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Lactic acid production from enzyme-thinned corn starch using *Lactobacillus amylovorus*

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SUMMARY

An alternative process for industrial lactic acid production was developed using a starch degrading lactic acid producing organism, *Lactobacillus amylovorus* B-4542. In this process, saccharification takes place during the fermentation, eliminating the need for complete hydrolysis of the starch to glucose prior to fermentation. The cost savings of this alternative are substantial since it eliminates the energy input, separate reactor tank, time, and enzyme associated with the typical pre-fermentation saccharification step. The only pre-treatment was gelatinization and enzyme-thinning of the starch to overcome viscosity problems associated with high starch concentrations and to make the starch more rapidly degradable. This fermentation process was optimized for temperature, substrate level, nitrogen source and level, mineral level, B-vitamins, volatile fatty acids, pH, and buffer source. The rate of the reaction and the final level of lactic acid obtained in the optimized liquefied starch process was similar to that obtained with *L. delbrueckii* B-445 using glucose as the substrate.

INTRODUCTION

The U.S. market for lactic acid, including both synthetic and natural fermented types, is estimated to be about 25 million pounds. Approximately half of this demand (12–13 million pounds) is met by importing lactic acid, with the Dutch CSM company supplying the fermented natural lactic acid [4]. New applications, such as degradable plastics made from poly(lactic) acid, have the potential to greatly expand the market for lactic acid, if more economical processes could be developed [12]. Industrial production of natural, fermented lactic acid is essentially non-existent in the United States due to an inability to compete economically with foreign production using current processes.

Industrial processes for the production of lactic acid typically use sugars, molasses, or starch hydrolysates as the fermentation substrate [23]. When these simple sugars occur naturally in the fermentation substrate, as lactose in whey or sucrose in beet or cane molasses, this is the most economical approach. However, when starch is used, these sugars must be produced by hydrolytic pretreatment of the starch in the natural substrate [20].

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The corn wet milling industry is the primary source of dextrose for fermentation in the U.S. The objective of industrial enzyme liquefaction/saccharification processes is to achieve a maximum glucose content in the final hydrolysate (>95% DE) [21]. The production of 95 DE dextrose typically involves a dual enzyme process of liquefication (1–2 h) and saccharification (24–96 h) [6]. These hydrolytic steps are necessary if the organism used in the fermentation does not have the hydrolytic enzymes required to use the starch directly. However, another approach which could significantly reduce the cost of the fermentation would be to use organisms with the ability to convert starch directly to lactic acid. Such a process could also utilize inexpensive waste materials containing starch, such as the 1.3 billion kg of potato-processing waste generated each year [22].

Lactobacillus amylovorus [15] and *Lactobacillus amylophilus* [17], isolated from corn-manure enrichments [7, 16], both produce an extracellular amylase which enables them to produce lactic acid from starch. *L. amylovorus* produces a racemic mixture of nearly equal proportions of L- and D-lactic acid [15], and *L. amylophilus* produces only L-lactic acid [17]. Border et al. developed a process for production of proprionic acid from wheat flour using a co-culture of *Lactobacillus amylophilus* and *Propionibacterium freudenreichii* [2]. However, the nutritional and cultural requirements of these starch-utilizing lactobacilli are not fully defined and their ability to produce lactic acid

under high substrate conditions has not been tested. The objective of this research was to optimize a process for the production of lactic acid from corn starch using *L. amylovorus*.

MATERIALS AND METHODS

Microorganisms. *Lactobacillus amylovorus* strains NRRL B-4542, B-4540 and *Lactobacillus delbrueckii* NRRL B-445 (now known to be *Lactobacilli casei* subsp. *rhamnosus*) were obtained from the USDA Northern Regional Research Center in Peoria, Illinois. *L. delbrueckii* B-445, a widely studied industrial organism, grown under optimum conditions on glucose was used as a basis by which to evaluate the performance of *L. amylovorus* fermentations. *L. delbrueckii* stock cultures were maintained in a 5% (w/v) glucose medium developed by Friedman and Gaden [5]. *L. amylovorus* stock cultures were maintained in the same media modified to contain 10% (w/v) enzyme-thinned corn starch (Table 1). Stock cultures were incubated at 37 °C and transferred at weekly intervals.

