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Simultaneous delignification and saccharification of rice straw as a lignocellulosic biomass by immobilized *Thrichoderma viride* sp. to enhance enzymatic sugar production

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ABSTRACT

Simultaneous biological delignification and saccharification of rice straw by the immobilized *Trichoderma viride* cells were studied in this work. Response surface methodology as a multiple responses optimization technique was utilized to optimize several important factors such as the biomass content of the medium (w/v), inoculum size and agitation rate. Results indicated that at the obtained optimum conditions the lignin removal efficiency of 74% and sugar concentration equal to 8.52 g/L could be achieved in ten days of pretreatment. In addition, the influence of initial glucose concentration in the medium on both the pretreatment saccharification and the subsequent enzymatic hydrolysis efficiencies was investigated. It was revealed that the higher initial glucose concentration is beneficial to obtain higher total saccharification efficiency from pretreatment and the following enzymatic hydrolysis and a total efficiency equal to 81% was obtained for 15 g/L initial glucose concentration. Accordingly, it can be concluded that the immobilized *Trichoderma viride* in this work can be considered as a potentially applicable strain to design a promising lignocellulosic materials bio-pretreatment process.

1. Introduction

Bioethanol can be considered as an alternative to petroleum-derived transportation fuels to reduce the overall contribution of greenhouse gases to the atmosphere, [1]. Lignocellulosic biomass such as agriculture and forest residue have been investigated as the low cost sources of substrate for the fermentative production of ethanol [1,2]. Rice straw is one of the abundant lignocellulosic waste materials in the world and Asia is responsible for 90% of its annual global production [3,4]. In terms of chemical composition, the straw predominantly contains cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%) [5]. However, lignocellulosic materials such as rice straw are difficult to hydrolyze due to their recalcitrant and heterogeneous structure [6]. As a result, a pretreatment process is required to make the lignocellulosic material more susceptible to the hydrolysis stage. In fact, the main purpose of a pretreatment process could be summarized in breaking down the shield formed by lignin and hemicellulose, disrupting the crystalline structure and reducing the degree of polymerization of the cellulose to maximize the saccharification efficiency in the sequential enzymatic hydrolysis [7].

An extensive research has been carried out on pretreatment methods to enhance the digestibility of lignocellulosic materials. Several pretreatment methods such as steam explosion, solvent extraction and thermal pretreatment as well as the biological pretreatment have been investigated [7–9]. Among these processes, the biological method has inherent merits like higher safety, lower cost and production of no harmful byproducts. However, low rate of the biodelignification prevents its integration to an industrial scale process [9]. This problem can be addressed partially by enhancing the rate of process through optimization of influential factors [10]. Combination of the biological pretreatment with the sub sequent enzymatic hydrolysis can be considered as another remedy. The process can be carried out through the addition of cellulolytic enzymes to the pretreatment bioreactor as described by Potumarthi et al. [11]. Cautiously, unfavorable interactions might be occurred between the various system components [12]. In this case, the coexistence of ligninolytic fungi with cellulolytic enzymes and reduced sugars should be studied in detail. Alternatively, employing the co-culture of fungi (i.e. ligninolytic and cellulolytic enzyme

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producers) has been suggested by Ma and Ruan and a synergetic effect has been reported for the final saccharification yield [13]. However, the optimum conditions for biodelignification and enzymatic saccharification will not be the same and this can adversely affect the overall efficiency. Moreover, the separation of fungi from the system after the pretreatment should be handled properly [14]. Immobilization of fungi can be employed to reduce the above mentioned interactions and facilitate the separation of microorganisms from the system. The immobilization of ligninolytic enzymes has been frequently reported as an effective method in their industrial applications [15]. One of the most widely used techniques for cell immobilization is entrapment, in which the living cells are enclosed in a polymeric matrix such as alginate which is porous enough to allow the diffusion of substrates and products [16].

In the present study, a *Trichoderma viride* species which is capable of producing both the ligninolytic and cellulolytic enzymes has been used to study the delignification and saccharification of rice straw at the same time. Clearly, employing a single microorganism to produce both of the required enzymes simplifies the process by eliminating some of the complex interactions and makes it possible to optimize the conditions more conveniently. The fungi have been immobilized in calcium alginate beads to append the reusability to the process and to facilitate the fungal removal in the subsequent stages. The important operating factors such as the biomass content of the system, inoculum size, and the agitation rate have been optimized simultaneously for both the delignification and saccharification through response surface methodology (RSM). The enzymatic hydrolysis of the pretreated biomass has been studied to determine the effect of pretreatment on sugar production efficiency.

