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ORIGINAL RESEARCH PAPER

Genetic modifications and introduction of heterologous *pdc* genes in *Enterococcus faecalis* for its use in production of bioethanol

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Abstract Genetically-modified *Enterococcus faecalis* has a potential of survival and can be used in ethanolic fermentations. Fermentation profiles of *E. faecalis* JH2-2 were assessed using glucose and lactose as carbon sources. Deletion of lactate dehydrogenase (*ldh*) genes increased the ethanol production from 0.25 to 0.82 g/l, which was further increased to 0.96 g/l by the insertion of a pyruvate decarboxylase (*pdc*) gene (from *Sarcina ventriculi* or *Clostridium acetobutylicum*) in place *ldh1*. When grown on lactose, the *pdcSv* and *pdcCa* showed 13.6 and 17.6 U mg^{-1} of *pdc* specific activity, respectively. Highest activity (47 U mg^{-1}) and ethanol concentration (2.3 g/l) were obtained with *pdcCa* using an expression plasmid. Formate and acetate were also produced in high quantities. Transcriptional analysis showed that aldehyde alcohol dehydrogenase gene was upregulated up to 16-fold. Further optimizations are required for higher ethanol production.

Keywords *Enterococcus faecalis* · Ethanol · Fermentation · Lactate dehydrogenase · Pyruvate decarboxylase

Introduction

Stress tolerance and the ability to convert several substrates into ethanol are the main limitations to the use of bacteria and yeasts as bioethanol producers on industrial level. Therefore in the last two decades, various microorganisms have been genetically engineered in order to improve their tolerance against different stresses and to widen the range of the substrates they can metabolize. But the improvement of biocatalysts for ethanol production remained one of the major barriers in the development of the successful bioethanol industry (Dien et al. 2003).

Among lactic acid bacteria there are several salient features which make *Enterococcus faecalis* a potential candidate for renewable bioethanol applications. *E. faecalis* is a lactic acid bacterium, well known for its survival against different type of stresses such as high temperature (62 °C), heavy metals (Laplace et al. 2000), acidic pH (3.2), osmotic stresses and high ethanol concentration (22 % v/v) (Giard et al. 2003).

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Two lactate dehydrogenase (*ldh*) genes are present in *E. faecalis* JH2-2 (personal communication: Noshreen Rana, Unité de Recherche Risques Microbiens, Caen, France) and V583 genomes (Jönsson et al. 2009). The purpose of this study was therefore to disrupt the lactic acid production pathway in *Enterococcus faecalis* JH2-2, through the deletion of lactate dehydrogenase genes, and then to enhance its ability to produce ethanol by introducing an heterologous *pdc* gene. The genes encoding lactate dehydrogenase were deleted and one of the *ldh* gene (*ldh*1) was replaced by a *pdc* gene from *Sarcina ventriculi* (*pdcSv*) or *Clostridium acetobutylicum* (*pdcCa*). No additional alcohol dehydrogenase (*adh*) gene was introduced. Further the *pdcCa* gene was also expressed in the *ldh* mutant (*Δldh1/Δldh2*) using an expression plasmid pMSP3535. Until now the *pdc* of *S. ventriculi* was considered as a good choice for engineering gram positive bacteria for ethanol production. However our results show that the *pdc* gene of *Clostridium acetobutylicum* is a new efficient candidate to carry out alcoholic fermentation by modified lactic acid bacteria such as *E. faecalis*.

Materials and methods

Bacterial strains and culture conditions

The strains used in this study (Supplementary Table 1), *Enterococcus faecalis* JH2-2 strains, *Sarcina ventriculi* Goodsir, *Clostridium acetobutylicum* ATCC 284 and *Eschericiha coli* TOP10F' were grown in the M17 (Terzaghi and Sandine 1975), MYA (Canale-Parola 1970), CGM (Roos et al. 1985) and LB (Lauria Bertani) media, respectively. For measurement of PDC activity and for the fermentation profiles, *E. faecalis* strains were grown on M17MOPS medium (pH 7.3) containing (g/l): glucose or lactose 5, peptone 5, soya 5, yeast extract 2.5, meat extract 5, ascorbic acid 0.5, $MgSO_4$ 0.25 and MOPS 42.

