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Simultaneous Saccharification and Fermentation of Lactic Acid from Empty Fruit Bunch at High Solids Loading

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The production of value-added chemicals from the bioconversion of lignocellulose biomass has been considered a promising venture. In this study, microwave, alkali-pretreated empty fruit bunch (EFB) was used as the substrate, utilizing pelletized filamentous *Rhizopus oryzae* NRRL 395 and cellulolytic enzymes for lactic acid production in a fed-batch simultaneous saccharification and fermentation (SSF) process. Insoluble solids generally do not affect the SSF process until a certain concentration is exceeded. To achieve a high lactic acid concentration in the broth, a high solids loading was required to allow a higher rate of glucose conversion. However, the results revealed a decrease in the final lactic acid yield when running SSF at a massive insoluble solids level. High osmotic pressure in the medium led to poor cellular performance and caused the *Rhizopus oryzae* pellets to break down, affecting the lactic acid production. To improve the process performance, a fed-batch operation mode was used. The fed-batch operation was shown to facilitate higher lactic acid yield, compared with the SSF batch mode. Enzyme feeding, as well as substrate feeding, was also investigated as a means of enabling a higher dry matter content, with a high glucose conversion in SSF of cellulose-rich EFB.

Keywords: Empty fruit bunch; Lactic acid; Fed-batch fermentation; Rhizopus oryzae

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INTRODUCTION

In Asia and the Pacific, the consumer demand for non-biodegradable plastics has increased sharply because of urbanization and economic growth. This trend means that more resources are required to meet the demand and that a high quantity of plastic waste is being generated, posing a serious threat to the environment and human health. Waste plastics do not biodegrade for hundreds of years. People around the world have begun to realize the magnitude of this problem, and countries are taking steps to reduce the use of non-biodegradable plastics. The negative impact of waste plastics could be addressed or minimized by finding an alternative plastic compound that is truly biodegradable and compostable. After years of research into principle compostable, bio-based plastics, polylactic acid (PLA) appears to be one of the superior choices. Moreover, this bio-plastic has the desired properties of strength, flexibility, and full degradability. Polylactic acid's physical properties and the degradation rate can be altered by manipulation of the manufacturing process, making it much more versatile.

In the past, the use of PLA as a biodegradable plastic lacked popularity because it was too expensive to produce in large-scale applications. The problem has been that the main component, lactic acid, has been too expensive. Today, most commercial lactic acid is produced from the fermentation of starch, glucose, and sucrose (Anuradha *et al*. 1999; Abdullatif and Shangtian 2002; Wakai *et al*. 2014). The fermentation of these sugars into lactic acid is well-established in terms of both the microbiology and the chemical processes.

To reduce the feedstock cost, considerable study has also focused on the fermentation of agro-industrial wastes, such as lignocellulosic carbohydrates, for lactic acid production (Park *et al*. 1998; Moldes *et al*. 2000; Miura *et al*. 2004; Pessionne *et al*. 2014). Lignocellulose biomass is viewed as a sustainable lactic acid feedstock. The beauty of this process lies in the opportunity given to the agricultural industry to solve both the economic and environmental problems of waste by turning it into a highly desirable product. The production of lactic acid using the simultaneous saccharification and fermentation (SSF) process is performed using bacterial or fungal cultures. There are several major reasons for using fungi instead of bacteria; among these, fungi are normally used when the substrate is a raw material or a waste material (Soccol *et al*. 1994; Miura *et al*. 2004; Park *et al*. 2004). Under such circumstances, there is no requirement for specific nutrients, and most fungi can tolerate very acidic conditions. Other advantages include the simple and cheap downstream processing, whereby the filamentous or pelleted biomass can easily be removed from the fermentation broth.

Despite many studies conducted on the bioconversion of lignocellulose biomass using solid substrates, most previous studies have focused on the use of bacteria, *e.g*., *Lactobacillus* spp. (Sreenath *et al*. 2001; Garde *et al*. 2002; Hu *et al*. 2015). Recently, a fungus producing lactic acid, *Rhizopus* spp., was used in the fermentation process and was shown to produce lactic acid from lignocellulose biomass. However, in most of the studies that produced lactic acid *via* SSF using *Rhizopus* spp., the process involved lignocellulose hydrolysate (liquid) as a substrate, which was accomplished in two stages (Miura *et al*. 2004; Hamzah and Idris 2008; Zhang *et al*. 2015). The metabolism of *Rhizopus* spp. for lactic acid production using a solid substrate has not been widely investigated. A detailed study on the mechanism and metabolism of *Rhizopus* spp. may contribute to the knowledge of the biochemistry and improvement of the product synthesis. Thus, in this study, an attempt was made to use *Rhizopus* spp. pellets and lignocellulose from empty fruit bunch (EFB) as a solid substrate to produce lactic acid using SSF in a 16-L bioreactor. This study will lead to the advancement of knowledge in the production of lactic acid from EFB fiber and the use of *Rhizopus* spp. pellets in the SSF process.

