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ORIGINAL PAPER

Physiological and fermentation properties of *Bacillus coagulans* and a mutant lacking fermentative lactate dehydrogenase activity

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Abstract *Bacillus coagulans*, a sporogenic lactic acid bacterium, grows optimally at 50–55°C and produces lactic acid as the primary fermentation product from both hexoses and pentoses. The amount of fungal cellulases required for simultaneous saccharification and fermentation (SSF) at 55°C was previously reported to be three to four times lower than for SSF at the optimum growth temperature for *Saccharomyces cerevisiae* of 35°C. An ethanologenic *B. coagulans* is expected to lower the cellulase loading and production cost of cellulosic ethanol due to SSF at 55°C. As a first step towards developing *B. coagulans* as an ethanologenic microbial biocatalyst, activity of the primary fermentation enzyme L-lactate dehydrogenase was removed by mutation (strain Suy27). Strain Suy27 produced ethanol as the main fermentation product from glucose during growth at pH 7.0 (0.33 g ethanol per g glucose fermented). Pyruvate dehydrogenase (PDH) and alcohol dehydrogenase (ADH) acting in series contributed to about 55% of the ethanol produced by this mutant while pyruvate formate lyase and ADH were responsible for the remainder. Due to the absence of PDH activity in *B. coagulans* during fermentative growth at pH 5.0, the *l-ldh* mutant failed to grow anaerobically at pH 5.0. Strain Suy27-13, a derivative of the *l-ldh* mutant strain Suy27, that produced PDH activity during anaerobic growth at pH 5.0 grew at this pH and also produced ethanol as the fermentation product (0.39 g per g glucose). These results show that construction of an ethanologenic *B. coagulans* requires optimal expression of PDH activity in addition to the removal of the LDH activity to support growth and ethanol production.

Keywords *Bacillus coagulans* · *ldh* mutant · Ethanol · Pyruvate dehydrogenase · Fermentation

Introduction

With the finite nature of the known petroleum reserves and the ever-increasing demand for energy due to a growing world population, alternate sources of energy are becoming critical needs for humankind. Renewable sources of energy are expected to play an increasing role in the future [5]. Due to the existing transportation infrastructure, need for liquid transportation fuels that can lower the demand for petroleum is increasingly apparent. At present, ethanol produced by fermentation from corn starch plays an important role in meeting this demand [1]. With corn starch and other edible carbohydrates as feedstocks for ethanol production, the issue of food vs. fuel and potential increase in food prices that can lead to hunger in the poor nations are major concerns [33]. Lignocellulosic biomass, a renewable non-food source of carbohydrate has the potential to contribute a significant part of the transportation fuel demand in a sustainable manner provided an economical process for conversion of the carbohydrates in biomass to ethanol (cellulosic ethanol) can be developed [5, 21, 37]. The biochemical process that is being envisioned for conversion of the sugars in biomass requires separation of the major carbohydrates followed by hydrolysis of the glycan by enzymes and/or dilute acid to release the sugars for fermentation by microbes. The projected cost

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of enzymes for hydrolysis of the cellulose in biomass in this process is reported to be about $0.50 per gallon of cellulosic ethanol produced at an industrial scale amounting to about 25% of the projected cost of ethanol production [19]. This is about ten times or higher than the cost of enzymes required for production of a gallon of ethanol from corn starch [10]. To reduce the overall cost of cellulosic ethanol production and make it competitive with petroleum-based liquid fuels, it is imperative that the cost of enzymes in the biochemical process is significantly reduced [7].

Fungal cellulases have a temperature optimum of about 50–55°C while *Saccharomyces cerevisiae*, favored by the industry for fermentation of glucose to ethanol, has an optimum temperature for growth and fermentation of less than 35°C [1, 22]. Simultaneous saccharification and fermentation (SSF) of cellulose to ethanol is probably a cost-effective process that requires rapid fermentation of glucose released by saccharification of cellulose to glucose by the enzymes [12]. The SSF process requires that the enzymes and the microbe operate in unison. However, due to the differences in the optimum conditions for yeast and enzyme activity, the process is normally carried out at temperatures that are closer to that of the yeast optimum than that of the enzymes contributing to the significant cost due to higher enzyme loading. We have previously demonstrated that the amount of cellulases required for SSF of cellulose to products can be reduced by three and four-fold by raising the temperature of the process to 55°C, compared to the requirement at 35°C [23]. SSF at 55°C requires a thermotolerant microbial biocatalyst that can ferment sugars to ethanol as the major product. However, a thermotolerant/thermophilic microbial biocatalyst that can ferment all the sugars in biomass to ethanol is yet to be isolated from nature. To circumvent this limitation, thermotolerant bacteria are being engineered to produce ethanol as the main product [3, 31]. An alternative approach is to isolate/engineer microbial biocatalysts that can produce cellulases to overcome the need for externally supplied expensive fungal cellulases [6]. Both these approaches are yet to yield a microbial biocatalyst for industrial scale ethanol production from biomass.

