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ORIGINAL PAPER

Lactobacillus buchneri strain NRRL B-30929 converts a concentrated mixture of xylose and glucose into ethanol and other products

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Abstract *Lactobacillus buchneri* strain NRRL B-30929 was isolated from a fuel ethanol production facility. This heterofermentative, facultative anaerobe can utilize xylose as a sole carbon source and tolerates up to 12% ethanol. Carbohydrate utilization (API, Biomerieux) and Phenotype Microarrays™ (PM, Biolog) analyses indicated that the strain is able to metabolize a broad spectrum of carbon sources including various monosaccharides (C5 and C6), disaccharides and oligosaccharides, with better rates under anaerobic conditions. In pH-controlled bioreactors, the bacterium consumed xylose and glucose simultaneously at high concentrations (125 g L^{-1}, pH 6.0). The major fermentation products were lactate (52 g L^{-1}), acetate (26 g L^{-1}) and ethanol (12 g L^{-1}). The strain ferments glucose alone (pH 4.0) into lactate and ethanol with a molar ratio of 1.03:1. This strain will be further explored via genetic engineering for potential applications in biomass conversion.

Keywords Ethanol tolerance · Mixed sugar fermentation · Xylose · Phenotype Microarray · *Lactobacillus buchneri* · Lactate

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the names by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Introduction

Efficient utilization of both five carbon (C5) and six carbon (C6) sugars derived from lignocellulosic biomass would greatly enhance the economic conversion of this material to biofuels and bioproducts. Fuel ethanol is conventionally produced by *Saccharomyces cerevisiae*, which is able to ferment only C6 sugars. Although a great deal of research is underway to develop novel ethanologens that utilize C5 sugars, many of these preferentially utilize C6 sugars and are sensitive to ethanol and fermentation inhibitors [26]. Gram-positive lactic acid bacteria (LAB) are attractive biocatalysts since they are fermentative and many are capable of metabolizing C5 and C6 sugars. LAB are generally robust organisms, tolerant of acidic pH and high concentrations of sugars and ethanol. Furthermore, they have GRAS (generally recognized as safe) status.

Lactobacillus buchneri is a heterofermentative, facultative anaerobe. Strains of *L. buchneri* have been described with diverse applications. Silage isolates of *L. buchneri* were approved by the FDA in 2001 to use as bacterial inoculants for ensilaging various grains [8–11, 22, 29]. These strains generally produce high levels of acetic acid with other unidentified products [1, 2, 18, 27, 32], although another silage isolate, *L. buchneri* strain NK01, metabolizes lactate with accumulation of substantial amounts of 1,2-propanediol [19]. A sour dough isolate, *L. buchneri* strain B190, produces 1,3-propanediol from glycerol [24, 30, 31]. *L. buchneri* strain LB, isolated from sauerkraut, produces an antibacterial peptide (buchnericin) that inhibits the growth of selected Gram-positive bacteria [35–37]. A recent study indicated that several isolates of *L. buchneri* can produce ferulate esterases, which break down the cross-links between lignin and hemicellulose [20]. These studies suggest novel applications of *L. buchneri* in lignocellulosic pretreatment and fermentation.

A previous study of industrial ethanol plants indicated that most bacterial contaminants are Gram-positive lactic acid bacteria [25]. In this study, we screen a number of the bacterial contaminants for their capacity to grow on and metabolize xylose, and report the identification and characterization of one specific strain, *L. buchneri* NRRL B-30929, which can ferment high concentrations of mixed C5 and C6 sugars to produce lactate, acetate and ethanol.

