REPEATED-BATCH PRODUCTION OF LACCASE BY *CERIPORIOPSIS SUBVERMISPORA*

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Abstract: The aim of this study was to set parameters of repeated-batch cultivation of *Ceriporiopsis subvermispora* for laccase production and evaluate the efficiency of this type of cultivation for production of selected enzyme. The suitable conditions for repeated-batch cultivation were designed on the base of study of batch cultivation of white-rot fungus *C. subvermispora*. *C. subvermispora* was cultivated in media with different concentration of casein hydrolysate as nitrogen source and glucose as carbon source. A suitable concentration of casein hydrolysate to stimulate the laccase production was 1.5 and 2.5 g/L. Laccase production was started at certain critical concentration of glucose (5 g/L). In order to improve laccase production by repeated-batch cultivation of *C. subvermispora*, glucose was tested in concentration 10 g/L and casein hydrolysate in concentration 1.5 g/L. During a repeated-batch cultivation was measured increase laccase activities from 177.8 to 266 U/L. It was also observed, the cultivation time needed to reach maximum laccase production was shortened to 10 days.

Keywords: *Ceriporiopsis subvermispora*, laccases, repeated-batch cultivation.

1. Introduction

Laccase is key enzyme participating in the breakdown of lignin during degradation lignocellulose in the nature. In comparison with other ligninolytic enzymes, laccase has wide substrate specificity for oxidation of various phenolic compounds, are more stable and not require the presence of cofactors (HAMMEL and CULLEN, 2008). Therefore, laccase founds more applications in various sectors. Laccase activity is essential for biodegradation of organic pollutants with phenyl ring such as polyamines, aminophenols, aryldiamines, polycyclic aromatic hydrocarbons (POZDNYAKOVA *et al.*, 2004), organophosphorus pesticides and synthetic dyes (TORRES *et al.*, 2003). Laccase can be produced by various organisms, but significant producers of this enzyme are white-rot fungi such as *Ceriporiopsis subvermispora* (RŰTTIMANN *et al.*, 1992).

Laccase production by *C. subvermispora* is affected by various factors such as temperature, pH, medium composition and presence of elicitors in cultivation medium (CHMELOVÁ *et al.*, 2011). Because laccase production starts in negative environmental conditions, parameters of fermentation process designed for laccase production are essential for achievement of sufficient yields. Significant factor influenced the production of laccase is an availability of carbon and nitrogen sources. Although easily utilizable carbon or nitrogen source allow an increase in laccase production, its production occurs only after exhaustion of the source (GALHAUP *et*

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al., 2002; THIRUCHELVAM and RAMSAY, 2007). In the literature, laccase was produced mostly by batch fermentation. This process does not allow sufficient using of sources for laccase production because most sources are used for biomass production. Repeated-batch cultivation was reported as a promising method for high laccase production (BIRHANLI and YESILADA, 2010). Fungal biomass can be reused for a long time in this cultivation (BIRHANLI *et al*., 2013).

The present study was carried out to investigate the laccase production by white-rot fungus *C. subvermispora* in batch cultivation media with different concentration of nitrogen or carbon sources, then the parameter selection of repeated-batch cultivation of *C. subvermispora* for laccase production and the evaluation of its effectiveness compared to batch cultivation.

2. Materials and methods

2.1 Chemicals

3,5-dinitrosalicylic acid 98 % (Acros Organics, USA), malt agar (Biomark, India), K_2HPO_4. 12 H_2O p.a., KH_2PO_4 p.a., NaCl p.a., $CaCl_2$. 2 H_2O p.a., $ZnCl_2$ p.a., glucose p.a., $FeSO_4$. 7 H_2O p.a., $MgSO_4$. 7 H_2O p.a., $CuSO_4$. 5 H_2O p.a., casein hydrolysate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) 98 % (Sigma Aldrich, G), $MnSO_4$. 4 H_2O p.a. (Slavus, SK).

2.2 Microorganism

Culture of *Ceriporiopsis subvermispora* ATTC 90467 was provided from the Centraalburea voor Schimmelcultures (Netherlands). The culture was maintained on malt agar and stored at 4 °C. In all cases, the suspension of fungal mycelium was prepared by scraping of plaque (1 cm^2) of the growth culture from agar plate using microbiological loop in sterile deionized water (10 mL).

