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Determination of agitation and aeration conditions for scale-up of cellulolytic enzymes production by *Trichoderma inhamatum* KSJ1

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Abstract−10 to 35 L jar fermentation scale-up cultures were performed to determine the optimum agitation and aeration rates in the cellulolytic enzymes production culture by *Trichoderma inhamatum* KSJ1. The optimum agitation rate in the 35 L jar fermenter was provisionally determined to be 150 rpm by using a geometrically resembled scale up method from the 10 L jar fermenter. The optimum aeration rate was determined to be 0.5 vvm by applying the mean values of superficial velocity and vvm. The DO (Dissolved Oxygen) concentration of the culture liquid was maintained below the critical DO concentration (2.336 mg/L) at 150 rpm in the 35 L jar fermenter. To increase the DO above the critical DO concentration, the agitation rate was increased from 150 to 200 rpm, with the aeration rate maintained at 0.5 vvm. As a result, the DO was maintained above critical DO concentration. The OUR (Oxygen Uptake Rate) and k_La values were 0.91 mg-DO/L·min and 11.1 hr^{-1}, respectively. The amylase and FPase (filter paper activity) activities were 4.48 and 0.74 U/mL, respectively, in the 35 L jar fermenter, which was comparable to that in the 10 L fermenter (4.2 and 0.5 U/mL, respectively). Therefore, the scale-up conditions, 0.5 vvm and 200 rpm, were concluded to be the optimum aeration and agitation rates in the 35 L jar fermenter.

Key words: Cellulolytic Enzyme Production, Scale-up, Agitation and Aeration Rate

INTRODUCTION

In our previous study, cellulolytic enzymes were produced at low cost from cellulosic wastes (rice straw, paper waste) [1], and food wastes were saccharified by cellulolytic enzymes [2], the latter (supernatant) being used as a medium source for BC (BC, bacterial cellulose) production [3] and ethanol fermentation [4]. BC has become known as a functionally advanced material, and ethanol as a clean energy. The concentration of organic substances in the residual solid produced by the saccharification reaction and the residual solution produced from the BC production process were reduced via methane fermentation. The residual compounds produced from methane fermentation were eventually collected as a soil conditioner for the "Development of a total resources recovery system of zero-emission of food wastes" project. Since BC has been utilized as a medical treatment, quality paper for records, as the diaphragm of high sensitivity speakers and diet materials [5-8], the mass production of cellulolytic enzymes from saccharifying food wastes is desirable for the low cost mass production of BC in our laboratory.

Previously, researchers identified and characterized *Trichoderma inhamatum* KSJ1 as a cellulolytic enzyme producer [9], and used modified Mandel's medium for cellulolytic enzyme production. In Mandel's medium, the carbon sources, Avicel and CMC (carboxymethyl cellulose), were substituted by 1% rice straw and paper wastes. The optimal conditions for enzyme production were evaluated in 500 mL flasks by using modified Mandel's medium with *T. inhamatum* KSJ1 [10]. Also, the optimal conditions were found in a 10 L jar fermenter scale-up by using the 500 mL flask conditions [2]. Therefore, in this study, the optimal aeration and agitation conditions were evaluated in a 35 L jar fermenter by using the optimized culture conditions found for the 10 L jar fermenter.

Trichoderma is an aerobic filamentous fungus, which absolutely needs oxygen as a substrate. Therefore, the k_La becomes a restricting element in liquid state fermentation by aerobic mycelium. Oxygen transfer is not easily built up, due to the morphology of mycelium and the addition of rice straw and paper wastes, and oxygen transfer to the culture liquid is also difficult as oxygen has low solubility. Increasing the agitation rate is generally used as a method to improve oxygen transfer; the oxygen transfer rate will be improved due to the formation of turbulent flow as a result of shear stress [11]. There are also other methods that will improve oxygen transfer, such as increasing the air flow rate or the partial pressure of oxygen [12]. When the air flow rate was increased, the enzyme productivity was found to be inefficient because more foam was produced. Also, since the partial pressure of oxygen was increased, the cost of enzyme production became higher.

In this study, the major scale-up factors of aeration and agitation rates were interpreted. The aeration rate was initially determined after the physical properties in the 35 L jar fermenter were investigated with the optimal agitation rate determined to improve the oxygen transfer rate under suitable k_La and OUR conditions. And then the final optimal agitation condition maintaining above critical dissolved oxygen concentration was determined.