In our previous work, an attempt was made to produce lactic acid from EFB in a laboratory-scale experiment (Hamzah and Idris 2008), using *Rhizopus* spp. pellets and cellulolytic enzymes. Unlike other studies (Miura *et al*. 2004; Ye e*t al*. 2014), which used lignocellulose hydrolysate (liquid) as the substrate, Hamzah and Idris (2008) used EFB, as solid substrate which was pretreated using a microwave-alkali method in the one stage SSF and the lactic acid yield was 11%. Thus, in the present work, a realistic commercial application was simulated by regulating the substrate concentration in a 16-L bioreactor, using a fed-batch mode. This stepwise procedure was aimed at achieving high SSF yields at lower enzyme loadings, together with high lactic acid concentration. The effect of adding fresh enzyme during the process was also assessed.

EXPERIMENTAL

Pretreatment of Empty Fruit Bunch

Empty fruit bunches were collected from Seri Ulu Langat Palm Oil Mill (Selangor, Malaysia) in a dry shredded-fiber form, as shown in Fig. 1. They were then ground to an average size of 2 to 3 mm. The biomass was then subjected to pretreatment to provide more available celluloses for enzymatic hydrolysis. The pretreatment was performed by immersing 2 kg of EFB in 20 L sodium hydroxide (2.5 M) at room temperature for 2 h. The slurry was then exposed to microwave irradiation for 1 h at 100 °C in a 30 L microwave reactor (Syno 30-L, Synotherm, China). After the reaction was completed, the outlet valve was opened and the slurry was transferred to the washing tank and washed with water so as to neutralize the pH. The fiber was then dried, and it was stored for subsequent experiments. The EFB chemical composition, after the pretreatment process, was analyzed to evaluate the effectiveness of the pretreatment.

Fig. 1. Shredded palm oil empty fruit bunch

Enzymatic Hydrolysis

A hydrolysis mixture consisted of 1 g treated EFB, cellulose, and 20 mL of 50 mM of citrate buffer (pH 5.0). The mixture was incubated at 50 °C in a waterbath shaker (Wisebath, Germany) at 150 rpm. 1 mL of sample was taken from reaction mixture at different time interval. The supernatant was used to determine the glucose concentration.

Microorganisms

Rhizopus oryzae NRRL 395, an L(+)-lactic acid producing strain, was obtained from the United States Department of Agriculture, Agriculture and Research Services Department culture collection (NRRL; National Center for Agricultural Utilization Research, Peoria, IL). Cultures of fungi were grown in agar slant tubes with potato dextrose (PDA) solution. The composition of the pre-cultured medium was as follows (g/L): xylose, 50; urea, 2; KH_2PO_4, 0.6; $MgSO_4$, 0.25; and $ZnSO_4$, 0.88. The medium was sterilized at 121 °C for 20 min. The *R. oryzae* spores were grown at 30 °C for seven days in the PDA agar slant tubes. Then, the fungi were harvested using a platinum loop and suspended in sterilized distilled water. Next, a small amount of the spore suspension was inoculated in a flask, with a final concentration of 10^6 spores/mL. Immediately thereafter,

the flask containing the spore suspension was incubated at 30 °C for 20 h in a rotary shaker, to allow the pellet cells to grow and form. The overnight culture was referred to as the seed culture and was subsequently used to initiate cell growth in the bioreactor. The SSF of microwave, alkali-pretreated EFB was conducted in a 16-L bioreactor, containing a 10-L final volume of the production media inoculated with 10% of the seed culture and cultivated for 48 h. Unless otherwise stated, the aeration rate and incubation temperature were set at 1.0 vvm and 35 °C, respectively. After 48 h in the batch culture, the aeration was stopped and the mycelial pellets were harvested.

SSF Experiments

Batch

Batch fermentation was carried out in a 16-L bioreactor (BioEngineering, Wald, Switzerland), with a total volume of 10 L of medium broth. The cultivation temperature inside the bioreactor was maintained at 35 °C throughout the experiments. Likewise, the aeration rate was set at 1.0 vvm, and the pH was controlled at 6.5 by adding sterile $CaCO_3$. The cultivation time of the experiments ranged from 96 to 120 h. In this work, a traditional method was applied to optimize the process performance by utilizing the "one-factor-at-a-time" rule. This technique involved varying one operation variable at a time while keeping all of the other operating conditions constant. In this manner, the effect of substrate loading and enzyme dosage were studied. Samples were taken periodically and analyzed using high-performance liquid chromatography.

