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Ethanol tolerance and carbohydrate metabolism in lactobacilli*

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SUMMARY

This paper describes the ethanol tolerance and metabolism of 31 strains of *Lactobacillus* on glucose, xylose, lactose, cellobiose and starch. The purpose of this work was to determine the suitability of the 31 strains as potential host for the ethanol producing genes, pyruvate decarboxylase and aldehyde dehydrogenase, from *Zymomonas mobilis*. The 31 strains were screened for their ability to grow in 0 to 8% v/v ethanol on all five carbohydrates. Those strains that were able to grow to an OD of 1.0 in 8% ethanol were evaluated at ethanol concentrations up to 16% v/v. The fermentative products from the five carbohydrates were analyzed to determine the ratios of lactic acid, ethanol, and acetic acid.

INTRODUCTION

The efficient biological conversion of biomass to ethanol requires isolation or development of microorganisms capable of fermenting an array of carbohydrates and tolerating high concentrations of ethanol. Recently, the genes coding for pyruvate decarboxylase and aldehyde dehydrogenase from *Zymomonas mobilis* were cloned and introduced into *Escherichia coli* [16]. Transformants produced 58, 52, and 42 g/l of ethanol from 12% glucose, 12% lactose, and 8% xylose at efficiencies of 95%, 80% and 102%, respectively [1]. The 102% conversion of xylose was attributed to the additional catabolism of amino acids. Production of higher levels of ethanol by *E. coli* would not be expected, however, since this organism does not tolerate greater than 7.5% ethanol [1].

One group of bacteria which may have potential as candidate recipients of the production of ethanol genes (PET) and as possible ethanol producers is the *Lactobacillus*. This genus has many desirable properties and has been recommended as the organism of choice for many industrial fermentations [6]. Many of the carbohydrates found in biomass, such as starch, cellobiose, lactose, glucose, and xylose, are fermented by various species of this genus [19,24]. Since metabolism by these bacteria occurs via glycolytic or pentose-phosphate pathways, both of which generate pyruvate, expression of cloned pyruvate decarboxylase and aldehyde dehydrogenase genes could result in high levels of ethanol production. In addition, several species possess a high level of ethanol tolerance which allows for their competitive success in fermentative environments [17]. For example, *Lactobacillus heterohiochii* and *Lactobacillus homohiochii*, spoilage organisms of the rice wine, sake, are reported to be the most ethanol tolerant organisms known, with an ability to grow in over 20% ethanol [20,29]. To our knowledge, however, a survey on ethanol tolerance by lactobacilli has not been reported.

In this study, 31 strains of heterofermentative and homofermentative lactobacilli were screened for their ability to ferment a wide range of biomass sugars and to tolerate high concentrations of ethanol. Hence, this study focused on the ability of these strains to use glucose, xylose, cellobiose, lactose and starch as carbon sources. In addition, the ability of these strains to grow on each substrate with the additional stress of up to 16% (v/v) ethanol was reported.

MATERIALS AND METHODS

The strains of *Lactobacillus* used in this study are listed in Table 1. All strains were grown in MRS broth (Difco Laboratories, Detroit, MI), at 37 °C, except for *Lactobacillus hilgardii* 8290, *Lactobacillus brevis* B-1127, and *Lactobacillus confusus* B-1064, which were grown at 26 °C. For growth studies, cells were grown in MRS [8] medium

