

Enhanced production of cellobiose dehydrogenase and β -glucosidase by *Phanerochaete chrysosporium*

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Abstract—The production of cellobiose dehydrogenase (CDH) and β -glucosidase by *Phanerochaete chrysosporium* ATCC 32629 was assessed during submerged fermentation. The maximum concentrations of CDH and β -glucosidase were obtained using rice straw as the carbon source. Organic nitrogen sources were more effective in enzyme production than inorganic nitrogen sources. Corn steep liquor (CSL) for CDH production and soy bean meal (SBM) for β -glucosidase production were the most appropriate organic nitrogen sources. Using optimum medium obtained by response surface methodology (RSM), the maximum concentrations of CDH and β -glucosidase achieved in the stirred-tank reactor (STR) were 204 U/L and 140 U/L, respectively. CDH productivity (22.7 U/L·day) was the highest at 9 days.

Key words: Cellobiose Dehydrogenase, β -Glucosidase, *Phanerochaete chrysosporium*, Rice Straw, Response Surface Method

INTRODUCTION

Cellobiose dehydrogenase (CDH; EC 1.1.99.18) is an extracellular enzyme produced by a number of wood-degrading fungi [1-4]. CDH oxidizes cellobiose, soluble cellodextrins, mannodextrins and lactose to their corresponding lactones using a wide spectrum of electron acceptors including quinines, phenoxyradicals, cytochrome C (cyt c), triiodide ions or metal ions such as Fe³⁺ and Cu²⁺ [5]. CDH is monomeric in two of its domains; a flavin domain containing FAD, and a heme domain containing a cytochrome b type heme connected by a fifteen-amino acid linker [6]. Typical CDH produced by Basidiomycetes is a monomeric protein of 90-110 kDa with glycosylation in the range of 10-20% [7]. Although CDH was first isolated and characterized more than 35 years ago [8], its role in wood decomposition has yet to be clearly defined. It has been suggested to the generation of other oxidative enzymes for the repolymerization of lignin prevented by CDH and the generation of highly active hydroxyl radicals that participate in the Fenton reaction to degrade cellulose, xylan, and lignin [9,10].

The actions of cellulase on both crystalline cellulose and wood components (carboxymethylcellulose, xylan, and synthetic lignin, for instance) have been enhanced by CDH in other studies, but there are still few studies to date on the direct role of CDH in cellulose modification with pulp as a substrate [1,11]. It has been suggested that CDH plays a biological role in fungal cellulose degradation, by which it activates cellulose for hydrolytic cellulases through depolymerization and disturbance of hydrogen bonding [12].

β -Glucosidase is produced by most microorganisms due to the

wide availability of cellobiose as a substrate. β -Glucosidase is a glycosidase enzyme that acts upon β 1 to 4 bonds linking two glucose or glucose-substituted molecules. β -Glucosidase exists as an extracellular, cell wall-associated, and intracellular enzyme. It is reported that the intracellular localization of β -glucosidase requires the transport of cellobiose into the cell [13].

Recently, the relationship between β -glucosidase and CDH has become a hot research topic. A previous investigation showed that β -glucosidase activity is competitively inhibited by three main factors: glucose, gluconolactone, and cellobionolactone. As already mentioned, cellobionolactone is the product of CDH oxidation of cellobiose. Therefore, it can be concluded that CDH indirectly influences β -glucosidase activity [3,14].

Analysis of variance (ANOVA) and response surface methodology (RSM) have been successfully used to evaluate the relationship between a set of controllable experimental factors and the observed results from medium and bioprocess optimization [15-19]. These statistical methods have been proven as powerful, useful tools. We employed a central composite design (CCD) in order to determine the optimal concentrations of medium components in the production medium.

Although the lactones produced by CDH indirectly inhibit the activity of β -glucosidase, cellobionolactone as the main product is subsequently hydrolyzed to carboxylic acid. Also, both β -glucosidase and CDH play an important role in cellulose degradation [3, 14]. Therefore, for further study about the synergism between two enzymes and Novozyme cellulases in the enzymatic saccharification of cellulose to glucose, the simultaneous production of CDH and β -glucosidase was performed in submerged culture. With regard to the production of CDH and β -glucosidase by *Phanerochaete chrysosporium*, there have been no reports concerning the optimization

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of production medium. In this study, different carbon and nitrogen sources were evaluated for the high-level production of CDH and β -glucosidase. The production medium was also optimized via RSM.

