EFFECT OF LACTIC ACID BACTERIA AND SOME BACTERIAL CONTAMINANT ON NON-STERILE YEAST CELL PRODUCTION

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ABSTRACT

Lactobacillus plantarum LF2, previously isolated from sugarcane juice, was used to assess its potential to reduce and prevent bacterial contamination in non-sterile yeast cell production using sugarcane based medium. The results showed that LF2 and *Saccharomyces cerevisiae* M30 coexisted, and that the maximum specific growth rate of the yeast was not affected by the presence of LF2, neither in sterile nor non-sterile batch cultures. The yeast specific growth rate was also not affected by the deliberate addition of gram-negative aerobic bacterial culture at the level of 10^7 CFU.ml^{-1} at the start of batch culture. However, compared to the pure yeast culture, the maximal cell dry weight decreased in all mixed cultures. During batch culture, growth of the bacterial contaminant was suppressed either when the pH fell below 4.0 or in the presence of LF2. During the prolonged growth phase of cyclic-fed batch culture (CFBC), the number of yeast M30 and LF2 remained coexistent. However, the bacterial contaminant exhibited growth competition during CFBC, resulting in a rapid increase in the cell number from approx. 10^5 to 10^8 CFU.ml^{-1} over the period of 24 hours while the number of LF2 tended to decrease slightly.

KEYWORDS: Lactic acid bacteria, yeast, fermentation, contamination

1. INRODUCTION

In commercial-scale fermentation, contamination with undesirable microorganisms is a serious problem and difficult to control. Medium sterilization is the most common and effective method to eliminate any microbial contaminants before start of fermentation. However, it is uneconomical for the process of low value products such as animal feed. In addition, it cannot prevent contamination that might occur during fermentation, particularly when the fermentation time is prolonged in continuous or fed-batch culture. In yeast growth and fermentation processes, maintaining a pH value of the culture broth between 4.0 and 4.5 is a common strategy used to control some bacterial contaminants that cannot grow in acidic environment [1]. Nevertheless, pH monitoring and control devices as well as regular maintenance of these instruments are required which can be expensive and may cause inconvenience in large-scale production. Furthermore, some contaminating microorganisms
may be able to survive in acidic condition but may not cause a serious damage to the process unless they exist in large quantities.

Due to extensive studies of lactic acid bacteria (LAB) and their renowned antimicrobial activity against some microorganisms including bacterial pathogens, it may be possible to develop a process utilizing LAB to control growth of other bacterial contaminants in non-sterile yeast cell production for animal feed. This may help reduce the cost of medium sterilization and pH control during fermentation. Moreover, LAB may contribute probiotic benefits to the animal host [2]. Commonly, lactobacilli were the only contaminants found at the end of yeast fermentation due to their ability to tolerate high alcohol level and temperature [3]. They fermented some sugars and other nutrients, which were not fermentable by yeast [4]. In addition, these contaminating LAB could grow and created acidic environment as well as may produce some antimicrobial substances that could prevent growth of other bacterial contaminants.

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Although some strains of LAB are known to interact with yeast and may cause some adverse effects on the yeast growth and production rate [3], selection of LAB strains that can coexist with yeast as well as a suitable mode of fermentation could make the process economically advantageous.

The objective of this research was to investigate the effect of LAB and some aerobic bacterial contaminant on growth of yeast in the process of yeast cell production. This was to assess potential for LAB to coexist with yeast and to control bacterial contamination during the process.

2. MATERIALS AND METHODS

2.1 Yeast and bacterial strains

Saccharomyces cerevisiae M30 was kindly provided by Dr. Jaroon Kumnuanta and Assoc. Prof. Dr. Savitree Limthong (Department of Microbiology, Faculty of Science, Kasetsart University, Thailand). The yeast strain was flocculent and high temperature tolerant (37°C) which would facilitate cell recovery and temperature control during the process. *Lactobacillus plantarum* LF2 and the bacterium A03 (aerobic, gram-negative short rod) were isolated from sugarcane juice in our laboratory (Faculty of Agricultural Industry, KMITL, Thailand). The LF2 did not inhibit the growth of yeast M30, when previously tested using agar spot method. The bacterium A03 was one of the most abundant contaminants found in raw sugarcane juice.

