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Enhancement of Lactic Acid Production from Kitchen Refuse by *Rhizopus oryzae* KPS 106 Immobilized on Loofa Sponge

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Kitchen refuse showed a good potential as substrate for lactic acid fermentation by *Rhizopus oryzae* in our previous study. For improved lactic acid production, immobilized cells of *R. oryzae* on loofa sponge (ICLS) were applied to exploit from kitchen refuse medium. Effects of size and conformation of loofa sponge were investigated in comparison with other carriers. ICLS stability and fermentation efficiency were also evaluated. Loofa sponge showed better immobilization than other carriers. Lactic acid yield was significantly increased ($p > 0.05$) with ICLS, $72.6 \pm 2.5\%$ (w/w) was obtained while $63.7 \pm 3.8\%$ (w/w) for free cells culture in shaking flask condition. The ICLS with $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ showed a great capability and successfully reused for few batches. The average lactic acid yield in 3 repeated batches was $73.3 \pm 1.9\%$ (w/w). After 72 h, lactic acid yield from $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ of ICLS (based on the amount of sugar consumed) with 69.2% (w/w) and 70.5 g/L of final concentration were obtained when conducted in 1-L of bioreactor under agitation speed of 100 rpm, aeration rate of 1.0 vvm and at 35 °C. These results demonstrate that lactic acid production from kitchen refuse medium by *R. oryzae* could be enhanced by immobilization on loofa sponge which is cheap and renewable material result in environment-friendly technology.

Key words: Immobilization, Kitchen refuse, Lactic acid, Loofa sponge, *Rhizopus oryzae*

1. Introduction

Lactic acid is an organic acid with a wide variety of industrial applications such as food, pharmaceutical, cosmetic and textile industries. Optically pure L(+)-lactic acid is important for the production of poly-lactic acid. The most effective way for L(+)-lactic acid syntheses is fermentation from renewable resources by microorganism. Certain types of microorganisms such as *Lactobacillus sp.* and *Rhizopus sp.* are capable to produce high concentration of lactic acid. Especially *R. oryzae* can produce not only high concentration but, high purity of L(+)-lactic acid from various substrate.

Previous studies in our work have proven that kitchen refuse after various pretreatments was successfully used as a substrate for lactic acid fermentation by *R. oryzae* KPS 106 [1]. However high amount of biomass leads to high viscosity of broth. It reduces the gas-liquid (GL) mass transfer and the homogeneity in fermentations [2]. This

phenomenon promotes *R. oryzae* to decrease lactic acid production. Therefore immobilization is well known technologies to solve these problems.

The big and heavy masses of mycelium can be reduced by immobilization, while oxygen mass transfer improved. It is easier to operate the bioreactor and separate the liquid medium from the fungus mycelia for recycle. Cell immobilization by passive cell adhesion on surface is an effective method to improve the efficiency of substrate utilization and productivities. But, carrier should be achieved with minimal additional cost and non-toxic. Loofa sponge is a highly porous and strong biomatrix, made of an open network of fibrous support from dry fruit of *Luffa cylindrica* [3]. It has been shown to be a very economical and excellent carrier for immobilizing fungus cells [4].

Currently, many researches have been increasing interests in lactic acid production by immobilized *R. oryzae* as shown in Table 1. Small cells pellets were investigated for produce lactic acid in different culture condition, 42 repeated batches in shaking flask condition throughout 22 days was demonstrated by Yang *et al.* [5]. By using air-lift

Table 1 Summary of lactic acid fermentation by immobilized *R. oryzae*.

