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Fermentation of starch hydrolysates by *Lactobacillus plantarum*

T.R. Shamala and K.R. Sreekantiah

Department of Microbiology and Sanitation, Central Food Technological Research Institute, Mysore, India

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SUMMARY

Lactic acid production by an isolate of *Lactobacillus plantarum* was standardised on enzyme-hydrolysed tapioca (*Manihot esculenta*) flour, tapioca starch and soluble starch. Calculated yields of lactic acid (g from 100 g reducing sugars used) in nutrient media containing the abovementioned hydrolysates (10% reducing sugars) were 21.8%, 16.2% and 16.2%, respectively. Higher yields (29–34%) were obtained in media containing 5% reducing sugars. A conversion efficiency of 80–99% was achieved when the acid produced in the broth was neutralised periodically. One hundred milliliters of the medium (5% sugars) yielded 4.0–4.5 g of calcium lactate. These results indicate that unrefined starchy material can be successfully employed for the economic production of lactic acid. The same substrate can also be utilised for biomass production, as viable lactobacilli are being used for therapy in medicine.

INTRODUCTION

Different sugars, molasses and starch hydrolysates have been tried as fermentation substrates for lactic acid production [1,5]. In recent years there has been an increased interest in utilising low-cost materials as media constituents in industrial fermentations [6]. The study reported here indicates the possibility of utilising a cheaper unrefined starchy material like tapioca flour for lactic acid fermentation. Tapioca is widely cultivated in certain parts of India and the annual production is 5–6 million tons [2]. Tapioca starch, soluble starch, glucose and lactose were used in the experiments for comparison.

MATERIALS AND METHODS

Substrates

Substrates used in the experiments were milled tapioca chips, commercial tapioca starch, soluble starch (analytical reagent grade (AR); BDH Chemicals), glucose (AR; BDH) and lactose (AR; BDH).

Enzymes

Bacterial α-amylase (10 000 U activity/g) and amyloglucosidase (3000 U/g) were obtained from M/S Anil Starch Products Ltd., Ahmedabad, India.

Correspondence: T.R. Shamala, Department of Microbiology and Sanitation, Central Food Technological Research Institute, Mysore 570 013, India.

Hydrolysis

Starchy material mixed with distilled water (20%, w/v) was liquefied by bacterial α-amylase (0.03%; pH 6.0, 80°C for 30 min) and hydrolysed by amyloglucosidase (0.2%; pH 4.5, 55°C for 48 h). The hydrolysate was filtered through nylon cloth (140 mesh) and the amount of reducing sugars present in the filtrate was estimated [4].

Lactic culture

Lactobacillus plantarum was obtained from the National Dairy Research Institute, Bangalore, India. The culture was maintained at 10°C in sterilised (1.1 kg/cm^2, for 15 min) skim milk (10%). The organism was subcultured at 14-day intervals.

Inoculum

Inoculum (1%, v/v) was from a 24-h-old culture which was grown in 10% skim milk.

Fermentation

(1) One hundred milliliters of media (in duplicate) prepared from hydrolysates of tapioca flour, tapioca starch or soluble starch only (adjusted to 10% reducing sugars, w/v) were autoclaved (1.1 kg/cm^2 for 15 min) in 250-ml conical flasks, cooled and inoculated with the bacterial culture. These cultures were used to determine: (a) optimum pH, temperature and growth period; (b) individual and combined effects of yeast extract (0.1 and 0.5%, w/v), $(NH_4)_2HPO_4$ (0.1 and 0.5%) and $MgSO_4 \cdot 7H_2O$ (0.01 and 0.05%) on lactic acid production (initial pH 6.0, 30°C for 96 h).

(2) The effect of different concentrations of hydrolysates (5–20% reducing sugars) on lactic acid production was studied in media (0.5% each of yeast extract and $(NH_4)_2HPO_4$) which had an initial pH of 6.0. Cultures were incubated at 30°C for 96 h.

(3) *L. plantarum* was cultivated in 2-liter conical flasks under standardised culture conditions (30°C, pH 6.0, 96 h) in 1 liter of sterile nutrient medium. The medium contained yeast extract 0.5%, $(NH_4)_2HPO_4$ 0.5% and reducing sugars 5% (glucose, lactose or starch hydrolysates). A sterile suspension of $Ca(OH)_2$ was used to adjust the pH of the broth to 6.0, once in 24 h. Lactic acid was precipitated as calcium lactate [8] from the fermented broth, dried (50°C) and weighed.

Analysis of fermented broth

During fermentation, a 10-ml sample was collected once in 24 h under aseptic conditions. Total titratable acidity (%) was estimated by titration with 0.1 N NaOH in the presence of phenolphthalein as indicator. The result is expressed as lactic acid and the yield (%) was calculated as the grams of acid that were produced from 100 g of reducing sugars.

Reducing sugars present in the broth were estimated by using dinitrosalicylic acid-reagent [4].

Viable cell counts were determined by surface plating of diluted broth on MRS-agar. Plates were incubated at 30°C for 48 h.