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TABLE 1

Growth of *Lactobaccillus* strains in ethanol after 48 h

Strain	Glucose			Xylose			Lactose			Cellobiose			Starch		
(% EtOH):	0	4	8	0	4	8	0	4	8	0	4	8	0	4	8
L. amylophilus															
NRRL[a] B-4437	0.97	0.82	0.34	NG[b]	NG	NG	NG	NG	NG	NG	NG	NG	0.97	0.65	NG
L. amylovorus															
NRRL B-4538	6.00	4.38	1.90	NG	NG	NG	NG	NG	NG	3.97	3.22	1.62	4.29	3.38	1.67
NRRL B-4540	5.80	5.36	2.18	NG	NG	NG	NG	NG	NG	4.88	4.66	2.59	4.16	3.86	2.22
NRRL B-4542	5.52	5.18	2.54	NG	NG	NG	NG	NG	NG	4.56	4.50	2.46	5.80	4.92	2.62
NRRL B-4549	5.86	5.08	1.78	NG	NG	NG	NG	NG	NG	4.38	3.50	1.48	5.10	4.30	1.56
L. brevis															
NRRL B-1127	1.08	0.83	NG	4.22	4.08	2.26	NG	NG	NG	NG	NG	NG	NG	NG	NG
IFO[c] 3960	6.24	6.20	3.74	3.28	3.08	0.34	4.40	3.80	2.46	5.40	5.32	2.26	3.64	3.34	2.29
IFO 12005	6.04	5.40	3.78	4.54	4.30	1.73	5.38	4.88	3.14	5.08	4.74	2.88	2.96	2.88	2.78
IFO 12520	5.96	5.54	3.94	3.70	3.44	0.62	6.14	5.28	3.78	4.64	3.70	2.14	3.34	3.02	2.90
IFO 13109	5.32	4.70	1.38	3.52	2.48	0.56	3.18	3.12	0.79	4.84	3.40	0.81	2.38	1.02	0.56
IFO 13110	6.86	6.14	4.56	3.82	3.52	0.47	3.88	3.78	2.00	5.08	4.32	2.65	2.74	2.46	0.83
IFO 27305	5.10	4.10	3.00	3.02	2.88	1.08	4.04	2.78	2.00	5.20	5.00	3.45	3.52	3.08	2.70
L. casei															
UNL[d] 685	4.40	3.60	2.60	3.34	3.20	0.18	3.60	3.38	2.24	1.84	0.38	NG	0.94	0.75	0.50
UNL 686	5.60	5.40	3.48	3.30	2.82	0.20	3.90	3.44	0.28	5.58	5.22	2.96	5.30	4.92	2.98
UNL 3532	6.66	5.44	3.44	0.60	0.48	0.33	6.20	5.14	1.66	6.04	5.56	3.14	0.62	0.54	0.34
IFO 3831	4.86	3.86	3.42	3.58	2.80	0.32	4.48	3.86	3.52	5.78	4.60	2.97	2.68	2.36	2.24
IFO 3953	5.70	5.60	4.28	3.00	2.80	0.35	5.60	4.12	2.74	5.26	4.08	0.95	0.54	NG	NG
IFO 12004	6.72	6.12	4.80	3.38	3.16	0.46	4.68	4.62	1.88	5.64	5.38	3.99	2.76	2.50	0.71
L. confusus															
NRRL B-1064	5.26	4.48	2.86	0.27	NG	NG	NG	NG	NG	1.34	0.53	NG	0.36	NG	NG
ATCC[e] 27646	4.04	3.40	1.94	4.88	3.18	0.86	8.80	8.24	3.90	0.20	NG	NG	0.30	NG	NG
L. delbrueckii															
NRRL B-1042	7.22	7.06	4.72	0.63	0.52	0.36	0.60	0.54	0.34	5.70	4.90	4.88	3.20	2.58	2.50
L. fermentum															
IFO 3956	5.60	5.20	4.24	2.66	2.56	0.32	3.94	3.08	0.89	4.96	4.86	2.98	2.84	2.80	2.35
IFO 3959	4.38	2.98	0.88	3.50	2.86	1.92	3.20	2.90	1.04	3.32	2.94	2.31	NG	NG	NG
L. hilgardii															
ATCC 8290	6.48	6.18	1.30	2.54	2.40	1.34	1.08	0.83	NG	NG	NG	NG	NG	NG	NG
L. sake															
IFO 3541	6.04	5.34	3.70	3.80	2.68	0.71	3.56	3.38	0.81	6.30	5.34	2.96	3.18	2.92	2.54
L. pentosus															
ATCC 8041	6.30	5.12	0.23	3.78	2.60	NG	5.52	5.18	0.63	5.00	4.40	1.30	3.66	3.44	1.40
L. plantarum															
NRRL 1195	5.58	5.38	3.92	0.67	0.53	0.29	3.90	3.06	0.62	5.44	4.60	3.42	3.38	2.72	2.58
IFO 3074	6.44	5.34	1.86	3.52	2.60	NG	5.00	3.60	3.46	5.02	4.24	1.70	5.26	4.82	3.04
IFO 12006	6.70	6.20	4.48	3.44	3.04	0.48	5.26	4.46	2.78	5.62	4.92	2.54	2.96	2.86	2.33
IFO 12011	5.86	5.50	0.46	3.58	3.40	0.25	4.46	3.34	2.24	4.64	4.22	0.39	3.30	3.08	2.46
L. sp.															
IFO 3954	3.24	3.00	0.27	4.48	2.88	NG	6.40	2.20	0.23	0.22	NG	NG	0.23	NG	NG

