

Direct conversion of starch to L(+) lactic acid by amylase producing *Lactobacillus amylophilus* GV6

C. Vishnu, G. Seenayya, Gopal Reddy

Abstract *Lactobacillus amylophilus* strain GV6, isolated from corn starch processing industrial wastes, was amylolytic and produced 0.96 g L(+) lactic acid per gram of soluble starch. The optimum temperature and pH for growth and L(+) lactic acid production were 37 °C and 6.5, respectively. At low substrate concentrations, the lactic acid production on corn starch was almost similar to soluble starch. The strain is fermenting various naturally available starches directly to lactic acid. The total amylase activity of the strain is 0.59 U/ml/min. The strain produced 49 and 76.2 g/l L(+) lactic acid from 60 g/l corn starch and 90 g/l soluble starch, respectively. This is the highest L(+) lactic acid among the wild strains of *L. amylophilus* reported so far.

1 Introduction

Lactic acid is widely used in food, pharmaceutical, leather, textile industries and as a chemical feed stock. Lactic acid is also the source of polylactic acid, a polymer used as specialty medical and environmentally biodegradable plastics, which substitute for synthetic plastics manufactured from petroleum derivatives [1–3]. The lactate polymer possess a higher melting point and crystallinity when L(+) lactic acid with higher optical purity is present in the lactate polymer [4].

Lactic acid is manufactured either by chemical synthesis or by carbohydrate fermentation. Through chemical synthesis only racemic (DL) lactic acid is produced, whereas, through carbohydrate fermentation technology a desired stereoisomer (L(+), D(–)) or racemic mixture (DL) of lactic acid can be produced [2, 3].

At present most widely used substrates for the production of lactic acid are refined sugars, which are expensive. Lactic acid is also produced from abundant and renewable substances such as starch by two step process of saccharification by acid or microbial amylase, followed by lactobacillus fermentation [1, 2]. The direct conversion of

starch to lactic acid by bacteria with both amylolytic and lactic acid producing characteristics will eliminate the liquefaction and saccharification processes [4].

Strains of *Lactobacillus amylophilus* produce extracellular amylase and ferment starch directly to L(+) lactic acid. All the wild strains of *L. amylophilus* reported so far produced more than 90% L(+) lactic acid at low starch concentrations. However, at high starch concentrations the lactic acid yield was low [4–6].

The starchy substrates available in the form of agricultural wastes, damaged grains and edible portions of grains and tubers serve as raw materials or feed stock materials. Among the various starches, cassava starch, sorghum starch and corn starch are the most abundant and relatively inexpensive raw materials [7]. As the demand for lactic acid is more than the production due to its increasing applications and finding of newer uses, it is necessary to exploit inexpensive and abundant starches for the large-scale production of lactic acid. For an economically viable process, it is also necessary to isolate or develop *L. amylophilus* strains with high conversion efficiency of starch to L(+) lactic acid. We report here the isolation of high L(+) lactic acid yielding *L. amylophilus* strain GV6, its amylase activity and ability to ferment various naturally available starches to L(+) lactic acid.

2 Materials and methods

2.1 Screening and isolation of strain GV6

The starch degrading strain GV6 was isolated from corn-starch processing industrial wastes after enrichment in serum vials of 120 ml capacity with 20 ml of pre-reduced modified MRS medium [8] containing g/l: beef extract, 10.0; protease peptone, 10.0; yeast extract, 5.0; soluble starch/corn starch, 10; tween 80, 1.0; diammonium citrate, 2.0; magnesium sulphate, 0.1; sodium acetate, 5.0; manganese sulphate, 0.05; dipotassium phosphate, 2.0 and resazurin, 0.002 in N₂ atmosphere. The vials were incubated at 37 °C for 2–4 days. As soon as the growth became evident by turbidity, subcultures were made into fresh starch containing medium. After few subcultures, 0.1 ml of 10-fold diluted culture was inoculated into agar roll tubes containing 5 ml of MRS medium with 1% of corn starch as the sole carbon source and rolled in cold water. The roll tubes were incubated for 2–4 days at 37 °C, the colonies showing larger zones of starch hydrolysis were picked up and transferred into soluble starch broth and incubated for

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1 day at 37 °C. From this 0.1 ml of the 10-fold diluted culture was again inoculated into starch agar roll tubes to get well isolated colonies. The process was repeated for a few times to get a well-defined pure culture [9].

