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Lactic acid production by immobilized *Rhizopus sp.* IIUM-G05 in air pulsed ALR using agriculture waste substrates

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Abstract

The demand for lactic acid (LA) in various fields has increased significantly due to its functional properties. *Rhizopus* sp. IIUM-G05 has been used before to produce lactic acid using various substrates, but the low production yield remains an issue. This is due to the difficulty in finding a suitable low-cost substrate and controlling the fungal morphology during fermentation to maintain high oxygen concentration. Therefore, this study aimed to address these issues by selecting the best substrate for higher LA yield and using loofah immobilized *Rhizopus* sp. to increase the yield through semi-continuous simultaneous saccharification and fermentation (SSF) in an air pulsed airlift reactor (ALR). The study compared banana peel (BaP) and beet pulp (BeP) as substrates and found that BaP produced a 3-fold higher yield than BeP. The study also tested different air pulsation frequencies in loofah immobilized ALR with BaP to increase LA yield. The results showed that the 0.0384 s^{-1} frequency was the best to increase LA yield to 0.091 g/g after three days of fermentation, which was 2.1-fold higher than the free-cell non-pulsed shake flask culture in the first part of the study. SEM images showed that the 0.1667 s^{-1} frequency resulted in oxygen transfer limitation in the ALR due to irregular formation and trapped BaP particles between mycelium. This study demonstrates that BaP can be used for LA production by *Rhizopus* sp. The air pulsation system with suitable pulsing frequency may help improve production yield by controlling fungal growth inside submerged cultures.

Key words: Lactic acid, *Rhizopus sp.*, air-pulsation, airlift reactor, loofah sponge, immobilization, simultaneous saccharification and fermentation

Introduction

Lactic acid (2-hydroxy propionic acid), is a high-quantity microbial and chemical compound with an annual global production volume of 370,000 metric tons. Historically, LA has been primarily used for acidity and preservation in food. However, in recent years, polylactic acid (PLA) synthesis as a polymer for biodegradable plastics manufacturing has gained more attention than other applications, such as in leather tanning, cosmetics, and pharmaceuticals (Shahri *et al.*, 2020).

The production strategies of LA can be divided into two: chemical synthesis and fermentation process. However, the biotechnological pathway is the favored approach to chemical manufacturing. A given microorganism can synthesize the most effective one shape of the LA isomer from the two optical isomers called D (-) and L (+) and for human consumption, only L-form is allowed (Shahri *et al.*, 2020). Compared to Lactobacillus, Lactococcus, Streptococcus, Bacillus, and Enterococcus bacteria, *Rhizopus* oryzae fungi are preferred for L-lactic acid production due to their ability to exclusively generate the L-isomer, simple nutritional requirements, and easy product recovery (Fu *et al.*, 2016; Hassan and Idris, 2016; Takano and Hoshino, 2016; Miller *et al.*, 2019). Despite the extensive investigation of *Rhizopus* fungi for LA production, several technical challenges are associated with this biotechnological process. One major challenge is the demand for fermentable sugars derived from low-cost materials, particularly lignocellulosic biomass, to produce various chemical products such as ethanol and lactic acid. Lignocellulosic substrates such as cassava starch, solid pineapple waste, potato pulp, sugar bagasse, corncob, and wheat straw have been used for LA production by *Rhizopus* fungi (Yuwa-Amompitak and Chookietwattana, 2014; Trakarnpaiboon *et al.*, 2018; Aziman *et al.*, 2015; Zain *et al.*, 2020; Peng and Feng, 2014; Ranjit and Srividya, 2016; Bai *et al.*, 2008; Guo *et al.*, 2010; Maas *et al.*, 2006; Vially *et al.*, 2010; Saito *et al.*, 2012). Despite the massive diversity of the agricultural wastes that can be considered rich carbohydrate sources, not all of them can be used by *Rhizopus* as a substrate for LA fermentation. Some of these wastes are preferable to be utilized by *Rhizopus* than others; some lignocellulosic materials release toxic elements inhibiting the LA fermentation like the elements that come off from lignin and carbohydrate degradation in a corn cob and stover hydrolysates (Zhang *et al.*, 2016). Therefore, one of the options to find the best substrate for LA fermentation by *Rhizopus sp.* is by testing every substrate and comparing the LA yield.