Materials and methods

Bacterial strains and growth conditions

Bacterial isolates [25] were maintained on solid MRS medium (Becton Dickinson, Sparks, MD, USA) under aerobic or anaerobic conditions (BBL GasPak anaerobic system, Becton Dickinson, Franklin Lakes, NJ, USA) at either 30 or 37°C. *L. buchneri* strain DSM 5987 was purchased from DSMZ (Braunschweig, Germany) and grown on MRS plates and static MRS broth cultures. For carbon source utilization testing, a simplified MRS medium (designated MRSsi) was developed by reducing or omitting several components from MRS. MRSsi contains 5 g of casamino acids, 5 g of peptone, 5 g of yeast extract, 0.5 ml of Tween 80, 0.05 g of MnSO4 · $4H_2O$, 0.2 g of $MgSO_4$ · $7H_2O$, 0.1 g of $CoCl_2$ · $6H_2O$, and 5 g of sodium acetate per liter. Concentrated sugars were autoclaved separately and added prior to use. For xylose utilization screening, 5 ml MRSsi cultures containing 4–6% xylose in 14 ml tubes were inoculated at 1% (v/v) from overnight MRS preinocula and incubated at 30°C without shaking. Growth was monitored by measuring the A600 periodically.

16S rDNA sequencing

Genomic DNA was isolated using the Gram-positive DNA purification kit (Epicentre, Madison, WI, USA). Genomic PCRs were performed by using 16 rDNA primers [33], and the PCR fragment was sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI Prism 310 DNA sequencer (Perkin–Elmer, Foster City, CA, USA). Sequence analyses were performed with the SDSC biology workbench (http://www.sdsc.edu/Research/biology/) and through the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/).

C-source utilization and phenotype microarray analyses

Profiles of sugar fermentation were determined by the API 50 CHL system (bioMerieux, Marcy I'Etoile, France) according to vendor's instructions. Phenotype MicroArrays™ (PM) analyses were performed by Biolog, Inc (Hayward, CA, USA). Two independent experiments were conducted with each strain under two growth conditions, and pairwise comparisons were performed using OmniLog® V. 1.5 Comparison Module (Biolog, Inc) to compare images of the carbon source profiles of the two submitted strains.

Bioreactor fermentations

Fermentations of *L. buchneri* strain NRRL 30929 were performed in 2 L fermentors (Biostat B, B. Braun International, Germany), at constant pH controlled using 4 M NaOH and 4 M phosphoric acid, and at 30°C with 100 rpm stirring. Duplicate fermentations were carried out in each experiment with conditions specified in figure legends. Samples were taken periodically during the course of fermentation. The concentrations of residual xylose and/or glucose, and fermentation products, including lactate, acetate, and ethanol, were measured by HPLC using a 300 mm Aminex HPX-87H column (Bio Rad, Richmond, CA, USA) and a refractive index detector (G1362A, Agilent Technologies, Palo Alto, CA, USA). Samples were run at 65°C and eluted at 0.6 ml min^{-1} with 5 mM sulfuric acid.

Results and discussion

Screening of bacterial contaminants from ethanol plants

In a previous study of fuel ethanol plants, numerous bacterial contaminants were isolated and identified as Gram-positive lactic acid bacteria [25]. Approximately 80 isolates from this study were tested for growth on xylose as a sole carbon source. Isolates were screened on a modified MRS medium (MRSsi) containing xylose. MRSsi is simplified MRS and could potentially be modified further with corn steep liquor and biomass hydrolysates for industrial fermentations.

One isolate clearly outperformed other strains for rapid growth on 4–6% xylose as a sole carbon source in MRSsi, reaching an OD600 of 8.50–10.00 in overnight cultures. This strain was identified as *L. buchneri* by NCBI analyses of the16S rDNA sequence (Genbank accession number: DQ987924), which showed 99.9% identity with that of *L. buchneri* JCM1115 (GenBank accession number: AB205055). The isolated strain was deposited in the ARS Patent Culture Collection as *L. buchneri* NRRL B-30929.

Carbon utilization profiles

API assays suggested that NRRL B-30929 ferments L-arabinose, D-ribose, D-xylose, galactose, glucose, fructose, D-maltose, D-lactose, D-melibiose, and D-rafinose while

L. buchneri strain DSM 5987, used for comparison, did not use L-arabinose and D-xylose (data not shown but see below for PM result).