Construction of mutants

For the deletion of the *ldh1* and *ldh2* genes, two fragments (size 1 kb)—one upstream, one downstream of the 300 bp region inside the genes-were cloned in pGEM-T (Supplementary Tables 1, 2). For the replacement of *ldh1* gene by the *pdc* gene, a *ldh1*up-*pdc*-*ldh1*down construct was prepared in the pGEM-T with two fragments one upstream and one downstream to the area of *ldh1* to be replaced by *pdc* gene. The constructs obtained were restricted and transferred in the pMAD (Arnaud et al. 2004). Verified constructs were extracted from the *E. coli* and concentrated 3–4 times before their transformation into *E. faecalis*. The crossing-over leading to gene deletion or replacement was done using the Hébert et al. (2007) method with some modifications. Integration of the plasmid was done at 44 °C and the second cross-over was done at 30 °C for 5 h followed by overnight incubation at 44 °C. The white colonies, sensitive to erythromycin, were verified by PCR.

Analysis of the fermentation products

Lactate, formate, acetate and ethanol concentrations were determined using HPLC and the results are the average of three independent experiments. The filtrates of 24 h cultures were injected (20 µl) in a Phenomenex PHM-monosaccharide column maintained at 65 °C. The mobile phase, 5 mM H_2SO_4, was at 0.5 ml/min. A refractometer detector was used and the signal was analyzed through the Jasco Borwin software (JMBS Developments, France).

Pyruvate decarboxylase (PDC) specific activity

Proteins were extracted from the anaerobic cultures at OD_{600nm} 0.5 (~1 g cell/l dry wt) and protein quantification was done by using a Bradford kit. The PDC activity assays were performed in a test-tube with 0.3 ml sodium phosphate buffer 0.2 M (pH 6.0), 0.2 ml pyruvate 0.1 M, 0.1 ml thiamin pyrophosphate 5 mM, 0.1 ml $MgCl_2$ 5 mM and 0.1 ml diluted protein extracts. After the addition of 2 ml dimedone reagent (Guglielminetti et al. 2001), the mixture was incubated for 10 min at 40 °C and then 6 min in boiling water. Fluorescence was measured at 365/460 nm. *T* test was used to compare the enzymatic activity in different mutants.

RNA extraction, DNase treatment, cDNA preparation and RT-PCR

RNA were extracted with Max enhancement reagent and Trizol (Invitrogen) from the anaerobic cultures at exponential phase (OD_{600nm} 0.5; ~1 g cells/l dry wt).

The RNAs were treated with DNase using a DNA-free kit (Ambion). 50 µl of reaction mixture, containing RNA (10 µg), 10× DNase buffer (5 µl) and DNase (1 µl), were incubated for 30 min at 37 °C. Then 5 µl DNase inactivator were added and after 2 min at 37 °C, samples were centrifuged at 10,000×*g* for 2 min. The supernatant containing the RNA was stored at −80 °C.

The preparation of cDNA and the RT-PCR were carried out by Omniscript enzyme and BioRad iCycler iQ, respectively. The size of amplicons was 99–101 bp and the primer size was 19–21 bp (Supplementary Table 3). All primers used for RT-PCR have hybridization temperature between 59 and 61 °C. To standardize the results, cDNA of 23S RNA were used for all samples.

The value used for comparison of gene expression among wild type and mutants was the number of PCR cycles required to reach the threshold cycle, Ct. To relate the Ct value to the abundance of a mRNA species, Ct was converted to '*n*-fold difference' by comparing mRNA abundance in the JH2-2 wild-type strain with that obtained with the mutant strain, where, $n = (E_{23S}{}^{Ct23S(wt)}/E_x^{Ctx(wt)})/(E_{23S}{}^{Ct23S(mutant)}/E_x^{Ctx(mutant)})$ and E represents the efficacy of PCR.

Results and discussion

Enterococcus faecalis JH2-2 was grown on two different carbon sources—glucose and lactose—under aerobic and anaerobic conditions. Although the fermentation was not strictly homolactic, lactate was the major end-product in all growth conditions tested (Fig. 1). The fermentation profiles show that under aerobic conditions, besides lactate, acetate was produced while under anaerobic conditions significant amounts of formate were found. However the production of ethanol appeared to be related to the anaerobic conditions.

