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ORIGINAL RESEARCH PAPER

L-Lactic acid fermentation by *Enterococcus faecium*: a new isolate from bovine rumen

Wentao Sun · Jianjun Liu · Hui Xu · Wenjing Li · Jing Zhang

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Abstract
Objectives To obtain a novel adaptable stain that can produce enantioselective L-lactic acid efficiently, reduce the operational cost of fermentation process and be suitable for scale production.
Results *Enterococcus faecium* S.156 produced 126 g L-lactic acid/l with high optical purity (99.7 %), high productivity (5.25 g/l.h) and a conversion ratio >90 %. L-lactic acid production remained steady from 32 to 40 °C and at pH values from 5.5 to 6.5. O_2 made no difference to both biomass growth and the L-lactic acid fermentation. 8 mg folic acid/l combined with 2 mM Fe^{2+} substituted for 6 g yeast extract/l, which is a cost-saving.
Conclusions The special characteristics and economic traits of *E. faecium* S.156 make it suitable for industrial-scale production of L-lactic acid.

Keywords Bovine rumen · *Enterococcus faecium* · High optical purity · L-lactic acid

W. Sun · J. Liu (✉) · H. Xu · W. Li · J. Zhang
Food and Fermentation Engineering Key Laboratory of Shandong Province, Jinan, Shandong, People's Republic of China
e-mail: liujianjun_2014@163.com

Introduction

Lactic acid is used widely in the food, leather and pharmaceutical industries (Abdel-Rahman et al. 2013). It is also used as a building block for producing biodegradable and biocompatible poly-lactic acid (Razavi et al. 2014). However, humans lack D-lactic acid dehydrogenase, so D-lactic acid cannot be digested by humans and L-lactic acid is preferred, especially in food and pharmaceutical industries. Furthermore, because of the amorphism of poly DL-lactic acid and the limitation in applications of D-lactic acid, optically-pure L-lactic acid becomes more important.

Optically-pure L-lactic acid can only be produced by enzymatic and microbial fermentations. But the enzymatic method is impractical for industrialized production because of its high cost and complex process. At present, about 90 % of the world's lactic acid is produced through microbial fermentation (Hofvendahl and Hahn-Hägerdal 2000).

Many microbes, such as *Lactobacillus rhamnosus* (Wang et al. 2010) and *Bacillus subtilis* MUR1 (Gao et al. 2012), produce lactic acid using different carbon sources. But, in most cases, these microbes have strict requirements on the fermentation process, of which process parameters, such as aeration and temperature, must be accurately controlled at the optimal values. In contrast, adaptable microbes that are unresponsive to the change of these process parameters to some extent will not face these problems. Adaptable microbes could permit these parameters changing in a broad

range and make the fermentation process more practicable and economical.

Although many efforts have been made to study L-lactic acid production, adaptable microbes remain rare. Differing from the frequently reported lactic acid bacteria (LAB) for L-lactic acid producing, such as *Lactobacillus* and *Bacillus* strains, studies of L-lactic acid produced by *Enterococcus faecium* are relatively scarce.

Enterococcus faecium S.156 was isolated from contents of bovine rumen. It showed an adaptable ability and superiority to produce abundant L-lactic acid with high optical purity and productivity.

Materials and methods

Microorganism

Enterococcus faecium S.156 was isolated from contents of bovine rumen and identified by 16S rDNA sequencing and physiological biochemistry experiments (mainly including Gram's stain, catalase test, VP test, growth temperature and sugar fermentation test). It was grown on (g/l): glucose 50, yeast extract 10.

Optimization of fermentation conditions

The effect of aeration, temperature and pH was studied and the experiments were carried out using a 5 l bioreactor containing 3 l fermentation medium.

Optimization of fermentation medium

The experiments were carried out using a 5 l bioreactor. The initial medium used was constituted of (g/l): glucose 120, yeast extract 10, Tween 80 1. Appropriate amounts of $CaCO_3$ were added to neutralize the lactic acid. The optimal amount for each component would be used in the next step for the optimization of the remaining components. The incubation time was 24 h.