Inoculum preparation. In preparation for an experiment, stock cultures (3 drops) were transferred to 3 ml of either 10% starch or 5% glucose Friedman's medium. After 24 h this procedure was repeated. From that point, 5% inoculum was used in successive transfers that were 24 h apart until sufficient inoculum was obtained to innoculate the final medium at the 5% level.

Enzyme-thinned starch. The starch used in these studies was gelatinized and enzyme-thinned. Pearl starch (A.E. Staley, food grade) was mixed with distilled water to make 20 liters of 30% (w/v) solution. The pH was adjusted to 7.0 with 5 N NaOH and 1.2 g of bacterial α-amylase in 80 ml of a 0.2% $CaCl_2 \cdot 2H_2O$ solution (54.5 ppm Ca^{2+}) was added (Amano; *Bacillus subtilis*, 176000 BAU/g). The starch/enzyme mixture was heated in a steam jacketed kettle (Groen, Model TDB/7) at a stirring dial setting of 2. The temperature was increased from room temperature to 87 °C over a 24 min period and then held constant for 10 min. The enzyme-thinned starch was allowed to air cool for 10 min and then dispensed into 600 ml bottles and stored in a freezer at − 15 °C. Microscopic examination indicated that all of the starch grains were disrupted and the starch was completely gelatinized. The final concentration of the enzyme-thinned starch solution was 300 g/l (30% w/v) as determined by drying a known volume in an oven at 95 °C for 24 h. This enzyme-thinned starch remained in suspension and was fluid when refrigerated at 4 °C. All media was autoclaved at 121 °C for a minimum of 30 min prior to inoculation to sterilize the media and ensure that the α-amylase used in enzyme-thinning of the starch was deactivated. The deactivation was confirmed by incubating uninoculated media containing 10% enzyme-thinned starch at 37 °C for 5 days. No significant change in starch level or oligosaccharide pattern occurred during this incubation as determined by the HPLC method described below.

TABLE 1

Basal medium[a]

Medium component	Amount (g/l)
Enzyme-thinned starch	100.0
Yeast extract	30.0
Sodium acetate	1.7
Minerals	*Amount (mg/l)*
$MgSO_4 \cdot 7H_2O$	1225.0
K_2HPO_4	500.0
KH_2PO_4	500.0
$FeSO_4 \cdot 7H_2O$	58.4
$MnSO_4 \cdot H_2O$	33.6
Distilled water	to volume

[a] Modified from Friedman and Gaden, 1970 [5].

Experimental protocols. The batch mode of fermentation was used for all studies. All experiments, with the exception of the pH optimization studies and the *L. delbrueckii* comparison were conducted in shake flasks containing 15 ml of medium and incubated in a temperature controlled shaker operated at 150 rpm (Psycrotherm; New Brunswick Scientific Co., Inc.). The shake flasks were 50 ml Erlenmeyer flasks fitted with a Bunsen valve to allow escape of CO_2 released as a result of lactic acid neutralization. The buffer used was an excess of $CaCO_3$ marble chips (3.3 g in 15 ml of medium; Mallinckrodt). The fermentor used for the pH optimization studies and the *L. delbrueckii* comparison was a New Brunswick Scientific Co., Inc. Model C30 bench top chemostat with a Model pH-40 pH controller. The working volume of the fermentor was 1.2 l with agitation at 400 rpm. The pH was controlled using 5 N NaOH.

An overview of the media composition used for the different experiments in shown in Table 2. Media preparation, inoculation and incubation were carried out using the anaerobic Hungate technique [3]. The gas was oxygen free CO_2 or N_2 (N_2 was used in fermentor studies). Two replicate shake flasks were used for each data point presented in figures. When a single sampling value is presented, the data were obtained after five days of incubation (nitrogen source studies).