Bacillus coagulans is a sporogenic lactic acid bacterium that optimally grows at 50–55°C [4, 25]. *B. coagulans* ferments various sugars in lignocellulosic biomass to L(+)-lactic acid as the primary product. *B. coagulans* is also a potential probiotic bacterium [9]. *B. coagulans* is one of the few members of the *Bacillus* group that can grow to high cell density under anaerobic condition. Due to its ability to grow anaerobically and at temperatures that are higher than the optima for lactic acid bacteria, *B. coagulans* is attracting attention as a potential bacterial biocatalyst for industrial production of optically pure lactic acid [26, 29] as well as other products, such as ethanol, after metabolic engineering.

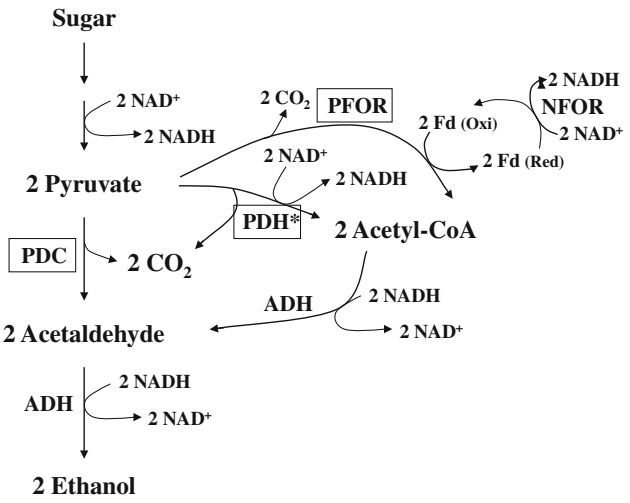

Fig. 1 Metabolic pathways that can lead to production of ethanol as the major fermentation product in microorganisms. The pyruvate decarboxylase (*PDC*) and alcohol dehydrogenase (*ADH*) pathway is the natural pathway contributing to ethanol production. The other two pathways can be constructed by modification of the enzymes such as pyruvate dehydrogenase complex (*PDH**) in facultative bacteria or by deleting the competing fermentation pathways in anaerobic bacteria that can channel the pyruvate through the pyruvate ferredoxin oxidoreductase (*PFOR*) to acetyl-CoA for further reduction to ethanol by ADH. NADH ferredoxin oxidoreductase (*NFOR*) converts the reductant in reduced ferredoxin to NADH, a substrate for ADH. *Fd* ferredoxin. The enzymes, PDC, PDH and PFOR/NFOR are the critical enzymes in the construction of homoethanologenic microbial biocatalysts

All naturally ethanol-producing microbes utilize pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) to convert pyruvic acid produced by glycolysis to ethanol (Fig. 1). This pathway has been successfully transferred to other bacteria for high rates of ethanol production from biomass-derived sugars [14, 16]. However, engineering thermotolerant microbes for ethanol production with this pathway is hampered by limitation in high-level production of the active protein combined with thermostability of the pyruvate decarboxylase at the required high temperature. Two other alternate pathways for ethanol production have been developed that utilize native genes; pyruvate dehydrogenase (PDH) and ADH pathway and pyruvate ferredoxin oxidoreductase (PFOR), NADH ferredoxin oxidoreductase (NFOR) and ADH pathway (Fig. 1) [17, 18, 31, 39]. These two pathways are mutually exclusive in nature; the PDH/ADH pathway can be developed in facultative anaerobes while the PFOR/NFOR/ADH pathway is exclusive for strict anaerobes. Engineering microbes for ethanol production by either of the alternate native pathways requires that competing fermentation pathways are deleted to divert all the pyruvate to ethanol production. An understanding of the metabolic control at the pyruvate node in such a microbial biocatalyst

is critical for success of such a metabolic engineering for cost-effective ethanol production from biomass-derived sugars [36].

In this communication, we have focused on *B. coagulans* as a thermotolerant microbial biocatalyst and evaluated the aerobic and anaerobic growth and fermentation characteristics of the wild-type strain 36D1 and an *ldh* mutant derivative as well as the critical enzymes at the pyruvate node during a shift to oxygen limitation and anaerobiosis as a prelude to engineering this bacterium for ethanol production.

Materials and methods

Materials

Biochemicals were acquired from Sigma Chemical Co. (St. Louis, MO) and organic and inorganic chemicals were from Fisher Scientific (Pittsburgh, PA). Molecular biology reagents and supplies were from New England Biolabs (Ipswich, MA), Invitrogen, or Bio-Rad Laboratories.

Bacteria, medium, and growth condition

Bacillus coagulans strain 36D1 used in this study was described previously [25]. Strain 36D1 and its *ldh* mutants were grown in L-broth (LB) [20] at 50°C and pH 5.0 or 7.0, as needed. Aerobic cultures were grown in a shaker at 200 RPM. Glucose or xylose was sterilized separately and added to the medium at the indicated concentration before inoculation. Rifampicin concentration in LB medium was 50 mg/l.

Fermentation

Fermentations were carried out in 250 ml of LB with sugar in 500-ml vessels as described previously [24]. Inoculum for these cultures was grown in the same medium aerobically at 50°C to mid-exponential phase of growth. Inoculum volume in all fermentations was 1% of the culture volume. Culture pH was maintained by automatic addition of 2N KOH. Samples were removed periodically for determination of fermentation products and sugar concentration.

