Scaling-up of Simultaneous Saccharification and Fermentation of Lactic Acid from Microwave-alkali-treated Empty Fruit Bunches

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The goal of this work was to scale up the simultaneous saccharification and fermentation (SSF) of lactic acid using microwave-alkali-pretreated empty fruit bunches (EFB) from a scale of 16 L to a scale of 150 L. To facilitate the scaling-up process of lactic acid production by *Rhizopus oryzae* NRRL 395, a scaling-up criterion of constant k_La value was applied. Operating conditions, such as aeration rate and superficial velocity, were varied and evaluated on both scales (16-L and 150-L). The highest lactic acid yield of 6.8 g/L was obtained under an operating condition of 1 vvm (0.061 s^{-1}). Parallel aeration rates were determined for the 150-L fermenter system to obtain the same k_La value as the 16-L fermenter. An operational condition of 0.5 vvm dissolved oxygen supply in the 150-L fermenter was optimal to support an identical value of k_La and production rate of lactic acid for both scales.

Keywords: Empty fruit bunch; Lactic acid; Rhizopus oryzae; Scaling-up

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INTRODUCTION

In Malaysia, palm oil trees are cultivated over an area of 5 million hectares. It has been predicted that the amount of biomass waste from empty fruit branches (EFB) will reach 2.8 million tons in 2020 (Ng *et al.* 2012). This huge accumulation of waste leads to several problems, as the waste is generally burnt for fuels or left to rot, with the rotting process causing serious environmental pollution. Alternatively, EFB could be used as a renewable and cheap substrate for lactic acid production as a lignocellulosic carbohydrate source. Lactic acid production from microwave-alkali-pretreated EFB by *Rhizopus oryzae* has been reported (Hamzah and Idris 2008). However, the study was mainly performed on a laboratory scale involving shake flask fermentation. There is currently no published data for the large-scale production of lactic acid from solid substrate (EFB) using *Rhizopus oryzae via* simultaneous saccharification and fermentation (SSF). Therefore, the present study aimed to scale up the production of lactic acid *via* SSF, taking into consideration various issues of cellulosic lactic acid production, such as pretreatments and inoculum development.

A scale-up strategy was also developed to contribute to the advancement in knowledge and provide an engineering aspect guidance towards the commercialization process for industrial-scale cellulosic lactic acid production from EFB. Effective scale-up is essential for the successful production of lactic acid in industrial-scale fermenters. Once a particular bioprocess is successfully optimized in lab-scale experiments, the scaling-up

process is required to increase the production capacity while maintaining the production rate.

Different scale-up criteria could result in different process conditions on a production scale. It is well known that the influence of oxygen gas-liquid mass transport is the most significant factor for the scale-up of aerobic fermentations. Therefore, the scaling-up of aerobic fermentation is frequently performed on the basis of keeping the volumetric oxygen mass transfer coefficient (k_La) constant in variously sized fermenters (Garcia-Ochoa *et al.* 2000; Qu *et al.* 2013; Labbeiki *et al.* 2014). Despite the detailed knowledge associated with lactic acid fermentation, there is still no published information regarding the engineering aspects of scaling-up fermentation for lactic acid production using SSF. As a result of the altered catalytic properties often caused by different fermentation environments, filamentous fungal cells usually exhibit different physiology and morphology during the scaling-up process.

The successful scaling up of aerobic fermentation requires a rigorous understanding of the oxygen transfer rate to the liquid, which is characterized by the lumped mass transfer coefficient (k_La). In aerobic fermentation, sufficient oxygen must be supplied to fulfill the needs of the respiratory cell. The aeration efficiency is an important scale-up criterion that must be evaluated during the scaling-up process. In this study, the aeration efficiency of the small-scale fermentation (16 L) at its optimum condition for product formation was measured using recognized methods for oxygen transfer coefficient (k_La) determination.

EXPERIMENTAL

Materials

The overall methodology of the experimental work is summarized in Fig. 1. Empty fruit bunch fibers (EFB) were collected from FELDA Palm Industries (Sdn Bhd, Kilang Sawit Felda Semenchu, Johor). The *Rhizopus oryzae* NRRL 395, a lactic acid-producing strain, was a gift from the United States Department of Agriculture, Agriculture and Research Services Department culture collection (NRRL, National Center for Agricultural Utilization Research, Peoria, IL). The EFB was subjected to a microwave alkali pretreatment in a 30-L microwave reactor. The operating conditions of the microwave pretreatment have been described in a previous study (Hassan and Idris 2016).

