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NEUTRALISING LACTIC ACID PRODUCED BY *LACTOBACILLUS CASEI* WITH CALCIUM CARBONATE

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Abstract: Maintaining pH by neutralising lactic acid with NaOH improves the growth, glucose consumption, and lactic acid production by *Lactobacillus casei*. However glucose consumption and lactic acid production were increased when the pH of the above medium was maintained with $CaCO_3$. When glucose concentration was increased leading to a decrease in growth rate, $CaCO_3$ seems to be effective at high glucose concentrations. Adding glucose and $CaCO_3$ at different growth phases did not alter glucose consumption and lactic acid production.

Key words: Calcium carbonate, lactic acid, *Lactobacillus casei*

INTRODUCTION

A major factor affecting lactic acid fermentation is pH. Fermentation of sugars to lactic acid proceeds best in acidic pH[1]. Continuous control of pH in lactic acid fermentation therefore increases yields and rate of production of lactic acid[2]. Different neutralising agents such as calcium carbonate[3], calcium hydroxide[3], NaOH[4], $(NH_4)OH$[5] and $(NH_4)_2CO_3$[5] have been described in lactic acid fermentation process involving *Lactobacillus delbrueckeii* [3,5], *Rhizopus oryzae*[6] etc. This paper describes the fermentation of glucose to lactic acid by *L. casei* under pH controlled conditions either using NaOH or $CaCO_3$. Studies were also made to improve lactic acid yield by altering glucose concentration under pH controlled conditions.

To our knowledge, calcium carbonate has not been used earlier for maintaining pH in *L. casei* or other *Lactobacillus cultures*.

METHODS AND MATERIALS

Microorganism

L. casei, a hormofermentative lactic acid producer was used. The strain was stored in glycerol (12%,w/v/) at -40°C .

Preparation of inoculum

MRS medium (25 ml, 52 gl^{-1}) was inoculated with 500 µl freezing medium [7] containing *L. casei* and incubated at 42°C while shaking (120 rpm) for 24 h.

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From this, 1.0 ml was transferred to culture medium (50 ml) and incubated at 42°C for 48 h while shaking (120 rpm).

Media

The medium used was modified from Roy *et al*[6] and consisted of the following in gl^{-1} : yeast extract, 10.0; K_2HPO_4,0.5,KH_2PO_4,0.5; sodium citrate, 1.0; and salts, 1.0 $ml1^{-1}$ ($MgSO_4.7H_2O$, 0.5 g;$MnSO_4H_2O$,0.31 g; $FeSO_4.7H_2O$,0.2g; and ascorbic acid , 0.5 g; dissolved in 100 ml distilled water). Glucose concentration was varied by mixing sterilized glucose solutions with other constituents of the medium.

Analytical methods

Cell concentrations- Cell density was monitored by measuring the optical density (OD) at 620 nm. Samples removed at different time intervals were centrifuged. Cells obtained by the centrifugation of 10 ml of medium were washed twice with 5.0 ml 0.02M phosphate buffer (pH 6.5) and homogenised with 0.5 g acid washed sand and 5.0 ml, 0.1% (v/v) Triton-0.02M phosphate buffer (pH 6.5) solution. Homogenate was centrifuged and supernatant was used for the measurement of NADPH[8] in a Fluorescence spectrophotometer (Perkin-Elmer, LS-3) at excitation and emission wave lengths of 360 and 450 nm respectively against 0.1% (v/v) Triton-0.02M phosphate buffer (pH 6.5) as blank. The supernatants were analysed for lactic acid [9] and glucose.[10]

Growth of and fermentation by *L. casei*

L. casei was cultivated batch wise in 500 ml conical flask containing 250 ml medium. Fermentation was carried out at 42°C in shaker water (120 rpm). The medium was inoculated with 10%(v/v) inoculum.

Effect of pH maintenance

Medium containing 50 g l^{-1} glucose was inoculated with *L. casei* and the pH was maintained at 6.5 either by the addition of 4N NaOH at 2 h intervals during fermentation or by 50 g l^{-1} $CaCO_3$ at the time of inoculation. The pH was not monitored in controls. The OD_{620},NADPH, pH, lactic acid and glucose were monitored.

