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Hydrolysis of lignocellulosic feedstock by novel cellulases originating from *Pseudomonas* sp. CL3 for fermentative hydrogen production

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ABSTRACT

A newly isolated indigenous bacterium *Pseudomonas* sp. CL3 was able to produce novel cellulases consisting of endo-β-1,4-D-glucanase (80 and 100 kDa), exo-β-1,4-D-glucanase (55 kDa) and β-1,4-D-glucosidase (65 kDa) characterized by enzyme assay and zymography analysis. In addition, the CL3 strain also produced xylanase with a molecular weight of 20 kDa. The optimal temperature for enzyme activity was 50, 45, 45 and 55 °C for endo-β-1,4-D-glucanase, exo-β-1,4-D-glucanase, β-1,4-D-glucosidase and xylanase, respectively. All the enzymes displayed optimal activity at pH 6.0. The cellulases/xylanase could hydrolyze cellulosic materials very effectively and were thus used to hydrolyze natural agricultural waste (i.e., bagasse) for clean energy (H_2) production by *Clostridium pasteurianum* CH4 using separate hydrolysis and fermentation process. The maximum hydrogen production rate and cumulative hydrogen production were 35 ml/L/h and 1420 ml/L, respectively, with a hydrogen yield of around 0.96 mol H_2/mol glucose.

1. Introduction

Biomass energy is a promising option of renewable energy but the feedstock used for producing biomass energy should come from non-food biomass or agricultural waste to avoid the competition with food source and arable land (Hui et al., 2010). Hence, lignocellulosic materials are utilized as feedstock for the production of liquid (i.e., ethanol, butanol) or gaseous (i.e., hydrogen or methane) fuels (Yu and Zhang, 2003; Lo et al., 2008a,b). Agricultural wastes such as wheat straw, bagasse, rice straw and Napiergrass were successfully converted to ethanol and hydrogen after hydrolysis (Lever et al., 2010; Lo et al., 2008b, 2009).

The rate limiting step of cellulosic bioenergy production processes is hydrolysis and saccharification of cellulosic materials. Most biofuels producing microorganisms (such as *Saccharomyces cerevisiae*) could only utilize simple cellulosic sugars (e.g., glucoses or cellubiose) for growth and biofuel production, whereas the rigid structure of cellulose fibers make them very difficult to be degraded or saccharified by the attack of cellulolytic enzymes (Chandra et al., 2007). Therefore, to accelerate enzymatic cellulose hydrolysis, pretreatment of cellulosic materials is usually employed by combined procedures of physical (e.g., milling, steam explosion) and chemical (alkali or acidic treatment) methods (Saha and Cotta, 2006; Zhang et al., 2007). For example, after pretreatment with alkaline peroxide and dilute sulfuric acid, the enzymatic saccharification efficiency of wheat straw and hardwood xylan was substantially enhanced to reach a 97% or 80–90% of theoretical value, respectively (Torget and Hsu, 1994; Taherzadeh and Karimi, 2008).

Cellulase is one kind of biocatalysts composed of endo-β-1,4-D-glucanase, exo-β-1,4-D-glucanase and β-1,4-D-glucosidase, which were used to convert cellulose fibers to soluble sugar for follow-up biofuels production by bacteria or yeast (Shanmughapriya et al., 2010). The endo-β-1,4-D-glucanase reacted on the amorphous part of cellulose, causing fragmentation of cellulosic materials. The exo-β-1,4-D-glucanase hydrolyzes crystalline cellulose into simple sugars such as cellobiose and cellodextrin. Finally the cellooligosaccharides were degraded to glucose by β-glucosidase (Li et al., 2009; Soni et al., 2010). Cellulases are often produced from fungal species, such as *Trichoderma* sp. and *Aspergillus* sp. Some bacteria are also effective in producing cellulolytic enzymes, such as *Cellulomonas* sp., *Bacillus* sp., and *Clostridium thermocellum* (Suto and Tomita, 2001). However, cellulolytic enzymes production by *Pseudomonas* sp. is still rarely documented in the literature (Subramaniyan and Prema, 2000; Xu et al., 2005; Her et al., 1999). In this work, we isolated a cellulolytic *Pseudomonas* sp. strain able to produce novel and effective cellulase and xylanase

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enzymes, which were used to hydrolyze agricultural waste to prepare carbon source for biohydrogen production.

Hydrogen is a clean, sustainable, and efficient energy carrier, which is expected to play a crucial role in the development of future energy sources. Producing hydrogen from cellulosic materials via dark fermentation is an economic, environmental-friendly and sustainable way of producing hydrogen. Cellulosic bioH_2 production has caught much attention recently, as more and more research work has been devoted to this field (Lo et al., 2008b, 2009). In this study, sugarcane bagasse was pretreated by NaOH and hydrolyzed by novel cellulases/xylanase produced form an indigenous isolate *Pseudomonas* sp. CL3. The bagasse hydrolysate was then fermented by *Clostridium pasteurianum* CH4 to produce biohydrogen using a separate hydrolysis and fermentation process.

2. Methods

2.1. Strain isolation

Pseudomonas sp. CL3 was isolated from forest soil in southern Taiwan. The soil sample was diluted in sterile dilution solution, and then spread on BHM agar plate incubated for 2 days at 37 °C. The BHM agar was used as selection medium consisting of (g/L): carboxymethyl-cellulose (CMC), 10.0; $MgSO_4{\cdot}7H_2O$, 0.2; K_2HPO_4, 1.0; KH_2PO_4, 1.0; NH_4NO_3, 1.0; $FeCl_3{\cdot}6H_2O$, 0.05; $CaCl_2$, 0.02; agar, 15.0. The Congo red staining method was utilized to screen the cellulase-producing strain, selecting the plate with a clear hydrolysis zone after washing off Congo red dye by 8 M NaCl (Wang et al., 2009a; Sakamoto and Toyohara, 2009). The selected colonies were incubated in liquid BHM medium (without agar) at 37 °C and 200 rpm for 2 days, and then samples were taken from the culture to evaluate the cellulase/xylanase activities by the amount of reducing sugars produced. Finally, the most effective strain was chosen as the target strain to be used in the present work for cellulase production.