Methods

Inoculum preparation

The production of lactic acid on a large scale is more complicated and demanding than laboratory-scale production. Industrial fermenters are usually large; thus, the inoculum is built up to fulfill the requirement in large quantities. Inoculum development, which involves various stages of build-up, has a large effect on the subsequent performance of the fermentation and process economics (Alam *et al.* 2011). For seed inoculum production in airlift fermentation, the spore suspension was inoculated into 50 mL of pre-culture medium contained in a 250-mL Erlenmeyer flask. Small, homogenous pellets of *Rhizopus oryzae* NRRL 395 germination from spores in a pre-cultivation stage, held on a rotary shaker at 150 rpm, 35 °C for 24 h, were used as inoculum. The inoculum build-up process for the fermentation of lactic acid is described in Fig. 2.

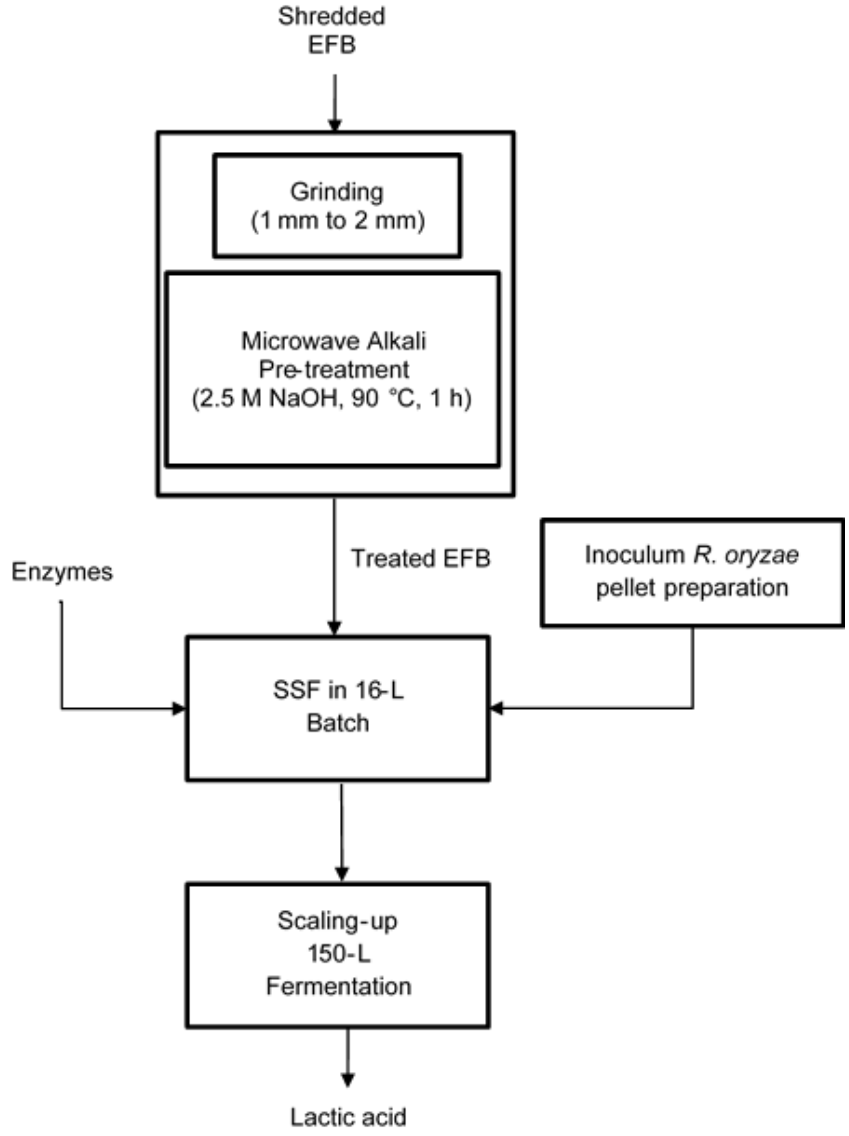

Fig. 1. The overall methodology of the experimental work

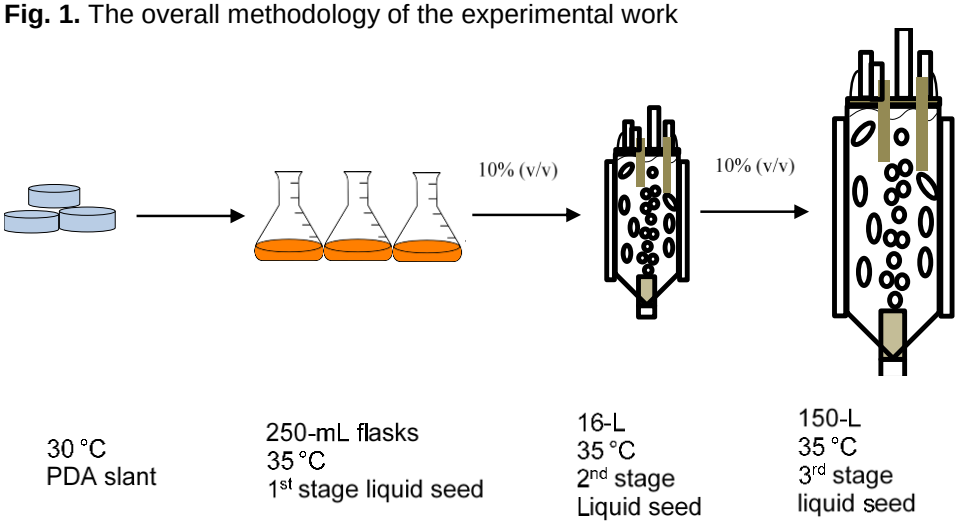

Fig. 2. Inoculation preparation procedure

In the process of preparing sufficient amounts of *R. oryzae* NRRL 395 pellets, inoculum from potato dextrose agar (PDA) slant was cultivated in 100 mL of pre-cultured liquid medium. The seed culture in these flasks was transferred directly to the 16-L bioreactor. The 16- and 150-L bioreactors that contained pre-culture medium were used as second- and third-stage liquid seed culture, respectively.

Specifications of 16- and 150-L fermenters

The fermentations for lactic acid production were performed in a 16-L fermenter (BioEngineering, Wald, Switzerland), and the scale-up fermentations were run in a 150-L pilot-scale fermenter (BioEngineering, Wald, Switzerland). The specifications for the fermenters are summarized in Table 1.

Table 1. Specifications of 16- and 150-L Fermenters

Dimension	16-L Bioreactor	150-L Bioreactor
Total Volume V_T (m^3)	0.0160	0.15
Working Volume V_L (m^3)	0.01	0.1
Vessel Height H_T (m)	0.507	1.143
Liquid Height H_L (m)	0.393	0.825
Vessel Diameter D_T (m)	0.2	0.41
Surface Area (m^2)	0.0005	0.132

Cultivation of Rhizopus oryzae NRRL 395 pellets in bioreactors

