RESEARCH LETTER

Production of 2-butanol through *meso*-2,3-butanediol consumption in lactic acid bacteria

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Abstract

2-Butanol has been an issue of industries in many areas, for example, biofuel production (as an advanced alternate fuel), fermented beverages, and food (as taste-altering component). Thus, its source of production, the biological pathway, and the enzymes involved are of high interest. In this study, 42 different isolates of lactic acid bacteria from nine different species were screened for their capability to consume *meso*-2,3-butanediol and produce 2-butanol. *Lactobacillus brevis* was the only species that showed any production of 2-butanol. Five of ten tested isolates of *L. brevis* were able to convert *meso*-2,3-butanediol to 2-butanol in a synthetic medium (SM2). However, none of them showed the same capability in a complex medium such as MRS indicating that the ability to produce 2-butanol is subject to some kind of repression mechanism. Furthermore, by evaluating the performance of the enzymes required to convert *meso*-2,3-butanediol to 2-butanol, that is, the secondary alcohol dehydrogenase and the diol dehydratase, it was shown that the latter needed the presence of a substrate to be expressed.

Introduction

The diminishing supply of fossil fuels has become a driving force for finding out new substitutes and novel chemical production paths from renewable sources. Different isomers of butanol (1-butanol, 2-butanol, iso-butanol) have been in focus as emerging biofuels among which 2-butanol is known to be produced by some *Lactobacilli* strains (Keen *et al.*, 1974; Radler & Zorg, 1986; Speranza *et al.*, 1996). 2-Butanol, compared to the other isomers, is less toxic to the cells due to its lower hydrophobicity which helps the cells to tolerate higher 2-butanol concentrations in the media (Paterson *et al.*, 1972; Grisham & Barnett, 1973; Hui & Barton, 1973; Ingram, 1976).

Furthermore, 2-butanol production by *Lactobacilli* has been a concern of the fermented beverages industries as it might affect the taste quality of the end products (Postel, 1982). It has also been shown how 2-butanol and 2-butanone formation in Cheddar cheese is linked to different *Lactobacillus* strains (Keen *et al.*, 1974). In some *Lactobacillus* strains (e.g. *L. brevis*), 2-butanol is known to be produced through *meso*-2,3-butanediol which is converted to 2-butanone by a diol dehydratase enzyme. 2-butanone is then converted to 2-butanol through the action of a secondary alcohol dehydrogenase (Radler & Zorg, 1986). The source of 2,3-butanediol is the pyruvate-diacetyl-acetoin pathway which is present in some lactic acid bacteria (Kandler, 1983) but also in a wide range of wine yeasts (Romano *et al.*, 1998, 2000, 2003). The diol dehydratase enzyme is usually a protein of three subunits, homologs of which are produced also in *Klebsiella oxytoca* (Abeles *et al.*, 1960), *Salmonella typhimurium* (Obradors *et al.*, 1988), *Propionibacterium* (Toraya *et al.*, 1980) and *Flavobacterium* (Willetts, 1979). The diol dehydratase from these bacteria is known to require adenosylcobalamin (AdoCbl) or coenzyme B_{12} as an essential activating component (Abeles *et al.*, 1960; Willetts, 1979; Toraya *et al.*, 1980; Radler & Zorg, 1986; Obradors *et al.*, 1988).

FEMS MICROBIOLOGY LETTERS

The very last enzyme of 2-butanol conversion in *Lactobacillus* strains, secondary alcohol dehydrogenase, is attractive due to its capacity for reducing ketones to the relevant alcohols enantioselectively, which makes it a promising biocatalyst (Leuchs & Greinera, 2011). The rising demand for pure enantiomer intermediates in pharmaceutical industries has also been a driving factor in this regard (Meyer, 2010). The secondary alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*SADH), in particular, shows affinity for an extensive range of substrates and it is also known to be solvent tolerant (Leuchs & Greinera, 2011).

In this study, a range of lactic acid bacteria were screened for 2-butanol production capability and the kinetic properties of the relevant enzymes were assessed for the two best 2-butanol producers.