2.3 Medium composition

For batch cultivation, 50 mL of basic mineral medium (Table 1) containing glucose as carbon source (5, 10, 25, 50, 75 and 100 g/L) and casein hydrolysate as nitrogen source (0.5, 1.5, 2.5 and 5.0 g/L) was inoculated with 5 mL of fungal mycelium suspension. Inoculated cultivation medium was shaken (min. 200 RPM) at 30 °C.

Table 1. Composition of basic mineral medium (AGUIAR *et al.*, 2006).

Components of medium	Concentration
$MgSO_4$. 7 H_2O	0.5 g/L
NaCl	0.1 g/L
$CaCl_2$. 2 H_2O	0.1 g/L
$CuSO_4$. 5 H_2O	0.1 mg/L
$FeSO_4$. 7 H_2O	0.2 mg/L
$MnSO_4$. 4 H_2O	0.02 mg/L
$ZnCl_2$	0.15 mg/L

For repeated-batch cultivation, 200 mL of basic mineral medium (Table 1) containing glucose (10 g/L) and casein hydrolysate (1.5 g/L) was inoculated with 20 mL of fungal mycelium suspension. Inoculated cultivation medium was maintained shaken (min. 200 RPM) at 30 °C. After achieving of maximal laccase activity, cultivation medium was replaced by fresh cultivation medium and biomass in fresh cultivation medium was cultivated at 30 °C with shaking (min. 200 RPM). Replacement of cultivation medium was repeated two times.

2.4 Concentration of glucose

Residual glucose was determined in cultivation media using DNS (3,5-dinitrosalicylic acid) method (MILLER, 1959). 0.8 mL of DNS reagent was added to 0.1 mL of supernatant. Reaction mixture was incubated in boiling water batch for 5 minutes. The mixture was cooled down to room temperature and 8 mL of distilled water was added. The absorbance of the reaction mixture (200 µL) was measured at 540 nm using a microplate reader (BioTek EL 800, Fisher, GE).

2.5 Activity of laccase

Activity of laccase was determined with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as the substrate. The assay mixture contained 150 µL of 50 mmol/L phosphate buffer (pH 5.0) with 1 mM ABTS and 50 µL of enzyme extracts. Oxidation of ABTS was monitored by measuring of absorbance at 405 nm (SHIN *et al*., 1987). Activity of laccase was expressed in unit (U) as the amount of enzymes able to convert 1 mg of ABTS per minute.

3. Results and discussion

Composition and availability of nutrients play a significant role in laccase production (GALHAUP *et al.*, 2002). Production of laccase is a part of secondary metabolism of white-rot fungi (HOWARD *et al.*, 2003). Secondary metabolism starts in response to negative environmental conditions as a lack of nutrients (carbon or nitrogen sources), the presence of specific organic compounds (catechol, syringaldazine, 2,5-xylidine, veratrylalcohol) (CHMELOVA *et al.*, 2011) or growth conditions (pH, temperature, pressure, ionic strength) (HOWARD *et al.*, 2003). In our study, we tested laccase production ability of white-rot fungus *C. subvermispora* under various concentrations of carbon and nitrogen sources.

C. subvermispora was cultivated in medium intended for the production of biomass (AGUIAR *et al.*, 2006). Cultivation medium was composed from basic mineral medium (Table 1), carbon source and nitrogen source. Glucose was selected as a readily consumed carbon source, because it is a cheap and easily available substrate (GALHAUP *et al.*, 2002). For efficient laccase production by fungi, the selection of suitable nitrogen source is essential (GALHAUP *et al.*, 2002; THIRUCHELVAM and RAMSAY, 2007). The organic nitrogen substrates such as peptone, casein hydrolysate or malt-extract supported better fungal biomass production and enzyme activities as

compared to the inorganic nitrogen substrates (LEVIN *et al.*, 2008; CHMELOVÁ *et al.*, 2011). Therefore, casein hydrolysate was used as nitrogen source. For determination of effect of carbon and nitrogen sources on laccase production, *C. subvermispora* was cultivated in medium with glucose (10 g/L) and variable concentrations of casein hydrolysate (0.5, 1.5, 2.5 and 5.0 g/L). During cultivation, glucose concentration as indicator of metabolic activity and laccase activity were determined (Fig. 1).

