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Microbial hydrolysis and fermentation of rice straw for ethanol production

Xiaodan Wu [a,b], Jinsheng Zhang [a,b,*], Erni Xu [a,b], Yuhuan Liu [a,b], Yanling Cheng [c], Min Addy [c], Wenguang Zhou [c], Richard Griffith [c], Paul Chen [c], Roger Ruan [a,b,c,*]

[a] MOE Biomass Energy Research Center and State Key Laboratory of Food Science, Nanchang University, Nanchang 330047, China
[b] School of Food Science and Technology, Nanchang University, Nanchang 330031, China
[c] Center for Biorefining and Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN 55108, USA

HIGHLIGHTS

- Two fungi could co-grow and excrete both lignin-degrading enzymes and cellulase.
- They could hydrolyze rice straw *in situ* and produce 22.74 g/L of reducing sugar.
- Yeasts were co-immobilized in suitable microenvironment for ethanol fermentation.
- The ethanol productivity of two co-immobilized yeasts reached up to 2.17 g/(L h).
- The co-immobilized beads remained intact after 4 cycles of use.

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ABSTRACT

In this work, *Trichoderma reesei* Aq-5b and *Trichoderma viride* NSW-XM, capable of excreting lignin-degrading enzymes and cellulase were used to create a mixed culture system to hydrolyze rice straw *in situ*. The results showed that Aq-5b and NSW-XM were able to co-grow and produce complementary lignin degrading enzymes, namely laccase and lignin peroxidase. The two microorganisms were able to produce 22.74 g/L of reducing sugar in the optimal condition. For fermentation of the hydrolysates produced, *Saccharomyces cerevisiae* and *Candida tropicalis* NSW-NW were co-immobilized in polymer beads composed of sodium alginate, polyvinyl alcohol, and silicon dioxide (SA–PVA–SiO_2). The beads remained intact after 4 cycles of use and provided satisfactory protection for the yeasts and a suitable microenvironment for ethanol fermentation, greatly improved the efficiency of ethanol production, shortened the fermentation cycle and achieved yeasts recycling for continuous or batch-fed fermentation. The productivity of ethanol reached up to 2.17 g/(L h).

1. Introduction

The fuel ethanol industry is booming [1,2] because gasohol (a mixture of gasoline and ethanol) is widely adopted for its advantages of full combustion, fewer pollutants, and less explosive. As an alternative to starch, abundant crop residues can provide a stable source of feedstock for ethanol production. Various lignocellulosic crop residues, such as wheat straw [3], rice straw [4], corn stalk [5], sunflower stalk [6], have been explored for ethanol production. However, any research in ethanol production from crop residues is confronted by at least three challenges: (1) high energy consumption, serious pollution, and special equipment requirements are constraining the pretreatment process aimed at removing the lignin that binds carbohydrate polymers; (2) costly cellulase is needed during the enzymatic saccharification process; (3) pentose utilization is low and yeast cells cannot be reused. The present research was aimed at reducing these three bottlenecks. Possible means of improvement in these aspects were explored in this paper using rice straw, the most abundant agriculture residue in Southern China, as feedstock.

Rice straw contains 20–25% lignin, 25–30% hemicellulose, and 30–35% cellulose. Prior to the enzymatic saccharification of rice straw, the steric hindrance of lignin and hemicelluloses must be

* Corresponding authors at: MOE Biomass Energy Research Center and State Key Laboratory of Food Science, Nanchang University, Nanchang 330047, China; School of Food Science and Technology, Nanchang University, Nanchang 330031, China; Center for Biorefining and Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN 55108, USA.
E-mail address: ruanx001@umn.edu (R. Ruan).

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reduced through pretreatment process. In recent years, a number of different methods, including physical methods (e.g. steam explosion [7], liquid hot water [8], microwave [9], etc.) and chemical methods (e.g. dilute acid, flow-through acid, lime, wet oxidation and ammonia, etc.) [10], have been developed for the pretreatment of lignocellulosic biomass. However, most of these pretreatment methods require high temperature, high pressure, or high doses of chemicals that may be toxic to the enzymes and fermentative microorganisms. Biological methods, which are considered mild, environment-friendly and low cost, normally involve fungi, certain actinomycetes, or bacteria which secrete lignin peroxidases and laccases that help remove a considerable amount of lignin from the biomass [11]. It is now clear that lignin degradation is a multi-enzymatic process because of its complex structure, involving an array of accessory enzymes [12]. So biological processes require long processing time [13], which constitute a serious limitation for large-scale applications. There are some differences in lignin degradation enzymes among microorganisms, so mixed fermentation without high-cost of enzyme extraction and purification is expected to improve the efficiency of microbial pretreatment by the synergy effect of various enzymes secreting by different strains [14–16].

The hydrolyzate of straw cellulose is composed of glucose that is regarded as the optimal fermentable monosaccharide while the hydrolyzate of straw hemicellulose contains 85–90% of xylose that is difficult to be fermented by ordinary *Saccharomyces cerevisiae*. Xylose fermentation has become a hot topic since Wang et al. proposed xylose could be converted into ethanol by certain microorganisms [17]. Hundreds of microorganisms, which can metabolize xylose to ethanol, have been identified, including bacteria (*Zymomonas mobilis*), filamentous fungi (*Neurospora crassa*), yeasts (*Pachysolen tannophilus*, *Pichia stipitis*, *Candida shehatae*, *Candida tropicalis*, *Hansenula polymorpha*). In addition to the recombination technique [18,19], mixed fermentation can be used to realize efficient use of all the hydrolysate [20,21].

