ISOLATION OF *Rhizopus oryzae* FROM ROTTEN FRUIT AND ITS POTENCY FOR LACTIC ACID PRODUCTION IN GLUCOSE MEDIUM WITH AND WITHOUT ADDITION OF CALCIUM CARBONATE

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ABSTRACT

Studies on lactic acid production by filamentous fungi *Rhizopus oryzae* have been explored in the world. Unfortunately, these studies are still limited in Indonesia, particularly studies in lactic acid production by indigenous strain *R. oryzae*. Four strains obtained from rotten avocado and guava were potential in producing lactic acid (AT1, JT1, AT2, and AT3). *Rhizopusoryzae* AT3 was used for lactic acid production using 100 g/l glucose medium with and without addition of 7.5 g/l calcium carbonate ($CaCO_3$) at initial fermentation. Addition of $CaCO_3$ increased lactic acid concentration of 59.30%, the concentrations were 11.61 g/l and 18.495 g/l in glucose medium and glucose medium with $CaCO_3$ addition, respectively. Glucose+$CaCO_3$ medium also showed higher productivity, reached continuously from 1 day (0.059 g/l/h) until 5 days fermentation (0.154 g/l/h), whereas highest productivity in glucose medium was reached at 1 day fermentation (0.124 g/l/h) and continued to decrease until 5 days fermentation (0.065 g/l/h).

Keywords: Lactic acid,*Rhizopus oryzae*, calcium carbonate, productivity

INTRODUCTION

Lactic acid ($C_3H_6O_3$) is a valuable organic acid because of its wide use in food and food-related product. Lactic acid studies nowadays become popular with go green campaign, due to its ability to form poly lactic acid (PLA) which can be used as a material for manufacturing biodegradable polymer, including biodegradable plastic to solve one of global warming causes.

Basically lactic acid exists in 2 isomers, D(-)-lactic acid and L(+)-lactic acid. However, Abeysekaraet al. (2007) reported that D(-)-lactic acid potentially caused neurotoxicity in calf model. WHO also mentioned that elevated levels of D-lactic acid are harmful for humans (Zhanget al., 2007). L-isomer is more desired in lactic acid production. L(+)-lactic acid is preferred for food-related product and pharmaceutical industries due to its safety and its highcrystallinity which is suitable for commercial use (Wee et al, 2006).

Lactic acid can be synthesized by chemical and biological ways. Chemical synthesis occurs through lactonitrile pathway which forms racemic acid (D- and L-isomer) (Narayanan et al., 2004; Buyukkileci, 2007). Biological synthesis occurs through fermentation using microorganism such as lactic acid bacteria (LAB) and *R. oryzae*. Lactic acid bacteria are well known as lactic acid producers due to its high productivity in producing lactic acid. However, LAB can produce both D- and L-isomer of lactic acid. On contrary, *R. oryzae* producesL(+)-lactic acid as the sole lactic acid in its metabolism. Besides, LAB are fastidious and requires complex nutrient for fermentation because of its limited ability to synthesize B-vitamins and amino acid (Yan et al., 2001). *R. oryzae* can grow better under limited nitrogen condition, if compared to LAB (Soccolet al., 2004). In addition, fermentation using *R. oryzae* will yield valuable by-product, fungal biomass, which can be used in biosorption processes for purification of contaminated effluent and as additive in animal feeds to improve the feed quality (Zhanget al., 2007). Comparing in downstream process, fermentation using *R. oryzae* has cheaper cost because of its easy separation between fungal biomass and fermentation broth. Ufortunately, studies on lactic acid production by *R. oryzae*in Indonesia are still limited, so exploration of its potency is needed.

In this study, *R. oryzae* used for lactic acid production was isolated from Indonesian fruit, since Indonesia is a tropical country which has many varieties of fruits and *R. oryzae* can easily grow in fruits (Pitt and Hocking, 2009). This

newly isolated *R. oryzae* indigenous strain required further investigation on its lactic acid production potency.

