

Production of Propionic Acid for Antifungal Activity by Calcium alginate Immobilization of *Propionibacterium acidipropionici* TISTR 442 Using Whey as Substrate

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ABSTRACT

The improvement of propionic acid production, as fermenting by calcium alginate immobilized cells of Propionibacterium acidipropionici TISTR 442 and using whey as substrate was investigated. The highest amount of propionic acid was produced by adjusting the distance between the tip of the tube and the surface of CaCl₂ solution to be 4 - 6 cm and the flow rate of the gel solution to be 7 ml/min. Fermentation by immobilized cells in 2-l fermentors using 1% CaCO₃ and 5 N KOH to control the pH at 6.5 gave the highest amount of propionic acid 29.24 g/l and consistent potential to recycle 2 rounds of fermentation by producing the total amount of 15.85±0.25 g/l and 13.39±0.25 g/l propionic acid from Batch 1 and Batch 2 fermentation, respectively. Which one amount of propionic acid was higher than that from free cells in 2-l fermentors at 216 h of the fermentation time. The fermented propionic acid as well as the commercial propionic acid from chemical processes were able to inhibit the growth of the fungal tested.

Key words: Immobilization, Whey, *Propionibacterium acidipropionici*, Antifungal activity

INTRODUCTION

The demand of consumers for natural preservatives has caused an interest in using the preserving capacity of bacteria, naturally occurring in food and feeds (Lind et al., 2005). Propionic acid, the biopreservative produced by *Propionibacterium* sp., is able to inhibit the growth of molds, bacteria and dairy-spoilage yeasts such as *Zygosaccharomyces bailii* and *Candida* sp. (Schwenninger and Meile, 2004). In addition, propionic acid and its salts are used as raw materials in perfume and plastic industries (Goswami and Srivastava, 2000).

Commercial production of propionic acid is chiefly carried out by chemical synthesis which is an expensive recovery process and harmful chemicals can occur. If higher yields of propionic acid are obtained, production by fermentation will become economically competitive and will offer several advantages over chemical synthesis (Himmi et al., 2000). The advantages include the ability to label the product as a “natural preservative” which responds to the consumer’s demands and the fermentation process that harmful chemicals will not be released.

Several processes have been patented for producing propionic acid by fermentation. Batch method using a variety of substrates produced 1 to 3% propionic acid in 7-14 days (Playne, 1985 ; Blanc and Goma, 1989) and other processes such as fed-batch and continuous system of the fermentation have been used to improve the yield of propionic acid. However, the major factor that limits the production of propionic acid during fermentation is the end-product inhibition by acid (Woskow and Glatz, 1991). Therefore, immobilized-cell reactors have been developed to protect cells from the end-product inhibition which could enhance the yield and productivity of the fermentation.

Immobilized cells have been used for production of organic acids, amino acids, antibiotics, enzymes and other compounds. By using immobilized cells, the process can be controlled much easier than using a batch system of free cells. In addition, immobilized cells are more stable than free biomass cells and the product separation is easier (Ates et al., 2002).

In this investigation, the fermentation by immobilized cells of *P. acidipropionici* TISTR 442, using whey as substrate, was studied in order to enhance the yield of propionic acid by reusing the immobilized cells (Jianlong, 2000) and using whey, the inexpensive substrate which was the by-product from dairy industry, in order to increase its commercial value.

MATERIALS AND METHODS

Microorganisms

Propionibacterium acidipropionici TISTR 442, *Aspergillus flavus* TISTR 3365, *A. niger* TISTR 3012, *A. oryzae* TISTR 3014, *Rhizopus* sp. TISTR 3024, *Penicillium* sp. TISTR 3046, *Fusarium moniliforme* TISTR 3175, *Trichoderma* sp. TISTR 3327, *Candida albican* TISTR 5239, *C. sake* TISTR 5143, *Pichia membranefaciens* TISTR 5072, *Saccharomyces cerevesiae* TISTR 5017, *Zygosaccharomyces rouxii* TISTR 5044, *Kluyveromyces marxianus* TISTR 5163 and

Rhodotorula glutinis TISTR 5159 were from TISTR Culture Collection Bangkok, Mircen, Thailand.

Propionibacterium acidipropionici TISTR 442 was grown statically on de Man Rogosa and Sharpe (MRS) broth at 30°C for 48 h and an inoculum of 5% (v/v) was used.

Culture conditions

5% (v/v) of the seed culture was transferred to a 250-ml flask containing 175-ml of each of the following six cultures;

- MRS broth (HiMedia Laboratories Pvt. Ltd. India).
- MRS broth plus 1% CaCO₃.
- Modified broth (modified from Gu et al., 1998 ; Goswami and Srivastava, 2000) containing 10 g/l yeast extract, 40 g/l lactose, 5 g/l trypticase soy broth, 0.25 g/l K₂HPO₄, 0.2 g/l MgSO₄.7H₂O and 0.05 g/l MnSO₄.4H₂O.
- Modified broth containing 10 g/l yeast extract, 40 g/l lactose, 5 g/l trypticase soy broth, 0.25 g/l K₂HPO₄, 0.2 g/l MgSO₄.7H₂O, 0.05 g/l MnSO₄.4H₂O and 1% CaCO₃.
- Whey (from Minor Dairy's Factory, Thailand) broth dissolved with 10 g/l yeast extract, 0.25 g/l K₂HPO₄, 0.2 g/l MgSO₄.7H₂O and 0.05 g/l MnSO₄.4H₂O.
- Whey broth dissolved with 10 g/l yeast extract, 0.25 g/l K₂HPO₄, 0.2 g/l MgSO₄.7H₂O, 0.05 g/l MnSO₄.4H₂O and 1% CaCO₃.