MATERIALS AND METHODS

1. Microorganism

Trichoderma inhamatum KSJ1, isolated from rotten wood by Kim et al. [9] in a previous study, was used for cellulolytic enzyme

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production.

2. Culture Medium and Inoculation

Trichoderma inhamatum KSJ1 was pre-incubated in YMEB (yeast extract 4 g/L, malt extract 10 g/L, glucose 4 g/L) medium, at 30 °C and 120 rpm for 3 days. Mandel's medium was also used as a cellulolytic enzyme production medium [13], with the carbon sources, Avicel and CMC, were substituted by 1% rice straw and 1% paper wastes. The composition of the modified Mandel's medium was: rice straw 10 g/L, paper wastes 10 g/L, bacto peptone 1 g/L, urea 0.3 g/L, $CaCl_2$ 0.3 g/L, KH_2PO_4 2 g/L, $(NH_4)_2SO_4$ 1.4 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, and trace elements solution ($FeSO_4 \cdot 7H_2O$ 50 mg/L, $MnSO_4 \cdot H_2O$ 16 mg/L, $ZnSO_4 \cdot 7H_2O$ 14 mg/L and $CoCl_2$ 20 mg/L). 2 and 3% pre-incubated cultures were inoculated into 10 and 35 L jar fermenters, respectively.

3. Determination of Dry Cell Weight

The DCW (dry cell weight) was determined by measuring the protein concentration of sonicated mycelium obtained in the culture broth with a sonicator (VC70, Sonics & materials INC., U.S.A.). A calibration curve was made by using the following procedure. First, *T. inhamatum* KSJ1 was incubated in YMEB medium at 30 °C and 120 rpm for three days, centrifuged (8,000 rpm, 20 min, 4 °C) and washed twice with distilled water. The collected mycelium was divided into two equal parts. One was used to measure the dry weight after drying at 80 °C in a drying oven for 24 hours. The other was suspended in 40 mL of distilled water, and then homogenized for 15 min at 8,000 rpm with a homogenizer (SSC811EA, Masustita Electric Industrial Co., Ltd, Japan). Homogenized mycelium was diluted 1 : 1 with distilled water, sonicated for 5 min at 8 W, and then centrifuged at 12,000×g for 10 min. The supernatant was used to quantify the protein concentration by using the Lowery method [14]. Accordingly, the calibration curve was obtained in [DCW (g/L) =5.3286×absorbance].

4. Enzyme Assay

After centrifugation (12,000×g, 10 min), the supernatant was used to test the enzyme activity. The amylase activity was assayed by measuring the reducing sugars, as described by Thomas [15] and Ji et al. [16]. The FPase (Filter paperase) activity was assessed by its ability to hydrolyze 50 mg of filter paper (Whatman No. 1). The reducing sugar concentration was measured according to the DNS method [15].

5. Determination of k_La

5-1. Static Method

Measurement of the k_La was performed in modified Mandel's medium containing 1% rice straw and 1% paper wastes. A DO electrode was soaked in the culture medium, with the oxygen in the medium substituted by nitrogen purging [17]. After the DO concentration had been reduced to 0, air was flowed, at 0.6, 1.2 and 1.8 vvm, respectively, into the fermenter, with the k_La determined by measuring the rate of increase of the DO concentration over time. The rate of increase of the DO concentration is shown in Eqs. (1) and (2). Therefore, a graph of the logarithm value of (C^*-C_L) against time could be gained by a linear relation. The slope of the linear graph will be $-k_La$.

$$\frac{dC_L}{dt} = k_La(C^* - C_L) \quad (1)$$

$$\ln(C^* - C_L) = -k_La t \quad (2)$$

where, C_L: DO concentration of culture liquid (mg/L)
dC_L/dt: oxygen transfer rate (mg/L min)
k_L: oxygen transfer rate coefficient on the liquid film (cm/hr)
a: gas-liquid interfacial area per unit volume (cm^2/cm^3)
C*: DO concentration in the equilibrium with partial oxygen pressure of gas phage (mg/L)
k_La: volumetric oxygen transfer coefficient (hr^{-1})