Materials and methods

Strains

The *Lactobacillus brevis* strains SE20 and SE31 together with 16 isolates of *L. plantarum*, eight isolates of *L. pantheris,* two isolates of *L. buchneri*, two isolates of *L. rossiae* and one isolate of *L. paracasei, L. fermentum* and *L. paracollinoids* were isolated from the ethanol pilot plant facility in Örnsköldsvik, Sweden. The strain *L. brevis* (LB 734, Centre National de Recherches Zootechniques – CNRZ 734) was kindly provided by Professor Giovanna Speranza, Department of Organic and Industrial Chemistry, University of Milan, Italy. *Lactobacillus brevis* strains LB 215, LB 219, LB 350, LB 368, LB 399, LB 443, LB 579 and one isolate of *L. malefermentas* were obtained from the Culture Collection, University of Gothenburg (CCUG).

Cultivation conditions

MRS (OXOID, UK) and SM2 medium were used to cultivate the strains. MRS medium was composed of peptone 10 g L^{-1}, 'Lab-Lemco' 8 g L^{-1}, yeast extract 4 g L^{-1}, glucose 20 g L^{-1}, 'Tween 80' 1 mL, di-potassium hydrogen phosphate 2 g L^{-1}, sodium acetate.$3H_2O$ 5 g L^{-1}, triammonium citrate 2 g L^{-1}, magnesium sulfate.$7H_2O$ 0.2 g L^{-1}, manganese sulfate. $4H_2O$ 0.05 g L^{-1}. SM2 medium was prepared according to Radler and Zorg (Radler & Zorg, 1986). The cells were grown in 50-mL Falcon tubes at 150 r.p.m. in 30 °C.

Enzyme extraction

Enzyme extraction was performed following the procedure of Schütz & Radler (1984), with some minor modifications. Cells were cultivated in SM2 medium for 48 h and harvested at 1500 ***g*** for 10 min. The cells were then washed twice in 10 mM potassium phosphate (pH 7.2) and 1 mM dithiothreitol buffer. Finally, cells were suspended in 2 mL of the same buffer. The suspension was transferred to lysing matrix E (MP$_{TM}$) tubes. Fastprep 24 (MP Biomedicals Solon) was used to disrupt the cells (five cycles of 20 s at 6 m s^{-1}, kept on ice for 1 min in between each cycle). Cell debris was then removed by centrifuging at 14 000 ***g*** for 30 min (4 °C). Samples were desalinated by use of a spinning column: amicon ultracel-4 10k device (Merk Millipore).

Total protein concentration

The protein concentration was determined using Nanodrop2000 spectrophotometer (Termo Scientific). Absorbance was measured at 280 nm, and concentration was calculated following the Beer's law.

Enzyme activity measurements

Secondary alcohol dehydrogenase – Enzyme assay was performed following Jo *et al.* (Jo *et al.*, 2008). A total volume of 1 mL including potassium phosphate buffer pH 7 (final concentration of 50 mM), dithiothreitol (final concentration of 10 mM), 0.5–100 mM substrate (2-butanol, 1-propanol, ethanol, or 2-butanone), NAD^+/NADH (final concentration of 2 mM/1 mM respectively), and crude extract was prepared. For the reducing reaction, 2-butanone was used as substrate and NADH as coenzyme. 2-butanol, 1-propanol, or ethanol was used as substrate for oxidation reaction, together with NAD^+ as coenzyme.

The activity of alcohol dehydrogenase was determined by measuring the reduction/oxidation of NAD^+/NADH at 340 nm using a plate reader (Fluostar Omega, BMG LabTech) at 30 °C.

Diol dehydratase – This assay was performed following Toraya *et al.* (1977). A mixture of 0.05 M potassium chloride, 0.035 M potassium phosphate buffer pH 8, 15 μM adenosylcobalamin, 0.05 M substrate (1,2-propanediol, 1,3-propanediol, *meso*-2,3-butanediol, glycerol), and enzyme crude extract was incubated at 37 °C for 10 min. Then, 0.5 mL 0.1 M potassium citrate buffer (pH 3.6) was added to terminate the reaction followed by 0.25 mL 0.1% MBTH hydrochloride. The aldehydes/ketones produced have the ability to react with MBTH, forming azine derivates which can be determined by spectrophotometer (Paz *et al.*, 1965). The mixture was then incubated at 37 °C, and 0.5 mL water was added before measuring its absorbance at

305 nm in plate reader (Fluostar Omega, BMG Lab-Tech).