Banana is a tropical fruit rich in many vitamins as they are classified as lignocellulosic. However, this fruit's wastes are the most plentiful agricultural residues determined withinside the equatorial region. For example, Makanan Ringan Mas Industry, a Small and Medium Industry (SMI) located in Parit Raja, Malaysia, is one of the industries producing food snacks like banana and tapioca chips. This industry produces 101.82 kg per month with a total density of 32303.04 kg m^{-3} per month of food waste. In this industry, BaP waste presents the highest rate of waste generated (27%), followed by tapioca peel waste rate

(25%) and shredded coconut waste (16%) (Abd Kadir *et al.*, 2017, Zain *et al.*, 2020). The problem faced by Makanan Ringan Mas Industry and other SMIs is inappropriate waste disposal from dumping the wastes into ditches to open burning, which causes surface and ground water contamination, atmospheric and hydrologic pollution, and negative environmental impact (Abd Kadir *et al.*, 2017).

On the other hand, sugar beets are root vegetables adapted to grow at temperate zones when days are warm (15 to 20^oC) and nights cool (10 to 15^oC). The sugar beet is considered the second source of sugar worldwide after sugarcane since it has a high sucrose level. According to the 2020 Turkish Statistical Institute (TÜİK) data, Turkey has produced approximately 21 million tons of sugar beet to occupy fifth place worldwide in sugar beet production. Nevertheless, the pulp, which is a by-product of this sugar beet, has the potential to create environmental pollution. Therefore, in this study due to their availability in Malaysia and Turkey, BaP and BeP was chosen to be tested for higher LA fermentation yield by *Rhizopus sp.*

Secondly, LA production by filamentous fungi like *Rhizopus* usually can be done through several methods like One-Step Fermentation (OSF) (Shahri *et al.*, 2020), Solid State Fermentation (SSF) (Aziman *et al.*, 2015), or Separate Hydrolysis and Fermentation (SHF) processes (Zhang *et al.*, 2015). The simultaneous saccharification and fermentation (SSF) process has several advantages over other methods. It improves the concentration and yield of Lactic Acid (LA) while reducing overall production costs. This is achieved by immediately converting released sugars from the saccharification stage into LA, thereby increasing productivity and reducing processing time. In addition, SSF minimizes the inhibitory effect of the product (glucose) on cellulose hydrolysis and reduces the osmotic pressure of cells caused by glucose accumulation. (Zhang *et al.*, 2015). On the other hand, the LA production by *Rhizopus* in submerged cultures results in excessive mycelium and fungal biomass growth, which seriously affects the metabolic rate, mass transfer, and product secretion. Also, fungal hyphae can cause mass and oxygen transfer limitations by wrapping around impellers and causing blockages, spreading into nutrient and sampling feed lines, as well as causing an increase in broth viscosity (Pimtong *et al.*, 2017). This is the main cause that reduced LA production yield in several studies faced by Shahri *et al.* (2020). Moreover, in long-term cultivation, high-density large pellets often have problems with oxygen starvation in the pellet core caused by a restriction of oxygen diffusion (Moreira *et al.*, 2003, Wang *et al.*, 2010, Pino *et al.*, 2018). For example, in a study by Shahri *et al.* (2020), the excessive growth of *Rhizopus* hyphae under the aeration part in ALR made managing and controlling the system harder and eventually decreased the LA production yield. Therefore, improving aeration efficiency in the submerged fermentation process is critical. Therefore, to minimize these problems, two procedures used in this study. Firstly, *Rhizopus* was immobilized on a non-reactive, non-toxic, and highly porous (> 90%) loofah sponge made of the dry fruit of *Luffa cylindrica* that grows widely in Malaysia. Immobilization is a technique that has been successfully used to bind various types of cells, including fungi, yeast, microalgae, bacteria, plants, and rat hepatocytes. In particular, immobilization of *Rhizopus* has been applied in several productions, such as Lactic Acid (LA), biodiesel, enzymatic production of biodiesel, and fumaric acid. (Saeed and Iqbal, 2013; Ganguly *et al.*, 2007; Shahri *et al.*, 2020; He *et al.*, 2016; Sattari *et al.*, 2015; Liu *et al.*, 2017). Secondly, an air pulsation system was added to the airlift reactor (ALR) to supply air at different frequencies (0.167-0.038 s^{-1}). The fungal morphology can be controlled during the submerged fermentation process, and the oxygen starvation that reduces the LA productivity can be prevented. This type of fungal morphology control was successfully used in continuous manganese peroxidase (MnP) production by free pellets of *Phanerochaete chrysosporium* (Moreira *et al.*, 2003). However, to our knowledge, the air pulsing system has not been employed in the LA production process using *Rhizopus*. So, it was the first time to apply this system in ALR to produce LA by *Rhizopus sp*. semi-continuously.