Clarified culture broths and their distillates were made alkaline with concentrated NH_4OH and spotted separately on Whatman No. 1 paper. They were developed in 1-butanol/formic acid/water (10:2:15) and 1-propanol/concentrated aqueous NH_3 (7:3), respectively, sprayed with bromocresol green reagent and observed for non-volatile and volatile organic acids [3].

RESULTS AND DISCUSSION

Rogosa [7] reported that most lactobacilli do not readily metabolise starch. In an attempt to utilise starchy substrates for lactic acid fermentation, enzyme-hydrolysed material was tried. Enzyme hydrolysis yielded 77, 95 and 99% of reducing sugars from tapioca flour, soluble starch and tapioca starch, respectively.

Optimum fermentation of starch hydrolysates by *L. plantarum* occurred at 30°C, initial pH 6.0 and after 96 h growth (Table 1). However, the yield of lactic acid in these media containing starch hydrolysates only was low. According to Aksu and Kutsal [1], nutrients such as yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4 \cdot 7H_2O$ increase the efficiency of lactic acid fermentation. In our study also, yeast extract and $(NH_4)_2HPO_4$ were found to enhance fermentation of starch hydrolysates by *L.*

Table 1

Effect of temperature, pH and fermentation period on lactic acid production by *L. plantarum*

Hydrolysates	Yield of lactic acid (%) in media[a]										
	temperature (°C)				pH			period (h)			
	30	35	37	45	5.5	6.0	6.5	24	48	72	96
Tapioca flour	6.4	6.3	6.2	6.0	5.5	6.2	5.7	2.7	4.6	5.8	7.0
Tapioca starch	1.6	1.6	1.5	0.7	1.4	1.6	1.4	0.2	0.9	1.1	1.7
Soluble starch	1.4	1.0	1.0	0.8	1.0	1.2	1.1	0.5	0.9	1.0	1.1

[a] Media contained starch hydrolysates only (10% reducing sugars) and, unless otherwise mentioned, the fermentation was carried out at pH 6.0 (initial), 37°C for 96 h.

plantarum but $MgSO_4 \cdot 7H_2O$ did not exert a significant effect on the yield of acid (Table 2). In media (yeast extract and $(NH_4)_2HPO_4$) containing starch hydrolysates, the yield of lactic acid was higher at a 5% sugar concentration (tapioca flour 34.6%, tapioca starch 29.7%, and soluble starch 30.4%) than at 10% (22.0%, 16.3% and 17.1%, respectively), 15% (14.7%, 11.5% and 12.1%, respectively) or 20% (11.8%, 9.0% and 9.4%, respectively) levels. Nutrient media containing 5% glucose or lactose gave a 21% yield. In all the experiments, the yield of acid was higher with tapioca flour hydrolysate than with other substrates. In addition to nutrients, the unrefined flour also contains protein (1.7%), which may also help in preventing a rapid drop in the pH of the fermenting broth. The efficiency of lactic acid fermentation is improved by the maintenance of a suitable pH [5]. By adjusting the pH to 6.0 in the fermenting broth (containing 5% sugars) periodically (once in 24 h), a yield of 99% (sugar utilised, 98%) was obtained with starch hydrolysates and glucose and 88% with lactose. Lactic acid yields recorded for tapioca flour hydrolysate on four consecutive days were 0.7, 1.1, 1.5 and 1.6%. Corresponding viable cell counts (cells $\times 10^7$/ml) were 80, 287, 401 and 332. The broth contained lactic acid as the only organic acid as revealed by paper chromatography. Lactic acid was separated from the broth as calcium lactate (4–4.5 g/100 ml).

Table 2

Effect of nutrients on lactic acid production by *L. plantarum*

Hydrolysates	Yield of lactic acid (%) in nutrient media[a]							
	$(NH_4)_2HPO_4$ (%)		yeast extract (%)		$MgSO_4 \cdot 7H_2O$ (%)		$(NH_4)_2HPO_4$ + yeast extract (%)	
	0.1	0.5	0.1	0.5	0.01	0.05	0.1 + 0.1	0.5 + 0.5
Tapioca flour	8.7	10.5	10.3	14.8	6.9	6.9	13.5	21.8
Tapioca starch	2.4	5.4	5.5	7.2	1.7	1.8	9.6	16.2
Soluble starch	2.3	6.7	5.8	9.6	2.0	2.7	9.7	16.2

[a] Media also contained starch hydrolysates (10% reducing sugars) and had an initial pH of 6.0. The fermentation was carried out at 30°C for 96 h.

Results obtained in this study indicate that cheaper, easily available materials can be efficiently utilised for lactic acid fermentation. In addition to tapioca, there are other materials such as ragi (*Eleusine coracana*), rice (*Oryza sativa*), sorghum (*Andropogan sorghum*) and corn (*Zea mays*) which can be hydrolysed to yield 70–80% reducing sugars. These materials can also be utilised as substrates for lactic acid production.

In addition to lactic acid production, these substrates appear to be potential alternate substrates for obtaining cells of lactobacilli which are finding extensive use in medicine.

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