In the growth stimulant experiment referred to in Table 2, 1 ml of vitamin complex solution, 1 ml of trace metal solution, 1 ml of volatile fatty acids (VFA) solution, and 0.1 ml of hemin solution were added to 100 ml of the basal medium with 0.5% yeast extract. The vitamin

TABLE 2

Summary of experimental protocols

Experiment	Starch (%)	Yeast extract (%)	Mineral (× Basal)	Temp. (°C)
Temperature optimum	5	3	1	30, 35, 40, 45, 50
Substrate optimum	7.5, 10, 12.5, 15, 20	3	1	37
Yeast ext. level	10	0.5, 1.5, 3	1	37
Nitrogen	10	3	1	37
Mineral level	10	3	0.5, 1, 1.5	37
Growth stimulant	10	0.5	1	37
pH optimum	10	3	1	37
Optimum cond.	10	3	1	40

solution contained the following vitamins (mg/100 ml): Thiamine · HCl, 20.0 mg; D-pantothenic acid (Calcium salt), 20.0 mg; niacinamide, 20.0 mg; riboflavin, 20.0 mg; pyridoxine · HCl, 20.0 mg; P-aminobenzoic acid (PABA), 1.0 mg; d-biotin, 0.5 mg; folic acid, 0.5 mg; and cyanocobalmin (vitamin B_{12}), 0.2 mg. The trace mineral solution contained the following (mg/1000 ml): Disodium EDTA, 500 mg; $FeSO_4 \cdot 7H_2O$, 200 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 3 mg; H_3BO_3, 30 mg; $CoCl_2 \cdot 6H_2O$, 20 mg; $CuCl_2 \cdot 2H_2O$, 1 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; and $Na_2MoO_4 \cdot 2H_2O$, 3 mg. The VFA solution contained the following (ml/100 ml in distilled water): acetic acid, 16.0 ml. propionic acid, 6.7 ml; *n*-butyric acid, 4.1 ml; iso-butyric acid, 0.84 ml; *n*-valeric acid, 0.98 ml; iso-valeric acid, 0.98 ml; 2-methyl butanoic acid, 0.98 ml. The hemin solution was made by dissolving 50 mg of hemin in 1 ml of 1 N NaOH and diluting to 100 ml with distilled water.

Assay procedures. Lactic acid was analyzed by HPLC using a Perkin Elmer Series 4 pump equipped with an RI detector (Model LC 25) and a computing integrator (Model LC 100). The column used was a Polypore H 10 mm (4.6 × 220 mm; Brownlee Labs, Inc) operated at room temperature. The mobile phase was diluted H_2SO_4 at pH 2 and the flow rate was 0.2 ml/min. When necessary, samples were heated to redissolve calcium lactate crystals and diluted prior to analysis (the linear range for the lactic acid calibration curve was 0–30 g/l). Appropriately diluted samples were centrifuged (Beckman J2-21M/E centrifuge) at 10000 rpm for 10 min and filtered through a 0.2 μ filter, prior to injection into the HPLC.

VFAs were detected using a Varian Model 3700 gas chromatograph equipped with a flame ionization detector as described previously [8].

Starch and oligosaccharides were also analyzed by HPLC with a carbohydrate column (Aminex HPX-42A; Bio-Rad) heated at 75 °C. The mobile phase was distilled water at a flow rate of 0.4 ml/min. Malto-oligosaccharide standards ranging from D-glucose to maltoheptaose were used to quantitate oligosaccharides in enzyme-thinned starch (Sigma Chemical Co.). The filterable starch peak (>G7) was quantified by the following procedure: (a) Five ml of enzyme-thinned starch solution was dried at 95 °C to determine the dry weight of starch; (b) a second 5 ml of enzyme-thinned starch solution was filtered through a pre-weighed 0.2 μ acetate filter; (c) starch retained on the filter was determined by dry weight measurement after drying at 95 °C; (d) the filtrate collected was analyzed by HPLC; (e) the filterable starch peak was the only unquantified peak, and was therefore determined as the total dry weight of starch minus the starch retained on the filter and the total of the oligosaccharides. Once quantified, the filtered liquefied starch was used as a standard for further starch/oligosaccharide analyses.

