Biotechnology and Bioprocess Engineering **2006**, 11: 306-312

Decolorization of Dye and Molasses by Continuous and Semi-Continuous Jar-Fermentor Cultures of *Geotrichum candidum* Dec 1

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Abstract Two culture modes, continuous and semi-continuous, of the decolorization fungus, *Geotrichum candidum* Dec 1, were compared to obtain a high treatment efficiency of molasses decolorization and a large productivity of peroxidase (DyP) to simultaneously decolorize dyes and molasses. The continuous culture of *G. candidum* Dec 1 using a 5-l jar-fermentor showed high DyP activity at a low dilution ratio of 0.005 h^{-1}, and decolorization ratio of molasses of 80% was obtained concomitantly. Therefore, a semi-continuous culture was performed by repeated refill and draw. In this mode, approximately 1.5 liters of the culture broth was replaced per cycle when the decolorization ratio of molasses was near 80%. The molasses medium (1.0 liter per day) was treated and the peroxidase productivity in the drawn culture broth was 26.6 U/day, whereas the peroxidase productivity was 17.9 U/day in the continuous culture with a dilution rate of 0.005 h^{-1}. The semi-continuous treatment system was an efficient decolorization method for the strain, *G. candidum* Dec 1.

Keywords: *Geotrichum candidum*, decolorization, dye, molasses, peroxidase, continuous, semi-continuous

INTRODUCTION

Geotrichum candidum Dec 1 was isolated and characterized as a novel decolorizing fungus for various colorants [1], and a decolorization enzyme (DyP, a peroxidase) and an aryl alcohol oxidase that could supply hydrogen peroxide to the DyP enzymatic reaction were subsequently purified [2,3]. The decolorization activity of Dec 1 appeared as a secondary metabolic effect when sugar was present in the culture broth [1]. Therefore, cultures capable of decolorizing dye and molasses simultaneously were tested in molasses medium using molasses as a sugar source [4]; these cultures were successfully scaled up in a jar-fermentor with a working volume of 5 liters, and the decolorization ratio of molasses and peroxidase activity were enhanced by a high oxygen tension of 60% [5].

On the other hand, cultures of the white-rot fungi, such as *Phanerochaete chrysosporium*, a well-known candidate for lignin degradation and decolorization of synthetic dyes, produced a series of extracellular peroxidases such as ligninases (LiP) and manganese peroxidase (MnP) that are responsible for dye degradation [6-10]. Large-scale cultures of *P. chrysosporium* have been attempted to enhance the production of LiP and/or MnP. Due to the sensitivity of peroxidase activity to agitation [11], bioreactors with low shear stress, such as the bubble column reactor, packed column reactor or air-lift reactor, [12-15] were employed, using various immobilizing materials like polyurethane, nylon-web and silicon tube [13]. However, the production of peroxidases by suspended cells in the jar-fermentor was lower, comparable to that observed with pneumatic bioreactors, because it was difficult to control the 1.0- to 2.0-mm pellet size with high activity [16,17] and the sensitivity to agitation [15,18].

As the decolorization activity and peroxidase production of *G. candidum* Dec 1 were not greatly affected by shear stress, high activity could be obtained in a jar-fermentor [5]. One possible method is a continuous culture to maintain peroxidase, which declined during the batch culture [5]. In order to further investigate more effective decolorization culture models, semi-continuous cultures were also performed by repeated fill and draw, and these two methods, continuous and semi-continuous cultures, were compared with respect to stable decolorizing activity.

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MATERIALS AND METHODS

Microorganism

The characteristics of the decolorizing fungus, *G. candidum* Dec 1 were described in the previous paper [1].

Medium

Potato dextrose (PD) (Difco) liquid medium adjusted to pH 6 was employed to obtain inoculum sources for the jar-fermentor cultivation [5].

