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ORIGINAL RESEARCH PAPER

Fermentative production of L-(+)-lactic acid by an alkaliphilic marine microorganism

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Abstract Of six strains of lactic acid-producing alkaliphilic microorganisms, *Halolactibacillus halophilus* was most efficient. It produced the highest concentration and yield of lactic acid, with minimal amounts of acetic and formic acid when sucrose and glucose were used as substrate. Mannose and xylose were poorly utilized. In batch fermentation at 30°C, pH 9 with 4 and 8% (w/v) sucrose, lactic acid was produced at 37.7 and 65.8 g l^{-1}, with yields of 95 and 83%, respectively. Likewise, when 4 and 8% (w/v) glucose were used, 33.4 and 59.6 g lactic acid l^{-1} were produced with 85 and 76% yields, respectively. L-(+)-lactic acid had an optical purity of 98.8% (from sucrose) and 98.3% (from glucose).

Keywords Alkaliphilic · Batch fermentation · Glucose · *Halolactibacillus halophilus* · Sucrose

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Introduction

Lactic acid is important for food, pharmaceutical, cosmetics and chemical production. The demand for it has steadily increased due to its use as raw material for the production of the biodegradable and renewable polymer, polylactic acid, to replace various petrochemical-based polymers. Lactic acid is produced either by chemical synthesis or by microbial fermentation. The latter is the more efficient producer of optically pure L- or D-isomers depending on the strain used, whereas chemical synthesis produces racemic mixture of lactic acid.

Several species of lactic acid bacteria (LAB) and some filamentous fungi can produce lactic acid (Litchfield 1996). The majority of LAB have optimum growth at pH 5.5–6.5 and are alkali intolerant. However, some alkaliphilic lactic acid-producing microorganisms are known: *Marinilactibacillus psychrotolerans* (Ishikawa et al. 2003), *Alkalibacterium psychrotolerans* (Yumoto et al. 2004), *M. piezotolerans* (Toffin et al. 2005), *A. iburiense* (Nakajima et al. 2005), *Halolactibacillus halophilus* (Ishikawa et al. 2005), *H. miurensis* (Ishikawa et al. 2005), *H. alkaliphilus* (Cao et al. 2008) and *A. indicireducens* (Yumoto et al. 2008). Nevertheless, the potential of these organisms for large scale production is uncertain. To our knowledge, this is the first attempt to explore the use of alkaliphilic microorganisms for lactic acid production.

Halolactibacillus halophilus is slightly halophilic and alkaliphilic, preferentially growing under the physicochemical conditions of seawater (Ishikawa et al. 2005). The use of this microorganism for lactic acid fermentation presents potential advantage over commonly used LAB. Its tolerance to high levels of salt and high pH values, for instance would minimize contamination problems during processing. The aim of this work is to investigate the ability of alkaliphiles, *M. psychrotolerans, H. halophilus,* and *H. miurensis*, to produce lactic acid and describe the optimum conditions for lactic acid production.

Materials and methods

Microorganisms and culture conditions

*Marinilactibacillus psychrotoleran*s (JCM 21451, JCM 21457 and JCM 21458), *M. piezotoleran*s (JCM 12337), *H. halophilus* (JCM 21694), and *H. miurensis* JCM 21699 were cultivated in the pre-culture medium containing (per liter) 20 g glucose, 5 g yeast extract, 10 g Bacto-peptone, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.005 g $MnSO_4{\cdot}H_2O$, 5 g sodium acetate, 25 g NaCl. The pH was adjusted to 7.0 before sterilization at 121° C, 15 min. The buffer solution ($NaHCO_3/Na_2CO_3$) was separately sterilized and added to obtain an initial pH of 9.0 in the medium.

Batch experiments were carried out in a 5 l jar fermentor with 2.5 l fermentation medium as previously described (Timbuntam et al. 2006), except for the addition of $NaHCO_3/Na_2CO_3$ (100 mM) and 2.5% (w/v) NaCl. Fermentation was initiated by adding 5% (v/v) inoculum. The pH was automatically controlled at 9 by adding 25% (w/v) NaOH. The temperature and agitation were maintained at 30°C and 250 rpm, respectively. No aeration was used.