2.2

Fermentation experiments

All experiments were conducted in serum vials of 120 ml capacity with 20 ml of MRS medium with 10 g starch/l. The medium was sterilized by autoclaving at 121 °C for 30 min. A 5% (v/v) inoculum, grown on 10 g starch/l for 24 h, was added to the MRS medium and incubated at 37 °C for 2 days. The effect of temperature was determined by incubating the serum vials at different temperatures ranging from 15 to 40 °C. To determine the effect of pH, the medium pH was adjusted between 4.5 and 9.5 using sterile 1 M HCl or 1 M NaOH. The pH of the culture medium during fermentation of varied concentrations of corn starch and soluble starch was maintained by addition of CaCO₃. The concentration of CaCO₃ added was 30% of the substrate concentration taken.

2.3

Substrates

Except soluble starch and corn starch, other starches were prepared with grains obtained from local market and used directly for lactic acid fermentation. Soluble starch and corn starches are procured from Himedia, Bombay, wheat bran and rice bran obtained from local mills.

2.4

Amylase production

The strain GV6 was grown on MRS medium with 0.5% soluble starch for 24 h. For extracellular amylase 20 ml of fermented broth was centrifuged at 10,000 rpm for 30 min at 4 °C, the supernatant was dialyzed against several changes of distilled water, and used as extracellular amylase enzyme [5]. The cell bound enzyme was obtained, from the pellet of the above centrifuge tubes, washed twice and suspended in 20 ml of distilled water.

2.5

Estimations

Cell growth was determined by measuring the absorbance of culture broth at 660 nm. The undegraded starch was determined on the basis of starch-iodine colour complex [5]. The total Lactic acid was estimated according to the method of Pryce (1969) [10] and Kimberley and Taylor (1996) [11]. The L(+) and D(-) lactic acid assay methods were adopted from the Sigma diagnostics procedure No.826UV (Sigma, St. Louis, MO). Amylase activity was measured by incubating 1 ml of the enzyme with 1 ml of 1% soluble starch in 0.1 M acetate buffer (pH 6.0) at 37 °C for 30 min [12]. Reducing sugars formed were measured by 3,5-dinitrosalicylic acid method described by Miller (1959) [13] using glucose as standard. One unit of amylase activity is defined as the amount of enzyme, which produces 1 µmol/min of reducing sugar with glucose as standard under the conditions described. Sugars formed by hydrolysis of starch were detected by thin layer chromatography (TLC) according to the method of Hansen (1975) [14].

The results reported here are the average of three experiments conducted in triplicate on different occasions.

3

Results and discussion

3.1

Isolation and identification of strain GV6

Eight starch hydrolyzing and lactic acid producing strains, isolated from corn starch industrial wastes, were screened for their potential to amylolytic activity and lactic acid yields [15]. They are further screened for substrate utilization, product stereospecificity, growth temperature and pH as per the methods suggested by Venus et al. (1992) [16] and Tsai et al. (1993) [17]. Among the eight strains, GV6 produced high amount of lactic acid. It was a facultatively anaerobic, Gram positive, non-sporeforming and non-motile rod. The colonies on MRS agar medium were minute, smooth, white and translucent with clear zone of starch hydrolysis around the colony. The strain produced L(+) lactic acid as the end product of starch fermentation with extracellular amylolytic activity. The strain had a growth temperature range of 15–40 °C and pH range between 4.5–9.5, with an optimum temperature and pH of 37 °C and 6.5, respectively (Figs. 1 and 2). The strain did not exhibit oxidase, catalase or nitrate reducing activities and produced only L(+) lactic acid. No gas was formed from glucose or starch. Based on the above characteristics, the strain GV6 was identified as *Lactobacillus amylophilus* [5, 18].