containing 2% glucose, xylose, lactose, cellobiose, or liquefied starch. Liquefied starch was prepared by the method of Cheng et al. using potato starch (Sigma Chemical Co. St. Louis, MO) [7]. The concentration of starch in solution was determined by the phenol-sulfuric acid method [10] using glucose as a standard. The starch solution was diluted to 4% with sterile distilled water and used as a stock solution.

Carbohydrate fermentation

Growth studies were performed on MRS medium containing xylose, lactose, glucose, cellobiose, and liquified starch. All carbohydrate solutions were sterilized separately. Each strain was inoculated at a 5% inoculation level and incubated at the optimal growth temperature. Cell growth was measured after 24 and 48 h by optical density (OD) measurement (DU-64, Beckman Instruments, Inc., Fullerton, CA) of appropriately diluted samples at 625 nm. Strains exhibiting the ability to utilize one or more of the tested carbohydrates (gauged by an observed $OD \geq 1.0$ after 48 h) were progressed to the next stage of the study in the respective carbohydrate medium.

Ethanol tolerance

Those strains which demonstrated sufficient growth in one or more of the tested carbohydrates were transferred twice, i.e. two successive transfers with overnight incubation, into MRS containing the respective carbohydrate, prior to inoculating into like media containing 4, 5, 6, 7, and 8% (v/v) ethanol. Strains inoculated into the ethanol-containing media were incubated at 35 °C, except for *L. hilgardii* 8290, *L. brevis* B-1127, and *L. confusus* B-1064, which were incubated at 24 °C. Strains preferred suboptimal growth temperatures in the presence of ethanol and were grown at 2 °C below the optimum temperature. OD readings were recorded at 24 and 48 h. Those strains which developed an OD above 1.0 after 48 h in 8% ethanol were then propagated in MRS media containing the appropriate carbohydrate and 6% ethanol for 3 days (daily transfers) and then inoculated into like media prepared with 8, 10, 12, 14, and 16% ethanol. As before, OD reading were recorded at 24 and 48 h.

Product analysis

Each strain which achieved an $OD \geq 1$ in the higher percent ethanol studies was subjected to a series of analyses designed to quantify the concentrations of lactic acid, acetic acid and ethanol produced during fermentation of each tested carbohydrate. Each strain was initially inoculated into MRS, followed by two daily transfers (overnight incubations) into MRS, without sodium acetate, containing the carbohydrate to be tested (cellobiose, lactose, glucose, xylose or starch). Cells were removed by centrifugation (Fisher Scientific Centrific Centrifuge, $12000 \times g$, 15 min) and the supernatant collected.

Acetic acid and ethanol concentrations were determined by gas-liquid chromatography (Hewlett Packard, HP 5890 series II) using an $^{80}/_{100}$ Chromosorb 101 1.8 m × 2 mm i.d. packed glass column (Supelco, Bellefonte, PA). Samples were diluted 1:1 with 100 mM isobutyric acid (internal standard) and 2 μl of this solution were then injected (column temp. 155 °C for 15 min, flow rate: 25–30 ml/min, He, detector: FID).

Lactic acid production was determined with a L-lactic acid and D-lactic acid enzymatic assay kit (Boehringer Mannheim, Indianapolis, IN). In addition, uninoculated MRS was tested for the presence of lactic acid, and this base amount was subtracted from each sample.