To further understand and analyze the carbon utilization and metabolic profile differences between *L. buchneri* NRRL B-30929 and *L. buchneri* DSM 5987, a pairwise comparison of Phenotype Microarrays™ (PM) was performed under aerobic and anaerobic growth conditions (Fig. 1). In this scheme, each coordinate represents a graph of substrate utilization over time (in this case, 96 h). PM1 and PM2 are separate sets of 96 substrates (the lists of substrates for PM1 and PM2 are available at www.biolog.com) tested under either aerobic (aeroPM) or anaerobic (anaeroPM) conditions. Substrate utilization by control strain DSM 5987 is represented by green, utilization by test strain NRRL B-30929 is represented by red, and overlapping utilization is represented by yellow. At a glance, the prevalence of red curves indicates that more substrates are utilized by NRRL B-30929 than by DSM 5987 (Fig. 1). Anaerobic conditions generally favored substrate utilization by both strains. Common carbon sources used by both NRRL B-30929 and DSM 5987 include L-arabinose (anaeroPM1, A2), D-galactose (anaeroPM1, A6), D-gluconic acid (anaeroPM1, B6), D-mannitol (anaeroPM1, B11), D-ribose (aeroPM1 and anaeroPM1, C4), D-fructose (anaeroPM1, C7), α-D-glucose (anaeroPM1, C9), maltose (anaeroPM1 C10), D-melibiose (anaeroPM1,C11), sucrose (anaeroPM1, D11), uridine (anaeroPM1, D12), adenosine (anaeroPM1, E12), inosine (anaeroPM1, F12),methyl pyruvate (anaeroPM1, G10), D-psicose (anaeroPM1, H5), L-xylose (anaeroPM1, H6), pyruvic acid (anaeroPM1, H8), D-melezitose (aero PM2, C4), D-glucosamine (aero and anaeroPM2, E5), oxalomalic acid (aero and anaeroPM2, F5) and dihydroxy acetone (aero and anaeroPM2 H9).

Although the use of L-arabinose as a sole C-source by DSM 5987 was not confirmed by API for unknown reasons, it has been reported that 90% or more of *L. buchneri* strains can use arabinose [34]. D-psicose and D-tagatose are stereoisomers of fructose (ketohexose) and L-lyxose is a stereoisomer of ribose (aldopentose), and thus may be metabolized by NRRL B-30929. *buchneri* strains can use arabinose [34]. Other C-sources described as commonly used by *L. buchneri* strains include fructose, glucose, maltose, melezitose, and D-melibiose [34]. About 11–89% of *L. buchneri* strains can also use galactose, ribose, sucrose and xylose [34]. D-glucosamine, methyl pyruvate, pyruvic acid and dihydroxy acetone can serve as precursors or analogs of glucose, pyruvate and DHAP, which are central metabolites in LAB. Similarly, oxalomalic acid and malic acid may be metabolized via the citrate fermentation shunt, mediated by citrate lyase and oxaloacetate decarboxylase, and/or via malolactic fermentation mediated by malate decarboxylase and malate permease [5, 16].

Carbon sources metabolized by NRRL B-30929 and not DSM 5987 include *N*-acetyl-D-glucosamine (aeroPM1 and anaeroPM1, A3), D-trehalose (aeroPM1 and anaeroPM1, A10), D-mannose (aeroPM1 and anaeroPM1, A11), thymidine (anaeroPM1, C12), D-lactose (aeroPM1 and anaeroPM1, D9), lactulose (aeroPM1 and anaeroPM1, D10), maltotriose (aeroPM1 and anaeroPM1, E10), 2-deoxy adenosine (anaeroPM1, E11), citric acid (aeroPM1 and anaeroPM1, F2), D-cellobiose (aeroPM1, F11), L-malic acid (anaeroPM1,G12), salicin (aeroPM2 and anaeroPM2, D2), D-tagatose (aeroPM2 and anaeroPM2, D6), and turanose

