1.2^{g}	0.35 ± 0.04^{i}	111.61 ± 7.4^{a}	33.79 ± 1.2^{f}	13.56 ± 1.3^{gh}

Data represents the mean and standard deviation of three replicates. Based on ANOVA, a, b, and c had the three highest activity levels. The different letters represent a significant difference of $p < 0.05$. The data were compared in all conditions.

Table 6. Optimal Conditions for *Z. mobilis* ZM4 and TISTR551 Intracellular and Extracellular Cellulases

Z. mobilis Strain	Cellulase	Optimum Temperature (°C)	Optimum pH
ZM4	Intracellular	30	7
	Extracellular	40	6
TISTR 551	Intracellular	40	6
	Extracellular	40	8

To determine the substrate specificities of the crude intracellular and extracellular cellulases from *Z. mobilis* ZM4 and TISTR 551, various substrates were used (CMC, rice bran, microcrystalline cellulose, and filter paper). Cellulose contains crystalline and amorphous structures mixed together in various degrees, and therefore various substrates have been used to compare the activities of crude intracellular and extracellular cellulases. CMC is a highly soluble cellulose that has been used as a substrate to study endoglucanase production. Microcrystalline cellulose, a nearly pure cellulose, is a most commonly used model substrate with a high degree of crystallinity. Microcrystalline cellulose is a good substrate to study exoglucanase activity because it is relatively inaccessible to attack by endoglucanases, despite some amorphous regions (Soares Junior *et al.* 2013). Filter paper and pretreated rice bran, which exhibit intermediate crystallinity, were included in the study of the total cellulase activity both as a comparison to the pure substrates and as examples of highly abundant by-products that can be diverted for further use (Lynd *et al.* 2002; Dashtban *et al.* 2010).

Cellulose hydrolysis requires three classes of cellulases: 1) endoglucanase that attacks regions of low crystallinity or internal amorphous site in the cellulose fiber to create free chain ends; 2) exoglucanase that acts in a processive manner from both reducing and non-reducing ends of cellulose polysaccharide chains to produce either glucose or cellobiose; and 3) β-glucosidase that hydrolyzes cellodextrin and cellobiose to glucose (Howard *et al.* 2004). This study of intracellular and extracellular crude extracts of ZM4 and TISTR 551 cellulase under standard assay conditions revealed that the enzyme exhibited the highest specific activity toward CMC substrates (Fig. 2). Significant enzymatic activity was detected in intracellular extracts of *Z. mobilis* strains ZM4 (837.98 ± 22.5 U/ mg) and TISTR 551 (667.30 ± 43.2 U/ mg) (Fig. 2a).