Fermentation conditions	Substrate Glucose	Lactic acid yield (%)	Productivity (g/L/h)	Product concn. (g/L)	Operation period mode	References
Immob. cells in calcium alginate	Glucose	72	2.5	62	9 repeated batches	[10]
Immob cells with polymer supports prepared by gamma-ray	Glucose	71	0.7	50	Batch	[11]
Immob. cells with calcium alginate in fluidized bed	Glucose	65	1.6	73	Batch	[9]
Small cell pellets from growth on xylose	Glucose	62~74	2.9~6.2	60	42 repeated batches in 22 days	[5]
Immob. cells with polyurethane foam cubes in fluidized bed	Glucose	~75	~5	40	10 repeated batches in 4 days	[13]
Cotton-liked flocs on support in an air-lift reactor	Glucose	87	1.8	104.6	58 h batch	[12]
Small cell pellets in air-lift bioreactor	Glucose	-	1.1	85.7	9 batch cycles in 14 days	[6]
Cells on a rotating-disc contactor coupled with product separation by ion exchange	Glucose	70	62.5 (g/h/m ²)	60	6 batches in 15 days	[14]
Small cell pellets in a bubble column	Glucose	86	1.5 (g/h/g)	66	3 repeated batches	[7]
Small cell pellets in a bubble column	Glucose	88	2.6	83	32 h batch	[8]
Cells immob. on cotton cloth in a rotating bed	Corn starch	100	1.7 (300g/h/m ²)	127	Repeated batches and fed-batches	[15]
Cells immob. on loofa sponge in shaking flask	Kitchen refuse	72~75	1.0	72	3 repeated batches	This work
Cells immob. on loofa sponge in jar-bioreactor	Kitchen refuse	69	1.0	70	Batch	This work

bioreactor, 1.1 g/L/h was obtained from 9 batch cycles in 14 days [6]. And in bubble column bioreactor, 86% and 88% lactic acid yield were achieved in repeated batches [7] and batch culture [8], respectively. Alginate salts were used as the carrier for immobilization of *R. oryzae* and tested the stability in repeated batch [9,10]. Tamada *et al.* [11] investigated the feasibility of polymer support induced by

(γ -ray, the lactic acid yield of 71% was obtained from batch culture. The same operation of batch culture, 87% of lactic acid yield was obtained from glucose when using cotton-liked flocs on support in an air-lift bioreactor [12]. In 1996, Dong *et al.* [13] succeeded to immobilize cells *R. oryzae* with polyurethane cubes and apply in fluidized bed. The average of lactic acid yield in 10 repeated batches

was 75%. Lin *et al.* [14] reported that it was possible to produce lactic acid by *R. oryzae* grown on a rotating-disc contractor coupled with product separation by ion-exchange. Almost 100% of lactic acid was synthesized from corn starch by immobilized *R. oryzae* on cotton cloth in a rotating bed [15]. From all literature, the application of loofa sponge to immobilize *R. oryzae* for lactic acid fermentation has not been investigated. Thus the objectives of this

work were to determine the feasibility of using the loofa sponge as an immobilized support of *R. oryzae* by comparison with other carriers and reported effects of loofa sponge conformation on lactic acid production from kitchen refuse. The stability of immobilized cells for a prolonged period by repeated batch was also investigated. At last, scale up of using immobilized *R. oryzae* was also demonstrated in 1-L of bioreactor.

Table 2 Composition of kitchen refuse for saccharification.

Kitchen refuse fractions	Percentage (w/w, wet weight)	Total of percentage (w/w, wet weight)
Carbohydrates group		
Rice	31.5	43
Noodle	5.67	
Bread	0.73	
Pasta	5.07	
Proteins group		
Chicken	6.93	19
Fish	6.20	
Beef	2.67	
Pork	1.47	
Fried egg	1.73	
Vegetables and fruits group		
Lettuce	6.67	38
Long with radish	2.80	
Pumpkin	3.47	
Onion	1.60	
Cabbage	2.00	
Japanese Leek	0.73	
Bell pepper	0.67	
Corn	0.47	
Apple peel	2.60	
Carrot	2.47	
Orange peel	2.00	
Watermelon peel	0.52	
Melon peel	3.60	
Broccoli	4.47	
Lime	1.07	
Potato peel	0.33	
Enoki mushroom	0.73	
Seaweed (Wakame)	1.47	
Cherry	0.33	

2. Materials and Methods

2.1 Pretreatment of kitchen refuse

The kitchen refuse was collected from Yahata Royal Hotel (Kitakyushu, Fukuoka, Japan). The main items of kitchen refuse were carbohydrates, proteins and vegetables mixed with fruits. Each item was separated and weighed before saccharification as shown in Table 2. Percentage of each item for saccharification was 43%, 19% and 38% (w/w), respectively. Fig. 1 shows process flow of kitchen refuse pretreatment as reported by Praneetrattananon *et al.* [1]. Finally kitchen refuse syrup obtained was supplied as substrate for lactic acid fermentation in this work.