2.2 Fermentation Medium

Sugarcane based medium (composed of sugarcane juice, containing 20 g. l^{-1} invert sugar; 1 g. l^{-1} urea; 3 g. l^{-1} KH_2PO_4; and 0.5 g. l^{-1} $MgSO_4.7H_2O$) was used throughout this study. This medium was previously tested for the yeast M30 growth and found superior than MY broth (data not shown).

2.3 Fermentation experiments

All fermentation experiments were conducted in an airlift fermenter (10 cm diameter × 50 cm height), with an internal draft tube (6 cm diameter). Batch cultures were performed with a working volume of 2.3 L and a constant aeration rate of 1.5 L. min^{-1}.

In cyclic fed-batch culture (CFBC) experiments, the initial working volume was 1.5 L. Following the end of exponential phase in batch culture, the medium was fed continuously at a constant flow rate of 0.3 L. h^{-1}. On reaching a maximal volume of 2.3 L, a part of the culture was then removed rapidly to remain at the initial volume while continuing feeding. The cyclic feeding regime resulted in the dilution rate fluctuations from 0.2 to 0.1 h^{-1} in each cycle, which were below the maximum specific growth rate of the yeast as determined from batch culture.

2.4 Determination of cell dry weight

Culture sample was centrifuged (10,000 rpm, 5 min) and the supernatant was discarded. The resulting cell pellet was washed once with distilled water and then dried (at 105°C for 16-18 h) to a constant weight. Cell dry weight was calculated in grams per liter.

2.5 Viable count determinations

Samples were diluted in 0.1% peptone solution and 0.1ml of an appropriate serial dilution was spread onto the suitable medium as follows:

For yeast count, MY agar supplemented with 40 μg. ml^{-1} streptomycin sulfate (Sigma) was used. Plates were incubated aerobically at 30° C for 24-48 hours.

For LAB count, MRS agar supplemented with 10 μg. ml^{-1} cycloheximide (Sigma) was used. Plates were incubated under microaerobic condition at room temperature for 48-72 hours.

For bacterial count, Nutrient agar (NA) supplemented with 10 μg. ml^{-1} cycloheximide was used. Plates were incubated aerobically at 30° C for 24 hours.

2.6 Determination of maximum specific growth rate

The maximum specific growth rate (μ_{max}) was determined by plotting the natural logarithm of cell concentration during the exponential growth in batch culture, against time. Since cells were growing at their constant and maximal rate when nutrient were in excess, the slope of the resulting straight line represents μ_{max} of the culture as described by the following equation:

$$\text{Ln } N = \text{Ln } N_0 + \mu t$$

where N is the number of viable cells (CFU. ml^{-1})

2.7 Sugar assay

Invert sugar concentration (g. l^{-1})was determined using Lane and Eynon method. Somogyi-Nelson metho [5] was also used to determine reducing sugar concentration (g. l^{-1}) in the culture.

3. RESULTS AND DISCUSSION

3.1 Effect of LAB on yeast growth in batch culture

The effect of *L. plantarum* LF2 on growth of *S. cerevisiae* M30 in batch culture using sterile and non-sterile sugarcane based medium was studied. The results showed that *L. plantarum* LF2 coexisted with the yeast without having an adverse effect on the yeast specific growth rate (Table 1). However, the maximum cell dry weight was reduced in the mixed culture when compared to that obtained in the yeast culture alone. This may be due to reduction of the nutrients available for yeast growth in the mixed culture. Moreover, the maximum cell dry weight was lower in the sterile mixed culture, compared to the non-sterile mixed culture. This may result from the higher initial pH of sterile medium that favored the growth of LF2 in such condition. On the other hand, a higher maximum cell dry weight obtained in the non-sterile medium may be accounted for the indigenous microorganisms in the non-sterile sugarcane juice since the raw material itself was a major source of the contaminating bacteria. Similar pH reduction was observed in the pure and mixed culture (Figure 1 and 2). However, the minimum pH was slightly lower in the mixed culture containing LF2. After the exponential growth phase, the culture pH tended to increase gradually. This may result from the release of by-products following the yeast autolysis during the stationary phase [6].