2.2. Identification of isolates

The isolated target strain was inoculated into LB medium and was cultivated at 37 °C and 200 rpm for 24 h. An appropriate amount of cells were taken from the culture and the genomic DNA of the cells was extracted by DNA Purification Kit (Viogene, Taiwan) for the sequence analysis. Amplification of the 16S rDNA gene was conducted by polymerase chain reaction (PCR) using F8 and R1510 primer (Wang et al., 2009b). The PCR products were sequenced (Mission Biotech, Taipei, Taiwan) and the obtained sequence was compared with the database available in NCBI GenBank to determine the identity of the isolated strain. The phylogenetic tree was also constructed using the MegAlign software (Wang et al., 2009a; Liang et al., 2009).

2.3. Enzyme activities assay

The activity of endoglucanase was analyzed according to the method reported by Nitisinprasert and Temmes (1991). The enzyme reaction solution contained 0.5 ml sample solution and 0.5 ml reactive reagent composed of 10 g/L CMC in 100 mM sodium acetate buffer (pH 5.0) at 40 °C for 30 min. The amount of reducing sugar was measured by using DNS method (Miller, 1959) using glucose (Sigma) as the standard for the quantification of reducing sugar. The unit of endoglucanase activity (U) was defined as the amount of enzyme required to release 1 μg of reducing sugar equivalent per min.

The activity of exoglucanase was assayed according to the method described by Nitisinprasert and Temmes (1991). The enzyme reaction solution contained 0.5 ml sample solution and 0.5 ml reactive reagent composed of 10 g/L Avicel in 100 mM sodium acetate buffer (pH 5.0) at 50 °C for 2 h. The amount of reducing sugar was measured by DNS method at OD_{540} using glucose as standard. The unit of exoglucanase activity (U) was defined as the amount of enzyme required to release 1 μg of reducing sugar equivalent per min.

The assay of β-glucosidase activity followed that reported by Nitisinprasert and Temmes (1991). The enzyme reaction solution contained 0.5 ml sample solution and 0.5 ml reactive reagent composed of 10 g/L cellobiose in 100 mM sodium acetate buffer (pH 5.0) at 40 °C for 30 min. Quantitative analysis of glucose was carried out using high performance liquid chromatography (HPLC) system equipped with a RI detector. The column used was ICSep ICE-COREGEL 87H3 (Transgenomic, USA). The mobile phase used was 0.008 N H_2SO_4 with a controlled flow rate of 0.4 ml/min. The unit of β-glucosidase activity (U) was defined as the amount of enzyme required to release 1 μg of glucose equivalent released per min.

The assay of xylanase activity was conducted according to the method reported by Nitisinprasert and Temmes (1991). The enzyme reaction contained 0.5 ml sample solution and 0.5 ml reactive reagent composed of 10 g/L xylan in 100 mM sodium acetate buffer (pH 5.0) at 50 °C for 10 min. The amount of reducing sugar was measured by DNS method at OD_{540} using xylose as the standard for quantitative analysis of reducing sugar. The unit of xylanase activity (U) was defined as the amount of enzyme required to release 1 μg of reducing sugar equivalent per min.

2.4. SDS–PAGE and zymography analysis

Pseudomonas sp. CL3 was grown on BHM medium at 37 °C and 200 rpm for 4 days. An appropriate amount of the culture broth was taken and centrifuged (4 °C, 9000*g*) for 20 min. The supernatant (containing extracellular cellulolytic enzymes) was subjected to precipitation by salting out method with ammonium sulfate at 50% saturation. The precipitate containing crude enzymes was collected after centrifugation (4 °C, 9000*g*) for 20 min and was re-dissolved in phosphate buffer (pH 7, 100 mM). The crude enzyme solution was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and zymography analysis. The SDS–PAGE analysis was conducted using a 12% polyacrylamide gel to determine the molecular weight of the existing enzymes. The zymography analysis for β-glucosidase (Joo et al., 2009) was performed on 12% polyacrylamide gels amended with 0.001% 4-methylumbelliferyl-β-D-gluco-pyranoside (MUG) as substrate but in the absence of SDS. After reaction for 30 min at 37 °C, MUG was hydrolyzed to fluorescent methyl lumbelliferone, which was visualized under ultraviolet light. For the zymography of exoglucanase, the procedures were similar to those used for β-glucosidase zymography, except that MUC (4-methylumbelliferyl-β-D-cellobioside) was used as substrate, which have the same reactive mechanism as MUG (Walter and Schrempf, 1996). For the zymography of endoglucanase, 0.5% CMC was used as substrate and the reaction was performed at 37 °C for 30 min and the clear zones were visualized by 0.1% Congo red solution (Sakamoto and Toyohara, 2009). The procedures for xylanase zymography analysis were similar to those used for endoglucanase zymography except that 0.5% xylan (instead of CMC) was used as the reaction substrate.

2.5. Pretreatment of cellulosic feedstock

In this study, acid and alkaline pretreatments of bagasse were conducted to breakdown the rigid structure of cellulose for easier enzymatic attack. For alkaline pretreatment, 1 g of bagasse was treated with 20 ml of alkaline solution (containing 15 g/L NaOH