This study consists of two objectives. The first objective is to investigate the ability of *Rhizopus* IIUM-G05 to produce higher LA yield from the banana peel (BaP) and beet pulp (BeP) and select one substrate with highest LA yield for the SSF process in ALR. Second objective is to study the effect of air pulsation in the SSF process conducted in ALR with immobilized *Rhizopus* on loofa sponge at different frequencies (0.167-0.038 s^{-1}) on the LA yield.

Materials and methods

Chemicals and preparation of BaP and BeP: Potato dextrose agar (Brand: Oxoid, UK), potato dextrose (Brand: BD Difco, United States), calcium carbonate (Brand: Chemiz, United Kingdom), Iron (III) chloride hexahydrate AR/ACS (Brand: HmbG, Germany), and DL-lactic acid (~ 90%, Brand: Sigma Aldrich, United States) were purchased from the local supplier (Bio3 Scientific Sdn. Bhd.). The BaP and loofah sponges were collected from the local greengrocer in Kuala Lumpur, Malaysia. The BeP was obtained from Turhal Sugar Factory in Tokat, Turkey. Each substrate was dried in an oven at 60^oC for three days, then milled and stored separately in containers for further use.

Microorganism cultivation and inoculum preparation: *Rhizopus sp.* IIUM-G05 previously isolated from ragi tapai was grown on potato dextrose agar (PDA) under an incubation temperature of 37°C for 7 days (Azmi *et al.*, 2016). After growth and sporulation, 10 mL distilled water was aseptically added to each agar plate, which then scraped to release spores (Guo *et al.*, 2010). This spore suspension was filtered with sterile gauze and counted by a hemocytometer (Sattari *et al.*, 2015). Then, each experiment used a 1 mL spore suspension containing about 10^7 spores to provide a constant quantity of spores.

Substrate selection for higher LA yield: Semicontinuous fermentation was carried out in 250 mL flasks containing 100 mL solutions with different concentrations (40, 60, 80, and 100 $g \cdot L^{-1}$) of BaP and BeP. Each flask was inoculated with the previously prepared spore suspension and incubated at 32°C in an incubator shaker at 80 rpm for 72 h. Then samples were collected at the end of the experiment to determine the LA concentration.

Semi-continuous production of LA in immobilized oxygen pulsed ALR: Lactic acid production was studied in a 1.5 L working volume of an oxygen pulsed ALR by loofah immobilized *Rhizopus sp*. IIUM-G05 semi-continuously for 8 days. A cylinder net draft (5 Mesh) was placed inside the reactor vessel to hold the loofah sponge. After that, the reactor was filled with potato dextrose media (0.2 M) with the loofah sponge inside the reactor vessel. Then, the ALR was sterilized at 121°C for 20 minutes. After cooling, the spore suspension was added to the ALR, and the immobilization process was conducted at an agitation speed of 100 rpm for 24 h. After that, a peristaltic pump replaced the potato dextrose media with sterile BaP media. The temperature was adjusted to 32.2°C (Zhang *et al.*, 2015, Zain *et al.*, 2020); pH was automatically controlled at 5.6 by adding 10% sterile calcium carbonate; three different air pulsation frequencies in three separate runs were tested: 0.1667, 0.0625, and 0.0384 s^{-1} with corresponding shutting times of 5, 15, and 25 s and constant opening time of 1 s (Moreira *et al.*, 2003). Each SSF run was carried out for 8 days, with sample collection once every day.