2.2 Fermentation

2.2.1 Microorganism and inoculum

R. oryzae KPS 106, mutative strain by UV irradiation [16] was used through out this study. This mutant strain was obtained from Kasetsart University (Bangkok, Thailand). The fungus was maintained on Difco potato-dextrose agar (PDA) slants, and transferred to fresh slants every week. Spores were produced on PDA after 8 days of culture at 35°C. They were collected in sterile distilled water containing 0.1% Tween 80 by platinum loop and filtrated through sterile cotton to eliminate mycelia. The spore suspension with 1×10^7 spores/mL was used as inoculum. The spore number in suspension was counted using a hemocytometer.

2.2.2 Culture medium

The composition of medium for lactic acid production contained the following (per liter): 100 g kitchen refuse syrup, 3.20 g $(\text{NH}_4)_2\text{SO}_4$, 0.30 g K_2HPO_4 , 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.04 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The pH of the media was not controlled and sterilized at 121°C for 15 min.

2.2.3 Immobilizing material and technique

The synthetic sponge of 2 types purchased from local stationary store, supplied for our study. Synthetic sponge type I and II were made from polyethylene glycol and polyvinyl alcohol, respectively. They were cut into cubic pieces of approximately $1.0 \times 1.0 \times 1.0 \text{ cm}^3$.

The reticulated loofa sponge was obtained by removing hard pericarp tissue of the ripened dried fruit of *Luffa cylindrica*. The fibrous sponge was cut into different size of cubic pieces approximately $0.5 \times 0.5 \times 1.0$, $1.0 \times 1.0 \times 1.0$ and $3.0 \times 3.0 \times 1.0 \text{ cm}^3$, respectively. Another pattern of loofa sponge was a single cylindrical loofa sponge (1.0 cm at height and about 3.0 cm at diameter).

Fig. 1 Pretreatment of kitchen refuse.

The immobilization of *R. oryzae* KPS 106 on loofa sponge were carried out as follows: The spore suspension (1.0 mL) was inoculated into 300-mL Erlenmeyer flasks containing 50 mL of the culture medium and loofa sponges. Each of these flasks contained with 1 g of loofa sponge or synthetic sponge type I or II (formation size with $0.5 \times 0.5 \times 1.0$ and $1.0 \times 1.0 \times 1.0 \text{ cm}^3$) as immobilizing matrix. In the case using $3.0 \times 3.0 \times 1.0 \text{ cm}^3$ and a single cylindrical loofa sponge, only one piece per flask was used as carrier. The inoculated flasks were incubated in rotary shaker 100 rpm at 35°C. After 24 h, 2.5 g of sterilized CaCO_3 was added to each flask as pH neutralizer and cultivation carried out until 72 h. Flasks with no loofa sponge in the medium for free cells, used as the control.

Table 3 Comparison of free cells and immobilized cells fermentation by *R. oryzae* KPS 106.

Yield [%]	Immobilized			Free cells
	Carrier type I	Carrier type II	Loofa sponge	
Sugar consumption	13.5 ± 6.5	7.9 ± 2.0	98.9 ± 1.5	99.4 ± 0.6
Glucose consumption	12.6 ± 3.8	6.9 ± 3.2	98.3 ± 2.0	99.3 ± 0.6
Succinic acid	0.5 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	0.5 ± 0.4
Lactic acid	5.2 ± 2.9	3.4 ± 3.4	72.6 ± 2.5	63.7 ± 3.8
Formic acid	0	0	0	0
Fumaric acid	0	0	0	0
Acetic acid	2.0 ± 0.7	1.2 ± 0.4	2.7 ± 1.0	2.6 ± 0.9
Ethanol	4.7 ± 2.7	2.4 ± 2.9	19.7 ± 3.9	21.4 ± 2.2

Mean ± standard deviations for $N = 5$

% Yield based on initial total sugar concentration

2.2.4 Repeated batch culture

Few batches were repeated to determine ICLS stability. On the completion of each cycle, the ICLS was washed with sterilized distilled water and transferred to a fresh medium for the next cycle, incubation followed as the last condition.