Preparation of crude enzyme extract

Cells were collected in the late growth phase by centrifugation (4000×*g*, 15 min) and washed three times with 0.1 M Tris/HCl buffer (pH 7.0). The collected cells were then suspended in 0.1 M Tris/HCl buffer (pH 7.0) and disrupted by sonication in ice-bath. The cell lysis solution was centrifuged for 15 min (12,000×*g*, 4 °C). The crude enzyme extract was the supernatant, which was collected and filtered through a filter membrane (0.22 μm). The protein content of the crude enzyme extract was measured by Coomassie Blue.

Analytical methods

Biomass was expressed as the OD_{600} value, and $CaCO_3$ was neutralized by HCl. The blank was the fresh culture with the same treatment. An OD_{600} value of 1 = 0.25 g cell dry weight (CDW)/l.

The culture was centrifuged (4000×*g*, 15 min) and the supernatant was diluted to the desired extent with deionized water for sugar and lactic acid assays. Lactic acid and other organic acid were quantified by HPLC using 0.1 M KH_2PO_4, with its pH adjusted to 2.5 by H_3PO_4, at 0.5 ml/min. The column was at 30 °C. Glucose and L-lactic acid were measured by an SBA-40D biosensor analyzer. The ratio of L-lactic acid to total lactic acid was used as an approximate value of its optical purity. The optical purity of L-lactic acid was also measured by D/L-lactate assay kit (Megazyme International Ireland, Ireland). L-LDH assay kit (Jiancheng, China) was used to analyze the activity of L-LDH.

Results and discussion

Identification of *Enterococcus faecium* S.156

Strain S.156 isolated from gut contents of bovine rumen was identified as *E. faecium* according to its 16S rDNA gene sequence (KM875593) showing 99.86 % identity to *E. faecium* (DQ411813). Other characteristics, such as Gram-positive, catalase-negative, growth at 45 °C, and sugar fermentation, also agreed with the identification. *E. faecium* S.156 produced L-lactic acid with a conversion ratio of 90 %, which suggested a homolactic fermentation. Optical purity of L-lactic acid was 99.7 % according to the result of assay kit. To our knowledge, this is the highest optical purity obtained by *E. faecium*.

Effects of different aeration conditions on lactic acid production

When the aeration increased from 0 to 4 l/min in the 5 l bioreactor, the final concentration and conversion ratio of L-lactic acid were maintained at 91 g/l and 90 % respectively. The CDW was maintained at 2.8 g/l (Table 1). This indicated that O_2 had no obvious detrimental effect on either biomass or L-lactic acid production. Consequently, *E. faecium* S.156 could be applied in anaerobic fermentation, which was more suitable for industrialization than aerobic fermentation by removing the energy consumption of aeration. *E. faecium* S.156 could also be applied in the equipment that was not tailor-made for anaerobic fermentation, while appropriate aeration could be used to maintain the pressure in the fermenter without any detrimental effect on L-lactic acid production. This was an advantage compared with *Lactobacillus* (Amanatidou et al. 2001), by which O_2 would result in lower conversion ratio and lower lactic acid concentration.

Small amounts of acetate, citric acid, and pyruvic acid were also detected by HPLC as byproducts, but formic acid, as a primary byproduct appearing with many other LAB, was not detected.

Effect of temperature and pH on lactic acid production

The L-lactic acid concentration was steady at 91 g/l from to 40 °C. But the CDW was higher at lower temperatures (Fig. 1).

The activity of L-LDH, which is a key enzyme of lactate metabolism, was also tested from 30 to 42 °C. It increased up to 40 °C (Fig. 2). Although the L-LDH activity was lower at lower temperatures, it was compensated by the higher biomass. So the L-lactic acid production could remain steady from 32 to 40 °C.

Table 2 showed that the L-lactic acid concentration, CDW, productivity and conversion ratio had no significant differences at pH values from 5.5 to 6.5. This differed from the existing strains, such as *Lactobacillus casei* G-02, *B. subtilis* MUR1 and *E. faecium* No. 78 (Ge et al. 2011; Gao et al. 2012; Shibata et al. 2007), which produced the highest yield and productivity of L-lactic acid at the optimal temperature and pH value. Above or below the optimal condition would result in lower efficiency of L-lactic acid fermentation by these strains. In contrast, *E. faecium* S.156 permitted the culture temperature and pH value to change in a broad range without any negative effect on L-lactic acid production. This made the fermentation easier to control and reduced the dosage of cooling or warming water as well as the neutralizer. These characteristics would make the fermentation process more economical by reducing the cost of temperature and pH control (Table 2).