Before inoculation of the seed culture, pH of the medium was adjusted to 6.5-7, then heat-sterilized at 121°C and 15 psi for 15 min (without lactose). Lactose was autoclaved separately and added aseptically to the medium. Samples were taken from cultures every 24 h until the 336 h to analyse for propionic acid by high performance liquid chromatography (HPLC : SHIMADZU C-R7 AE PLUS, JAPAN)

Cell Immobilization

The seed culture (final absorbant ≈ 0.5 with a total population of 7.50×10^6 cfu ml⁻¹) was centrifuged at 10,000 g for 10 min and the spun broth was decanted. Pelleted cells were resuspended in sterile 0.85% NaCl solution and re-centrifuged. After the NaCl solution was decanted, the pelleted cells were mixed with sterile 0.85% NaCl solution and 2% sterile sodium alginate at a volumetric ratio of 10:3:20 (cells:saline:alginate). The mixture was extruded with a peristaltic pump through the tube into 0.1 M CaCl₂ solution to form beads. The beads were suspended in 0.1 M CaCl₂ solution at 4°C for 4 hours and were washed thoroughly twice with sterile distilled water before being used.

Distance optimization

The immobilized cells with various distances of 2, 4, 6, 8, 5, 10, 15 and 20 cm from the end of the tube to the surface of CaCl₂ solution were investigated for gel formation and the optimal distance was selected for further studies.

Flow rates optimization

Immobilized cells on the selected distance were experimented with various flow rates of the gel solution at 5, 6 and 7 ml/min, and the optimal flow rate was selected.

Fermentation and cells recycling

The content of the 2-l fermentor containing 1.4 liters of selected medium was maintained at a constant pH of 6.5, 30°C and 100 rpm agitation. Samples were taken from the culture every 24 h until at 216 h. when the medium was then drained out of the fermentor and a fresh medium was added to the beads before the repeated batch fermentation was started.

Antifungal activity

Propionic acid was extracted from the culture (Gu et al., 1998) and a comparison test with commercial propionic acid for antifungal effect by agar disc diffusion method was then performed (Faria et al., 2006). Concentration of 10^6 spores/ml solutions of tasted mold and 10^6 cells/ml solutions of yeast were prepared. One hundred μ l of each of the solutions were pipetted and spread on sterile petri dishes containing potato-dextrose-agar (PDA) medium. One hundred μ l of the fermented and the commercial propionic acid were then pipetted onto sterile Whatmann No. 1 paper discs which were in turn placed on the center of the PDA agar plates inoculated with a fresh fungal spores or cell suspension (one microorganism per Petri dish). The Petri dishes were then incubated at 30°C for 4 days. The degree of inhibition was measured as the inhibited growth area of the Petri dish. Distilled water instead of the two types of propionic acid was used as a control.

Assay methods

The supernatants of the samples which had been 20 min at 5,000 g centrifuged samples were detected by HPLC and the propionic acid was quantified by filtering the samples through 0.45 μ m cellulose membranes in an Inertsil C8-3 column. The wave length of the UV detector was 210 nm and the flow rate was 0.1 mm/min. The total sugar was determined by the Dubois's process (Dubois et al., 1956).

Statistical analyses

All fermentation experiments were run in triplicate. Statistical analyses were performed by Duncan's New Multiple Range Test while antifungal activity was performed by Pair Sample T-Test.

RESULTS AND DISCUSSION

Typical medium and acid production

Propionic acid production by *Propionibacterium acidipropionici* TISTR 442 grown in 6 cultures are shown in Figure 1. In whey broth added with 1% CaCO₃, *P. acidipropionici* TISTR 442 produced the highest amount of propionic acid, 14.26±0.16 g/l (Table 1) with the reason that whey is composed of nutrient supplement and high lactose which are necessary for propionic acid production. These results were similar to those of Yang et al., (1994) who studied the continuous production of propionate from whey lactose by *P. acidipropionici* ATCC 4875, immobilized in a fibrous bed bioreactor, and it was found that 2% (w/v) propionic acid was obtained from 4.2% lactose fed at a retention time of 35-45 hours and the propionic acid yield was 46% (w/v) from lactose.

It was also found that in the comparisons of the media added with 1% CaCO₃ and without CaCO₃, the media added with 1% CaCO₃ had more acid occurred than that without CaCO₃ with the reason that CaCO₃ can reduce the acidity of the accumulated propionic acid in broth, therefore, free cells of *P. acidipropionici* could grow and produce propionic acid continuously. Because of having higher productivity, whey broth added with 1% CaCO₃ was selected for fermentation in the next step. Calculations of the propionic acid used for statistical analyses on various broths are shown in Table 1. The concentration of propionic acid from whey broth added with 1% CaCO₃ was significantly higher than that of the other broths at 95% confidence level.

