Journal of Applied Sciences

ISSN 1812-5654

ANSInet
an open access publisher
http://ansinet.com

Journal of Applied Sciences 9 (17): 3086-3091, 2009
ISSN 1812-5654

Lactic Acid Production from Microwave-Alkali Pre-Treated Empty Fruit Bunches Fibre using *Rhizopus oryzae* Pellet

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Abstract: Microwave-alkali (MW-A) pre-treated EFB consisting of 74% cellulose, 16% hemicellulose and 8% lignin was subjected to Simultaneous Saccharification and Fermentation (SSF) of lactic acid using *Rhizopus oryzae* NRRL 395. Two different morphologies of *Rhizopus* were studied for SSF of lactic acids; i.e., the clump and pellet form. Aspect Ratio (AR) was introduced to classify the pellet morphology, since cultivation of Rhizopus sp. produced non-uniform size pellets. Pellet with AR = 1 indicated a perfect pellet, while AR = 1.5 represented an ellipsoidal pellet. The AR of *Rhizopus* pellet used in the SSF was controlled to 1. The results showed that lactic acid production from MW-A pre-treated EFB using pellet exhibits higher lactate yield as compared to the clump *Rhizopus*. The lactate yield was 0.77 g g^{-1} EFB used after 96 h cultivation. Meanwhile, productivity of the lactic acid obtained from SSF of MW-A pre-treated EFB using pellet (AR = 1), clump (AR = 1.5) and pellet (AR = 1.5) *Rhizopus oryzae* NRRL 395 were 0.12, 0.089, 0.099 g L^{-1}, respectively.

Key words: Microwave, EFB, Rhizopus, pellet, simultaneous sacharification and fermentation, cellulose

INTRODUCTION

Empty Fruit Bunches (EFB) fibre, one of the lignocellulosic material consist primary of cellulose and hemicellulose, is an alternative to the traditional feedstock for lactic acid production. Theoretically, utilization of the lignocellulosic material as a feedstock in the organic acid production can decrease production cost, since it is inexpensive and widely available renewable carbon source that has no competing food value. The choice of the feedstock depends on its price, availability and on the respective product recovery and purification costs (Datta *et al*., 1995). According to Tejayadi and Cheryan (1995), the cost for raw material possessed 68% of the total cost for lactic acid production. Previous study on the simulation of lactic acid fermentation process by Akerberg and Zacchi (2000) reported that the operational cost including raw material, neutralizing agent, hydrolyzing enzyme and membrane for electrodialysis contributed to approximately 80% of the total cost for lactic acid production. Since, raw material cost cannot be reduced by scaling up process, EFB has been considered as attractive substrate for lactic acid production.

The main pathway to derive fermentable sugar from EFB is through enzymatic hydrolysis by cellulolytic and hemicellulolytic enzyme. A mechanical and chemical pre-treatment of the lignocellulose is required to reduce particle size, to modify and or to remove lignin and with that to enhance the accessibility of the polysaccharide for enzymatic hydrolysis (Akerberg and Zacchi, 2000; Mosier *et al*., 2005). Various pre-treatment methods of lignocellulose have been studied for conversion of lignocellulose to organic acid. One of the promising technologies is microwave alkaline (MW-A) pre-treatment. Through this pre-treatment technique, enzymatic digestibility of the fibre is increased due to the disruption of the recalcitrant structure of the fibre (Hu and Wen, 2008). Therefore, the cellulose molecule is more exposed to the enzymatic attack and thus encouraged the conversion to soluble sugar that can be used in the fermentation of lactic acid.

Lactic acid has gained prominence in research and industry because of its potential for biodegradable and biocompatible lactic acid polymers. Polylactate polymers such as Poly Lactic Acid (PLA) could be an environmental friendly alternative to plastics derived from petrochemical materials. Due to the unique properties of PLA, lactic acid has the potential to be a substitute for biodegradable plastics and becomes a very large volume commodity chemical intermediate (Huang *et al*., 2005). On the other hand, antitumor and antimicrobial effects of PLA have also been reported.