Ten kg of molasses was diluted two-fold with 7,042 mL H_2O and preserved at 4°C to unify the composition of the medium during continuous culture at various dilution rates. Molasses medium for continuous cultures contained 70 mL molasses, 3 g glucose, 0.5 g ammonium tartrate, 1 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ in 1 liter H_2O, and was adjusted to pH 5.10~5.15. For the initial batch stage before continuous cultivation, the medium was modified with 7 g glucose. In the two-stage continuous culture, the molasses medium was modified with 10 and 15 g glucose per liter for continuous and initial batch cultivations, respectively.

Molasses medium for semi-continuous culture used 50 g molasses, rather than the diluted molasses described above. The initial batch and semi-continuous cultures were modified with 20 and 10 g of glucose per liter molasses medium, respectively. Other components were added to the medium in the same amounts.

Cultivation

Continuous Culture at Various Dilution Rates

The molasses medium in a jar-fermentor with a 5-L working volume was inoculated with 3-old day preculture broth in PD medium at an agitation speed of 180 rpm, 60% oxygen tension with 0.4 vvm, and 30°C. Twenty liters of fresh medium were aerated with air at 2 L/min in a 36-liter stainless tank to maintain a high dissolved oxygen concentration. Fresh medium was periodically supplied to the jar fermentor with a flow rate of 200 mL/min, with the supply term controlled according to the dilution rate. At dilution rates of 0.01 and 0.005 h^{-1}, the cultures were terminated after 8 days when steady states were observed, due to the decreases in the decolorization activity as a result of long-term incubation. At dilution rates, from 0.05 to 0.2 h^{-1}, another culture that started at 0.05 h^{-1} was changed to higher dilution rates in stages after data of steady states were obtained. To assess decolorization activity, cell mass, and sugar concentration, 35 mL of culture broth was harvested everyday from the cultures with dilution rates from 0.005 to 0.03 h^{-1}. In the cultures that lacked decolorization activity, namely dilution rates from 0.05 to 0.2 h^{-1}, 200 mL of culture broth was collected and analyzed.

Transient washout experiments at dilution rates of 0.1 and 0.2 h^{-1} were performed to investigate the maximum specific growth rate (μ_{max}), although more experiments need to be done with other intermediate dilution rates between 0.1 to 0.2 h^{-1}. Cell mass was measured from pre-steady state to steady state of the continuous culture, and μ_{max} was evaluated according to methods employed by Pinelli *et al.* [19].

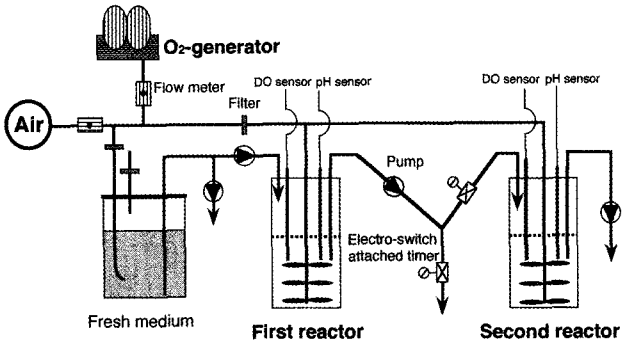


Fig. 1. Systematic profile of two-stage continuous culture of Dec 1.

Two-Stage Continuous Culture

The two-stage continuous culture system was devised so that growth would mainly occur in the first vessel, while decolorization activity would be maximized in the second vessel; a longer retention time in this vessel was designed to obtain high activity. Half of the efflux from the first vessel was supplied into the second vessel under the control of a timer, as shown in Fig. 1. For the initial 3 days in the first vessel, the culture was maintained at 180 rpm and 60% oxygen tension of 0.6 vvm; thereafter, it was changed to continuous culture, in which the first and second vessels were operated at dilution rates of 0.02 and 0.01 h^{-1}, respectively, for 6 days (stage II). Then in stage III, the dilution rates were lowered to 0.01 and 0.005 h^{-1}. Additionally, the agitation speed of the second vessel in stage III was elevated to 250 rpm, because higher decolorization activity is obtained by increasing the active mycelia that result from the formation of smaller pellets.