Effect of glucose concentration

Medium containing 150, 85 and 50 gl^{-1} glucose was inoculated with *L. casei* and pH was maintained at 6.5 by the addition of NaOH at 2 h interval or $CaCO_3$ (150 or 50 gl^{-1} respectively)

Intermittent addition of $CaCO_3$ or $CaCO_3$ and glucose to the medium during fermentation

Medium containing 150 gl^{-1} glucose was inoculated with *L. casei* and pH was maintained either by initial (150 gl^{-1}) or intermittent (50 gl^{-1} at each instant and total 150 g l^{-1}) addition of $CaCO_3$. In another set of experiments, medium containing glucose (50 gl^{-1}) and $CaCO_3$ (50gl^{-1}) was inoculated with *L.casei* and $CaCO_3$ (50 gl^{-1}) and glucose (50 g l^{-1}) were added intermittently until the total amount of glucose and $CaCO_3$ added were each of 150 gl^{-1}.

RESULTS

Effect of pH maintenance

Maintenance of pH with NaOH improved the optical density from 3.1 to 8.2 (Figure 1.) When the pH was not maintained, the cells had shorter log phase of 4 h duration than under pH controlled conditions, where the log phase was 12 h. Glucose consumption and lactic acid production were more (30 and 24% respectively) when pH was maintained at 6.5 than otherwise (Figure 1). When pH was maintained by the addition of NaOH or $CaCO_3$, NADPH levels increased steadily up to 14 h and then started to decline (Figure 2).

Effect of glucose concentration

An inverse relationship between initial glucose concentration in the medium and the growth of *L. casei* was observed (Figure 3A). Although the cell growth decreased with increase in glucose concentration from 50 to 150 gl^{-1}, amount of lactic acid produced increased with the increase in glucose concentration. Increase in lactic acid production with 150 gl^{-1} glucose was observed after 40 h of fermentation (Figure 3B). However lactic acid production was delayed when glucose concentration in the medium was increased above 85 g l^{-1} under our experimental conditions. Increase in glucose concentration from 50 to 150 gl^{-1} increased the cellular NADPH level (Figure 4A). Production of lactic acid was delayed initially with lower glucose concentration and the rate increased after 8h (Figure 4B).

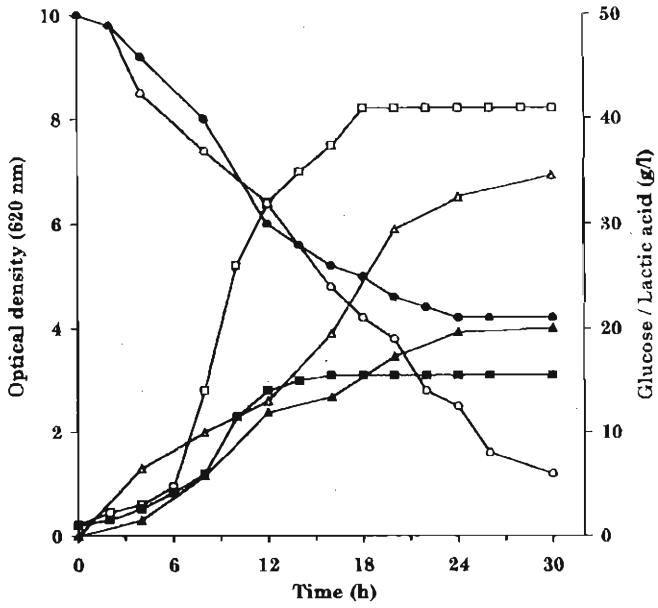


Figure 1 : Effect of pH maintenance with 4N NaOH on growth (square), glucose consumption (circle) and lactic acid (triangle) by *L. casei*. Open symbols for pH controlled conditions and closed for pH uncontrolled conditions.