Conversion calculations. The conversion percentage was calculated as follows: Conversion% = [lactic acid produced (final lactic acid concentration – initial lactic acid concentration due to innoculation carryover) / total initial carbohydrate expressed as glucose] × 100. Complete hydrolysis of 10 g of starch yields 11.1 g of glucose due to the addition of water during hydrolysis, as shown below:

$$(C_6H_{10}O_5)_n + (n-1)H_2O \rightarrow n\,(C_6H_{12}O_6)$$

The conversion factor for starch to glucose is therefore:

$$\text{conversion factor} = \frac{180 \times n}{180 \times n - (n-1) \times 18}$$

As n becomes large, as in the starch molecule, this factor becomes 1.11. Thus, complete conversion of 100 g/l starch to lactic acid would yield 111.0 g/l of lactic acid.

RESULTS AND DISCUSSION

Temperature optimum

In preliminary tests, *L. amylovorus* strain NRRL B-4542 produced higher final levels of lactic acid than the type strain NRRL B-4540, so it was selected for further study. Nakamura [15] reported a minimum growth temperature of 20–25 °C, an optimum of 37–45 °C and a maximum of 45–50 °C for the type strain of *L. amylovorus* NRRL B-4540. In order to determine the effect of temperature on the rate and final level of lactic acid produced by strain B-4542 when grown at higher substrate levels, a range of temperatures were studied. The temperatures selected were 30, 35, 40, 45, and 50 °C (Fig. 1). *L. amylovorus* strain NRRL B-4542 was highly sensitive to changes in temperature out of its optimum temperature range. The final concentration lactic acid produced from 5% enzyme-thinned starch was similar for 35, 40, and 45 °C (58.6, 55.4, and 57.0 g/l, respectively). The lactic acid production rate increased as temperature increased from 30 to 40 °C, remained the same between 40 and 45 °C, and decreased dramatically above 45 °C. The maximum lactic acid production rate achieved was 8.6 g lactate/l/h at 40 °C. Although there was a marked decrease in product formation rate at 30 and 50 °C, the final lactic acid level at 30 °C was similar (57.8 g/l) to that at 35–45 °C. From these studies, the optimum temperature would appear to be between 40 and 45 °C.

Substrate concentration

In order to determine the effect of starch concentration on the final concentration of lactic acid produced, 7.5%, 10%, 12.5%, 15% or 20% enzyme-thinned starch was added to the basal medium. Final lactic acid levels increased with each increase in starch level, with the final levels of lactic acid in flasks with 7.5, 10, 12.5, 15 and 20% initial starch concentrations being 75.2, 103.8, 120.3, 148.3, and 165.3 g/l, respectively (Fig. 2). The corresponding starch to lactic acid conversion percentages were 91.1, 94.1, 89.2, 89.3, and 74.2%. Thus conversion percentage remained relatively constant (between 90 and 95%) up to 15% starch, with a substantial drop in conversion percentage at the 20% starch level. The iodine test was used to determine whether residual starch remained at the end of each experiment. The results indicated that up to 15% starch was completely hydrolyzed by *L. amylovorus*; however, residual starch was observed in flasks with 20% initial starch concentration.

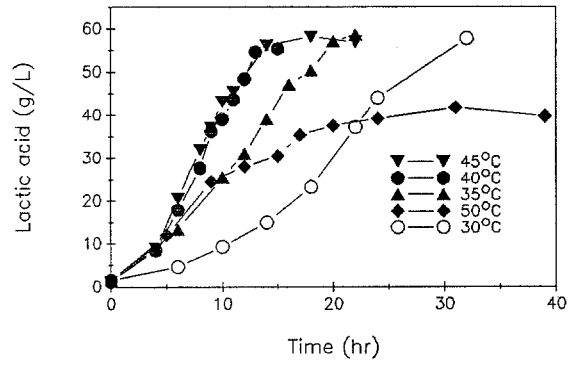

Fig. 1. Effect of fermentation temperature on the rate of product formation and final level of lactic acid produced by *L. amylovorus*.

Yeast extract level

The concentration of yeast extract in the basal medium with 10% starch was varied from 0.5%, 1.5%, to 3% (w/v) in order to determine the optimum level of yeast extract. The rate of lactic acid production decreased significantly when the yeast extract level was decreased below 3% (Fig. 3). However, the final levels of lactic acid produced for 0.5, 1.5 and 3.0% yeast extract were not significantly different (101.1, 107.3 and 110.5 g/l, respectively). This yeast extract requirement is higher than one would expect simply to supply nitrogen for growth. This suggests that yeast extract contains a specific peptide or other growth factor present at low levels which increases the growth rate and product formation rate of *L. amylovorus*. *L. delbrueckii* is stimulated by peptides [18,19] and Berg et al. [1] have isolated an octapeptide from fresh yeast extract which greatly stimulated growth of *L. sanfrancisco*.