Table 1 Maximum specific growth rate (μ_{max}), pH and maximum cell dry weight (CDW) in batch cultures

Batch culture*	μ_{max} (per hour)			Initial pH	Min. pH	Max. CDW (g/l)
	M30	LF2	A03			
M30 (sterile medium)	0.38	-	-	6.35	3.72	5.3
M30+LF2 (sterile medium)	0.41	0.28	-	6.71	3.56	2.9
M30+LF2 (non-sterile medium)	0.37	0.16	-	4.90	3.56	3.7
M30+A03 (non-sterile medium)	0.41	-	N/A	5.04	3.18	3.7
M30+LF2+A03 (non-sterile medium)	0.40	0.17	0.30#	5.24	3.43	3.4

*Up to triplicate individual experiments

#Estimated from the data during the first 6 h in Figure 4

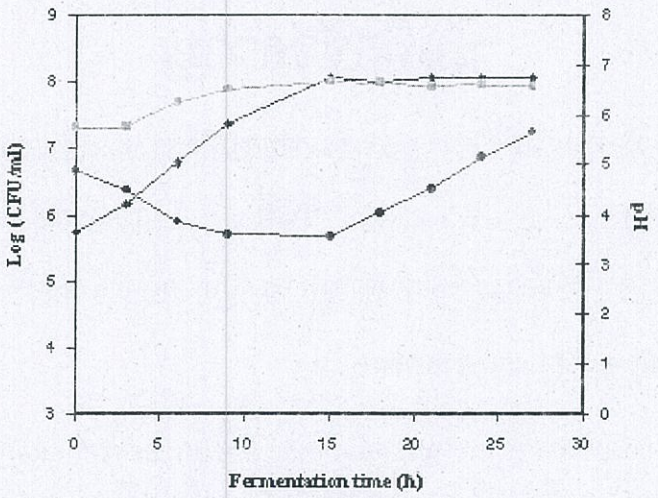

Figure 1 Growth profiles of *S. cerevisiae* M30 (◆) and *L. plantarum* LF2 (■) and change in pH (●) during sterile batch culture.

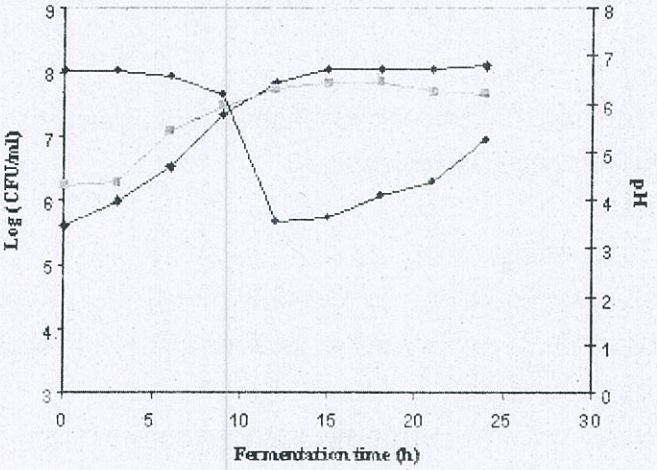

Figure 2 Growth profiles of *S. cerevisiae* M30 (◆) and *L. plantarum* LF2 (■) and change in pH (●) during non-sterile batch culture