Fed-batch

The fermenter (containing nutrient and solid substrates) was autoclaved at 121 °C for 20 min prior to SSF. Then, SSF was initiated by adding the pelleted *R. oryzae* (Fig. 2) and cellulase.
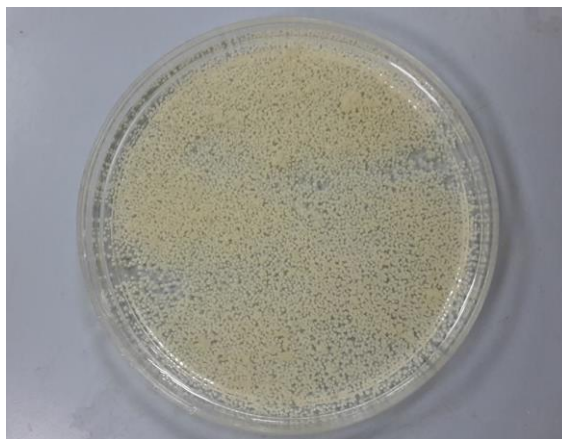

Fig. 2. Photograph of uniform, small pellets of *R.oryzae* formed during the SSF fermentation of lactic acid

Throughout the fermentation process, the pH of the substrate was maintained at 6. Fresh substrate (the same amount of EFB fiber that was used initially) was added during fermentation. In the same way, an amount of enzyme (the same amount of enzyme that was used initially) was also fed into the bioreactor during the SSF reaction in selected experiments. The experiment was divided into three strategies, *i.e.*, A, B, and C.

i) For strategy A, the SSF operation was initiated with 15 g/L of EFB fiber and a cellulase dosage of 10 FPU/g of biomass (substrate and enzyme were added initially). After 24 h, an additional 15 g/L of solids, with no additional enzyme, was aseptically added.

ii) In strategy B, the SSF operation was initiated with 15 g/L of EFB fiber and a cellulase dosage of 10 FPU/g of biomass. Additional fresh substrate was added twice after 24 h (15 g/L) and 48 h (15 g/L).

iii) In strategy C, the SSF operation was initiated with 15 g/L of EFB fiber and a cellulase dosage of 10 FPU/g of biomass. A combination of fresh substrate (15 g/L of EFB) and enzyme (cellulase at 10 FPU/g of biomass) was added at 24 h and 48 h of fermentation.

Analytical Procedure

The samples were analyzed for sugars and lactic acid production using high-performance liquid chromatography (HPLC; Agilent 1220, Agilent Technologies, Palo Alto, CA, USA) with a Hi-plex H-column. 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.

RESULTS AND DISCUSSION

Chemical Composition of EFB Substrates

Table 1 depicts the chemical composition of raw EFB and EFB after Microwave (Mw)-Alkali pretreatment. The chemical pretreatment resulted in an increase in cellulose content and a reduction in lignin, hemicellulose, and extractives. The results are in line with those reported in previous studies (Lai and Idris 2013; Akhtar e*t al*. 2014).

Table. 1. Chemical Composition of Palm Oil EFB before and after Pretreatment

Component	% Dry basis	
	Raw EFB	Mw-alkali pretreated EFB
Cellulose	41.68 ± 1.12	69.86 ± 0.14
Hemicellulose	28.45 ± 1.23	9.01 ± 0.21
Lignin	19.85 ± 0.07	17.60 ± 0.02
Extractives	10.02 ± 0.04	3.53 ± 0.3