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Lactic acid occurs naturally in two isomers, L-lactic acid and D-lactic acid. However, elevated levels of the D-isomer are harmful to human. Thus, L-lactic acid is the preferred isomer in food and pharmaceutical industries. The most commonly used lactic acid is synthesised from chemical route which involved the hydrolysis of lactonitrile. Unfortunately, lactic acid produced is in the racemic form whose resolution is difficult. Thus, the direct microbial synthesis of the pure isomer is preferable.

One of the potential microbe for this purpose is filamentous fungi *Rhizopus oryzae* because of its outstanding ability to directly produce almost optically pure L(+)-lactic acid with low nutrient requirement and high yield (Yin *et al.*,1997; Rosenberg and Kristofikova, 1995). *Rhizopus oryzae* can be grown in submerged cultures in several different morphological forms such as; pellet, suspended mycelium or clump. Morphological differences may have a significant influence on the formation of metabolic product (Couri *et al.*, 2003). If the fungal cells grow as mycelia or large pellets or clumps, large scale production of lactic acid could be difficult because the growing mycelia will result in highly viscous broth and thus limit the oxygen transfer inside the cell (Zhou *et al.*, 1999).

Thus, an attempt was made to investigate the productivity and yield of lactic acid from MW-A pre-treated EFB in SSF using two different morphologies of *Rhizopus* sp. Performance of clumps and pellets *Rhizopus* on lactic acid was compared. Since, the size of the pellet produced is not uniform for all of the samples, thus in the present study the Aspect Ratio (AR) is introduced to identify the perfect pellet produced during cultivation.

MATERIALS AND METHODS

Substrate preparation: EFB obtained from Seri Ulu Langat's palm oil milling, Dengkil, Selangor, Malaysia was dried and grinded into 1 mm particle size using IKAE grinder (German). The composition of hemicellulose and lignin was determined by triplicates using Acid Detergent Fibre (ADF), Acid Detergent Lignin (ADL) and Neutral Detergent Fibre (NDF). Meanwhile, the composition of cellulose was calculated from the deduction of ADF with ADL value.

Microwave pre-treatment: Pre-treatment of EFB particles was conducted in a microwave oven model (NN-5626F) with the following specification: rated power output (240v-50 Hz) operation. In the pre-treatment process, 2.5 M of sodium hydroxide solution was used and the process was performed in a round bottom glass vessel set up complete with a stirring (Ani and Iqbal, 2008). During the pre-treatment, the agitator was set at 150 rpm. Prior to microwave treatment, the EFB were pre-soaked with sodium hydroxide solution at room temperature for 2 h. The pre-soaked slurry was then transferred to the round bottom flask reactor vessel and treated in the microwave oven. The power setting was set to medium. The EFB were exposed to irradiation for 1 h reaction time. When the microwave irradiation pre-treatment was completed, the reaction vessel was removed from the microwave oven and cooled to the room temperature. The slurry in the reaction vessel was then filtered through a Whatman filter paper No. 1. The filter cake was washed with deionized water for a few times to neutralize the pH to 7.0, dried and stored for the enzymatic hydrolysis.

Pellet preparation: *Rhizopus oryzae* NRRL 395, a (L)-lactic acid producing strain was a gift from USDA (Northern Regional Research Centre, United State Department of Agriculture, Peoria, Illinois). The strain was maintained on Potato Dextrose Agar (PDA) for fermentation study, agar slant containing fungus was washed by sterile water to obtain spore suspension. Spore suspension of 1×10^2 spores mL^{-1} was used for production of the pellet *Rhizopus.* The pre-culture composition of the pellet formation medium was as follows (g L^{-1}): glucose, 50; KH_2PO_4, 0.2, $ZnSO_4.7H_2O$, 0.04; $MgSO_4.7H_2$, O 0.25 and $(NH_4)_2SO_4$, 2.0. The pH of the medium was adjusted to 6.0 before steam sterilization. The cultivation temperature was set to 37°C with agitation of 170 rpm. This cultivation process is continued for 3 days so as to allow the formation of pellet/clump *Rhizopus*.