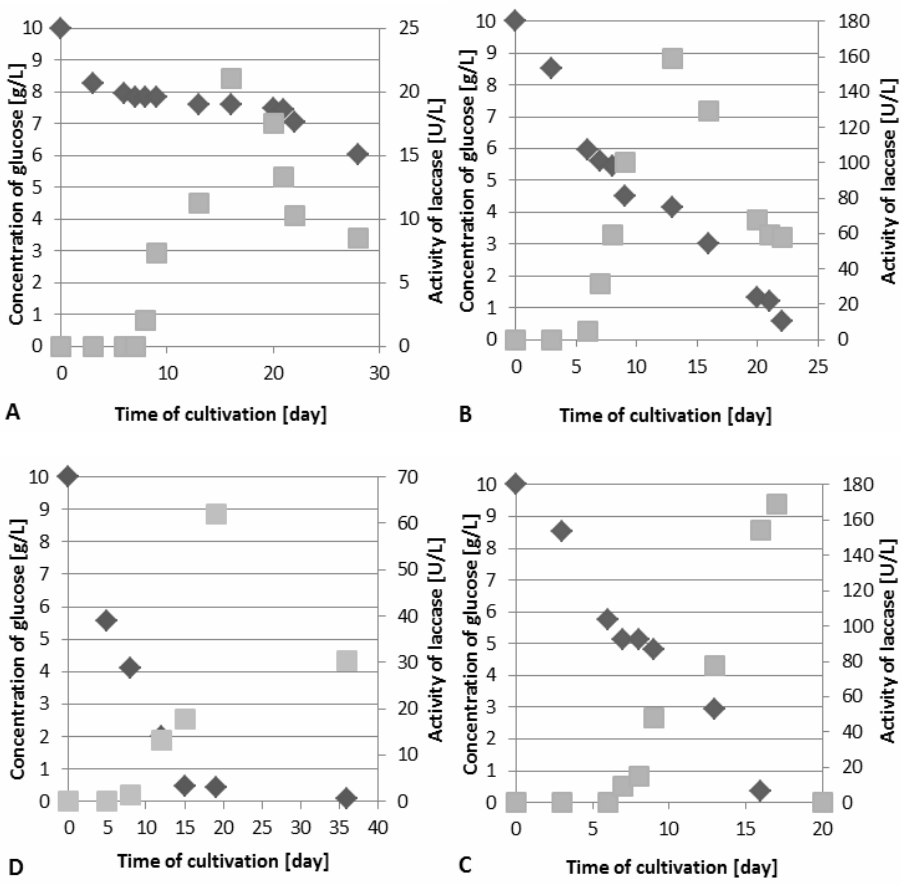

Fig. 1. Concentration of glucose and activity of laccase during growth of white-rot fungus *C. subvermispora* in cultivation medium with glucose as carbon source (10 g/L) and casein hydrolysate as nitrogen source in a concentration A – 0.5 g/L, B – 1.5 g/L, C – 2.5 g/L and D – 5.0 g/L; ♦ - concentration of glucose and ■ – activity of laccase.

From Fig. 1, laccase activity in cultivation media with different concentrations of casein hydrolysate varied considerably (from 21.1 to 169 U/L). The highest laccase activity (169 U/L) was determined in medium with 2.5 g/L of casein hydrolysate (Fig. 1-C). In medium with higher concentration of casein hydrolysate (5.0 g/L), laccase

production was lower (Fig. 1-D). Time for maximum laccase production increased with increasing of casein hydrolysate concentration. Laccase production achieved maximum enzyme activity in 13th (159 U/L), 17th (169 U/L) and 19th (62 U/L) day of cultivation at casein hydrolysate concentration 1.5, 2.5 and 5 g/L, respectively. Production of laccase was observed when a glucose concentration in the medium declined to critical level (approximately 5 g/L) with the exception of the medium with the lowest concentration of casein hydrolysate (0.5 g/L). In this medium, the laccase activity was achieved the maximum after 16th day of cultivation, while the glucose concentration did not decrease to critical level (5 g/L) and maximum laccase activity was only 21 U/L. Low casein concentration led to deficient laccase production. In this case, laccase production was started probably due to lack of nitrogen not carbon source. Similar results were described in other studies (GALHAUP *et al.*, 2002; LEVIN *et al.*, 2010), when laccase activity was mainly detectable in the phase when glucose in culture medium was almost exhausted. These results suggest that suitable a carbon:nitrogen ratio for an effective laccase production is 6.7:1. Carbon:nitrogen ratio depend on fungal species, for example *Phanerochaete flavido-alba* requires low carbon:nitrogen ratio (PÉREZ *et al*., 1996) and *Pycnoporus cinnabarinus* high C/N ratio (EGGERT *et al.*, 1996).