Unlike in *Lactococcus lactis* (Gaspar et al. 2007), in *E. faecalis* both *ldh* genes (*ldh1* and *ldh2*) were functional. However, *ldh2* expression was dependent on the presence or absence of O_2 and also on the carbon source. No difference of growth was observed between the wild type and the mutant strains (data not shown). This is in contrast to the deletion of *ldh* in *Bacillus subtilis*, where the absence of LDH leads to a growth retardation in the mutant strains which was not improved by addition of the stoichiometricaly equivalent pathway (Romero et al. 2007). Therefore *E. faecalis* has more survival potential in the absence of functional LDH as compare to *B. subtilis.*

The deletion of *ldh1* led to a more important decrease in the production of lactate compare to that of *ldh2* gene. This difference was more prominent in case of growth on lactose where lactate production was decreased up to 80 % whereas the main fermentation product was formate (Fig. 1). Thus in the *ldh2* mutant, we have undertaken to replace the *ldh1* gene by a heterologous *pdc* gene in order to determine whether it was possible to divert the metabolism of *E. faecalis* towards the production of ethanol. Indeed a *pdc* gene encodes a pyruvate decarboxylase that catalyses the non-oxidative decarboxylation of pyruvate to acetaldehyde that can be further reduced into ethanol. Since *pdc* expression is dependent on codon usage, in the current study two *pdc* gene sources were included from *S. ventriculi (pdcSv)* and from *C. acetobutylicum* (*pdcCa*).

Like *Δldh1/Δldh2* mutant, the *Δldh2/Δldh1*::*pdc* mutants produced formate and acetate in increased quantities as compared to the wild type strain (Fig. 1). The increased ethanol production in the *Δldh2/Δldh1*::*pdc* mutants was an indication of the PDC activity. In order to confirm that *pdcSv* or *pdcCa* were really responsible for an increased ethanol production, the PDC activities were measured in these mutants. Our results show that both *pdc* genes were functional in *E. faecalis* with no significant difference between their activities. The PDC specific activities in glucose cultures were significantly lower than those observed in lactose culture (Table 1).

Using an expression plasmid, 0.5 µg nisin/ml led to optimum activity of PDC as well as higher ethanol yield. Under anaerobic conditions using 5 g lactose/l as substrate, the higher ethanol concentrations obtained were 2.1 g/l and 2.3 g/l with 0.5 µg nisin/ml and 1 µg nisin/ml respectively (Fig. 2). High level of *pdc* transcript (Ct: 15.6) suggest that the *pdcCa* is suitable to obtain heterologous *pdc* expression in gram positive bacteria.

The transcriptional analysis showed that most of the pyruvate catabolic genes were down-regulated except *pflB* and *adhE* (Table 2). However, after deletion of the *ldh*, an upregulation of *pdh* and *alsS* was expected as described by Mehmeti et al. (2011). PDH enzymes are typically inhibited by NADH and are inactive under anaerobic conditions (Condon 1987). During