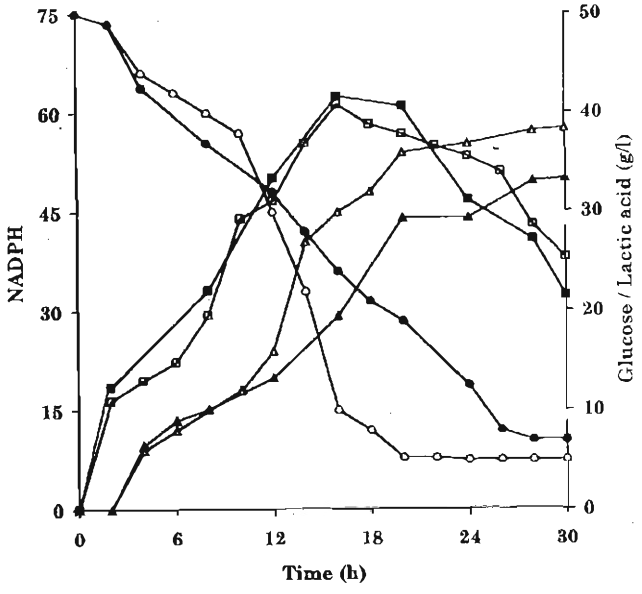
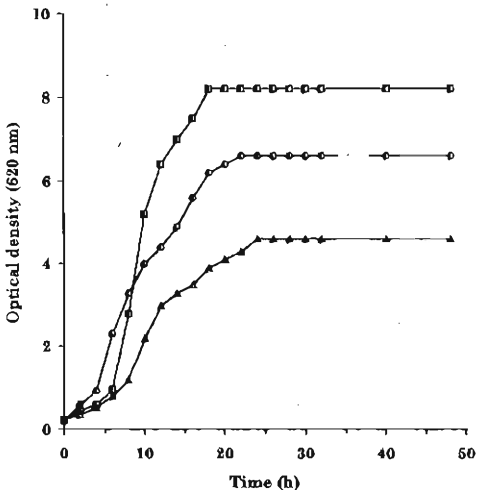
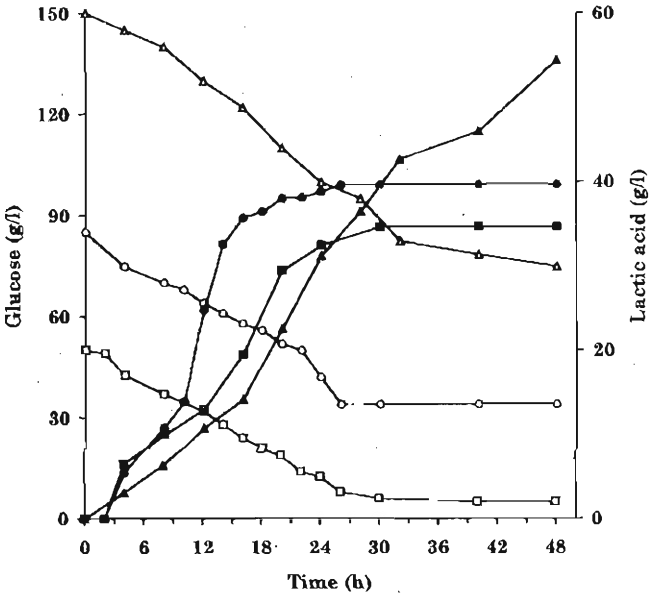


Figure 2 : The effect of maintaining pH with 4N NaOH and $CaCO_3$ (50 g l^{-1}) on growth (NADPH, square), glucose consumption (circle) and lactic acid production (triangle) by *L. casei*. Open symbols for pH maintained with $CaCO_3$ and closed for pH maintained with 4N NaOH