The effect of carbon source concentration on laccase production was studied in media with variable glucose concentrations (5; 10; 25; 50; 75 and 100 g/L) and with maintained carbon:nitrogen ratio (6.7:1). During cultivation of *C. subvermispora,* glucose concentration and laccase activity were determined. Results are shown in Fig. 2.

Activity of laccase was increased with increasing glucose concentration until a glucose concentration 50 g/L. Activity of laccase reached a maximum at 15th (50.6 U/L), 12th (221 U/L), 26th (239.1 U/L) and 35th (352 U/L) days in cultivation media with glucose concentration 5, 10, 25 and 50 g/L, respectively (Fig. 2A-D). Laccase activity was not measured throughout the cultivation in media with 75 and 100 g/L of glucose (Fig. 2E,F). This could be caused by an excessive concentration of glucose which prevents the start of secondary metabolism of *C. subvermispora* (LEE *et al.*, 2004). In these media, it was not observed decreasing of glucose concentration under critical level neither after 50 days. This could be due to the exhaustion of nitrogen source and stopping the growth of white-rot fungus *C. subvermispora*. We found that, although laccase production increased with increasing glucose also cultivation time required to maximum laccase production was extended. Therefore, in repeated-batch process, cultivation media were composed of glucose in concentration 10 g/L and casein hydrolysate in concentration 1.5 g/L.

Repeated-batch cultivation represents a potential alternative mode of cultivation, in which medium or some part of the medium is drawn and fresh medium is refilled periodically without changing fungal biomass (WESENBERG *et al.*, 2003). This process allows the use of fungal biomass repeatedly and achieves better results, compared with batch cultivation (BIRHANLI and YESILADA, 2006). The best selected conditions were used for the following repeated-batch cultivation of fugal biomass. In order to improve laccase production by *C. subvermispora,* glucose was tested as a carbon source (10 g/L) and casein hydrolysate as nitrogen source (1.5 g/L). This medium was added to biomass of *C. subvermispora*. During cultivation was

determined glucose concentration as indicator of metabolic activity and laccase activity. After achieving of maximal laccase activity, the cultivation medium was replace by fresh medium with starting concentration of glucose and casein hydrolysate. The results of repeated-batch cultivation of *C. subvermispora* are shown in Fig. 3.