2.2.5 Batch culture in jar-bioreactor

The batch fermentations were performed in a 1-L jar-bioreactor with operating volume of 800 mL. Agitation speed, aeration rate and temperature were controlled at 100 rpm, 1.0 vvm and 35°C, respectively. Air was sterilized by passing through a membrane filter (0.2 µm). The pH of the medium was kept at 6.5 by the addition of 25% NH₃ solution.

2.3 Analytical methods

The concentration of lactic acid and other organic acids were determined by HPLC, using a Shim-pack SCR-102H column (Shimadzu, Japan). The eluent, 951 mg/mL of p-toluenesulfonic acid, was used at a flow rate of 0.8 mL/min. The column temperature was maintained at 40°C and used refractive index (RI) detector. Glucose concentration was enzymatically measured by the glucose oxydase-peroxydase enzyme (Glucose test kit, Toyobo, Japan). The amount of total sugar was measured by the phenol sulfuric acid method [17]. Concentration of ethanol was determined using an Ethanol test kit (Boehringer, Germany).

ICLS were collected to observe morphology with a scanning electron microscope, Hitachi S-3000N (Hitachi Science Systems, Ltd., Japan) after vacuum dried at 70°C for 12 h and platinum coating.

Fig. 2 Morphology of immobilized *R. oryzae* KPS 106 on loofa sponge of cubical formation (size $1.0 \times 1.0 \times 1.0 \text{ cm}^3$): (A) At 0 h; (B) At 24 h (after CaCO₃ addition); (C) At 72 h.

2.4 Statistical analysis

The standard error of the mean (SD) was calculated. Differences among means were tested for significance ($P < 0.05$) by Fisher's pairwise comparisons [18].

3. Results

3.1 Comparison of free cells and immobilized cells on lactic acid fermentation from kitchen refuse by *R. oryzae* KPS 106

A comparison of lactic acid production from kitchen refuse by free cells and immobilized cells of *R. oryzae* KPS 106 was shown in Table 3. Significant difference in lactic acid yield was observed. A little amount of lactic acid was produced when synthetic sponge type I and II used as immobilized carrier. Only 5.2 ± 2.9 and $3.4 \pm 3.4\%$ of lactic acid yield were obtained, respectively. The fungus showed poor growth on both synthetic sponges as can be seen from the sugar consumption results. On the other hand when *R. oryzae* KPS 106 immobilized on loofa

Fig. 3 Scanning electron micrographs of immobilized *R. oryzae* KPS 106 on loofa sponge (atmagnification 300 K ×): (A) Thread of loofa sponge view before; (B) After *R. oryzae* cells immobilized along loofa sponge fibers.

Fig. 4 Kinetics of lactic acid production from kitchen refuse by *R. oryzae* KPS 106.

(■ Sugar consumption by immobilized cells, □ Sugar consumption by free cells, ▲ Lactic acid production by immobilized cells, ● Lactic acid production by free cells, ○ Ethanol production by immobilized cells, △ Ethanol production by free cells)

sponge, sugar was completely consumed as same as free cells culture. The lactic acid yields with 72.6 ± 2.5 and $63.7 \pm 3.8\%$ were obtained from immobilized cells of *R. oryzae* on loofa sponge (ICLS) and free cells of *R. oryzae*, respectively. The morphology of ICLS during cultivation in shaking flask was shown in Fig. 2. No leakage of mycelia from ICLS was observed, as shown in Fig. 2C (cloudy flocculation in Fig. 2B, CaCO_3 was added as neutralizer).