Simultaneous saccharification and fermentation of lactic acid from MW-A pre-treated EFB: For production of lactic acid, 15 g L^{-1} of MW-A pre-treated fibre was used in 250 mL Erlenmeyer flasks containing 50 mL of fresh medium. The medium (g L^{-1}) used in SSF process consisted of KH_2PO_4, 0.2, $ZnSO_4.7H_2O$, 0.04; $MgSO_4.7H_2O$, 0.25 and $(NH_4)_2SO_4$, 1.35. The pH of the medium was adjusted to pH 6.0. All flasks were incubated in the orbital shaker with agitation of 170 rpm. The culture temperature was maintained at 37°C throughout the experiments. The reduction in pH of the medium was controlled by addition of 5% $CaCO_3$. Culture was carried out for 4 days and the sampling was done every 24 h. Each experiment was repeated in triplicate and presented as an average.

Analysis: At specific intervals of time, samples were taken and then heated in boiling water to denature the enzyme. The heated samples were centrifuged at 5000 rpm for 5 min and supernatants were filtered through 0.45 μm syringe filter. Finally, the glucose and lactate concentration were measured by glucose analyzer (YSL instrument). Lactate yield was defined as amount of lactate produced divided by total amount of pre-treated EFB used and productivity as lactate concentration divided by fermentation time.

Aspect ratio determination: The morphology of the cultures was determined by examining submerged cultures dispersed on Petri dishes. A microphotographer (Olympus) was used to observe the pellet morphology and measured the size of the pellets. Prior to processing of images, care was taken to assure that all pellets were detected as single entities. The maximal diameter (D_{max}), minimal diameter (D_{min}) and average diameter of the pellets was determined. The values obtained were used to calculate the Aspect Ratio (AR) of the pellet using the following equation.

$$AR = D_{max}/D_{min}$$

The dry biomass was determined by neutralizing the excess $CaCO_3$ using 6 N HCL, washed using distilled water and dried at 80°C for 24 h before weight analysis.

RESULTS AND DISCUSSION

EFB composition after MW-A pre-treatment: The composition of the raw and MW-A pre-treated EFB were shown in Table 1. The results revealed that the EFB fibre which was subjected to MW-A pre-treatment showed a reduction in the composition of the hemicellulose and lignin; however the percentage of cellulose obtained was increased to 74.3%. The percentage of the hemicellulose was reduced from 35% in raw EFB to 16.8%, while, the lignin decreased from 16.4 to 7.6% when subjected to MW-A pre-treatment. The increment in cellulose percentage obtained is probably because of the microwave heating technique which contributed to the effective removal of lignin and hemicellulose.

In microwave, heating results from the interaction of the electromagnetic wave with the irradiated medium. Principle of microwave heating is based on molecular friction or dielectric loss. The material molecules are stimulated and rotated millions of times a second in response to electromagnetic field and these rotations quickly generate heat in the material in a manner similar to friction. Such rapid intense heating caused the rupture of the lignin and hemicellulose structure. It is a mass heating where heat transfer occurs from the treated medium to the outside.

Table 1: Compositions of raw and EFB pre-treated with MW-A

	Compositions (%)			
	Extractives	Hemicellulose	Cellulose	Lignin
Raw EFB	4.80±1.03	35.00±0.59	43.80±0.02	16.4±0.23
Microwave (MW-A)	1.35±1.59	16.77±0.95	74.33±0.12	7.56±0.64

During microwave pre-treatment, the heat produced selectively heats the more polar (lossy) part and creates hot spots within the inhomogeneous EFB. In addition, the presence of agitator in microwave cavity distributed a uniform heating within the reaction vessel. It is hypothesized that this unique heating feature results in an explosion effect among the particles and improves the disruption of the recalcitrant structures of lignocellulose. Hydrogen bonds between hemicellulose and cellulose were ruptured during the treatment and thus, reduced the stability form between lignin-hemicellulose-cellulose matrixes.