3.2 Effect of aerobic bacterial contaminant on yeast growth in batch culture

To evaluate the effect of aerobic bacterial contaminant on the growth of yeast M30, the bacterium A03 (an aerobic gram-negative bacterium previously isolated from sugarcane juice) was deliberately added at the level of 10^7 CFU.ml^{-1} to the yeast culture. The result showed that the bacterium A03 had no influence on the yeast specific growth rate (Table1). On the other hand, it was found that the decrease in culture pH to less than 4.0 had a negative effect on the growth of bacterium A03. The number of A03 increased in the first 3 hours of fermentation where the culture pH was about 5.0. Thereafter, the number of A03 decreased as the culture pH reduced to approximately 3.2, and remained almost constant at about 10^6 CFU.ml^{-1} towards the end of batch culture (Figure 3). However, the result showed that the number of A03 tended to recover when the culture pH gradually increased at the end of batch culture. This is possibly because the bacteria may use some residual sugars available in the medium that could not be utilized by the yeast, as well as by-products from the yeast autolysis. It is worth noting that the minimum pH obtained in this batch experiment was the lowest, implying that the bacterium A03 may be an acid-producer, which could not tolerate the low pH condition.

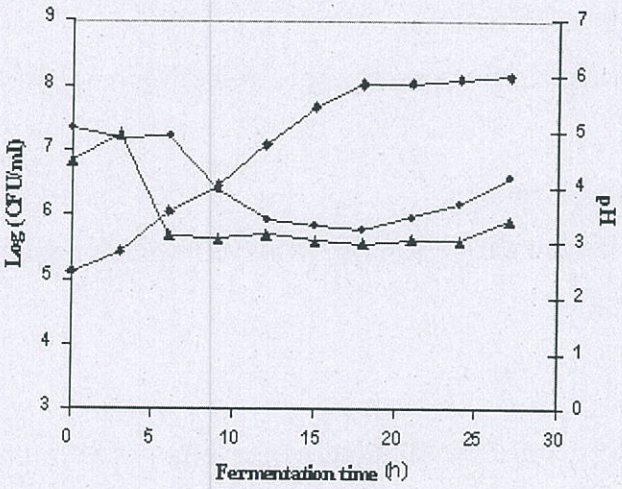

Figure 3 Growth profiles of *S. cerevisiae* M30 (◆) in the presence of bacterium A03 (▲) and change in pH (●) during non-sterile batch culture

3.3 Bacteria and yeast interaction in batch culture

In this batch experiment, interaction of *L. plantarum* LF2 and A03 during the yeast cell production was investigated. The result showed that the yeast specific growth rate was not affected by the presence of both LF2 and A03 in the culture (Table 1). This can be explained by the highest specific growth rate of the yeast amongst these microorganisms. At the beginning of fermentation when the culture pH was above 4.0, the number of A03 increased as rapidly as that of the yeast M30 (Figure 4). However, the growth of bacterium A03 was retarded when the culture pH was decreasing. Then, the number of A03 decreased rapidly when the pH dropped to less than 4.0. This was similar to the previous case (Figure 3) where the decrease in pH below 4.0 affected the growth of bacterium A03. This result would confirm that growth of bacterium A03 could be inhibited at a pH less than 4.0. Moreover, it is worth noting that the number of A03 continued to decrease although the pH was increasing gradually towards the end of batch culture. This result implied that *L. plantarum* LF2 may be able to produce some antibacterial substance that could inhibit the growth of bacterium A03 in the culture, apart from the effect of low pH as discussed previously.

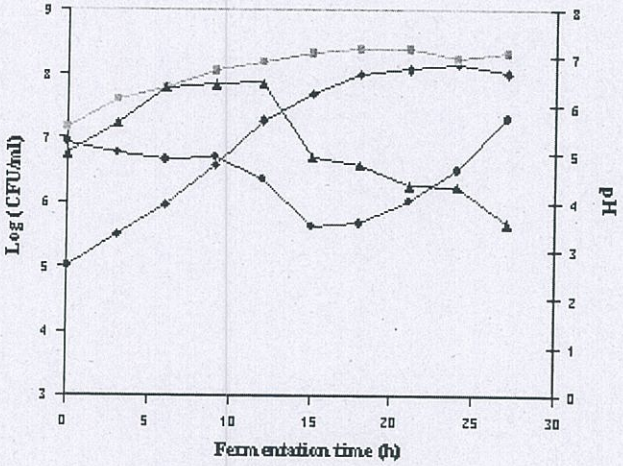

Figure 4 Growth profiles of *S. cerevisiae* M30 (◆) and *L. plantarum* LF2 (■) in the presence of bacterium A03 (▲) and change in pH (●) during non-sterile batch culture

3.4 Coexistence of LAB and yeast during CFBC

The effect of *L. plantarum* LF2 on growth of the yeast M30 in CFBC using sterile and non-sterile sugarcane based medium were illustrated in Figure 5 and 6, respectively. The number of yeast and LF2 remained almost constant during the prolonged growth phase in CFBC, both in sterile and non-sterile conditions, indicating that the yeast M30 and LF2 were able to coexist during CFBC. The culture pH gradually decreased from the end of previous batch culture, and could be maintained at approx. 4.0 throughout CFBC.

