

# Comparison of Lactic Acid Isomers Produced by Fungal and Bacterial Strains

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## ABSTRACT

Many organisms produce lactic acid by fermentation, but most industrially important strains are from the genus *Lactobacillus* and *Rhizopus oryzae*. L(+)-Lactic acid is the only optical isomer for use in pharmaceutical and food industries because human body is only adapted to assimilate this form. In this research, six strains of *Lactobacillus* and four strains of *R. oryzae* (known as high producer) were examined for optical isomers of lactic acid. The production of lactic acid was improved and lactic acid produced in submerged media on rotary shaker incubator. The optical isomers of lactic acid were examined by L(+) and D(-) lactate dehydrogenase kit. All the *R. oryzae* strains tested produced only L(+) isomer of lactic acid. The highest fungal and bacterial producer strains were *R. oryzae* PTCC 5263, *Lactobacillus plantarum* PTCC 1058, *L. Bulgaricus* PTCC 1332 and *L. delbruekii subsp delbruekii* PTCC 1333. *Lactobacilli* strains produced combination of both optical isomers of lactic acid. Among them, *L. casei subsp. Casei* produced the low amount of D(-)-lactic acid (2%). The optimum concentration of glucose for lactic acid production by *R. oryzae* and *Lactobacillus* strains were 180 g/l and 80–120 g/l, respectively. *Iran. Biomed. J.* 6 (2 & 3): 69-75, 2002

Keywords: Lactic acid, Production, Optical isomers

## INTRODUCTION

Lactic acid is produced by humans, animals, plants and microorganisms [1, 2]. Lactic acid molecule has two optical active isomers, D(-) and L(+) forms [3]. Lactic acid is an organic acid with a wide variety of industrial applications. The most important application as a preservative and acidulant in foods [3], as a prosthetic device, controlled delivery of drugs in pharmaceutical agents, as a precursor for production of polymers like polylactic acid [4] and as a moisture agents in cosmetics [4, 5].

Optically pure lactic acid is important for the production of polylactide, because the physical properties of the polylactide are dependent on the stereochemistry of individual lactic acid molecule [5]. The L(+) form of lactic acid is used for food and drug industry, because human body is only adapted to assimilate this form and only produced L-Lactate dehydrogenase [5]. The most effective way for L-lactic acid syntheses is through biosynthesis rather than chemical processes [6, 7].

In fact, the only source for producing optically pure lactic acid isomers is microbial fermentation [2].

Certain types of microorganisms such as *Lactobacillus spp.* [8, 9] and *Rhizopus spp.* [10,11] are capable of producing lactic acid in high concentrations. *Rhizopus spp.*, especially *R. oryzae*, produce L(+)-lactic acid from glucose, starch [12] and molasses, in the presence of CaCO<sub>3</sub> [13, 14].

The objectives of this study were to screen several *Lactobacilli* and *R. oryzae* strains to determine optical active isomer producers of lactic acid in the fermentation broth and to provide a complete profile of substrate utilization by these microorganisms.

## MATERIALS AND METHODS

**Microbial strains.** *Lactobacillus casei subsp casei* PTCC 1608, *L. lactis subsp lactis* PTCC 1403, *L. delbruekii subsp delbruekii* PTCC 1333, *L. plantarum* PTCC 1058, *L. leichmannii* PTCC 1057, *L. bulgaricus* PTCC 1332, were supplied from PTCC (Persian Type Culture Collection, Iran).

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They were cultured on MRS agar (Man, Rogosa and Sharpe medium) slants and incubated at 37°C for 24 h. Cells were suspended in skim milk containing 15% glycerol and maintained at -70°C [15]. Working bacterial strains were subcultured every two weeks [16].

*R. oryzae* PTCC 5263, 5174, 5175 and 5176 were also obtained from PTCC and cultured on potato dextrose agar (PDA) slants [17]. The slants were incubated at 28°C for 7 days. Spore suspension ( $10^7$  spores/ml) was prepared in broth medium containing 15% glycerol and maintained at -70°C [18]. Working fungal strains were cultured on PDA slants and subcultured every 4 weeks [16].

All media and chemicals were obtained from Merck. Lactic Acid Enzyme Kit was obtained from Boehringer Mannheim Biochemica (Germany).