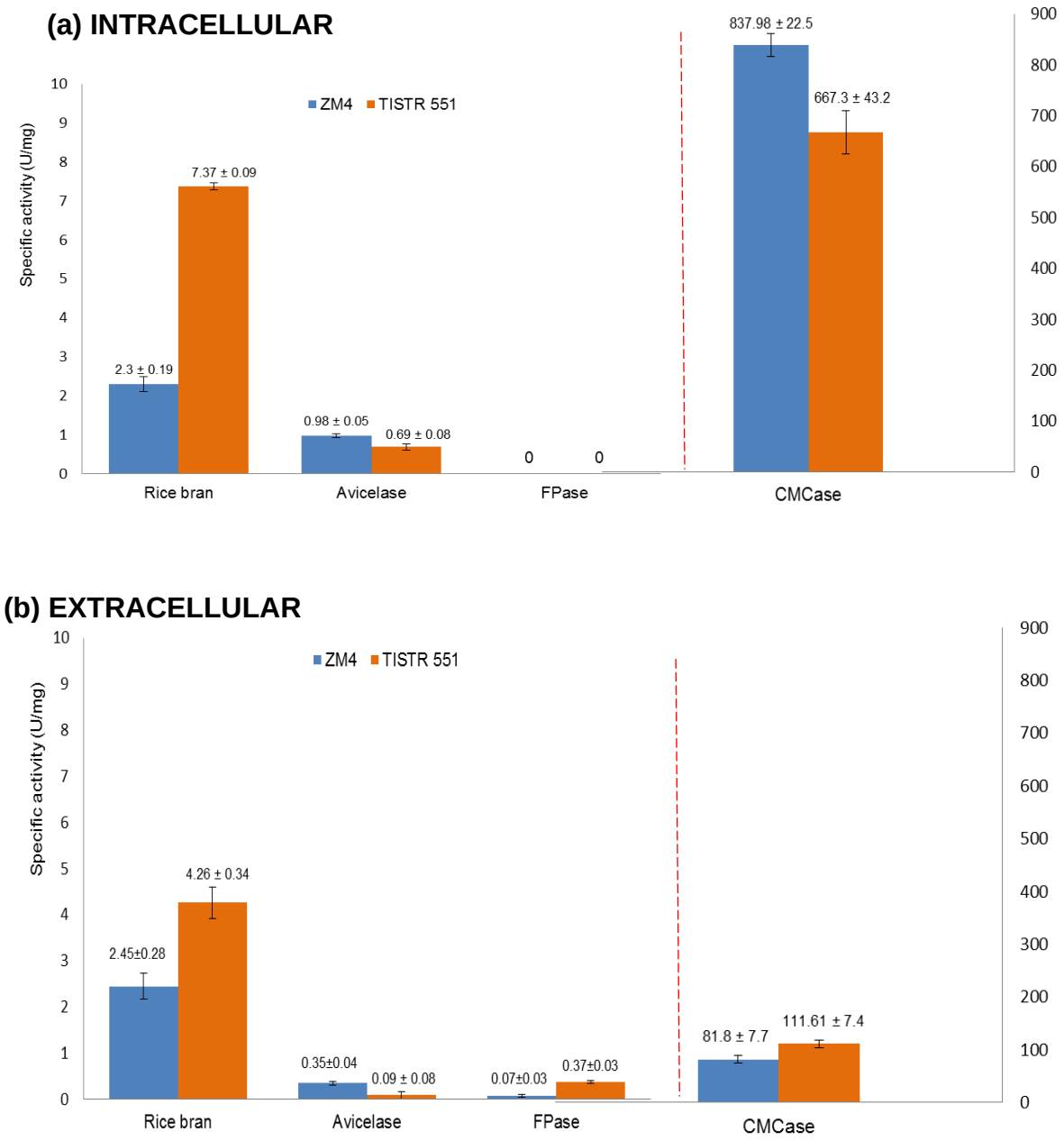


Fig. 2. Substrate-specific cellulose activities of *Z. mobilis* ZM4 and TISTR 551 intracellular (a) and extracellular (b) crude extracts on different types of substrates; CMC (CMCase), treated rice bran, microcrystalline cellulose, and filter paper (FPase) (Left scale for Rice bran, microcrystalline cellulose, and FPase while right scale for CMCase)

Intracellular cellulase activities of both strains were significantly higher than extracellular cellulase activities, implying that *Z. mobilis* maintains cellulase inside cells rather than secreting it. Hence, *Z. mobilis* cellulase is an endoglucanase, as its highest enzyme activity was against CMC substrates (Adsul *et al.* 2007). Several *Z. mobilis* strains (ATCC 39676 and CP4) natively produce endoglucanase activity against CMC (He *et al*.

2014). *Z. mobilis* ZM4 (ATCC 31821) encodes a cellulase gene (endoglucanase) named ZMO 1086 (Cel A); *Z. mobilis* ATCC 29192 also encodes a cellulase (endoglucanase), Zymop_0203. There is no genomic database available for *Z. mobilis* strain TISTR 551. Other *Z. mobilis* genome sequences available in GenBank do not include predicted cellulase genes (Table 7) (Seo *et al.* 2005; Kouvelis *et al.* 2009; Kouvelis *et al.* 2011; Pappas *et al.* 2011; Desiniotis *et al.* 2012; Kouvelis *et al.* 2014). ZMO 1086 from *Z. mobilis* has been cloned and successfully expressed in *Escherichia coli* (Rajnish *et al.* 2008). However, the expression and secretion of native endoglucanase gene ZMO1086 that represented signal peptide SP1086 had facilitated its own secretion in only 12% of the expressed enzyme (Lou and Bao 2015). This finding correlated to our result that native endoglucanases from *Z. mobilis* ZM4 and TISTR551 represented the intracellular activity with 6-8 folds higher than extracellular activity or highly presented inside the cell rather than secreted out. Future development on the cellulase secretions can be processed by engineering the effective secretion system SecB-dependent and twin arginine translocation (TAT) to the native cellulases (Linger *et al.* 2010).