Fermentations were also carried out in 1.5 l of LB with glucose in a 3.0-l vessel of a BioFlo 110 fermenter (New Brunswick Scientific, New Brunswick, NJ). Inoculum was prepared as described above and the fermentations were started with 1% (v/v) inoculum. Although the cultures started growth aerobically, dissolved oxygen in the broth decreased to below the detection limit for the probe before the culture density reached an OD of about 0.5 (420 nm; DU640 spectrophotometer, Beckman, Brea, CA).

Isolation of an *ldh* mutant

For isolation of an *ldh* mutant, *B. coagulans* strain 36D1 was mutagenized with ethyl methane sulfonate (EMS) using a procedure described previously with modifications [30]. Ten ml of LB (pH 5.0) in a 125-ml flask was inoculated with 0.1 ml of an overnight culture grown without shaking in LB (pH 5.0; 50°C). The culture was grown in a shaker at 50°C (200 RPM) until it reached early exponential phase of growth (OD 420 nm of about 0.4). To the 10-ml culture, 0.1 ml of EMS was added and incubation continued in the shaker for another 30 min. Mutagenized cells were collected by centrifugation at room temperature (1,700 × *g*; 15 min) and washed twice with same volume of LB, pH 5.0. Cells were resuspended in 10 ml of LB, pH 5.0 and incubated at 48°C for 40 min without shaking. During this mutagenesis, the cell number increased by twofold. After the 48°C incubation, cells were collected by centrifugation at room temperature and resuspended in 10 ml of LB, pH 5.0. The cells were incubated at 50°C in a shaker for 2 h for segregation of mutation(s). Cells were harvested by centrifugation at room temperature and stored at −75°C in 20% (v/v) glycerol in LB, pH 5.0. This mutagenesis protocol increased rifampicin resistance of *B. coagulans* by about 40-fold to 1.2×10^{-6}.

The mutagenized culture was serially diluted and plated on LB-agar medium that was supplemented with glucose (2%, w/v) and overlayed with 2.5 ml of $CaCO_3$ agar (solid $CaCO_3$ suspended in water (1% w/v) with 1.5% agar). These $CaCO_3$ overlay plates with mutagenized culture were incubated at 50°C in an anaerobic jar with GasPak for 2 days. Colonies producing relatively smaller zone of clearing of $CaCO_3$ compared to the parent strain were selected and their fermentation profiles were determined in batch fermentations using LB + glucose (1%, w/v). One mutant that produced very low level of lactate was selected for further study (strain Suy27).

Enzyme assay

To determine the level of PDH and LDH activity, cells were cultured in LB until the culture reached mid-exponential phase of growth. Cells were harvested by centrifugation (10,000 × *g*, 10 min; room temperature), washed once with 10 ml of phosphate buffer (60 mM, pH 7.5) and resuspended in 5.0 ml of same phosphate buffer. Cells were broken by passage through a French pressure cell (20,000 PSI). All operations after this step were at 4°C. The cell extract was centrifuged at 12,000 × *g* for 15 min to remove the cell debris and the supernatant was centrifuged again at 100,000 × *g* for 1 h to remove large particulates and membrane vesicles. Supernatant was used for enzyme assay.

PDH activity was assayed as described previously [35]. A standard assay was based on pyruvate-dependent reduction of NAD^+ at 340 nm ($\varepsilon = 6{,}220\ M^{-1}\ cm^{-1}$) at 50°C. Each 1 ml of reaction mixture contained thiamine pyrophosphate (0.4 mM), CoA (0.13 mM), $MgCl_2\ 6H_2O$ (2 mM), dithiothreitol (2.6 mM), NAD^+ (0.73 mM) and crude extract in 60 mM potassium phosphate buffer (pH 7.5). The reaction was started by addition of pyruvate (5 mM). One unit of enzyme activity is expressed as 1 μmole of NADH produced min^{-1} mg $protein^{-1}$.

LDH activity was assayed as described previously [38] as the oxidation of NADH in the presence of pyruvate. Each 1 ml of reaction mixture contained NADH (0.1 mM) and crude extract in 60 mM potassium phosphate buffer (pH 6.0). The reaction was started by addition of pyruvate (0.1 mM). One unit of enzyme activity is expressed as 1 μmole of NADH oxidized min^{-1} mg $protein^{-1}$.

Quantitative RT-PCR

For determination of the level of various mRNA coding for the enzymes at the pyruvate node, *Bacillus coagulans* strains were harvested from LB + glucose (3%) fermentations at 50°C. Cells were collected by centrifugation (16,000 × *g*; 30 s, room temperature) and resuspended in 0.1 ml of phenol and 0.1 ml of lysis buffer (SDS, 1%; sodium acetate, 30 mM; EDTA, 3 mM). After the addition of 60 mg of lysing matrix (glass beads; MP Biochemicals), the cells were disrupted by vortex (Fastprep-24®, MP Biomedicals). RNA was isolated using the phenol extraction method described before [32]. Contaminating genomic DNA was removed from RNA after hydrolysis with DNase I (Ambion) and RNA was purified using Qiagen RNeasy Mini Kit. RNA concentration was determined from the absorbance at 260 nm (NanoVue, GE). Using appropriate gene-specific primers, a cDNA copy was prepared with Superscript III reverse transcriptase (Invitrogen) from a starting concentration of 1 μg of total RNA. The cDNA was amplified using SYBR-green containing PCR reaction mix (Bio-Rad Laboratories, Hercules, CA). The threshold cycle for each of the PCR reaction with different concentrations of cDNA was determined and compared against a standard DNA that was also run at the same time [18]. From these results, a ratio of the concentration of gene-specific mRNA present in the sample was calculated. Reported results are the average of at least three experiments.