Nitrogen sources

Various nitrogen sources were compared to yeast extract by replacing yeast extract in the basal medium with 10% starch. The amount of each nitrogen source was

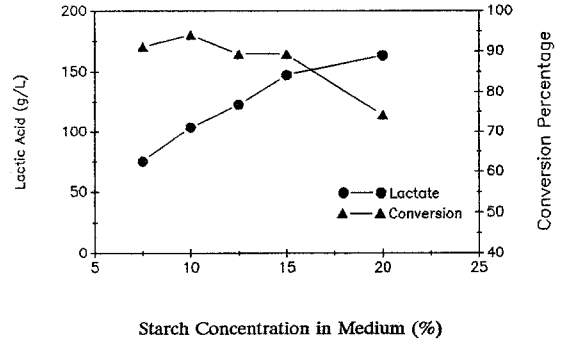

Fig. 2. Effect of enzyme-thinned starch concentration on conversion of starch to lactic acid by *L. amylovorus*.

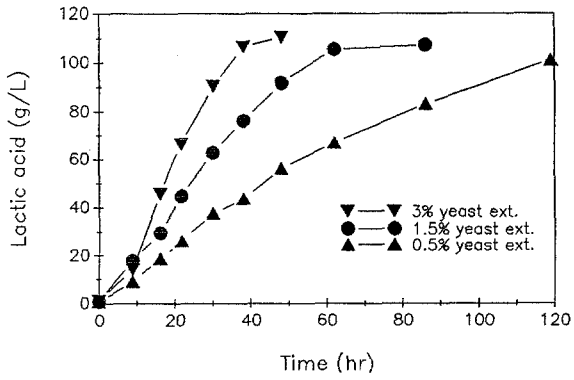

Fig. 3. Effect of yeast extract concentration on rate of product formation and final level of lactic acid produced by *L. amylovorus*.

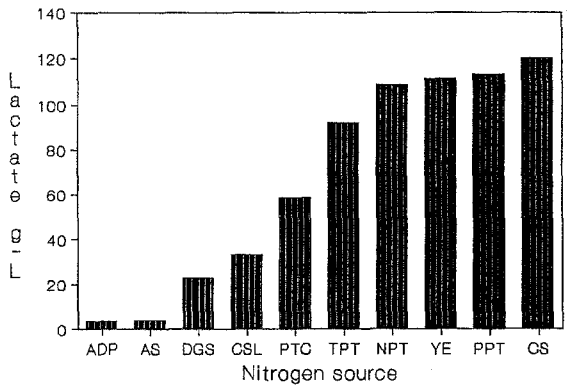

Fig. 4. Final lactic acid level produced by *L. amylovorus* on different nitrogen sources. Abbreviations are as follows: ADP, $(NH_4)_2HPO_4$; AS, $(NH_4)_2SO_4$; DGS, distillers grain and solubles; CSL, corn steep liquor; PTC, pepticase; TPT, trypticase; NPT, neopeptone; YE, yeast extract; PPT, phytone peptone; and CS, cottonseed hydrolysate.

calculated to give the equivalent nitrogen of 3% yeast extract; if the nitrogen content was unknown, 3% (w/v) of the nitrogen source was added. The amounts used were $(NH_4)_2HPO_4$, 1.36%; $(NH_4)_2SO_4$, 1.36%; cottonseed hydrolysate (Sheffield Products), 3.48%; neopeptone (Difco), 3%; corn steep liquor (Sigma), 1.36%; trypticase (BBL), 2.64%; pepticase (Sheffield Products), 2.32%; phytone peptone (papaic digest of soybean meal; BBL), 3%; distiller grains and solubles (Sigma), 3%; or yeast extract (Difco), 3%. As anticipated, the inorganic nitrogen sources tested were not utilized by *L. amylovorus* (Fig. 4). Corn steep liquor, distillers grains and solubles, pepticase and trypticase were utilized, but yielded lower final levels than neopeptone, yeast extract, phytone peptone and cottonseed hydrolysate. Starch was completely consumed in media with each of the four best nitrogen sources.