Analytical methods

Cell growth was determined as c.f.u. using the pre-culture medium agar. Fermentation products were analyzed by HPLC using UV (210 nm) and RI detectors with a Shim-pack SPR-H (G) (Shimadzu, Japan) column at 40°C and eluted with 4 mM perchloric acid at 0.6 ml min^{-1}. HPLC analysis for residual sugar was performed on a Tosoh LC-8010 apparatus (column: Shodex Asahipak NH2P-50 4E), mobile phase, acetonitrile/H_2O 75/25 (v/v) at 0.5 ml min^{-1}, injection 0.1 ml, detection at 210 nm. The D/L ratio of produced lactic acid was analyzed using an enzymatic bioanalysis kit (Boehringer Mannheim GmbH).

Results and discussion

Among the six alkaliphiles screened for fermentation, *H. halophilus* produced the highest lactic acid concentration at pH 9 ($P < 0.05$) using 2% (w/v) glucose (Fig. 1).

Figure 2 shows the time course of lactic acid production from sucrose, glucose, mannose and xylose at 4% (w/v). *H. halophilus* preferred sucrose and glucose as carbon sources, with the former being more efficiently utilized after 36 h (Fig. 2, Table 1). Lactic acid production at 3.6 and 1.7 mol for every mol of sucrose and glucose, respectively, suggests a homolactic type of fermentation mediated by *H. halophilus.* This strain produced L-lactic acid with high optical purity of 98.8% (from sucrose) and 98.3% (from glucose). Lactic acid production increased with increasing sucrose and glucose concentration up to 8% (w/v).

However, when the concentration of these substrates was increased to 10% (w/v), viable cell count, lactic acid production and yield decreased (Fig. 3,

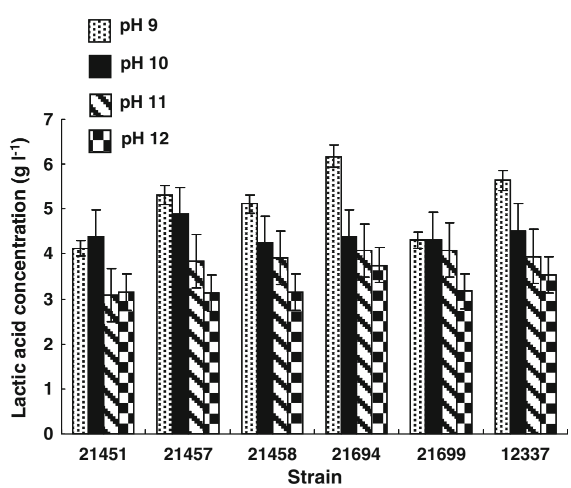

Fig. 1 Screening of alkaliphilic microorganisms for lactic acid production at different pH without pH control. Experiments were conducted in test tubes containing 10 ml pre-culture medium and 200 μl of inoculum. The tubes were statically incubated at 30°C for 24 h. *M. psychrotoleran*s (JCM 21451, JCM 21457, and JCM 21458), *H. halophilus* (JCM 21694), *H. miurensis* (JCM 21699) and *M. piezotoleran*s (JCM 12337). Data are means ± SD of duplicate samples

