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FUNGAL PRETREATMENT OF RAPESEED STRAW BY SOLID-STATE FERMENTATION OF *PHANEROCHAETE CHRYSOSPORIUM* TO PRODUCE REDUCING SUGARS

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ABSTRACT

A white rot fungus, *Phanerochaete Chrysosporium* (ATCC 24725) was used for the biological pretreatment of rapeseed straw to produce reducing sugars. The reducing sugars are normally produced within two stages in biological pretreatments: Solid state fermentation for delignification and then enzymatic hydrolysis of the treated biomass. The biological pretreatment of rapeseed straw resulted in production of MnP and LiP enzymes. These enzymes reached their maximum level after 15 days (198.5 U/L for MnP and 304 U/L for LiP). At the same time, maximum level of glucose and reducing sugars was 78.4 mg/ml and 91.5 mg/ml, respectively. Contrary to other pretreatment methods, this method can be a suitable alternative to avoid additional costs for washout and elimination of inhibitors.

Keywords: Biological Pretreatment, *Phanerochaete Chrysosporium*, Reducing Sugars, Ligninolytic Enzymes, Rapeseed Straw

INTRODUCTION

The global energy is declining due to the increasing population growth and development of industrial communities and increased demand for energy. The reduction of fossil energies and environmental concerns drew more focus on agricultural wastes as a source for lignocellulosic materials[1]. These materials can be used as an important source for production of biofuels [2]. Lignocellulosic materials

contain cellulose and hemicellulose that are bound together by lignin. The conversion of lignocellulosic materials to biofuels usually comprises three main steps: 1) the pretreatment that deconstructs lignin and changes cellulose crystalline structure; 2) enzymatic hydrolysis of cellulose and its conversion to glucose; and 3) microbial conversion of glucose to ethanol through fermentation [3]. The pretreatment of lignocellulosic materials is an important stage of their economical conversion into biofuels [4].

Rapeseed is produced to make animal feed and vegetable oils for human consumption. After separating the grains, the remaining rapeseed straw should be used. This apparently worthless material is usually burnt, but it can be used as a lignocellulosic material to produce bioethanol[5]. Its seeds can be used to produce biodiesel while the rapeseed straw is good for bioethanol and biohydrogen production [6].

The polymer structure of lignin causes limitations for degradation of lignocellulosic materials and their conversion into ethanol [7, 8]. Lignin is a polymer found in cell walls of all woody plants. This aromatic polymer contains phenyl propanoid internal units that bind to various covalent bonds, such as aryl-ether, aryl-aryl, and carbon-carbon[7, 8]. Removal of lignin and hemicelluloses and reduction of cellulose crystalline structure during the pretreatment can improve the enzymatic hydrolysis of lignocellulosic materials[9, 10]. Therefore, the objective of pretreatment is to make cellulose more accessible to the enzyme, convert it to sugar, and ferment it to biofuels.

Pretreatment can be mainly classified as physical, chemical, and biological. So far, different pretreatments have been examined for lignocellulosic materials, including pretreatment with hot water[11], diluted and concentrated acid[12-14], alkaline[15-17], and ammonia fiber explosion (AFEX)[18]. Generally, these pretreatments have major disadvantages, including: a) the need to the equipment resistant to corrosion and pressure; b) the production of environmentally high-risk effluent by most of these processes, and c) high consumption of energy.

Today, unlike the physical and chemical pretreatments, the biological pretreatments have received more attention because they do not need the high consumption of energy, are biocompatible, and do not produce toxic substances [19-21]. The enzymes produced in the biological pretreatment can degrade the lignin and expose cellulose. These enzymes mainly include lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) [8, 21-23]. White-rot fungi can produce some of

these enzymes [21, 22]. *Ph. Chrysosporium* is one of the best candidates for this purpose and many studies have been conducted on it. This fungus can produce most of the ligninolytic enzymes and consequently has received attention as a suitable model for development and identification of enzyme-producing systems [7]. So far, researchers have used *Ph. Chrysosporium* for different pretreatments [3, 4, 7, 8, 19, 23, 24]. However, it cannot be still used as a major process in the industry due to its slow degradation.