In order to determine the effect of the degree of hydrolysis of the protein source on lactic acid production, nitrogen sources with varying degrees of hydrolysis were tested (Table 3). The amount of the nitrogen source added to the 10% starch basal medium was calculated to provide nitrogen equivalent to 3% yeast extract. Yeast extract yielded the highest final level of lactic acid when compared to the other nitrogen sources. However, soy protein hydrolysates (Hy-Soy and N-Z-Soy BL) yielded lactic acid levels nearly as high as yeast extract. The casein hydrolysates tested all yielded considerably lower levels of lactic acid.

Within nitrogen source, the degree of hydrolysis significantly affected the final level of lactic acid produced. Nitrogen sources which were nearly completely hydrolysed (high levels of free amino acids, high amino/total N% and low molecular weight (MW)) yielded less lactic acid than sources with less extensive hydrolysis. These results are consistent with studies on the effect of whey hydrolysates on lactic acid production by *L. bulgaricus*, in which hydrolysates of average MW of 700 appeared to be most stimulatory [10, 11]. These results further suggest that *L. amylovorus* may be stimulated by peptides of a certain length or of a certain amino acid composition. This aspect of the nutrition of *L. amylovorus* needs to be studied further.

Macro-mineral level

In order to determine whether the level of minerals in the basal medium was optimal for high substrate levels, the mineral solution concentration was included at 0.5, 1.0, or 1.5 fold basal levels. The rate of product formation was nearly identical and the final levels of lactic acid produced from 10% enzyme-thinned starch for the 0.5-, 1.0-, and 1.5-fold mineral levels were 115.1, 110.5, and 108.2 g/l, respectively. These results indicated that the level of minerals in the basal medium was more than sufficient and could possibly be lowered if the optimal level of individual minerals was determined.

Growth stimulants

In an effort to determine if the requirement for yeast extract could be reduced by the addition of known nutrients to the medium, B-vitamins, trace minerals, volatile fatty acids, and hemin were added to the basal medium containing 0.5% yeast extract. The addition of these known growth stimulants to media containing 0.5% yeast extract did not increase the rate or final level of lactic acid produced significantly. It is possible that one component may have been stimulatory and another component inhibitory. This needs to be tested further by single addition experiments.

TABLE 3

Effect of protein source and extent of hydrolysis[a] on lactic acid production by *L. amylovorus*

Protein type	Product name[b]	Amino N/total N (%)	Free amino acids (%)	Di- and tripeptides (%)	Larger peptides (%)	Avg. MW	Lactic acid (G/L)
Soy	Amisoy	69.0	76	24	–	178	2.6
	Hy-Soy	20.0	17	51	32	521	88.9
	N-Z-Soy BL	18.7	10	30	60	735	91.1
Casein	Hy-Case SF	79.8	72	28	–	185	1.4
	Amicase	76.6	71	U[c]	U[c]	U[c]	1.6
	N-Z-Amine A	49.6	58	32	10	283	30.0
	N-Z-Case M	35.1	27	60	13	365	30.6
	Pepticase	30.1	14	61	25	476	29.8
Yeast	Yeast ext.	39.4	U[c]	U[c]	U[c]	U[c]	97.2

[a] Values for extent of hydrolysis of Sheffield products are from the Sheffield Product Technical Manual on Hydrolyzed Proteins. Yeast extract Amino N/Total N percent is from BBL manual.

[b] Registered trade names of Sheffield Products, a subsidiary of Kraft, Inc. Yeast extract was Difco Bacto Yeast Extract.

[c] U, data were unavailable.

pH Optimum

The lactic acid production rate of lactobacilli is known to be sensitive to culture pH [9]. In order to determine the optimum pH for lactic acid production from enzyme-thinned starch for *L. amylovorus*, pH was continously controlled at 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 with 5 N NaOH in a 1.2 l (working volume) fermentor. Maximum lactic acid production rate was obtained between pH 5–6 in these studies (Fig. 5). The rate of product formation decreased slightly at 4.5, and was greatly reduced at pH 4.0 and 6.5.