3.2 SEM photographs of immobilized *R. oryzae* KPS 106 on loofa sponge

Representative scanning electron micrographs focusing single fiber of loofa sponge before and after the immobilization of *R. oryzae*, were presented in Fig. 3. The SEM micrograph clearly showed mycelia of *R. oryzae*

grew on the surface of loofa sponge. The mycelium was not localized at a single point but, covered whole surface on loofa fiber. They along grew the surface of the fibrous thread, eventually covered the entire surface of the loofa sponge, as shown in Fig. 3B.

3.3 Kinetics of lactic acid fermentation from kitchen refuse by immobilized cells on loofa sponge and free cells of *R. oryzae* KPS 106 in shaking flask

The kinetic profiles of lactic acid production from kitchen refuse by immobilized and free cells of *R. oryzae* KPS 106 were shown in Fig. 4. The lactic acid production rate of ICLS and free cells were same in the first 24 h. After that, lactic acid production rate of ICLS was higher than free cells. At the same condition, the maximum lactic acid concentration was obtained from ICLS with 72.1 g/L, while 63.7 g/L from free cells. Sugar was completely consumed and its consumption rate did not show any difference in both culture conditions. Ethanol was the main by-product. The ethanol production rate was same at both immobilization and free cells culture of *R. oryzae* KPS 106, about 20 g/L of ethanol was produced.

3.4 Repeated batch efficiency of immobilized *R. oryzae* KPS 106 on loofa sponge for lactic acid production from kitchen refuse

The results of repeated lactic acid fermentation by ICLS were shown in Table 4. As in the case with cubical size of $1.0 \times 1.0 \times 1.0 \text{ cm}^3$, the activity of *R. oryzae* cells was stable throughout 3 batches. The average lactic acid yield was 73.3 ± 1.9 , while using cubical loofa sponge with size $0.5 \times 0.5 \times 1.0 \text{ cm}^3$, the lactic acid yield was slightly decreased to second and third batch (from 74.9 ± 1.9 to 74.0 ± 2.6 to 71.3 ± 1.8). A comparison of the lactic acid production with other conformations by cutting the loofa sponge in square size $3.0 \times 3.0 \text{ cm}^2$ (thickness 1.0 cm), the average lactic acid yield was 70.3 ± 2.1 in 3 repeated

Table 4 Repeated batch efficiency of immobilized *R. oryzae* KPS 106 on loofa sponge for lactic acid production from kitchen refuse.

Formation of Loofa sponge	Batch No.	Total sugar consumption [%]	Glucose consumption [%]	Succinic acid yield [%]	Lactic acid yield [%]	Formic acid yield [%]	Fumaric acid yield [%]	Acetic acid yield [%]	Ethanol yield [%]
0.5×0.5×1.0 cm ³	1	98.3 ± 0.5	100	0.2 ± 0	74.9 ± 1.9	0	0	0.1 ± 0	20.3 ± 1.9
	2	98.1 ± 0.3	100	0.2 ± 0.1	74.0 ± 2.6	0	0	0.3 ± 0.2	20.8 ± 1.4
	3	98.1 ± 0.3	100	0.4 ± 0.2	71.3 ± 1.8	0	0	0.4 ± 0.2	23.3 ± 2.7
1.0×1.0×1.0 cm ³	1	98.1 ± 0.4	100	0.4 ± 0.1	73.4 ± 2.1	0	0	0.2 ± 0.1	19.4 ± 1.4
	2	98.2 ± 0.4	100	0.3 ± 0.1	72.9 ± 1.8	0.1 ± 0	0	0.3 ± 0.2	20.3 ± 2.5
	3	98.0 ± 0.3	100	0.3 ± 0.1	73.6 ± 2.0	0	0	0.4 ± 0.1	21.1 ± 2.9
3.0×3.0×1.0 cm ³	1	98.1 ± 0.4	100	0.4 ± 0.1	72.3 ± 2.3	0	0	0.4 ± 0.2	19.6 ± 2.7
	2	98.3 ± 0.4	100	0.4 ± 0.1	70.6 ± 2.3	0	0	0.4 ± 0.1	20.6 ± 1.6
	3	98.1 ± 0.4	100	0.4 ± 0.1	67.9 ± 1.7	0	0	0.5 ± 0.2	21.2 ± 1.7
Cylinder with 1.0 cm length	1	98.2 ± 0.5	100	0.3 ± 0	74.6 ± 1.4	0	0	0.3 ± 0.1	20.2 ± 2.1
	2	98.4 ± 0.3	100	0.4 ± 0.1	70.8 ± 1.7	0	0	0.4 ± 0.1	20.3 ± 1.7
	3	98.1 ± 0.7	100	0.4 ± 0.1	70.0 ± 1.6	0	0	0.4 ± 0.2	19.9 ± 2.4