Semi-Continuous Culture

The culture system is the same as that of batch culture described previously [5]. The culture broth was partially replaced with fresh molasses medium when the decolorization ratio of molasses at 407 nm reached 80%. The amount of molasses medium exchanged per batch was approximately 1.5 liters, but the precise amount was calculated by changes in the absorbance at 407 nm before and after exchange. The culture was sustained by partial refill and draw a total of 9 times over 21 days.

Decolorization Activity and Peroxidase Assay

Decolorization of molasses was measured at 475 nm as described previously [5]. In order to measure the decolorization activity in continuous culture, although it can be predicted by peroxidase (DyP) activity, 200 mL of culture broth was drawn into an autoclaved 500-mL flask; 500 ppm Reactive Blue 5 (RB5) was then added to the culture broth and incubated at 120 spm at 30°C. The

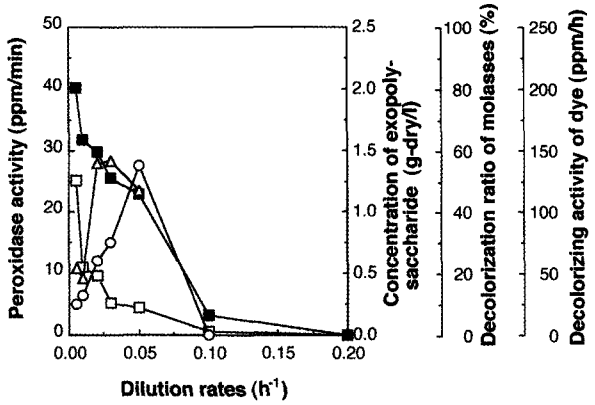


Fig. 2. Peroxidase activity (□), decolorization of molasses (■) and dye (Δ) and concentration of exopolysaccharide (○) at steady state under various dilution rates.

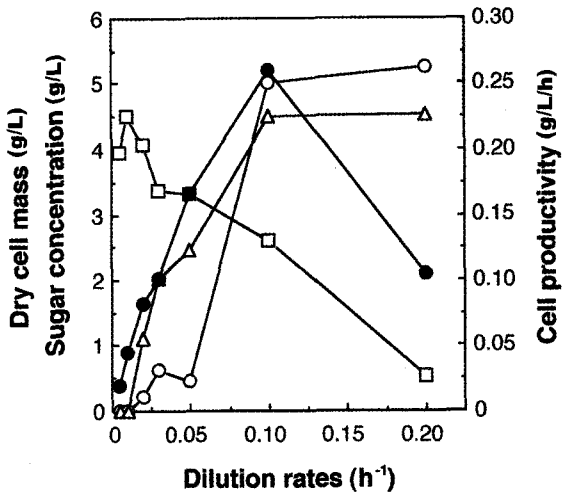


Fig. 3. Changes in cell mass (□), cell productivity (●), and glucose (○) and fructose (Δ) concentrations at steady state under various dilution rates.

activity was assayed by the initial decolorization rate at 600 nm as shown [5]. The decolorizing activities of other cultures were represented by DyP activity involved in the decolorization of various dyes [1,2]. The peroxidase activity was assayed with RB5 using 6-fold diluted supernatant at pH 3.2 as described [5]. A peroxidase activity of 1 ppm/min in the enzyme assay using supernatant is calculated to equal 1.19 U/L, when RB5 is used as the substrate.

Exopolysaccharide Measurement

Two hundred mL of the culture broth from each dilution rate was centrifuged at 8,000 g for 15 min, and the resulting supernatant was frozen at −20°C to separate exopolysaccharides and non-polymeric components. The frozen supernatant was melted at room temperature, and then precipitates containing the polysaccharides were obtained by centrifugation (8,000 g × 15 min). The polysaccharides were washed two times with H_2O and dried at 100°C for 24 h.