5-2. Dynamic Method

The dynamic method proposed by Taguchi et al. [18] was followed in this study. This method measures the rate of change of the DO concentration using only living mycelium. The OUR measurement was carried out in modified Mandel's medium containing 1% rice straw and 1% paper wastes, with 3% of *T. inhamatum* KSJ1 inoculated into the culture medium. After 24 hours cultivation, the airflow to the fermenter was blocked, and the OUR (oxygen uptake rate) then determined by measuring the rate of change in the DO concentration over time (dC_L/dt). The airflow into the fermenter was rapidly blocked, and the DO concentration maintained at a constant value. Therefore, the DO concentration was linearly reduced due to mycelium respiration, the rate of which could be evaluated from the slope of the linear graph. The reduction in the DO concentration was calculated using the formulation expressed in Eq. (3).

$$\frac{dC_L}{dt} = -xQ_{O2} \quad (3)$$

where, C_L: DO concentration of culture liquid (mg/L)
Q_{O2}: specific respiration rate (DO-mg/g·cell·min)
x: dry cell mass (g/L)

In this situation, when aeration was resumed, the DO concentration could be increased to the initial equilibrium value. At this time, the increase of the DO concentration is the value of oxygen transfer rate into the cultivation medium and can be calculated by subtracting the value of the rate of DO uptake due to mycelium respiration, as shown in Eq. (4).

$$\frac{dC_L}{dt} = k_La(C^* - C_L) - xQ_{O2} \quad (4)$$

Here, xQ_{O2} can be indicated by the slope of the curve from the DO concentration change from the time of air cut-off to air resumption. Eq. (4) can be rearranged to give Eq. (5).

$$C_L = \frac{1}{k_La}\left(\frac{dC_L}{dt} + xQ_{O2}\right) + C^* \quad (5)$$

From Eq. (5), the graph of $dC_L/dt+xQ_{O2}$ against C_L gives a linear relation, with the slope of the equation equal to $1/k_La$. Therefore, the k_La value was determined.

RESULTS AND DISCUSSION

1. Determination of Aeration and Agitation Rates in 35 L Jar Fermenter

The major factors for scale-up are the agitation rate, linear velocity, OUR (oxygen uptake rate), k_La (oxygen transfer rate), configuration (size and diameter) of the fermenter, and the diameter and shape of the agitation blade. The cultivations were performed in a

10 L stirred tank bioreactor (Bio-G, Hanil Co., Korea) containing 5 L of culture medium. The design of the 10 L bioreactor allowed adjustment of the DO, pH, temperature and aeration rate. The optimal conditions in the 10 L jar fermenter were found from previous researchers [2], including 2% inoculation, a temperature of 30 °C, an agitation rate of 200 rpm and an aeration rate of 0.6 vvm. Therefore, based on these conditions, the 10 L jar fermenter was scaled up to 35 L.

The 35 L jar fermenter (total volume: 35 L, working volume: 18 L) was composed of a stainless cylindrical incubator, and the experiment was performed at 30 °C in a water bath. The 35 L jar fermenter was designed with a DO electrode (InPro 6820/12/230, Mettler Toledo, Switzerland), pH sensor and airflow injection port installed.

The cultivations were performed with 3% inoculation and an aeration rate of 0.5 vvm, with the agitation rate determined by using a geometrically resembling scale up method for the same power supply per unit liquor capacity. The agitation rate was operated between 150-200 rpm, because the agitation rate in filamentous mycelium cultivation should be kept low to reduce the effect of shear stress [19].

$$N_L = N_{sm}\left(\frac{V_{sm}}{V_L}\right)^{2/9} \quad (6)$$

Increasing the aeration rate leads to an increase of the airflow, resulting in a much greater amount of foam in the fermenter. Therefore, the aeration rate was maintained at 0.5 vvm during the 35 L fermentation. Increasing the vvm will cause foaming; therefore, the vvm was determined by applying the mean values of the superficial velocity (air flow rate divided by the internal cross section of the reactor) and vvm (volume of air added to liquid volume per minute).