Mean ± standard deviations for *N* = 5

% Yield based on initial total sugar concentration

Fig. 5 Kinetics of lactic acid fermentation from kitchen refuse by *R. oryzae* KPS 106 in jar-bioreactor.

(■ Total sugar, □ Lactic acid, △ Succinic acid,
○ Acetic acid, ▲ Ethanol)

batch culture. The lactic acid yield was gradually decreased from $72.3 \pm 2.3\%$ in the first to $67.9 \pm 1.7\%$ in the third batch. The similar results were obtained when used cylindrical sponge length with 1.0 cm as carrier for immobilization. In this case, the average lactic acid yield was $70.5 \pm 1.6\%$ and lactic acid yield was decreased from $72.6 \pm 1.4\%$ in the first batch to $68.0 \pm 1.6\%$ in the third batch. From visual observation of all conditions, the cells continued to grow and increase on the sponge matrix during cultivation, without mycelium release from the loofa sponge matrix. According to these results, $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ loofa sponge was used for the further experiment.

3.5 Kinetics of lactic acid fermentation from kitchen refuse by *R. oryzae* immobilized on loofa sponge in jar-bioreactor

The time course of the sugar consumption and lactic acid production by ICLS in jar-bioreactor were shown in Fig. 5. After 72 h, sugar was completely consumed, accumulating 70.5 g/L of lactic acid concentration. By-products were succinic acid, acetic acid, fumaric acid and ethanol as same as flask condition. Lactic acid production rate was calculated to be 1.0 g/L/h; and the yield, 69.2%.

4. Discussion

ICLS had some positive influence on increasing lactic acid production from kitchen refuse and showed an excellent carrier for immobilization of *R. oryzae* when compared with other carriers in this study, as data presented in Table 3. It showed significant difference ($P < 0.05$) in the lactic acid production by ICLS and free cells culture. By ICLS, the lactic acid yield reached to $72.6 \pm 2.5\%$ (w/w), while $63.7 \pm 3.8\%$ (w/w) from free cells culture. In both synthetic sponges, *R. oryzae* grew very poor and a little amount of lactic acid was produced. Some toxic chemicals leached from synthetic sponge during sterilization might affect. As Wada *et al.* [19] proposed about toxicity associated with synthetic polymeric materials to immobilize yeast cells.

Immobilizing technique by entrapping cells in polymer

Fig. 6 SEM photographs of *R. oryzae* grown on the intervold matrix of loofa sponge with different size of cubical formation: (A) $0.5 \times 0.5 \times 1.0 \text{ cm}^3$; (B) $1.0 \times 1.0 \times 1.0 \text{ cm}^3$; (C) mycelium of *R. oryzae* grew on the network of $0.5 \times 0.5 \times 1.0 \text{ cm}^3$ ICLS in 2nd repeated batch culture and (D) mycelium of *R. oryzae* grew on the network of $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ ICLS in 2nd repeated batch culture (Magnification : (A) (B); 50 K $\times$ and (C) (D); 300 K $\times$).

gels such as alginate beads, carrageenan and polyurethane foam cubes have been reported about leakage during lactic acid fermentation [20]. Conversely, there were no free mycelia releasing from ICLS or grew on other surfaces of the flask as shown in Fig. 2. And scanning electron micrographs in Fig. 3 showed that all *R. oryzae* mycelia attached on the surface of loofa sponges.