Enzymatic Hydrolysis of EFB

Figure 3 shows the time course of enzymatic hydrolysis of untreated and microwave-alkali pretreated EFB using the same cellulase concentration. From the results, Mw-alkali treated EFB gave higher hydrolysis rate and glucose production as compared to the untreated EFB. After 100 h hydrolysis, 0.35 g glucose per g EFB was formed from Mw-alkali pretreated EFB, whereas with untreated EFB only 0.24 g glucose per g EFB was produced. The result indicated that the Mw-alkali treated method was capable of improving the morphology of the EFB and make cellulose more accessible to the enzymatic attack.
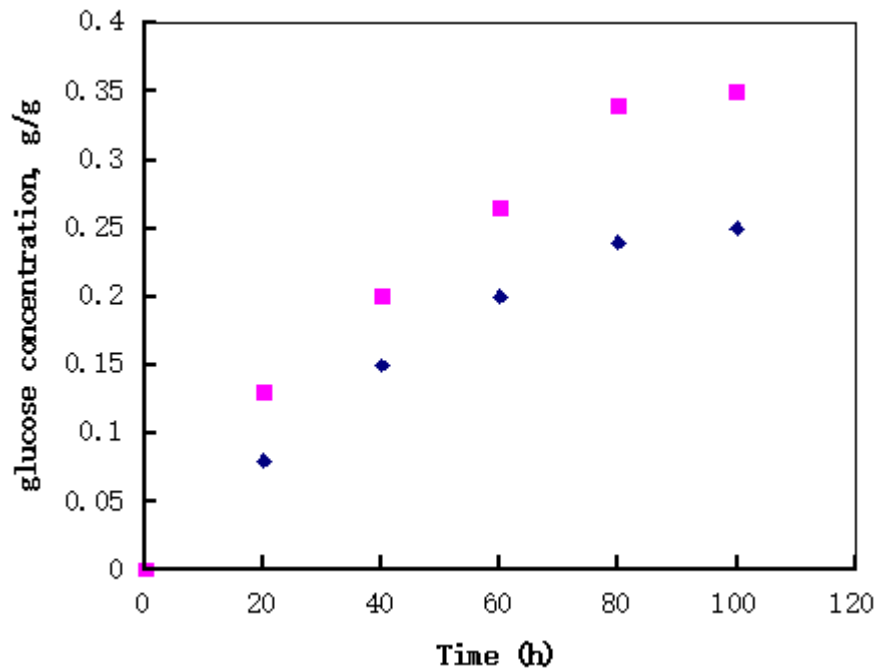
Fig. 3. Glucose accumulation in enzymatic hydrolysis of EFB:(♦) raw EFB, (■) Mw-alkali treated EFB

Effect of Enzyme Dosage on Lactic Acid Production

Taking into consideration the cost of the cellulosic lactic acid process, many strategies have been developed to improve the process from an economic standpoint. Pretreatment of the biomass and usage of cellulase enzymes becomes important for improving the process economically. For that reason, the usage of enzyme should be kept minimal at the optimum pretreatment condition, so as to provide easy digestion of the carbohydrate polymer. The effect of cellulase loading on SSF of EFB is presented in Fig. 4.
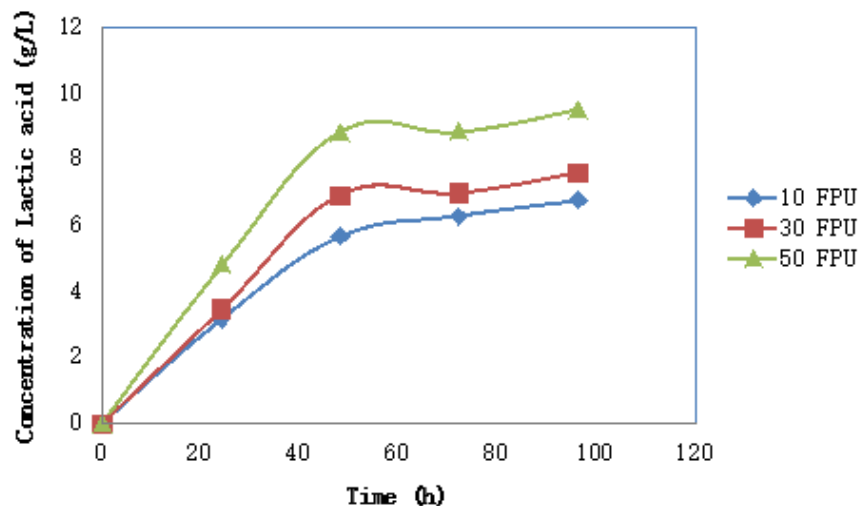

Fig. 4. Effect of enzyme loading on lactic acid yield as a function of time during batch SSF at 35 °C, with a 1.5% substrate concentration: (♦) 10 FPU, (■) 30 FPU, and (▲) 50 FPU

Three different enzyme dosages, 10, 30, and 50 FPU/g of biomass, were used for this experiment. It was observed from the graph that the lactic acid production was quite low when 10 FPU enzymes were added, resulting in only 6.8 g/L of lactic acid. When the enzyme dosage was increased by a factor of 3, the lactic acid yield was slightly higher, at around 13% with 7.63 g/L of lactic acid. Increasing the enzyme load to 50 FPU led to a maximal lactic acid production of 9.8 g/L. According to Fig. 4, at lower enzyme loading (10 FPU/g of biomass), the reaction time appeared to be prolonged; however, at higher enzyme loading, the fermentation seemed to finish after 72 h. Thus, the amount of cellulase used in the SSF of microwave, alkali-pretreated EFB influenced the final lactic acid concentration during the SSF processes. This finding was consistent with the results reported by Rudolf *et al*. (2005).

It was concluded that higher enzyme loading into the medium will increase the hydrolysis rate because of an increase in the active sites available for cellulose molecules, resulting in more lactic acid production. The trend in Fig. 4 shows that maximal lactic acid yield could be higher than what was demonstrated in this experiment. From these observations, no saturation of cellulase loading was found in the investigated region (10 to 50 FPU/g of biomass). However, the addition of large amounts of enzyme is an uneconomical approach, as the enzymes are expensive and unable to be recycled. Overall, the process demonstrated an economic improvement when the cellulase usage was reduced to an acceptable lactic acid production.