MATERIALS AND METHODS

1. Microorganisms

P. chrysosporium ATCC 32629 (*Sporotrichum pulverulentum*) obtained from the Korean Culture Center of Microorganisms (KCCM, Korea) was cultured at 27 °C for 7 days and maintained on slants of potato dextrose agar (PDA).

2. Production of CDH and β -Glucosidase in Flask and STR

Thirty milliliters of 0.2% Tween 80 solution was added to test tubes containing PDA media. After shaking, each flask was inoculated with 20 mL of spore solution and incubated at 27 °C with rotary shaking at 150 rpm for three days. Seed culture was carried out in a 500 mL Erlenmeyer flask containing 200 mL of YM broth. The compositions of the main media were as follows (per liter): 2.28 g of $(\text{NH}_4)_2\text{HPO}_4$, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.74 g of CaCl_2 , 0.01 g of FeCl_3 , 0.0158 g of NaNO_3 , 6.6 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.8 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 mg of thiamine-HCl, 6.75 g of succinic acid, and 10 g of microcrystalline cellulose. The seed culture broth (10%) was inoculated in a 500 mL Erlenmeyer flask containing 200 mL of main medium and incubated at 27 °C with shaking as described above for 12 days [1]. Fermentation was performed in 5 L stirred-tank reactor (STR) with an operation volume of 3 L containing the medium optimized in the flask culture. Culture temperature was controlled at 27 °C. Impeller speed and aeration rate were maintained at 250 rpm and 1.5 vvm, respectively. Samples were taken regularly on a daily basis during the culture period. Anti-foam agent was added as necessary.

3. Experimental Design and Statistical Analysis

Response surface methodology (RSM) consists of a set of experimental techniques designed to evaluate the relationships between a cluster of controlled experimental variables and measured responses (y), according to one or more selected criteria [20]. Central composite design (CCD) is the most extensively utilized experimental design for determining maximum or minimum response points. CCD consists of a factorial portion (± 1 levels) that is increased via the addition of axial or star points [$\alpha = (2^n)^{1/4}$] as well as several center points (0). The variables were assigned in accordance with the following Eq. (1):

$$x_i = (X_i - X_0) / \Delta X_i, i = 1, 2, 3, \dots, j \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value. The behavior of the system was explained by the following second-order polynomial Eq. (2):

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where y is the predicted response, β_0 (offset term), β_i (linear effect), β_{ii} (squared effect), and β_{ij} (interaction effect) are constant coefficients, and x represents the coded level of the independent variable. The SAS 9.1 package program was employed for regression analysis of the obtained experimental data, and also to estimate the coefficients of the regression equation. Maximum enzyme produc-

tion value was the response of the design experiments.

4. Analytical Methods

Cell packed volume (PCV) was analyzed using sediment of *P. chrysosporium* culture broth in a 100 mL mass cylinder for 30 min, and pH was measured by using the supernatant. Cellobiose dehydrogenase (CDH) activity was measured by the reduction of 2,6-dichloro-indophenol (DCIP) at 55 °C. Activity was monitored by absorbance at 520 nm using a UV/VIS spectrophotometer. The 1 mL reaction mixture contained 100 μL of 30 mM lactose, 100 μL of 100 mM Na-acetate buffer (pH 4), 100 μL of 0.3 mM DCIP, and 700 μL of diluted enzyme solution. One unit of enzyme activity is equivalent to the reduction of 1 μmol of DCIP per min [21]. The β -glucosidase activity was assayed in 1 mL of reaction mixture containing 0.1 mL of diluted enzyme solution and 0.9 mL of 1 mM p-nitrophenyl- β -D-glucoside (PNPG) in 0.05 M citrate buffer (pH 4.8) at 50 °C, 150 rpm for 20 min. After incubation, 1 mL of 1 M Na_2CO_3 solution was added to the mixture, which was allowed to develop in color. Then, 10 mL of distilled water was added and the release of p-nitrophenol determined spectrophotometrically at 400 nm. One unit of β -glucosidase activity is defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min under the above reaction conditions.