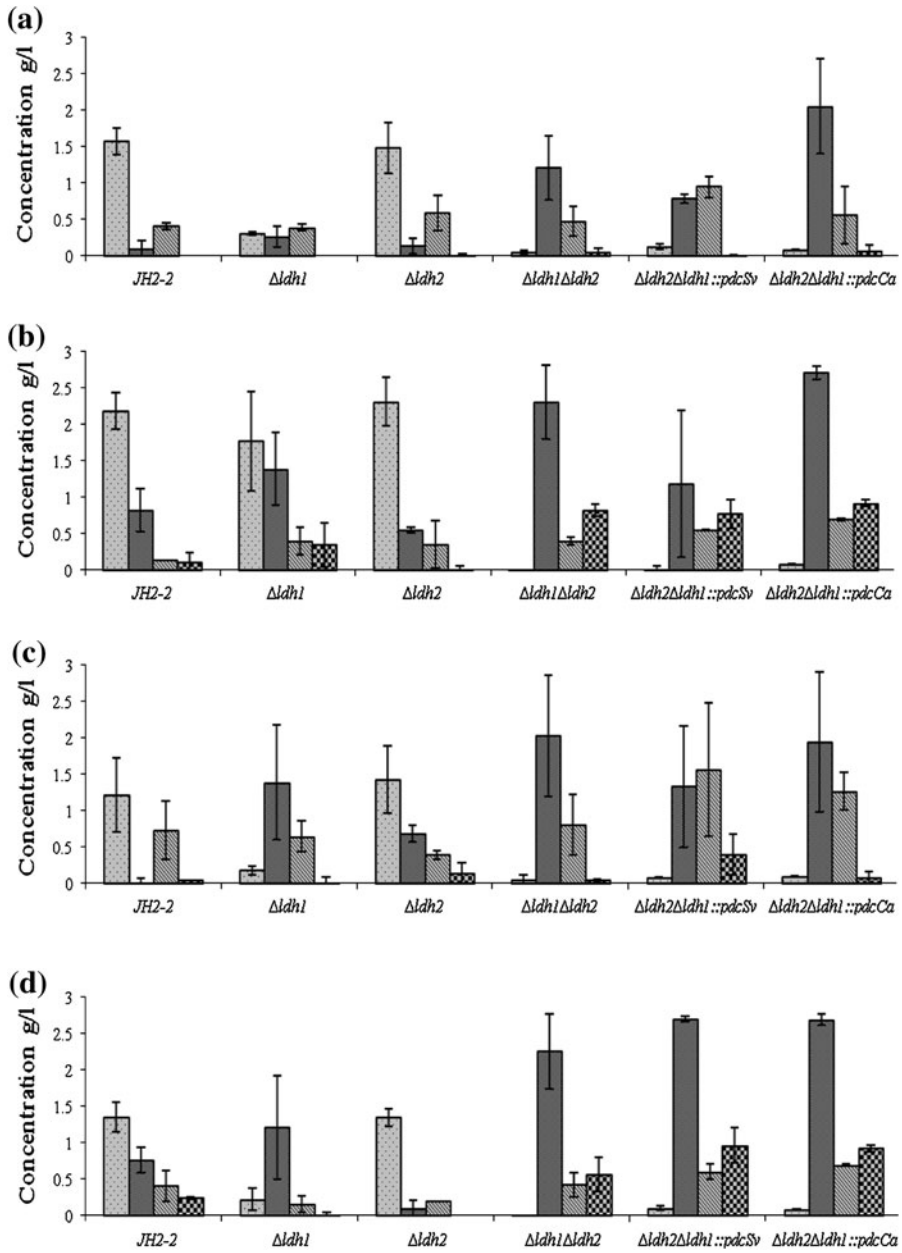

Fig. 1 Fermentation profiles of *E. faecalis* JH2-2, *ldh*-mutants and *pdc-ldh* mutants after 24 h of fermentation on **a** glucose under aerobiosis; **b** glucose under anaerobiosis; **c** lactose under aerobiosis and **d** lactose under anaerobiosis (*gray square with dots* lactate, *small square with diagonal cross hatch fill* formate, *square with upper left to lower right fill* acetate, *medium square with diagonal cross hatch fill* ethanol)

Table 1 PDC specific activity (U mg^{-1}) measured in anaerobic cultures of different recombinant mutants of *E. faecalis*

	Δldh2/ldh1::pdcSv	*Δldh2/ldh1::pdcCa*	*Δldh1/Δldh2* (*pdcC*ap3535) 0.25 μg nisin/ml	Δ*ldh1/Δldh2* (*pdcC*ap3535) 0.5 μg nisin/ml	Δ*ldh1/Δldh2* (*pdcC*ap3535) 1 μg nisin/ml
Glucose	6.1 ± 3.1	9.1 ± 1.7	21.5 ± 13.3	34.1 ± 10.1	23.7 ± 13.3
Lactose	13.7 ± 0.3	17.7 ± 3.6	31.0 ± 19.3	46.7 ± 18.1	29.6 ± 4.7

the conversion of pyruvate to acetylCo-A by PDH, NAD^+ is consumed. Also, due to the lack of functional LDH, the intracellular NADH/NAD^+ ratio becomes high and PDH is sensitive to high NADH/NAD^+ ratios since the dihydrolipoamide dehydrogenase (E3) component of pyruvate dehydrogenase complex is reduced