**Bacterial cultures.** The *Lactobacillus* inoculum was prepared by transfer of cultures into MRS broth and incubated at 37°C 24 h. Five ml of  $10^8$  cell/ml of bacteria was transferred into 500 ml flask containing 100 ml of lactic acid production medium (6 g Glucose, 1.5 g yeast extract, 0.1 g sodium acetate, 0.05 g  $\text{KH}_2\text{PO}_4$ , 0.05 g  $\text{K}_2\text{HPO}_4$ , 0.02 g  $\text{MgSO}_4$ , 0.003 g  $\text{MnSO}_4$ , 0.003 g  $\text{FeSO}_4$ ) [19]. Cultures were incubated at 37°C on rotary shaker (150 rpm), for 100 h. After 8 h, the pH was maintained between 5.5-6 by addition of 2%  $\text{CaCO}_3$  [17]. The production of lactic acid were determined every 12 h.

**Fungal culture.** *R. oryzae* strains were cultured on PDA slants and incubated at 28°C for 7 days. Spore suspension ( $10^7$  spores/ml) was inoculated into 500 ml Arleen Meyer flask containing 100 ml production medium (12g Glucose, 0.015 g  $\text{KH}_2\text{PO}_4$ , 0.03 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.025 g  $\text{MgSO}_4$  &  $7\text{H}_2\text{O}$ , 0.004 g  $\text{ZnSO}_4$  &  $7\text{H}_2\text{O}$ ) [13]. All flasks were incubated at 30°C on rotary shaker (180 rpm) for 60 h. After 24 h, the pH was maintained between 5.5–6.5 by addition of 2%  $\text{CaCO}_3$  [20, 21]. The production of lactic acid was assayed every 12 hours.

**Analytical methods.** Bacterial cell concentration was determined at 610 nm and calibrated into colony forming units (CFU) by colony count method and into dry mass weight [22]. Fungal cell concentration was determined by dry mass weight [19, 23]. The dry mass weight of bacterial and fungal cells was determined by centrifugation of the fermentation broth and freeze dried sediments [19, 23].

The concentration of lactic acid was measured based on colorimetric determination by Kimberly and Taylor method [25]. In this method, known amounts of production medium were taken during fermentation and centrifuged at  $3000 \times g$  for 10 min. The supernatant was used directly for determination of lactic acid concentration.

Assay mixture containing 0.5 ml of supernatant (10-50 mg/l lactic acid) were well mixed with 3 ml of sulfuric acid and heated in boiling water bath. In this method, acetaldehyde is released from lactic acid by hot sulfuric acid effects. After 10 min, the mixture was cooled at room temperature and mixed with 50  $\mu\text{l}$  of  $\text{CuSO}_4$  (4% in distilled water) and 100  $\mu\text{l}$  of -Phenyl phenol reagent (1.5% in ethanol 95%). The acetaldehyde is reacted with copper and -phenyl phenol and produces an chromogenic complex. After 20 min, the absorbance was read against blank at 570 nm (Unicam 8620 uv/vis spectrometer).

Glucose concentration was measured by using glucose oxidase method (Enzyme Kit) [24]. The D(-) and L(+)-lactic acid were also determined by enzyme test kit according to the manufacture instruction. (Boehringer Mannheim Biochemica) [24].

## RESULTS AND DISCUSSION

Many organisms produce lactic acid by fermentation but most industrially important strains are from genus *Lactobacillus* and *Rhizopus* [1]. Six *Lactobacillus* and four *R. oryzae* strains, known as lactic acid producer were examined for the lactic acid production and also for the differential quality of the L(+) and D(-) isomers [6, 7, 22, 23].

**Lactic acid production by lactobacillus strains.** As shown in Table 1, *L. bulgaricus*, *L. plantarum*, *L. delbruekii* and *L. casei subsp casei* produced lactic acid higher than *L. lactis subsp. Lactis* and *L. lechmannii*. All *lactobacilli* strains grew similarly in MRS broth and lactic acid production medium. They produced different amounts of lactic acid optical isomers ( $Y_{p/s}$ ). Specific glucose consumption rate in these strains was examined (Fig. 1A). The curve of the specific glucose consumption rate showed the same pattern as the specific production rate (Table 1).

In contrast to productivity (g product/lh), the growth rate ( $Y_{x/s}$ ) of *Lactobacillus* strains were

**Table 1.** Comparison of lactic acid production by fungal and bacterial producers.

( $Y_{x/s}$  : g biomass produced/g substrate consumed ,  $Y_{p/s}$  : g product formed/g substrate consumed , productivity : g product formed/h fermentation time  $\cdot$  l volume of fermentation medium )