Lactic acid production can extremely decrease the pH of fermentation medium, out of the optimum pH range for *R. oryzae* growth (pH = 3.4-6). Neutralizing agents are usually used to solve this problem. Some studies used calcium carbonate ($CaCO_3$) as neutralizing agent (Buyukkileci, 2007; Xiao et al., 2011; Gangulyet al., 2007). Effect of $CaCO_3$ addition in fermentation process was also investigated in this study.

MATERIAL AND METHODS

The chemicals and media components employed in this study were purchased from Oxoid (Basingstoke, England), Sigma-Aldrich (St. Louis, MO, USA), and Merck (Darmstadt, Germany).

Isolation and Identification of *R. oryzae*

Isolation and identification of *R. oryzae* was carried out using direct plating method in Dichloran Rose Bengal Chloramphenicol (DRBC) and Malt Extract Agar (MEA) medium, respectively. Avocado and guava were used as samples to obtain the indigenous *R. oryzae*. In isolation step, the incubation was in 30°C for 4-6 days, while in identification step it was 37°C for 7 days. Identification at species level was refered to Samson et al. (1984).

Screening of Acid-Producing Fungi

Bromocresol Purple (BCP) agar medium was used for screening the *R. oryzae* isolates, in 30°C for 24 hours. BCP agar contains BCP indicator which shows yellow color in pH=<5.2 and purple color in neutral pH (pH=6.8). Acid production was observed every 2 hours, by calculating the total diameter formed (colony and yellow zone around) minus diameter of colony (purple zone). The most potential isolate was used for lactic acid production.

Lactic Acid Production

A spore solution of 4-7x10^4 spores/ml was inoculated into 250 ml Erlenmeyer flask containing 100 ml of the culture medium (glucose 100g/l; $(NH)_2SO_4$ 1.35g/l; $MgSO_4.H_2O$ 0.15g/l; $ZnSO_4.7H_2O$ 0.04g/l; KH_2PO_4 0.25g/l; $CaCO_3$ was added at the beginning of fermentation process 7.5g/l). The culture temperature was ambient temperature (±29°C), agitated in reciprocal shaker with agitation rate of 130 strokes per min (spm) for 5 days.

Analytical Method

After fermentation, samples were filtered using filter paper to separate medium from fungal biomass. Biomass concentration was given as dried mycelium weight. Biomass was dried in 90-95°C until constant weight reached (±24 h) (Sardjono et al., 1992). pH meter was used for measuring the pH of medium right after fermentation. To measure lactic acid concentration, titrable acidity method (AOAC, 1995 in Ramadzanti, 2006) and HPLC was used with a MetaCarb H Plus column and 0.005 M H_2SO_4 as the eluent (flow rate 0,5 ml/min). Column temperature was 68°C and detected by UV light 215 nm. Glucose consumption was measured by DNS method (Miller, 1959).

RESULT AND DISCUSSION

Isolation, Identification, and Screening of *R. oryzae*

Colony of suspected *Rhizopuss pp* (brownish grey colony) started to grow on guava and avocado in 2 days

Table 1. Identification result of four fungal isolates

Parameters	Isolate				Literature (Samson et al., 1984)
	AT1	JT1	AT2	AT3	
Sporangiophore number in a group	3	4-5	3-4	3-5	Solitaire or group, up to 5 sporangiophores in a group.
Sporangiophore height Sporangiophore diameter	612-1980 µm 12.5 µm	624-1020 µm 12.5 µm	250-632 µm 12.5 µm	612-836µm 12.5 µm	150 – 2000 µm high with 6 – 14 µmin diameter
Sporangium diameter	57.5-175 µm	85-150 µm	72.5-183 µm	55-150 µm	50 – 200 µm diameter.
Columella diameter	37.5-120 µm	50-87.5 µm	50-102 µm	37.5-57.7 µm	Ovoid orglobose with 30 – 120 µmin diameter.
Sporangiospores diameter	5-7.5 µm	6.25-7.5 µm	6.25 µm	6.25-7.5 µm	Globose, ovoid, or irregular with 4 – 10 µmin diameter.
Chlamidospores existence and their size	Exist d=22.5 µm and 15x20 µm	Exist 7.5x20-15x25µm	Exist 7.5x15-10x15µm	Exist 7.5x12.5-12.5x25 µm	Chlamidospores exist with 8x13 – 16x24 µm size (cylindrical) or 10 -35 µmin diameter.
Species	Suspected *Rhizopusoryzae*	Suspected *Rhizopusoryzae*	Suspected *Rhizopusoryzae*	Suspected *Rhizopusoryzae*	*Rhizopusoryzae*