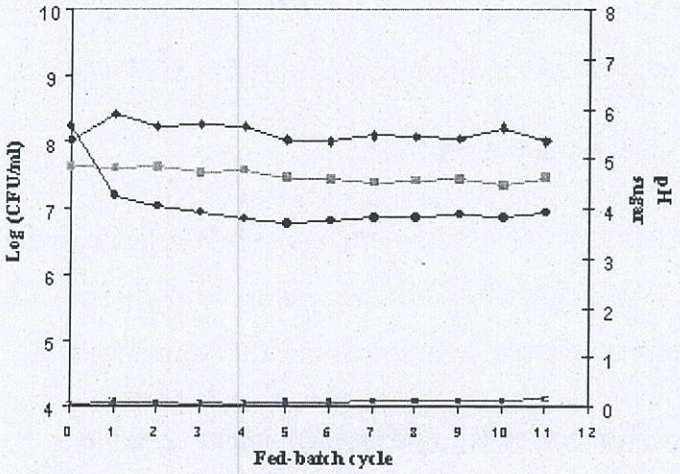

Figure 5 Growth profiles of *S. cerevisiae* M30 (◆) and *L. plantarum* LF2 (■) and changes in pH (●) and sugar concentration (✳) during sterile CFBC.

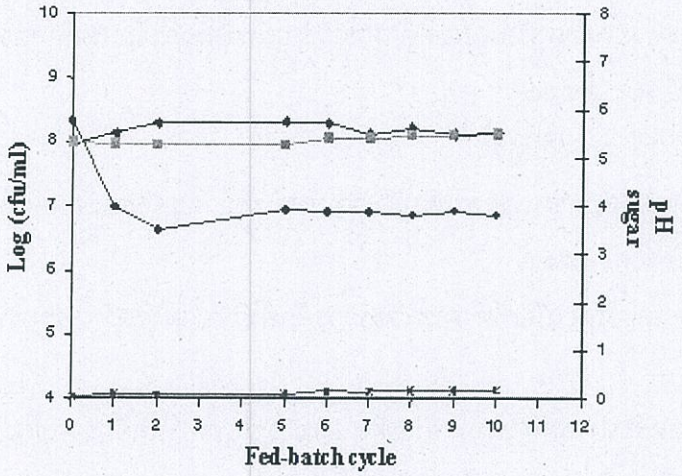

Figure 6 Growth profiles of *S. cerevisiae* M30 (◆) and *L. plantarum* LF2 (■) and changes in pH (●) and sugar concentration (✳) during non-sterile CFBC.

3.5 Bacteria and yeast competition in CFBC

The effect of bacterium A03 on the yeast M30 was studied in CFBC. Figure 7 shows that the number of A03 increased continuously from approx. 6 to 8 log CFU.ml^{-1}, indicating that the bacterium A03 was competitive with the yeast M30 during CFBC. The culture pH that maintained at approx. 4.0 was not able to inhibit the growth of A03 in CFBC. This result implied that a small quantity of bacterial contaminants could cause a serious problem in continuous process or cell recycle system.

