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Production of laccase by *Coriolus versicolor* and its application in decolorization of dyestuffs:
(Ⅰ) Production of laccase by batch and repeated-batch processes

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Abstract: The production of laccase by *Coriolus versicolor* was studied. The effect of cultivation conditions on laccase production by *Coriolus versicolor* was examined to obtain optimal medium and cultivation conditions. Both batch and repeated-batch processes were performed for laccase production. In repeated-batch fermentation with self-immobilized mycelia, total of 14 cycles were performed with laccase activity in the range between 3.4 and 14.8 U/ml.

Keywords: laccase; textile dyes; immobilized mycelium; decolorization; *Coriolus versicolor*

Introduction

Non-specific oxidation of polyaromatic compounds by ligninolytic enzymes from lignin-degrading white-rot fungi has increasingly attracted attentions because of its ability to metabolize a wide range of organic contaminants(Kacstner, 2000). Among them, laccase, also called phenolase or polyphenol oxidase, is an important one. Unlike lignin peroxidase and Mn-dependent peroxidase, those need H_2O_2 as the second substrate in the reaction, the degrading reaction catalyzed by laccase requires O_2 only, without H_2O_2 participation.

Coriolus versicolor is the most commonly used microbe to produce laccase. The fermentation can be carried out at room temperature. Comparing with lignin peroxidase and Mn-peroxidase production via *P. Chrysosporium*, the laccase fermentation does not need to limit carbon or nitrogen source and to supply pure oxygen occasionally. Also *C. Versicolor* is not very sensitive to the shearing stress.

Laccases from various white-rot fungi can be exploited for a number of environmental applications including bleaching of kraft pulp(Bajpai, 1999), decolorization of different synthetic textile dyes(Kirby, 2000; Wong, 1999), removal of phenolic compounds in industrial wastewater(Atlow, 1984) and soil bioremediation(Kaestner, 2000).

In this work, the medium composition and cultivation conditions will be optimized for improving the production of laccase by the white-rot fungus *Coriolus versicolor*, and the repeated-batch strategy is to be performed to increase the laccase productivity.

1 Materials and methods

1.1 Microorganisms

The strain of *Coriolus versicolor* was maintained at 4℃ on YMS agar slant (glucose 20 g/L, malt extract 20 ml/L, peptone 1 g/L, and agar 20 g/L).

1.2 Medium

The fundamental medium used for the production of laccase contains (g/L): glucose 2.2, diammonium tartrate 0.94, NaH_2PO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.5, and trace element solution 1 ml/L. Medium is adjusted to pH 4.5.

The trace element solution contains(mg/L): vitamin B_1 100, $CaCl_2$ 100, $FeSO_4 \cdot 7H_2O$ 100, $MnSO_4 \cdot H_2O$ 5, $ZnSO_4 \cdot 7H_2O$ 10, and $CuSO_4 \cdot 5H_2O$ 20.

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1.3 Chemicals

ABTS, diammonium-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate), used for enzyme assays was from Sigma. The ABTS solution was prepared by dissolving 0.55g ABTS into 1L sodium phosphate buffer (2 mol/L).

1.4 Enzyme assays

The fermentation broth was filtered to remove mycelia, the filtrate was then diluted to reach proper enzyme concentration for analysis. Both sample and ABTS solution were preheated to 30℃ for 5 min, then mixing 1 ml diluted sample with 1 ml ABTS solution in a 1 cm cuvette to start reaction. The reaction was continued for 5 min and the OD_{420} values were recorded on-line by the HP 8452A spectrometer. The linear region of OD_{420} was used to determine the enzymatic activity of laccase. One unit of activity was defined as the amount of enzyme causing 1 unit change of OD_{420} in 1 min.

1.5 Analytical methods

Glucose concentration was analyzed by DNS method(Miller, 1959).

2 Results and discussion

2.1 Production of laccase

Fig.1 shows the time courses of batch fermentation of *Coriolus versicolor* for laccase production. It indicated that the growth rate of *Coriolus versicolor* was very slow and six days was required for the maximum dried-cell weight. After another two days, the laccase activity reached the maximum and fall down fast afterward. It could also be noticed that the laccase production was partially growth-rate related. The pH value during batch culture was relative stable. Table 1 lists the effects of initial glucose concentration on the production of laccase. It seems that higher initial glucose concentration did not guarantee higher laccase production. Though laccase is not a typical secondary metabolite (Archibald, 1992), glucose does show inhibition in cell growth. Most of laccase were produced at the end of growth phase. Initial glucose concentration of 5.4 g/L was suitable for laccase production.

Fig.2 shows the effects of initial pH values on laccase production. It indicated that the optimal initial pH value for laccase production was pH 4.5, where the laccase activity at the ninth day was 80% higher than that at pH 3 or 6.