The solid state fermentation refers to the fermentation of substrate in the absence of free water. However, a little moisture in the substrate is essential for growth of microorganisms and production of metabolites. The solid state fermentation has received more attention for its lower energy consumption [7, 25]. This study was conducted to examine the biological pretreatment of rapeseed straw using solid state fermentation by *Ph. chrysosporium*. So the objective of this work is to assess the possibilities of using rapeseed straw as a source of fermentable sugars.

MATERIALS AND METHODS

Biomass and microorganism

The rapeseed straw was supplied from rapeseed farms in Quchan, Khorasan-e Razavi Province, Iran. To remove the soil particles and unwanted materials, the rapeseed straw was washed with water and dried in the laboratory environment for one week. Then, it was dried using an oven at 60°C for 24 hours to reach a constant weight. The dried rapeseed straw was grounded, sieved through mesh 18, packed in nylon containers for further use, and stored at room temperature.

The lyophilized *Ph. chrysosporium*(ATCC 24725) was purchased from Iran's regional center for collection of industrial fungi and bacteria. Once the fungus was cultured on the potato dextrose agar (PDA) medium at 30°C for one week, the spores were kept in dark at 4°C until use. The inoculum was prepared from a spore suspension in distilled water, with absorption rate of 0.5 at 650 nm. There were 2×10^6 spores per milliliter of the suspension solution. To ensure the appropriate function of the fungus, a new culture medium was prepared every two months. All the chemical materials used in this study were purchased from Merck and Sigma-Aldrich companies.

Inoculation and performing the pretreatment

5 g of the rapeseed straw was poured in a capped 250 ml jar and autoclaved at 121°C for 15 min. Of the spore suspension, 2.5 ml was added to the jar, and moisture of the medium was increased to $80\%\frac{\text{w water}}{\text{w total}}$ using sterile distilled water. The jar was

capped and incubated at 37°C for maximum 15 days.

Assay of the produced enzymes

100 ml of 0.1M citrate phosphate buffer was added to each jar and stored at room temperature for 24 hours. Then, the content of each jar was filtered, and the solution was used to measure the activity of extracellular enzymes. Once the solid materials were dried, they were stored at 4°C for other measurements and also enzymatic hydrolysis. The lignin peroxidase (LiP) was measured on the basis of the oxidation of veratryl alcohol to veratryl aldehyde at the wavelength of 310 nm. One unit of the activity was defined as Lip amount that can oxidize one micromole of veratryl alcohol into veratryl aldehyde at room temperature within one minute[26]. The activity of manganese peroxidase (MnP) was determined on the basis of the oxidation of manganese sulfate at wavelength of 270 nm. The manganese trivalent cations (Mn III) resulting from the oxidation of Mn II by the enzyme forms a complex with the malonate that has absorbance at 270 nm. One unit of manganese peroxidase is the amount that can catalyze the formation of one micromole of the complex within one minute [27].

Enzymatic hydrolysis of cellulose

1 g of the pretreated sample plus 100 ml of 0.05M sodium citrate buffer were poured into a 250 ml Erlenmeyer flask. A certain amount of cellulase (10 FPU/g) was added to the flask, and the flask was incubated in the shaker incubator at 50°C and 150 rpm for maximum 56 hours. The cellulase was purchased from Sigma Company.

Measurement of reducing sugars

The total sugar was measured using dinitrosalicylic acid (DNS) method [28]. An amount of 0.1 ml of the enzymatic hydrolysis solution and 0.9 ml of distilled water were poured into the test tube, and then, 3 ml of DNS solution was added to the tube. The compound was placed in hot water bath for 5 min and cooled down to the room temperature. Then, 7 ml of distilled water was added to the tube, and the absorption occurred at wavelength of 540 nm. The level of glucose was measured using the glucose kit. All the tests were repeated three times.