Optimization of N source

Yeast extract is the most commonly applied N source in current L-lactic acid production. Our study showed that the L-lactic acid concentration increased with the concentration of yeast extract (data not shown). The highest L-lactic acid concentration (126 g/l) and

Table 1 L-Lactic acid production, CDW, productivity, conversion ratio and byproduct formation by *Enterococcus faecium* S.156 under different aeration levels

Aeration (l/min)	Agitation (rpm)	L-Lactic acid (g/l)	CDW (g/l)	Conversion ratio (%)	Productivity (g/l h)	Critic acid (g/l)	Acetate (g/l)	Pyruvate (g/l)
0	300	91.3 ± 1.1	2.8 ± 0	90 ± 0.12	3.81	3.9 ± 0	1.2 ± 0	0.7 ± 0
2	300	91.3 ± 1.1	2.8 ± 0	90 ± 0.12	3.81	3.9 ± 0	1.2 ± 0	0.7 ± 0
4	300	90.7 ± 1.1	2.8 ± 0	90 ± 0.12	3.78	3.9 ± 0	1.2 ± 0	0.7 ± 0
4	500	91.3 ± 1.1	2.8 ± 0	90 ± 2.1	3.81	3.9 ± 0	1.2 ± 0	0.7 ± 0

Values are the average ± standard deviation of three repeated fermentations

The experiments were carried out in a 5 l bioreactor at 37 °C, containing 3 l fermentation medium with addition of $CaCO_3$ sterilized separately to neutralize the lactic acid. The initial pH value was 6.55. The fermentation medium was constituted of (g/l): glucose 120, yeast extract 10, Tween 80 1. The incubation time was 24 h

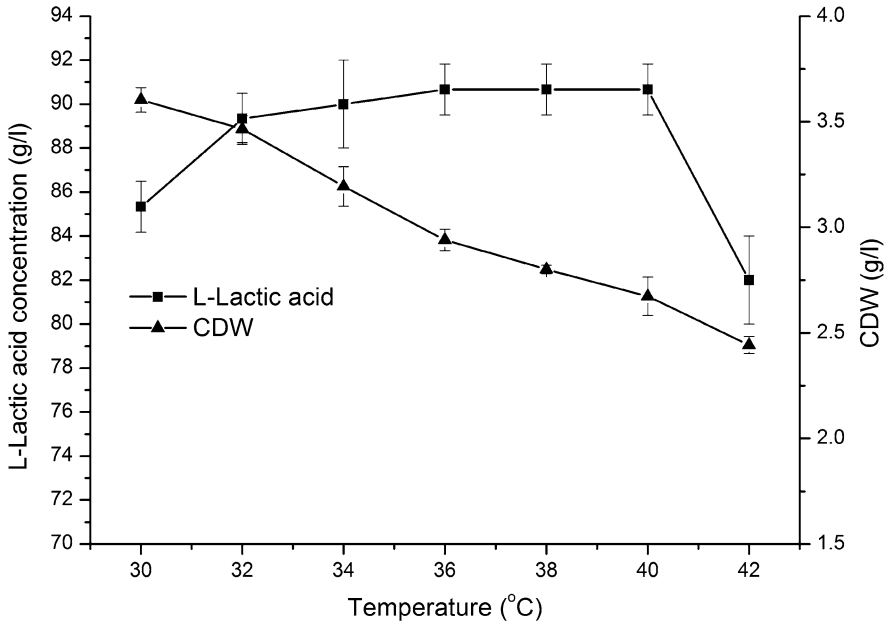

Fig. 1 Effect of temperature on L-lactic acid production (*square*) and CDW (*triangle*) during L-lactic acid fermentation by *Enterococcus faecium* S.156. The experiments were carried out in a 5 l bioreactor containing 3 l fermentation medium with addition of appropriate amounts of $CaCO_3$. The initial pH value was 6.55. The fermentation medium was constituted of (g/l): glucose 120, yeast extract 10, Tween 80 1. The incubation time was 24 h