Analytical methods: A scanning electron microscope (SEM) (JSM-IT100, JEOL, Ltd., Japan) was used to evaluate the microstructures of the loofah immobilized *Rhizopus sp.* and the BaP after sputtering the samples with a gold/palladium layer. LA concentration was determined by spectrometry, as described by Borshchevskaya *et al.* (2016). Briefly, each fermentation sample was centrifuged at 10,000 rpm for 10 minutes, and a 50 µL supernatant was added to 2 mL of 0.2% iron (III) chloride, stirred and absorbance was measured at 390 nm (A = 0.1374B -0.01, R^2 = 0.9987, where A is absorbance and B is LA concentration). The LA concentration was calculated based on the previously obtained calibration curve (A = 0.1374B - 0.01, R^2 = 0.9987, A: Absorbance, B: LA concentration). Then the LA yield was calculated using Eq. 1 (Shahri *et al.*, 2020).

$$\text{LA yield} = \frac{(C_p)_t}{(C_s)_i} \tag{1}$$

Where $(C_p)_t$ ($g \cdot L^{-1}$) is the concentration of LA produced at any time *t* during the experiment and $(C_s)_t$ ($g \cdot L^{-1}$) is the initial amount of substrate.

The LA volumetric productivity ($g \cdot L^{-1} \cdot h^{-1}$) was calculated using Eq. 2.

$$\text{LA volumetric productivity} = \frac{(C_p)_t}{t} \tag{2}$$

The LA areal productivity ($g \cdot m^{-2} \cdot h^{-1}$) was calculated using Eq. 3.

$$\text{LA areal productivity} = \frac{(C_p)_t * (V_w)}{(A_w) * l} \tag{3}$$

where (V_w) (L) is the working volume and (A_w) (m^2) is the used loofah sponge area in each SSF process.

Statistical analysis: Data were statistically analysed for regression analysis in EXCEL ver. 2110 at *P* = 0.05, and the differences among means were determined using ANOVA, followed by Tukey's test at *P* = 0.05.

Results and discussion

LA yield from BaP and BeP and substrate selection: The produced LA yield from BaP after 72 h of incubation was about 3-fold higher than BeP as shown in Fig. 1. The highest LA yield produced from BaP and BeP was 0.044 and 0.0146 $g \cdot g^{-1}$ at a concentration of 80 and 100 $g \cdot L^{-1}$, respectively. In Peng and Feng (2014) study, the LA yield produced from potato pulp was 0.0112 $g \cdot g^{-1}$ after 6 days in a shake flask using *R. oryzae* IFO5740. This indicates that BaP and BeP can potentially be used as a lignocellulosic substrate for LA using *Rhizopus sp.* On the other hand, the higher LA yield produced by BaP compared to BeP could be due to the lower content of lignin in BaP which has only 8% of lignin (Anhwange *et al.*, 2009), while BeP has around 20% lignin content (Sheridan, 2019). Furthermore, according

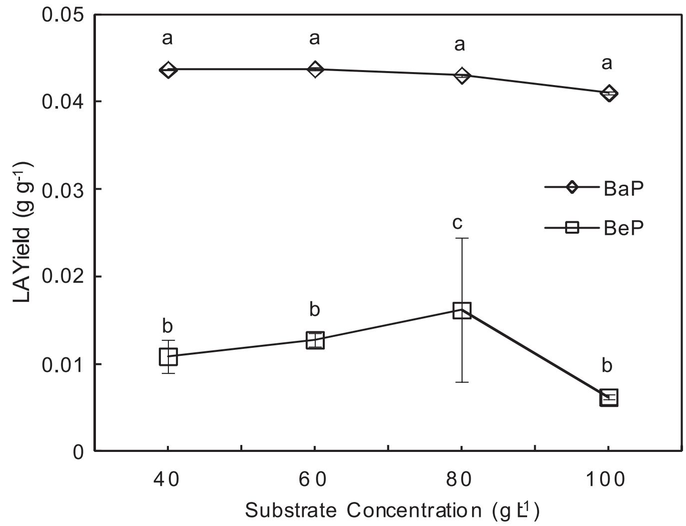

Fig. 1. LA yield from beet pulp and banana peel in shake flask after 72 h using *Rhizopus* sp.