In addition, whey contains high lactose which results in more products due to the reason that lactose was fermented to become propionic acid by the propionibacteria. Using whey as substrate reduced the cost and increased the values of the by-products from dairy industry.

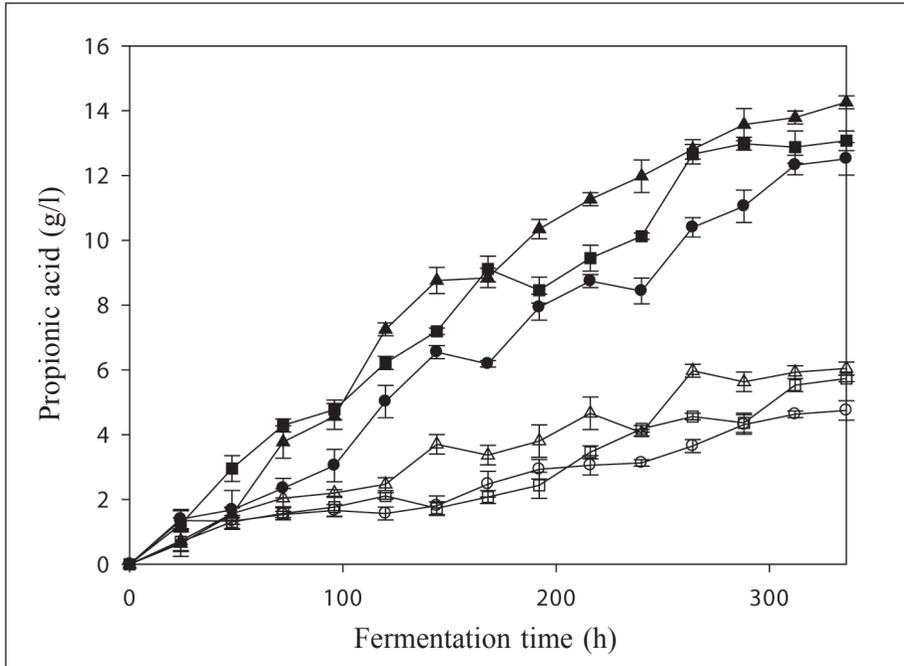


Figure 1. Propionic acid production by free cells of *P.acidipropionici* TISTR 442 on various broths at 336 h:

(▲) whey broth added with 1% CaCO₃, (■) modified broth added with 1% CaCO₃, (●) MRS broth added with 1% CaCO₃, (Δ) whey broth, (□) modified broth, (○) MRS broth

Table 1. Comparisons of various broths for propionic acid production and for total sugar consumption at 336 h

Type of broth	Propionic acid (g/l)	Total sugar consumption (%)
MRS	4.75 ^d ± 0.25	74.13
MRS+CaCO ₃	12.52 ^b ± 0.41	94.23
modified	5.73 ^c ± 0.08	75.22
modified+CaCO ₃	13.0 ^b ± 0.33	98.79
whey	6.04 ^c ± 0.16	75.29
whey+CaCO ₃	14.26 ^a ± 0.16	98.96

Batch fermentation of free cells was performed in 250-ml flasks with a 175-ml working volume at a static state of 30°C for 336 h. Data are presented as means of three replications ± standard deviations

Batch fermentation by free cells of *P. acidipropionici* TISTR 442 in 2-l flasks and 2-l fermentors

Propionic acid production and total sugar consumption by free cells of *P. acidipropionici* TISTR 442 in 2-l flasks and 2-l fermentors are shown in Figure 2. After 216 h fermentation, the acid in the fermentors was nearly in a static level due to the lack of total sugar. However, slightly slow cell growth in the flasks and the total sugar are remained and the propionic acid was therefore produced continuously in the flasks. In the 2-l flasks, free cells gave 14.33 ± 0.25 g/l propionic acid at 316 h. However, more propionic acid (15.16 ± 0.24 g/l) in the 2-l fermentors was produced in a more rapid time (216 h) with a pH of 6.5 maintained with KOH. Agitation was done in order to mix the cultures and the microorganisms occurred through fermentation and made up an optimal condition for propionic acid production. Champagne et al., (1989) studied about whey fermentation by immobilized cells of *P. shermanii* and found that agitation increased propionic acid fermentation rates but lowered the ratio of propionic acid to acetic acid.