The primers used for RT–PCR are listed below. The *ldh* primers (GGTGTTGCAGAAGAGCTTGT and GTGCCG CAATCGGAATAATC) amplified a 137-bp region starting at +79 from the translation start site. The *pdhA* (E1α) primers (CCCGCCGCAAATCATTATCG and TAAAAG CACCCGCAAAGTT) amplified a 161-bp region starting at +414 from the translation start site. The *pdhD* primers (GGAAAACGGCGTAGTTGTCA and GCCCCTGTCTG TCATCTTTA) amplified a 145-bp region starting at +741 from the translation start site. The *polA* primers (TTG GAGGCGAACAAAGAACA and CGGCAATGGAAAAA GAAATG) amplified a 229-bp DNA between +685 and +913 from the translation start site.

Analytical methods

Protein concentration was determined by Bradford method with bovine serum albumin as standard [2]. Sugars and fermentation products were determined by HPLC as described previously [34]. DNA sequence was determined using the DNA sequencing core facility at the Interdisciplinary Center for Biotechnology Research at the University of Florida with custom-made primers. DNA primers were synthesized by Invitrogen (Carlsbad, CA).

Results and discussion

Fermentation profile of parent strain 36D1

B. coagulans strain 36D1 is a sporogenic lactic acid bacterium that produces lactate as the primary fermentation product (Table 1) irrespective of the growth pH [25]. Although small amounts of acetate, formate, and ethanol can be detected in the fermentation broth of strain 36D1 cultured at pH 7.0, contribution from the PFL pathway to overall fermentation products is less than 10%. However, when the pH of the fermentation was lowered to 5.0, lactate accounted for more than 95% of the fermentation products produced from glucose. The lactic acid produced by strain 36D1 is L(+)-lactic acid irrespective of the pH of the culture [25]. Although a lactate dehydrogenase that catalyzes the production of D(−)-lactic acid has been identified and the corresponding gene cloned and expressed in *E. coli* (data not presented), contribution of this enzyme to the total lactic acid produced appears to be minimal, if any, in strain 36D1 fermentation. These results show that in order to construct an ethanologenic mutant derivative of *B. coagulans*, the *l-ldh* gene encoding L(+)-lactate dehydrogenase needs to be deleted to divert pyruvate to ethanol pathways.

Isolation of an *l-ldh* mutant

Strain 36D1 was mutated with EMS and a set of mutants producing less acid than the parent was isolated using $CaCO_3$ overlay medium. One of the mutants, strain Suy27, produced a very low level of lactic acid during glucose fermentation in rich medium (Table 1). The *l-ldh* gene

Table 1 Fermentation profiles of *ldh* mutants of *B. coagulans*[a]

Strain	Culture pH	Cell yield[b] (g/l)	Glucose consumed (mM)	Product (mM)					Lactate fraction[c]	Ethanol fraction[c]	Ethanol yield[d]
				Lactate	Formate	Acetate	2,3-butanediol	Ethanol			
36D1	7.0	2.30	120	207	25	14	UD	32	0.82	0.13	0.13
36D1	7.0	2.69	268	381	42	10	UD	82	0.81	0.17	0.15
Suy27	7.0	2.34	116	2	112	24	41	146	0.01	0.57	0.63
Suy27-13	7.0	2.73	124	2	166	74	4	191	0.01	0.69	0.77
Suy27-13	7.0	3.16	272	13	196	44	17	412	0.03	0.82	0.76
Suy27-13	5.0	1.40	102	3	40	10	35	105	0.02	0.56	0.51
Suy27-13	5.0	2.15	179	9	36	6	83	155	0.03	0.46	0.43
36D1	5.0	1.44	150	266	UD	UD	UD	UD	1.00	0.00	0.00
36D1	5.0	1.54	288	530	UD	UD	UD	26	0.95	0.05	0.05

UD undetectable (less than 0.5 mM)

[a] Batch fermentations with pH control were conducted in LB with glucose and the product profile at the end of fermentation when all the sugar was consumed is presented. Strain Suy27-13 grown at pH 5.0, did not completely ferment all the glucose in the medium when the concentration was 50 g l^{-1}. Results are from a typical experiment after the isolation of the mutants

[b] The highest cell yield of the culture in dry weight

[c] Fraction of lactate or ethanol among the total fermentation products (minus formate; 2× of 2,3-butanediol). This value provides the fraction of glucose-derived pyruvate that is converted to lactate or ethanol

[d] Mole fraction of consumed glucose recovered as ethanol

from this strain and the parent, strain 36D1, were amplified by PCR and the PCR products were sequenced. The DNA sequence revealed that the *l-ldh* gene of strain Suy27 carries an alteration of guanine to adenine at position 142 (starting with A in ATG as position 1). This alteration of G142A resulted in an amino acid change of aspartate to asparagine at position 48 of the L-LDH protein (D48N). Absence of lactate in the fermentation broth in strain Suy27 indicates that the D48N mutation abolished the L-LDH activity and aspartate at this position is critical for activity. This is confirmed by the lack of LDH activity in the crude extract of strain Suy27 grown in LB + glucose in fermenters. Aspartate at this position was also found to be conserved in L-LDH from various bacteria, such as, *Geobacillus stearothermophilus, Lactobacillus pentosus, Thermatoga maritima* as well as in humans.