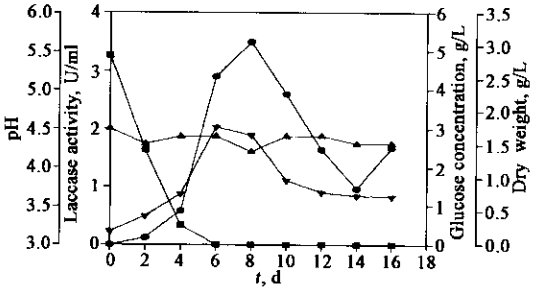

Fig.1 Time courses of batch fermentation of *Coriolus versicolor* ● laccase activity; ■ glucose concentration; ▲ pH; ▼ dried weight of *Coriolus versicolor*

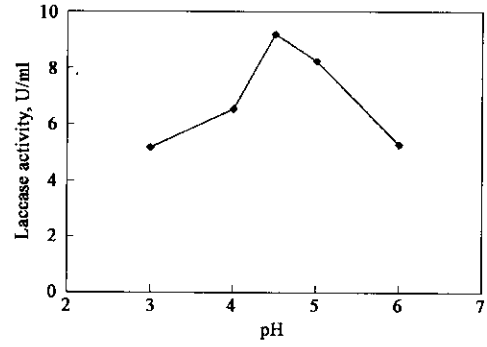

Fig.2 Effect of initial pH values on laccase activity after nine days' cultivation

The effects of trace element solution on laccase production are shown in Fig.3. When the addition of trace element solution increased from 1 ml to 10 ml, the laccase activity increased by 25%, further increase of trace element did not mean better lacasse production.

Table 1 Effects of initial glucose concentration on the production of laccase

Initial glucose concentration, g/L	Maximum laccase activity, U/ml	Days to reach maximum laccase activity, d	Days for complete glucose consumption, d
4.8	3.6	8.0	6.0
5.4	4.3	8.0	6.0
23.0	4.6	13.0	10.0

In other lignin degrading enzyme production, such as lignin peroxidase and Mn-dependent peroxidase, veratryl alcohol must be added as an inducer for enzyme production. The effect of veratryl alcohol on laccase production was also examined and the results are shown in Fig.4. It can be seen that veratryl alcohol is not essential for laccase production, but do improve the laccase productivity. When 5 mmol/L of veratryl alcohol was added, the laccase production was increased from 10.8 U/ml to 14.2 U/ml.

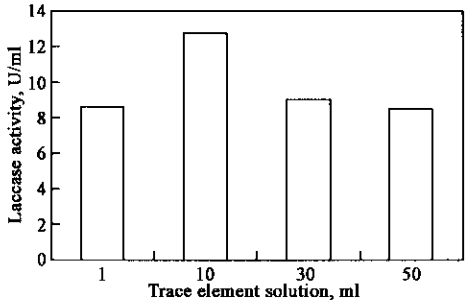

Fig.3 Effect of metal elements on laccase activity after nine days' cultivation

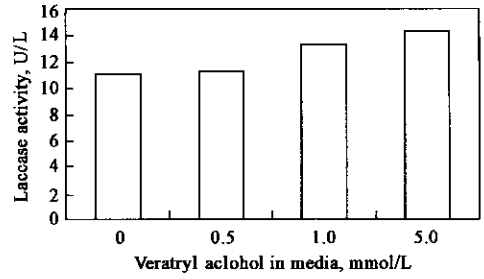

Fig.4 Effects of the supplement of veratryl alcohol on laccase production

The effects of inoculation volume on laccase production are shown in Fig.5. It is favorable for laccase production with the increase in inoculation volume, but when inoculation volume is greater than 30 ml/L, the laccase activity will not increase further or even fall down.

2.2 Self-immobilization of *Coriolus versicolor* and repeated-batch production of laccase

During *Coriolus versicolor* cultivation, the mycelium can be self-aggregated to form spherical mycelium pellets with a diameter about 1—3 mm. The pellets were very stable in size and activity. From Fig.1, it is obvious that the growth rate of *Coriolus versicolor* is very slow. It takes six and eight days to reach maximum cell concentration and laccase activity, respectively. Therefore it is desired to use the self-immobilized *Coriolus versicolor* pellets to produce laccase continuously or repeatedly. The repeated batch fermentation was performed as following: when one batch fermentation was ended, harvest the broth containing laccase and then add the fresh medium into the fermenter to start a new batch.