incubation. Dense colony formed after 5 days incubation. Four isolates were obtained and identified as *Rhizopusoryzae* based on keys of identification according to Samson et al. (1984). Identification result was shown in Table 1 and fungal microscopy observation was shown in Figure 1.

Identification of isolates above were carried out after cultivating isolates in MEA medium at 37°C for 7 days incubation, according to identification keys Samson et al. (1984).

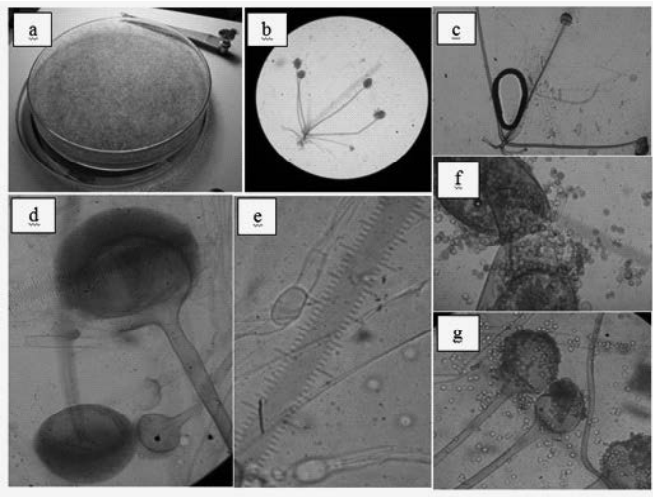

Figure 1. Result of *R. oryzae* microscopy observation
a) Colony of *R. oryzae* on MEA medium after 7 days incubation at 37°C
b) 5 sporangiophores formed in a group (JT1)
c) Sporangiophore with 663 µm long (AT1)
d) Smooth sporangium with 112.5 µm diameter (JT1)
e) Chlamidospore in cylindrical shape with 8x15 µm size (AT2)
f) Globose sporangiospores 5-10 µm diameter (AT3)
g) Globose columella with 62.5 µm diameter (AT2)

Among the isolates obtained, screening was undertaken to select the most potential isolate in producing lactic acid. Using BCP agar, isolate with the widest acid zone (yellow zone) was determined as the most potential isolate in acid production. Figure 2 shows that the best acid producer was observed in the culture of AT3 isolate (2.90 cm), followed by JT 1 and AT 1, which was 2.75 cm and 2.25 cm, respectively. *Rhizopus oryzae* AT 1 produced wide acid zone (2.95 cm acid zone) in 18 hours,then decreased. The different phenomenon was obtained in isolate AT3, which was obtained from avocado, performed more stable in increasing of acid zone during 24 hours incubation. Since AT3 showed the best acid production, this isolate was used for lactic acid production in glucose medium.

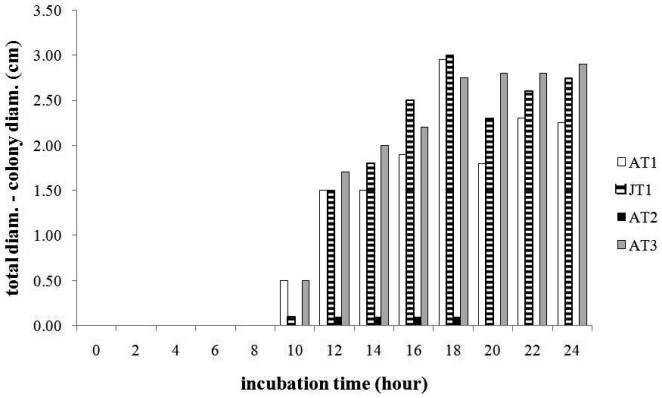

Figure 2. Acid zone (total diameter-colony diameter in cm) produced by isolate *R. oryzae* AT1, JT1, AT2, and AT3 on BCP medium during 24 hours incubation at ambient temperature (±29°C)