Intracellular and extracellular crude cellulases from strains ZM4 and TISTR 551 displayed only trace exoglucanase activities against microcrystalline cellulose, filter paper (FPase), and rice bran, indicating a preference for amorphous rather than crystalline cellulose (Fig. 2). The hydrolysis activities of all crude extracts were slightly higher in pretreated rice bran substrate than microcrystalline cellulose and filter paper substrates. These results are in agreement with studies showing that the alkaline pretreatment of lignocellulosic materials as rice bran eliminates non-cellulosic polysaccharides, and efficiently releases maximal cellulose content from microfibrils, and further facilitates enzyme efficiencies with crystalline cellulose (Lynd *et al.* 2002; Vasan *et al.* 2011). Intracellular and extracellular cellulases did not show any significant differences in enzymatic activity against pretreated rice bran, microcrystalline cellulose, or filter paper. However, crude intracellular enzymes from *Z. mobilis* TISTR 551 demonstrated a slightly higher specific enzyme activity against treated rice bran than the other highly crystalline substrates. Interestingly, substrate pretreatment with steam explosion, diluted acid, or lime does reduce the crystallinity of lignocellulosic substrates but does result in high hydrolysis yield (Thompson *et al.* 1992; Alvira *et al.* 2010). This is the first report on the level of native cellulolytic activity of *Z. mobilis* against various cellulosic substrates. The level of cellulolytic activities has been dramatically reported for recombinant *Escherichia coli*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* through the engineered cellulase obtaining from native *Bacillus* sp., *Clostridium thermocellum* and fungal sources (Garvey *et al.* 2013; Gefen *et al.* 2012). The recombinant strains elaborated significant enzyme activity level against CMC and microcrystalline cellulose. Thus, this study indicated that *Z. mobilis* ZM4 and TISTR 551 represented their potential to be further developed as CBP microbes for the ethanol production using lignocellulosic material as a substrate.

One future goal is to engineer the native strain to express higher levels of exoglucanase either from the native strain or engineered foreign exoglucanase. An additional goal is to engineer the strains to secrete the available endoglucanase and exoglucanase enzymes to synergistically increase cellulose degradation, as both ZM4 and TISTR 551 demonstrated highly intracellular cellulase activities. An effective CBP process requires extracellular enzymes to hydrolyze lignocellulosic materials.

Table 7. Summary of Genomics, Proteins, and Gene Encoded for Cellulases with GenBank Accession Number in Different *Z. mobilis* Strains

***Z. mobilis* strain**	**Genome Assembly Information (GenBank Accession Number)**	**Reference for Genome**	**Type of Cellulase in *Z. mobilis* Strain and GenBank Accession Number for the Protein**	**Gene encoded for Cellulase and GenBank Accession Number**	**Reference for Enzyme Activity**
ZM4 (ATCC 31821)	NC_006526.2	(Seo *et al.* 2005)	Endoglucanase, AAV89710.1	ZMO1086, AE008692.2	(Rajnish *et al.* 2008)
NCIMB 11163	NC_013355.1	(Kouvelis *et al.* 2009)	-	-	-
ATCC 29191	NC_08145.1	(Desiniotis *et al.* 2012)	-	-	-
ATCC 29192	NC_015709.1	(Kouvelis *et al.* 2011)	Endoglucanase, AE137106.1	Zymop_0203, CP002865.1	NCBI
ATCC 10988	NC_017262.1	(Pappas *et al.* 2011)	-	-	-
CP 4 (NRRL B-14023)	NC_022900.1	(Kouvelis *et al.* 2014)	-	-	-

CONCLUSIONS

1. Crude intracellular extracts from *Z. mobilis* ZM4 and TISTR 551 showed significant endoglucanase activity in the utilization of amorphous cellulose, such as CMC.
2. The optimum temperature and pH for the enzymatic activities were 30 to 40 °C and pH 6 to 8.
3. Of the crystalline cellulose substrates used, lignocellulosic material treated with KOH was most highly hydrolyzed compared with the other crystalline containing substrates (filter paper and microcrystalline cellulose).

ACKNOWLEDGEMENTS

This work was supported by Assumption University Fund, under the grant number P570337, Thailand. Briana M. Young is gratefully acknowledged for her proofreading of the manuscript.

