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Lignocellulose degrading enzyme production from *Irpex lacteus* and *Fusarium solani*

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Abstract*—Lignocellulose-degrading fungi are considered as the most promising strategy for biomass utilization. In this work two different fungal isolates were investigated for their ability to produce lignocellulose-degrading enzyme during cultivation process using hay substrate as a carbon source. The extracted enzymes were compared with commercially available cellulotic enzyme mixture Viscozyme L (Sigma-Aldrich). Filter paper units (FPU ml^{-1}) were determined. The results demonstrated that *I. lacteus* produced a lignocellulose-degrading enzyme with an activity of 1.195 U ml^{-1} and was able to release almost 30% from available reducing sugars while no enzyme activity was detected from *F. solani.

Keywords—Lignocellulose-degrading enzymes; Irpex lacteus; Fusarium solani

I. INTRODUCTION

Over the last few decades, there has been an urgent need to develop alternative fuels to the extensively used fossil fuels due to the world's growing energy demand, environmental pollution and resource decrease. Renewable lignocellulosic biomass, like, agricultural waste and wood processing products, is rich in carbohydrates (55–75% dry basis) and, thus, is regarded as a very attractive and widely available feedstock for bioenergy production [1, 2, 3, 4].

Lignocellulosic biomass is composed of three mayor components – cellulose, hemicellulose and lignin [5]. The conversion of lignocellulosic biomass to bioenergy involves the following steps: biomass pre-treatment/hydrolysis to produce reducing sugars, fermentation of the sugars and alcohol recovery/purification. The cost-effective and high yield pre-treatment/hydrolysis process is a major challenge to increase the production of fermentable sugars [6]. Thermal and chemical pre-treatment methods have been studied as the current leading technologies, however, they require expensive technologies, environmentally unfriendly reagents and/or generate large volumes of waste. Thus, there is an increasing interest to use biological methods, particularly, enzymatic hydrolysis, due to its low energy requirement and mild environmental conditions [2, 7, 8].

Microorganisms producing lignocellulosic enzymes are widespread, and include not only anaerobic bacterial species (e.g. *Orpinomyces* spp. in the gastrointestinal tracts of ruminant animals), but also fungal species from the ascomycetes (e.g. *Trichoderma reesei*) and basidiomycete's phyla, such as, white-rot fungi (e.g. *Phanerochaete chrysosporium*) and brown-rot fungi (e.g. *Fomitopsis palustris*) [9, 10, 11]. Since anaerobes have a very low growth rate and require oxygen free growth conditions, most research on lignocellulosic enzyme production has been focused on fungi [12]. In general, white-rot fungi are known to be one of the most efficient lignocellulose-degraders, for instance, white-rot fungi *Ceriporiopsis subvermispora*, *Phlebia spp*., *Physisporinus rivulosus, Dichomitus squalens* can selectively attack lignin while *Trametes versicolor*, *Heterobasidium annosum*, and *Irpex lacteus* simultaneously degrade all cell wall components [2, 7, 9].

In contrast to the white-rot fungi, very few studies have considered the involvement and application of other environmental fungi in lignocellulose degradation. There are several reports on plant pathogenic *Fusarium solani* ability to produce lignocellulosic enzymes. For example, Obruca et al. [13] showed that *F. solani* was capable of secreting enzyme cocktail for lignocellulose degradation, thus, showing the high potential of this species.

The aim of this study was to compare the efficiency and activity of lignocellulose-degrading fungal enzymes extracted from *Irpex lacteus* and *Fusarium solani*. The results were compared with commercially available cellulolytic enzyme mixture Viscozyme L. Within the research lignocellulose-degrading enzyme activity of *I. lacteus* and *F. solani* was investigated and reducing sugar yields after enzymatic hydrolysis were determined.