Strain Suy27 grew aerobically at rates that are comparable to the parent strain 36D1. In fermentations of glucose in LB medium at pH 7.0 strain Suy27 had several hours of lag before growth started. The growth rate of strain Suy27 was also lower than the parent although the final cell density was comparable to the parent. In these fermentations, ethanol was the major product (about 60% of the products) and lactate was present only in a trace amount (Table 1). Formate, acetate, and 2,3-butanediol were the other products of fermentation of glucose. Formate is produced by the enzyme pyruvate formate lyase (PFL). In order to maintain redox balance, the PFL-produced acetyl-CoA needs to be converted to acetate and ethanol in equi-molar amounts. The six-times higher ethanol yield compared to acetate suggests the availability of NADH from another pathway to reduce the PFL-generated acetyl-CoA to ethanol (Fig. 2). Strain Suy27 also produced 2,3-butanediol that is not detected in the parent strain in any of the growth conditions. Production of 2,3-butanediol by the acetolactate, acetoin pathway without diacetyl as an intermediate would consume only one NADH per two pyruvates. This would leave an extra NADH per 2,3-butanediol produced and this NADH could be used in the reduction of acetyl-CoA to ethanol. Combination of the 2,3-butanediol pathway and the PFL/ADH pathway can partly account for the observed ethanol-to-acetate ratio. The PDH/ADH pathway discussed above (Fig. 1) can also contribute to the yield of ethanol. Analysis of fermentation profiles of strain Suy27 suggests that about 55% of the ethanol may be derived from the PDH/ADH pathway and the remainder from the PFL/ADH pathway. These results show that the *l-ldh* mutant strain Suy27 has the ability to produce ethanol as a major fermentation product when cultured at pH 7.0.

However, in pH 5.0 fermentations of glucose, growth of strain Suy27 was very poor and led to selection of LDH-plus revertants. The *l-ldh* gene from three of the revertants were PCR-amplified and sequenced. The DNA sequence of *l-ldh* in all three revertants revealed that the G142A mutation reverted back to G. In an attempt to isolate a stable *l-ldh* mutant, strain Suy27 was mutagenized again with EMS and mutants which produced smaller zone of clearing in $CaCO_3$ overlay medium were selected. One such strain, Suy27-13, was selected for further study.

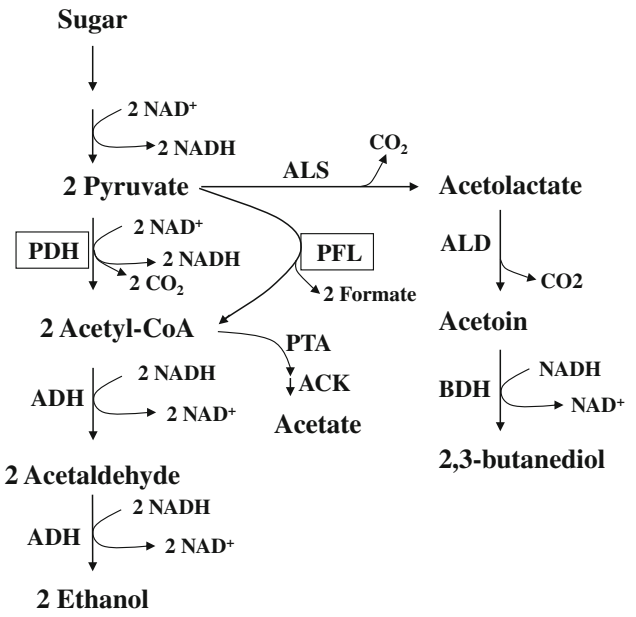

Fig. 2 Fermentation pathways of *B. coagulans l-ldh* mutants. *PDH* pyruvate dehydrogenase complex, *ADH* alcohol dehydrogenase, *PFL* pyruvate formate lyase, *PTA* phosphotransacetylase, *ACK* acetate kinase, *ALS* acetolactate synthase, *ALD* acetolactate decarboxylase, *BDH* 2,3-butanediol dehydrogenase

Properties of strain Suy27-13

During fermentation of glucose, at pH 7.0, strain Suy27-13 converted about 70–80% of the pyruvate to ethanol at an ethanol yield from glucose of about 75% (0.39 g ethanol per gram glucose fermented) (Table 1; Fig. 3a). Strain Suy27-13 fermented 50 g l^{-1} in about 3 days. As the case with strain Suy27, PDH/ADH pathway contributed to about 55% of the ethanol produced by strain Suy27-13. In contrast to strain Suy27, strain Suy27-13 grew in pH 5.0 fermentations and produced ethanol as a major fermentation product (Fig. 3b). With an increase in glucose concentration, 2,3-butanediol level in the broth also increased. However, the yield of ethanol from glucose was ≤50% at pH 5.0 compared to over 75% in pH 7.0 fermentations. PDH/ADH pathway is also a significant contributor of this ethanol in pH 5.0 fermentations.