The kinetics of lactic acid production by ICLS and free cells of *R. oryzae* showed no significant differences in sugar consumption rate and ethanol production rate only except lactic acid production rate, as presented in Fig. 4. Higher lactic acid yield by ICLS as compared to free cells was due to decline of shear stress and increase of surface area that enhancing oxygen transfer in fermentation broth. Since Du *et al.*, [7] reported that *R. oryzae* required high oxygen transfer for lactic acid production. The similar results of high ethanol production by ICLS and free cells of *R. oryzae* (about 20 g/L) were probably because of the complex composition of kitchen refuse medium. This observation was consistent with the finding of Taherzadeh *et al.* [21]. They concluded that ethanol yield produced by *R. oryzae* was high in rich media.

From the Table 3, these results suggested that surface area of loofa sponge had effect on lactic acid production by *R. oryzae* KPS 106 in repeated batch culture. The lactic acid yield was stable and high in 3 repeated batches when ICLS of cubical formation with size $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ was

used, while in the case of using sliced and cylindrical loofa sponge, lactic acid yield was slightly decreased in the second and third batch of repeat batch culture. We explained that surface area of fungus exposed to medium was extended when small cubic of loofa sponge was used, compared with sliced and cylindrical loofa sponge.

ICLS with size $0.5 \times 0.5 \times 1.0 \text{ cm}^3$ was smaller than $1.0 \times 1.0 \times 1.0 \text{ cm}^3$, but lactic acid production in repeated batch culture were not same. It decreased from 74.9 ± 1.9 to 74.0 ± 2.6 to $71.3 \pm 1.8\%$ (w/w) in first to second to third batch of repeated culture, respectively. The possible reason for explain should be the diffusive limitation of nutrients and oxygen at the deep mycelium inside the matrix, whereas the dense mycelium which grew on $0.5 \times 0.5 \times 1.0 \text{ cm}^3$ of ICLS piece was plenty and compact than $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ of ICLS, as illustration by scanning electron microscope shown in Fig. 6, resulted in prevention of nutrient and oxygen diffusion. This dilemmatic phenomenon was similar with lactic acid production by immobilized *R. oryzae* on cotton cloth in repeated batch fermentation [15]. Although a longer period of fermentation might be required to determine the stability of this process, the fermentation was stopped after three batches because of a limited sample supply. However, it has previously been reported that cells immobilized on loofa sponge were stable for more than 35 batches in repeated batch processing of ethanol production [22].

When lactic acid production from kitchen refuse medium by ICLS was carried out in 1-L of jar-bioreactor, the lactic acid yield was relatively low when compared with the shaking flask condition. This might be due to impeller of jar-bioreactor damaged and fragmented ICLS. High local shearing forces, present especially in the regions near impellers, may prevail over the tensile strength of ICLS. Therefore, the newly formed hyphal tips of ICLS did not grow, which results in decreasing lactic acid fermentation. Based on these results, we suggested that gentle mixing and excellent circulation system of bubble column or airlift bioreactor will be more suitable for lactic acid fermentation by ICLS.

For enhanced lactic acid production, loofa sponge was also applied as the skeletal support to immobilize *Lactococcus lactis* spp. *lactis* [23]. The average lactic acid yield and productivity from raw cassava starch by simultaneous saccharification and fermentation with immobilized *Aspergillus awamori* and *Lactococcus lactis* in a loofa sponge were 0.76 g lactic acid/g starch and 1.6 g lactic acid/ (L·h), respectively at 120 - 130 g/L lactic acid concentration in repeat batch culture. Type of microorganism might be the reason of high lactic acid production because, lactic acid production by bacteria are generally 90% or greater [24]. While lactic acid production from kitchen refuse medium by immobilized *R. oryzae* was $73.3 \pm 1.9\%$ (w/w) since high ethanol production as main by-product was induced. This is due to some component included in rich and complex composition of kitchen refuse medium. However, among all literatures of lactic acid fermentation by immobilized *R. oryzae* published so far, this work will be the first report to use *R. oryzae* immobilized on loofa sponge to improve lactic acid production. ICLS provided many advantages over conventional fermentation process. The simple and cheap immobilization method, reuse of fungal cells for long-term period and easy separation of cells from fermentation broth were achieved to provide a new economical process for lactic acid production.