to Pothiraj *et al.* (2006) study, *Rhizopus stolonifer* can produce good amounts of cellulolytic enzymes, but very low levels of lignin-degrading enzymes. Therefore, it prefers high cellulose and low lignin-containing substrates. In this stage of study, BaP was selected to proceed with second objective at concentration of 60 $g \cdot L^{-1}$. The selected concentration is due to a small LA yield difference in 60 and 80 $g \cdot L^{-1}$ of BaP. Furthermore, the medium circulation in the ALR at 60 $g \cdot L^{-1}$ is more manageable compared to 80 $g \cdot L^{-1}$.

Semi-continuous SSF process in ALR: Further experiments in this study were conducted to improve the LA yield in the loofah immobilized air pulsed ALR using 60 $g \cdot L^{-1}$ BaP through the SSF process. The results obtained from this objective is later compared to the LA yield using free cells in the first objective as the base LA yield. The cell immobilization strategy was applied to the ALR which was based on the optimum conditions from our previous study (Alasali *et al.*, 2022) (*i.e.*, potato dextrose concentration of 0.2 M, incubation time of 24 h and 100 rpm of agitation speed).

The effect of different air pulsation frequencies on the LA production during the SSF processes was examined, and results are presented in Fig. 2. It can be seen from Fig. 2 that at 0.1667 s^{-1} air pulsation frequency, the LA yield has reached a maximum of 0.087 $g \cdot g^{-1}$ after one day of fermentation, but it dropped significantly to 0.038 $g \cdot g^{-1}$ the next day (Table 1). This problem occurred because *Rhizopus sp.* is an obligate aerobe fungus requiring high oxygen concentration to grow and produce LA; hence, *Rhizopus sp.* in high air pulsation frequency (0.1667 s^{-1}) synthesized LA rapidly after only one day of fermentation. However, at the same time, the mycelia were also grown fast and caused substrate circulation and oxygen starvation problems inside the ALR on the second day of fermentation (Fig. 3b). Therefore, oxygen starvation might be the main reason that caused a decrease in LA production by triggering the LDH enzyme to induce a reversible reaction between pyruvate and lactate (Kurniawati and Indrati, 2014).

On the other hand, even though the LA yield at 0.0625 s^{-1} frequency was lower (0.067 $g \cdot g^{-1}$) than it was at 0.1667 s^{-1} after one day of fermentation (0.087 g g^{-1}), but it kept increasing for three days of fermentation to reach 0.075 g g^{-1}. This result indicates that decreasing the pulsation frequency to 0.0625 s^{-1}

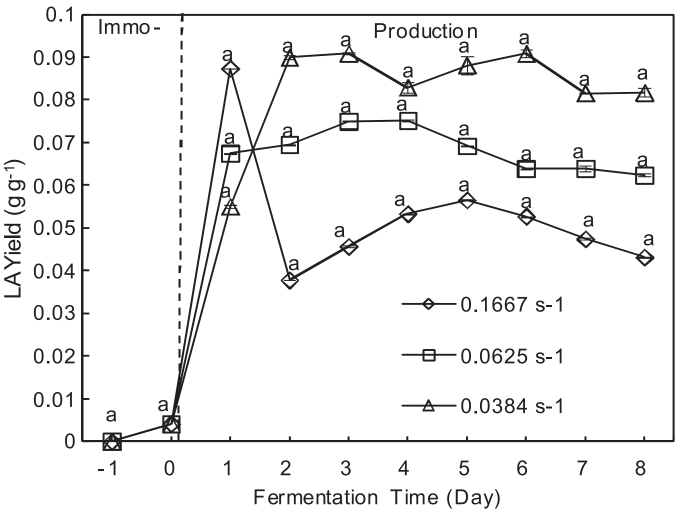

Fig. 2. LA yield during immobilization and BaP fermentation using *Rhizopus* sp. under different air pulsation frequencies.

helped reduce the substrate circulation and oxygen limitation problems inside the ALR (Fig. 3c), yet to obtain LA yield as much as produced on the first day with 0.1667 s^{-1} frequency.