Consequently, more cellulose was exposed after microwave pre-treatment process as observed in Table 1. Additionally, combination of NaOH with microwave pre-treatment reduced the crystallinity of the cellulose in the EFB. According to the Thostenson and Chou (1999), the effectiveness of the microwave pre-treatment is dependent on the material behavior. In fact, the crystallinity of the fibre affects the dielectric properties in the microwave pre-treatment. Degrees of crystallinity above 45% are essentially transparent to microwaves due to the restriction of dipoles. Addition of NaOH as a swelling agent during microwave treatment helps in the formation of more disorganized amorphous cellulose structure. As been reported by Jacobsen and Wyman (2000), approximately 50-90% of the total cellulose is the crystalline structure where the remainder is the disorganized amorphous cellulose. However, the amorphous cellulose is more rapidly hydrolyzed in enzymatic reaction than the crystalline regions. Here, NaOH solution acts an intracrystalline swelling agent that is capable to penetrate and swell both the accessible amorphous and crystalline region (Shujun *et al*., 2007). At the same time, destruction of cellulose crystalline structure occurred and the highly ordered fibrils in cellulose were distorted. As a consequence, the microfibrils were separated from the initial connected structure and fully exposed, thus increasing the external surface and the porosity of the cellulose.

Aspect ratio and morphology: In cultivation of filamentous *Rhizopus*, the spores tend to aggregate and grow as pellets which have a variety of compactness. Pellets are spherical or ellipsoidal masses of hyphae with variable internal structure, ranging from loosely packed hyphae, forming fluffy pellets, to tightly packed, compact, dense pellets. In the present study, AR was used to

Fig. 1: *Rhizopus* pellet with AR = 1.0

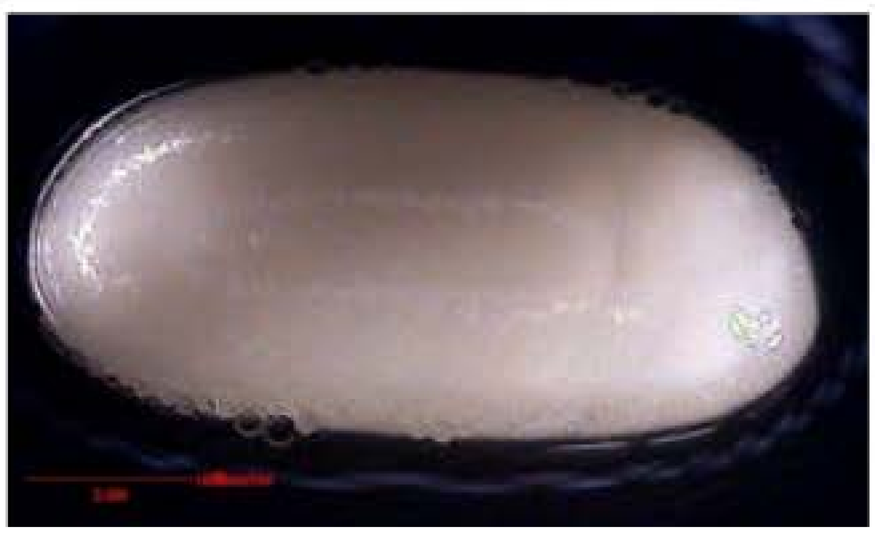

Fig. 2: *Rhizopus* pellet with AR = 1.5

characterize pellet morphology since the size of the pellet produced during cultivation is not uniform in size. AR of 1 indicated a prefect pellet whereas a value closer to 1 indicated rounder pellets. The perfect *Rhizopus* pellet with AR = 1 obtained is illustrated in Fig. 1. On the other hand, AR of 1.5 mostly showed the ellipsoidal pellet as shown in Fig. 2. The results indicated that the ellipsoidal pellet is bigger in size (>3 mm) compared to the spherical pellet.