RESULTS AND DISCUSSION

1. Effect of Carbon Source on Enzyme Production

There have been no reports concerning the optimization of medium for the production of cellobiose dehydrogenase (CDH) and β -glucosidase by *P. chrysosporium* ATCC 32629. First, we evaluated different carbon and nitrogen sources for the high-level production of CDH and β -glucosidase. The effects of various carbon sources for the production of CDH and β -glucosidase were investi-

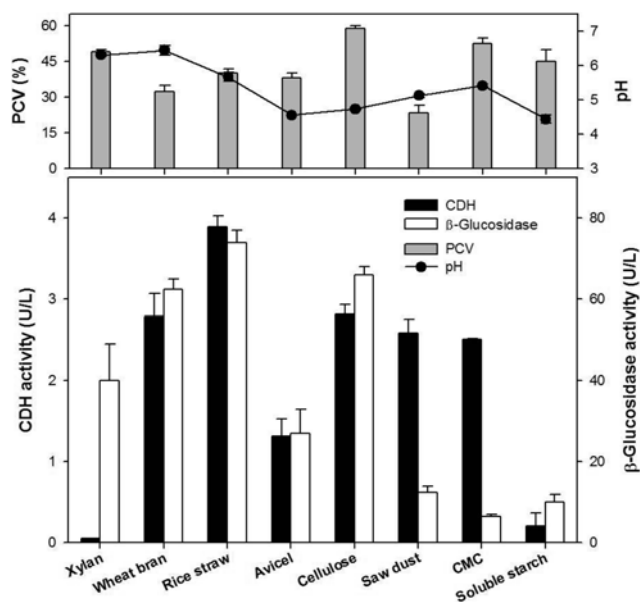


Fig. 1. Effects of carbon source on the production of enzyme by *P. chrysosporium* ATCC 32629. The concentration of carbon source was fixed at 1.0% in the main medium. Cultures were carried out at 27 °C and 150 rpm for 12 days. The data shown are the mean of two independent experiments.

gated as shown in Fig. 1. The concentration of the carbon source remained constant at 1.0% (w/v). Compared to microcrystalline cellulose as a carbon source in the main medium, the level of CDH production was enhanced when rice straw and wheat bran were used in a shake-flask culture, and was highly decreased when using xylan and soluble starch. Among the carbon sources tested, the highest CDH concentration was achieved with rice straw. The level of β -glucosidase production also increased when rice straw was used and decreased in the case of any other source. The differences in the production levels of enzymes concerning cellulose degradation in the presence of various biomasses perhaps result from their ability to induce enzymes. The pH levels of the culture broth differed from 4.3-6.6 according to the carbon source used, and the highest cell concentration was obtained in cellulose. When rice straw was used as a carbon source, activities of CDH (3.9 U/L) and β -glucosidase (74 U/L) increased 1.4-fold and 1.1-fold compared to main media containing microcrystalline cellulose. These results demonstrate that carbon source was crucial for the control of CDH and β -glucosidase production, and cheaper carbon sources such as rice straw and wheat bran could be used to produce these enzymes. Therefore, rice straw was employed for further study.

2. Effect of Nitrogen Source on Enzyme Production

Enzyme activity often changes according to nitrogen source or amino acid composition in the production medium. The effects of various nitrogen sources on the production of CDH and β -glucosidase were assessed in this study (Fig. 2). When nitrogen sources (0.5%, w/v) were added to the main medium, the activities of CDH (2.4-4.5 U/L) were obtained using soy bean meal (SBM), yeast extract, bacto-peptone, cotton seed flour, corn steep liquor (CSL), and ammonium nitrate. CDH activity was detected at a low level in the

