Lactic Acid Fermentation From Enzyme-Thinned Starch With Immobilized *Lactobacillus amylovorus*

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In memoriam Prof. Emeritus Vera Johanides

Immobilized *Lactobacillus amylovorus* converts enzyme-thinned starch (ETS) to lactic acid. ETS was gelatinized and thinned with α-amylase. The standard medium contained w = 3 % (w/v) Yeast Extract, ETS and j = 10 % (v/v) Friedman's mineral solution. As in free-cell fermentation, increasing levels of yeast extract resulted in faster production rates and higher cell numbers in alginate beads but not in increased productivity per cell. In immobilized-cell batch fermentation, the yeast extract requirement could be reduced to 0.75 %. Lactic acid productivity could be maintained in repeated batch fermentations.

Keywords

Fermentation, immobilization, lactic acid, *Lactobacillus*, starch, yeast extract.

Introduction

Lactic acid fermentation has received extensive attention for over a long time[1,2,3]. If by-product streams are excluded, the starting raw-material for most lactic-acid fermentations is starch. Herein, starch is first enzymatically liquefied and saccharified to glucose, which is fermented by microorganisms to lactic acid. Of the two pretreatment steps, saccharification takes a longer time (24–96 h) than liquefaction (1–2 h)[4]. The production of glucose from starch adds cost and time to the process. Hence, use of starch directly in the fermentation medium is desirable in order to reduce the processing cost as well as time. We have earlier optimized a process for production of lactic acid from enzyme-thinned starch (ETS) by *Lactobacillus amylovorus* to reduce substrate costs[5].

Lactobacillus amylovorus, an amylolytic lactic acid producing bacterium, utilizes raw corn, rice and wheat, and to a lesser extent cassava and potato starch[6, 7]. *L. amylovorus* rapidly adheres to cornstarch granules and releases an α-amylase which results in hollow and fragmented granules[8, 9]. However, use of starch granules causes problems in pumping and flowing continuous systems. Using enzyme-thinned corn starch in fermentation eliminates the need for complete hydrolysis of the starch to glucose prior to fermentation, and avoids the problems associated with handling a solid substrate. Starch saccharification takes place during the fermentation and results in kinetics similar to that obtained with organisms using glucose as the substrate[5].

The productivity of many lactobacilli is significantly improved by the addition of yeast extract (YE), protein concentrates and hydrolysates, single amino acids, vitamins, minerals, etc[5,7,10–19]. *Lactobacillus amylovorus* like other lactic acid bacteria require a complex mixture of nutrients for optimal growth and product formation[5, 19]. YE is amongst the best nitrogen sources that also include $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, distillers' grain and solubles, pepticase, phytone peptone, cotton seed hydrolysate, corn steep liquor, trypticase, and neopeptone[5]. The highest production rates required a relatively high level of YE which supplies peptones, a nucleic acid, and other growth factors, that have not been identified (C. M. Hsieh, 1994, 'Optimization of Growth of Lactic Acid Bacteria', Ph.D. Thesis, University of Missouri, Columbia, MO, USA). YE would be the single most expensive component in the fermentation medium.

Cell immobilization can improve production rates of lactic acid while reducing medium requirements, inhibitions, and problems in downstream recovery[20–27]. The major disadvantages are limited diffusion rates, cell leakage, and build up of the end product in the beads; also, the small void space of polymer matrix and the leakage of cells limit the final cell loading in the beads[28–29]. In addition, the beads with microbial cells are not strong enough for long-term use. There might be further problems with *Lactobacillus amylovorus*. The diffusion of

amylases out of beads, and diffusion of yet to be identified nutrients into the beads might make it not practical to immobilize this organism.

The goal of these studies was to determine the feasibility of immobilization of *L. amylovorus* in an alginate matrix for the production of lactic acid, and to increase our understanding of YE requirement through determining its effect on cell numbers, and product formation by immobilized cells.