REFERENCES CITED

Adsul, M. G., Bastawde, K. B., Varma, A. J., and Gokhale, D. V. (2007). "Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production," *Bioresource Technol.* 98, 1467-1473. DOI: 10.1016/j.biortech.2006.02.036

Alvira, P., Tomas-Pejo, E., Ballesteros, M., and Negro, M. J. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review," *Bioresource Technol.* 101, 4851-4861. DOI: 10.1016/j.biortech.2009.11.093

Antoni, D., Zverlov, V. V., and Schwarz, W. H. (2007). "Biofuels from microbes," *Appl. Microbiol. Biotechnol.* 77, 23-35. DOI: 10.1007/s00253-007-1163-x

Apiwatanapiwat, W., Murata, Y., Kosugi, A.,Yamada, R., Kondo, A., Arai, T., Rugthaworn, P., and Mori, Y. (2011). "Direct ethanol production from cassava pulp using a surface engineered yeast strain co-displaying two amylases, two cellulases, and β- glucosidase," *Appl. Microbiol. Biotechnol*. 90, 377-384. DOI: 10.1007/s00253-011-3115-8

Cardona, C. A., and Sanchez, O. J. (2007). "Fuel ethanol production: Process design trends and integration opportunities," *Bioresource Technol.* 98, 2415-2457. DOI: 10.1016/j.biortech.2007.01.002

Carere, C. R., Sparling, R., Cicek, N., and Levin, D. B. (2008). "Third generation biofuels *via* direct cellulose fermentation," *Int. J. Mol. Sci.* 9, 1342-1360. DOI: 10.3390/ijms9071342

Dashtban, M., Maki, M., Leung, K.T., Mao, C., and Qin, W. (2010). "Cellulase activities in biomass conversion: Measurement methods and comparison," *Crit. Rev. Biotechnol.* 30, 302-309. DOI: 10.3109/07388551.2010.490938

Davis, J. J., and Olsen, G. J. (2011). "Characterizing the native codon usages of a genome: An axis projection approach," *Mol. Biol. Evol.* 28(1), 211-221. DOI: 10.1093/molbev/msq185

Den, H.R., Van, R. E., Rose, S. H., Görgens, J. F., and Van Zyl, W. H. (2015). "Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing," *Curr. Opin. Biotechnol*. 33, 32-38.DOI: 10.1016/j.copbio.2014.10.003

Desiniotis, A., Kouvelis, V. N., Davenport, K., Bruce, D., Detter, C., Tapia, R., Han, C., Goodwin, L. A., Woyke, T., and Kyrpides, N. C. (2012). "Complete genome sequence of the ethanol-producing *Zymomonas mobilis* subsp. *mobilis centrotype* ATCC 29191," *J. Bacteriol.* 194, 5966-5967. DOI: 10.1128/JB.01398-12

Garvey, M., Klose, H., Fischer, R., Lambertz, C., and Commandeur, U. (2013). "Cellulases for biomass degradation: Comparing recombinant cellulase expression platforms," *Trends Biotechnol*. 31(10), 581-593. DOI: 10.1016/j.tibtech.2013.06.006

Gefen, G., Anbar, M., Morag, E., Lamed, R., and Bayer, E. A. (2012). "Enhanced cellulose degradation by targeted integration of a cohesin-fused β-glucosidase into the *Clostridium thermocellum* cellulosome," *Proc. Natl. Acad. Sci*. 109(26), 10298-10303. DOI: 10.1073/pnas.1202747109

Gonçalves, F. A., Ruiz, H. A., Dos Santos, E. S., Teixeira, J. A., and De Macedo, G. R. (2016). "Bioethanol production by *Saccharomyces cerevisiae*, *Pichia stipitis* and *Zymomonas mobilis* from delignified coconut fibre mature and lignin extraction according to biorefinery concept," *Renew. Energy* 94, 353-365. DOI: 10.1016/j.renene.2016.03.045

Gu, H., Zhang, J., and Bao, J. (2015). "High tolerance and physiological mechanism of *Zymomonas mobilis* to phenolic inhibitors in ethanol fermentation of corncob residue," *Biotechnol. Bioeng.* 112(9), 1770-1782. DOI: 10.1002/bit.25603

Gunasekaran, P., and Raj, K. C. (1999). "Ethanol fermentation technology - *Zymomonas*," *Curr. Sci.* 77 (1), 56- 68.