Fig.6 and Table 2 show the experimental data of repeated-batch production of laccase by self-immobilized mycelium pellets. Three parallel experiments were performed as shown in Fig.6. The only difference is the inoculation volume. The parallel experiments showed the similar results. Total of 14 cycles

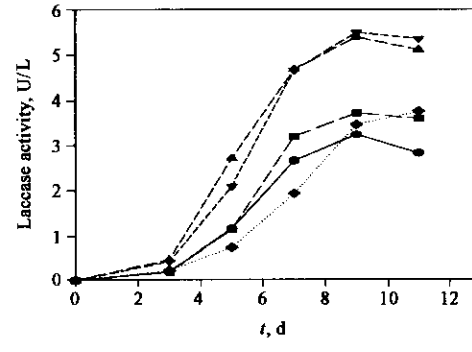

Fig. 5 Effects of the inoculation volume on laccase production

inoculation volume: ● 10 ml/L; ■ 20 ml/L; ▲ 30 ml/L; ▼ 40 ml/L; ◆ 50 ml/L

Table 2 Repeated-batch production of laccase by self-immobilized mycelium pellets(according to Fig.6a)

Repeated number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cycle period, d	11.0	6.0	4.0	6.0	6.0	6.0	5.0	3.0	2.0	2.0	1.5	1.5	1.5	1.5
Laccase activity, U/ml	5.0	3.6	4.0	3.0	2.5	3.7	6.2	4.2	5.9	4.9	11.5	11.8	12.3	7.9

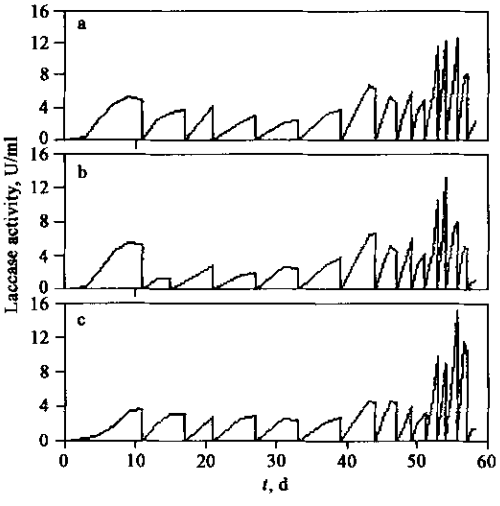

Fig.6 Repeated-batch fermentation of *Coriolus versicolor* for laccase production (inoculation volume, a: 3%; b: 4%; c: 5%)

were performed from the same pellets and lasted for 57 days. It is a surprise to notice that with the increase of cycle number, the period for each cycle will be reduced greatly from 11 days in the first cycle to 1.5 days in the 14th cycle. The average laccase activity for the last four cycle was 10.9 U/ml, doubled in comparison with the first cycle. The number of pellets did not change and no noticeable increase in the size of pellets was found.

After 14 cycles, the color of mycelium pellets became yellowish. Under microscope, it was observed that the inside of pellets was almost hollow, which indicated that only the outside shell of pellet was of active mycelia.

3 Conclusions

Laccase can be produced by batch, and more effectively by repeated-batch fermentation with *Coriolus versicolor* mycelia pellets. High concentration of glucose showed some inhibition in the cell growth and laccase production. The optimal initial pH value for laccase production was pH 4.5. Suitable increase of trace element solution benefitted the laccase production. Veratryl alcohol is not essential for laccase production, but does improve the productivity. During repeated-batch fermentation, with the increase of cycle number, the period for each cycle is reduced from 11 days to 1.5 days and the laccase activity has been doubled.

References:

Archibald F, Roy B, 1992. Production of manganic chelates by laccase from the lignin degrading fungus *Trametes* (*Coriolus*) *versicolor*[J]. Appl Environ Microbiol, 58(5): 1496—1499.

Atlow S C, Bonadonna-Aparo L, Klibanov A M, 1984. Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase[J]. Biotechnol Bioeng, 26: 599—603.

Bajpai P, 1999. Application of enzymes in the pulp and paper industry[J]. Biotechnol Prog, 15: 147—157.

Kaestner M, 2000. Degradation of aromatic and polyaromatic compounds[M]. in Biotechnology (Volume 11b, J Klein ed.), 2nd (H-J Rehm and G Reed eds.). Heidelberg: VCH, 212—239.

Kirby N, Marchant R, McMullan G, 2000. Decolorization of synthetic textile dyes by *phlebia tremellosa*[J]. FEMS Microbiolgy Letters, 188: 93—96.

Miller G L, 1959. Dinitrosalicylic acid reagent for determination of reducing sugar[J]. Anal Chem, 31: 426—428.

Wong Y, Yu J, 1999. Laccase-catalyzed decolorization of synthetic dyes[J]. Wat Res, 33: 3512—3520.

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