Strain	Dry Mass g/l	Production g/l	$Y_{x/s}$ g/g	Productivity g/lh	$Y_{p/s}$ g/g
<i>L. bulgaricus</i> PTCC 1332	9.00	24.00	0.15	0.33	0.40
<i>L. plantarum</i> PTCC 1058	9.12	29.00	0.15	0.40	0.83
<i>L. delbrueckii subsp delbrueckii</i> PTCC 1333	8.76	15.00	0.14	0.21	0.25
<i>L. lichmannii</i> PTCC 1057	7.86	1.68	0.13	0.02	0.02
<i>L. casei</i> PTCC 1055	8.22	6.18	0.13	0.08	0.10
<i>L. casei subsp casei</i> PTCC 1608	8.40	19.00	0.14	0.26	0.31
<i>L. lactis subsp lactis</i> PTCC 1403	7.80	2.91	0.13	0.04	0.04
<i>R. oryzae</i> PTCC 5263	22.28	55.00	0.18	0.92	0.46
<i>R. oryzae</i> PTCC 5174	19.25	41.00	0.16	0.59	0.34
<i>R. oryzae</i> PTCC 5175	21.22	51.00	0.17	0.85	0.45
<i>R. oryzae</i> PTCC 5176	20.35	31.00	0.17	0.45	0.26

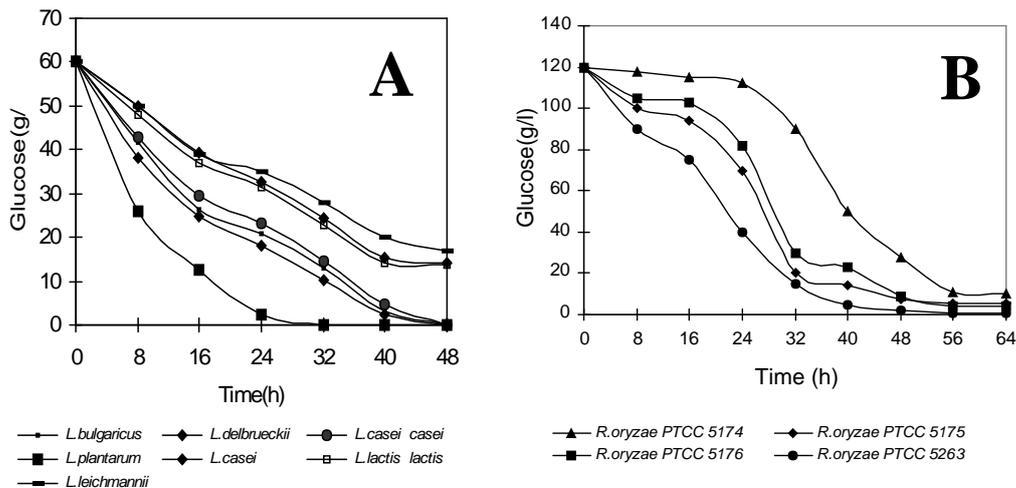
approximately the same (Table 1). It means that medium was suitable for mass production. All bacterial strains grew rapidly, but ability of lactic acid production in some bacterial strains was low.

On the other hands, pH of fermentation medium in all bacterial strains was dropped rapidly, but production of lactic acid was varied in different strains. *L. bulgaricus* and *L. delbreukii* are obligate hemofermentative and produce lactic acid without any other organic acids but *L. plantarum*, and *L. casei* are facultatively heterofermentative [26]. In uncontrolled conditions facultative heterofermentative lactobacilli produce acetate and formate more than lactate in fermentation medium [24]. Hence, decreasing of pH might be related to

different ability of other organic acid production by these strains.

#### Lactic acid production by *R. oryzae* strains.

Specific growth rate of all fungal strains was similar and growth was completed after 48-60 h in the culture medium. As shown in Table 1, the highest lactic acid producing fungal strain was *R. oryzae* PTCC 5263. This strain produced higher lactic acid in shorter fermentation time. The volumetric productivity of this strain was 0.92 g/lh. The production of this strain in fermentation broth was 55 g/l. The second high producer fungal strain was *R. oryzae* PTCC 5175 with 51 g/l lactic acid production.

**Fig. 1.** Glucose consumption by A: *R. oryzae* and B: *Lactobacillus* strains in fermentation medium.

The curve of specific glucose consumption rate in fungal cultures (Fig. 1B) showed the same pattern as the specific growth rate. In fact, growth rate and yield of biomass ( $Y_{x/s}$ ) in all fungal strains were the same (0.170–0.186 g mass/g glucose), but production yield ( $Y_{p/s}$ ) was different.

Lactic acid production depends on the strain used. Some strains of *R. oryzae* produced fumaric acid even more than lactic acid, so lower production of some strains in this research, in spite of decreasing pH indicated production of other organic acids such as fumaric acid [17].