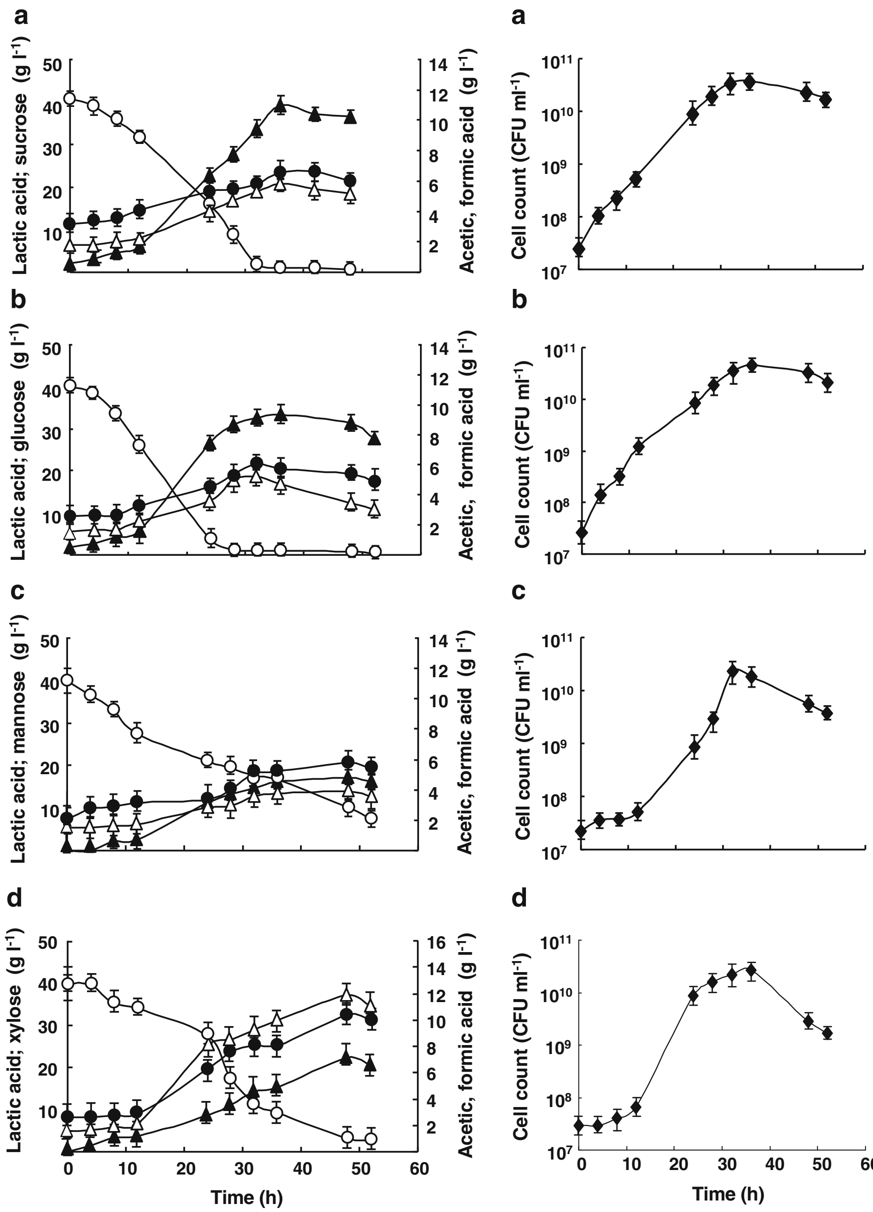

Fig. 2 Time course of lactic acid fermentation by *H. halophilus* using 4% (w/v) sugars. Sugar concentration g l^{-1} (*open circle*); lactic acid g l^{-1} (*closed triangle*); acetic acid g l^{-1} (*closed circle*); formic acid g l^{-1} (*open triangle*); viable cell count c.f.u. (*closed diamond*). Each point represents the mean ± SD of the results of two independent experiments

Table 1). Bulut et al. (2004) also reported similarly. Likewise, 10% (w/v) sucrose decreased lactic acid production possibly due to the increase in medium viscosity. Furthermore, the substrates were not completely metabolized and approximately 50% of the initial sugar remained in the fermentation medium.

Mannose and xylose were poorly utilized with very low lactic acid production (Fig. 2c, d). Tanaka et al. (2002) also reported low yields from xylose. Moreover with xylose, the undesirable production of formic acid and acetic acid was as high as previously reported (Taniguchi et al. 2004).

Lactic acid production and yield using sucrose and glucose were comparable to other work using the common lactic acid bacteria: *L. casei* (Yoo et al. 1996), *L. lactis* (Akerberg et al. 1998), *L. rhamnosus* (Berry et al. 1999) and the fungus *Rhizopus oryzae* (Bulut et al. 2004). The lactic acid yield from these previous reports ranged from 79 to 90% with the L-lactic acid optical purity from 95 to 99%.