The similar result was obtained in Figure 8 where the effect of A03 on the mixed culture of yeast M30 and LF2 was investigated in CFBC. That is the number of A03 increased continuously and reached approx. 8 log CFU.ml^{-1} over the period of 9 fed-batch cycles (equivalent to approx. 24 h), while the number of LF2 tended to decrease slightly. In this experiment, the dilution rate of the CFBC was controlled between 0.1 and 0.2 h^{-1}, which was lower than the maximum specific growth rate of the yeast. This led to a nutrient limited condition, and hence growth competition among these microorganisms in the mixed culture. It is therefore obvious that the yeast M30 and bacterium A03 having a higher specific growth rate than LF2 in this medium (see Table 1) would grow faster than LF2 in such condition. Moreover, it was

noticeable that yeast flocculation was strongly developed during the CFBC. This is known to limit the substrate and oxygen transfer to the inner part of the flocs, resulting in some residual sugar (as shown in Figure 5 and 6) that could be utilized by the bacterium A03, and hence supported its growth to be competitive with the yeast. These results suggested that CFBC might not be an ideal continuous process for non-sterile yeast cell production, unless sufficiently high-dilution rates would be operated to avoid nutrient limitation [7].

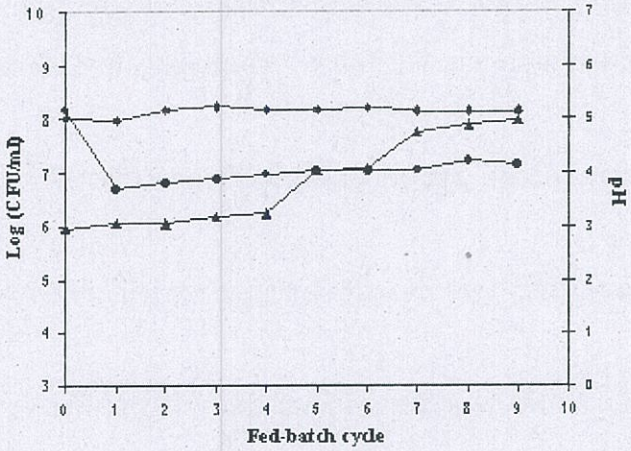

Figure 7 Growth profiles of *S. cerevisiae* M30 (◆) and the bacterium A03 (▲) and change in pH (●) during non-sterile CFBC.

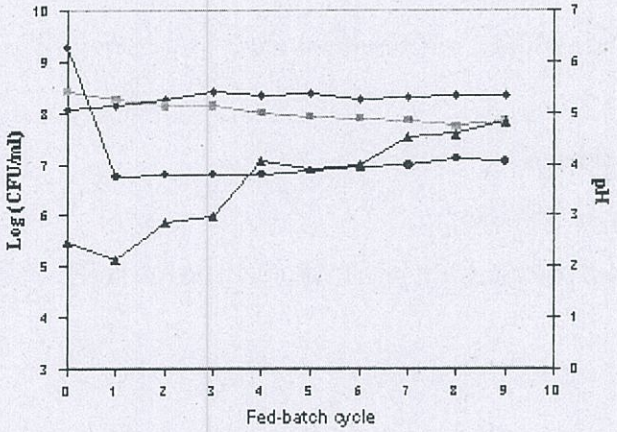

Figure 8 Growth profiles of *S. cerevisiae* M30 (◆) and *L. plantarum* LF2 (■) and the bacterium A03 (▲) and change in pH (●) during non-sterile CFBC.

4. CONCLUSIONS

Effects of lactic acid bacteria (*L. plantarum* LF2) and aerobic bacterial contaminant (A03) on non-sterile yeast cell production were studied. The result indicated that yeast M30 and *L. plantarum* LF2 were able to coexist in both batch and cyclic fed-batch culture using sterile or non-sterile sugarcane based medium. The maximum specific growth rate of the yeast was not affected by the presence of LF2 and A03 in batch culture. The growth of bacterium A03 could be suppressed in batch culture when the pH dropped below 4.0 or in the presence of LF2. However, under the controlled dilution rates in CFBC, the bacterium A03 could grow rapidly and competed with the yeast. The pH that was maintained at approx. 4.0 during CFBC did not affect the growth of bacterial contaminant. From these results, it may be postulated that cyclic (repeated) batch culture, where the nutrient limitation is theoretically not established, might be an alternative continuous process for non-sterile yeast cell production. This should be further investigated.

5. ACKNOWLEDGEMENTS

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