Materials and methods

Organisms and media

Lactobacillus amylovorus NRRL B4542 was maintained on Friedman's simplified medium with $w = 0.5$ % enzyme thinned starch (ETS) and $w = 1$ % agar[30]. Unless stated otherwise, the medium contained 2.5 % (w/v) ETS, 3 % (w/v) YE, and 10 % (v/v) Friedman's mineral solution (0.6 g L^{-1} $MgSO_4.7H_2O$, 0.03 g L^{-1} $MnSO_4.H_2O$, 1.0 g L^{-1} CH_3COONa, 0.03 g L^{-1} $FeSO_4$. $7H_2O$, 0.5 g L^{-1} K_2HPO_4, and 0.5 g L^{-1} KH_2PO_4). The standard incubation temperature was 37 °C.

ETS stock solution was prepared by adding pearl starch (A.E. Staley, Food grade) in 20 liter distilled water to make $w = 30$ % solution[5]. The pH was adjusted to 7.0 with 5 mol L^{-1} NaOH and 1.2 g of *Bacillus subtilis* α-amylase (176000 BAU/gm) in 80 ml 0.2 % $CaCl_2 \cdot 2H_2O$ solution was added. The mixture was heated in a steam jacketed kettle while stirring. The temperature was increased from room temperature to 87 °C over a 24 min. period and then held constant for 10 minutes. The cooled solution was dispensed into 600 ml bottles and frozen. The final concentration of ETS in stock solution was 268 g L^{-1}.

Cells for immobilization were obtained by transferring the stock culture (3 loops) from a slant to 100 ml 1 % ETS medium, incubating for 24 hours at room temperature, followed by subculturing once again for 24 hours. After 24 hr of subculturing, the cells were harvested by centrifugation. The final cell slurry contained 10 – 30 g dry mass/100 ml.

Immobilization

Equal volumes of $w = 2$ % (w/v) solution of sodium alginate (Kelco division of Merck & Co., Inc.) in distilled water and microbial cells were stirred for 5 minutes, and transferred to a stainless steel pressure vessel connected to an atomizer. The vessel was pressurized until the cell suspension started flowing through the atomizer needle at a rate of 90 drops/minute. After stabilizing the flow for 1 minute, the drops were collected in 0.2 mol L^{-1} $CaCl_2$. The beads were hardened for 30 minutes before storage. The radius of the beads used in the fermentation were between 1.1–1.25 mm (> 95 %). Hence, the volume of a bead was between 5.6–8.2 mm^3. The beads were aged for two days in the growth medium containing 2.5 % (w/v) ETS and 3 % (w/v) YE in Friedman's mineral solution at 37 °C in order to increase cell numbers in beads, before experiments.

Unless stated otherwise, all solutions used in this research were autoclaved at 121 °C for 15 minutes and all the processes, except during production of beads, were carried out under oxygen free CO_2[31]. The canister used for production of beads was purged and pressurized with N_2.

Fermentations were carried out with 300 beads in 250 ml shake flasks with 100 ml medium. The shaking rate was 150 rpm. Marble chips (20 g) were added to the flask to buffer the solution. Using the above volume range, the volume fraction of the beads to the media was between 1.7–2.5 %.