The fermenters were autoclaved twice at 121 °C for 60 min, with an overnight interval before operation. The bioreactor that contained the growth medium was prepared and autoclaved at 121 °C for 60 min. After cooling to room temperature, the bioreactor was connected to the controller system. Unless otherwise stated, the fermenter was operated at 35 °C, 1 vvm, and without pH control during the mycelial cell growth. The air was filtered and sterilized by passing through a filter (Pall llfrancombe, Fribourg, Switzerland). Then, 0.5 mL/L of antifoam was added to the medium to prevent foaming during fermentation, and 10% of the seed culture was pumped and cultivated in the bioreactor (Miura *et al*. 2003). The overnight culture, as seed culture, was used to initiate growth in the bioreactor and was cultivated for 48 h. The aeration was then stopped, and the batch culture containing the mycelial pellets was precipitated.

Simultaneous saccharification and fermentation (SSF) of EFB into lactic acid

Simultaneous saccharification and fermentation was performed in both bioreactors: 16-L (lab-scale) and 150-L (pilot-scale). The cultivation temperature inside the bioreactor was kept constant at 37 °C throughout the experiments, while the aeration rate was varied, as specified separately. The pH was controlled at 6.5 by adding sterile excess calcium carbonate ($CaCO_3$). The cultivation time of the experiments ranged from 24 h to 100 h.

Measurement of dry cell weight and lactic acid concentration

Pre-dried and pre-weighed filter paper (Whatman No. 1, Maidstone, England) was used to filter the culture before the biomass was weighed. The mycelia were collected and oven-dried until a constant weight was achieved. The dried sample was cooled in a dessicator before its weight was recorded.

The lactic acid concentration was determined using high-performance liquid chromatography (HPLC; Agilent 1220, Agilent Technologies, Palo Alto, CA, USA) and a Hi-plex H-column (Agilent, Santa Clara, CA, USA), as described previously (Hassan and Idris 2016). The analysis was conducted at 50 °C. A sulfuric acid solution (0.005 M) was used as the mobile phase, and the flow rate was set at 0.6 mL/min.