The rate of lactic acid production by *L. amylovorus* grown on 10% enzyme-thinned starch with pH maintained at 6.0 (7.36 g/l/h) compared favorably with the rate of lactic acid production by *L. delbrueckii* B-445 (7.34 g/l/h) grown in medium with 5% glucose at its optimum growth temperature (45 °C) and pH (6.0) (Fig. 6).

These results under controlled pH conditions were somewhat surprising in light of data obtained in shake flask experiments. In shake flasks in which pH was controlled by $CaCO_3$ marble chips, the pH of the broth rapidly dropped from initial levels of 5.8 to approximately 4.2. Thus, the $CaCO_3$ was effective in preventing the pH from dropping lower than pH 4. Yet there was no significant difference in lactic acid production rates in fermentation with continuously controlled pH (pH 6.0) and in poorly controlled shake flask cultures buffered by $CaCO_3$ in which the pH dropped to approximately 4.2 (Fig. 7).

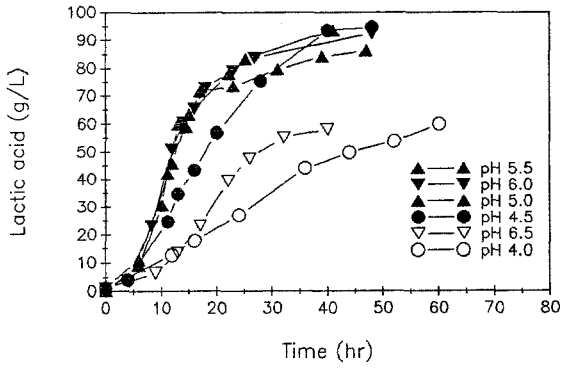

Fig. 5. Effect of fermentation pH on the rate of product formation and final level of lactic acid produced by *L. amylovorus*. pH was continuously controlled with 5 N NaOH.

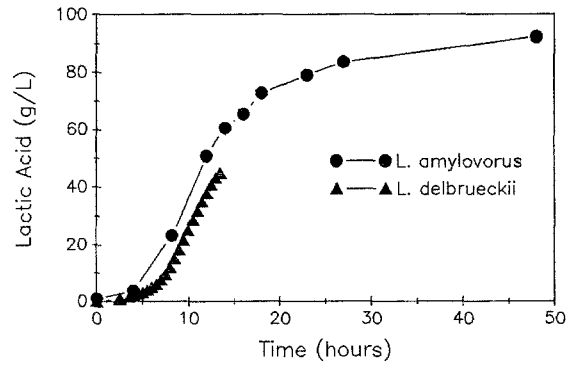

Fig. 6. Comparison of lactic acid production by *L. amylovorus* grown on 10% enzyme-thinned starch versus *L. delbrueckii* B445 grown on 5% glucose media. Growth temperature was 37 °C for *L. amylovorus* and 45 °C for *L. delbrueckii*; pH was controlled at 6.0 with NaOH for both organisms. Data for *L. delbrueckii* was obtained from Luedeking and Piret [13], Table II, p. 402.

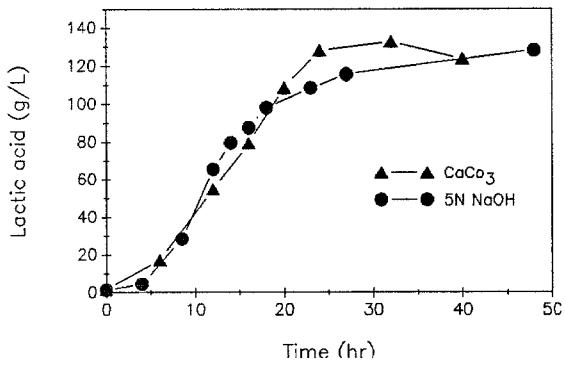

Fig. 7. Comparison of product formation rate and final level of lactic acid produced by *L. amylovorus* with pH controlled by either one-time addition of $CaCO_3$ chips or by controlled addition of 5 N NaOH to maintain a constant pH of 6.0.