RESULTS AND DISCUSSION

Substrates

The five different carbohydrates were chosen based on their potential as a low-valued feedstock for ethanol production. The extent of growth of the 31 strains on the five carbohydrates is presented in Table 1, with glucose chosen as a reference carbohydrate for all of the strains.

Twenty-three of the strains were able to grow on liquefied starch. *Lactobacillus amylovorus* and *Lactobacillus amylophilus* are the only known *Lactobacillus* that are able to grow on unliquefied starch [21,22]. The composition of the liquefied starch was maltose, maltotriose, and higher oligosaccharides as determined by HPLC (data not shown), with virtually no glucose present. It was not unexpected to see growth by many of the strains on liquefied starch, since most of the strains are able to metabolize maltose [19].

Fifteen of the 31 strains were able to ferment lactose, a sugar not used by the ethanol producer, *Saccharomyces cerevisiae* and *Z. mobilis*. Their ethanol tolerance and lactose metabolism make them prime candidates for geneticly engineering them to produce ethanol. Twenty-one strains

Table 1 (see opposite page).

[a] National Regional Research Laboratory, Peoria, IL.

[b] No growth observed.

[c] Institute for Fermentation, Osaka, Japan.

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[e] American Type Culture Collection, Rockville, MD.

were able to ferment cellobiose, a disaccharide derived from cellulose. Three enzymes are necessary to hydrolyze cellulose to glucose, endocellulase, cellobiohydrolase, and β-glucosidase. The first two enzymes, cellobiohydrolase and endoglucanase are inhibited by the accumulation of cellobiose and the commonly used cellulolytic enzyme systems are deficient in β-glucosidase [25,26,30]. Direct conversion of cellobiose to ethanol would eliminate the need for supplementation of β-glucosidase and reduce production costs.

Twenty-five of the 31 strains investigated were able to ferment xylose. Xylose, which is derived from xylan, an abundant plant polysaccharide, is converted to ethanol by *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophillus*. Unfortunately, each of these organisms is intolerant to ethanol above 2 to 3% [23]. Increased ethanol tolerance of xylose-fermenting organisms would have a significant impact on the process economics of a biomass-to-ethanol facility.

Ethanol tolerance

Thirty-one strains of *Lactobacillus* were evaluated for their ability to utilize five different carbohydrates in the presence of ethanol from 0–16% v/v. The effect of ethanol on growth varied depending on the strain and the carbon source. Table 1 lists the combined results of the carbohydrate fermentation study and the first stage of the ethanol tolerance tests (through 8% ethanol.) In general, the majority of the strains were inhibited only slightly by 4% ethanol, demonstrating significant tolerance by the genus *Lactobacillus* as a whole. The graduation from 4% to 8% ethanol exposed a wide range of threshold levels. Table 1 summarizes these data by listing optical densities after 48 h for the 4% and 8% ethanol levels. The extent of growth of all of the strains was reduced in the presence of 8% v/v exogenous ethanol based on comparison to the OD at 0% ethanol after 48 h.

The data suggested that ethanol tolerance was both strain and substrate dependent. Relative ethanol tolerance was significantly reduced with xylose as the carbon source. Twenty-two of the strains grew well on xylose and glucose (at 0% ethanol, OD > 2.5). At 8% ethanol 20 of the 22 strains had a higher relative ethanol tolerance on glucose than xylose when expressed as:

$$\%\ OD_{Rel} = \frac{OD \text{ at } 8\% \text{ EtOH (Substrate)}}{OD \text{ at } 0\% \text{ EtOH (Substrate)}} \times 100\%$$

The 20 strains on xylose at 8% ethanol had an average $\%OD_{Rel}$ of 13% ± 12% (S.D.), while the average for glucose was 58% ± 22%. The exceptions were *L. brevis* NRRL B-1127 and *L. fermentum* IFO 3959, which were 53 and 54%, respectively.

The utilization of the other substrates, lactose, cellobiose, and starch was not as sensitive to ethanol as compared to the utilization on xylose. The two exceptions were *L. casei* UNL 686 and *L. plantarum* NRRL-1195, which did not grow well in 8% EtOH on lactose ($\%OD_{Rel}$ of 7% and 16%, respectively), but grew well on glucose, starch, and cellobiose in 8% EtOH (all > 50%).