Table 1 Summary of fermentation parameters of L-lactic acid production from different carbon sources

Carbon source	Sucrose			Glucose			Mannose	Xylose
Initial sugar (g l^{-1})	40.7	80.6	102.6	40.1	80.6	101.3	39.8	39.9
Maximum c.f.u ($\times 10^{-10}$)	3.7	2.9	0.5	3.5	2.9	0.5	2.3	2.7
Residual sugar (g l^{-1})	1.0	1.4	61.7	0.9	1.4	52.4	9.9	2.8
Sugar consumption (g l^{-1})	39.7	79.2	40.9	39.2	79.2	48.9	29.9	37.1
Total lactic acid, Pmax (g l^{-1})	37.7	65.8	28.2	33.4	65.8	34.5	17.2	22.3
% Yield (g g^{-1})	95	83	69	85	76	70.6	57.5	60

Experiments were carried out twice and the average data are shown

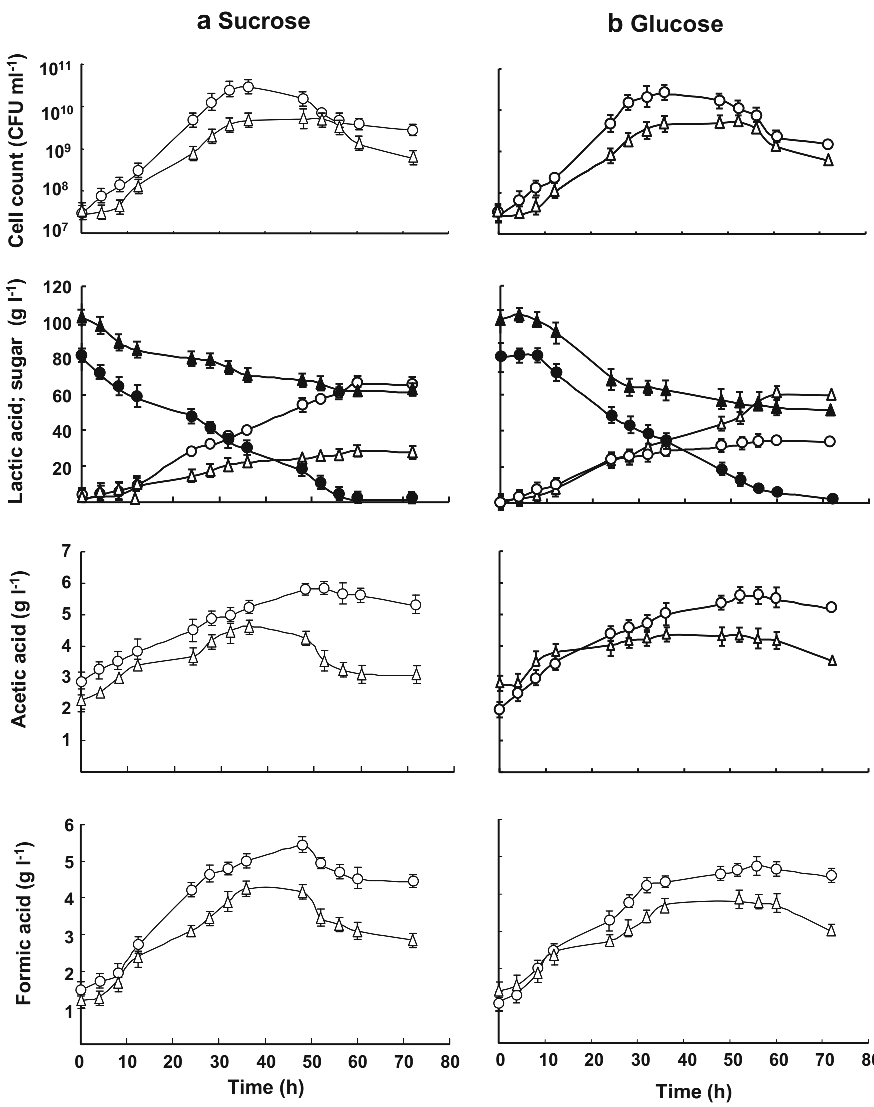

Fig. 3 Profiles of cell viability, organic acid production and utilization of 8% (w/v) (*open circle*, *closed circle*) or 10% (w/v) (*open triangle*, *closed triangle*) glucose or sucrose by batch fermentation using *H. halophilus*. Each point represents the mean ± SD of the results of two independent experiments

For the first time, this study has demonstrated that an alkaliphilic strain, *H. halophilus,* is effective for the direct production of L-lactic acid with high optical purity from sucrose and glucose.

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