Effect of Substrate Concentration on Lactic Acid Production

The experimental relationship between the lactic acid production and the concentration of solid loading is shown in Fig. 5. Figure 5 shows that the lactic acid concentration increased when the amount of solids loading increased. When the EFB fiber loading into bioreactor was increased from 10 to 15 g/L, the lactic acid concentration increased and achieved a maximum lactic acid yield of 7 g/L. However, this represented a non-linear relationship, because further increases in solid loading resulted in a decrease in the lactic acid concentration. This was attributed to the high viscosity at higher solid loadings.
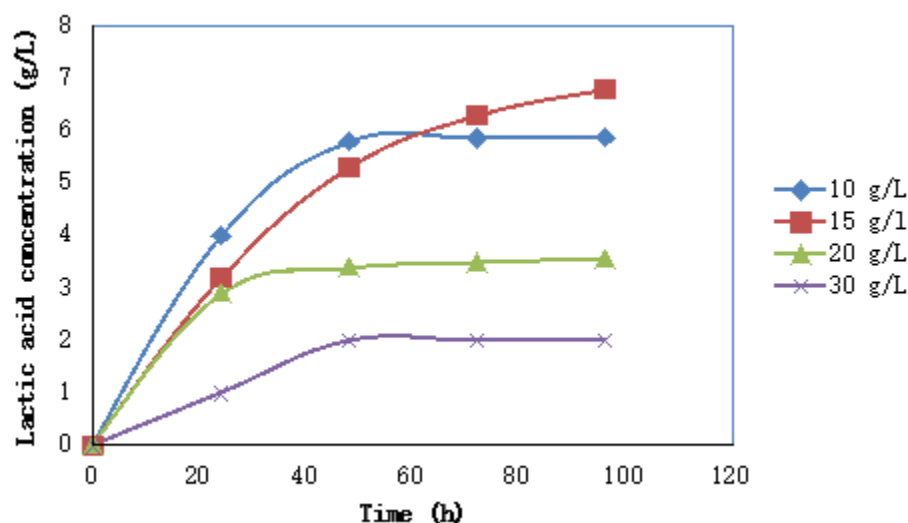
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Fig. 5. Effect of substrate loading on the lactic acid yield as a function of time during batch SSF at 35 °C: (♦) 10 g/, (■) 15 g/L, (▲) 20 g/L, and (x) 30 g/L

However, further increases in EFB loading beyond 15 g/L decreased the final lactic acid concentration. At 20 and 30 g/L EFB fiber loading, the final lactic acid amounts obtained were only 3.5 and 1.8 g/L, respectively. The increment of solid loadings led to an increase in fermentation broth viscosity, which hindered proper mixing. The high viscosity medium in the bioreactor created an obstacle for the mixing process, poor accessibility of enzymes to substrates, and high inhibitors concentration that reduces the cell viability and metabolism (Koppram and Olsson 2012). The results obtained were consistent with the previous literature, where high substrate concentration led to a poor saccharification rate, which resulted in a low lactic acid yield (Stenberg *et al*. 2000; Varga *et al*. 2004). Furthermore, 20 g/L of solid loading changed the morphology of *R. oryzae* from a distinct pellet to a cotton-like mycelia form, which subsequently lowered the production yield of lactic acid. A further increment of solid loading (30 g/L) deteriorated the fermentation conditions, leading to poor cellular performance and cell damage.

SSF with Fed-Batch Feeding

Many SSF studies revealed that a lower yield of the final product was detected when the batch mode of high solids loading SSF was performed. In the batch operation mode, all of the substrates were being added in the initial process. In this study, gradual solids loading, also known as the fed-batch approach was used. It was noted that a high solids loading rate in the fed-batch mode led to poor mixing and poor cellular performance. In our preliminary study of SSF, the mixing problem, at high solids content, was tackled using a stirred tank bioreactor. However, poor cellular performance and cell viability problems still existed in the stirred tank filled with EFB fiber and *R. oryzae* pellets.

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Cells were damaged, and no pellets were visible during the course of the fermentation, as well as the fermentation products (data not shown). Shear stress arising from mechanical agitation of the stirred reactor damaged the cell pellets. Therefore, a reactor without mechanical agitation (airlift bioreactor) was employed in this study. To solve the associated problems at high solids loading, a dual feeding system, *i.e.*, a combination of both substrate feeding and enzyme, were applied to provide good mixing and maintain cell viability.