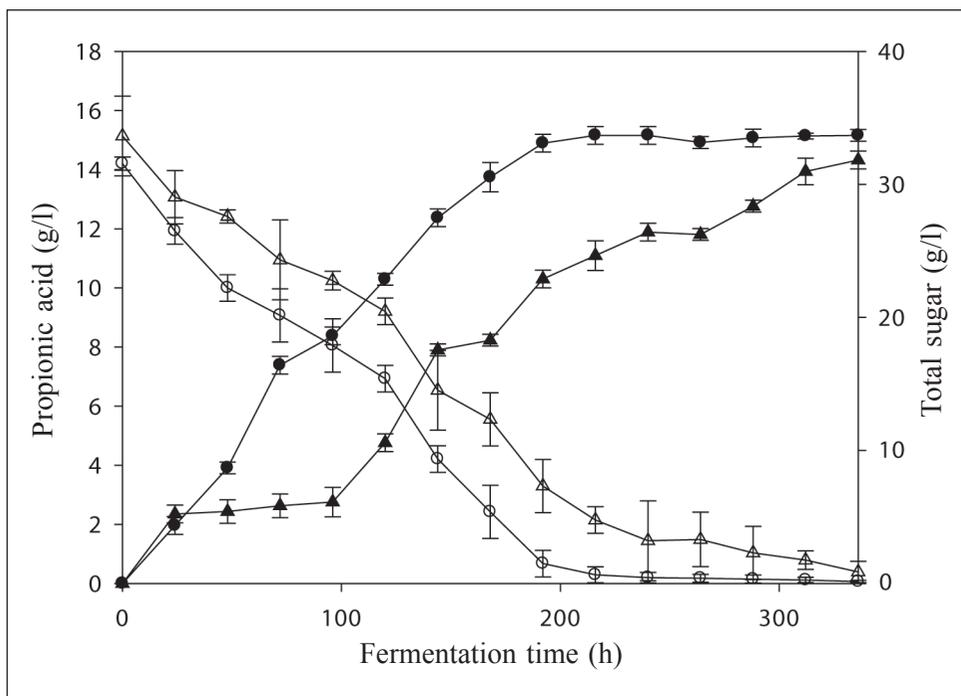


Figure 2. Comparisons of propionic acid production and total sugar consumption in 2-l fermentors and 2-l flasks by free cells of *P. acidipropionici* TISTR 442 : (▲) propionic acid in 2-l flasks, (●) propionic acid in 2-l fermentors, (Δ) total sugar in 2-l flasks, (○) total sugar in 2-l fermentors

Optimal conditions of gel formation

The conditions of gel formation were studied by varying the distance from the end of the tube to the surface of the CaCl_2 solution in order to determine the optimal values. Propionic acid productions by immobilized cells in the distances of 4-6 cm from the end of the tube to the surface of CaCl_2 solution were significantly higher than those at other distances at 95% confidence level (Table 2). The propionic acid production was significantly decreased when the distance increased.

Table 2. Comparisons of distances from the end of the tube to the surface of CaCl_2 solution on immobilization for propionic acid production and for total sugar consumption

Distance (cm.)	Propionic acid (g/l)	Total sugar consumption (%)
2	17.68 ^{bc} ± 0.49	98.95
4	18.37 ^a ± 0.16	99.2
5	18.53 ^a ± 0.15	99.26
6	18.49 ^a ± 0.32	99.22
8	18.23 ^{ab} ± 0.16	98.93
10	17.38 ^c ± 0.24	98.9
15	17.20 ^{cd} ± 0.09	98.87
20	16.67 ^d ± 0.48	98.74

Batch fermentation of immobilized cells was performed in 250-ml flasks with a 175-ml working volume at a static state of 30°C for 336 h. Data are presented as means of three replications ± standard deviations

Fixing flow rates of gel solutions at 5, 6 and 7 ml/min produced 18.97±0.25, 19.19±0.08 and 19.22±0.15 g/l propionic acid, respectively (Table 3). The highest propionic acid was produced by controlling the flow rate of the gel solution to be 6 or 7 ml/min. Although the productions of propionic acid at the 6 and 7 ml/min flow rates of the gel solution were not significantly different at 95% confidence level, the 7 ml/min flow rate which was faster saved up time in the immobilization process. Therefore, the fixing of flow rate of the gel solution at 7 ml/min was selected as the optimal condition of gel formation in further investigations.

Table 3. Comparisons of flow rates of gel solutions on immobilization for propionic acid production and for total sugar consumption

Flow rate (ml/min)	Propionic acid (g/l)	Total sugar consumption (%)
5	18.97 ^b ± 0.25	97.54
6	19.19 ^a ± 0.08	97.66
7	19.22 ^a ± 0.15	97.68

Batch fermentations of immobilized cells were performed in 250-ml flasks with a 175-ml working volume at a static state of 30°C for 336 h. Data are presented as means of three replicate ions ± standard deviations

Fermentation by immobilized cells of *P. acidipropionici* TISTR 442 in 2-l fermentors supplemented with 5 N KOH and without KOH

The fermentation of immobilized cells of *P. acidipropionici* TISTR 442 in 2-l fermentors, added with 1% CaCO₃ but without KOH, gave 15.1 ± 0.08 g/l propionic acid and 98.80% total sugar consumption at 336 h while that in 2-l fermentors, added with 1% CaCO₃ and 5 N KOH, gave 15.85 ± 0.25 g/l propionic acid and 98.54% total sugar consumption at 192 h. After 192 h incubation, the propionic acid did not increase due to the lack of total sugar in the culture. Therefore, it could be said that the maximum propionic acid in the fermentors occurred at 192 h. When fermentations in the two conditions of the fermentors were completed, it was found that the fermentation in the 2-l fermentors with 1% CaCO₃ and 5 N KOH gave more propionic acid than that in the 2-l fermentors added only with 1% CaCO₃ (Figure 3).