In term of quantities, the pellets with AR more than 1.5 were produced in fewer quantities. Meanwhile, AR failed to classify the clump morphology because the clump morphology tends to aggregate with other segment thus forming a long structure (Fig. 3).

The morphology of a filamentous fungus developed in any fermentation system could be considered as a final result of competing influence, equilibrium between forces of cohesion and disintegration. Shear forces may be unambiguously assigned the role as disintegrating factors. At pH value above 5.5, cell walls of most microorganisms are negatively charge, tending to cause separation or cell aggregation by electrostatic repulsion.

Fig. 3: Clump morphology of *Rhizopus oryzae* NRRL 395

This may be suppressed by an increase in ionic strength or bridging cells with Ca^{2+} ions. Addition of polycations usually induces aggregation whereas polyanion suppress it (Domingues *et al.*, 2000). The surface thermodynamic balance fungal cell and liquid medium was found to be responsible for pellet formation since Gibbs free energy of pellet formation of the initial culture media -73 to -81 ergs cm^{-2} were increased to -13 to -46 ergs cm^{-2} at 48 h. The factors inducing pellet formation, simultaneously increased the cell wall hydrophobicity (Papagianni, 2004).

L-lactic acid concentration and productivity: In the present study, SSF of the lactic acid from MW-A pre-treated EFB using *Rhizopus oryzae* was studied. The results indicated that *Rhizopus oryzae* NRRL 395 has an ability to perform a single stage SSF process for lactic acid production using cellulosic material. SSF with clump or pellet *Rhizopus* selectively produced L-lactic acid with a high concentration. The production of lactic acid in SSF took place simultaneously with the hydrolyzed cellulose to soluble sugar. *Rhizopus* sp. has a high enzymatic and metabolic capability to perform an SSF process and also utilize cellulose as a carbon source for lactic acid production (Karimi *et al.*, 2006).

The MW-A pre-treatment of the EFB made cellulose molecule in the fibre easily accessible to metabolite activities of the fungus. From the experiments performed it was observed that after 24 h of fermentation, the cultivation medium became clear when most of the EFB fibre was coagulated with the pellets. Along with this mechanism, the diameter and density of the pellet increased with the time and this condition represent the increment in the biomass produced during fermentation. As been reported by Huang *et al.* (2005) fungal cell growth appeared to be very fast, resulting to a sharp increase in fungal biomass production. Associated with the fungal biomass produced, the lactate yield was excreted as a metabolite product and the results are illustrated in Fig. 4.

Under similar cultivation conditions, the pellet *Rhizopus* demonstrated a higher lactate yield than clump *Rhizopus*. Lactate production in the pellet cultivation is

Table 2: Productivity of L-lactic acid in SSF using clump and different AR *Rhizopus* pellet

Clump and pellet	Productivity of SSF (g Lh^{-1})
Clump	0.089
Pellet (AR>1.5)	0.099
Pellet (AR = 1)	0.120