II. MATERIALS AND METHODS

A. Fungal Strains and Culture Conditions

The white-rot fungus *Irpex lacteus* IBB 104 and *Sordariomycetes* filamentous fungus *Fusarium solani* (environmental isolate from a pharmaceutical wastewater treatment plant located in Riga, Latvia) was grown in fungal liquid media (0.8 g KH_2PO_4, 0.4 g K_2HPO_4, 0.5 g $MgSO_4*7H_2O$, 2 g NH_4NO_3, 2 g yeast extract, 10 g glucose in 1 l H_2O; pH 5.5 – 5.8) for 72 h and 24 h at 30°C, respectively.

B. Feedstock Material

Biomass was obtained from lowland hay meadows (6510, Latvia). After collection the biomass was dried, milled with a Retsch GM200 grinder (< 0.5 cm) and used as a reference material. The maximum theoretical cellulose and hemicellulose yield were accepted 55% of the 1 g dry hay biomass [14].

C. Enzyme Extraction

Laboratory scale preparation of cellulolytic enzymes was performed according to the methodology described by Mezule et al. [15]. In brief, liquid fungal cultures were homogenized with a grinder (Retsch GM200) and transferred into liquid media (0.8 g KH_2PO_4, 0.2 g K_2HPO_4, 0.5 g $MgSO_4*7H_2O$, 3 g NH_4NO_3, 2 g yeast extract in 1 L H_2O; pH 5.5 – 5.8) containing hay as a sole carbohydrate source and incubated at 30ºC. After 14 days solid material and fungal biomass was separated by centrifugation (8500 rpm, 10 min). Then 0.5 kg l^{-1} $(NH_4)_2SO_4$ was added to the supernatant, dissolved and incubated for 24 h at 4˚C. Finally, the enzyme was extracted by centrifugation (8500 rpm, 10 min) and stored in 0.05 M sodium citrate buffer (pH 4, mono-sodium citrate pure, AppliChem) for further use.

D. Enzymatic Hydrolysis

To evaluate sugar production yields with prepared fungal enzymes batch scale tests were performed. In brief, the biomass was diluted in a 0.05 M sodium citrate buffer (3% w v^{-1}, pH 4) and boiled for 5 min (1 atm) to eliminate unnecessary microorganisms. After cooling 1% v v^{-1} of prepared enzymes or cellulolytic enzyme mixture (Viscozyme L, Sigma-Aldrich) was added to the diluted substrates and incubated on a shaker for 24 h at 30˚C. For total reducing sugar determination at least two identical samples were collected from each preparation.

E. Analysis of Total Reducing Sugars

Sugar concentration with dinitrosalicylic acid (DNS) method was performed according to Ghose [16]. All samples were centrifuged (6600 g, 10 min) and 0.1 ml of citrate buffer and 0.6 ml of DNS (3, 5-dinitrosalicylic acid, Sigma-Aldrich). For blank control, distilled water was used instead of the sample. Then the samples were boiled for 5 min and transferred to the cold-water bath. Further 4 ml of distilled water was added. Spectrophotometry at 540 nm with UV-Vis spectrophotometer M501 (SpectronicCamspec, UK) and NanoDrop 200c (Thermo Fisher Scientific, USA) was performed for all samples to compare and determine the accuracy of the instruments. To obtain absolute concentrations, a standard curve against glucose was constructed.

F. Filter Paper Assay

Filter paper assay (FPA) was performed according to the International Union of Pure and Applied Chemistry (IUPAC) [17]. To define filter paper units (FPU) of each of the laboratory prepared enzymes, Whatman No.1 filter paper strips (1 x 6 cm) were incubated with an enzyme sample in 0.05 M sodium citrate buffer for 24 h at 30˚C. After the incubation, 3 ml of DNS reagent was added to stop the reaction and sample was boiled for 5 min. The reducing ability was measured according to the dinitrosalicylic acid method [16].