3A

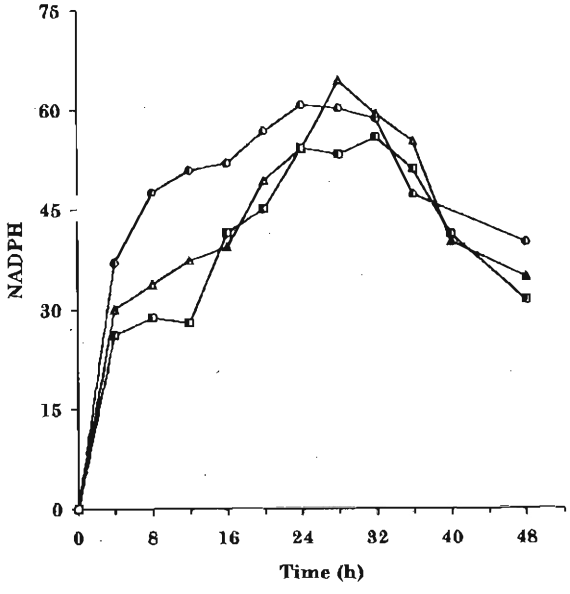


3B

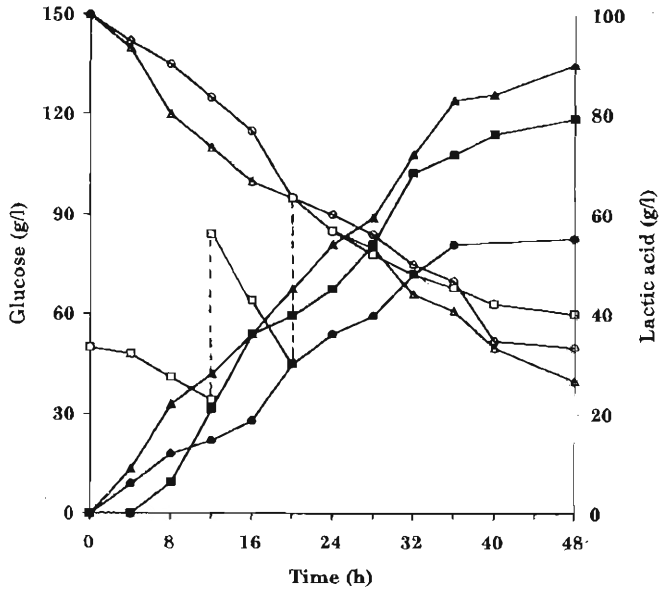
Figure 3 : Effect of glucose concentration A: on growth (half closed symbols) B: glucose consumption (open symbols) and lactic acid production (closed symbols) by *L. casei*. The pH was controlled manually with 4N NaOH. Glucose concentrations in the medium were (square) 50 gl^{-1}, (circle) 85 gl^{-1}, (triangle) 150 g l^{-1}.

Intermittent addition of $CaCO_3$ with initial addition of glucose or intermittent addition of both $CaCO_3$ and glucose

In this set of experiments glucose and $CaCO_3$ each of total 150 gl^{-1} were added to the medium. In the first set, 150 gl^{-1} of glucose and $CaCO_3$ were added at the beginning of the experiment. In the second set, 150 gl^{-1} glucose and 50 g l^{-1} $CaCO_3$ were taken initially and then 50,30 and 20 gl^{-1} $CaCO_3$ was added at 12,24 and 36 h respectively. In the third set 50 gl^{-1} glucose and $CaCO_3$ were taken initially and then 50 gl^{-1} glucose was added at 12 and 20 h while 50,30 and 20 gl^{-1} $CaCO_3$ was added at 12,20 and 36 h respectively (Addition of $CaCO_3$ at different periods to the medium depended on the decrease in pH). Increase in NADPH value was faster when both 150 gl^{-1} glucose and $CaCO_3$ were added at the beginning of the experiment (Figure 4A) However initial addition of 150 gl^{-1} glucose to the medium containing 50 gl^{-1} $CaCO_3$ showed a delayed increase in NADPH value. Similar delay with the third set of experiments to which glucose and $CaCO_3$ were added intermittently, was observed (Figure 4A).



4A



4B

Figure 4 : Effect of glucose and $CaCO_3$ addition at various time intervals during fermention. A: on growth of *L. casei* (half open symbols), B: glucose consumption (open symbols) and lactic acid production (closed symbols), by *L casei*. Initial addition of glucose and $CaCO_3$ (circle), initial addtion of glucose with intermittent addition of $CaCO_3$ (triangle), intermittent addition of glucose and $CaCO_3$ (square). A total amount of 150 g l^{-1} glucose and 150 gl^{-1} $CaCO_3$ were added in these experiments.

DISCUSSION

The pH maintenance studies with NaOH indicated that it is essential to maintain the pH of the fermentation medium. If the lactic acid produced is not neutralised the viability of *L. casei* was decreased. Further efficiency of lactic acid production under controlled pH at 6.5 was 82% and this decreased to 68.4% when pH was not controlled. When pH of the medium was maintained by the addition of $CaCO_3$ instead of NaOH (Figure 2), growth was monitored by the measurement of NADPH level in the cells instead of OD measurement, because of the turbidity developed by $CaCO_3$, in the medium. The NADPH level reflects the metabolic status of the cells. Initial addition of 50gl^{-1} $CaCO_3$ compared with manual addition of 4N NaOH increased the glucose consumption and lactic acid production. When $CaCO_3$ was added to the medium, CO_2 was continuously released which could have created semiaerobic conditions to the cells, that is optimal for the process These results indicated the importance of pH maintenance in lactic acid producing fermentation processes and $CaCO_3$ is a suitable alternative for NaOH where continuous addition of NaOH could be replaced by the addition of $CaCO_3$ in portions.