RESULTS AND DISCUSSION

Continuous Culture in Various Dilution Ratios

These experiments were designed to optimize the dilution rates between 0.005 and 0.2 h^{-1} for optimal decolorization of molasses and peroxidase activity. As shown in Fig. 2, the observed molasses decolorization rate was 80% at 0.005 h^{-1} and decreased as the dilution rate increased to 0.1 h^{-1}. The dye decolorization activity was 50 ppm/h at 0.005 h^{-1}, due to almost complete exhaustion of sugar in culture broth, but the addition of 10 g/L glucose in a 500-mL flask to the assay culture elevated the activity to 140~150 ppm/h. At dilution rates of 0.02 and 0.03 h^{-1}, the dye was decolorized with a rate of 140 ppm/h, and at 0.05 h^{-1} with a rate of 120 ppm/h. The decolorization of dye in continuous culture using molasses medium was predominantly observed at dilution ratios below 0.05 h^{-1}. Similarly, peroxidase activity exhibited the same trend as the molasses decolorization activity under various dilution rates (Fig. 2). Therefore, the longer retention times exhibited higher molasses decolorization ratios and higher peroxidase activity. The concentration of exopolysaccharide increased as the dilution rate increased up to 0.05 h^{-1}, but disappeared at 0.1 h^{-1} at which the decolorization of molasses and dye and the peroxidase activity were scarcely observed, suggesting that exopolysaccharide is probably secreted as a secondary metabolite together with peroxidase (DyP) activity. In addition, the polysaccharide from Dec 1 culture was largely produced under low nitrogen concentrations and high oxygen tension. This polysaccharide did not affect the oxidation of guaiacol, which is a substrate for DyP *in vitro* (data not shown), although another anionic polysaccharide from *P. chrysosporium* has been shown to inhibit LiP activity [20].

Cell mass decreased almost linearly as the dilution ratio increased, and cell productivity was at its maximum at 0.1 h^{-1} (Fig. 3). On the other hand, the μ_{max}, as evaluated by a transient washout experiment with dilution ratios of 0.1 and 0.2 h^{-1} was 0.14 h^{-1}. However, cell mass existed even at 0.2 h^{-1}, showing that the washout dilution rate is not sharply observed caused by the rheology or growth pattern of mycelia of Dec 1 different from that of bacterial growth. That is, the mycelia can be grown even at a washout dilution rate; this notion is supported by the decrease in glucose concentration from 7 to 5.2 g/L at the dilution rate of 0.2 h^{-1}. Therefore, μ_{max} = 0.14 h^{-1} determined by the transient washout experiments is thought to be valid. On the other hand, no decolorization activities were found at 0.14 h^{-1}, which generated the highest cell productivity (Fig. 2). This indicates that molasses decolorization activity and peroxidase activity from Dec 1 require longer retention times in continuous culture.

The results of the continuous cultures at dilution rates of 0.01 h^{-1} and 0.005 h^{-1} were shown in Fig. 4. Both cul-

