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Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae*

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Abstract

Ethanol production from rice straw by simultaneous saccharification and fermentation (SSF) with *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae* was investigated and compared with pure cellulose, Avicel, as a reference. The straw was pretreated with dilute-acid hydrolysis. The SSF experiments were carried out aerobically and anaerobically at 38 °C, 50 g/l dry matter (DM) solid substrate concentration and 15 or 30 filter paper unit (FPU)/g DM of a commercial cellulase. The experiments were ended after 7 days, while an average of 2–3 days were usually enough to achieve the maximum ethanol yield. All the strains were able to produce ethanol from the pretreated rice straw with an overall yield of 40–74% of the maximum theoretical SSF yield, based on the glucan available in the solid substrate. *R. oryzae* had the best ethanol yield as 74% from rice straw followed by *M. indicus* with an overall yield of 68% with 15 FPU/g DM of cellulase. Glycerol was the main byproduct of the SSF by *M. indicus* and *S. cerevisiae* with yields 117 and 90 mg/g of equivalent glucose in the pretreated straw, respectively, while *R. oryzae* produced lactic acid as the major byproduct with yield 60 mg/g glucose equivalent in pretreated rice straw under anaerobic conditions.

Keywords: Ethanol; Simultaneous saccharification and fermentation; *Mucor indicus*; *Rhizopus oryzae*; *Saccharomyces cerevisiae*; Rice straw; Dilute-acid hydrolysis

1. Introduction

Ethanol from renewable resources has been of interest in recent decades as an alternative fuel or oxygenate additive to the current fossil fuels. Lignocellulosic materials are cheap renewable resources, available in large quantities [1]. Rice straw is one of the abundant lignocellulosic waste materials in the world. It is annually produced about 731 million tonnes which is distributed in Africa (20.9 million tonnes), Asia (667.6 million tonnes), Europe (3.9 million tonnes), America (37.2 million tonnes) and Oceania (1.7 million tonnes). This amount of rice straw can potentially produce 205 billion l bioethanol per year, which is the largest amount from a single biomass feedstock [2,3].

Cellulose, the major fraction of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes. This hydrolysis can be affected by porosity (accessible surface area) of lignocellulosic biomass, cellulose fiber crystallinity, and lignin and hemicellulose content. A pretreatment process is therefore essential in order to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. This pretreatment method gives high reaction rates and significantly improves cellulose hydrolysis [4,5]. Depending on the substrate and the conditions used, up to 95% of the hemicellulosic sugars can be recovered by dilute-acid hydrolysis from the lignocellulosic feedstock [6].

The cellulose fraction of lignocelluloses can be converted to ethanol by either simultaneous saccharification and fermentation (SSF) or separate enzymatic hydrolysis and fermentation (SHF) processes. SSF is more favored because of its low potential costs [5]. It results in higher yield of ethanol compared to SHF by minimizing product inhibition. On the other hand, SSF suffers from a drawback, which is different optimum temperature of the hydrolyzing enzymes and fermenting microorganisms. Large

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number of publication stated that the optimum temperature for enzymatic hydrolysis is at 40–50 °C, while the microorganisms with good ethanol productivity and yield do not usually tolerate this high temperature. This problem has usually been tackled by applying thermotolerant microorganisms such as *Kluyveromyces marxianus*, *Candida lusitaniae*, and *Zymomonas mobilis* or mixed culture of some microorganisms like *Brettanomyces clausenii* and *Saccharomyces cerevisiae* [7,8]. Another method to stand against this problem was reported to add a prehydrolysis step at, e.g. 50 °C for 24 h, followed by the addition of microorganisms at a suitable lower temperature [9].

Although rice straw is generally considered attractive for an ethanol feedstock [10], we detected no publication on ethanol production from rice straw with SSF during the last decade. Punnapayak and Emert [11] studied SSF of alkali-pretreated rice straw with *Pachysolen tannophilus* and *Candida brassicae*, where *P. tannophilus* resulted in higher ethanol yields than *C. brassicae* in all the experiment. However, they achieved only less than 30% of theoretical ethanol yield from rice straw.

The capability of zygomycetes was recently explored for production of ethanol. This class of filamentous fungi are saprophytic organisms, which are able to produce several metabolites including ethanol. Among three genera of *Rhizopus*, *Mucor* and *Rhizomucor*, *Mucor indicus* (formerly *M. rouxii*) and *Rhizopus oryzae* showed good performances on ethanol productivity from glucose, xylose and wood hydrolyzate [12]. They reported ethanol yield 0.39, 0.22, and 0.44 g/g on glucose, xylose, and dilute-acid hydrolyzate of spruce at 37 °C by *M. indicus* under aerobic conditions, respectively. The corresponding yield by *R. oryzae* was higher on xylose, but lower on glucose and the hydrolyzate than by *M. indicus*. Sues et al. [13] obtained 0.48 g/g ethanol from glucose under anaerobic condition by *M. indicus*, while *R. oryzae* gave 0.43 g/g ethanol from glucose [12]. These fungi may have several industrial advantages compared to baker's yeast such as (a) capability of utilizing xylose, the major fraction of hemicellulose of rice straw, (b) having a valuable biomass for, e.g. production of chitosan and (c) optimum temperature of the baker's yeast is in the range of 28–35 °C, where as these fungi showed ethanol production with comparable yield and productivity at 37 °C [14,15].

The current work deals with ethanol production from the cellulose fraction of rice straw by simultaneous saccharification and fermentation with *M. indicus*, *R. oryzae* in comparison with a thermotolerant strain of *S. cerevisiae*.