Three different feeding strategies, labeled A, B, and C, were applied and evaluated in this experiment. As illustrated in Fig. 6, the subsequent addition of fresh EFB fiber into the bioreactor after 24 h of fermentation (strategy A), led to a final lactic acid concentration of 7.64 g/L, corresponding to 0.51 g of lactic acid per g of biomass, which was higher than that obtained in the one-time addition of high solids loading (7.0 g/L). In strategy A, the addition of solid substrate was done in time intervals; therefore, the solids content was gradually degraded. This stepwise feeding strategy allows the slurry inside of the bioreactor to have ample time to be digested; thus, the viscosity remains relatively unchanged and more solids could be introduced into the bioreactor.

Strategy B shows some remarkable findings. There was an increase in the final lactic acid concentration when a second 15 g/L of EFB was added intermittently after 48 h of fermentation, without adding the enzyme. It took 120 h to reach 9.0 g/L of lactic acid concentration. The reaction was slower compared to strategy A.

Strategy C illustrated the best strategy because a sharp increase in lactic acid production was observed. This strategy encouraged enzymatic hydrolysis and an optimum lactic acid yield. The addition of both fresh substrate and enzyme at 24 and 48 h of fermentation resulted in an increase in the final lactic acid concentration to 12 g/L.
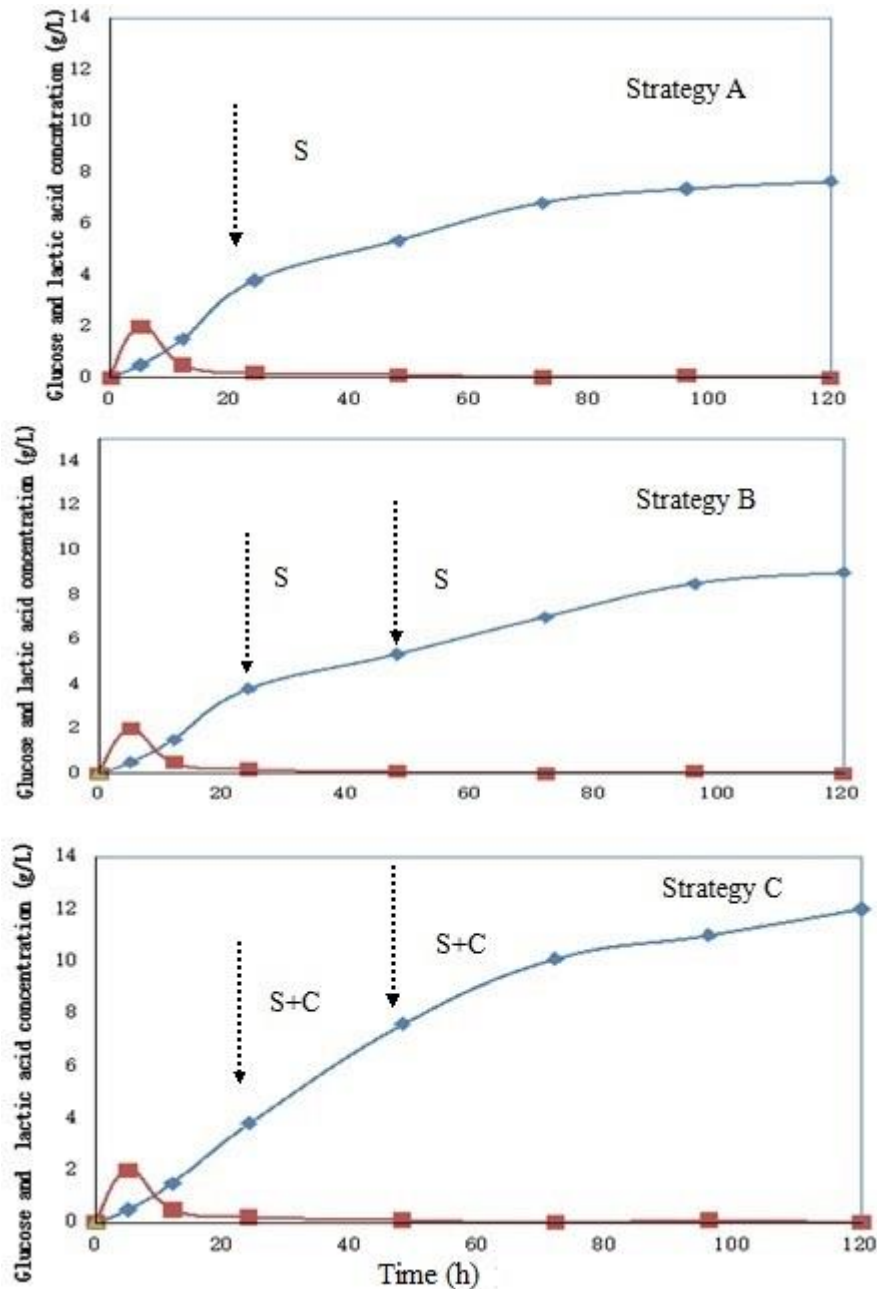
Fig. 6. Profiles of glucose and lactic acid in an operated, fed-batch SSF mode: (♦) lactic acid, (■) glucose. Fermentation conditions: 37 °C, pH: 6.5, initial loading: 15 g/L of EFB, and 10 FPU/g of biomass enzyme. Strategy A: one subsequent substrate feed; Strategy B: two subsequent substrate feeds (feed 1 and feed 2); Strategy C: two subsequent substrate and enzyme feeds (feed 1 and feed 2)