Eventually, as Fig. 2 shows, by decreasing the pulsation frequency to 0.0384 s^{-1}, the LA yield has reached a maximum of 0.091 $g{\cdot}g^{-1}$ after three days of fermentation without a significant drop in LA concentration until the end of the SSF process (Table 1). These results demonstrate the advantages of the air pulsation system in; improving the LA yield by 2.1-fold, from 0.043 $g{\cdot}g^{-1}$ (Fig. 1) without pulsation to 0.091 $g{\cdot}g^{-1}$ under 0.0384 s^{-1} air pulsation frequency (Fig. 2); and preventing the mass and oxygen transfer limitation through controlling the fungal growth by keeping fresh, active biofilm surface with a controlled thickness (Fig. 3d). This result indicates that the air pulsation system has efficiently helped increasing the LA yield by controlling the fungal growth which means that this system might help, not only the LA production using different substrates, but also might be used in other fermentation process using filamentous fungus.

Various studies on LA production from low-cost substrates have been reported and summarized in Table 2 for comparison. Yuwa-amornpitak and Chookietwatana, (2018) reported the highest LA production of 4.03 $g{\cdot}L^{-1}$ from waste cooking oil glycerol in 80 h fed-batch culture, whereas the productivity and yield were 0.05 $g{\cdot}L^{-1}{\cdot}h^{-1}$ and 0.24 $g{\cdot}g^{-1}$. Compared to these LA production studies with different substrates and fermentation methods, comparable LA concentration yield and productivity were obtained in this study.

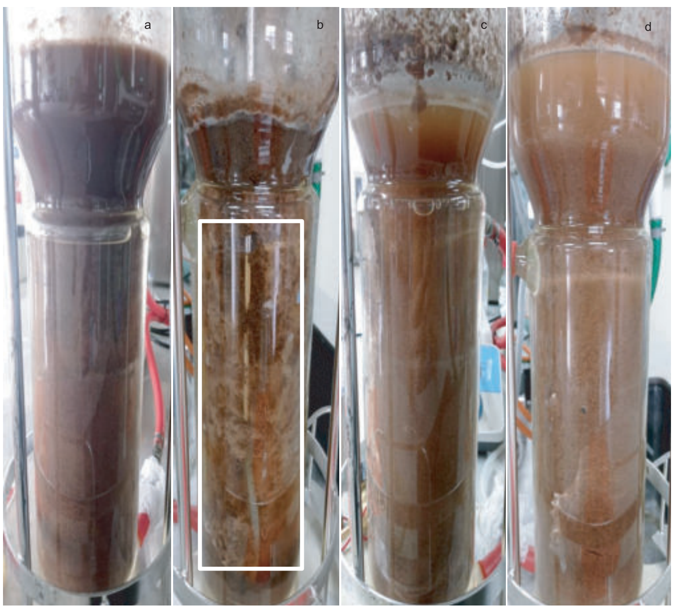

Fig. 3. ALR images at (a) the beginning of the SSF process and (b), (c), and (d) the fourth day of the SSF process under 0.1667, 0.0625, and 0.0385 s'1 air pulsation frequency, respectively. The white square marks the excessively grown fungal hyphae.

Table 1. Production of LA from BaP by *Rhizopus sp.* under different air pulsation frequencies

Fermentation time (Day)	LA yield (g g^{-1})	Volumetric productivity (g L^{-1} h^{-1})	Areal productivity (g m^{-2} h^{-1})	LA concentration (g L^{-1})
		0.1667 s^{-1}		
1	0.087	0.218	5.966	5.240
2	0.038	0.047	1.288	2.263
3	0.046	0.038	1.047	2.758
4	0.053	0.033	0.907	3.188
5	0.056	0.028	0.769	3.377
6	0.053	0.022	0.603	3.180
7	0.047	0.017	0.460	2.831
8	0.043	0.014	0.370	2.598
		0.0625 s^{-1}		
1	0.067	0.168	6.848	4.032
2	0.070	0.087	3.554	4.185
3	0.075	0.063	2.555	4.512
4	0.075	0.047	1.922	4.527
5	0.069	0.035	1.414	4.163
6	0.064	0.027	1.086	3.836
7	0.063	0.022	0.915	3.770
8	0.062	0.019	0.788	3.712
		0.0384 s^{-1}		
1	0.053	0.133	5.414	3.188
2	0.090	0.112	4.561	5.371
3	0.091	0.076	3.082	5.444
4	0.085	0.053	2.169	5.109
5	0.086	0.043	1.760	5.182
6	0.089	0.037	1.512	5.342
7	0.082	0.029	1.188	4.898
8	0.081	0.025	1.027	4.840