Those strains which utilized xylose, lactose, cellobiose and/or starch at the 8% level (gauged arbitrarily by an observed OD over 1.0 after 48 h) were progressed to the higher ethanol levels in the respective carbohydrate medium. A total of 28 strains; 25 on glucose, four on xylose, 15 on lactose, 16 on liquefied starch, and 21 on cellobiose were tested at the higher ethanol concentrations. These results are presented in Table 2.

All the strains tested grew in the presence of 10% ethanol. At 12% ethanol 21 strains were capable of achieving an OD of 0.39 or greater (the initial OD from the 5% inoculum was 0.1 to 0.2). It was impressive to note that at least some growth (OD ≥ 0.39) was observed for eight strains in media containing 16% ethanol. As was seen earlier, ethanol tolerance was substrate dependent. This was observed with xylose, but also with cellobiose and lactose when compared to glucose. Examples include *L. amylovorus* NRRL B-4538, *L. brevis* IFO 3960, and *L. fermentum* IFO-3959.

Ethanol tolerance is typically associated with a change in the fatty acid content of the cell membrane resulting in a change in the fluidity of the membrane [9]. Uchida and Mogi studied the cellular fatty acid profile of ethanol tolerant lactobacilli isolated from sake and found that the alcoholphilic bacterium *L. heterohiochii* produced unusually long-chain (C_{20}–C_{30}) fatty acids [29]. Others have found in *L. homohiochii* [27], *L. heterohiochii* [28], *E. coli* [15,18], and *S. cerevisiae* [2] that an ethanol-induced change will occur in the fatty acid profile from saturated to mono-unsaturated fatty acids. Buttke and Ingram determined that ethanol affected the composition of fatty acids in *E. coli* at the level of biosynthesis by altering the type of fatty acids that were synthesized and assembled into phospholipids [4]. Buttke and Ingram used mutants that lacked enzymes necessary for fatty acid synthesis to identify β-ketoacyl ACP synthetase II enzyme as the most likely enzyme affected by the presence of ethanol [5].

The fact that ethanol tolerance is substrate dependent, especially in the case of xylose, suggests that ethanol inhibits certain enzymes involved in metabolism. It is certainly possible that ethanol may be affecting specific membrane-bound proteins that are responsible for carbohydrate transport, although other alcohols, such as butanol, appear to have a more general chaotropic effect [14]. At this time there is no clear understanding why xylose metabolism is so adversely affected by ethanol.

TABLE 2

Growth of *Lactobacillus* strains in 10% to 16% ethanol after 48 h

Strain	Substrate	% Ethanol			
		10	12	14	16
L. amylovorus					
NRRL B-4538	cellobiose	1.08	0.39	0.23	NG
	glucose	0.27	NG[a]	NG	NG
	starch	1.07	0.37	0.23	0.21
NRRL B-4540	cellobiose	0.28	0.21	0.20	NG
	glucose	0.27	0.26	0.23	NG
	starch	1.15	0.27	0.22	0.22
NRRL B-4542	cellobiose	0.27	0.20	NG	NG
	glucose	0.83	0.48	0.34	0.27
	starch	1.05	0.54	0.21	NG
NRRL B-4549	cellobiose	0.27	NG	NG	NG
	glucose	0.22	NG	NG	NG
	starch	0.45	0.20	NG	NG
L. brevis					
NRRL B-1127	xylose	0.35	0.20	NG	NG
IFO 3960	cellobiose	1.16	0.45	0.27	NG
	glucose	0.80	NG	NG	NG
	lactose	0.34	0.30	NG	NG
	starch	1.12	0.64	0.43	0.23
IFO 12005	cellobiose	1.16	0.45	0.27	NG
	glucose	2.27	1.08	0.63	0.50
	lactose	2.76	1.62	0.22	NG
	starch	1.57	1.02	0.59	0.46
	xylose	0.27	NG	NG	NG
IFO 12520	cellobiose	1.76	0.51	0.32	0.20
	glucose	3.21	1.10	0.76	0.47
	lactose	2.76	1.62	0.22	NG
	starch	1.73	0.81	0.54	0.35
IFO 13109	glucose	0.98	0.23	NG	NG
IFO 13110	cellobiose	0.38	0.47	0.31	0.23
	glucose	1.45	0.90	0.56	0.40
	lactose	1.51	0.56	0.20	NG
IFO 27305	cellobiose	1.87	0.44	0.24	NG
	glucose	1.84	0.86	0.51	0.31
	lactose	2.00	1.90	0.23	0.20
	starch	1.39	0.50	0.40	0.39
	xylose	1.56	0.32	0.24	NG
L. casei					
UNL 685	glucose	0.43	0.33	0.30	0.27
	lactose	0.30	0.20	NG	NG
UNL 686	cellobiose	1.05	0.48	0.26	NG
	glucose	0.70	0.41	0.33	0.31