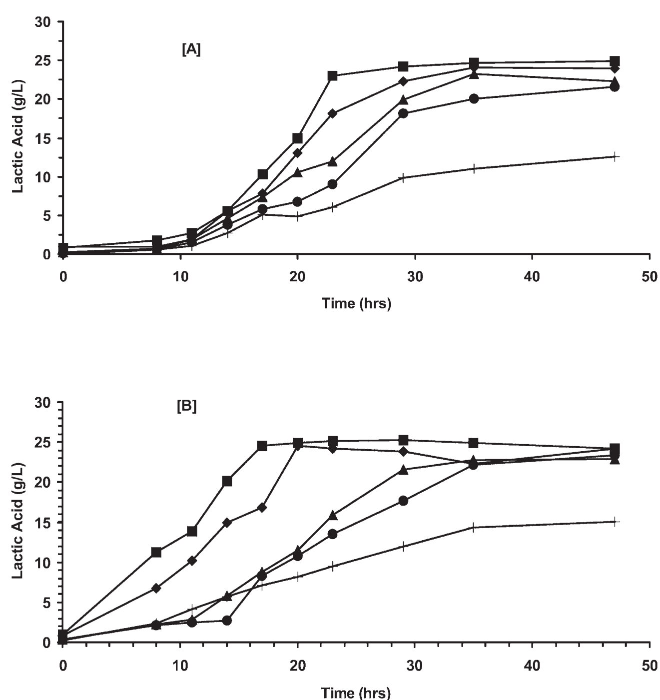
In order to determine the cell number in the beads, 10 beads were rinsed 3 times with 10 ml saline solution. The beads were solubilized by adding 5 ml of a solution fraction $w = 1.5$ % (w/v) tryptone, $w = 0.5$ % (w/v) NaCl and $w = 1$ % (w/v) sodium citrate with vigorous mixing. Cell numbers were determined by plate count on the standard medium with 1.5 % agar after serial dilution.

Lactic acid was analyzed by HPLC using a Perkin Elmer LC 250 isocratic pump equipped with Perkin Elmer LC 25 RI detector and Perkin Elmer LC 100 computing integrator. The mobile phase was H_2SO_4-acidified water with at pH 2 and the flow rate was 0.6 ml min^{-1}. The samples were centrifuged (Beckman J2-21M/E Centrifuge) at 10 050 rpm for 10 minutes and filtered through a 0.2 μm filter before injecting into HPLC. Oligosaccharides were also analyzed by HPLC under the same conditions.

Results and discussion

Effect of YE concentration on lactic acid production

Five sequential 2-d fermentations were carried out with different yeast extract (YE) mass fraction (0.5, 0.75, 1, 3, 4 %). At the end of each cycle, the beads were washed twice with 20 ml saline solution, and 100 ml fresh medium was added. As shown in Figure 1, immobilized *Lactobacillus amylovorus* converts enzyme-thinned starch (ETS) to lactic acid. YE was an important factor for lactic acid production. Higher YE fraction resulted in a higher production rates, and in some cases higher final lactic acid concentration. The greatest differences



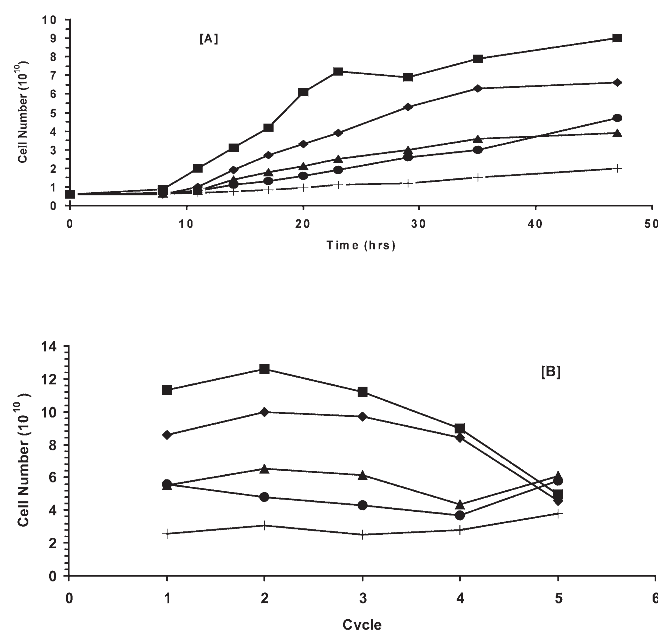
F i g . 1 – *Effect of yeast extract (0.5% –+–; 0.75% –●–; 1% –▲–; 3% –◆–; 4% –■–) on lactic acid production by Lactobacillus amylovorus immobilized in alginate beads during the first (A) and fifth cycle (B) of growth in 2.5% enzyme-thinned starch with 300 beads / 100 ml medium, at 37 °C and stirring at 150 rpm.*