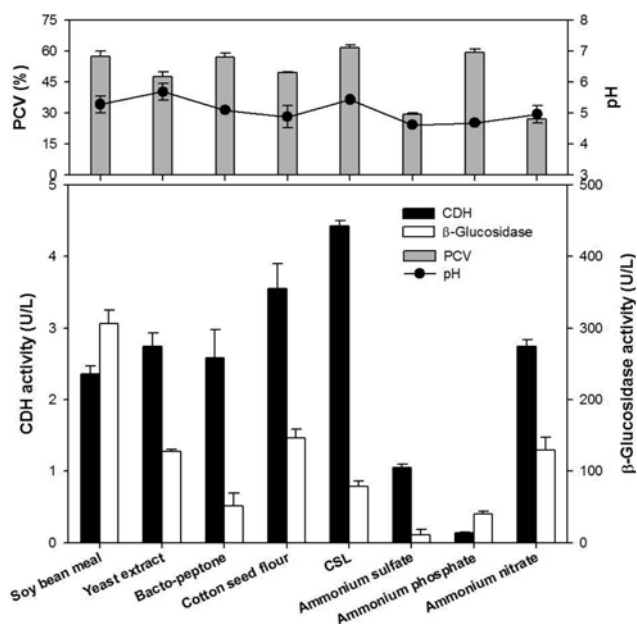


Fig. 2. Effects of nitrogen source on the production of enzyme by *P. chrysosporium* ATCC 32629. The concentration of nitrogen source was fixed at 0.5% in the main medium. Cultures were carried out at 27 °C and 150 rpm for 12 days. The data shown are the mean of two independent experiments.

cases of ammonium sulfate and ammonium phosphate. Organic nitrogen sources were more effective than inorganic nitrogen sources for CDH production. When CSL was used as a nitrogen source, the activity of CDH (4.4 U/L) was increased 4.2-fold compared to that of ammonium sulfate. When soy bean meal (SBM) was utilized, the highest level of β -glucosidase (about 300 U/L) was detected, which was 7.5-fold higher than that of ammonium phosphate. Cell growth and pH were different according to the nitrogen source used. These results indicate that CSL for CDH production and SBM for β -glucosidase production were the most appropriate organic nitrogen sources. When inorganic nitrogen sources were employed, the levels of enzyme production were very low. These findings indicate that the selection of a nitrogen source was essential to CDH and β -glucosidase production. Therefore, CSL and SBM were employed for further study.

3. Effect of Inoculation Method on Enzyme Production

The effect of inoculation method on the production of CDH and β -glucosidase was estimated using two methods. Method I was the same as described in Materials and Method. That is, 30 mL of 0.2% Tween 80 solution was added to test tubes containing fungi grown in PDA media. After shaking, the seed culture flask was inoculated directly by 20 mL of spore solution. In Method II, 20 mL of 0.2% Tween 80 solution was added to test tubes containing fungi grown in PDA media. After shaking, the spore suspensions were transferred to a beaker and stirred for 30 min, after which 20 mL of spore solution was inoculated into the seed culture flask. The effect of mixing in Method II resulted in the effective dispersion of single spores. Therefore, the pellet size of Method II was smaller than that of Method I in seed culture. Method II showed higher enzyme production due to the difference between Method I and II in terms of the concentration and dispersion of spores (Table 1). The production levels of CDH (29.5 U/L) and β -glucosidase (169 U/L) were increased about 7.6-fold and 2.3-fold, respectively, compared to those of Method I when rice straw was used as a carbon source in the main medium. The production of CDH was enhanced about 4.4-fold in the case of microcrystalline cellulose.

4. Optimization of Production Medium through RSM

In a previous study, we found that rice straw did not bring about catabolite repression even though the concentration of rice straw

Table 1. Comparison of enzyme production according to inoculation method with different carbon sources

Inoculation method	Cellulose		Rice straw	
	CDH (U/L)	β -Glucosidase (U/L)	CDH (U/L)	β -Glucosidase (U/L)
I	2.8	66	3.9	74
II	12.3	62	29.5	169

The concentration of carbon sources was fixed at 1.0% in the main medium. In Method I, 0.2% Tween 80 solution was added to test tubes, and after shaking, seed culture flask was inoculated directly with 20 mL of spore solution. In Method II, after shaking, the spore suspensions were transferred to a beaker and stirred for 30 min. The 20 mL of spore solution was inoculated into the seed culture flask. Cultures were carried out at 27 °C and 150 rpm for 12 days. The data shown are the mean of two independent experiments