(Table continued on following page)

TABLE 2 *(continued)*

Growth of *Lactobacillus* strains in 10% to 16% ethanol after 48 h

Strain	Substrate	% Ethanol			
		10	12	14	16
UNL 3532	cellobiose	1.68	0.45	0.21	NG
	glucose	0.75	0.48	0.38	0.31
	lactose	0.54	0.39	NG	NG
IFO 3831	cellobiose	0.95	0.37	0.20	NG
	glucose	2.17	0.99	0.54	0.30
	lactose	1.70	0.65	0.27	NG
	starch	1.56	0.50	0.25	0.22
IFO 3953	glucose	1.76	1.46	1.15	0.28
	lactose	1.36	0.41	NG	NG
IFO 12004	cellobiose	2.30	0.95	0.49	0.29
	glucose	2.38	1.58	0.70	0.48
	lactose	1.87	0.97	0.34	NG
L. confusus					
ATCC 27646	glucose	1.66	0.83	0.50	0.40
	lactose	1.90	0.75	0.31	NG
L. delbrueckii					
NRRL B-1042	cellobiose	1.89	0.47	0.20	NG
	glucose	0.85	0.53	0.26	0.25
	starch	1.67	0.55	0.31	0.25
L. fermentum					
IFO 3956	cellobiose	1.59	0.37	NG	NG
	glucose	1.84	0.87	0.42	0.24
IFO 3959	cellobiose	2.16	0.80	0.39	0.22
	lactose	0.82	0.38	0.22	NG
	xylose	0.20	NG	NG	NG
L. hilgardii					
ATCC 8290	glucose	0.38	0.36	0.31	0.22
	xylose	2.72	1.38	0.35	0.21
L. sake					
IFO 3541	cellobiose	1.46	0.46	0.23	NG
	glucose	2.56	0.90	0.33	0.20
	starch	2.12	1.18	0.52	0.29
L. pentosus					
ATCC 8041	cellobiose	0.23	NG	NG	NG
	starch	0.37	0.28	0.23	0.20
L. plantarum					
NRRL 1195	cellobiose	1.77	0.92	0.26	NG
	glucose	2.02	0.67	0.64	0.29
	starch	1.72	0.69	0.52	0.31
IFO 3074	cellobiose	0.54	0.29	0.25	NG

(Table continued on opposite page)

TABLE 2 *(continued)*

Growth of *Lactobacillus* strains in 10% to 16% ethanol after 48 h

Strain	Substrate	% Ethanol			
		10	12	14	16
	glucose	0.43	0.34	0.27	0.25
	lactose	0.73	0.57	0.35	0.24
	starch	2.70	0.70	0.52	0.37
IFO 12006	cellobiose	1.94	0.93	0.46	0.26
	glucose	2.27	1.11	0.58	0.47
	lactose	2.01	0.90	0.22	NG
	starch	1.62	0.72	0.50	0.25
IFO 12011	glucose	2.21	1.15	0.66	0.40
	lactose	1.10	0.73	NG	NG
	starch	1.55	0.35	0.20	NG

[1] No growth observed.