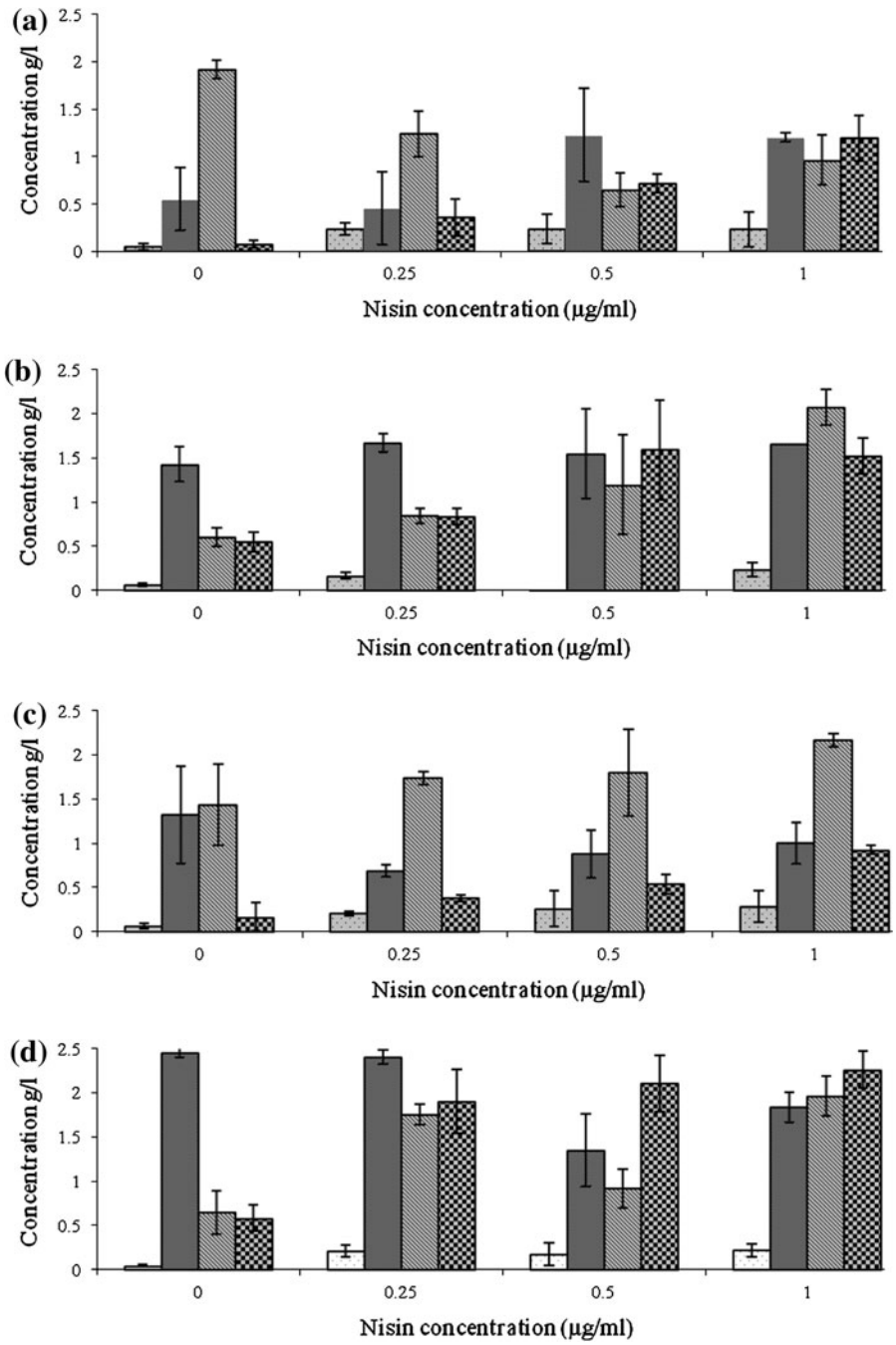

Fig. 2 Fermentation profiles of *E. facalis Δldh1/Δldh2,* carrying *pdcCa*p3535, induction by nisin, after 24 h of fermentation **a** glucose under aerobiosis; **b** glucose under anaerobiosis; **c** lactose under aerobiosis and **d** lactose under anaerobiosis (*gray square with dots* lactate, *small square with diagonal cross hatch fill* formate, *square with upper left to lower right fill* acetate, *medium square with diagonal cross hatch fill* ethanol)