Dynamic technique in k_La measurement

The mass balance equations used to express the change in dissolved oxygen concentration in the liquid medium, C_L, with time for the dissolved oxygen in the batch fermentation are shown below in Eqs. 1 and 2,

$$\frac{dC_L}{dt} = k_L a\left(C^* - C_L\right) - r_{o_2} C_X \quad (1)$$

where $\frac{dC_L}{dt}$ is the oxygen transfer rate (mmoles O_2/L/h), $k_L a$ is the liquid-phase oxygen transfer coefficient (h^{-1}), C^* is the oxygen concentration in the equilibrium with the gas phase (g mol/m^3 s), C_L is the actual dissolved oxygen concentration in the broth (g mol/m^3 s), ro_2 is the specific respiration rate (mmoles O_2/g.h), and C_X is the dry weight of cells per volume (g /L). The first term on the right hand side of Eq. 1 is the oxygen transfer rate (OTR) (mmoles O_2/L/h), and the second term is the oxygen uptake rate of the culture (OUR) (mmoles O_2/L/h). The OUR and OTR were determined using the dynamic technique suggested by Bandyopadhyay *et al.* (1967) in two stages. In the first stage, the aeration was stopped to allow the dissolved oxygen concentration in the medium to decrease as the result of cellular respiration. The OUR was determined by the change in the dissolved oxygen concentration after stopping air flow. Under these conditions, Eq. 1 can be simplified to Eq. 2:

$$\frac{dC_L}{dt} = -r_{o_2} C_x \quad (2)$$

The dissolved oxygen (DO) concentration of the liquid medium was maintained at greater than 20% of the saturation value of 80% DO to avoid the microorganisms being damaged because of lack of oxygen. The OUR was obtained from the slope of the linear regression of the change in dissolved oxygen concentration, C_L, against time. The first stage was employed to obtain the value of the specific respiration rate by dividing the OUR by the biomass concentration. In the second stage, the aeration was resumed at predetermined values, and the dissolved oxygen concentration increased. To calculate the $k_L a$, Eq. 1 was rearranged to result in a linear relationship (Eq. 3):

$$C_L = C^* - \left(\frac{1}{k_L a}\right)\left(\frac{dC_L}{dt} + r_{o_2} C_x\right) \quad (3)$$

The $k_L a$ value was then determined by reciprocating the slope obtained from the semi-logarithmic plot of C_L *versus* $\left(\frac{dC_L}{dt} + ro_2 C_x\right)$. This technique was employed at various aeration rates.

RESULTS AND DISCUSSION

Effect of Superficial Velocity (V_s) on k_La

The influence of superficial velocity on the volumetric oxygen mass transfer coefficient (k_La) was examined by investigating the effects of aeration rate on the 16-L and 150-L fermentation systems. These studies were undertaken for an effective scale-up of the fermentation process, with the goal of successful production of lactic acid in a 150-L pilot-scale fermenter. The scaling-up of aerobic fermentation is usually performed on the basis of a constant oxygen mass transfer coefficient (k_La) to ensure an optimal supply of dissolved oxygen in the large fermenter system.

The dependence of k_La values on superficial velocity for the 16- and 150-L airlift bioreactors are illustrated in Fig. 3. The k_La measurement was performed by employing the dynamic gassing-out method. From the logarithmic plots, it can be seen that the trend of k_La dependency on superficial velocity was nearly identical for both scales, with similar slopes observed in the trend of both scales. A linear correlation between both bioreactors was expressed by the following empirical equation,

$$k_L a = 0.0747 V_s \quad (4)$$

where V_s is superficial air velocity based on the empty cross-sectional area of the vessel (cm/s).

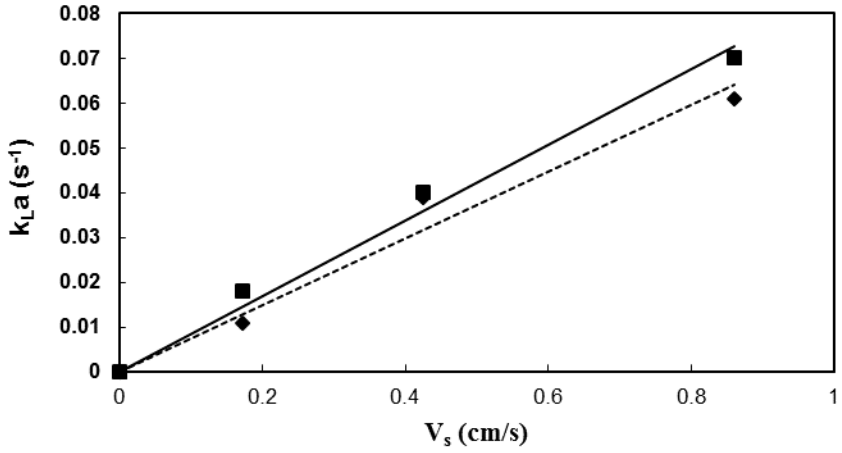

Fig. 3. A correlation between and in the 16-L and 150-L airlift bioreactors; (♦) 16-L bioreactor, (■) 150-L bioreactor