Table 2. Real and coded values of the factors and experimental design and results for response surface methodology (RSM)

Run	CSL (x_1 , mL/L)	SBM (x_2 , g/L)	CDH activity (U/L)
1	-1 (10)	-1 (5)	26.6
2	+1 (50)	-1 (5)	65.9
3	-1 (10)	+1 (15)	40.3
4	+1 (50)	+1 (15)	63.7
5	1.414 (58)	0 (10)	55.9
6	-1.414 (2)	0 (10)	31.6
7	0 (30)	1.414 (17)	62.7
8	0 (30)	-1.414 (3)	33.9
9	0 (30)	0 (10)	44.0
10	0 (30)	0 (10)	67.3

The concentration of rice straw was fixed at 1.0%. The concentrations of CSL and SBM were varied according to the experimental design. Cultures were carried out at 27 °C and 150 rpm for 12 days

increased in the statistical experiment. That is, the optimal concentration of rice straw increased successively as the F -value of rice straw increased in analysis of variance (ANOVA) [22]. However, the high viscosity of the culture broth containing higher than 3% rice straw posed handling problems in submerged culture. Therefore, we had to determine the concentration of rice straw before the experimental design. When rice straw was tested at concentrations of 1, 2, 3 and 4%, the levels of CDH were all similar in the range of 27-30 U/L (data not shown). Hence, the concentration of rice straw as a carbon source was fixed at 1.0%. RSM was conducted to determine the optimal concentrations of medium components affecting the production of CDH. Nitrogen components were selected on the basis of preliminary experiments in shake-flask cultures. The independent variables and their levels are provided in Table 2, and the experiment was conducted using two independent variables, corn steep liquor (CSL, X_1) and soy bean meal (SBM, X_2). Ten runs were tested using a 2^2 full factorial design experiment with four star points ($\alpha = \pm 1.414$) and two replicates at the center point (Table 2). Regression analysis was conducted to fit the response function with the experimental data, and the results are provided in Table 3. The value of the determination coefficient ($R^2 = 0.774$), a measure of the goodness of fit of the model, indicates that 77.4% of the variability in the response could be explained by the model. Additionally, the F -value and P -value were 2.74 and 0.175, respectively. This indicates that the response equation provided a suitable model for the response surface of the CDH production experiment. The response equation obtained via multiple regression analysis is as follows:

$$y = 55.65 + 12.13x_1 + 6.53x_2 - 5.18x_1x_1 - 2.9x_2x_2 + 3.98x_1x_2 \quad (3)$$

Table 3. Analysis of variance (ANOVA) for the production of CDH by *P. chrysosporium* ATCC 32629

Source	Sum of squares	Degrees of freedom	Mean square	F -value	P -value
Model	1706.9	5	341.4	2.74	0.175
Error	497.8	4	124.5		
Corrected total	2204.8	9			

Coefficient of determination ($R^2 = 0.774$)

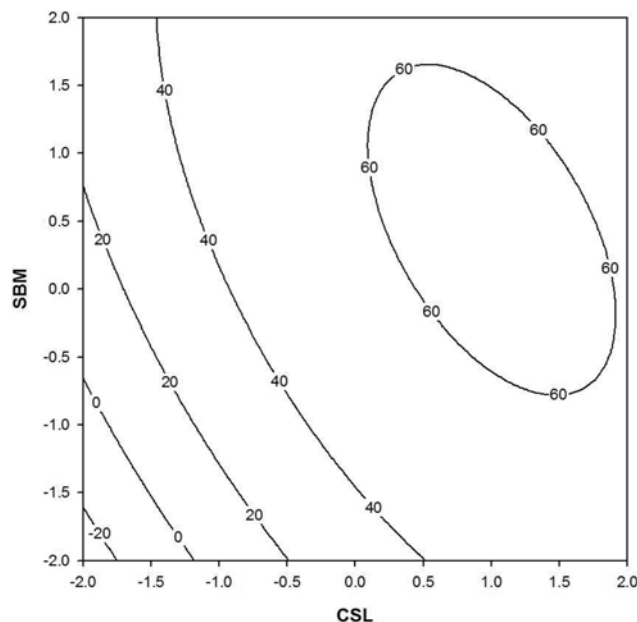


Fig. 3. Contour plot showing the effect of corn steep liquor (CSL) and soy bean meal (SBM) on CDH production. The concentration of rice straw was fixed at 1.0%. Cultures were carried out at 27 °C and 150 rpm for 12 days.

where x_1 = coded value of CSL, x_2 = coded value of SBM.