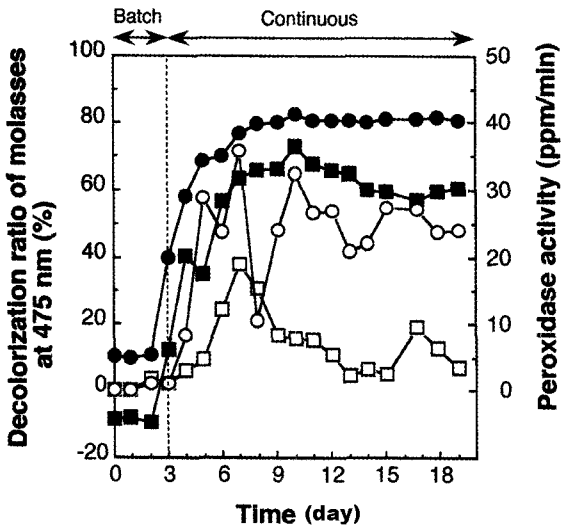


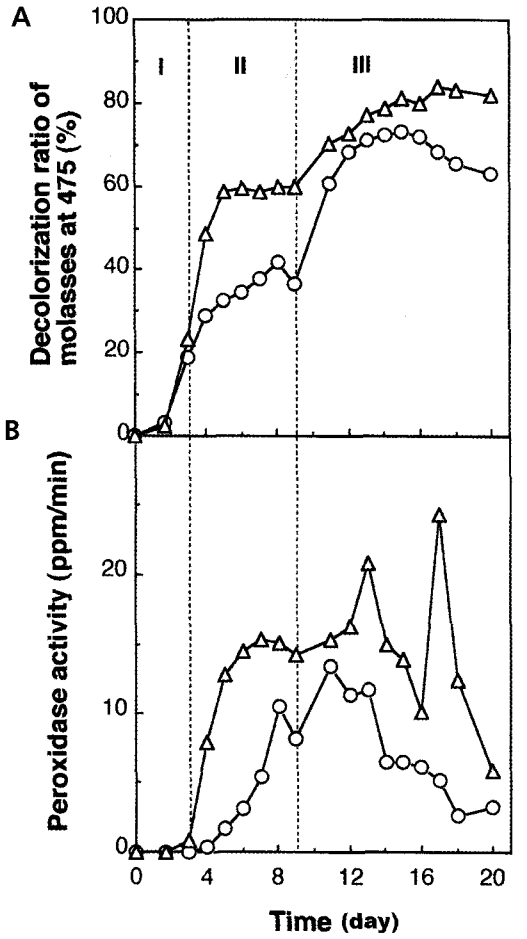
Fig. 4. Changes in decolorization of molasses and peroxidase activity at dilution rates of 0.01 h^{-1} and 0.005 h^{-1}. Symbols, ■ and ● show decolorization ratios of molasses, and □ and ○ show peroxidase activities at 0.01 h^{-1} and 0.005 h^{-1}, respectively.

tures reached steady states on day 7 and 11, respectively. At steady state, the decolorization of molasses in these dilution rates was uniformly maintained at 65 and 80%, respectively. However, peroxidase activity fluctuated significantly, probably as a result of varying productivity, variance of inhibitor components to peroxidase, or variance of active mycelia caused by the repeated adhesion to and slippage from the apparatus in the culture broth with high viscosity. On the other hand, we verified that significant inhibitory components on peroxidase activity of Dec 1 exist in molasses in our previous report [5]. The average activities of peroxidase at 0.01 h^{-1} and 0.005 h^{-1} were determined to be 8 and 25 ppm/min, respectively (Fig. 4). To obtain higher peroxidase activities, the continuous culture of Dec 1 should be operated at lower dilution rates.

Two-Stage Continuous Culture

In continuous cultures at various dilution rates, the culture maintained at 0.005 h^{-1} showed the highest molasses decolorization activity and peroxidase activity. Therefore, a two-stage continuous culture system, in which the second vessel had a two-fold longer retention time than the first, was employed (Fig. 1). The maximum decolorization ratio of molasses under optimal operating conditions of a jar-fermentor was 80% where irregular pellets of 2~3 mm diameter were formed; this was lower than the 87% previously reported for flask cultures [4,5]. This is presumably due to the proportion of active cells to total cell mass forming the pellet, as only the inner cell parts at depths of less than 0.2 mm from the surface are involved in the activity [21]. Hence, the agitation speed in the two-stage continuous culture was raised to 250 rpm in the second vessel on day 16 of the culture to form smaller pellets, thereby obtaining more active cell parts.

In stage I, a batch culture was conducted for the initial



Vessel	Symbol	Change of dilution rates (h^{-1})		
		I	II	III
First	○	Batch	0.02	0.01
Second	△	Batch	0.01	0.005

Fig. 5. Variation of decolorization ratio of molasses (A) and peroxidase activity (B) at various dilution rates in the two-stage continuous culture of Dec 1.

