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Growth Characteristics of a Pyruvate Decarboxylase Mutant Strain of *Zymomonas mobilis*

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Studies of the inactivation of a gene encoding pyruvate decarboxylase, *pdc*, in an ethanol-producing bacterium, *Zymomonas mobilis,* identified a mutant strain with 50% reduced PDC activity. To evaluate the possibility of a carbon-flux shift from an ethanol pathway toward higher value fermentation products, including pyruvate, succinate, and lactate, fermentation studies were carried out. Despite attempts to silence *pdc* expression in the wild-type strain ZM4 using *cat*-inserted *pdc* and *pdc*-deleted homologs by electroporation, the strain isolated showed partial gene activation. Fermentation experiments with the PDC mutant strain showed that the reduced expression level of PDC activity resulted in decreased rates of substrate uptake and ethanol production, together with increased pyruvate accumulation of 2.5 g l^{-1}, although lactate and succinate concentrations were not significantly enhanced in these modified strains. Despite numerous attempts, no strains were isolated in which complete *pdc* inactivation occurred. This result indicates that the ethanol fermentation pathway of this bacterium is totally dependent on the activity of the PDC enzyme. To ensure a redox balance of intracellular NAD and NADH levels, other enzymes, such as lactate dehydrogenase for lactate, and enzymes involved in the production of succinic acid, such as pyruvate dehydrogenase (PDH) and malic enzymes, may be needed for their increased end-product production.

Key words : Fermentation, gene inactivation, higher value products, pyruvate decarboxylase, *Zymomonas mobilis*

Introduction

There is current interest in the facultative anaerobic bacterium, *Zymomonas mobilis*, not only for ethanol production from lignocellulosic materials, but also for its potential for production of higher value chemicals [7, 12, 13, 16, 17, 18]. This results from its higher specific rates of sugar uptake, ethanol production and lower biomass yields when compared to yeasts or other ethanologenic bacteria [4, 19, 26]. Furthermore, the successful genetic engineering of *Z. mobilis* has become more feasible following characterization of its DNA restriction and modification system [10], its improved genome annotation [28] and genome-scale *in silico* analysis [12, 27] based on the earlier publication of the genome of *Z. mobilis* ZM4 by Seo *et al*. [22].

Pyruvate, succinic and lactic acids are potential target chemicals that might be produced via the successful metabolic engineering of *Z. mobilis,* as they are industrially important intermediates in the food, pharmaceutical and plastics industries. Wild-type strains of *Z. mobilis* produce very small amounts of these organic acids [9, 29] as glucose taken up via the Entner-Doudoroff (ED) pathway, or xylose metabolized via a modified pentose phosphate pathway, is usually rapidly and efficiently converted into ethanol.

Pyruvate decarboxylase (PDC) is a member of a large family of enzymes that catalyze the decarboxylation of pyruvate producing CO_2 and acetaldehyde. The latter is subsequently reduced by alcohol dehydrogenase to ethanol as the major end product of *Z. mobilis* fermentation [2]. It comprises upto 4-6% of soluble cell protein in *Z. mobilis* [15] and influences upto 95% of the carbon metabolites through its activity. Other authors have reported on high expression level of pyruvate decarboxylase (PDC) in *Z. mobilis* as well as its high substrate affinity for pyruvate [2]. Through the reduced PDC

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activity in this ethanologen, it may be possible to redirect significant carbon flux from ethanol and towards several higher value metabolites.

Here, we report the growth characteristics of such mutant strains with modified PDC activities on *Z. mobilis*, including its effect on ethanol production and related fermentation products.

Materials and Methods

Microbial strains and plasmids

The bacterial strains used were *Zymomonas mobilis* ZM4 (ATCC31821), *E. coli* strain BL21 (DE3) pLysS (Invitrogen) and JM109. *Z. mobilis* cultures were incubated at 30°C in RM medium [5] without shaking. When appropriate, chloramphenicol and/or tetracycline were added at 100 μg ml^{-1} and/or 20 μg ml^{-1}, respectively to isolate PDC mutant strains of *Z. mobilis*. The plasmids used were pNSW812 (a suicide plasmid harboring a homolog of *pdc* insertionally inactivated by a *cat* gene encoding chloramphenicol acetyltransferase) and pNSW813 (a suicide plasmid harbouring a *pdc*-deleted homolog replaced with *tet* gene coding for tetracycline resistance) derived with the pGEM-T Easy vector (Promega, Madison, USA).