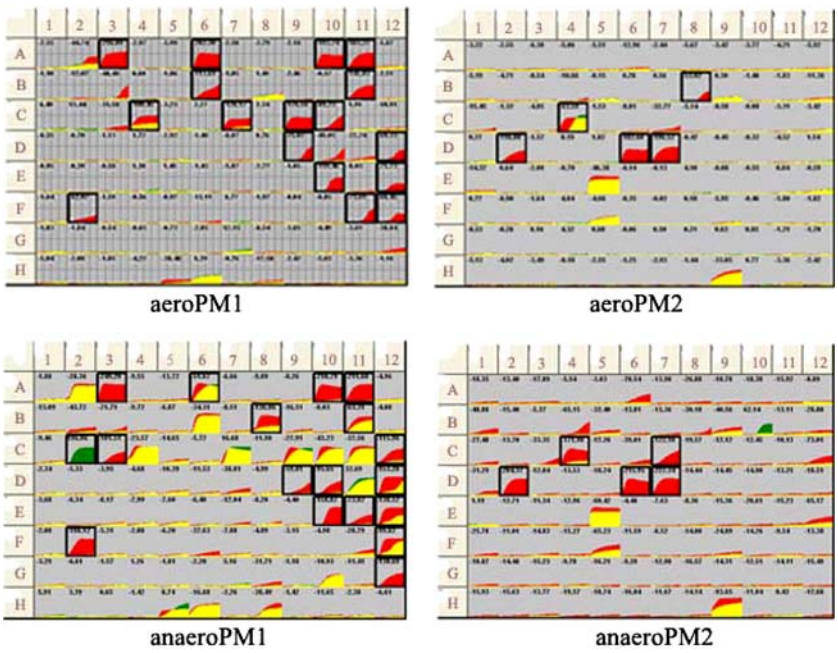

Fig. 1 Phenotypic microarray (*PM*) comparison of *L. buchneri* strain NRRL B-30929 with *L. buchneri* strain DSM 5987 growing under aerobic (*upper panel left* aeroPM1, *right* aeroPM2) and anaerobic (*lower panel left* anaeroPM1, *right* anaeroPM2) conditions, *green* DSM5987, *red* B-30929, *yellow* overlap. PM1 and PM2 are separate sets of substrates that are provided by Biolog Inc. Plates were read after 48 and 96 h of incubation. Only 96 h data are shown here. Duplicate data set showed perfect positive correlation by scatter plots (Biolog Inc)

(aeroPM2 and anaeroPM2, D7) (Fig. 1). The only carbon utilized by DSM 5987 but not B-30929 as shown (Fig. 1) is D-galactonic acid-γ-lactone (anaeroPM1, C2).

N-acetyl-glucosamine is a monosaccharide derivative of glucose and one of the main components of the cell walls of fungi and bacteria and also is found in exoskeletons of insects and other animals. NRRL B-30929 may be capable of utilizing *N*-acetyl-glucosamine originating from these natural polymers.

The capacity of utilizing *N*-acetyl-glucosamine, mannose, and mannitol distinguishes NRRL B-30929 from the majority (more than 90%) of other *L. buchneri* strains [34]. This indicates that the genome of this organism contains specific genes for unique transporters such as PTS-*N*-acetyl-D glucosamine, PTS-mannose, and PTS-mannitol, which were described when 12 genomes of lactic acid bacteria were analyzed and compared [14]. The utilization of mannitol by both B-30929 and DSM 5987 suggests the presence of a specific mannitol dehydrogenase that converts mannitol to fructose, or vice versa, as was reported in a closely related species *L. brevis* [12].

NRRL B-30929 probably produces various hydrolytic enzymes to break down specific disaccharides or polysaccharides. The utilization of D-trehalose implies the presence of β-amylase required to break down the substrate. Other enzymes may include: β-glucosidase (EC 3.2.1.21) (cellobiose, melibiose and salicin), β-galactosidase (EC 3.2.1.23) (lactose and lactulose), β-fructofuranosidase (EC 3.2.1.26) (ribofuranose: inosine, adenosine, melezitose and uridine), α-glucosidase (EC 3.2.1.20) (trehalose, turanose, sucrose, maltose, melezitose and maltotriose), and α-galactosidase (EC 3.2.1.22) (melibiose or related oligosaccharides containing α 1,6 linked galactose). Previous enzymatic studies indicated that a *L. buchneri* strain can hydrolyze melibiose, raffinose, and stachyose [17].