2. Materials and methods

2.1. Microorganism and media

M. indicus CCUG 22424 and *R. oryzae* CCUG 28958, obtained from Culture Collection, University of Göteborg (Göteborg, Sweden) were used in the experiments. These fungi were maintained on agar slants containing (g/l): D-glucose, 40; soy peptone, 10; agar, 20 at pH 5.5 and 30 ± 1 °C. Spore suspension was prepared by addition of 10 ml 0.05 M buffer citrate to slant and shaking it vigorously with tube shaker. One milliliter of the suspension, which contained $5(\pm1) \times 10^6$ spores/ml of *M. indicus* or *R. oryzae* was added to each flask. The strain of *S. cerevisiae* used was Thermosacc®, a stress-tolerant yeast for high alcohol, and high-temperature fermentations (Alltech Denmark A/S). It was grown at 30 ± 0.5 °C and maintained at 4 °C on agar slants containing (g/l): glucose, 20; yeast extract, 3; malt extract, 3; agar, 20.

2.2. Inoculum preparation

The inoculums were prepared using media containing (g/l): glucose, 50; yeast extract, 5; $(NH_4)SO_4$, 7.5; K_2HPO_4, 3.5; $MgSO_4 \cdot 7H_2O$, 0.75; $CaCl_2 \cdot 2H_2O$, 1 and 0.05 M buffer citrate at pH 5.5 ± 0.1. Volumes of 50 ml media were autoclaved at 121 °C and inoculated in 250 ml cotton-plugged Erlenmeyer flasks, and then incubated for 30 h at 35 ± 0.5 °C and shaked at 130 rpm for *R. oryzae* and 150 rpm for *M. indicus* and *S. cerevisiae*. At the end of incubation, the contents of these flasks were aseptically centrifuged and used for SSF. It resulted in inoculation of SSF by 1.0 ± 0.3 g biomass (base on the dry biomass) of *M. indicus*, 1.2 ± 0.3 g biomass of *R. oryzae*, and 0.98 ± 0.3 g biomass of *S. cerevisiae* in 100 ml working volume in all the SSF experiments.

2.3. Rice straw

Rice straw used in the experiments was obtained from Lenjan field (Isfahan, Iran). The straw was milled and screened to achieve the size of less than 833 μm (20 mesh) prior to pretreatment and contained 27(±0.5)% hemicellulose, 39(±1)% cellulose, 12(±0.5)% lignin and 11(±0.5)% ash, measured according to Sun et al [16]. The glucan and xylan of the straw were measured as 37.5(±0.5)% and 20.6(±0.3)%, respectively, based on a method described in the NREL Chemical Analysis & Testing Procedure [17]. This straw was pretreated by dilute-acid hydrolysis. The pretreatment was carried out by soaking 600 g of rice straw into 4 l of 0.5% sulfuric acid solution for 20 h. The mixture was then introduced into a 10 l reactor, steam heated for 1.5 min to achieve 15 bar pressure, and remained at this pressure for 10 min. It was then cooled within 3 min to reach 2 bar pressure, and the materials were then collected from the reactor. The solid fraction was washed five times with tap water at 50 °C, filtered and stored at 4 °C prior to SSF.

2.4. Enzymes

A commercial cellulase enzyme (BTXL) supplied from ASA Spezialenzyme GmbH (Germany) was used in all the experiments as sole enzymatic complex. The enzyme was originated by *Trichoderma reesei*. We have analyzed it by measuring its activity as FPU, endoglucanase, and β-glycosidase. According to the method presented by Adney and Baker [18], the enzyme showed activity of 55 FPU/ml. Endoglucanase activity was determined by its activity against carboxymethyl cellulose (CMC) as previously described [19] and showed CMCase activity of 101 IU/ml. β-Glycosidase activity was measured as 112 IU/ml according to the method presented by Ximenes et al. [20]. One unit of activity in each case is equal to 1 μmol of glucose produced per minute per milliliter of the enzyme.

2.5. Simultaneous saccharification and fermentation (SSF)

SSF was performed either under aerobic or anaerobic condition. A media contains (g/l): yeast extract, 5; $(NH_4)_2SO_4$, 7.5; K_2HPO_4, 3.5; $MgSO_4 \cdot 7H_2O$, 0.75; $CaCl_2 \cdot 2H_2O$, 1; pretreated rice straw or pure cellulose (Avicel), 50 and 0.05 M buffer citrate were used in 250 ml Erlenmeyer flasks. Medium pH was adjusted to 5.5 ± 0.1 for the filamentous fungi and 5 ± 0.1 for *S. cerevisiae* by NaOH (2 M). It was then autoclaved and the centrifuged microorganisms, the required enzyme, and 0.1 g Tween 80 (1 g/l) were added to each flask aseptically. The final volume in each flask was 100 ml. All the SSF experiments were performed at 38 °C. The enzyme loading was 15 or 30 FPU/g DM, which corresponds to 25 and 50 FPU/g cellulose of the rice straw.

In aerobic conditions, the flasks were covered with cotton, whereas they were equipped with a setup in anaerobic conditions. This setup involved a loop-trap containing glycerol to allow the gas outlet and preventing entrance of air, and two needle for sample removal and sparging nitrogen gas to the flasks, as described earlier [21]. Pure nitrogen gas was sparged into the media at the beginning of the fermentation and during the sampling. After 7 days SSF, the cultures were examined for contamination by microscope.