by NADH (Kim et al. 2008). Thus PDH is not used as an alternate enzyme for the further conversion of pyruvate. This regulation is prominent in the anaerobic conditions as shown in these results. But under aerobic conditions, the regulation of *pdh* in the *ldh* mutants could be different as E3 component of the PDH can react with oxygen and thus it will not be over reduced (Snoep et al. 1992). The down-regulation of *alsS* is unusual. The α-ALS has a low affinity for pyruvate and is inactive in vivo under energy-source-limiting conditions (Snoep et al. 1992). The down regulation of both *pdh* and *alsS* genes means that in *ldh* deficient mutant Δ*ldh1*/Δ*ldh2* of *E. faecalis* JH2-2 (high NADH, H^+/NAD^+ ratio) the regulation of

Table 2 Transcriptional analysis of *E. faecalis* JH2-2 mutants grown in anaerobic condition

Gene	Putative function	Regulation									
		Δldh1/Δldh2		*Δldh2/ ldh1::pdcSv*		*Δldh2/ ldh1::pdcCa*		*Δldh1/Δldh2 (pdcCa*p3535) without induction		*Δldh1/Δldh2 (pdcCap3535)* with induction	
		Lactose	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose	Glucose
ldh1	Lactate dehydrogenase	×	×	×	×	×	×	×	×	×	×
ldh2	Lactate dehydrogenase	×	×	×	×	×	×	×	×	×	×
pflb	Pyruvate formate lyase	2.9	9.1	2.4	5.2	6	5.3	5.3	5.8	4.7	8.5
pta	Pyruvate transacetylase	NC	5.4	3.4	4.1	4.4	5.3	4.4	2.7	2.5	2
alsS	Alpha-lactosynthase	−2.7	−2.6	NC	−2.1	2.7	−3.1	−3.3	−5.1	−2.2	−4
pdh	Pyruvate dehydrogenase	−2.1	−2.5	−2.5	−2.9	NC	−2.2	−4.9	−7.3	−2.7	−4.2
adhE	Aldehyde-alcohol dehydrogenase	2	7.6	3.3	20.6	6.6	17.3	3.8	2.7	2.1	16.6
adhA	Alcohol dehydrogenase	×	×	×	×	×	×	×	×	×	×
qdhZn	Alcohol dehydrogenase	2	2.3	−2	NC	NC	NC	NC	NC	−2.5	−3.1
adhFe	Alcohol dehydrogenase	×	×	×	×	×	×	×	×	×	×

Values indicate fold change in expression of genes in mutants as compare to the wild type *E. faecalis* JH2-2 strain. No change (NC) in the level of gene expression compared to the wild type and no gene expression (×)

fermentation enzymes is dependent on their affinity for their substrate as well as on the keeping of the redox balance since no regeneration of NAD^+ is obtained during the formation of acetoin. Out of three alcohol dehydrogenase genes only *adhZn* has shown expression (Table 2). ADHE is a bifunctional aldehyde-alcohol dehydrogenase responsible for the production of ethanol. The up-regulation of this enzyme in all mutant strains is involved in the rapid conversion of acetyl-CoA into ethanol thus eliminating the need to clone any heterologous *adh* gene in *E. faecalis* for ethanol production.

Genetic engineering of Gram-positive bacteria for the production of ethanol has limited success because of the low level of heterologous expression of *pdc* and *adh* genes in the lactic acid bacteria and *Bacillus* species (Hillman et al. 1996; Nichols et al. 2003). For *pdc* gene expression, codon usage is an important criterion to be considered in order to obtain successful cloning of this gene. The PDC of *S. ventriculi* had good activity in *Bacillus megaterium* (5.3 U mg^{-1}) as compared to the PDCs from Gram-negative bacteria (*Acetobacter pasteurianus, Zymomonas mobilis*) and yeast (*Saccharomyces cerevisiae*) (Talarico et al. 2005). Although the deletion of the lactate dehydrogenase pathway was not sufficient to divert completely the pyruvate flow towards ethanol, increased ethanol concentrations were obtained specially when using an expression plasmid. Our results suggest that PDC from *C. acetobutylicum* may have some potential for the engineering of Gram-positive bacteria to produce ethanol.

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