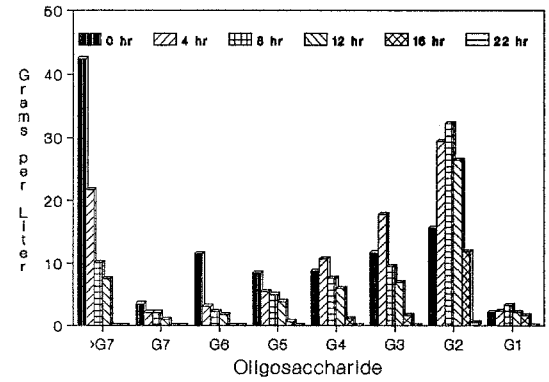

Fig. 8. Initial oligosaccharide concentrations in media containing 10% enzyme-thinned starch and changes with time during fermentation to lactic acid by *L. amylovorus*. Oligosaccharides measured were G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5; maltopentaose; G6, maltohexaose; G7, maltoheptaose; and > G7, higher saccharides including filterable enzyme-thinned starch.

The pH-controlled data shown in Fig. 6 have been corrected for dilution by base addition.

Possible explanations for such anomalous behavior may be the broad pH optima for this organism, or possibly calcium has a modulating influence upon pH effect. When grown in shake flasks with $CaCO_3$ buffer and high substrate levels (15–20% enzyme-thinned starch), *L. amylovorus* was able to continue to produce lactic acid until the calcium lactate formed a milky white gel of such a consistency that the flask could be inverted without any material flowing down the side of the flask. Indeed, it seems that the maximum level of lactic acid achievable in this shake flask system is limited by crystal formation and precipitation of calcium lactate.

Optimum conditions

The optimum conditions for temperature, substrate concentration, yeast extract concentration, buffer, etc. as determined in the above studies were selected to determine the rate of lactic acid production under these conditions. Since there was no difference in product formation rate between the shake flasks buffered with $CaCO_3$ and the fermentor with pH controlled by NaOH, the shake flask system was chosen for the optimum conditions experiment. The level of enzyme-thinned starch was 10% with 3% yeast extract and basal medium levels of minerals. The temperature optimum is between 40 and 45 °C and although a high fermentation temperature reduces the chance of contamination, it requires more energy to maintain the higher temperature. The temperature chosen for the optimum fermentation conditions was therefore 40 °C. With pH dropping to near 4.0, contamination should not be a significant problem in this system.

There was a parallel relationship between growth of *L. amylovorus* and product formation through the first 16 h of the fermentation (data not shown). Cell numbers reached a maximum of 6.8×10^9 per ml at 16 h after initiation. Bacterial numbers decreased after 16 h, presumably due to cell lysis, as a result of the accumulation of toxic materials and the depletion of energy sources. Similar results have been reported for *L. delbrueckii* [14]. The final level of lactic acid was 120.7 g/l after 35 h. The only other detectable endproduct was acetic acid (3.58 g/l). A mass balance calculation made on this fermentation under optimal conditions indicated that conversion of starch to lactic acid was 98.1%. This extremely high conversion percentage may be explained by the utilization of carbohydrates present in yeast extract. Approximately 16.6% of yeast extract is carbohydrate and total conversion of this material to lactic acid could contribute 5.0 g of lactic acid per liter. This assumption would lower the conversion percentage to approximately 94.0%; since some substrate was utilized for cell growth, which was not accounted for in the calculation, this is a reasonably good conversion percentage.

The initial levels of oligosaccharides in the medium and changes with time during fermentation under optimal conditions are shown in Fig. 8. All of the starch was converted to oligosaccharides by 16 h and no oligosaccharides were detectable after 22 h. The concentrations of glucose, maltose, maltotriose and maltotetraose increased initially and then decreased. The accumulation of these oligosaccharide intermediates is significant, as it indicates that the rate limiting step in this fermentation is not the conversion of starch to oligosaccharides, but the conversion of oligosaccharides to lactic acid. The increase in the concentrations of these four oligosaccharides agrees with the results of Nakamura [15], which indicated that these oligosaccharides are the major endproducts of

starch degradation by α-amylase obtained from *L. amylovorus* culture broth.

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