Based on the trend shown in Fig. 3, V_s had a proportional effect on the k_La value. As the V_s was increased from 0.1 cm/s to 0.8 cm/s, the k_La values also increased proportionally. The increase of superficial velocity in the riser resulted in an increase of gas build-up in the system, and thus, provided more available area for oxygen transfer. Moreover, an increase in the superficial gas velocity would promote an increase in the liquid velocity. This process reduces the mass transfer resistance by reducing the boundary layer thickness of the gas-liquid phase. According to Shukla *et al*. (2001), the aeration rate also displayed a noticeable effect on k_La values, as the shearing and dispersing of gas bubbles was found to promote an increase in k_La values and contact times. The results of

this study were in accordance with the results of Miura *et al.* (2004), Qu *et al.* (2013), and McClure *et al.* (2015).

Effect of $k_L a$ on Lactic Acid Fermentation on 16-L Scale

The effect of the oxygen supply condition, $k_L a$, on the cell morphology and lactic acid production by *Rhizopus oryzae* NRRL 395 was investigated by varying the $k_L a$ from 0.011 s^{-1} to 0.061 s^{-1} in the 16-L bioreactor. The results indicated that the morphology of *R. oryzae* and the lactic acid formation were governed by the $k_L a$ value, as shown in Table 2. At a $k_L a$ value of 0.011 s^{-1}, the production rate of lactic acid was low, and it was observed that cotton-like, loose mycelia formed in the fermentation medium. At values of 0.011 s^{-1} or lower, the oxygen transfer to the inner region of the pellet biomass was rather inadequate, and thus, limited the production of lactic acid. In addition, the cotton-like, loose mycelia morphology did not assist in the oxygen diffusion process. The maximum lactic acid concentration of 6.81 g/L was obtained at a $k_L a$ value of 0.061 s^{-1}. This result indicated that the oxygen supply at a $k_L a$ value of 0.061 s^{-1} was optimum for achieving the highest lactic acid yield and encouraging the formation of cell pellets.

Table 2. Effect of Aeration Rate on Lactic Acid Production and Morphology of *R. oryzae* NRRL 395 in 16-L Fermenter

Aeration Rate (vvm)	Superficial Velocity (cm/s)	$k_L a$ (s^{-1})	Lactic Acid Production (g/L)	Morphology of *R.oryzae* NRRL 395
0.2	0.172	0.011	3.31	Cotton-like Mycelia
0.5	0.425	0.039	4.64	Pellet
1	0.849	0.061	6.81	Pellet

It was important to maintain an appropriate $k_L a$ value to obtain a high lactic acid concentration and control cell morphology. Different fermentation processes have different $k_L a$ values, as the $k_L a$ value depends on several factors, such as the geometric and operating conditions of the bioreactor (agitation speed and airflow rate), media composition and properties, concentration, and the microorganism's morphology (Moutafchieva *et al.* 2013). There are limited studies available on detailed $k_L a$ values during the scaling-up process. The optimum $k_L a$ value in this study was lower than that found in the study conducted by Miura *et al.* (2003). In their study, lactic acid was produced by *Rhizopus* sp. using the hydrolysate of corncobs, which is a liquid substrate. Their optimum $k_L a$ value for lactic acid production was 0.83 s^{-1}.

The presence of EFB as a solid substrate may have decreased the superficial velocity of the slurry, resulting in an increase of coalescence of the bubbles and a change of the distribution range of the bubbles. According to Jin *et al.* (2004), the size of the bubbles in the medium depends primarily on such factors as the properties of the liquid phase and the gas velocity. Shin *et al.* (2013) used a scale-up criterion of constant oxygen transfer for the scale-up of itaconic acid production, and found that appropriate $k_L a$ values for the process were 0.03 s^{-1} to 0.05 s^{-1}. Their results are comparable to the $k_L a$ value found in the present study. In the SSF of lactic acid, the rate of oxygen transfer to microbial cells significantly affects the formation of product by influencing the metabolic pathways of the cell, as mentioned by Jin *et al.* (2004).