Effect of $CaCO_3$ Addition on Macro-morphological Growth of *R. oryzae* during Fermentation

This study revealed that $CaCO_3$ addition affected macro-morphological growth of *R. oryzae* during the fermentation. Filamentous form was observed in glucose+$CaCO_3$ medium at 1 and 2 days fermentation, whereas ribbon-like clumps were formed after 3, 4, and 5 days fermentation. In this medium, ribbon-like compact clump with about 1 cm diameter was formed after 5 days fermentation. Fermentation in glucose medium (without $CaCO_3$ addition) showed cotton-like clump with ±1 cm diameter at 1 day fermentation and increased in size until 5 days fermentation (±4 cm). $CaCO_3$ addition might cause different mycelia growth due to its low solubility in high pH, vice versa. In acid condition, $CaCO_3$ dissociates to Ca^{2+} and CO_3^{2-}, this CO_3^{2-} binds H^+ from the acid environment to form H_2CO_3 (carbonate acid), which is unstable and easily degraded into CO_2 and H_2O (Benedictus, 2010). As a consequence, CO_3^{2-} ion concentration becomes smaller and chemical reaction shift to the product (CO_3^{2-}), which causes higher solubility of $CaCO_3$. The presence of insoluble $CaCO_3$ particles might cause filamentous form mycelia in medium because these particles block mycelia to bind each other. After 2 days to 5 days fermentation, pH of medium decreased, reach 4.22 and 3.79 at day 3 and 5, respectively (Figure 3). This low pH increased $CaCO_3$ solubility, followed by the forming of clumps.

This finding is important, since larger size of clumps may limit the rate of nutrient and oxygen transfer, resulting in a low reaction rate and a decrease in production rate (Taskinet al., 2012). Bigger clump formed in glucose medium

might cause the lower lactic acid yield, compared to ribbon-like clump form in glucose+$CaCO_3$ medium. The highest lactic acid concentration in glucose medium was reached in 4 days fermentation (13.095 g/l) which is lower than that in glucose+$CaCO_3$ medium (18.495 g/l reached in 5 days fermentation).

Effect of $CaCO_3$Addition on Lactic Acid Concentration

Lactic acid concentration data is shown in Figure 3. Highest lactic acid concentration in glucose medium and glucose+$CaCO_3$ medium was reached at 4 days (11.61 g/l) and 5 days (18.495 g/l) fermentation, respectively. Lactic acid concentration in glucose+$CaCO_3$ medium was 59.3% higher than that in glucose medium. Addition of $CaCO_3$ decelerated the extreme decline of medium acidity. Initial pH of this medium was 6, and then slowly decreased until day 5 (pH=3.79), which was not too acidic compared to pH of glucose medium without $CaCO_3$ addition (pH=2.23). This condition supported *R. oryzae* AT3 to keep growing and producing lactic acid, since *R. oryzae* can grow at pH of 3.4 – 6 (Sorenson and Hesseltine in Permana, 2012). As shown by metabolic pathway in *R. oryzae* (Figure 4), Lactate Dehydrogenase (LDH) enzyme has an important role in changing pyruvate into lactic acid.

This enzyme shows optimum activity at neutral pH and poor activity at low pH (Davies et al in Tadegeet al, 1999). Glucose+$CaCO_3$ medium with nearly neutral pH made LDH possible to actively convert pyruvate into lactate until 5 days fermentation, as seen in Table 2, the lactic acid productivity tended to increase.Glucose was totally consumed by *R. oryzae* AT3 in this medium until 5 days fermentation, but the efficiency of glucose utilization tended todecrease, shown by Yp/s data (0.7688 and 0.18495 g lactic acid/g glucose at 1 and 5 days fermentation as seen in Table 2). The decreasing efficiency might be caused by pH of medium which extremely decreased at day 3 (pH=4,22). This acidic condition activated Pyruvate Decarboxilase (PDC) enzyme, which plays role in changing pyruvate into ethanol, leading to competition of pyruvate utilization.