Strain constructions

Genomic DNA was isolated from *Z. mobilis* cultures using a Puregene® genomic DNA isolation kit (Qiagen, USA).

In the first method for inactivation of PDC expression in *Z. mobilis*, insertional inactivation of *pdc* was carried out by introduction of the gene encoding chloramphenicol acetyltransferase (*cat*) via homologous recombination. PCR primers used for the cloning of *pdc* were pdcF (5′ ATGAGTTATACTGTCGGTAC 3′) and pdcR (5′ CTAGAGGAGCTTGTTAACAG 3′). PCR primers used for *cat* cloning were catFncoI (5′CCATGGTTGCTTTCGAATTTCTGCCATTC3′) and catRncoI (5′CCATGGTGACGGAAGATCACTTCGCAG 3′) with *Nco*I restriction recognition sequence included at the 5′ ends of the each primer. In this procedure, an 1,800 base pair (bp) fragment of *pdc* was isolated from the chromosome of *Z. mobilis* ZM4 by PCR using the pdcF and pdcR primers. The PCR product was cloned into pGEM®T-Easy via T/A cloning. To construct the *pdc*-disrupted gene, the 1,800 bp *pdc* gene in the pGEM T-Easy plasmid was cleaved by *Nco*I. The cleaved *pdc* fragment was ligated with a 1 kb-*cat* *Nco*I fragment from PCR using the catFncoI and catRncoI primers and was derived from the plasmid pLys S (Novogen, Co., USA). The constructed plasmid for insertion inactivation of *pdc* was named pNSW812.

The other suicide vector, pNSW813 with a *pdc* deleted homolog, was also constructed by means of a deletion-inactivation strategy. For the construction of the gene deletion homolog, the PCR primers pdcHomFor (5′GTCGGATAGGAAGTATAGCGAGG3′) and pdcHomRev (5′GATCCGGTATGTTCTTGCTTT3′) were used. For *cat* cloning, the PCR primers catFor (5′ GTTAACTTGCTTTCGAATTTCTGCCATTC3′) and catRev (5′ GGTACCTGACGGAAGATCACTTCGCAG3′) were used. A 3811 base pair-DNA homolog fragment including the *pdc* gene together with each of the 5′ and 3′ ends of the *pdc* flanking regions, was amplified by PCR from ZM4 genomic DNA using the pdcHomFor and pdcHomRev primers, and the amplicon was cloned into the pGEM-T Easy vector. *Kpn*I and *Hpa*I were used to delete the *pdc* gene from the intermediate plasmid and the *Kpn*I/*Hpa*I cleaved plasmid was subsequently ligated together with the *Kpn*I/*Hpa*I cleaved *cat* gene cassette amplified from *pdc-cat*. Consequently in the constructed plasmid, pNSW813, the marker *cat* gene was substituted with *pdc* and was linked to a 1 kb *pdc* homologous flanking region.

Electroporation was performed according to methods described by Kerr *et al*. [10] to disrupt *pdc* with these two suicide vectors. DNA manipulation techniques were performed as described by Sambrook *et al*. [20].

Confirmation for *pdc* gene inactivation

Recombinant colonies isolated after electroporation were subsequently purified three times on RM plates containing 100 μg chloramphenicol ml^{-1} at 30°C to avoid contamination with the background cells prior to the genotyping assay with PCR. Individual colonies were cultured on RM broth containing the selective concentrations of antibiotics at 30°C overnight and genomic DNA extracted from these cultures were used as a template for PCR analysis. For confirmation of insertional inactivation of *pdc*, PCR analysis was carried out using a primer set of pdcUPGF(5′AGCTGTCATTTATGTTTCAATAAGAC3′) and pdcDWGR (5′CAGAAGAGGTTCATCATGAACA3′) which hybridize outside the homologous region in the genome of mutant strains not present in the pNSW812 vector. For confirmation of deletion inactivation of *pdc*, PCR analysis was carried out using a primer set of pdc5cFor (5′GCGTCAGGTCATTACGAAAA3′) and pdc6cRev (5′TTGAAGCCTAAATAACAGACTCAAA

3′). Percent similarity and identity of DNA sequences of *pdc* found in ZM4812 and ZM4813 were determined using Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI, NIH).