**pH control of fermentation broth.** Undissociated lactic acid is known to be a strong inhibitor for both growth and lactic acid production, but lactate is not a strong inhibitor [25]. In fermentation without pH control, the pH of fermentation broth dropped to below 3, and the volumetric productivity was slowed by the inhibitory effect of lactic acid. In our experiments, yield of lactic acid production in uncontrolled pH of fermentation broth decreased significantly (30–50% of yield in controlled pH condition). For these reasons, rapid decrease in pH of fermentation medium depends on fumaric acid and lactic acid accumulation.

In order to produce more lactic acid, calcium carbonate, sodium hydroxide or ammonium hydroxide is usually added to neutralize the acid and maintain the fermentation broth at a controlled pH [27]. Calcium carbonate is the best neutralizing agents, Due to the highest glucose consumption rate and lactic acid production yield [5], So, In this research, pH was controlled at 5–6 by addition of  $\text{CaCO}_3$ , and the productivity increased significantly by production of calcium lactate instead of lactic acid.

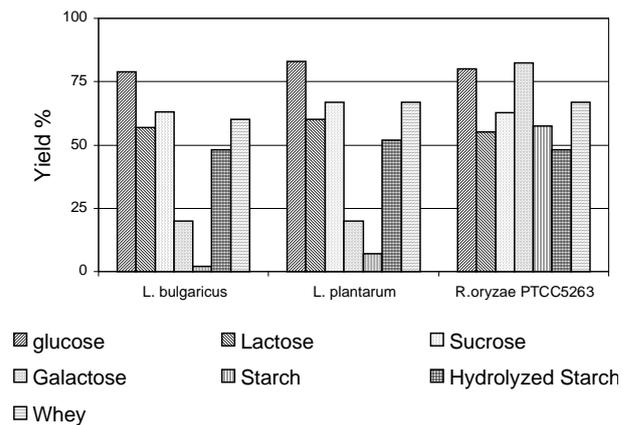
Du *et al.* [10] used the same condition as we used and showed that fungal mycelium grow filamentous and the pellet formation inhibited completely in the presence of  $\text{CaCO}_3$ . In contrast, in our cultures, mycelial pellets were formed in the present of  $\text{CaCO}_3$ .

These pellets were composed of highly interwoven hyphae and generally spherical in shape. Such pellets were inhibited mass transfer which were produced gradients of solutes (substrates and products) through the spheres [28, 29]. Mycellia at the center of the pellet became nutrient limited as the pellet increased in size. The growth was eventually continued in a shell of limited thickness at the surface of the pellet, and the production was decreased significantly [28, 29]. We inhibited pellet

formation completely by optimization of the inoculum size (5 ml of  $10^7$  spores/ml) and rpm of shaker incubator (180 rpm) in the presence of glucose and  $\text{CaCO}_3$ .

In contrast, in the presence of 20 g/l  $\text{CaCO}_3$ , the growth of *Lactobacilli* strains was stopped completely. To overcome this problem, we added  $\text{CaCO}_3$  slowly during the fermentation time (2 g/l every 8 h).

**Substrate optimization.** Several carbon sources were examined to increase the yield of lactic acid production in the optimized fermentation conditions. All strains of *R. oryzae* grew on poor medium contained one carbon source such as sucrose, starch, lactose, galactose and glucose, but *Lactobacillus* strains did not grow on starch medium. Figure 2 shows Lactic acid production yields ( $Y_{x/s}$ ) of the highest producers on different substrates.



**Fig. 2.** Lactic acid production yield (Y%) comparison in the presence of different substrate among high producer strains.

The production of lactic acid by *R. oryzae* on corn starch medium and ground corn medium were reported [12, 14, 17]. Yin *et al.* [17] reported significant increasing yield of lactic acid production on corn starch by *R. oryzae* [17], but in this research glucose medium was better than corn starch (Fig. 2).

Yang *et al.* [27] were compared glucose and xylose as carbon sources in fermentation medium [27]. They found higher productivity by glucose as we observed. *R. oryzae* produced lactic acid from all the carbon sources that we studied, (Fig. 2) and the glucose was the best carbon source. *Lactobacilli*