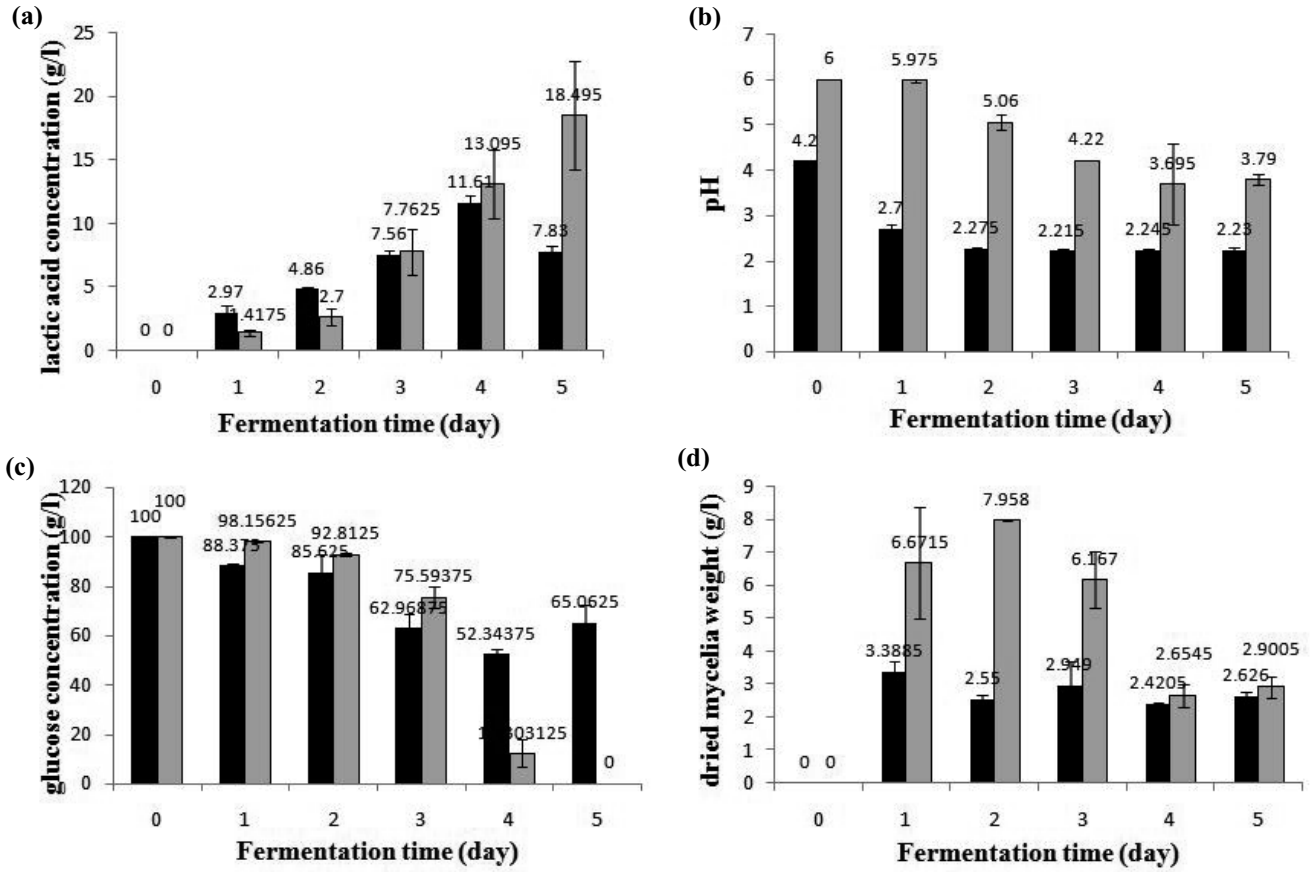

Figure 3. Effect of $CaCO_3$ addition on lactic acid production (a), pH changes (b), glucose consumption (c), and dried mycelia weight (d) during fermentation of glucose by *R. oryzae* AT3 at ambient temperature (±29°C).
Each data point represents mean ± SD (n=2 for pH and dried mycelia weight, n=4 for lactic acid concentration and glucose concentration). Black bars represent data from glucose medium, grey bars represent data from glucose+$CaCO_3$ medium.