among the different cycles were between the first and the second cycle (not shown); the second cycle through fifth were similar. The total amounts of lactic acid produced in the first and the fifth cycle were very similar (Figures 1A and B). After the first cycle, the higher the YE fraction the shorter the log phase and the higher overall production rate (Figure 1B). Nevertheless, the YE requirement could be significantly reduced to 0.75 %; at the 0.75 % YE level, the fermentation would require the time slightly twice over required with 4 % YE. The conversions of ETS to lactic acid were close to theoretical values.

The concentration of maltose increased up to 7 g L^{-1} in all the fermentations with the exception of those with the highest YE fraction, and then often decreased to undetectable levels over the 2 d. The fermentation with 0.5 % YE often had maltose remaining at the end of the fermentation. A fermentation without YE had 0.93 g L^{-1} lactic acid at the end of 2 d in cycle 1 and 0.2 g L^{-1} in cycle 2. The lactic acid production was probably due to carrying over a required nutrient from the storage medium by the beads.

Marble chips and powder did not maintain a stable pH in the broth. The pH decreased to approximately 4.5 in the first 20 h, then increased to about 6 by the end of the 2nd day.

After bead preparation, the number of cells / bead was approximately 2.2 × 10^5. After two day-storage in a nutrient medium (1 % ETS, 3 % YE), the count was 2 × 10^7 for a total of 0.6 × 10^{10} cells per flask. The cell numbers increased during the first fermentation cycle (Figure 2A). The number of cells increased further by the end of the second cycle, but then began to decrease (Figure 2B). This indicates that the cell number did not reach a maximum during the storage phase. The longer lag in lactic acid production in the first cycle was probably due to a low number of cells in the beads. The higher the YE level, the higher was the concentration of cells; the average number of cells in the fermentation with 4 % YE was close to being 4 times higher than that in the medium with 0.5 %. During the second through fourth cycle, the rate of lactic acid production per cell was 1.07 ± 0.034 × 10^{-11} / L · h · cell. There was no significant correlation with YE. At the end of the fermentation, 27 ± 3.6 % of the cells were free in the medium.



F i g . 2 – *Effect of yeast extract (0.5% –+–; 0.75% –●–; 1% –▲–; 3% –◆–; 4% –■–) on numbers of Lactobacillus amylovorus immobilized in alginate beads during the first cycle of growth (A) and at the end of each cycle in 2.5% enzyme-thinned starch with 300 beads / 100 ml medium, at 37 °C with stirring at 150 rpm (B).*

The beads were rigid after hardening in $CaCl_2$ solution for 30 minutes. Storage in the nutrient medium and the subsequent cycles resulted in a softening of the beads. Peeling of the outer surface was observed microscopically at the end of 4th cycle. This phenomenon became significant in the 5th cycle. This was probably due to loss of Ca^{2+} ions and

mechanical damage caused by the marble chips. Another possibility is that excessive cell growth and lactic acid production on the surface of the beads resulted in reduced calcium. Determination of the number of organisms / bead was more variable during the 5th cycle which might indicate that many of the cells were in the outer layers of the beads. Others have found that profiles in beads are not uniform and that the beads change shape during fermentation.[32–35]

Maintenance of organisms

Storage of *L. amylovorus* in calcium solution resulted in a decline of cell activity. The cells were inactive by the end of third week. Changing the solution to the fermentation medium improved subsequent fermentations (Figure 3). Cells stored in 2.5 % enzyme-thinned starch (ETS) resulted in shorter lag phases then those stored in 1% ETS or without nutrients in the storage medium. The activity of the immobilized cells could be maintained by weekly transfer in the fermentation medium. These results probably reflect the concentration of cells at the end of the storage period, but we need to know more about the effect of YE on the cell maintenance.