The velocity in a 10 L jar fermenter is given by:

$$\frac{3000\ \text{cm}^3}{\text{min}}\left|\frac{4}{\gamma\times(19\ \text{cm})^2}\right. = 10.58\ \text{cm/min}\ \text{ so,}$$

According to a velocity of 10.58 cm/min, the scale-up value will be:

$$\frac{10.58\ \text{cm}}{\text{min}}\left|\frac{\gamma\times(30\ \text{cm})^2}{4}\right. = 7478.56\ \text{cm}^3/\text{min} = 7.5\ \text{L/min}$$

Also, the vvm in the 10 L jar fermenter was 0.6 vvm; hence:

$$18\ \text{L}\times 0.6\ \text{vvm} = 10.8\ \text{L/min}$$

Applying the mean value of the airflow for the superficial velocity and vvm, the aeration rate would be 9 L/min:

$$\frac{(10.8+7.5)\text{L/min}}{2} = 9.15\ \text{L/min}$$

$$\frac{9\ \text{L/min}}{18\ \text{L}} = 0.5\ \text{vvm}$$

Eventually, aeration and agitation rates of 0.5 vvm and 150 rpm, respectively, were chosen as provisional optimal conditions in the 35 L jar fermenter.

2. Enzyme Activities at the Conditions of 0.5 vvm and 150 rpm in a 35 L Jar Fermenter

The cultivation in the 35 L jar fermenter was performed at 30 °C, 0.5 vvm and 150 rpm, with 18 L of culture medium. The pH changes and the amylase and FPase activities were evaluated from day 0 to 5 at 12 hour intervals. The pH decreased rapidly from an initial value of 6.18 to 4.08 after 2 days incubation, and was maintained around 4 until the third day, after which it gradually increased to 5.26 between 3 and 5 days of cultivation (data not shown). The pH value is inclined to rapidly decrease in most cultures of filamentous fungi that use a carbon source as the substrate [20,21], and then increase again after consuming most of the carbon source. The amylase and FPase activities gradually increased from the initial to terminal points over the 5 days of incubation, progressively increasing to 3.96, 0.38 U/mL after 3.5 days of cultivation, and then increased slightly until day 5 (data not shown). From this result, it is suggested that the culture solution had become concentrated due to evaporation and outflow by foaming and sampling; therefore, the enzyme activities were slightly increased from 3.5 to 5 days. Therefore, it was concluded that 3.5 days was an appropriate incubation time. The pH value rapidly decreased from day 1 of cultivation; accordingly, the amylase and FPase activities also rapidly increased from day 1 of cultivation. Eventually, the entry point of the logarithmic growth phase was considered to be between 1-1.5 days; therefore, the OUR measurement was performed during 1-1.5 days of cultivation.

3. DO Monitoring in 35 L Jar Fermenter at 150 and 200 rpm

The DO concentration according to the incubation time was monitored in modified Mandel's medium containing 1% rice straw and 1% paper wastes as carbon sources (Fig. 1). 3% of pre-cultured *T. inhamatum* KSJ1 in YMEB medium was inoculated into Mandel's medium. The DO concentration rapidly decreased, with a steep slope from 0 to 10 hours of incubation at 0.5 vvm and 150 rpm, and changed irregularly from 13 to 16 hours. It is proposed as the growing mycelium became successively attached and detached from the DO electrode that this would cause a problem with the response time of the electrode. The DO concentration was observed to be maintained below 2.336 mg/L [22], which is the critical DO concentration value for the *Trichoderma* genus after 10 hours of cultivation, indicating that the mycelium had grown under oxygen-limited con-

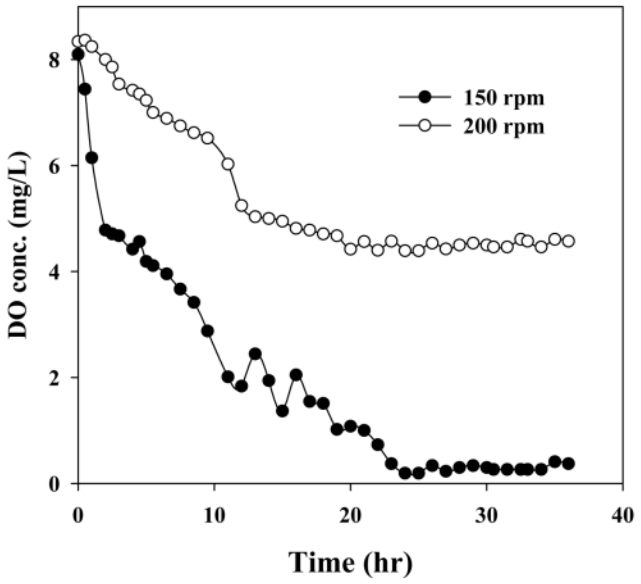

Fig. 1. Changes of DO concentration at 0.5 vvm in 35 L jar fermenter (150 rpm, 200 rpm).