Carbon sources metabolized by NRRL B-30929 under anaerobic conditions but not aerobic conditions (Fig. 1) include: D-xylose (anaeroPM1, B8), D, L-malic acid, (anaeroPM1, C3), thymidine (anaeroPM1, C12), L-malic acid (anaeroPM1, G12) and turanose (anaeroPM2, C7). However, no strict anaerobic growth condition was applied in the PM assays other than that the media was prepared anaerobically.

The PM results indicate that *L. buchneri* NRRL B-30929 is metabolically flexible and can use a broad spectrum of carbohydrates and derivatives. The strain not only possesses most common traits associated with the defined *L. buchneri* species, but also appears to have additional unique enzymes specific to this strain. Recently, the DOE-Joint Genome Institute announced plans to sequence the genome of *L. buchneri* NRRL B-30929. The genome data will enable us to verify the presence of genes coding specific enzymes and aid to clone and characterize individual enzymes.

Bioreactor fermentations of xylose and glucose

Batch fermentations in pH-controlled bioreactors showed that strain NRRL B-30929 can ferment up to 90 g L^{-1} xylose, producing approximately 53 g L^{-1} lactate (Fig. 2a). The yield of lactate is 0.5–0.6 g/g xylose (theoretical is 0.6 g/g xylose). By using fed-batch cultures, lactate production was increased to approximately 83 g L^{-1} (Fig. 2b). When xylose was used as a sole carbon source, the molar ratio of lactate and acetate was close to 1:1, suggesting the operation of the phosphoketolase pathway [15]. The PM results showed that strain NRRL B-30929 uses ribose, galactose, and arabinose, likely via the phosphoketolase pathway.

Lactobacillus buchneri NRRL B-30929 compared favorably (Table 1) with several reported microorganisms [3, 6, 13, 21, 28] for lactate production from xylose in terms of final yields and culture times required.

In fermentations with high concentrations of xylose and glucose (125 g L^{-1} each), the strain utilized both sugars simultaneously (Fig. 3). This suggests that the organism maintains an active xylose utilization pathway, and the xylose consumption is not repressed by the presence of glucose, similar to observations reported in *L. brevis* (personal communication). The molecular mechanisms for simultaneous utilization of xylose and glucose remain to be studied.

When glucose was used as the sole substrate at pH 4.0 (Fig. 4), the major fermentation products were lactate (240 mM), ethanol (232 mM) and CO_2, plus a small

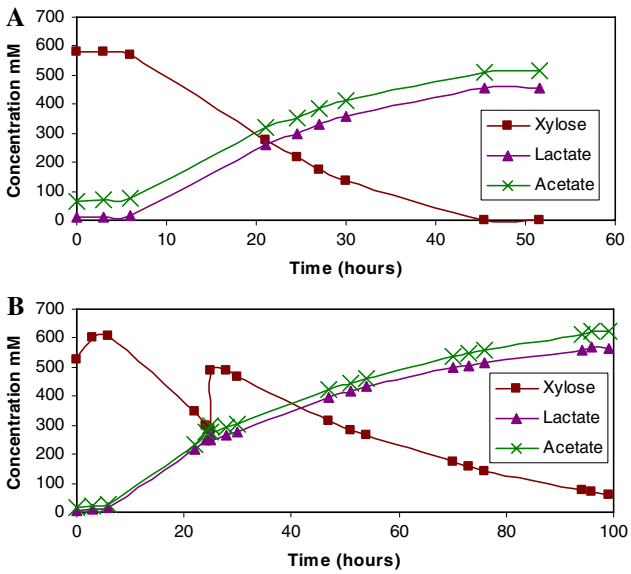

Fig. 2 Xylose fermentations by *L. buchneri* strain NRRL B-30929 using a bioreactor with 100 rpm stirring and constant pH 6.0. Initial volume is 1,000 ml, final concentrations are calculated after volume adjustment. **a** Batch fermentation of xylose alone, **b** Fed-batch fermentation:more xylose was added at 24 h. Data represent the mean of duplicate experiments