To calculate the laboratory prepared enzyme activity (FPU ml^{-1}), a standard glucose curve was constructed and the relationship between the real glucose concentration and their respective enzyme dilution rare (EDR) was determined. FPU $ml^{-1}$was calculated according to Equation (1):

$$Filter\ Paper\ Activity = \frac{\left(\frac{2\ mg\ glucose}{0.18\ \frac{mg\ glucose}{\mu mol}}\right)}{\frac{(0.5\ ml\ enzyme\ dilution\ x\ incubation\ time)}{EDR}}\ units\ ml^{-1} \quad (1)$$

G. Statistical Analysis

All tests were carried out in triplicate. The mean values, standard deviations and t-test (probabilities < 0.05 were regarded as significant) were calculated using Microsoft Excel software.

III. Results and Discussion

A. Lignocellulose Degrading Enzyme Production by Irpex lacteus and Fusarium solani

It has been shown that *F. solani* can colonize, modify and degrade lignocellulose [13, 18]. However, some studies have demonstrated a physiological variability and different production rates of lignocellulose-degrading enzymes among various strains of *F. solani* [19, 20]. Therefore, the capability of producing lignocellulose-degrading enzymes from *F. solani* isolate of pharmaceutical wastewater treatment plant was examined in this study and results were compared with white-rot fungi *I. lacteus* and commercially available enzyme mixture Viscozyme L. Wastewater isolate was selected due to its increased potential in synthesis of various chemical-degrading substances.

The results showed that enzyme mixture from *I. lacteus* and Viscozyme L produced lignocellulose-degrading enzymes with an activity of 1.122 and 1.195 U ml^{-1}, respectively, and there was no significant difference among the obtained results ($p > 0.05$). At the same time, no cellulose enzyme activity was detected from *F. solani* isolate. Thus, to evaluate overall sugar production potential from biomass, batch hydrolysis tests were performed.

To determine the total reducing sugar concentration dinitrosalicylic acid (DNS) method was used. No specific discrimination of the produced sugars was performed, since the overall activity of the enzyme preparations was assessed. To guarantee the overall accuracy of the results, simultaneous spectrophotometry at 540 nm with UV-Vis spectrophotometer M501 (SpectronicCamspec, UK) and NanoDrop 200c (Thermo Fisher Scientific, USA) was performed for all samples. As expected, both spectrophotometer instruments gave similar results according to the manufacturer specification of measurements accuracy and stability of the

relative error (better than 3%) [21, 22], and no significant differences ($p>0.05$) were observed between the results.

Batch scale studies with hay and lignocellulose-degrading enzymes generated 12% - 53% of sugar from theoretically available. The results with DNS method showed that the highest sugar yields after 24 h hydrolysis were obtained from enzyme mixture of Viscozyme L (290.07 mg $g_{dry\ substrate}^{-1}$) and were statistically different ($p < 0.05$) from the detected sugar yields from *I. lacteus* (161.61mg $g_{dry\ substrate}^{-1}$) and *F. solani* (65.64mg $g_{dry\ substrate}^{-1}$). At the same time, *F. solani* generated significantly lower ($p < 0.05$) amount of sugar *I. lacteus*, indicating on low potential of the produced enzyme mix (Table I).

To assess the impact of enzymatic hydrolysis, the released sugar concentration was measured directly after milling (before boiling) and after boiling. It has been reported that initial biomass heating might release more than 15% of sugar from total sugar yield [23]. This was confirmed in this study too, where only boiling generated from 11% (Viscozyme L) to 50% (*F. solani* enzyme mix) of all produced sugars (Table I). The effect of milling accounted for 4 to 30% from all produced sugars. The highest percent yield during pre-treatment was obtained from samples with *F. solani* enzyme mix where enzymatic hydrolysis produced a mere 20% of sugar and only 65.64 mg $g_{dry\ substrate}^{-1}$ in total. At the same time Viscozyme L and *I. lacteus* generated additional 85% and 69% of sugar during hydrolysis and the increase was reported as significant ($p < 0.05$). To exclude any potential limitations during *F. solani* enzyme production, corrections in production methodology were introduced.