Analysis of extracellular metabolites

HPLC (Ultimate 3000, Dionex) was used to analyze metabolites such as glucose, ethanol, glycerol, acetate, *meso*-2,3-butanediol, 2-butanone, and 2-butanol. Aminex® HPX-87H column (300 × 7.8 mm - Bio-Rad) was used, and 5 mM H_2SO_4 was the eluent (at 0.6 mL min^{-1}). The running temperature was 45 °C. VWD-3100 detector (Thermo Scientific Dionex) was connected to the column, and each metabolite was quantified based on a standard curve drawn for each metabolite. The concentration range of standards was as follow: acetate (0.1–2 g L^{-1}), glycerol (0.1–2 g L^{-1}), ethanol (1–15 g L^{-1}), glucose (1–20 g L^{-1}), 2-butanol and butanone (0.1–2 g L^{-1}), *meso*-2,3-butanediol (0.5–5 g L^{-1}). Rezextm ROA – organic acid H^+ (300 × 7.8 mm – Phenomenex) was the second column used to reconfirm the methanol peak in the medium. 5 mM H_2SO_4 was the eluent (at 0.8 mL min^{-1}), and the running temperature was 80 °C.

Results and discussion

2-Butanol production

As an initial screen, we tested the ability of 42 different isolates of lactic acid bacteria for their ability to consume *meso*-2,3-butanediol. The following species were included in this first screen, *L. plantarum* (16 isolates), *L. brevis* (10 isolates), *L. pantheris* (eight isolates), *L. buchneri* (two isolates), *L. rossiae* (two isolates) and *L. paracasei, L. fermentum, L. paracollinoids, L. malefermentas* (one isolate each). Among these the only species that showed any capacity for *meso*-2,3-butanediol consumption were *L. brevis* and *L. buchneri* (data not shown). It was therefore decided to include only these two species in the next step of investigations. Ten strains of *L. brevis* and two strains of *L. buchneri* were screened for their 2-butanol production capability during growth in MRS medium with addition of either *meso*.2,3-butanediol or butanone (Table 1). The origin of these strains were ethanol pilot plant (Albers *et al.*, 2011), fermenting olives (CCUG 21531), wine (CCUG 21959), industrial fermentation process (CCUG 35039), carrot (CCUG 39980), cider (CCUG 44317), silage (CCUG 36840), pickles (CCUG 57950), and French cheese (CNRZ 734, previously reported as a 2-butanol producer from *meso*-2,3-butanediol). All the tested strains were capable of consuming *meso*-2,3-butanediol and converting 2-butanone to 2-butanol, while grown in MRS medium (Table 1). However, none of them showed any production of 2-butanol from *meso*-2,3-butanediol.Instead, a clear peak of methanol was identified in the HPLC spectrum. Therefore, we assume provision of *meso*-2,3-butanediol in MRS medium led to methanol production by the tested LB strains.

The fact that even the previously reported butanol producing strain (LB 734) failed to show any butanediol–buta-

Table 1. List of different *Lactobacillus brevis* and *L. buchneri* strains tested for production of 2-butanol during growth in MRS medium with addition of *meso*-2,3-butanediol (5 g L^{-1}) or 2-butanone (0.8 g L^{-1})

Strain	Source	*meso*-2,3-butanediol consumption	2-butanol from *meso*-2,3-butanediol	2-butanol from butanone (g L^{-1})
L. buchneri				
LB 12	Ethanol pilot plant, Örnsköldsvik	Yes	No	0.04
LB 16	Ethanol pilot plant, Örnsköldsvik	Yes	No	0.03
L. brevis				
SE 20	Ethanol pilot plant, Örnsköldsvik	Yes	No	0.44
SE 31	Ethanol pilot plant, Örnsköldsvik	Yes	No	0.59
LB 215	CCUG 21531	Yes	No	0.72
LB 219	CCUG 21959	Yes	No	0.68
LB 350	CCUG 35039	Yes	No	0.16
LB 368	CCUG 36840	Yes	No	0.59
LB 399	CCUG 39980	Yes	No	0.49
LB 443	CCUG 44317	Yes	No	0.55
LB 579	CCUG 57950	Yes	No	0.66
LB 734	CNRZ 734	Yes	No	ND

ND, not determined.