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As shown in Fig. 5, at 24 and 48 h of fermentation, the lactic acid concentration increased considerably because of the addition of fresh cellulose and EFB fiber. On the other hand, the concentration of glucose was tremendously depleted and remained at zero. This was attributed to simultaneous utilization of glucose in the fermentation process. The addition of fresh enzyme three times periodically favored pellet cell viability and increased the cell performance, as the fermentation broth became less viscous and the insoluble solids content was reduced. The solid residues, produced during strategy C (Fig. 7), consisted of unconverted substrate and microbial biomass, proving that the pellet cells were unchanged in morphology during the fermentation process.

Fig. 7. Solid residues of unconverted substrate and pellet, *R. oryzae* (NRRL 395)

The findings of these three different case studies suggested that strategy C resulted in the highest lactic acid concentration, compared to strategies A and B. The addition of fresh enzyme and substrate into the fed-batch mode exhibited a positive effect on the lactic acid concentration.

From the results, the SSF of microwave alkali-pretreated EFB, with high solids loading, appears to pose a challenge primarily to the fermentation of the microorganism. However, the feeding strategy of substrate and enzyme in a fed-batch mode has the potential to further improve the yield of lactic acid. In this study, strategy C offered the best solution, where the substrate and enzyme were fed intermittently every 24 h intervals. The fed-batch addition of solid substrate feeding reduces high gravity of solid substrate loading. Thus, the problem with cell viability in high solids loading can be eliminated.

CONCLUSIONS

1. The batch fermentation process yielded a maximum lactic acid production of 7.63 g/L from low solids loading (15 g/L) at an enzyme dosage of 10 FPU/g of biomass. Increasing the solids content to 50 g/L did not increase the lactic acid yield. However, increasing the enzyme concentration to 50 FPU/g of biomass increased the lactic acid yield to 9.8 g/L. Nonetheless, low solids loading and high enzyme concentrations would not be economically favorable because of the high capital cost of equipment and operation costs to reach a certain lactic acid production capacity. Thus, high solids loading is the best strategy and very practical economically, compared with low solids loading.

2. To introduce high solids loading, a fed-batch mode was introduced using three different feed strategies. The results revealed that strategy C (substrates and enzymes added intermittently every 24 h) provided the highest lactic acid yield of 12 g/L. This feeding strategy enabled the addition of a higher solids loading, because the insoluble solids have ample time to be digested by *R. oryzae*, thus, maintaining the viscosity of the fermentation broth. This work demonstrated that the large-scale fermentation of lactic acid using solid lignocellulosic substrates by *R. oryzae* is viable and can be sustained using a fed-batch mode.

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REFERENCES CITED

Abdullatif, T., and Shangtian, Y. (2002). "Production of L(+)-lactic acid from glucose and starch by immobilized cells of *Rhizopus oryzae* in a rotating fibrous bed bioreactor," *Biotechnol. Bioeng.* 80(1), 1-12. DOI: 10.1002/bit.10340

Akhtar, J., Teo, C. L., Lai, L. W., Hassan, N., Idris, A., and Aziz, R. A. (2014). "Factors affecting delignification of oil palm empty fruit bunch by microwave-assisted dilute acid/alkali pretreatment," *BioResources* 10(1), 588-596.

Anuradha, R., Suresh, A. K., and Venkatesh, K. V. (1999). "Simultaneous saccharification and fermentation of starch to lactic acid," *Process Biochem.* 35(3-4), 367-375. DOI: 10.1016/S0032-9592(99)00080-1

Garde, A., Jonsson, G., Schmidt, A. S., and Ahring, B. K. (2002). "Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentosus* and *Lactobacillus brevis*," *Bioresour. Technol.* 18(3), 217-223. DOI: 10.1016/S0960-8524(01)00135-3

Hamzah, F., and Idris, A. (2008). "Enzymatic hydrolysis of treated palm oil empty fruit bunches fibre (EFB) using combination alkali-microwave techniques," *J. Biotechnol.* 136(Supp 1), S426-S427. DOI:10.1016/j.jbiotec.2008.07.938

Hu, J., Zhang, Z., Lin, Y., Zhao, S., Mei, Y., Liang, Y., and Peng, N. (2015). "High-titer lactic acid production from NaOH-pretreated corn stover by *Bacillus*

coagulans LA204 using fed-batch simultaneous saccharification and fermentation under non-sterile condition," *Bioresour. Technol.* 182, 251-257. DOI: 10.1016/j.biortech.2015.02.008

Koppram, R., and Olsson, L. (2012). "Challenges and possibilities of second generation bioethanol production process at high substrate," *Science and Technology Day 2012,* March 27, 2012, Göteborg, Sweden.