TABLE 3

The molar product ratio of lactic acid/ethanol/acetic acid of *Lactobacillus* strains on different carbohydrates standardized to lactic acid

Strain	Glucose	Xylose	Lactose	Cellobiose	Starch
L. amylovorus					
NRRL B-4538	1:0.39:0.06 (0.96)[1]	ND[2]	ND	1:0.04:0.11 (0.91)	1:0.22:0.05 (0.96)
NRRL B-4540	1:0.70:0.02 (0.99)	ND	ND	1:0.07:0.11 (0.91)	1:0.68:0.08 (0.95)
NRRL B-4542	1:0.41:0.02 (0.99)	ND	ND	1:0.05:0.07 (0.93)	1:0.23:0.1 (0.93)
NRRL B-4549	1:1.23:0.03 (0.99)	ND	ND	1:0.03:0.02 (0.98)	1:0.27:0.03 (0.98)
L. brevis					
NRRL B-1127	ND	1:0.05:0.99 (0.52)	ND	ND	ND
IFO 3960	1:1.25:0.1 (0.96)	1:0.02:0.28 (0.79)	1:0.74:0.31 (0.85)	1:0.15:0.44 (0.73)	1:0.57:0.38 (0.80)
IFO 12005	1:0.52:0.2 (0.88)	1:0.05:1.15 (0.48)	ND	ND	1:2.9:1.94 (0.67)
IFO 12520	1:0.52:0.2 (0.88)	1:0.05:0.94 (0.53)	1:0.55:0.23 (0.87)	ND	1:0.69:1.83 (0.48)
IFO 13109	1:0.98:0.08 (0.96)	1:0.04:1.0 (0.51)	ND	ND	ND
IFO 13110	1:1.15:0.14 (0.94)	1:0.09:1.78 (0.38)	1:1.09:0.21 (0.91)	1:0.12:0.66 (0.63)	1:1.85:0.94 (0.75)
IFO 27305	1:0.76:0.32 (0.85)	1:0.05:0.87 (0.55)	1:1.1:0.15 (0.93)	1:0.08:0.56 (0.66)	1:1.53:5.0 (0.34)
L. casei					
UNL 685	1:1.9:0.2 (0.94)	1:0.04:0.87 (0.54)	ND	ND	ND
UNL 686	1:1.27:0.05 (0.98)	1:0.07:1.07 (0.50)	ND	ND	ND

(Table continued on following page)

TABLE 3 *(continued)*

The molar product ratio of lactic acid/ethanol/acetic acid of *Lactobacillus* strains on different carbohydrates standardized to lactic acid

Strain	Glucose	Xylose	Lactose	Cellobiose	Starch
UNL 3532	1:1.02:0.09 (0.96)	1:0:2.82 (0.26)	1:0.29:1.04 (0.55)	1:0.17:2.69 (0.30)	ND
IFO 3831	1:0.92:0.06 (0.97)	1:0.07:1.15 (0.48)	ND	ND	1:12.26:4.02 (0.77)
IFO 3953	1:0.54:0.09 (0.95)	1:0.05:1.56 (0.40)	1:0.93:0.27 (0.88)	1:0.1:0.66 (0.63)	ND
IFO 12004	1:0.7:0.1 (0.94)	1:0.06:1.18 (0.47)	1:0.69:1.01 (0.63)	1:0.13:0.53 (0.68)	ND
L. confusus					
NRRL B-1064	1:1.5:0.14 (0.95)	ND	ND	ND	ND
ATCC 27646	1:1.38:0.11 (0.96)	1:0.05:1.04 (0.50)	1:1.4:0.23 (0.91)	ND	ND
L. delbrueckii					
NRRL B-1042	1:0.82:0.06 (0.97)	ND	ND	ND	1:4.85:7.82 (0.43)
L. fermentum					
IFO 3956	1:0.27:0.16 (0.89)	1:0.06:1.18 (0.47)	1:1.11:0.4 (0.84)	1:0.06:0.34 (0.76)	1:0.18:0.33 (0.78)
IFO 3959	1:1.11:0.3 (0.88)	1:0.05:1.0 (0.51)	1:0.65:0.34 (0.83)	1:0.06:0.42 (0.72)	ND
L. hilgardii					
ATCC 8290	1:1.73:0.34 (0.89)	ND	ND	ND	ND
L. sake					
IFO 3541	1:0.44:0.38 (0.79)	1:0.03:0.63 (0.62)	1:0.87:1.63 (0.54)	1:0.03:0.79 (0.56)	1:0.55:0.34 (0.82)
L. pentosus					
ATCC 8041	1:0.64:0.14 (0.92)	1:0.07:1.01 (0.51)	ND	ND	ND
L. plantarum					
NRRL 1195	1:0.29:0.09 (0.94)	ND	1:0.68:0.47 (0.78)	1:0.2:4.1 (0.23)	1:0.32:0.66 (0.67)
IFO 3074	1:1.2:0.7 (0.76)	1:0.24:1.45 (0.46)	1:1.02:0.73 (0.74)	ND	ND
IFO 12006	1:1.27:0.22 (0.91)	1:0.07:1.02 (0.51)	1:0.20:0.16 (0.88)	1:0.23:0.62 (0.66)	1:0.29:0.33 (0.80)
IFO 12011	1:1.27:0.15 (0.94)	1:0.06:1.1 (0.49)	1:1.36:0.16 (0.94)	ND	1:5.71:1.98 (0.77)