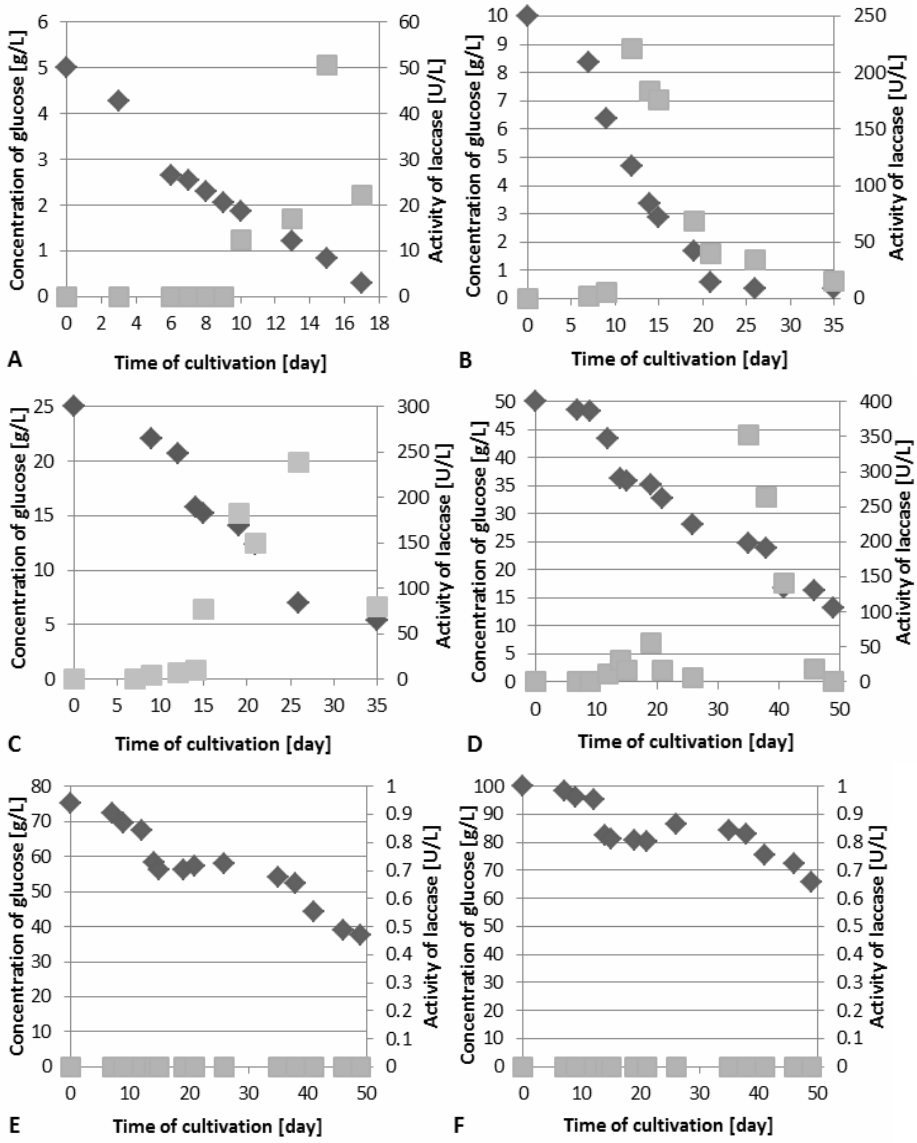

Fig. 2. Concentration of glucose and activity of laccase during growth of white-rot fungus *C. subvermispora* in cultivation medium with casein hydrolysate as nitrogen source and glucose as carbon source in carbon:nitrogen ratio (6.7:1; w/w) with glucose concentration A – 5 g/L, B – 10 g/L, C – 25 g/L, D – 50 g/L; E – 75 g/L and F – 100 g/L; ♦ - concentration of glucose; ■ – activity of laccase.

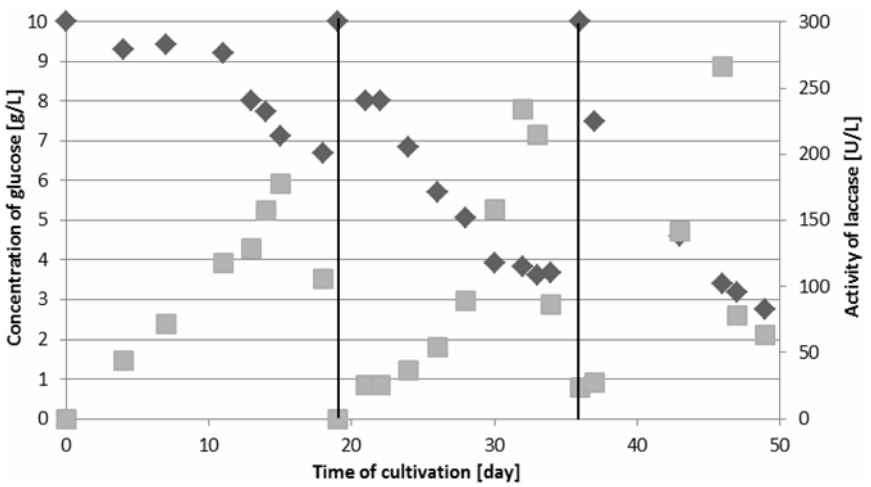

Fig. 3. Repeated-batch cultivation of *C. subvermispora* in cultivation medium with 10 g/L of glucose and 1.5 g/L of casein hydrolysate at 30 °C and pH 5.0; ♦ - concentration of glucose and ■ – activity of laccase.

From Fig. 3, the laccase production can be periodically increased during long-term cultivation by periodical replacing the cultivation medium for medium with started concentration of glucose and casein hydrolysate. Maximum laccase activity obtained by repeated-batch cultures of *C. subvermispora* was 177.8 U/L during first cultivation phase after 15 days, 233.5 U/L during second cultivation phase after 13 days and 266 U/L during third cultivation phase after 10 days. It was also observed, the cultivation time needed to reach maximum laccase production during repeated-batch cultivation was shortened and maximum laccase activity was increased. The advantage of this process is the reuse of fungal biomass for laccase production. Similar results were observed in other studies (BIRHANLI and YESILADA, 2006; BIRHANLI and YESILADA, 2010).

4. Conclusions

The stimulatory effect of some components of cultivation medium on laccase production during batch cultivation was described. High amount of laccase could be obtained with repeated-batch cultivation by selecting the most appropriate culture conditions for laccase production (glucose concentration 10 g/L, casein hydrolysate concentration 1.5 g/L). Our results show that repeated-batch cultivation is an easy and suitable process for high amount of laccase without a need for a new fungal biomass.

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