Obruca et al. [13] has demonstrated that *F. solani* is capable of producing cellulases, xylanases, and proteinases even in the simple medium without any inducer. Furthermore, tit has been demonstrated that the most efficient cultivation time for *F. solani* was 3 days instead of 14 as reported for *I. lacteus* and the introduction of the hydrogen peroxide was very efficient and simple strategy for improvement of lignocellulose-degrading enzyme production [13]. Therefore, to improve the yield and rate of the enzymatic hydrolysis, the incubation time of *F. solani* enzyme production was decreased to 3 days and the hydrogen peroxide (10 mmol l^{-1}) was applied into the medium at the second day of cultivation. The obtained results were compared with previously obtained enzyme extraction protocol where cultivation time was 14 days (Table II).

TABLE I. THE AMOUNT OF REDUCING SUGARS PRODUCED FROM FUNGAL ENZYMATIC HYDROLYSIS

	Irpex lacteus, mg g dry substrate^{-1}	***Fusarium solani,*** mg g dry substrate^{-1}	**Viscozyme L,** mg g dry substrate^{-1}
Before boiling	17.83 ±5.91	19.96 ±4.01	12.23 ±2.27
After boiling	51.52 ±3.74	52.85 ±7.05	46.17 ±3.49
After 24 h hydrolysis	161.61±16.79	65.64 ±7.05	290.07 ±4,16

Standard deviation represents the average values at least from 3 replicates

TABLE II. THE AMOUNT OF REDUCING SUGARS PRODUCED FROM *FUSARIUM SOLANI*

	14 days of cultivation, mg g dry substrate^{-1}	**3 days of cultivation,** mg g dry substrate^{-1}	**3 days of cultivation + H_2O_2,** mg g dry substrate^{-1}
Before boiling	5.29±1.40	17.12 ±0.99	22.91 ±2.49
After boiling	50.69 ±1.27	47.60 ±3.74	43.46 ±2.79
24 h	59.74±1.54	53.03 ±2.08	49.05 ±0.54

Standard deviation represents the average values at least from 3 replicates

The introduced modifications did not produced any increase in sugar yields, on the contrary a 12% decrease for 3 days incubated enzyme preparation was observed ($p < 0.05$). Simultaneously, addition of hydrogen peroxide decreased the amount of produced sugar for another 8%. Similarly as observed before, biomass pre-treatment generated most of the all produced sugars. Thus, the results demonstrated that the improvements did not produced any increase in yields. Moreover, a negative effect was obtained. Similarly, Saparrat et al. [19] in his study tested five strains of *F. solani* in a carbon-limited medium and results demonstrated a variable production of ligninolytic enzymatic activity. No lignin peroxidase activity and low levels of manganese-dependent peroxidases was observed, thus, confirming the high variations in enzyme production potential among various *F. solani* strains. The choice of culture conditions could have a great impact on the final amount of enzyme produced [12]. Moreover, under inappropriate conditions, *F. solani* might be found in idiophase or stationary phase of growth under carbon-limited conditions, restricting the trophophase of active growth where primary metabolites are formed [19].

Variations among strains, growth conditions and enzyme extraction represent an essential problem of lignocellulose-degrading enzyme production from various fungal isolates reported as potential producers. Moreover, correct enzyme production technology (purification, concentration, storage) could have a serious impact on final product quality [24]. Nevertheless, this study has shown that laboratory enzyme preparations could have a comparable quality than commercial products.

IV. CONCLUSIONS

The results showed that from the selected fungal strains only *Irpex lacteus* IBB 104 was able to produce cellulolytic enzymes and release fermentable sugars. Despite similar cellulolytic enzyme activity, the reducing sugar yields obtained with *I. lacteus* reached only 55% when compared to commercial extractions. To enhance the hydrolysability of the biomass, additional enzyme purification could be introduced.

Despite the reported potential of *Fusarium solani*, the selected strain did not generate any significant reducing sugar amount.

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