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Artificial construction and characterization of a fungal consortium that produces cellulolytic enzyme system with strong wheat straw saccharification

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ABSTRACT

A consortium-APcT2 composed of 92% of *Trichoderma* sp. T-1, 6.7% of *P. chrysosporium* and 1.3% of *A. oryzae* A-4 that produces cellulolytic enzyme system with strong wheat straw saccharification was constructed using Taguchi design combined with variance analysis (ANOVA). Among 32 consortia constructed from 6 fungi using Taguchi design, consortium 20 with the strongest composition was selected. The inhibitive fungal constituent in consortium 20 was subsequently removed according to the ANOVA results. The finally optimized consortium-APcT2 yielded 805.12 mg gds^{-1} sugars, 26.98% higher than the pure *Trichoderma* sp. T-1. Protein profile analysis of the cellulolytic enzyme systems, sugar composition analysis of the hydrolysates and compatibility evaluation of the fungal constituents showed that the enhanced straw saccharification of the consortium-APcT2 could be mainly attributed to the enhancement of the co-cultivation for enzyme production and the synergistic action of different types of enzymes in the hydrolysis process.

1. Introduction

Renewable resources are becoming increasingly important recently. It is estimated that more than one billion tons of lignocellulosic biomass per year, replacing as much as 30% of the total US gasoline consumption, could be sustainably harvested in the form of crop and forestry residues in the USA alone (Merino and Cherry, 2007). Bioconversion of this abundant biomass for the production of fuels and chemicals, natural and man-made, has the potential to change the world economically, socially, and environmentally (Detroy and Stjulian, 1983). To initiate the production of fuels and chemicals from lignocellulosic biomass, hydrolysis of the cellulosic components into fermentable sugars is necessary. However, the biomass hydrolysis process requires a large quantity of cellulolytic enzymes in the present bioconversion system. The high cost of cellulolytic enzymes is the primary hindrance in the cost-effective processing of lignocellulosic biomass. Significant efforts have been expended to reduce cellulase cost by focusing on the improvement of the efficiency of known cellulase, identification of new, more active cellulase, creation of multicomponent cellulase system optimized for selected pretreated substrates, and minimization of costs for cellulase production (Kumar et al., 2008b; Merino and Cherry, 2007).

Despite these efforts, the bioconversion of lignocellulosic biomass is still challenged by its low conversion efficiency. More and more researches proved that single component cellulase or cellulase from pure cultures could not convert highly ordered polymer into monomer efficiently (Han and Chen, 2007; Irwin et al., 1993; Kim et al., 2009). Mixing of several types of enzymes acting cooperatively has been proven to be an effective strategy for improving cellulosic wastes digestion (Boisset et al., 2001). Since no single microorganism could produce all the enzymes necessary for complete bioconversion of lignocellulose, use of microbial consortia which act synergistically for rapid bioconversion of lignocellulosic biomass is attractive (Kumar et al., 2008a; Wang et al., 2006). Besides, compared with conventional fermentations using pure cultures, the fermentations using consortia allow to obtain higher product yield and growth rate, to utilize cheap wastes, to overcome nutritional limitations in poor conditions, and to strengthen the protection of the culture from contamination (Feng et al., 2007; Guevara and Zambrano, 2006; Kumar et al., 2008a; Yang et al., 2004).

Conventional microbial consortia were obtained directly from nature by simple domestication. The lignocellulose-degrading ability of these kinds of microbial consortia is usually strong, but easy to degenerate due to the complex and unknown composition. Since extensive microorganisms capable of lignocellulose degradation have been isolated and purified, it is feasible for artificial design of complete cellulolytic enzymes producing-consortia with strong lignocellulose saccharification (Garcia-Kirchner et al., 2002;

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Guevara and Zambrano, 2006; Wang et al., 2006). Some artificial consortia with excellent characteristics have been successfully applied in industry, although the relationship among the strains in mixed cultures is unknown. Since the isolation and design of efficient consortia are a random process till now (Xu et al., 2009), the standard factorial design, which is time-consuming and laborious, is still a frequently used method.

In the recent years, experimental design such as Taguchi design, Plackett–Burman design, and Box–Behnken design have received much attention in the scientific researches (Montgomery, 2007). Taguchi design is a simple statistical tool utilizing orthogonal arrays to obtain extensive parameter data from only a few experiments. In this design, variables or factors are arranged in an orthogonal array (OA), in which each combination of levels (or variables) between each pair of factors appears an equal number of times. Due to an orthogonal layout, the number of test runs in the experiment is minimized with pair-wise balancing property (Rao et al., 2008). Compared with other experimental designs, the number of factors in Taguchi design is flexible (NF $\geqslant$ 1), and factors both with and without interaction could be tested. The objective of this study was to construct an efficient cellulolytic enzymes producing-consortium for fermentable sugars preparation from pretreated wheat straw, which is a world-wide ligocellulosic biomass feedstock. In this study, Taguchi design was firstly applied in the consortium construction to simplify the design and isolation procedure of efficient consortia.

2. Methods

2.1. Microorganisms

Aspergillus oryzae A-4, *Penicillium* sp. P-2, *Trichoderma* sp. T-1, *Penicillium* sp. zhwb-1 and *Aspergillus niger* A-1 were isolated from the African jungle soil samples collected from Republic of Cameroon and preserved in Lab of Microbiology, Zhejiang University. *Phanerochaete chrysosporium* was supplied from the College of Life Science and Technology, Huazhong University of Science and Technology, China. An oleaginous yeast *Trichosporon fermentans* CICC 1368 capable of co-fermenting xylose and glucose was supplied for single cell oils (SCOs) production from the China Center of Industrial Culture Collection.