Table 2. Fermentation profile (productivity, Yp/s, and Yx/s) of *R. oryzae* AT3 in room temperature (±29°C) in glucose medium with and without addition of $CaCO_3$

Fermentation Medium	Incubation Time (hour)	pH	Lactic Acid Concentration (g/l)	Glucose Concentration in Medium (g/l)	Dry Biomass Weight (g/l)	Glucose Consumed (g/l)	Productivity (g/l.h)	Yp/s	Yx/s
Glucose	0	4.2	0	100	0	0	0	0	0
	24	2.7 ± 0.085	2.97 ± 0.624	88.375 ± 1.021	3.389 ± 0.309	11.625 ± 1.021	0.124	0.255	0.291
	48	2.275 ± 0.035	4.86 ± 0.220	85.625 ± 7.036	2.550 ± 0.132	14.375 ± 7.036	0.101	0.338	0.177
	72	2.215 ± 0.001	7.56 ± 0.382	62.969 ± 5.837	2.949 ± 0.751	37.031 ± 5.837	0.105	0.204	0.080
	96	2.245 ± 0.884	11.61 ± 0.583	52.344 ± 2.362	2.421 ± 0.018	47.656 ± 2.362	0.121	0.244	0.051
	120	2.23 ± 0.113	7.83 ± 0.382	65.062 ± 7.433	2.626 ± 0.126	34.938 ± 7.433	0.065	0.224	0.075
Glucose + $CaCO_3$	0	6.0	0	100	0	0	0	0	0
	24	5.975 ± 0.035	1.4175 ± 0.258	98.15 ± 0.120	6.672 ± 1.698*	1.85 ± 0.120	0.059	0.769	3.606*
	48	5.06 ± 0.156	2.7 ± 0.661	92.812 ± 0.794	7.958 ± 0.006*	7.188 ± 0.794	0.056	0.376	1.1072*
	72	4.22 ± 0.001	7.7625 ± 1.810	75.594 ± 4.237	6.167 ± 0.844*	24.406 ± 4.237	0.108	0.318	0.253*
	96	3.695 ± 0.884	13.095 ± 2.659	11.678± 5.700	2.655 ± 0.361	88.322 ± 5.700	0.136	0.149	0.030
	120	3.79 ± 0.113	18.495 ± 4.215	0	2.901 ± 0.323	100	0.154	0.185	0.029

* Notes: Dry micellia weights which are marked as (*) could not represent the real data because some $CaCO_3$ was trapped inside micellia, which was .difficult to filter, bothering dry biomass weighing.

Lactic acid production in glucose medium (without $CaCO_3$) showed lower lactic acid concentration, compared to glucose+$CaCO_3$ medium. pH of medium might play important role on it because it affected lactic acid production strongly, due to its role in activating enzyme in *R. oryzae* metabolism. Initial pH of glucose medium was 4.2 and decreased to 2.7 in 1 day fermentation then slightly decreased at 2 until 5 days fermentation (pH=2.23 at day 5). This acidic medium did not support *R. oryzae* to grow well, also LDH to optimally convert pyruvate into lactate. Pyruvate was utilized by PDC enzyme better than LDH enzyme in this condition, so that lactic acid was produced slowly, as shown by its productivity which tended to decrease (Table 2). This slow productivity also related to inhibition activity which was mentioned bySkory (2003) that at pH near or below pK of lactic acid (pK=3.8), inhibition on lactic acid production may occur. Highest lactic acid concentration in glucose medium was reached in 4 days fermentation (11.61 g/l), then decreased at day 5 (7.83 g/l). It might be caused by reversible reaction between pyruvate and lactate, catalyzed by LDH enzyme (Moore and Landeckerin Hidayat, 2006). In this acidic environment, PDC enzyme activity was higher than LDH enzyme activity, causing ethanol produced better than lactate. Lactate which had been formed tended to reverse back into pyruvate then utilized by PDC to form ethanol.