Lai, L. W., and Idris, A. (2013). "Disruption of oil palm trunks and fronds by microwave-alkali pretreatment," *BioResources* 8(2), 2792-2804.

Miura, S., Arimura, T., Itoda, N., Dwiarti, L., Feng, J. B., Bin, C. H., and Okabe, M. (2004). "Production of L-lactic acid from corncob," *J. Biosci. Bioeng.* 97(3), 153-157. DOI: 10.1016/S1389-1723(04)70184-X

Moldes, A. B., Alonso, J. L., and Parajo, J. C. (2000). "Multi-step feeding systems for lactic acid production by simultaneous saccharification and fermentation of processed wood," *Bioproc. Eng.* 22(2), 175-180. DOI: 10.1007/s004490050028

Park, E. Y., Kosakai, Y., and Okabe, M. (1998). "Efficient production of L(+)- lactic acid using mycelial cotton-like flocs of *Rhizopus oryzae* in an air-lift bioreactor," *Biotechnol. Progress* 14(5), 699-704. DOI: 10.1021/bp9800642

Park, E. Y., Anh, P. N., and Okuda, N. (2004). "Bioconversion of waste office paper to L (+)-lactic acid by the filamentous fungus *Rhizopus oryzae*," *Bioresour. Technol.* 93(1), 77-83. DOI: 10.1016/j.biortech.2003.08.017

Pessione, A., Zapponi, M., Mandili, G., Fattori, P., Mangiapane, E., Mazzoli, R., and Pessione, E. (2014). "Enantioselective lactic acid production by an *Enterococcus faecium* strain showing potential in agro-industrial waste bioconversion: physiological and proteomic studies," *J. Biotechnol.* 173, 41-40. DOI: 10.1016/j.jbiotec.2014.01.014

Rudolf, A., Alkasrawi, M., Zacchi, G., and Lidén, G. (2005). "A comparison of batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce," *J. Enzyme and Microbial Tech.* 37(2), 195-204. DOI: 10.1016/j.enzmictec.2005.02.013

Soccol, C. R., Marin, B., Raimbault, M., and Lebeault, J. M. (1994). "Potential of solid state fermentation for production of L(+)-lactic acid by *Rhizopus oryzae*," *Appl. Microbiol. Biotechnol.* 41(3), 286-290. DOI: 10.1007/BF00221220

Sreenath, H. K., Moldes, A. B., Koegel, R. G., and Straub, R. J. (2001). "Lactic acid production by simultaneous saccharification and fermentation of alfalfa fiber," *J. Biosci. Bioeng.* 92(6), 518-523. DOI: 10.1016/S1389-1723(01)80309-1

Stenberg, K., Bollók, M., Réczey, K., Galbe, M., Zacchi, G. (2000). "Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production," *Biotechnol. Bioeng.* 68(2), 201-210. DOI: 10.1002/(SICI)1097-0290(20000420)68:2<204::AID-BIT9>3.0.CO;2-4

Varga, E., Klinke, H. B., Reczey, K., and Thomsen, A. B. (2004). "High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol," *Biotech. Bioeng.* 88(5), 567-574. DOI: 10.1002/bit.20222

Wakai, S., Yoshie, T., Nakashima, N. A., Yamada, R., Ogino, C., Tsutsumi, H., Hata, Y., and Kondo, A. (2014). "L-lactic acid production from starch by simultaneous saccharification and fermentation in a genetically engineered *Aspergillus oryzae* pure culture," *Bioresour. Technol*. 173, 376-383. DOI: 10.1016/j.biortech.2014.09.094

Ye, L., Hudairi, M. S., Li, Z., and Wu, J. C. (2014). "Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for L-lactic acid by *Bacillus coagulans* JI 12," *Biochem. Eng. J*. 83, 16-21. DOI: 10.1016/j.bej.2013.12.005

Zhang, Li., Li, X., Yong, Q., Yang, S. T., Ouyanga, J., and Yua, S. Y. (2015). "Simultaneous saccharification and fermentation of *xylo*-oligosaccharides manufacturing waste residue for l-lactic acid production by *Rhizopus oryzae*," *Biochem. Eng. J.* 94, 92-99. DOI: 10.1016/j.bej.2014.11.020