Higher initial glucose concentration shortened the log phase, which led to a decrease in growth rate. Higher initial glucose concentration also gave rise to an increase in log phase and a decrease in specific growth rate[1]. This results emphasise the inhibition of cell growth due to increased osmolarity created in the medium by increased sugar concentration. This is further evidenced by the delay in the commencement of lactic acid production. Further these results also indicated that the cells have begun to adopt to the environment with high osmolarity and then have started to produce lactic acid. Although the lactic acid yield decreased with increasing glucose concentration, efficiency of lactic acid production did not decline proportionally (Table 1).

Table 1 : Effect of glucose concentration on the yield and efficiency of lactic acid production by *L. casei*. The pH was maintained by the manual discontinuous addition of 4N NaOH or initial addition of $CaCO_3$.

	Glucose (gl^{-1})				
	50		85	150	
	4N NaOH	$CaCO_3$ (50 gl^{-1})	4N NaOH	4N NaOH	$CaCO_3$ (50 gl^{-1})
Lactic acid (gl^{-1})	34.5	38.2	42.7	53.5	83.0
Yield (%)	69.0	76.4	50.5	35.7	55.4
Efficiency (%)	82.1	83.0	76.4	73.3	82.8

$$\text{Efficiency (\%)} = \frac{\text{Lactic acid produced (g } l^{-1})}{\text{Glucose consumed (gl}^{-1})} \times 100$$

$$\text{Yield (\%)} = \frac{\text{Lactic acid produced (g } l^{-1})}{\text{Theoretical yield on the basis of glucose supplied (g } l^{-1})} \times 100$$

The results in Figure 4B show that increase in glucose concentration from 50 to 150 gl^{-1} while maintaining the pH by the addition of $CaCO_3$, had led to a decrease in the glucose consumption from 90 to 66%, but there was no significant decrease in the efficiency (84.8 and 82.8% respectively). In contrast, when pH was maintained using 4N NaOH (Figure 3B) and glucose concentration was increased from 50 to 150 gl^{-1} the glucose consumption decreased from 84 to 50%.

As the organism has the tendency to ferment glucose of 150 gl^{-1}, it was decided to study the effect of glucose concentration while maintaining the pH with $CaCO_3$. $CaCO_3$ is preferred here because the facilities for pH maintenance in the laboratories of developing countries are poor and if $CaCO_3$ gives better results, it would enable lactic acid production without expensive pH sensors and controllers.

When 50 and 150 gl^{-1} glucose was used in the medium for lactic acid production and pH was maintained by adding proportionate amounts of $CaCO_3$ increase in the glucose concentrations seemed to increase the NADPH level. The metabolic action of the cells were not proportionately increased but were almost two times higher in than the lowest concentration of glucose indicating the advantages of using $CaCO_3$ instead of NaOH as the neutralisng agent. These results indicated that the maintenance of pH with 4N NaOH was not effective at high glucose concentration in the medium. Further the lactic acid production was also higher when $CaCO_3$ was used as neutralising agent. Lactic acid produced from 150 gl^{-1} glucose was 83 and 51 gl^{-1} at 48 h when $CaCO_3$ and NaOH were used respectively as neutralising agents.

Addition of $CaCO_3$ as neutralising agent and addition of 150 gl^{-1} totally or in portions to the medium for lactic acid production indicated that the organism adopts to the medium with 150 gl^{-1} glucose and 150 gl^{-1} $CaCO_3$ (Figure 4A) by showing a regular increase in the NADPH level. Delay in attaining higher NADPH values with the second and third sets of experiments were not due to the decrease in pH because the pH of the media was well maintained and no difference in pH values was noted. Glucose consumption was almost the same in the first (control) second and third sets of experiments,. However the organism showed different behavior with respect to lactic acid production. The results showed that the organism did not prefer the addition of total amount of $CaCO_3$(150 gl^{-1}) at one instant. Thus the results indicated that the organism adopted well to 150 gl^{-1} glucose but prefers less $CaCO_3$ to be present in the medium. Therefore the experiment would be planned to take 150 gl^{-1} glucose initially and to add the $CaCO_3$ in three instalments. Addition of $CaCO_3$ maintained the pH better than the manual addition of 4N NaOH. However continuous maintenance of pH using automatic equipment gave better results for glucose consumption and lactic acid production [12]. When pH was maintained with automatic equipment, 150 gl^{-1} glucose was completely consumed and 140.2 gl^{-1} lactic acid was produced at

36 h where control of pH was affected with 8N NH_4OH. Hence it can be concluded that in developing countries where facilities for automated equipment are inadequate, pH could be better maintained with $CaCO_3$ rather than by manual discontinuous addition of 4N NaOH.

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