He, M. X., Wu, B., Qin, H., Ruan, Z. Y., Tan, F. R., Wang, J. L., Shui, Z. X., Dai, L. C., Zhu, Q. L., and Pan, K. (2014). "*Zymomonas mobilis*: A novel platform for future biorefineries," *Biotechnol. Biofuels* 7, 101. DOI: 10.1186/1754-6834-7-101

Howard, R. L., Abotsi, E., Van R. E. L. J., and Howard, S. (2004). "Lignocellulose biotechnology: Issues of bioconversion and enzyme production," *Afr. J. Biotechnol.* 2, 602-619. DOI: 10.5897/AJB2003.000-1115

King, F. G., and Hossain, M. A. (1982). "The effect of temperature, pH, and initial glucose concentration on the kinetics of ethanol production by *Zymomonas mobilis* in batch fermentation," *Biotechnol. Lett.* 4, 531-536. DOI: 10.1007/BF00131577

Kojima, M., Okamoto, K., and Yanase, H. (2013). "Direct ethanol production from cellulosic materials by *Zymobacter palmae* carrying *Cellulomonas endoglucanase* and *Ruminococcus beta glucosidase* genes," *Appl. Microbiol. Biotechnol.* 97, 5137-5147. DOI: 10.1007/s00253-0134874-1

Konig, J., Grasser, R., Pikor, H., and Vogel, K. (2002). "Determination of xylanase, β-glucanase, and cellulase activity," *Anal. Bioanal. Chem.* 374, 80-87. DOI: 10.1007/s00216002-1379-7

Kouvelis, V. N., Davenport, K. W., Brettin, T. S., Bruce, D., Detter, C., Han, C. S., Nolan, M., Tapia, R., Damoulaki, A., and Kyrpides, N. C. (2011). "Genome sequence of the ethanol-producing *Zymomonas mobilis* subsp. *pomaceae lectotype* strain ATCC 29192," *J. Bacteriol*. 193, 5049-5050. DOI: 10.1128/JB.05273-11

Kouvelis, V. N., Saunders, E., Brettin, T. S., Bruce, D., Detter, C., Han, C., Typas, M. A., and Pappas, K. M. (2009). "Complete genome sequence of the ethanol producer *Zymomonas mobilis* NCIMB 11163," *J. Bacteriol.* 191, 7140-7141. DOI: 10.1128/JB.01084-09

Kouvelis, V. N., Bruce, D., Detter, C., Tapia, R., Han, C., Tampakopoulou, V., Goodwin, L., Woyke, T., and Kyrpides, N. C. (2014). "Finished genome of *Zymomonas mobilis* subsp. *mobilis* strain CP4, an applied ethanol producer," Genome announcements 2:e00845-00813

Linger, J. G., Adney, W. S, and Darzins, A. (2010). "Heterologous expression and extracellular secretion of cellulolytic enzymes by *Zymomonas mobilis*," *Appl. Environ. Microbiol.* 76, 6360-6369. DOI: 10.1128/AEM.00230-10

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). "Protein measurement with the Folin phenol reagent," *J. Biol.* 193, 265-275.

Luo, Z. and Bao, J. (2015). "Secretive expression of heterologous β-glucosidase in *Zymomonas mobilis*," *Bioresource Bioprocess*. 2(1), 29-35. DOI: 10.1186/s40643-015-0053-9

Lynd, L. R., Van, Z. W. H., McBride, J. E., and Laser, M. (2005). "Consolidated bioprocessing of cellulosic biomass: An update," *Curr. Opin. Biotechnol.* 16, 577-583. DOI: 10.1016/j.copbio.2005.08.009

Lynd, L. R., Weimer, P. J., Van, Z. W. H., and Pretorius, I. S. (2002). "Microbial cellulose utilization: Fundamentals and biotechnology," *Microbiol. Mol. Biol. Rev.* 66, 506-577. DOI: 10.1128/MMBR.66.3.506-577.2002

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31, 426-428.

Pappas, K. M., Kouvelis, V. N., Saunders, E., Brettin, T. S., Bruce, D., Detter, C., Balakireva, M., Han, C. S., Savvakis, G., and Kyrpides, N. C. (2011). "Genome sequence of the ethanol-producing *Zymomonas mobilis* subsp. *mobilis lectotype* strain ATCC 10988," *J. Bacteriol.* 193, 5051-5052. DOI: 10.1128/JB.05273-11

Piriya, P. S., Vasan, P. T., Padma, V. S., Vidhyadevi, U., Archana, K., and Vennison, S. J. (2012). "Cellulosic ethanol production by recombinant cellulolytic bacteria harbouring pdc and adh II genes of *Zymomonas mobilis*," *Biotechnol. Res. Inter.* 2012. DOI:10.1155/2012/817549