ditions. Thus, the DO concentration should be maintained above the critical DO concentration to increase the oxygen transfer rate during the entire fermentation. The DO concentration was reduced to 0.32 mg/L after 24 hours of cultivation, at which time the mycelium approached the logarithmic growth phase; accordingly, the DO concentration was reduced by mycelium growth, and the rate controlling step of oxygen transfer was introduced into the culture medium. Then, to avoid this oxygen-limited phenomenon, an experiment to increase the oxygen concentration was carried out. The agitation rate was increased from 150 to 200 rpm, with the same aeration rate of 0.5 vvm; as a result, the fermentation proceeded with a DO concentration above 4.3 mg/L.

4. Effects of k_La According to Agitation Rate of 35 L Jar Fermenter

To find the change in the k_La according to the aeration rate in a 35 L jar fermenter, the k_La was measured, by using a static method, in distilled water and cellulolytic enzyme production medium containing 1% rice straw and 1% paper wastes. First, the oxygen in the cultivation medium was substituted by nitrogen purging until the DO was 0, and then a DO electrode was used at an agitation rate of 150 rpm, with aeration rates of 0.6, 1.2 and 1.8 vvm. The DO concentration was plotted against the cultivation time using Eq. (2). The k_La values were 27 and 29 hr^{-1} at 0.6 and 1.2 vvm, respectively, in distilled water. As these values were similar at 0.6 and 1.2 vvm, the k_La at 0.6 vvm was considered sufficient. Also, the turbulence with an air supply of 1.8 vvm was sufficiently well formed in distilled water, with a k_La value of 44.3 hr^{-1}, which was 1.5-fold the values obtained with 0.6 and 1.2 vvm (Table 1).

The k_La value was somewhat different in the cellulolytic enzyme production medium: modified Mandel's medium containing 1% rice straw and 1% paper wastes. The k_La at 0.6 vvm was low, 7.7 hr^{-1}, but was similar at 1.2 and 1.8 vvm, 20.9 and 22.8 hr^{-1}, respectively, which is threefold that at 0.6 vvm (Table 1). It was suggested that the cellulolytic enzyme production medium showed characteristics of a non-Newtonian fluid, whereas distilled water showed characteristics of a Newtonian fluid. To improve the oxygen transfer rate in the 35 L jar fermenter, both the aeration and agitation rates need to be increased. However, the aeration rate should be maintained in 0.5 vvm, as any increase will lead to the generation of more foam and result in culture loss. Therefore, it was considered the agitation rate should be increased from 150 to 200 rpm to improve the oxygen transfer rate.

5. Measurement of OUR and k_La Using Dynamic Method in 35 L Jar Fermenter

The k_La value was determined by using the Dynamic method between 24-36 hours of cultivation, which is the time *T. inhamatum* KSJ1 enters its logarithmic growth phase. The graph of DO concentration against cultivation time was plotted by using the Dynamic method during the OUR measurement, as shown in Fig. 2. During the cultivation process, the slope was slightly reduced (OUR could be calculated from the reduced slope of the straight line). When the DO concentration reached the critical concentration, aeration was resumed, and the DO concentration then increased and stabilized at a constant value. The DCW at this point was 3.94 and 2.00 g/L at 150 and 200 rpm, respectively. This difference was due to the different times the OUR measurement were taken.

Table 1. k_La values of DW and Mandel's medium according to aeration rate in 35 L jar fermenter

	k_La (hr^{-1})	
	Distilled water	Mandel's medium*
0.6 vvm	27.0	7.7
1.2 vvm	29.1	20.9
1.8 vvm	44.3	22.8