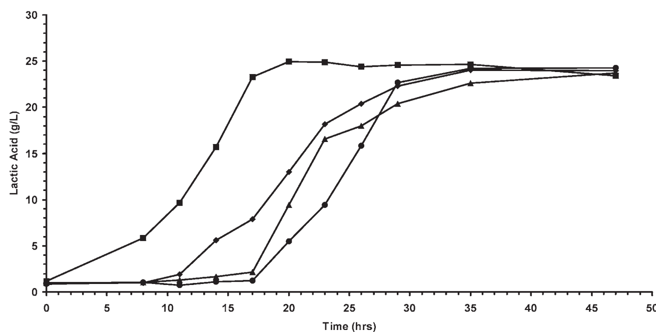


Fig. 3 – *Effect of storage of Lactobacillus amylovorus immobilized in alginate beads in 1.0 or 2.5% enzyme-thinned starch (ETS) for 0, 2, or 6 days on subsequent lactic acid production with 300 beads / 100 ml medium in a medium with 2.5 % ETS and 3% yeast extract at 37 °C with stirring at 150 rpm. (2 d in 2.5% ETS –■–) (2 d in 1% ETS –◆–) (0 d storage –▲–) (6 d in 2.5% ETS –●–)*

After development of high cell numbers in 2.5 % ETS and 3 % YE, the beads were transferred to media with equal or less (0.5 or 1 %) YE. The cell number decreased in the media with less YE (Figure 4). The data could be explained by dynamics of cell growth and death rates established by the concentration of YE. In other words, the cell numbers tend to reach a steady state level that depends on the level of YE.

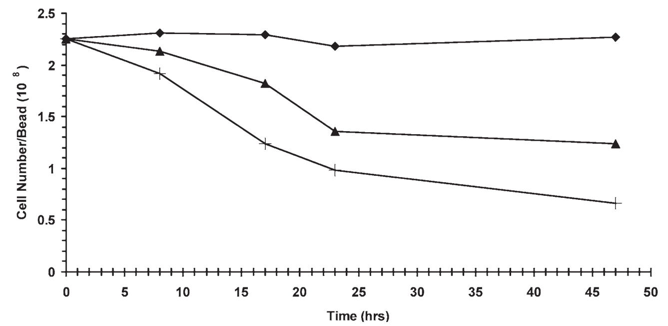


Fig. 4 – *Changes in cell numbers after shifting Lactobacillus amylovorus immobilized in alginate beads from a medium with 3% yeast extract to one with 0.5 % (–+–), 1% (–▲–), or 3% (–◆–)*

Effect of other parameters

Changing the number of beads did not make large changes in the fermentation. Twice (600) or two-thirds (200) the number of beads resulted in 11 % increase and decrease, respectively, in the concentration of lactic acid after 1 d which is the time that distinguishes the fermentations most. The higher the number of beads the shorter the lag phase.

Increasing the fermentation temperature from 37 to 40 °C, with immobilized cells, improved the 1-d lactic acid concentration by 14 %. Decreasing the temperature to 31 °C resulted in only 13 % of with the level of lactic acid achieved at 37 °C.

There was no significant difference between the 1-d lactic acid levels in the fermentation with immobilized and free cells. The lag phase was shorter with free cells; however we did not determine the initial numbers of free cells. Others have found increased rates and levels of lactic acid with immobilized organisms[24, 36–37]. Our results probably reflected the small number of beads used.

In summary, this study demonstrated the feasibility of immobilizing *Lactobacillus amylovorus* for conversion of enzyme-thinned starch (ETS) to lactic acid. Future studies will seek to improve the process by examining other matrices and increasing the concentration of the cells. More work is necessary to understand the role of YE on this organism.

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