Rajnish, K. N., Choudhary, G. M., and Gunasekaran, P. (2008). "Functional characterization of a putative endoglucanase gene in the genome of *Zymomonas mobilis*," *Biotechnol. Lett.* 30, 1461-1467. DOI: 10.1007/s10529-008-9716-3

Rogers, P. L., Jeon, Y. J., Lee, K. J., and Lawford, H. G (2007). "*Zymomonas mobilis* for fuel ethanol and higher value products." *Adv. Biochem. Eng. Biotechnol.* 108, 263-288. DOI: 10.1007/10_2007_060

Ruiz, E., Romero, I., Moya, M., Sanchez, S., Bravo, V., and Castro, E. (2007). "Sugar fermentation by *Fusarium oxysporum* to produce ethanol," *World J. Microbiol. Biotechnol.* 23, 259-267. DOI: 10.1007/s11274-006-9222-5

Sanchez, O. J., and Cardona, C. A. (2008). "Trends in biotechnological production of fuel ethanol from different feedstocks," *Bioresource Technol*. 99, 5270-5295. DOI: 10.1016/j.biortech.2007.11.013

Sarks, C., Bals, B. D., Wynn, J., Teymouri, F., Schwegmann, S., Sanders, K., Jin, M., Balan, V., and Dale, B. E. (2016). "Scaling up and benchmarking of ethanol production from pelletized pilot scale AFEX treated corn stover using *Zymomonas mobilis* 8b," *Biofuels*, 1-15. DOI: 10.1080/17597269.2015.1132368

Seo, J. S., Chong, H., Park, H. S., Yoon, K. O., Jung, C., Kim, J. J., Hong, J. H., Kim, H., Kim, J. H., Kil, J.-I., *et al.* (2005). "The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4," *Nat. Biotechnol.* 23, 63-68. DOI: 10.1038/nbt1045

Soares, J. F. L., Dias, A. C. F., Fasanella, C. C., Taketani, R. G., Lima, A. O. S., Melo, I. S., and Andreote, F. D. (2013). "Endo-and exoglucanase activities in bacteria from mangrove sediment," *Braz. J. Microbiol.* 44, 969-976. DOI: 10.1590/S1517 83822013000300048

Thompson, D. N., Chen, H. C., and Grethlein, H. E. (1992). "Comparison of pretreatment methods on the basis of available surface area," *Bioresource Technol.* 39, 155-163. DOI: 10.1016/09608524(92)90135-K

Van, Z. W. H., Lynd, L. R., Den, H. R., and McBride, J. E. (2007). "Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*," in: *Biofuels*, Springer, 205-235. DOI: 10.1007/10_2007_061

Vasan, P. T., Piriya, P. S., Prabhu, D., and Vennison, S. J. (2011). "Cellulosic ethanol production by *Zymomonas mobilis* harboring an endoglucanase gene from *Enterobactercloacae*," *Bioresource Technol.* 102, 2585-2589. DOI: 10.1016/j.biortech.2010.09.110

Wood, P. J. (1980). "Specificity in the interaction of direct dyes with polysaccharides," *Carbohydrate Research* 85, 271-287.

Xu, Q., Singh, A., and Himmel, M. E. (2009). "Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose," *Curr. Opin. Biotechnol*. 20, 364-371. DOI: 10.1016/j.copbio.2009.05.006

Yang, S., Pan, C., Hurst, G. B., Dice, L., Davison, B. H., and Brown, S. D. (2014). "Elucidation of *Zymomonas mobilis* physiology and stress responses by quantitative proteomics and transcriptomics," *Front. Microbiol.* 5, 246-259. DOI: 10.3389/fmicb.2014.00246

Yi, X., Gu, H., Gao, Q., Liu, Z. L., and Bao, J. (2015). "Transcriptome analysis of *Zymomonas mobilis* ZM4 reveals mechanisms of tolerance and detoxification of phenolic aldehyde inhibitors from lignocellulose pretreatment," *Biotechnol. Biofuels*. 8(1), 153-168. DOI: 10.1186/s13068-015-0333-9

Article submitted: October 4, 2016; Peer review completed: December 4, 2016; Revised version received and accepted: December 15, 2016; Published: December 19, 2016.
DOI: 10.15376/biores.12.1.1165-1178