The total sugar of the 2-l fermentors, with pH controlled by 1% CaCO₃ and 5 N KOH, decreased more than that of the 2-l fermentors with pH controlled by only 1% CaCO₃ because by using both of 1% CaCO₃ and 5 N KOH, the controlled pH was constant at 6.5 which is optimal for propionic acid production from whey lactose by immobilized cells of *P. acidipropionici* (Yang et al., 1994). Therefore, propionibacteria would continue to grow and produce propionic acid. Moreover, the constant pH would reduce the microorganism inhibition from the acid which is the product from propionibacteria.

Yang et al., (1994) studied on propionic acid from whey by immobilized cell of *P. acidipropionici* found that produced propionic acid 20 g/l in 55 h which similar to this study.

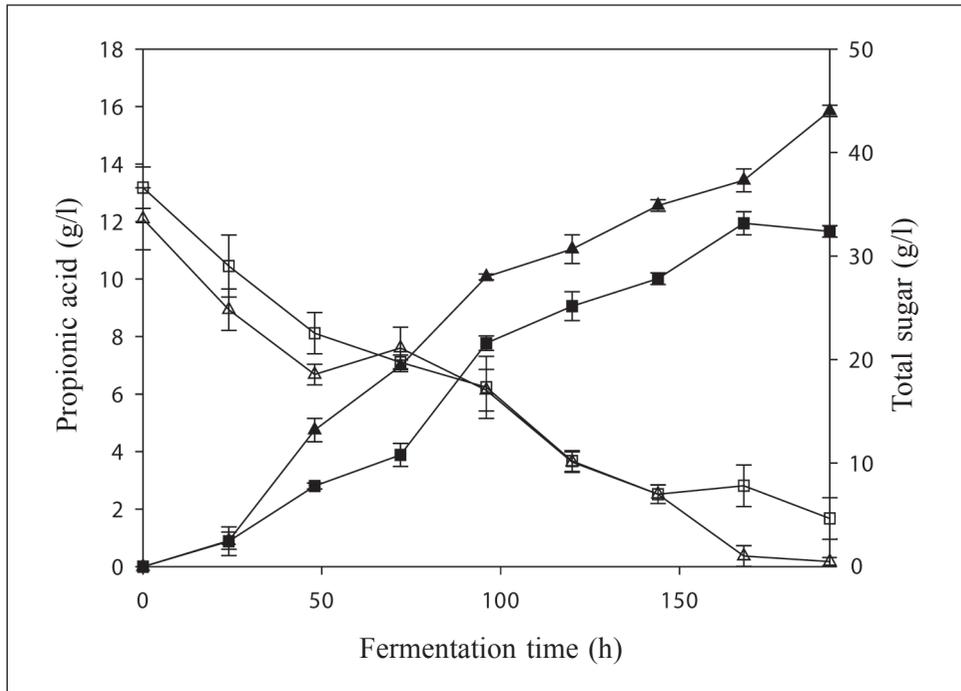


Figure 3. Comparison of propionic acid production by immobilized cells of *P. acidipropionici* TISTR 442 in 2-l fermentors with and without 5 N KOH added at 192 h : (▲) Propionic acid by immobilized cells in 2-l fermentors with KOH added, (■) Propionic acid by immobilized cells in 2-l fermentors without KOH added, (△) Total sugar by immobilized cells in 2-l fermentors with KOH added, (□) Total sugar by immobilized cells in 2-l fermentors without KOH added

Cell recycling of fermentation by immobilized cells of *P. acidipropionici* TISTR 442 in 2-l fermentors

Figure 4 shows propionic acid production in 2-l fermentors at 216 h. In the first cycle, maximum propionic acid, 15.85 ± 0.25 g/l, was produced by immobilized cells at 192 h which was higher than that produced by free cells (15.16 ± 0.24 g/l at 216 h.). Therefore, it was shown that immobilized cells enhanced more propionic acid production than free cells.

In the second cycle, propionic acid production decreased with a value of 13.39 ± 0.25 g/l at 216 h and the beads collapsed and no fermentation could occur in the next cycle because the beads became less resistant to the acidity of the accumulated propionic acid. In addition, K^+ from KOH would have replaced Ca^{2+} which decreased gel stability (Klinkenberg et al., 2001). Damirel et al., (2005) studied about the citric acid production by immobilized *Aspergillus niger* A-9 and found that the citric acid production decreased when numbers of reused immobilized *A. niger* cells increased and this process could be repeated 4 days per fermentation. Suwannakham and Yang (2005) compared the fed-batch

and free cells fermentations of glucose by *P. acidipropionici* ATCC 4875 in a fibrous-bed bioreactor (FBB) and found that the FBB culture produced 20-59% more propionate than that by free cells fermentation.