Table 1 Summary of recently reported microorganisms that use xylose to produce lactate

Organisms	*Lactococcus lactis* I0-1 Japanese Collection JCM 7638	*Lactobacillus casei* subsp. *rhamnosus* ATCC 10863	Recombinant *Lactobacillus* MONT4sup pLP3537-xyl	Recombinant *Escherichia coli* FBR9, FBR11	*Rhizopus oryzae* CBS 112.07	*Lactobacillus buchneri* NRRL B-30929
Journal ref	[28]	[6]	[21]	[3]	[13]	This study
Yield g/g lactate/xylose	60%	80%	86%	78%	36–40%	60%
Total lactate	33 g L^{-1}	25 g L^{-1}	15 g L^{-1}	56–63 g L^{-1}	7–15 g L^{-1}	40–83 g L^{-1}
Tm	37°C	45°C	37°C	35°C	37°C	30°C
pH	6.0	5.7	6.5	7.0	6.5	5–6.0
Culture time	Continuous	144 h	144 h	86–150 h	60 h	40–100 hrs
Initial xylose	7%	3%	2%	10%	3%	8–12.5%
source	Isolated by this group	Culture collection	Recombinant required 10 µg/ml erythromycin	Recombinant	Culture collection	Isolated from ethanol plant
By-products	LAB biomass Acetate, CO_2 trace formate, ethanol	LAB biomass Acetate, CO_2	*E. coli* biomass Acetate, CO_2 succinic acid Residual erythromycin	*E. coli* biomass Acetate succinic acid, CO_2	Fungal biomass Xylitol, glycerol, CO_2	LAB biomass Acetate, CO_2 buchnericin

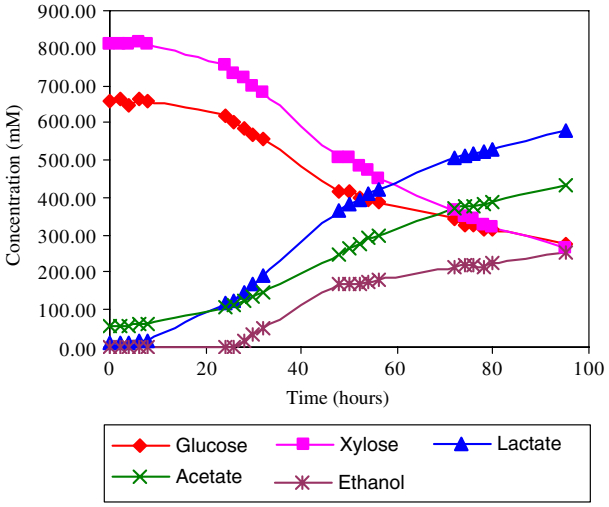

Fig. 3 Bioreactor fermentation of mixed sugars (about 10–12.5% w/v each, glucose and xylose) by *L. buchneri* strain NRRL B-30929 with 100 rpm stirring and constant pH 5.0. Initial volume is 1,000 ml, final concentrations are calculated after volume adjustment. Data represent the mean of duplicate fermentation experiments

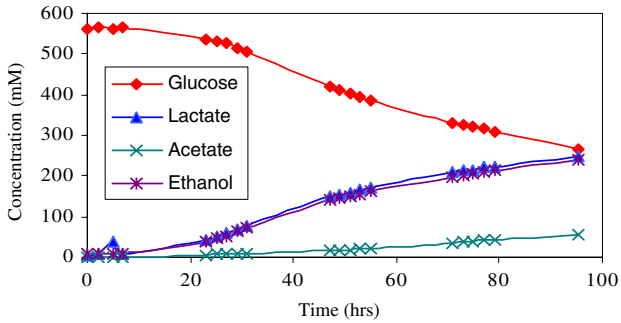

Fig. 4 Bioreactor fermentation of glucose (about 10% w/v) fermentation by *L. buchneri* strain NRRL B-30929 with 100 rpm stirring and constant pH 4.0. Initial volume is 1,000 ml, final concentrations are calculated after volume adjustment. Data represent the mean of duplicate fermentation experiments