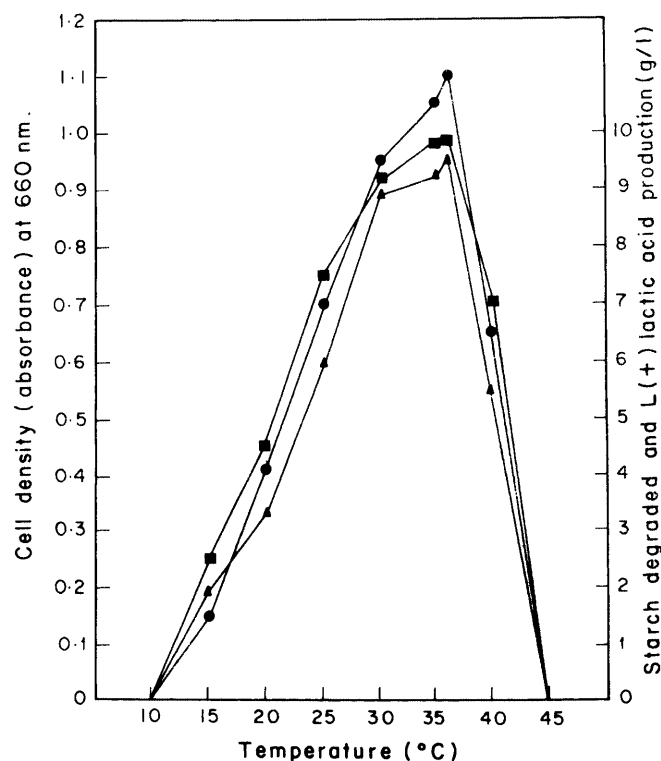


Fig. 1. Effect of temperature on the growth (A_{660}), starch degradation and lactic acid production by *L. amylophilus* GV6. An A_{660} value of 1.0 corresponded to 0.16 mg cell dry wt/ml; ●, absorbance at 660 nm; ▲, lactic acid production; ■, degraded starch

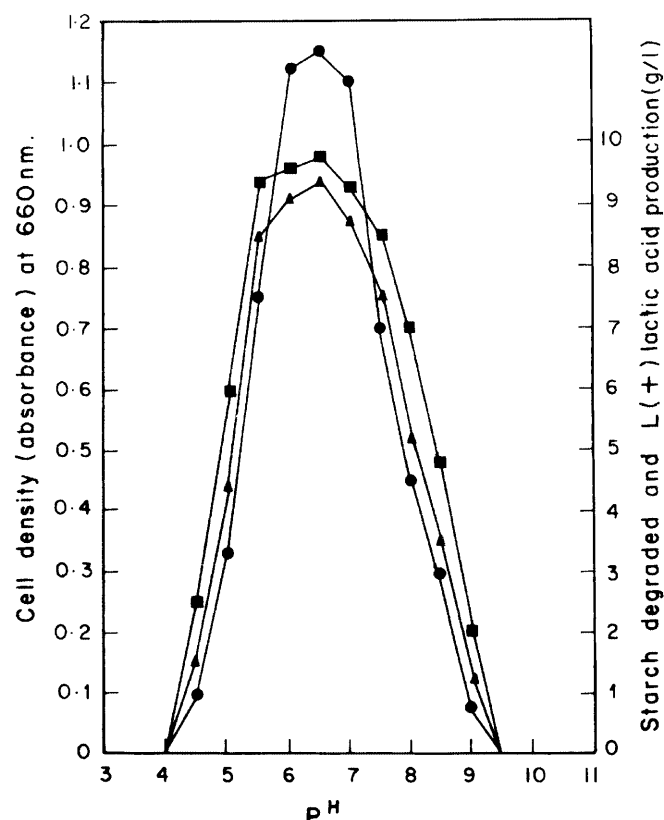


Fig. 2. Effect of initial medium pH on the growth (A_{660}), starch degradation and lactic acid production by *L. amylophilus* GV6. An A_{660} value of 1.0 corresponded to 0.16 mg cell dry wt/ml; ●, absorbance at 660 nm; ▲, lactic acid production; ■, degraded starch

3.2

Amylolytic activity

The extracellular and cell bound amylase activity of the strain GV6 was 0.28 and 0.31 U/ml/min, respectively. The hydrolysis products were glucose, maltose and maltotriose. The strain GV6 fermented various processed and natural starches and produced L(+) lactic acid resulting in a fall of pH of the medium (Table 1). Lactic acid yields and substrate fermentation by strain *L. amylophilus* GV6 and

Table 1. Fermentation of various starches by *Lactobacillus amylophilus* Strain GV6

Sr. No.	Substrate	Growth	Final pH of the fermentation broth
1	Soluble starch	+++	4.07
2	Corn starch	+++	4.29
3	Wheat starch	+++	4.32
4	Rice starch	+++	4.40
5	Sorghum starch	+++	4.43
6	Cassava starch	+++	4.61
7	Barley starch	++	5.69
8	Potato starch	++	5.72
9	Wheat bran	+	6.10
10	Rice bran	+	6.13

+++ , Good growth; ++, moderate growth; +, low growth; initial pH of fermentation medium, 6.5; Incubated at 37 °C for 48 h, substrate concentration 10 g/l

other reported strains of *L. amylophilus* are shown in Table 2. It is clear from the table that the strain GV6 produced higher yields of lactic acid and requires shorter incubation period than other reported strains of *L. amylophilus* (Table 2). This may be due to high starch degrading property of amylase enzyme produced by the strain GV6. Therefore, the strain can be further exploited for the fermentation production of L(+) lactic acid.