The type of yeasts and the cell concentration mainly determine the ethanol fermentation ability. There are problems with ethanol fermentation using free form yeasts, such as low initial cell concentration, long proliferation period, and difficulty to collect cells at the end of fermentation. By comparison, the immobilized cell technology developed in the 1960s has characteristics of high resistance, continuous and repeated use, high cell density cultivation, and simple product separation [22–24]. The cell immobilization may be achieved through embedding, chemical bonding, and physical adsorbing. The embedding method, which has the advantages of high ethanol yield, strong acetic acid toleration and pollution resistance brought by the immobilized microenvironment is very suitable for anaerobic fermentation. The insoluble gel of calcium alginate is formed when sodium alginate (SA) is calcified by calcium chloride. The preparation of calcium alginate is simple, inexpensive, but the phosphate in the medium will gradually cause the gel to rupture and disintegrate. In recent years, polyvinyl alcohol (PVA) is widely used as the immobilization material because of the advantages of strong adhesion, film flexibility, smoothness, and abrasion resistance conferred by its unique molecular chain structure containing a large number of hydroxyl groups [25], whereas the moisture content is low because of the strong tendency to agglomerate. It was presumed that PVA might contribute to improving the durability and strength of the beads, while sodium alginate might improve the surface properties of the beads, reducing the tendency to agglomerate [26]. Further, SiO_2 can increase the strength by combining SA through the chemical bonds [27]. However, little literature on Silica-PVA-alginate beads as an immobilization material for yeasts co-immobilization is available. As a result of our work, our research group obtained stable immobilized yeasts capable of continuously fermenting for more than 14 batches, when we used 2% SA, 6% PVA, 1% SiO_2 as the embedding solution, and 3% $CaCl_2$, 5% H_3BO_3 as the fixing solution.

In this study, a mixed culture system (system I) based on *Trichoderma viride* NSW-XM and *Trichoderma reesei* Aq-5b was constructed for enzymatic hydrolysis of rice straw. Another mixed culture system (system II) was built through co-immobilization for fermentation of the hydrolysate of rice straw. System II was based on the ethanol fermentation capacity of *S. cerevisiae* and *C. tropicalis* NSW-NW. The objective of this study was to evaluate the performance and compatibility of the two *Trichoderma* on solid culture media and to examine their performance on actual rice straw substrates in term of saccharification, and to evaluate the performance of the co-immobilized yeasts in term of ethanol production.

2. Material and methods

2.1. Materials

2.1.1. Microorganisms

T. reesei Aq-5b and *T. viride* NSW-XM were separated from the local deadwood, *C. tropicalis* NSW-NW was separated from the rumen of cattle. *S. cerevisiae* and *Pleurotus ostreatus* were procured from the Department of Microbiology, Nanchang University, Nanchang, China. The stock culture was maintained on potato dextrose agar (PDA) slant. The slants were incubated at 28 °C for 2–4 d and then stored at 4 °C.

2.1.2. Raw materials

Rice straw used in the experiments was harvested at maturity from a local farm in Nanchang, China. The air-dried rice straw was milled to powder that was passed through a 40-mesh sieve and stored in a sealed pot at room temperature. The main composition of the rice straw was determined to be 35% cellulose and 21% lignin according to the procedures of Agu et al. [28], and 28% hemicellulose according to a two-brominating method [29].

2.1.3. Media

Selection medium used for screening of laccase production is based on oxidation of guaiacol. The guaiacol-PDA medium was prepared according to Krishnaveni and Kowsalya [30]. The medium was made by adding 0.04% guaiacol to standard PDA medium (0.04 g guaiacol was added directly into 100 g standard PDA medium before sterilized at 121 °C for 25 min). Selection medium used for screening of lignin peroxidase production is based on decolorization of aniline blue. The aniline-blue-PDA medium was prepared according to Gao et al. [31] with minor modification. The medium was made by adding 0.01% aniline blue to standard PDA medium (0.01 g aniline blue was added directly into 100 g standard PDA medium before sterilized at 121 °C for 25 min). Potato-glucose medium (potato 200 g, glucose 20 g in 1 L water) was used as seed medium. The fermentation medium consisted of 6 g straw powder, 3 g wheat bran, 3 g soybean meal, 0.5 g KH_2PO_4, 0.3 g $CaCl_2$, and 100 g H_2O.

2.2. Methods

2.2.1. Performance evaluation and compatibility investigation of strains

P. ostreatus is commonly used in lignin degradation because it can excrete laccase and lignin peroxidase [32,33]. The reactions catalyzed by laccase can cause browning of guaiacol-PDA medium, forming a reddish-brown oxidized zone around the colony, while lignin peroxidase can cause fading of aniline-blue-PDA medium, forming a faded ring around the colony. In this study, *P. ostreatus*