3 days. In stage II, continuous culture was conducted for 6 days, and finally, in stage III, different dilution rates were applied for 11 days as shown in Fig. 5. The maximum decolorization ratio of molasses in stage II, in which the first and second vessel were operated at dilution rates of 0.02 and 0.01 h^{-1}, respectively, was about 60% in the second vessel (Fig. 5A). Simultaneously, the peroxidase activity was about 15 ppm/min in the second vessel (Fig. 5B). Subsequently, in stage III after steady state, the decolorization ratios of molasses in the first and second vessels were 70 and 83%, respectively (Fig. 5A), while the peroxidase activities in both vessels fluctuated significantly (Fig. 5B). In particular, after changing the agitation conditions to 250 rpm, the peroxidase activity of the second vessel increased to about 25 ppm/min, but thereafter decreased sharply, mainly due to exhaustion of sugar as shown in Fig. 6B. However, the peroxidase ac-

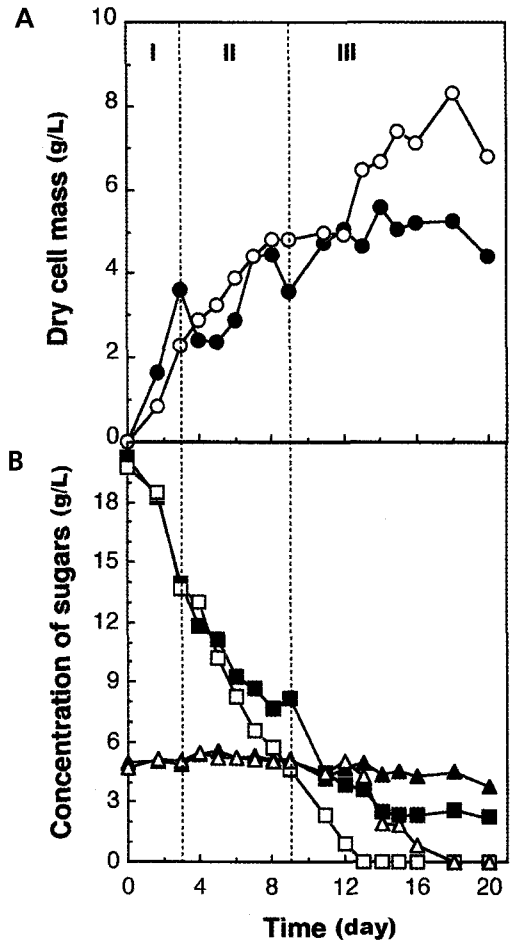


Fig. 6. Variation of cell mass (A) and sugar concentration (B) at various dilution rates in two-stage continuous culture of Dec 1. Changes in dilution rates in stages I, II, and III are the same as that in Fig. 5. Symbols indicate cell mass (●), glucose (■), and fructose (▲) in the first vessel, and cell mass (○), glucose (□), and fructose (Δ) in the second vessel.

tivities in both vessels decreased.

Semi-Continuous Culture by Repeated Fill and Draw

The semi-continuous culture was performed by repeated fill and draw. A batch culture was initially maintained for 9 days, after which approximately 1.5 liters of culture broth was replaced with fresh molasses medium (Fig. 7). Every 2 days from day 9 to 17, partial culture was replaced when the decolorization rate of molasses reached 80%. From day 17 to 21, the replacement was carried out every day 21st day due to high activity. The average amounts of molasses medium exchanged and the peroxidase productivity obtained per day in semi-continuous culture and continuous culture are shown in Table 1. The amount of culture broth exchanged in continuous culture at steady state of 0.005 h^{-1} from the day 11 was 0.6 liter per one day, while that in semi-continuous culture averaged to an exchange of 1.0 liter per day from day 9 to 21. The peroxidase productivities in