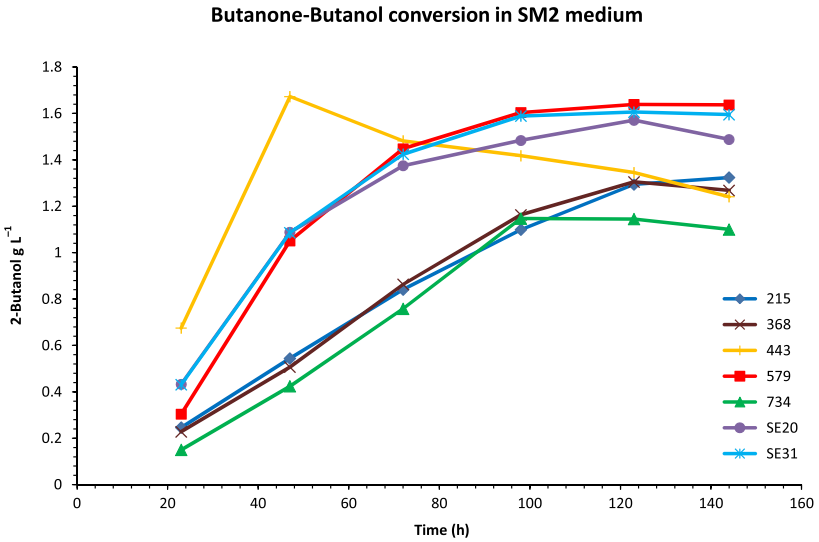

Fig. 1. Conversion of 2-butanone to 2-butanol by different *Lactobacillus brevis* strains grown in SM2 medium. SE31 (asterisk), SE20 (filled circle), LB 579 (filled square), LB 215 (filled diamond), LB 734 (filled triangle), LB 368 (cross), and LB 443 (plus). Cells were cultivated in SM2 medium with addition of 0.8 g L^{-1} butanone.

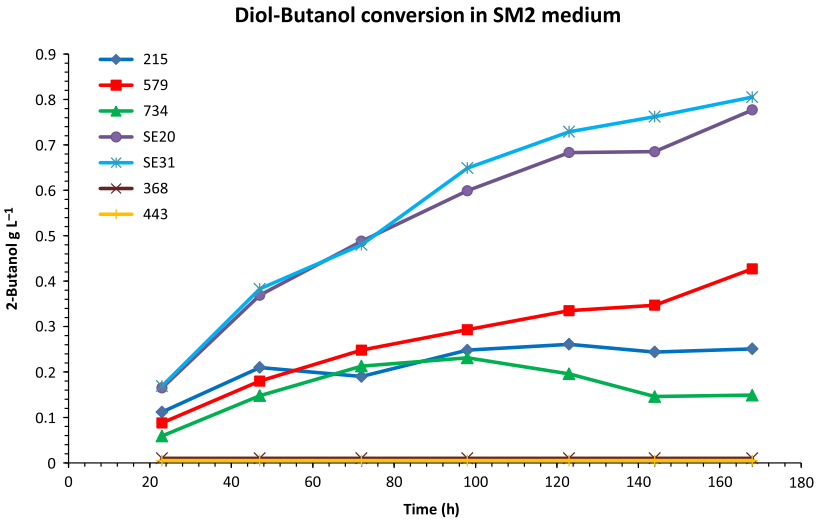

Fig. 2. Conversion of *meso*-2,3-butanediol to 2-butanol by different *Lactobacillus brevis* strains grown in SM2 medium. SE31 (asterisk), SE20 (filled circle), LB 579 (filled square), LB 215 (filled diamond), and LB 734 (filled triangle). Cells were cultivated in SM2 medium with addition of 3 g L^{-1} *meso*-2,3-butanediol.