**Table 2.** Effects of glucose concentration on lactic acid production in optimized medium.

Glucose g/l	<i>R. oryzae</i> PTCC 5263			<i>L. bulgaricus</i> PTCC 1332			<i>L. plantarum</i> PTCC 1058		
	Production g/l	Productivity g/lh	Yield %	Production g/l	Productivity g/lh	Yield %	Production g/l	Productivity g/lh	Yield %
40	33	1.37	83.3	35.8	1.50	89.55	38.10	1.60	95.67
60	49	2.00	81.0	51.8	2.20	86.34	55.90	2.33	93.24
80	64	2.60	80.0	76.6	2.10	95.83	77.80	2.40	97.31
100	80	3.30	80.0	89.6	2.00	89.69	89.10	2.80	89.19
120	106	3.40	85.0	76.6	1.60	63.39	100.00	2.10	83.33
140	116	3.80	82.0	63.6	1.33	45.46	66.60	1.40	47.57
160	133	4.00	83.0	50.0	1.00	31.25	42.00	0.87	26.25
180	160	4.10	84.0	30.0	0.62	16.67	27.00	0.56	15.00
200	85	3.50	42.0	17.0	0.35	11.76	15.00	0.31	7.500

strains did not grow in poor medium. They are fastidious strains and their medium must have several growth factors [26, 30]. We found that, *Lactobacilli* didn't grow on medium contained starch as carbon source, but they grew on medium contained glucose, lactose, galactose and sucrose. The best carbon sources for lactic acid production were lactose and glucose. Lactic acid production on glucose was a little better than lactose.

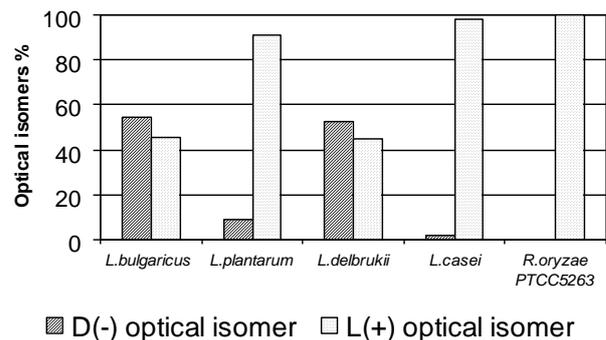
Table 2 shows the best concentration of glucose in fermentation broth. The high production of lactic acid by *R. oryzae* PTCC 5263 was occurred in medium contained 180 g/l glucose, but *Lactobacillus* strains did not produce high concentration of lactic acid in medium contained more than 100g/l glucose. The best concentration of glucose for lactic acid production by *L. plantarum* and *L. bulgaricus* (higher bacterial producers) was 80-100 g/l. In contrast of bacterial strains, lactic acid production by fungal strains in viscose medium with low water activity is possible. For this reason, many reports published about lactic acid production on solid state medium. In higher concentration (120 g/l glucose), production time increased and therefore volumetric productivity of *R. oryzae* did not change significantly.

**Optical isomer of lactic acid comparison.** L(+)-Lactic acid is more important for pharmaceutical and food industries [26]. So produced lactic acid was used for optical isomers determination. This was examined by lactate dehydrogenase kit enzyme test. The results of optimum isomers of lactic acid produced by best strains are shown in Figure 3.

The basic difference between *Rhizopus* and *Lactobacillus* fermentation is that the former is an aerobic fermentation and only L(+)-lactic acid is produced, whereas the latter fermentation is

anaerobic and L(+)-, D(-)-, DL-lactate is produced. L(+)-lactic acid production by *R. oryzae* occurs in 1 to 2 days in a nutritionally poor medium.

The purity of monomers is highly critical in the synthesis of polylactides and a purity of 99% or greater is usually required with the starting lactide material [26].



**Fig. 3.** Lactic acid optical isomers comparison produced by high producer strains.

Among *lactobacilli* strains, *L.casei subsp casei* PTCC 1608 produced high concentration of L(+)-lactic acid with 98% purity. Other *lactobacilli* strains were produced combination of both optical isomers of lactic acid as shown in Table 2. This confirmed experimental work of Vaccari *et al.* [19]. They compared several strains of *L. casei* and reported that lactic acid produced by *L.casei subsp casei* were 97.6% optically L(+) form. In this research, high purity of L(+)-lactic acid was produced by *R. oryzae* strains and *Lactobacilli* strains were produced both optical isomers of lactic acid.

In spite of higher productivity, higher purity of optical isomers, and lower cost of fermentation medium for *R. oryzae*, lactic acid production by *Lactobacilli* strains are easier in submerged fermentation. In bioreactors, the mycelial growth typical of filamentous fungi results in the formation of pellets or in highly viscose mycelial suspension. High sheer stress in stirred tank reactors, solute gradients formation through the pellets and mass transfer limitation are some problems for fungal submerged fermentation [28]. Therefore, the production of pure L(+)-lactic acid by immobilized *R. oryzae* is recommended by some researchers. [3, 8, 11, 17]

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