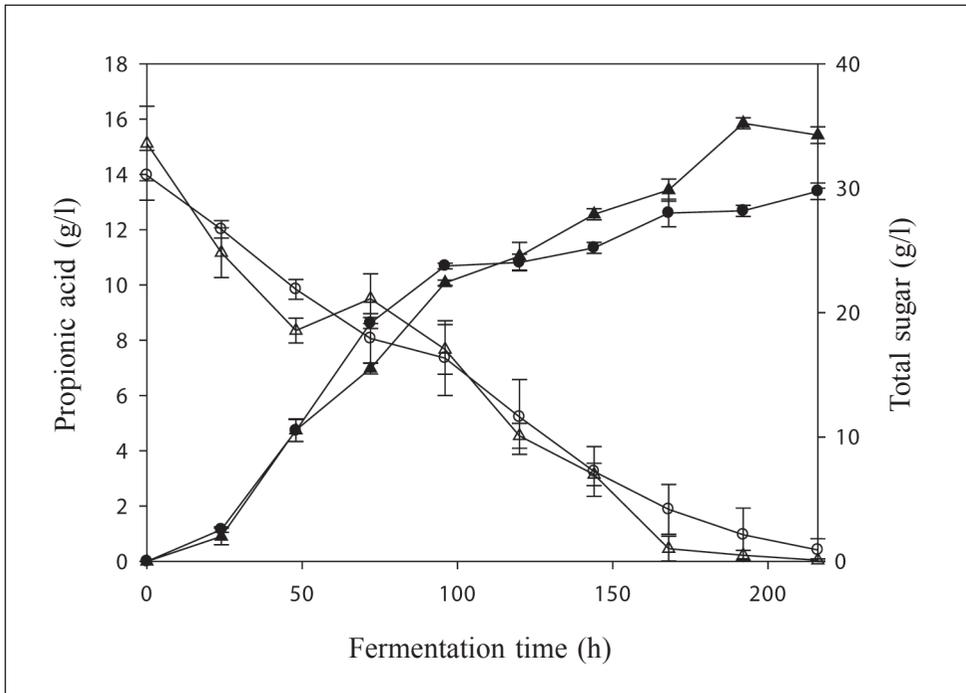


Figure 4. Cells recycling of propionic acid production by immobilized cells of *P.acidipropionici* TISTR 442 in 2-l fermentor : (▲) Propionic acid by immobilized cells in 2-l fermentors Batch 1, (■) Propionic acid by immobilized cells in 2-l fermentors Batch 2, (△) Total sugar – Batch 1, (◻) Total sugar – Batch 2

It was found in the case of the comparisons between fermentations by free cells and immobilized cells in 2-l fermentor, using both 1% CaCO₃ and 5 N KOH to control the pH, that immobilized cells gave more propionic acid than free cells at the same time (Table 4) with the reason that the entrapping method by alginate protected the cells from high acidity caused by the end-products (Ates et al., 2002). In addition, immobilization protects cells from excess oxygen, therefore, the oxygen tension inside alginate beads is lower than that in the surrounding medium which is suitable for anaerobic fermentation. Moreover, immobilized cells could be reused which would reduce the cost and time in preparing fresh seed culture.

Table 4. Comparisons of fermentation time, propionic acid, yield, productivity and total sugar consumption in 2-l fermentors fermented by immobilized cells and free cells of *Propionibacterium acidipropionici* TISTR 442 at 192 h incubation

2-l Fermentors	Fermentation time (h)	Propionic acid (g/l)	Yield (g/g)	Productivity (g/l h)	Total sugar consumption (%)
Immobilized cells					
Batch 1	192	15.85 ± 0.25	0.48 ± 0.01	0.083 ± 0.0008	98.54
Immobilized cells					
Batch 2	192	12.68 ± 0.25	0.44 ± 0.01	0.066 ± 0.001	93.11
Free cells	192	14.89 ± 0.24	0.50 ± 0.008	0.078 ± 0.0008	95.27

Batch fermentation was performed on 2-l fermentors with a 1.4-l working volume at 30°C, 100 rpm and pH 6.5. Data are presented as means of three replications ± standard deviations

Antifungal effects

The effects of propionic acid in inhibiting growth of molds and yeasts are shown in Figure 5 and Figure 6, respectively. The antifungal activities of fermented propionic acid were shown by having inhibition zones with *Aspergillus niger*, *A. oryzae*, *Rhizopus* sp., *Penicillium* sp. and *Zygosaccharomyces rouxii* with the values of 2.38, 1.94, 1.58, 3.14 and 1.68 cm, respectively. These results were similar to those of the commercial propionic acid with the inhibition zones of 2.42, 1.92, 1.68, 3.28 and 1.72 cm, respectively. Table 5 shows the statistical analyses of mold inhibition zones as affected by commercial and fermented propionic acids which were not significantly different at 95% confidence level.