nol conversion capability, made us hypothesize that a rich complex media such as MRS cause repression of genes required for conversion of 2,3-butanediol to butanol. Therefore, six of the *L. brevis* strains showing the highest *meso*-2,3-butanediol consumption rate together with LB 734 (previously reported as 2-butanol producer) were tested for their ability to produce 2-butanol from butanone and *meso*-2,3-butanediol in a synthetic medium (SM2). As expected all the tested strains could convert butanone to 2-butanol also in this media (Fig. 1), and the final concentration was higher compared to growth in MRS (Table 1, Fig. 1). Furthermore, five strains of seven could also convert *meso*-2,3-butanediol to 2-butanol in this medium (Fig. 2). The isolates, SE20 and SE31, were found to be the best 2-butanol producers with about 0.8 g L^{-1} 2-butanol produced after 7 days (equivalent to conversion yield of 0.8 g g^{-1} of 2,3-butanediol), while the strain reported to show the highest production in a previous study (Speranza

Table 2. Secondary alcohol dehydrogenase kinetics for SE20 and SE31 in both reducing and oxidizing directions

	2-butanone + NADH → 2-butanol + NAD^+		2-butanol + NAD^+ → 2-butanone + NADH	
	K_m (mM)	V_{max} (μmol min^{-1} g^{-1})	K_m (mM)	V_{max} (μmol min^{-1} g^{-1})
SE 20	0.22 ± 0.65	1.60 ± 0.26	1.26 ± 0.82	2.50 ± 0.32
SE 31	0.51 ± 0.08	7.90 ± 0.30	1.36 ± 0.36	10.0 ± 0.55

Table 3. Specific activity of SE20 diol dehydratase toward different substrates. Cells were cultivated in SM2 medium without or with addition of 1,2-propanediol or 2,3-butanediol

	Specific activity (μmol min^{-1} g^{-1})			
Grown in	1,2-propanediol	2,3-butanediol	Glycerol	1,3-propanediol
SM2	< 0.1	< 0.1	< 0.1	< 0.1
SM2 + 1,2-propanediol	0.19 ± 0.001	0.08 ± 0.001	0.10 ± 0.001	0.11 ± 0.001
SM2 + 2,3-butanediol	0.13 ± 0.001	0.10 ± 0.002	0.22 ± 0.001	0.16 ± 0.001

et al., 1996), LB 734, produced 0.29 g L^{-1}. Major part of growth occurred within the first 24 h while butanol production continued for up to 7 days.

Enzyme kinetics

The two best producers of 2-butanol, SE20 and SE31, were chosen for characterization of their secondary alcohol dehydrogenase and diol dehydratase kinetics.

The kinetics of the secondary alcohol dehydrogenases were measured in both reduction and oxidation directions (2-butanone + NADH → 2-butanol + NAD^+). Eadie-Hofstee plot was used to calculate K_m and V_{max} values which are shown in Table 2. SE31 shows an about fourfold higher V_{max} value for both reduction and oxidization reactions compared to SE20 while K_m values were not significantly different for the two strains. Furthermore, the V_{max} values for butanone reduction or butanol oxidation were not very different, but the K_m for butanol seemed to be higher than for butanone (Table 2).

Concerning the diol dehydratase, it was not possible to make a proper kinetic characterization of this enzyme. The activity at saturated substrate concentrations (0.05 M) was measured (Table 3). We could observe that the expression, as judged from the activity of the enzyme, was induced in the presence of its substrate in the growth medium. This could be due to either transcriptional or post-transcriptional processes. When cells were grown in the presence of 1,2-propanediol or 2,3-butanediol, the extracted diol dehydratase showed activity toward a range of substrates; 1,2-propanediol, *meso*-2,3-butanediol, 1,3-propanediol and glycerol (Table 3). The highest activity for 1,2-propanediol and 2,3-butanediol, respectively, was recorded when the same substrate was present in the medium during cultivation (Table 3). No diol dehydratase activity was observed when cells were grown in SM2 without any substrate added. This is consistent with the findings of Ailion & Roth (1997) which showed that in *Salmonella*, the presence of 1,2-propanediol induced transcription of expression from the pdu and cob operons encoding the propanediol dehydratase and adenosylcobalamin biosynthesis genes, respectively.

The ability to produce 2-butanol from 2,3-butanediol seems to be widespread among *L. brevis* strains. However, the conversion process seems to be repressed during optimal growth conditions in rich laboratory media. Furthermore, the presence of proper substrate is a prerequisite for induction of the diol dehydratase. Hence, this enzyme is regulated both via a repression and an induction mechanism.

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