The *l-ldh* structural gene of strain Suy27-13 still contained only the G142A mutation and no mutation was detected in the promoter region of *l-ldh*. DNA sequence did not reveal a mutation in the *pdhD* (*lpd*) gene or in the promoter region of the *pdh* operon that could increase the activity of PDH complex in the anaerobic cell as seen in ethanologenic *E. coli* strains [17, 18]. An *fnr* homolog of strain Suy27-13 that could influence anaerobic growth [15, 27] was also found to be unaltered. The apparent mutation that increased the growth of Suy27-13 in pH 5.0 fermentations is yet to be identified.

Anaerobic culture of strain Suy27-13 produced PDH

Although the presence of PFL activity in various fermentations can be confirmed by the production of formate, the role of PDH in ethanol production could not be directly identified. The ethanol concentration over and above the formate level in the broth is an indication of an alternate pathway responsible for ethanol production as seen with *E. coli* strains [17, 18]. To confirm the presence of PDH activity in strain Suy27-13 during fermentation, especially at pH 5.0, cells were collected during mid-exponential phase of growth and the level of PDH activity in the crude extracts was determined. Crude extracts of wild-type strain 36D1 from pH 5.0 fermentations (LB + glucose) had no detectable PDH activity while strain 27-13 had about 0.07 unit of PDH activity. The PDH activity of strains 36D1 and 27-13 grown in LB with aeration were 0.03 and 0.045 unit, respectively. These results show that the *l-ldh* mutant, strain Suy27-13, produced PDH activity during anaerobic growth in support of the contribution of PDH/ADH pathway in ethanol production in strain 27-13 (Fig. 1). The unidentified second mutation in strain Suy27-13 apparently

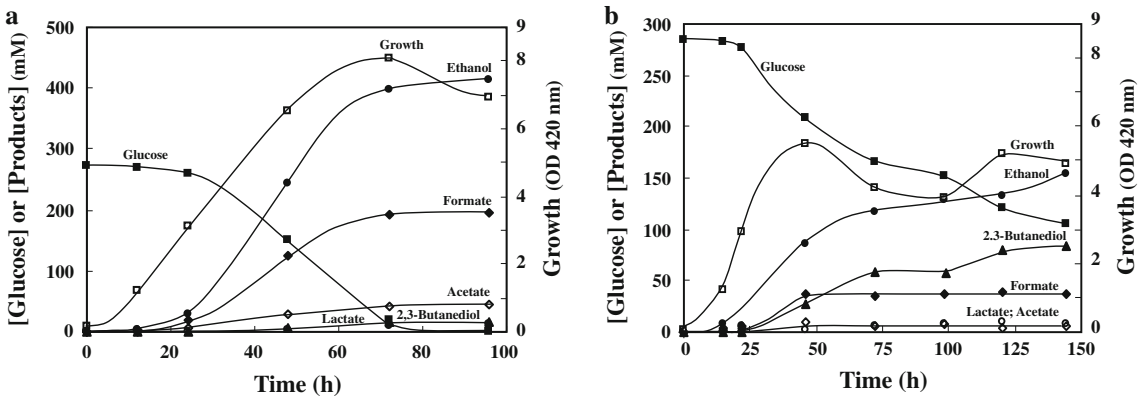

Fig. 3 Growth and fermentation profile of *B. coagulans l-ldh* mutant, strain Suy27-13 at 50°C in LB + glucose (50 g l^{-1}). **a** pH 7.0; **b** pH 5.0

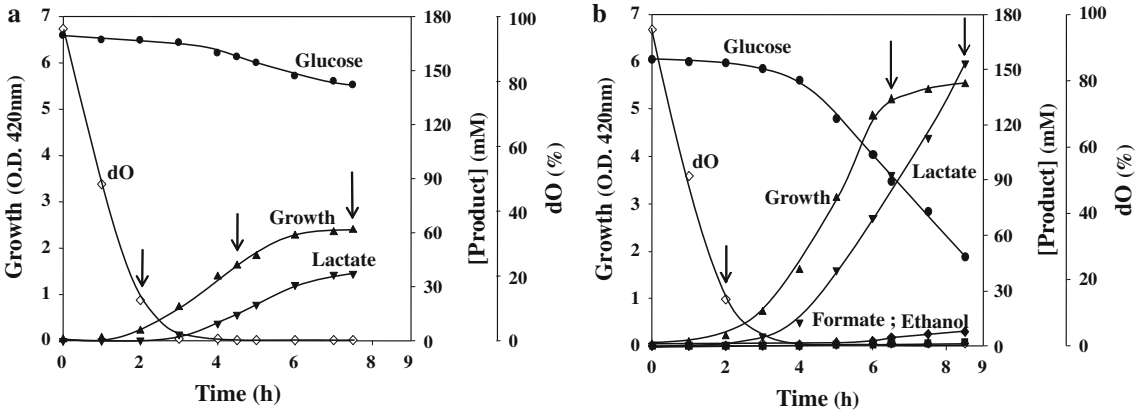

Fig. 4 Growth and fermentation profile of *B. coagulans* strain 36D1 in LB + glucose (30 g l^{-1}) at 50°C. **a** pH 5.0; **b** pH 7.0. *Vertical arrows* indicate the time of sampling for determination of enzyme activity and mRNA level. *dO* dissolved oxygen concentration

altered the control of PDH synthesis or activity of the enzyme during anaerobic growth at pH 5.0. It appears that the inability of the *l-ldh* mutant, strain Suy27, to produce active PDH during pH 5.0 fermentations is the cause of the growth defect.