* Mandel's medium: including 1% rice straw and 1% pulp waste as carbon source

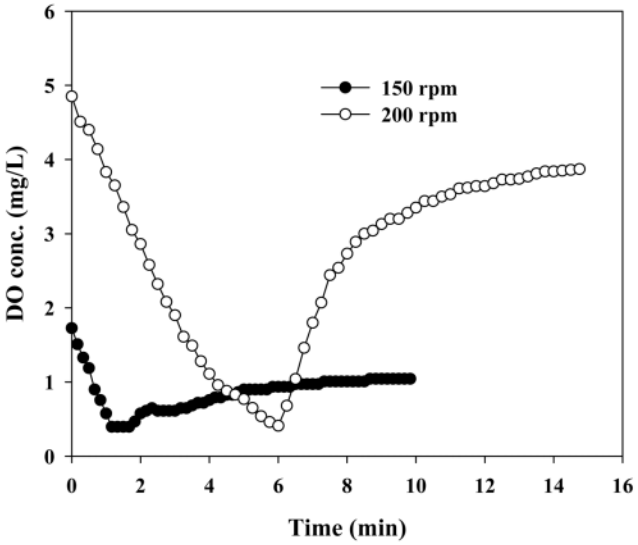

Fig. 2. Changes of the DO concentration for OUR determination at 0.5 vvm in the 35 L jar fermenter (150 rpm, 200 rpm).

The curve for the changes of the DO concentration over time was fitted to a quadric polynomial: $C_L=-2.668+0.823t-0.0247t^2$, which when differentiated gave; $dC_L/dt=0.823-0.04944t$. The linear relationship of C_L and ($dC_L/dt+xQ_{O2}$) in the 0-2.5 min time zone gave dC_L/dt plus OUR(xQ_{O2}), with a slope of $-1/k_La$, from which the k_La value was calculated, and found to be 11.1 hr^{-1}.

Using the same method, the OUR was calculated as 0.91 mg-DO/L·min at 200 rpm, with 0.46 mg-DO/g-cell·min of Q_{O2}, and the non-respiratory rate of the mycelium was obtained from the value of OUR divided by the DCW.

$$\frac{-dC_L}{dt} = OUR = -\frac{(4.14-0.96)\text{mgDO/L}}{(0.75-4.25)\text{min}} = 0.91\ \text{mgDO/L}\cdot\text{min}$$

$$OUR = q_{O_2}\cdot X$$

$$q_{O_2} = \frac{OUR}{X} = \frac{0.91\ \text{DOmg/L}\cdot\text{min}}{2\ \text{g cells/L}} = 0.46\ \text{mgDO/g cell}\cdot\text{min}$$

The graph of the DO concentration against cultivation time was plotted by the Dynamic method during the OUR measurement, as shown in Figs. 3 and 4. A k_La of 8.8 hr^{-1} was obtained from the slope of the graph for cultivation at 0.5 vvm and 200 rpm from the above method. It was observed that the time taken to attain a constant DO concentration on resuming aeration after the critical DO concentration had been reached for cultivation at 150 rpm was too long under this condition. Therefore, the oxygen transfer rate was improved by increasing the aeration rate from 150 to 200 rpm. As a result, a

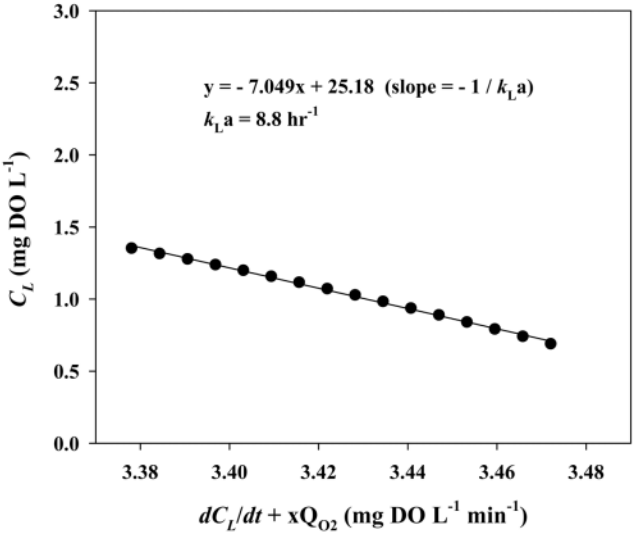

Fig. 3. Determination of k_La using the dynamic method (0.5 vvm, 150 rpm).