amount of acetate (57 mM). The molar ratio of lactate:ethanol is 1.03:1.00. When only hexose is available, the strain seems to use the pentose phosphate pathway to produce equimolar amounts of ethanol and lactate [23, 28]. Probably, at late stationary phase, cells need additional ATP, therefore acetate was accumulated. The PM results indicated that NRRL B-30929 can utilize gluconic acid, one of the intermediates for the pentose phosphate pathway, suggesting the operation of this pathway [28]. Furthermore, one of the key enzymes for this pathway, glucose 6-phosphate dehydrogenase, was purified from *L. buchneri* IFO 3961 [7]. The genome analyses will provide tools to study potential interactions of each pathway under various environmental conditions, and thus, may help to explain the ability of the strain to adapt and grow in a commercial ethanol production plant.

Ethanol tolerance

Like most other LAB, strain NRRL B-30929 produces large amounts of lactic acid from pyruvate, and carbon flow from pyruvate to ethanol is relatively weak. Ethanol is likely produced by alcohol dehydrogenase conversion of acetyl-CoA to acetaldehyde, and not directly from pyruvate due to the lack of a pyruvate decarboxylase gene. Nevertheless, the strain exhibited a high tolerance for ethanol, with no inhibition of growth on 6% xylose as sole carbon source at up to 6% ethanol (Fig. 5).

Growth was reduced approximately 30–70% by10–14% ethanol content. A maximum OD600 reached around 4.0 with 10% ethanol (Fig. 5), and no growth was observed in 16–20% ethanol. In a survey of 31 strains of *Lactobacillus* [4] with five different carbon sources tested (glucose, xylose, lactose, cellobiose and starch) in the presence of up to 16% ethanol, ethanol tolerance was reported both strain and substrate dependent. However, the ethanol tolerance was significantly reduced with xylose as the carbon source. In their study, the best xylose utilizing strains were *L. hilgardii* ATCC 8290 and *L. brevis* IFO 27305, which reached a maximum OD600 (after 48 h growth) of 2.72 and 1.56, respectively, in 10% ethanol [4]. Notice that their survey did not include any strains of *L. buchneri*.

In summary, we have isolated and identified *L. buchneri* strain NRRL B-30929 that can be used for simultaneous production of lactate, ethanol, and acetate from xylose, xylose plus glucose and glucose. This strain is robust, ethanol tolerant, and able to utilize diverse carbon sources. The strain will be further explored for potential applications in a bio-based refinery platform.

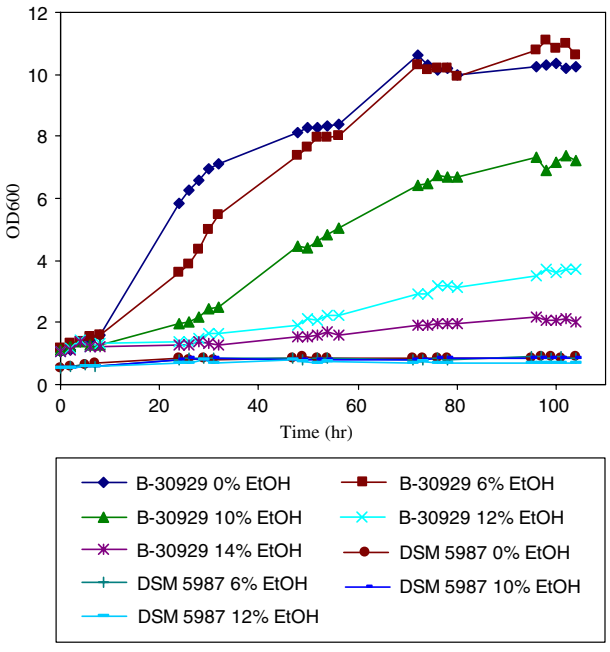

Fig. 5 Growth analyses and ethanol tolerance analyses of *L. buchneri* NRRL B-30929 grown on 6% xylose as sole sugar, plus 0–16% ethanol content. OD600 were recorded over 50 h time period

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