[1] The molar ratios of (lactic acid + ethanol)/(lactic acid + ethanol + acetic acid).

[2] ND, not determined.

Metabolism

Lactobacilli species can be either homofermentative, producing only lactate from glucose via the Embden-Meyerhof-Parnas pathway, or heterofermentative, producing lactate, ethanol, acetate, and CO_2 via the pentose phosphate pathway. Pentoses are metabolized heterofermentatively, and acetate production is energetically favored over ethanol, since conversion to lactate and acetate results in net 2 ATP/xylose vs. 1 ATP/xylose for lactate and ethanol [12,13].

The objective of this work was to screen *Lactobacillus* sp. for the ability to ferment certain carbohydrates in the presence of high concentrations of ethanol and to select suitable candidates for molecular cloning of the PET genes from *Z. mobilis* into *Lactobacillus*. For pyruvate decarboxylase (PDC) and aldehyde dehydrogenase (ADH) to produce ethanol there must be a pool of pyruvate, and PDC will have to out-compete lactate dehydrogenase (LDH), which converts pyruvate to lactate. The K_m values of LDH on pyruvate from lactobacilli vary from 0.37 to 10 mM [11], while the K_m of PDC from *Z. mobilis* is 0.4 mM [3], indicating that PDC has the potential to out-compete LDH for pyruvate.

Each of the strains that grew well in ethanol were analyzed for endproducts in their respective carbohydrate at 0% ethanol. The results are presented in Table 3 as molar ratios of lactic acid/ethanol/acetic acid with values standardized to 1 mol of lactic acid. Also in parentheses is the molar ratio (lactic acid + ethanol)/(lactic acid + ethanol + acetic acid), which is a ratio of the metabolites that are or can be converted to ethanol to the total amount of metabolites produced from a given substrate. The ratio is an indication of the maximum potential of the host to convert a substrate to ethanol. The preferred host for the PET genes would be homofermentative, but another acceptable host could be heterofermentative as long as the final products of fermentation are lactic acid and ethanol.

The media that was used to grow the strains for product analysis was devoid of acetate, since this interfered with sample analysis. Although the extent of growth was not the same as with acetate because of the lost buffering capacity, the product ratio is indicative of the strain's metabolism. The criteria for a good host is defined as 90% conversion of the substrate to lactic acid and/or ethanol. There were a total of 21 strains that met this criteria; 14 of those strains met the criteria on glucose, four on cellobiose (*L. amylovorus* B-4549, B-4540, B-4542, B-4538), three on lactose (*L. brevis* IFO sp. 13110, *L. plantarum* IFO sp. 12011, and *L. confusus* ATCC 27646), and four on starch (*L. amylovorus* B-4549, B-4540, B-4542, and B-4538). There were only seven strains that met the criteria and were able to metabolize a substrate other than glucose. There were no strains that met the criteria for xylose.

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