Scanning electron microscopy (SEM) images: The structure and attachment of the immobilized fungus on loofah was captured using SEM. The images were observed under 50× and 200× magnifications. It can be clearly seen in Fig. 4(a)(b) at 0.1667 s^{-1} of air pulsation frequency, the thick layer of irregular particles attached or trapped in between fungus hypha on the loofah sponge. These trapped particles are similar to the raw BaP inserted image in Fig. 4(g). However, when the pulsation frequencies were set at 0.0625 s^{-1} and 0.0385 s^{-1}, no attached or trapped BaP particles were observed as respectively shown in Figs. 4(b), (c) and 4(d), (e). The images also shown the fungus mycelia uniformly formed a thin smooth layer on the loofah sponge.

These images could explain the limitation of oxygen transfer to the fungi which could occur due to the thick biomass growth and the trapped BaP particles. At frequency 0.1667 s^{-1} of air

Table 2 Comparison of lactic acid production from low-cost substrates by *Rhizopus*

Substrate	Strain	Concentration ($g \cdot L^{-1}$)	Yield ($g \cdot g^{-1}$)	Productivity ($g \cdot L^{-1} \cdot h^{-1}$)	References
			SSF		
Banana peel	*Rhizopus sp.*	5.4	0.09	0.112	This study
Wheat straw	*R. oryzae* NBRC 5378	6	0.23	0.063	Saito *et al.*, 2012
			SHF		
Wheat straw	*R. oryzae* CBS 112.07	6.8	0.23	0.28	Maas *et al.*, 2006
Wheat straw	*R. oryzae* NBRC 5378	2	-	0.04	Saito *et al.*, 2012
			Continues		
Cassava mill effluent	*R. oryzae*	8.54	-	0.06	Kamal and Zain, 2015
Potato pulp	*R. oryzae* IFO5740	-	0.0112	-	Peng and Feng, 2014
			Fed-batch		
Waste cooking oil glycerol	*R. microsporus* LTH23	4.03	0.24	0.05	Yuwa-amornpitak and Chookietwatana, 2018
			One step fermentation		
Soluble potato starch	*R. oryzae* PTCC 5263	5	0.5	0.1	Shahri *et al.*, 2020
			Batch		
Mango peel	*R. oryzae* NCIM 1009	7.25	0.65	0.12	Mannepula *et al.*, 2015