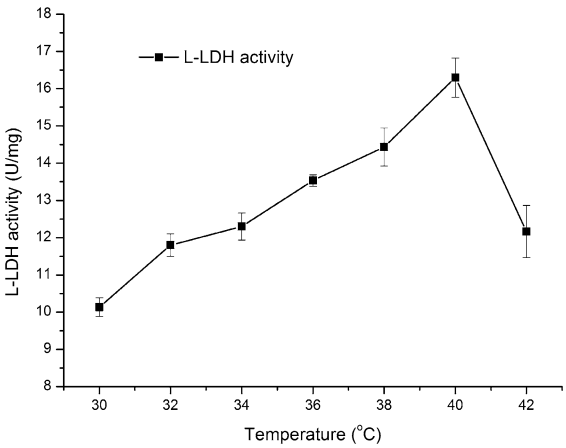

Fig. 2 Effect of temperature on L-LDH activity of *Enterococcus faecium* S.156

productivity (5.25 g/l h) was achieved with 18 g yeast extract/l. However, increasing yeast extract beyond 18 g/l did not increase the L-lactic acid concentration and productivity. So 18 g yeast extract/l was used in the fermentation medium, and the fermentation medium was constituted of (g/l): glucose 150, yeast extract 18, Tween 80 1.

Although yeast extract is an efficient N source, using high concentration of yeast extract is uneconomical in an industrial process. The cost of yeast extract was considered to account for 38 % of the total medium cost in lactic acid production (Altaf et al. 2007). Therefore, a cheaper alternative is needed.

The effect of yeast extract is possibly due to its B vitamin content (Nancib et al. 2005). Three B vitamins, folic acid, niacin acid and calcium pantothenate, were therefore evaluated for L-lactic acid production by *E. faecium* S.156 with 12 g yeast extract/l. Only folic acid was effective. Compared with the control (12 g yeast extract/l, 101 g L-lactic acid/l), the addition of 8 mg folic acid/l in fermentation medium improved L-lactic acid production by 10 %. The medium containing 8 mg folic acid/l and 12 g yeast extract/l was named as F8. The F8 medium was constituted of (g/l): glucose 150, yeast extract 12, folic acid 0.008 and Tween 80 1.

Effect of metal ions on L-lactic acid production

The effect of divalent metal ions, Mg^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+}, on *E. faecium* S.156 was investigated using F8 medium. L-Lactic acid increased significantly only with the addition of Fe^{2+}. The maximal concentration of L-lactic acid (121.3 g/l) and productivity (5.06 g/l.h) was obtained with 2 mM Fe^{2+}. Moreover, as the L-lactic acid concentration was close to that obtained with 18 g yeast extract/l (126 g/l), we concluded that 8 mg folic acid/l combined with Fe^{2+} substituted for 6 g yeast extract/l. It was also an efficient cost-saving

Table 2 L-Lactic acid production, CDW, productivity, conversion ratio by *Enterococcus faecium* S.156 under different pH values

pH	L-Lactic acid (g/l)	CDW (g/l)	Conversion ratio (%)	Productivity (g/l.h)
5.0	84.7 ± 1.15	2.7 ± 0.04	89 ± 0.11	3.53
5.5	90.7 ± 1.15	2.8 ± 0.05	91 ± 1.15	3.78
6.0	91.3 ± 1.15	2.8 ± 0.02	91 ± 1.1	3.81
6.5	91.3 ± 1.15	2.8 ± 0.04	91 ± 0.92	3.81
7.0	86 ± 2	2.7 ± 0.01	90 ± 0.25	3.58

Values are the average ± standard deviation of three repeated fermentations

The experiments were carried out in a 5 l bioreactor containing 3 l fermentation medium using $Ca(OH)_2$ as neutralizer at 37 °C. The fermentation medium was constituted of (g/l): glucose 120, yeast extract 10, Tween 80 1. The incubation time was 24 h

of L-lactic acid production. The optimized medium was constituted of (g/l): glucose 150, yeast extract 12, folic acid 0.008, $FeSO_4$ 0.3, and Tween 80 1.

Conclusions

A novel enantioselective L-lactic acid producer was isolated from bovine rumen and identified as *E. faecium* named S.156, which could produce abundant L-lactic acid with high optical purity, productivity and conversion ratio. Differing from other reported LAB, *E. faecium* S.156 was unresponsive to the change of temperature, pH and aeration. Trace folic acid combined with Fe^{2+} substituted for part of the yeast extract, which is a cost-saving. Considering the special characteristics and economic traits, *E. faecium* S.156 is suitable for industrial-scale production of L-lactic acid.

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