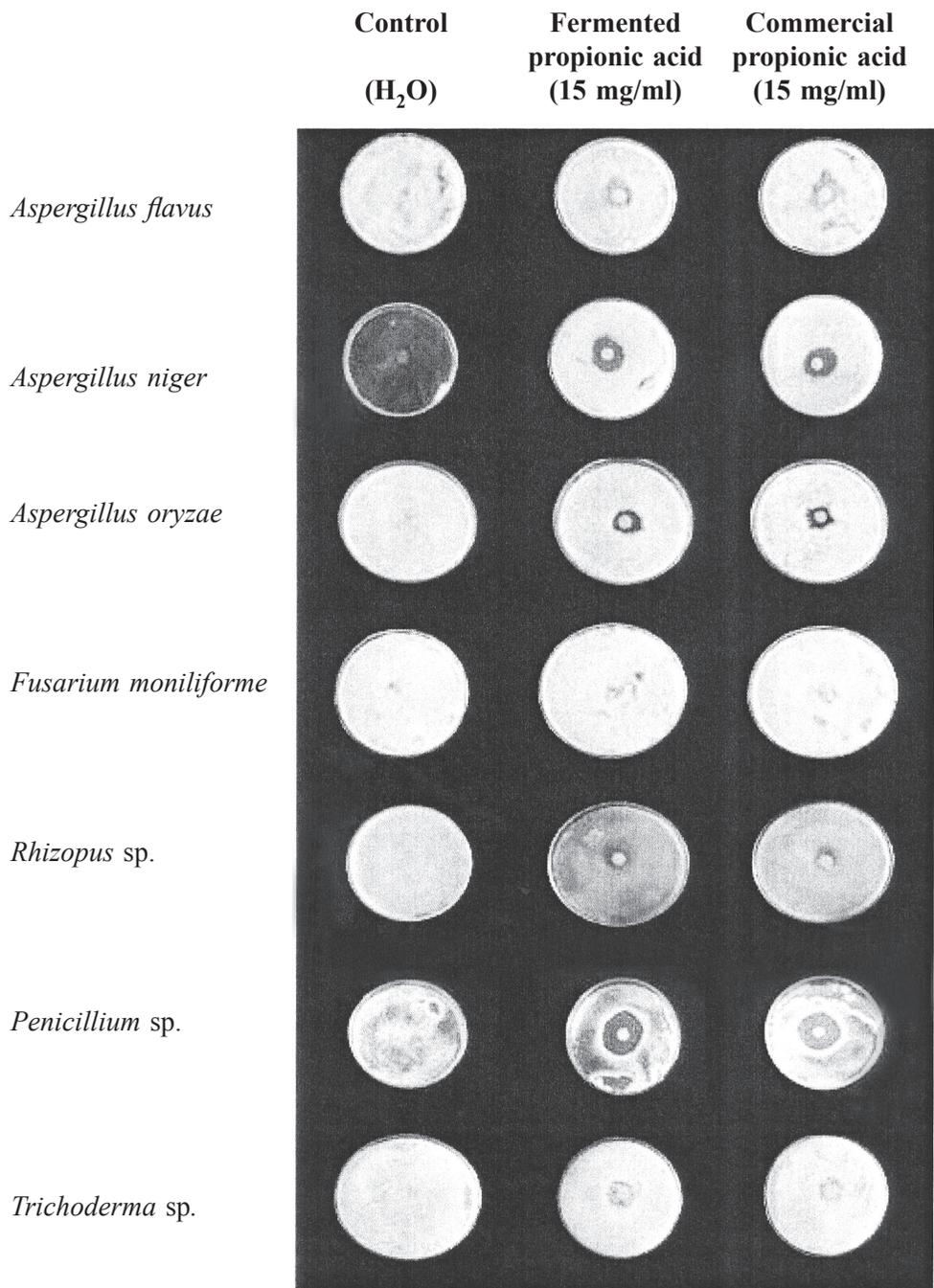


Figure 5. Comparisons of mold inhibitions as affected by the fermented and the commercial propionic acids