RESULTS AND DISCUSSION

The enzymatic activity in pretreated rapeseed straw

The enzymatic activity of LiP and MnP produced by the *Ph. chrysosporium* in the rapeseed straw is shown in Figure 1. The enzymatic activity of LiP and MnP reached its maximum after 15 days (198.5 U/L for MnP and 304 U/L for LiP). The *Ph. chrysosporium* could produce lignin-

degrading enzymes in the absence of nutrients[3, 29]. Previous studies also showed the production of these enzymes in solid state fermentation with other substrates. Kumar et al.[7] reported the maximum production of LiP and MnP, respectively as 2100 U/L and 1200 U/L at 37°C and moisture of 60% using zapota. Yao *et al.*, [21] also could reach maximum production of LiP and MnP in solid state fermentation of corn wastes using *Ph. chrysosporium*. The production of LiP and MnP began simultaneous to the growth of mycelia on the substrate. Therefore, LiP and MnP were not produced at early stages of the fungal growth (Figure 1). Moreover, after the production of these enzymes, the release of hydrogen peroxide resulted in degradation of lignin although MnP was sensitive to high concentrations of hydrogen peroxide that would inactivate the enzyme[30]. In this study, the deactivation of MnP was not observed during the culturing, and the level of MnP increased continuously up to the 15th day (Figure 1). However, Kumar et al. reported that the activity of MnP stopped completely 21 days after solid state culture of zapota at 37°C and moisture of 60% [7].

The reduced sugars

The level of glucose and total sugar was measured within 56 hours of rapeseed straw hydrolysis in 5-day, 10-day, and 15-day treatments (Figures 2, 3 & 4). As shown in the figures, the production rate of glucose and total sugar in all three treatments increased with time, but the production rate of glucose and total sugar in the 15-day treatment was higher than that in the 5-day and 10-day treatments. Maximum level of glucose (78.4 mg/ml) and total sugar (91.5 mg/ml) was achieved in the 15-day treatment (Figure 4). The degradation of lignin following the ligninolytic activities increased the accessibility of the fungus to cellulose and hemicelluloses, and consequently, the production of glucose and total sugar increased following the enzymatic hydrolysis[4, 8, 31]. The production rate of LiP and MnP enzymes reached its maximum on the 15th day (198.5 U/L for MnP and 304 U/L for LiP) (Figure 1). Therefore, further degradation of lignin increased the accessibility of the fungus to cellulose and hemicelluloses, and consequently, more glucose and total sugar were produced (Figure 4).

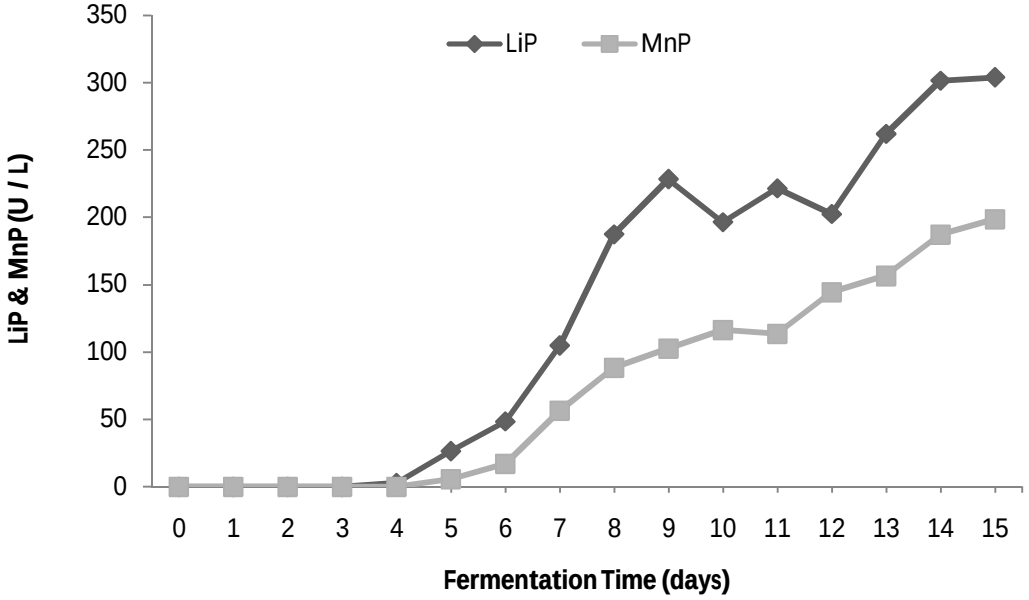

Figure 1: Production of LiP and MnP as a function of time by *Ph. chrysosporium* during SSF (80% Wet basis) using rapeseed straw at temperature 37 ℃. All data points were obtained from triplicate experiments and are presented as the mean value