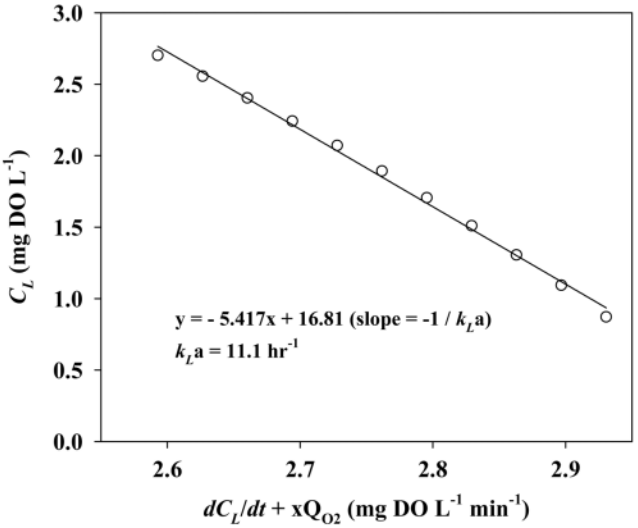

Fig. 4. Determination of k_La using the dynamic method (0.5 vvm, 200 rpm).

k_La value of 11.1 hr^{-1} was obtained for cultivation at 200 rpm, which was higher than that at 150 rpm (Fig. 4). By increasing the agitation rate to 200 rpm, the oxygen transfer rate was increased, with the cultivation performed above the critical DO concentration for *Trichoderma*.

6. Enzyme Activities at the Conditions of 0.5 vvm and 200 rpm in 35 L Jar Fermenter

The enzyme activities were measured after 3.5 days of cultivation in the 35 L jar fermenter under conditions of 0.5 vvm and 200 rpm. The amylase and FPase activities after 3.5 days of cultivation were 4.48 and 0.74 U/mL, respectively, under conditions of 0.5 vvm and 200 rpm in the 35 L jar fermenter, compared to 4.2 and 0.5 U/mL under conditions of 0.6 vvm and 200 rpm in the 10 L jar fermenter, as reported in our previous study (Fig. 5).

Both the amylase and FPase activities were high in the 35 L cultivation under conditions of 0.5 vvm and 200 rpm. The amylase activity was slightly increased, but the FPase activity was about 2-fold higher in the 35 compared to the 10 L cultivation. It is suggested that the increased oxygen mass transfer rate due to increasing the agitation rate from 150 to 200 rpm contributed to increasing the enzyme activities. From these results, it was concluded that the optimal conditions for enzyme production were 0.5 vvm and 200 rpm in the 35 L jar fermenter cultivation from the scale-up of the conditions used in the 10 L jar fermenter.

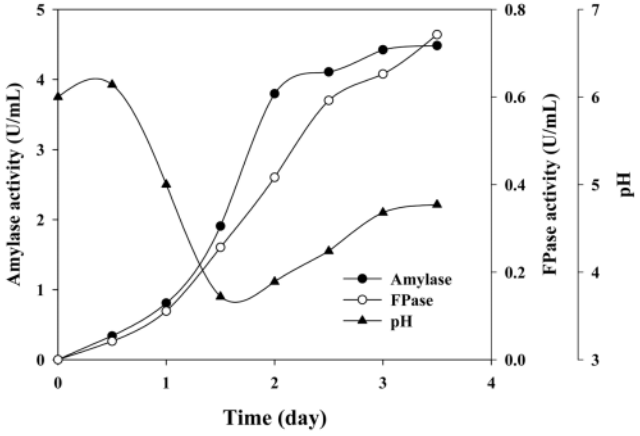

Fig. 5. Time course of pH and enzyme activities in the 35 L jar fermenter (0.5 vvm, 200 rpm).

CONCLUSIONS

To scale up the cellulolytic enzyme production from 10 to 35 L cultivation, the optimum agitation and aeration rates were determined to be 150 rpm and 0.5 vvm in the 35 L jar fermenter. At these conditions, the DO concentration of the culture solution was maintained below the critical DO concentration. Then, in order to maintain the DO concentration of the culture solution above the critical DO concentration, the aeration rate was kept in 0.5 vvm and the agitation rate was increased from 150 to 200 rpm. Consequently, the DO concentration of the culture solution was maintained above the critical DO concentration during the entire fermentation. And, amylase and FPase activities were slightly increased compared with 10 L fermentation. Therefore, the scale-up conditions of 0.5 vvm and 200 rpm were concluded to be the optimum aeration and agitation rates for the cellulolytic enzyme production by *Trichoderma inhamatum* KSJ1 in the 35 L jar fermenter.

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