2. Materials and methods

2.1. Rice straw

The rice straw used in this study was obtained from a local farm near Gorgan city in Iran. It was washed thoroughly with tap water to be clean and colorless and dried at 50 °C for 48 h. The dried materials were ground into fine pieces using an electric blade grinder and stored in glass bottles at room temperature. Cellulose content of the biomass was measured by the method of Sluiter et al. [17] and reported equal to 36.2 ± 1.3%. The hemicellulose and lignin content of the biomass were equal to 29% and 21% respectively.

2.2. Biological pretreatment

2.2.1. Microorganism and culturing conditions

The fungus used for biological pretreatment in the present study was *Trichoderma viride* BBRC9070. This strain which has been originally isolated from a decayed wood sample is capable of producing both the ligninolytic and cellulolytic enzymes simultaneously [10]. To prepare the inocula, the strain was cultivated on sterile Potato Dextrose Agar plates (PDA) for 4 days at 37 °C and the plates were stored at 4 °C.

The fungus was grown in a liquid culturing medium (pH: 4 ± 0.1) containing 10 g/L peptone, 5 g/L yeast extract, 15 g/L glucose, 1 g/L Tween 80. The solution was then enriched by copper ($CuSO_4 \cdot H_2O$) and manganese ($MnSO_4 \cdot H_2O$) ions with the final concentrations equal to 2.5 μM and 0.1 mM, respectively. Volumes of 100 mL from this medium were autoclaved at 121 °C in 250 mL Erlenmeyer flasks and inoculated by four plugs (0.5-mm diameter) of the PDA fungal culturing medium and incubated in an orbital shaker (30 °C, 160 rpm) for 2 days.

2.2.2. Fungus immobilization

The fungi immobilization was carried out in calcium alginate-polyvinyl alcohol (CA-PVA) beads [18]. Beads were produced by adding the sodium alginate-PVA solution to a $CaCl_2$ and Boric acid mixture. 1.35 g of polyvinyl alcohol was added to 45 mL water and heated at 60 °C to completely dissolve the PVA. Then 2.25 g of sodium alginate was gently added to stirring PVA solution. Next, it was sterilized and 100 μL of Tween 80 were added to the flask aseptically and cooled down to 30°C. This solution was thoroughly mixed with 10 mL of the fungi suspension containing 6.6 ± 0.4 g of the dry biomass. The resultant mixture was extruded through a thin niddel into 100 mL of an aqueous solution containing saturated boric acid (7 gr) and 2gr $CaC1_2$. The beads were stirred in the solution at 4°C for 24 h and then rinsed with distilled water to remove any excess amounts of boric acid, alginate and PVA.

The diameter of the beads was approximately between 2 and 3 mm. The produced beads in each 100 mL flask were used to inoculate the biodelignification culturing medium at the next steps. The inoculation size of the immobilized fungi in the produced beads in a 100 mL flask was presumed equal to 1 in the following experiments.

2.2.3. Rice straw pretreatment by immobilized fungi

100 mL of the above mentioned liquid culturing medium was used to investigate the biological pretreatment efficiency of immobilized fungi. The glucose concentration in the medium was equal to 5 g/L. Then, 4 g of sterilized rice straw and the immobilized beads were added to each flask aseptically. Flasks were incubated in an orbital shaker (30 °C, 160 rpm) up to 10 days. Within the intervals of 48 h, delignification efficiency and the produced sugar in the solution were measured.

2.3. Enzymatic hydrolysis of pretreated rice straw

Cellulase Celluclast 1.5 L (Novozymes, Denmark) was utilized for enzymatic saccharification of the biomass after biological pretreatment. The enzyme activity was measured and reported equal to 41 FPU/mL [19]. After removing of the alginate beads and the culturing medium from the system, the remained biomass was washed and suspended in 100 mL of a citrate buffer solution (pH = 4.8) and then, the hydrolysis of the pretreated biomass was carried out by addition of the enzyme to each flask at a final activity of 15 FPU per gram of cellulose. Next, flasks were incubated in an orbital shaker (40 °C, 160 rpm) up to 4 days. After this time, the suspension was centrifuged for 10 min (5000 rpm, 4 °C) and the concentration of the produced glucose was measured through DNS method at 540 nm [20].

2.4. Experimental design

The effects of several important operational factors on the lignin removal efficiency as well as the glucose production from rice straw in fungal pretreatment process were investigated through Box–Behnken Response Surface Methodology (RSM). Factors and their levels were selected based on the previously reported results in the literature and a wide range of variation was covered. Three factors namely biomass content of the system (2%, 4% and 6% w/v), inoculum size (0.5, 1 and 1.5) and the agitation rate (120,160 and 200 rpm) were studied. In fact, high biomass content is economically favorable while it has an inhibitory effect on hydrolase enzymes activity, which significantly reduces the hydrolysis rate [21]. The inhibition depends on the ratio of lignocellulosic substrate to the produced enzyme in the medium. The inoculum size is another important factor which is effective on the amounts of the produced enzymes in the system. The agitation rate is important because of