3.3

Effect of temperature and pH on starch fermentation

The strain had a temperature range of 15–40 °C and pH range of 4.5–9.5. When experiments were conducted at the narrow range of temperature and pH, maximum growth, starch degradation and lactic acid production were observed at 37 °C and pH of 6.5 (Figs. 1 and 2). The temperature optimum of the strain GV6 is higher than other strains of *L. amylophilus* (Table 2) and the pH optimum remained the same as those of other reported strains [5, 6].

3.4

Fermentation of various natural starches

The strain GV6 fermented variety of starches and also by-products of starch industry such as wheat bran, rice bran and produced L(+) lactic acid (Table 1). Acid production

Table 2. Comparison of L(+) lactic acid production by *L. amylophilus* strains

Strain	Substrate	Substrate concentration (g/l)	Number of days	Temperature (°C)	Lactic acid yield (g/g)	References
NRRL B4437	Soluble starch	10	2	28	0.90	Nakamura and Crowell, 1979
NRRL B4437	Glucose	20	1	30	0.93	Mercier et al., 1992
	Corn starch	45	3		0.68	
JCM 1125	Soluble starch	50	6	28–35	0.60	Yumoto and Ikeda, 1995
GV6	Glucose	20	1	37	0.96	Present study
	Soluble starch	50	3		0.90	
		90	4		0.76	
	Corn starch	50	3		0.82	
		60	4		0.81	

g/g – Gram lactic acid per gram substrate taken

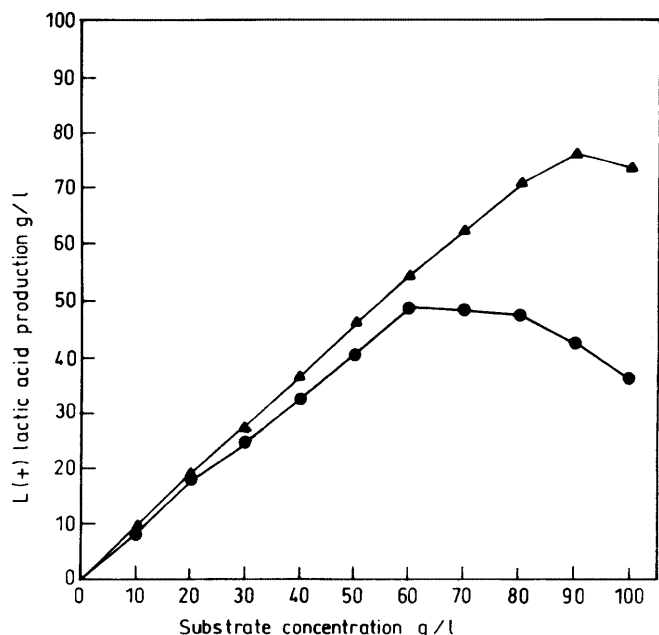


Fig. 3. Comparison of substrate degradation and L(+) lactic acid production by *L. amylophilus* GV6 from corn starch and soluble starch; ●, corn starch; ▲, soluble starch

was more on soluble starch, corn starch, sorghum starch, wheat starch, rice starch and cassava starch. It was moderate on potato and barley starches and very less on rice and wheat brans. The L(+) lactic acid production by *L. amylophilus* has been reported by different authors from soluble starch (processed) and corn starch (purified and unprocessed) [3, 4] and no reports have been made from natural starches (unpurified and unprocessed).

When fermentation experiments were conducted with increasing concentrations of soluble starch and corn starch, the lactic acid production was almost similar both on soluble starch and corn starch at low substrate concentrations (Fig. 3) and increased with increasing substrate concentrations upto 60 g/l corn starch and 90 g/l soluble starch and produced 49 g/l and 76.2 g/l lactic acid, respectively (Fig. 3). Mercier et al. (1992) reported lactic acid production of 29 g/l from 45 g/l of corn starch and Yumoto and Ikeda (1995) reported 30 g/l lactic acid production from 50 g/l soluble starch. It is clear from Tables 1 and 2 that the most attractive features of *L. amylophilus* GV6 are its ability to ferment different processed (liquefied starch/soluble starch) and unprocessed (natural starches) starches and produce high yields of L(+) lactic acid.

To the best of our knowledge, production of 49 g/l and 76.2 g/l L(+) lactic acid from 60 g/l corn starch and 90 g/l soluble starch, respectively by *L. amylophilus* GV6 is the

highest among the wild type strains of *L. amylophilus* reported so far. Therefore, the strain GV6 has considerable potential in the direct conversion of starch to L(+) lactic acid.

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