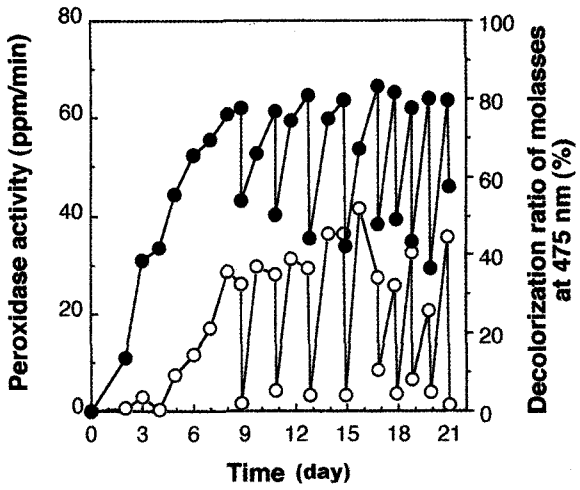


Fig. 7. Peroxidase activity (○) and molasses decolorization (●) in semi-continuous culture by partial refill and draw method using molasses medium, under conditions of 100%-oxygen tension of 0.4 vvm and 180 rpm.

Table 1. Comparisons of decolorization treatment efficiency and peroxidase productivity between continuous culture and semi-continuous culture in a 5-l working volume

	Continuous	Semi-continuous
Replaced amount (liter/day)	0.6[a]	1.0[b]
Peroxidase productivity in culture broth drawn (U/day)	17.9[c]	26.6[d]

[a]Amount of culture broth replaced per day at a dilution ratio of 0.005 h^{-1}.

[b]Exchanged amount evaluated by the variance of absorbance at 475 nm before and after partial exchange of the culture broth. Approximately 1.5 liter was replaced per cycle.

[c]Average peroxidase activity of 25 ppm/min evaluated from day 11 to 19 at 0.005 h^{-1} in Fig. 4 was used to calibrate the productivity per day. Activity of 1 ppm/min with RB5 could be calibrated to 1.19 U/L.

[d]Total peroxidase activity in culture broth drawn from day 9 to 21 in Fig. 4 was adjusted to determine the productivity per day.

culture broth exchanged in the continuous and semi-continuous culture modes were evaluated as 17.9 and 26.6 U, respectively. The semi-continuous culture mode was superior in terms of treatment efficiency by the exchanged amount. In addition, the productivity of peroxidase activity, which decolorizes various dyes, in semi-continuous culture was about 50% higher than in the continuous culture per day. Therefore, the semi-continuous culture obtained by repeated partial fill and draw was revealed as an efficient decolorization treatment method for Dec 1. The semi-continuous culture has also been used in the LiP production from immobilized white-rot fungi, because it is difficult to continuously control

their growth and production phases [22-24]. On the other hand, Ryu reported semi-continuous decolorization of azo dyes with a rotating disc contactor containing immobilized *Aspergillus sojae* B-10 [25].

CONCLUSION

In order to investigate an efficient decolorization treatment method for molasses and dye, we performed continuous culture and semi-continuous culture modes of the decolorizing fungus, *G. candidum* Dec 1, using a jar-fermentor with a 5-l working volume. In continuous culture, high decolorization of molasses and peroxidase activity were obtained at a dilution ratio of 0.005 h^{-1}, but the culture was not an efficient treatment due to its long retention time. When the goal for the decolorization ratio of molasses was defined as 80%, the semi-continuous culture by repeated fill and draw was determined to be an efficient treatment method for decolorization of molasses and peroxidase productivity.

Further studies will be needed to maximize the active mycelia with secondary metabolism in the semi-continuous culture system.

Acknowledgement This research was supported by the Program for the Training of Graduate Students in Regional Innovation which was conducted by the Ministry of Commerce Industry and Energy of the Korean Government.

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[Received December 13, 2005; accepted August 4, 2006]