Low yield of lactic acid in SSF fermentation when using solid substrate is quite normal as reported by other researchers. This could be because the pretreated EFB has low cellulose accessibility or there are high inhibitors produced during pretreatment. It has been reported that the amount of lactic acid produced was 6 g/L when using chipped spruce (Stenberg *et al.* 2000), 13.8 g/L when using wheat straw (Garde *et al.* 2002), and 0.59 g/L when using waste office paper (Park *et al*. 2004) in SSF. In our previous study (Hassan and Idris 2016), the lactic acid yield was 6.8 g/L when substrate and enzyme were added once in the beginning of the fermentation process. However upon introducing the fed batch mode, the lactic acid yield increased to 12 g/L, which demonstrated that the large scale fermentation of lactic acid using solid lignocellulosic can be sustained.

Scale-up of SSF of Lactic Acid Based on the Oxygen Mass Transfer Coefficient ($k_L a$)

During the process of scaling-up lactic acid fermentation from 16-L to 150-L, the strategy was to provide an almost equivalent k_La value to each fermentation system, thus ensuring the same volumetric transfer rate (OTR) into the respective fermenter. A k_La value of 0.061 s^{-1} resulted in the greatest lactic acid production in the 16-L fermenter. Several sets of 150-L fermentation experiments at various k_La values were conducted. Superficial air velocity was manipulated to obtain the desired value of k_La. The 150-L fermentation was performed at various V_s values (0.2, 0.5, and 0.9 cm/s), which were equivalent to the calculated k_La values of 0.011, 0.039, and 0.061 s^{-1}, respectively. The experimental conditions in the 150-L culture, such as temperature, pH, inoculum size, and substrate loading, were similar to those in the 16-L culture. Figure 4 shows the lactic acid fermentation profiles on the 150-L scale at different k_La values.

A decrease in oxygen concentration was observed in the medium, particularly in the experiments with lower values of k_La (0.011 s^{-1} and 0.039 s^{-1}). At a k_La value of 0.011 s^{-1}, a depletion of dissolved oxygen in the medium was observed during the first 20 h of fermentation, and the DO level remained at 0% throughout the process, which explains the lower lactic acid concentrations obtained under these conditions.

In the experiments with an initial k_La value of 0.039 s^{-1}, the dissolved oxygen concentration dropped to 40% in the first 20 h, and stabilized at approximately 10% throughout the process. For a k_La value of 0.061 s^{-1}, the oxygen concentration in the medium did not drop to 0%, but stabilized at approximately 30% throughout the process. The results indicated that conditions of 0.5 vvm and a V_s of 0.9 cm/s in the 150-L system were optimal for providing a comparable volumetric amount of dissolved oxygen as the 16-L fermentation. An aeration rate of 0.5 vvm in the 150-L scale was sufficient to provide oxygen for the cells' requirement and maintain a constant oxygen level during fermentation.

Fermentation Kinetics of 16-L and 150-L Fermenters with Same $k_L a$ Value

Time-course profiles on cell growth, lactic acid production, sugar consumption, DO, and pH in the 16- and 150-L systems were compared to observe any substantial differences that occurred while scaling-up at a constant k_La value. The fermentation profiles reflecting cell growth, glucose utilization, and DO concentration obtained from the 16-L and 150-L bioreactors operated under an environment of nearly equivalent k_La are illustrated in Fig. 5.