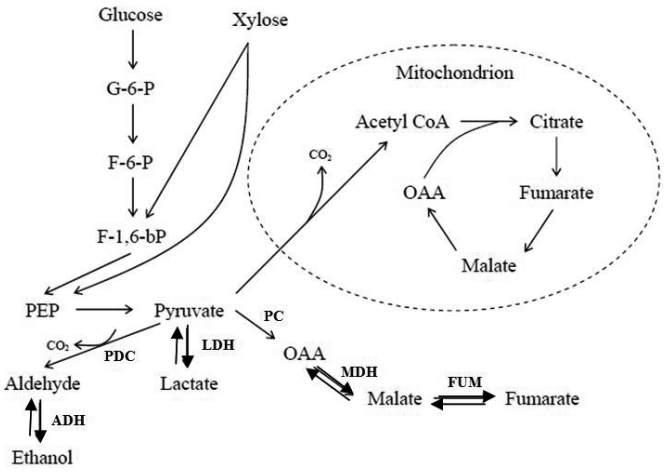

Figure 4. Metabolic pathway in *R. oryzae*and the enzymes (Thongchul, 2005)

According to stoichiometry of lactic acid synthesis, 1 mol of glucose could yield 1.5 mol of lactic acid in *R. oryzae* metabolism (Litchfield in Hidayat, 2006), in other words, 1 gram of glucose could maximally yield 0.75 gram of lactic acid. In glucose medium, the highest lactic acid produced was 1.161 g/100 ml medium by consuming 4.7656 g/100 ml glucose (32.48 % of the maximum yield). In glucose+$CaCO_3$ medium, the highest lactic acid produced was 1.8495 g/100 ml medium by consuming 10 g/100 ml glucose (24.66 %

of the maximum yield). It showed the weakness of direct fermentation. Many uncontrolled condition occurred, such as pH, temperature, and size of mycelial aggregate. Fermentation using fermentor might be a solution. Fermentor may result in a better process because of the well-controlled condition (pH and temperature) so that *R. oryzae* can grow well in fermentation medium. Controlling size of aggregate is also important due to the nutrient and oxygen transfer. Pellet form with small size is usually desired. Certain size of pellets can be obtained by immobilizing spores *R. oryzae* in certain materials, such as loofa sponge (Gangulyet al., 2007) or honeycomb matrix (Wang et al., 2010). Composition of fermentation medium and source of carbon, nitrogen, metal ion, and neutralizing agent are also potentially able to control *R. oryzae* mycelial growth (Liao et al., 2007).

Organic Acid Composition in Fermentation Medium

Organic acid analysis was carried out using HPLC. In glucose medium, maximum acid concentration was reached in 4 days fermentation with lactic acid concentration of 80.84 ppm. In glucose+$CaCO_3$ medium, maximum acid was attained at day 5 with lactic acid concentration of 450.01 ppm. This result showed that lactic acid concentration in fermentation medium with $CaCO_3$ addition was 5 times higher than that in glucose medium without $CaCO_3$. Either using titrable acidity or HPLC, lactic acid concentration in glucose+$CaCO_3$ medium was higher than which in glucose medium. The discussion was already mentioned in point 3.3.

Other organic acids were produced, such as fumaric acid with the concentration of 1.58 ppm and 1815 ppm in glucose medium and glucose+$CaCO_3$ medium, respectively. Citric acid was also detected in low concentration, which was less than 9.45 ppm in both medium. Considering this data, strain *R. oryzae* AT3 is also potential in producing fumaric acid.

CONCLUSION

Four isolates of *R. oryzae* were found in this research. *R. oryzae* AT3 showed the best acid production in BCP medium and when used in glucose fermentation, this isolate could produce lactic acid with concentration of 11.610 g/l and 18.495 g/l in glucose medium and glucose+$CaCO_3$ medium, respectively. Addition of $CaCO_3$ at initial fermentation potentially increased lactic acid concentration, 59.30% higher than without addition of $CaCO_3$.

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