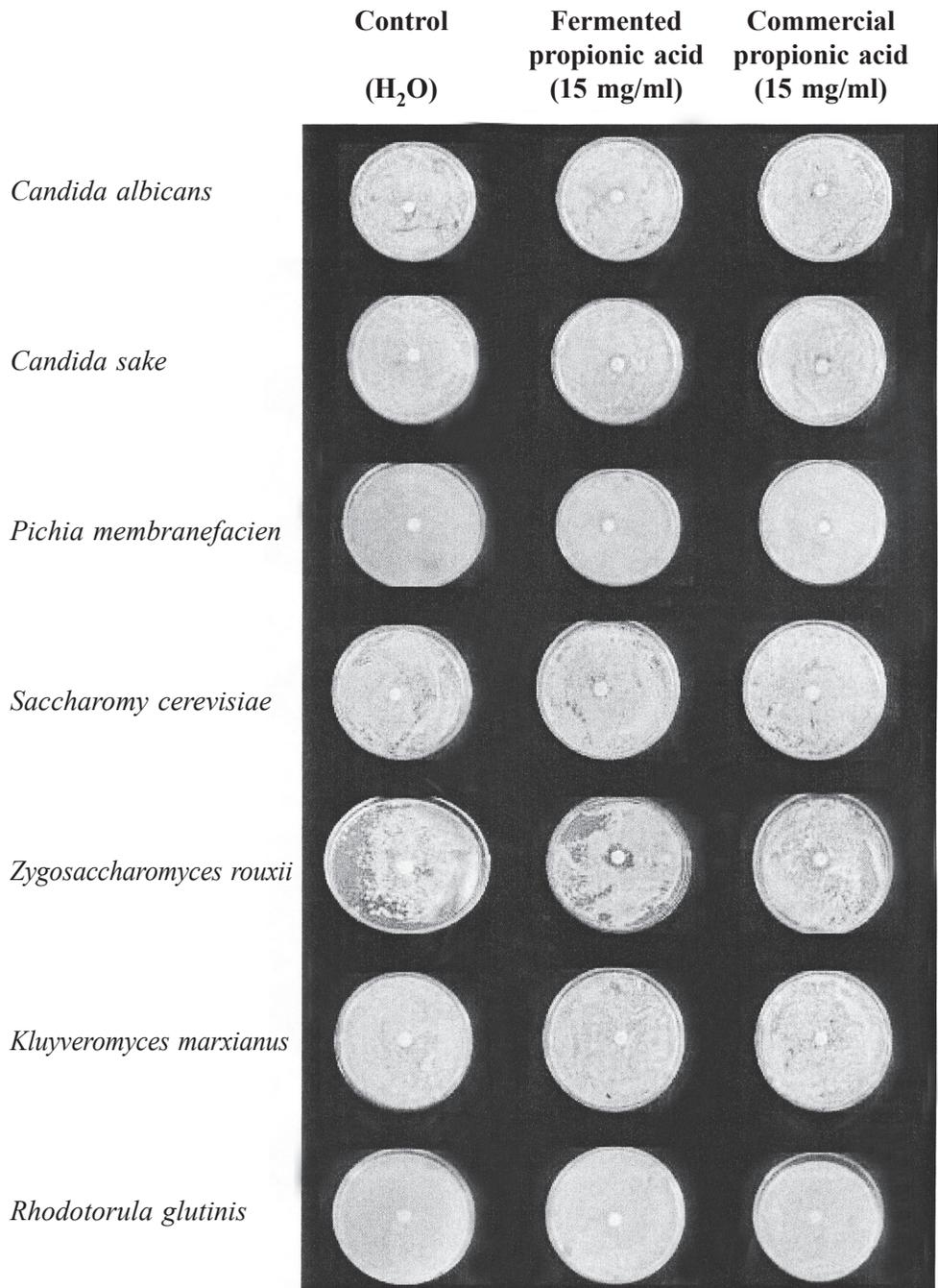


Figure 6. Comparisons of yeast inhibitions as affected by the fermented and the commercial propionic acids

Table 5. Comparisons of antifungal activity by the commercial and the fermented propionic acids using T-test

Type of fungus	Inhibition zone by commercial propionic acid (cm.)	Inhibition zone by fermented propionic acid (cm.)	T-test
<i>Aspergillus niger</i>	2.42	2.38	0.622 ^{ns}
<i>Aspergillus oryzae</i>	1.92	1.94	0.56 ^{ns}
<i>Rhizopus</i> sp.	1.68	1.58	0.392 ^{ns}
<i>Penicillium</i> sp.	3.28	3.14	0.31 ^{ns}
<i>Zygosaccharomyces rouxii</i>	1.72	1.68	0.147 ^{ns}

Data are presented as means of five independent inhibition zones and statistical analyses were performed by pair sample T-test ns : P-value > 0.05, means are not significantly different at 99% confidence level * : 0.01 < P-value < 0.05, means are significantly different at 95% confidence level.

Although the fermented propionic acid was able to inhibit the growth of molds and yeasts similar to the commercial propionic acid at the same concentration, the fermented propionic acid is better than the commercial propionic acid because harmful chemicals would not occur. Lind et al., (2005) evaluated the antifungal activities of different strains of propionibacteria, e.g., *Propionibacterium acidipropionici*, *P. freudenreichii subsp.shermanii*, *P. freudenreichii subsp. freudenreichii*, *P. thoenii* and *P. jensenii* on sodium lactate medium at pH 7.0 and found that the growth of *Rhodotorula mucilaginosa*, *Penicillium roqueforti* and *Aspergillus fumigatus* was inhibited.

CONCLUSION

Whey broth supplemented with 1% CaCO₃ was the optimal medium in producing propionic acid by *P. acidipropionici* TISTR 442 as 15.16±0.24 g/l propionic acid was produced by free cells of the bacterium. The fermentation times in 2-l fermentors were faster than those in flasks. The total sugar in broth was the important factor for propionic acid production as it was shown that the remaining total sugar in broth had affected the increasing of the amount of propionic acid. For propionic acid production by immobilized cells of *P. acidipropionici* TISTR 442, the optimal conditions of gel formation were the controlled distances between the end of the tube to the surface of CaCl₂ solution which were 4-6 cm and the flow rate of the gel solution which was 7 ml/min. The fermentation by immobilized cells in 2-l fermentors with the controlled pH by 1% CaCO₃ and 5 N KOH produced more propionic acid than that with the controlled pH by 1% CaCO₃ without 5 N KOH because the constant pH in the culture obtained by the 5N KOH reduced the acidity produced by the propionibacteria. However, K⁺ from KOH replaced Ca²⁺ causing the gel stability to decrease. In this experiment, the consistent potential of immobilized cells could be maintained for 2 cycles of

fermentations and 15.85 ± 0.25 g/l and 13.39 ± 0.25 g/l propionic acids were produced on the first and second cycle, respectively, and these values were higher than those of the free cells (15.16 ± 0.24 g/l) in 2-l fermentors at 216 h of the fermentation time. The fermented propionic acid as well as the commercial propionic acid from chemical processes were able to inhibit the growth of the fungi.

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