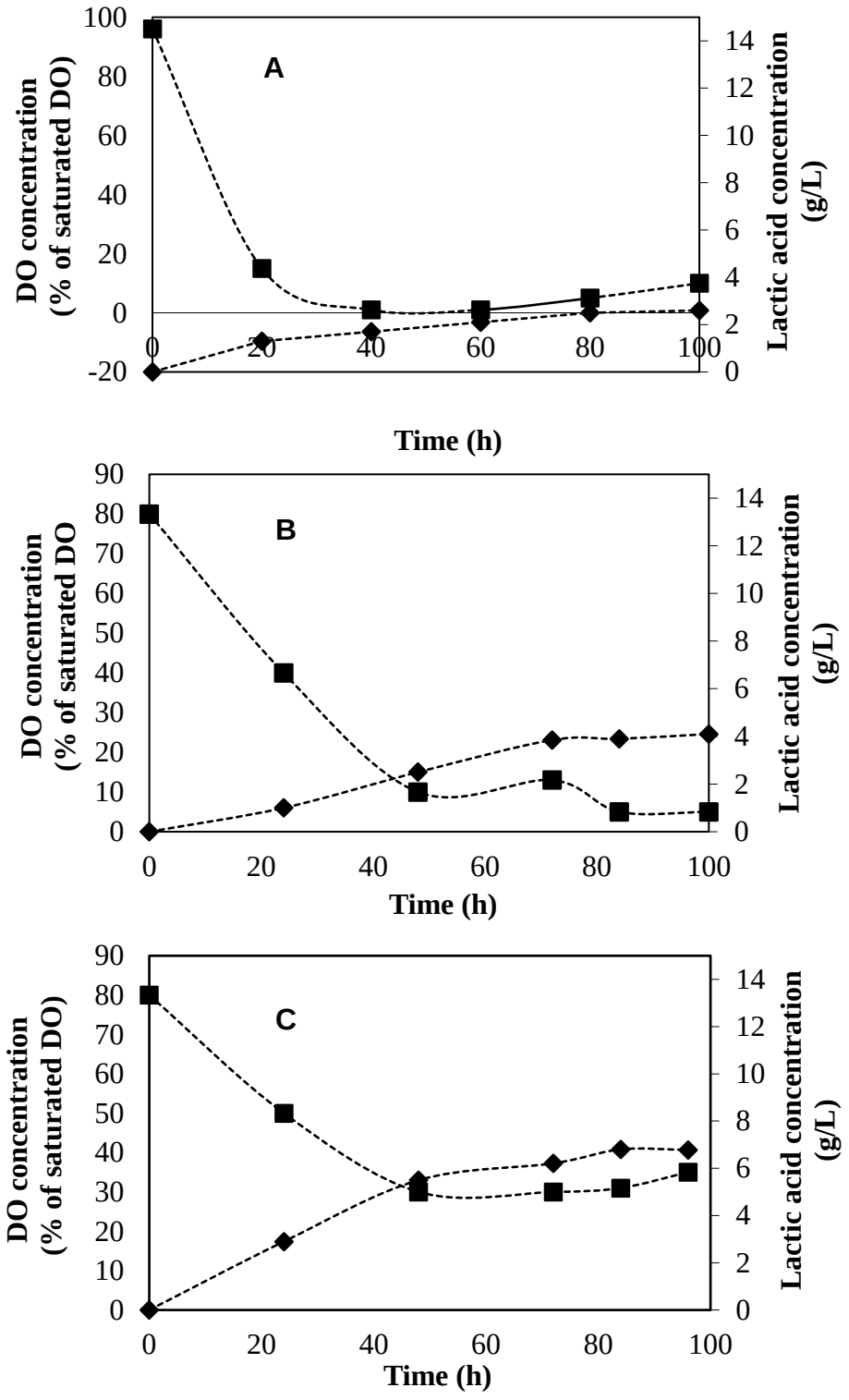

Fig. 4. Lactic acid fermentation profiles in 150-L scale at different V_s values: (A) V_s = 0.2 cm/s, $k_L a$ = 0.011, (B) V_s = 0.5 cm/s, $k_L a$ = 0.039 and (C) V_s = 0.9 cm/s, $k_L a$ = 0.061. (♦) Lactic acid concentration, (■) DO concentration

Under these respective conditions (1.0 vvm for the 16-L system and 0.5 vvm for the 150-L system), comparable time-course profiles of cell growth, lactic acid production, sugar consumption, and DO were obtained. As depicted in Fig. 5, the amount of lactic acid produced in the 16-L and 150-L bioreactors were very similar, with maximum values of 6.8 g/L and 6.78 g/L, respectively.

In the 16-L scale bioreactor, lactic acid formation began during the first 24 h of fermentation and continued to increase for the next 24 h and 48 h. During the same period, the glucose concentration decreased, as the cells simultaneously consumed glucose and produced lactic acid effectively after 24 h. The same response was observed when the production capacity was sized up to 150 L. Similar kinetics of lactic acid yield were obtained in both the 16-L and 150-L bioreactors, as reflected by the similar k_La value upon scale-up.