2.2. Solid state fermentation (SSF) medium

SSF medium in a Petri dishes (U = 9 cm) for cellulolytic enzymes production consisted of 3 g of wheat straw, 1 g of wheat bran and 4 ml of Mandel's medium (Mandels et al., 1976) with slight modification. Wheat straw was obtained from Anhui province in China, while wheat bran was collected from a local market. All of the solid substrates were dried and milled to 20–40 mesh. The modified Mandel's medium contained the following chemicals ($g\,l^{-1}$): $NaNO_3$ 2, KH_2PO_4 1.5, $CaCl_2$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.005, $MnSO_4 \cdot H_2O$ 0.0016, $ZnSO_4 \cdot H_2O$ 0.0014, $CoCl_2$ 0.0005, and its pH was adjusted to 6.

2.3. Alkaline peroxide pretreatment of wheat straw

For alkaline peroxide pretreatment, wheat straw (10%, w/v) was slurried in water containing 2.5% (w/v) H_2O_2, adjusted to pH 11.5 using 10 M NaOH, and shaken at 180 rpm and 37 °C for 24 h (Saha and Cotta, 2010). The pretreated solids were washed with deionized water and then dried. Compositional analysis (Van Soest et al., 1991) showed that the alkaline peroxide pretreated wheat straw contained 66.75% cellulose, 16.44% hemicellulose, 6.65% lignin and 2.06% ash.

2.4. The enzymatic hydrolysate (EH) preparation system integrating cellulolytic enzymes production and enzymatic hydrolysis

To simplify the preparation of fermentable sugar and the evaluation of the hydrolytic ability of fungi, the enzyme production and pretreated straw saccharification were combined together in a system without enzyme purification and concentration. For enzyme production, 2 ml of spore suspension, which was produced by releasing spores from PDA plate (3–4 days old) with deionized water containing 0.1% Tween-80, was inoculated into the SSF medium. SSF was performed at 28 °C and 50%–80% humidity for 4 days. After fermentation, the residue was transferred to a 100 ml flask and mixed with 80 ml of citric acid buffer (pH 4.8, 0.05 M). The mixture was shaken at 37 °C for 2 h, and then centrifuged to obtain a cell-free culture filtrates. The cell-free culture filtrate (40 ml) was further mixed with 1 g of pretreated wheat straw, and the slurry was shaken gently (120 rpm) at 50 °C for 20 h for overall sugar release. The final EH containing high concentration of sugar was obtained by vacuum filtration, which could be used as carbon source for the production of SCOs.

2.5. Artificial construction of consortia using Taguchi design

A standard orthogonal array (OA) L32 (2^{31}) was used to construct consortia. Six cellulolytic fungi were selected as six factors and the impact of two-way interactions were also studied. Spore concentration of each fungus in 2 ml of working spore suspensions were chosen as levels. Each factor was assigned with 0 spores ml^{-1} of spore concentration as low level and 1×10^6 spores ml^{-1} of spore concentration (1×10^5 spores ml^{-1} for *A. oryzae* A-4) as high level. Then, main effect and interaction effect of different strains on straw saccharification were analyzed using the analysis of variance (ANOVA).

2.6. Production of SCOs using wheat straw hydrolysate

Wheat straw sulphuric acid hydrolysate (SAWSH) was prepared as described before (Huang et al., 2009). Wheat straw was mixed with dilute sulphuric acid (1.5%, v/v) to give a mixture with a solid loading of 10% (w/v). The mixture was treated in an autoclave at 121 °C for 90 min. After cooling and adjusting the pH to 6.5, the liquid fraction (SAWSH) was separated by vacuum filtration. Both the SAWSH and EH were concentrated till the reducing sugar concentration reached to 50 $g\,l^{-1}$. In addition to the concentrated hydrolysate (SAWSH and EH), the fermentation medium for SCOs production also contained ($g\,l^{-1}$): yeast extract 0.5, peptone 1.0, $MgSO_4 \cdot 7H_2O$ 0.4, KH_2PO_4 2.0, $MnSO_4 \cdot H_2O$ 0.003, $CuSO_4 \cdot 5H_2O$ 0.0001. The cultivation of *T. fermentans* was performed in a 250 ml conical flask containing 50 ml fermentation medium in a rotary shaker at 28 °C and 180 rpm (Zhu et al., 2008).

2.7. Analytical methods

2.7.1. Enzyme assay

Filter paper enzyme activity (FPAase) was analyzed using the method of Ghose (1987). Endoglucanase activity (CMCase) was determined using 2% (w/v) carboxymethyl cellulose solution in citrate buffer (Ghose, 1987). β-glucosidase activity was assayed using p-nitrophenol-β-1, 4-D-glucoside as the substrate (Wood and Bhat, 1988). Xylanase activity was measured using the method of Bailey et al. (1992) by mixing 0.25 ml of an appropriately diluted enzyme solution with 0.5 ml of 1.0% oat spelt xylan in 0.05 M citric acid buffer (pH 4.8) at 50 °C for 30 min. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar from the substrates per minute under assay condi-