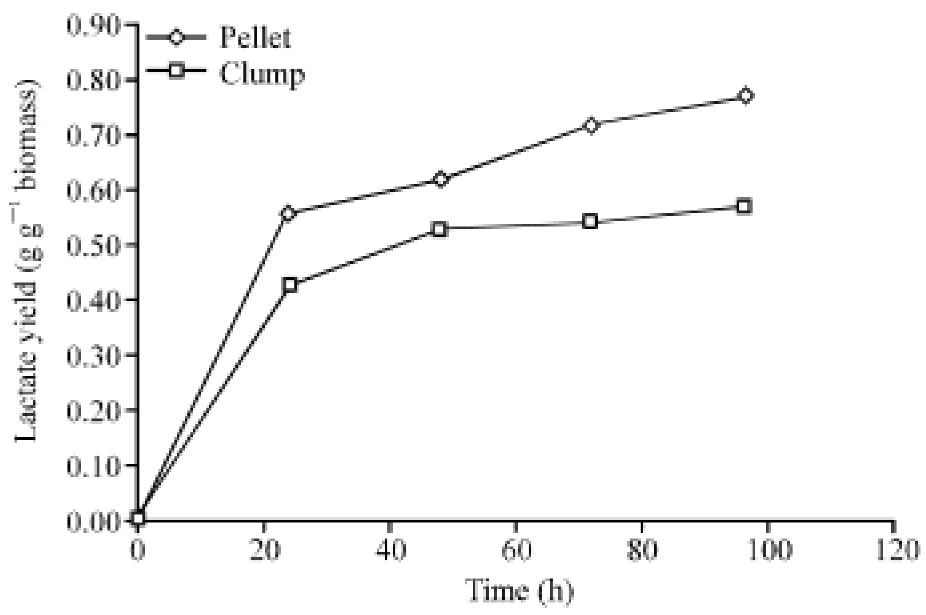

Fig. 4: Lactate yield in the SSF of MW-A treated EFB using pellet (AR = 1) and clump rhizopus

almost 30% higher than in clump system. In the cultivation of medium containing approximately 15 g L^{-1} MW-A pre-treated EFB, pellet *Rhizopus* with AR = 1 produced lactate at 0.77 g g^{-1} EFB within 96 h, while with clump *Rhizopus,* yield of the lactate is approximately 0.57 g g^{-1} EFB. Pellet *Rhizopus* with AR = 1 also gave the highest productivity of lactic acid as compared to the clump and pellet with AR>1.5 (Table 2, Fig. 4). Productivity of the lactic acid obtained using pellet with AR = 1 was 0.12 g Lh^{-1} Meanwhile, clump and pellet with AR 1.5 cultivation gave productivity of 0.089 and 0.099 g Lh^{-1}, respectively.

Size of the pellet is an important factor in metabolite activity of the fungus. As reported by EL-Enshasy *et al.* (1999) smaller pellets were more efficient with respect to the production of exocellular glucose oxidase by *Aspergillus niger*. Generally, the pellets with AR = 1 are smaller in size compared to the pellets with AR = 1.5. The decreasing pellet size also correlated with an increased mycelia density, indicating an improvement of the transport of nutrient to the inner part of the pellet. According to Couri *et al*. (2003), higher diameter pellets tend to suffer autolysis of the cells in their inner diameter, particularly if their cores are also large making it difficult for the nutrients to be transport to the core.

It is known that mineral or nutrient deficiency in fungi growth cause a decrease in the activity of several enzymes in the fungi metabolite. Meanwhile, for the clump morphology, the high hyphae entanglement had probably produced a compact core where the inner part was lysed due to a deficient nutrient transportation. The high annular area of the pellet could not compensate this effect and the enzyme activity was low (Hermersdorfer *et al*., 1987). Thus, the metabolite activity is reduced, thus reducing the productivity of the lactic acid in SSF reaction.

CONCLUSION

Microwave-alkali (MW-A) pre-treated EFB has been used as a carbon source in the SSF of the lactic acid using *Rhizopus oryzae* NRRL 395. Cellulose molecules in the EFB were converted by *Rhizopus* metabolite into lactic acid. The lactic acid production from SSF of MW-A pre-treated EFB is very much influenced by the different morphologies of *Rhizopus* used. Pellet with as AR = 1 indicated a perfect pellet while aspect ratio of 1.5 represented an ellipsoidal pellet. Lactic acid yield and productivity was the highest when SSF was cultivated with pellet *Rhizopus* than clump *Rhizopus*. The maximum lactate yield was 0.77 g g^{-1} EFB used after 96 h cultivation. Highest productivity of the lactic acid (0.12 g L^{-1}) was obtained from SSF of MW-A pre-treated EFB using pellet (AR = 1).

ACKNOWLEDGMENTS

The present research was made possible through IRPA grant sponsored by the Ministry of Science, Technology and Innovation of Malaysia (MOSTI), UiTM scholarship and also facilities in Faculty of Chemical Engineering and Natural Resources Engineering, Universiti Teknologi Malaysia (UTM).

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