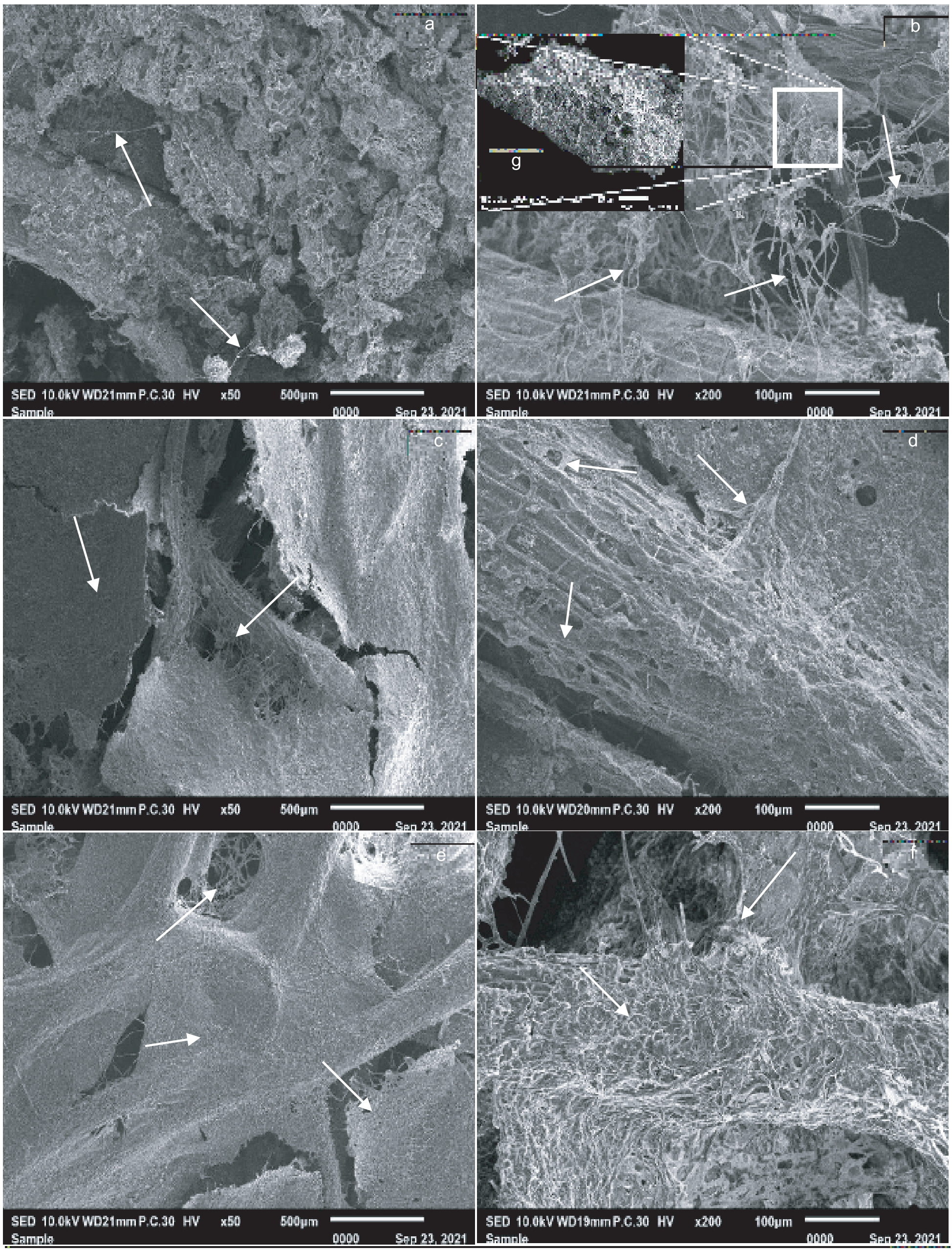

Fig. 4. Scanning electron microscopy images of loofah sponge after LA fermentation by *Rhizopus* sp. using 6 % BaP at air pulsation frequency of (a)and (b) 0.1667 s'^{1} (c) and (d) 0.0625 s'^{1}, and (e) and (f) 0.0385 s'^{1}and (g): the raw BaP particle. Mycelium networks are marked with arrows.

pulsation, the attachment of BaP to the loofah trapped between fungus hypha can only happen if the BaP were static inside the ALR which could occurr because the high oxygen concentration at the beginning of the SSF that led the fungus to grow excessively. The excessive growth and the trapped particles prevent the liquid and air circulation inside the ALR. Similar problem was also faced by Shahri *et al.* (2020) in their study. Nevertheless, decreasing the frequency to 0.0625 s^{-1} and 0.0385 s^{-1} not only controlled the thickness of the mycelia but also kept it fresh and active to produce LA (Table 1).

This study showed that *Rhizopus* fungus produces a higher yield of LA using BaP than BeP. Lignocellulosic material of loofah sponge was used as the support matrix to immobilize *Rhizopus* for SSF fermentation. To increase the LA yield from BaP, three SSF processes in ALR under different air pulsation frequencies were tested, and the pulsation frequency of 0.0385 s^{-1} with a shutting period of 25 s was found to increase 2.1-fold of the LA yield. This yield was achieved by controlling the fungal hypha growth that decreased the substrate and oxygen transfer limitation. These results may help improve the LA production using different substrates, and the air pulsation system results might help control the fungal growth of different filamentous fungi in other fermentation processes.

Acknowledgments

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