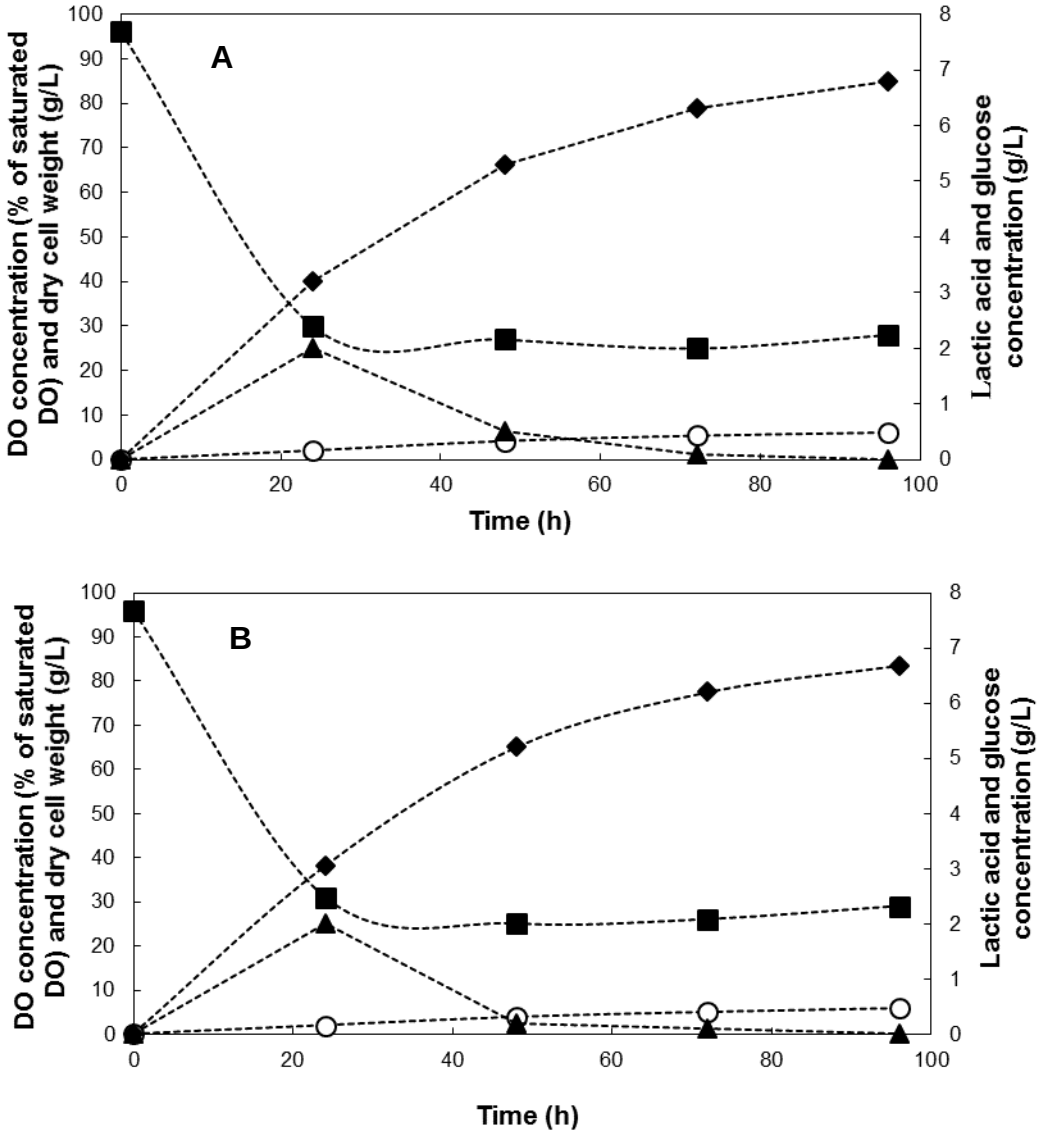

Fig. 5. Time-course profiles of cell growth, sugar consumption, DO concentration, and lactic acid production at the same k_La value, (A) in the 16-L fermenter and (B) in the 150-L pilot-scale fermenter; (♦) Lactic acid, (■) DO, (▲) Glucose, (○) Dry cell weight

The DO level on both scales (16-L and 150-L) remained greater than 20% of the saturated DO concentration throughout the entire fermentation period. According to Miura *et al*. (2003), in lactic acid fermentation, it is important to keep the DO level above its critical level, which is around 15% of the saturated DO. These DO environments are necessary, because the oxygen uptake rate is largely influenced by the DO concentration of the fermentation broth, especially when it is below a critical DO level, leading to reduced production of secondary metabolites. *Rhizopus oryzae* NRRL 395 exhibited an identical growth profile on both scales. The maximum cell density achieved on the 16-L and 150-L scales were 6.09 g/L and 5.96 g/L, respectively.

CONCLUSIONS

1. The multiple stages of inoculum proposed in this study noticeably reduced the time constraint in the inoculum preparation by shortening the lag phase. This technique also eliminated the requirement of a large quantity of spore suspension, and improved fermentation consistency.
2. Maintaining the k_La value at a constant level in both bioreactors (16-L and 150-L) resulted in an almost identical production rate of lactic acid.
3. Similar trends of fermentation physiology were observed, as expressed in terms of lactic acid production, maximum cell growth which reflect the successful scale-up of the lactic acid fermentation process.
4. In summary, all of these comparative results imply that the application of the criterion of constant oxygen mass transfer coefficient (k_La) was successful for the scale-up of the lactic acid fermentation process (the scale-up ratio of 1:10).

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