The absence of PDH activity in anaerobically grown wild-type strain (pH 5.0) is in agreement with the role of PDH in cellular physiology. PDH produces one NADH during the oxidative decarboxylation of pyruvate and this additional reductant is not expected to be oxidized by the fermentation reactions of this bacterium causing a redox imbalance with a significant growth defect. Conversion of acetyl-CoA generated by PDH to ethanol in a two-step reduction is one way to restore redox balance (Fig. 2) and this is accomplished only in the mutant and not in the wild-type that produces lactate (Table 1). We have previously demonstrated that an *E. coli* mutant with a PDH complex that was active during anaerobic growth failed to utilize the PDH/ADH pathway in the pyruvate metabolism when both LDH and PFL activities are present [36]. Only in the absence of either PFL or LDH activity due to mutation, pyruvate flux through the PDH/ADH pathway in *E. coli* was detected. However, wild-type strains of *E. coli* and *B. coagulans* differ in their levels of PDH activity during anaerobic growth. Crude extracts of *E. coli* grown under anaerobic conditions did contain significant level of PDH activity and the level of mRNA corresponding to the PDH operon was comparable in both aerobic and anaerobic cell [18]. In contrast, anaerobically grown *B. coagulans* strain 36D1 at pH 5.0 had no detectable PDH activity.

PDH operon expression in wild-type strain 36D1

To understand the regulation of PDH, a fermenter culture of strain 36D1 was started aerobically and oxygen was allowed to deplete with the growth of the culture. Under this condition, when the cell density reached an OD 420 nm of about 0.5, dissolved oxygen concentration of the culture was below the level of detection (Fig. 4). Samples were removed from this culture during the early-, mid- and late-exponential phase of growth for determination of PDH and LDH activity and corresponding mRNA levels. When strain 36D1 was cultured at 50°C and pH 7.0, the level of PDH activity or the *pdhA* mRNA representing E1α subunit did not change significantly during growth (Table 2). Both the PDH activity and *pdhA* mRNA level did increase as the culture reached stationary phase. However, a threefold increase in mRNA level only increased the PDH activity by less than 1.4-fold.

The PDH activity of a pH 5.0 culture of strain 36D1 at an early exponential phase of growth representing an aerobic culture at 50°C was about twice the level of the activity of the pH 7.0 culture at about the same cell density and oxygen concentration (Table 2; Fig. 4a). However, the culture lost 95% of PDH activity as it entered oxygen limitation condition. This decrease in PDH activity did not correlate with only a 30% reduction in the *pdhA* mRNA level of the cells at that stage of growth. Similar results were also obtained for the level of the *pdhD* mRNA encoding dihydrolipoamide dehydrogenase (LPD), another component of the PDH complex (data not presented). As a control, the LDH activity of the culture was not significantly influenced by the growth pH or the age of the culture. A slight increase in LDH activity was observed as the culture entered stationary phase at both pH values. The *l-ldh* mRNA level also increased during late-exponential to early stationary phase of growth supporting this increase in LDH activity. These results show that the level of PDH activity in the anaerobic culture of *B. coagulans* is dependent on the culture pH.

The loss of PDH activity as the pH 5.0 culture entered oxygen limitation suggests that the PDH complex is

Table 2 Level of PDH and LDH activities and mRNA of *B. coagulans* wild-type strain 36D1 grown in LB + glucose (30 g l^{-1}) at pH 5.0 or pH 7.0[a]

Culture pH	OD 420 nm	Enzyme activity[b]		mRNA level (ng ml^{-1})[c]	
		PDH	LDH	*pdhA*	*ldh*
pH 5.0	0.21	33.2	63.0	0.26	10.62
	1.62	0.4	66.5	0.18	34.12
	2.39	1.4	87.0	0.18	13.61
pH 7.0	0.20	18.3	63.5	0.29	9.95
	5.19	18.0	75.5	0.24	45.39
	5.51	24.8	83.5	0.72	26.06

[a] Samples for determination of PDH and LDH activities and mRNA levels were removed at the times indicated by *arrows* in Fig. 4

[b] Enzyme activity was determined in crude extracts and expressed as nmoles min^{-1} (mg cell protein)$^{-1}$

[c] The level of each specific mRNA in total RNA isolated from these cultures is presented after normalizing to the level of *polA* mRNA that changed very little during growth under these conditions. The *pdhA* represents the mRNA level of the gene encoding E1α subunit of the PDH complex

Table 3 Level of PDH activity and *pdhA* mRNA of *B. coagulans* wild-type strain 36D1 after 1 h exposure to anaerobic condition[a]