5. Conclusions

The above results have demonstrated that loofa sponge is an effective carrier for immobilized *R. oryzae* KPS 106 mycelia to improve lactic acid production from kitchen refuse. In addition, ICLS could be reused at least for a few cycle of lactic acid fermentation. The high performance was shown when using cubic form of loofa sponge with size of $1.0 \times 1.0 \times 1.0 \text{ cm}^3$ as carrier. The average lactic

acid yield from 3 repeated batches was $73.3 \pm 1.9\%$ (w/w). No leaking free cells or breakage of ICLS was found during repeated culture without diminishing lactic acid production. When lactic acid production from kitchen refuse by ICLS was carried out in 1-L of jar-bioreactor, 69.2% of lactic acid yield lower than of shaking flask condition was obtained after 72 h batch culture. For reaching maximum lactic acid production from kitchen refuse by ICLS, proper bioreactor that enables tender mixing and high efficiency of air circulation such as bubble column and airlift bioreactor shall be extended.

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◆◆◆ 和文要約 ◆◆◆

ヘチマ担体に固定化した *Rhizopus oryzae* KPS106 株による 食品ゴミからの乳酸発酵

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食品ゴミなどの廃棄物系バイオマスから、糖化→乳酸発酵→乳酸精製→重合の工程を経て、生分解性プラスチックであるポリ乳酸を製造する研究を行っている。ポリ乳酸の品質には、乳酸の光学純度が大きく影響する。*Rhizopus oryzae* は、選択的に高い L(+)-乳酸生産能を示すことが知られている。前報で、食品ゴミを基質として、*Rhizopus oryzae* KPS106 株を用いて、高濃度ならびに高純度の L(+)-乳酸を生産出来ることを報告した。

本研究では、*Rhizopus oryzae* KPS106 株を用いる食品ゴミを基質とした L(+)-乳酸発酵において、固定化による生産性向上について検討した。固定化担体として、廉価に入手かつ再生可能なバイオマスであるヘチマに着目した。まず、ポリビニルアルコール (PVA) やポリウレタン (PU) 製の担体との比較を行った。また、ヘチマ固定化担体について、形状やサイズの影響、さらに、繰り返し利用時の安定性についても検討した。さらに、フラスコ培養と 1 L のリアクターを用いた培養時の基質と代謝産物に関する速度論的解析を行った。

PVA や PU 製のスポンジへの固定化と比較して、高い乳酸収率を示した。これは、ヘチマ担体において、良好に酸素および基質が供給されたためと考える。また、菌

体の遊離もみられないことから、ヘチマスポンジは、*R. oryzae* の固定化に適した担体であることが示唆された。固定化担体としてのヘチマの形状やサイズについて検討したところ、表面積の大きい 1 cm 角の立方体状としたものが、最も高い乳酸収率を示した。フラスコによる回分振とう培養の結果、固定化を行わない場合には、 $63.7 \pm 3.8\%$ (w/w) の乳酸収率であるのに対して、 $72.6 \pm 2.5\%$ (w/w) とより高い乳酸収率が得られた。さらに、ヘチマ固定化菌体を 3 回の回分培養で繰り返し利用できることを確認した。3 回の回分培養における乳酸収率は、平均 $73.3 \pm 1.9\%$ (w/w) であり、繰り返しにより乳酸収率が大きく損なわれることはなかった。1 cm 角のヘチマ固定化担体を用いて、1 L のジャーファーマンターによる培養（攪拌速度 100 rpm, 通気速度 100 vvm, 温度 35°C）を行ったところ、乳酸収率は、69.2% (w/w) であり、72 時間後の到達乳酸濃度は、70.5g/L であった。

以上のとおり本研究の結果から、ヘチマ担体への *R. oryzae* の固定化が、食品ゴミを基質とした L(+)-乳酸発酵の生産性向上に有効であることが明らかとなった。