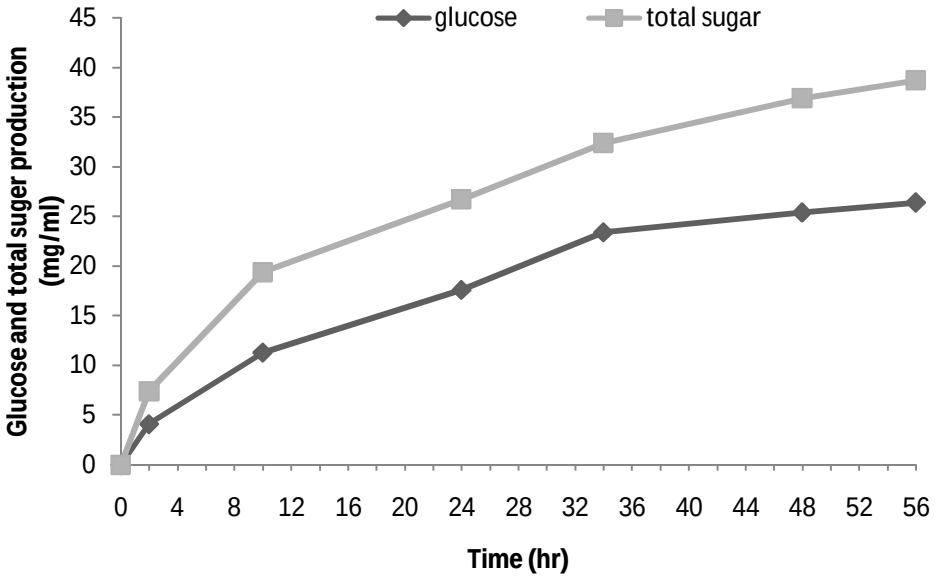

Figure 2: Glucose and total sugar produced in enzymatic hydrolysis of 5-day pretreated rapeseed straw during SSF with *Ph. chrysosporium*. Conditions: cellulase (10 FPU/g), 50°C, 150 rpm

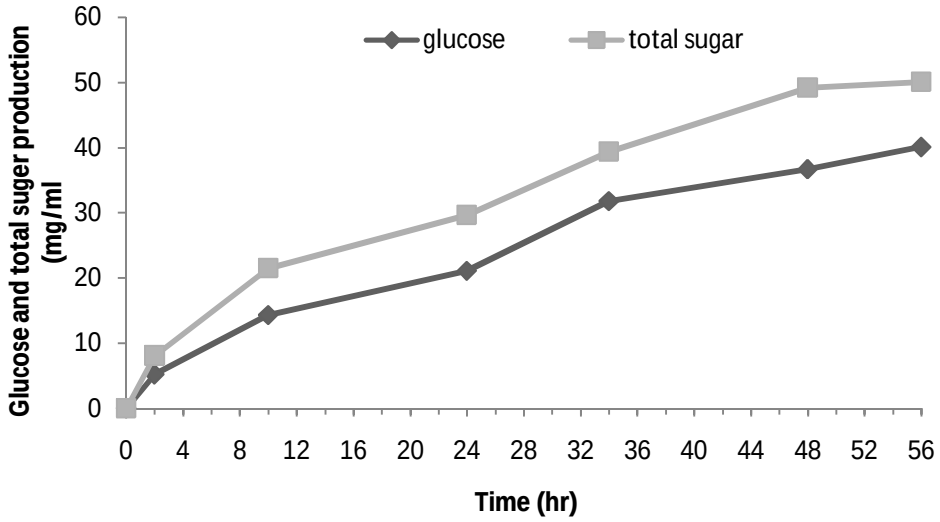

Figure 3: Glucose and total sugar produced in enzymatic hydrolysis of 10-day pretreated rapeseed straw during SSF with Ph. chrysosporium. Conditions: cellulase (10 FPU/g), 50°C, 150 rpm