Response surface plots provide a method by which responses for different test values of variables can be predicted, and the contours of the plots help identify the type of interaction between the test variables. The effect of each variable can be observed by analysis of the two-dimensional (2D) contour plots. As shown in Fig. 3, the 2D-contour plot was elliptical, and a rapid change in the level of CDH production was observed as the CSL concentration was varied. Thus, CSL was identified as a factor highly affecting the production of CDH. This result was in agreement with the effect of nitrogen source on the production of CDH. Therefore, according to our analysis of 2D-contour plots, the optimum points of each variable yielding maximal production of CDH were 4.42% CSL ($x_1 = 0.71$) and 1.15% SBM ($x_2 = 0.31$), respectively, and the maximum value of CDH predicted from the model was 63.2 U/L.

5. Production of Enzyme Using Optimized Medium in Stirred-tank Reactor

The optimal concentration of medium components was determined through RSM, and the composition of the optimized media was 1.0% (w/v) rice straw, 4.42% (v/v) CSL, and 1.15% (w/v) SBM. Fermentation was performed in a 5 L stirred tank reactor (STR) with an operation volume of 3 L containing the optimized medium. Impeller speed and aeration rate were maintained at 250 rpm and 1.5 vvm,

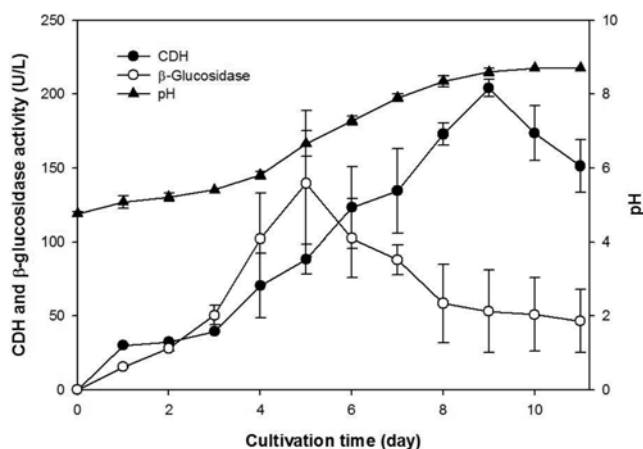


Fig. 4. The time course profiles of enzyme production by *P. chrysosporium* ATCC 32629 using optimized medium in STR. The data shown are the mean of two independent experiments.

respectively. Culture temperature was controlled at 27 °C. Fig. 4 shows the time course profiles of enzyme production by *P. chrysosporium* ATCC 32629 using the optimized medium. Maximum CDH (204 U/L) and β -glucosidase (140 U/L) production were obtained at 9 days and 5 days of cultivation, respectively. The CDH productivity was 22.7 U/L-day in the optimized medium containing rice straw. The pH of the culture broth was gradually increased to pH 8.7 until fermentation ceased. Compared to the flask culture, a sufficient supply of oxygen in the STR resulted in increased CDH production as well as decreased cultivation time.

CONCLUSION

P. chrysosporium represents a potential biological source of cellobiose dehydrogenase (CDH) for use in scientific study and industrial applications. We evaluated the effects of carbon and nitrogen sources in the main medium on the high-level production of CDH and β -glucosidase. The results of this study show that lignocellulose as the carbon source and an organic nitrogen source were the most significant factors affecting the production of enzymes. We also used a statistical method for optimization of the production media. In STR using the optimized medium, the highest levels of CDH and β -glucosidase obtained from *P. chrysosporium* ATCC 32629 were 204 U/L and 140 U/L, respectively. Further studies should estimate the effect of the operating conditions on increased enzyme production by *P. chrysosporium* in STR.

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