Culture pH	Gas phase	PDH activity[b]	mRNA level (ng ml^{-1})[c]
pH 5.0	Air	13.84 ± 1.22	0.17 ± 0.02
	N_2	2.02 ± 0.33	0.19 ± 0.01
pH 7.0	Air	19.43 ± 3.77	0.35 ± 0.03
	N_2	9.87 ± 1.02	0.14 ± 0.02

[a] Cultures were grown at the indicated pH in LB + glucose (30 g l^{-1}) at pH 5.0 or pH 7.0 in a fermenter at 50°C with air (0.7 l min^{-1}). After the cultures reached mid-exponential phase of growth, N_2 was sparged through the culture at 0.2 l min^{-1}. Samples were removed after 1 h under N_2 for determination of PDH activity and *pdhA* mRNA level

[b] Enzyme activity was determined in crude extracts and expressed as nmoles min^{-1} (mg cell protein)$^{-1}$

[c] The level of mRNA corresponding to the *pdhA* (E1α subunit of PDH complex) in total RNA is presented after normalizing to the level of *polA* mRNA

biochemically regulated either by rapid degradation, modification or dissociation as suggested for the PDH complex of aerobic *B. subtilis* entering sporulation [11]. To evaluate the effect of oxygen on PDH activity, strain 36D1 was cultured in fermenters with aeration. After the culture reached mid-exponential phase of growth, air was replaced by nitrogen. Both PDH activity and *pdhA* mRNA levels were monitored at the end of 1 h of anaerobiosis (Table 3). The mRNA level of the pH 7.0 culture declined by about 60% while the PDH activity decreased by about 50% after 1 h of anaerobic condition. However, when strain 36D1 was grown at pH 5.0, the level of *pdhA* mRNA did not change significantly but the PDH activity decreased by about 85% at the end of 1 h of anaerobiosis. These results show that the level of PDH activity is influenced by the culture pH and oxygen concentration. To evaluate potential dissociation of PDH complex, as suggested for sporulating *B. subtilis* [11], PDH complex in the crude extract from the pH 5.0 and pH 7.0 culture was pelleted by centrifugation at 150,000 × *g* for 3 h. The supernatant was assayed for LPD activity that could have been released during dissociation of the PDH complex. The LPD dimer is expected to be too small (anhydrous molecular mass of 99,918 Da for the dimer) to be sedimented at the 150,000 × *g* centrifugation and should be detectable in the supernatant. However, no significant free LPD activity was found in any of the 150,000 × *g* supernatants before and after centrifugation suggesting that the loss of PDH activity during anaerobic condition induced by nitrogen sparging may not be due to dissociation of the PDH complex. It is possible a biochemical modification of the PDH complex could contribute to the loss of activity. Further biochemical experiments are needed to identify the mechanism of this control.

Conclusions

The *l-ldh* mutant of *B. coagulans*, strain Suy27, produced ethanol during fermentation of glucose at pH 7.0 and the ethanol yield from glucose was 0.32 (g ethanol per gram of glucose). About 55% of the ethanol was produced by the PDH/ADH pathway and the remainder from the PFL/ADH pathway. Coupling the 2,3-butanediol pathway to the PFL/ADH pathway appears to support redox balance while increasing the overall ethanol yield. Although this mutant grew aerobically at pH 5.0, it failed to grow fermentatively at this pH. This inability of the *l-ldh* mutant to grow anaerobically at pH 5.0 is probably due to the absence of PDH and PFL activities as seen with the parent strain 36D1 grown at pH 5.0. A derivative of the *l-ldh* mutant, strain Suy27-13, that produced PDH activity during anaerobic growth at pH 5.0, also produced ethanol as the main fermentation product from glucose during growth at pH 7.0 (0.39 g ethanol g^{-1} sugar) and pH 5.0 (0.26 g ethanol g^{-1} sugar). Although this ethanol yield of *B. coagulans* strain 27-13 at pH 7.0 is lower than the theoretical yield of 0.51 g per gram glucose fermented, this value is comparable to the ethanol yield of batch cultures of other *Bacillus* and *Geobacillus* strains engineered for ethanol production by the PDH/ADH pathway (Fig. 1); *B. stearothermophilus* strain LLD-15 (0.34 g per g sugar) [13], *G. thermoglucosidasius* strain DL44 (0.42 g per g glucose) [3], and a non-recombinant ethanologenic *E. coli* strain SE2378

(0.41 g per g glucose) [17]. Although a *B. subtilis* with *Z. mobilis* PDC activity (strain BS37) produced a significantly higher ethanol yield from glucose (0.45 g per g glucose), this strain required about 9 days to ferment 20 g l^{-1} glucose [28]. Ethanol yield of an *ldh* mutant of a strictly anaerobic *Thermoanaerobacterium saccharolyticum*, strain TD1, was not significantly different from its parent strain (0.36 g per g glucose) [8]. However, deleting phosphotransacetylase and acetate kinase activities also (strain ALK2) led to an ethanol yield of 0.46 (g per g xylose fermented) [31]. This bacterium is an example of the PFOR/NFOR/ADH pathway of sugar conversion to ethanol (Fig. 1). Further metabolic engineering of strain Suy27-13 that deleted the competing 2,3-butanediol pathway and increased the PDH activity is expected to elevate the ethanol yield from glucose and pave the way for cellulosic ethanol production at 50–55°C with a lower cellulase loading compared to SSF at 30–37°C.

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