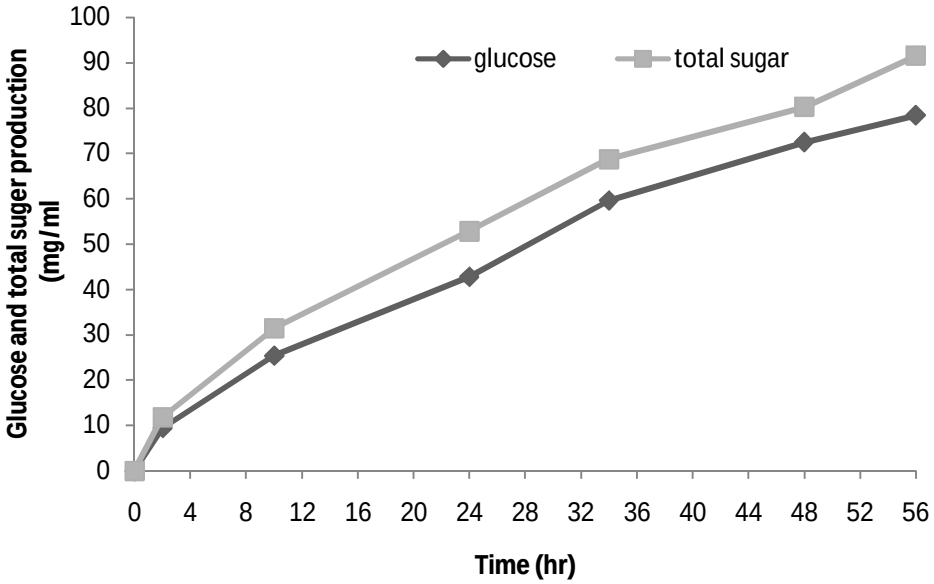

Figure 4: Glucose and total sugar produced in enzymatic hydrolysis of 15-day pretreated rapeseed straw during SSF with *Ph. chrysosporium*. Conditions: cellulase (10 FPU/g), 50°C, 150 rpm

Although the mechanism of lignin degradation has not been known thoroughly, it seems that the degradation of lignin by *Ph. chrysosporium is* due to the consequent breakdown of large molecules of lignin by free radicals ions produced by the hydrogen peroxide and substrate [22]. Furthermore, some reports revealed the production of cellulose and xylanase by *Ph. chrysosporium* during the solid state or submerged fermentation [25, 32]. However, the hydrolyzed cellulose was used by the fungus itself. Therefore, it can be concluded that the produced glucose and the total sugar in this study was the result of substrate degradation.

Some researchers believe in toxic effect of secondary metabolites produced by

Ph. chrysosporium. They believe that not only the production of ligninolytic enzymes, LiP and MnP, stopped but also other metabolites with toxic effects on the enzymatic hydrolysis might reduce enzymatic hydrolysis[3, 10, 33]. This study did not examine the above problem; however, the activity of LiP and MnP and, subsequently, the production of glucose and total sugar might be reduced if the solid state fermentation was continued.

CONCLUSION

Ph. chrysosporium can produce sugar out of the rapeseed straw in a single step pretreatment without adding nutrients. The activity of the ligninolytic enzymes, LiP and MnP, was also observed in the biological pretreatment of the rapeseed straw for production of sugar. Regarding the low production rate of these enzymes, the growth rate of *Ph. chrysosporium* and, consequently, production rate of the ligninolytic enzymes can be increased through changing the culture medium and adding appropriate nutrients. Contrary to other pretreatment methods, this method can be a suitable alternative to avoid additional costs for washout and elimination of inhibitors.

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