Measurement of PDC activity

Cells of *Z. mobilis* were harvested in the exponential phase of growth (0.8~1.0 OD_{660} nm). The harvested cells were resuspended in lysis buffer containing 30 mM sodium citrate buffer (pH= 6.0), 20 mM $MgCl_2$ and 1.5 mM thiamine pyrophosphate. The resuspended cells were then lysed using 0.5 g of 0.15 mm glass-beads by vortexing for 1 min followed by incubation for 1 min on ice. This cycle was repeated 5 times to achieve effective cell lysis. The crude cell extract was separated by centrifugation at 4°C, 16,000× *g* for 5 min. The extracted supernatant was collected for subsequent enzymatic assay and total protein determination. Determination of pyruvate decarboxylase activity was performed using previously reported methods [23]. The enzymatic assay for PDC (EC: 4.1.1.1) activity was based on the principle of coupling the decarboxylation reaction with an alcohol dehydrogenase-mediated reaction and monitoring a decrease of NADH at a wavelength of 340 nm. One unit of enzyme activity is defined as the formation of 1μmol of acetaldehyde per min. The protein concentrations of the cell free crude extracts were quantified according to Smith *et al*. [24] with bovine serum albumin (Promega, U.S.A) as a standard reference.

Fermentation studies

Fermentation studies were carried out using medium composed of 50 g glucose l^{-1}, 5 g yeast extract l^{-1}, 2 g KH_2PO_4 l^{-1}, 2 g $(NH4)_2SO_4$ l^{-1}, and 1 g $MgSO_4 \cdot 7H_2O$ l^{-1} as described previously by Jeon *et al*., [8]. Seed cultures were prepared in10ml of medium containing 20 g glucose l^{-1}, 10 g yeast extract l^{-1}, 2 g KH_2PO_4 l^{-1}, 2 g $(NH4)_2SO_4$ l^{-1}, 1 g $MgSO_4 \cdot 7H_2O$ l^{-1}. When an optical density of A_{660}= 1.0 was reached, a 10%(v/v) aliquot of the seed culture was used for inoculation. Fermentations were carried out in 200 ml Erlenmeyer flasks with working volumes of 100 ml and performed at 30°C and an initial pH 5.0, without agitation. Samples were taken at various times to determine biomass, as well as glucose, pyruvate, lactate, succinate and ethanol concentrations.

Analytical procedures

Samples taken from fermentation flasks were analyzed for glucose, ethanol, pyruvate, succinate and lactate concentrations by HPLC using an Aminex column HPX-87H (300 ×7.8) (Bio-Rad, Richmond, CA) equipped with a refractive index detector and a computer interfaced electronic integrator. Separations were performed at 50°C with elution at 0.6 ml min^{-1} using 5 mM H_2SO_4. Growth was measured turbidometrically at 660 nm (1 cm light path). Dry cell mass was determined by conversion of optical density values at 660 nm to dry cell weight concentrations using a conversion constant for ZM4 ($OD_{660\ nm}$ 0.05= 15 mg l^{-1}) [11]. Standards containing analytical grade components were used periodically to confirm the calibration accuracy for the HPLC analyses. The concentration of succinic acid was checked with an enzymatic assay from a commercial kit (Megazyme, Ireland) according to the Manufacturer's Instructions.

Calculation of kinetic parameters

The maximum specific growth rates were calculated from the exponential phase. The maximum specific glucose uptake rates ($q_{s\ max,glucose}$) and maximum specific ethanol production rates ($q_{pmax,}$) were calculated over the exponential phase of growth and based on the following formulae: $q_{smax,glucose} = (1/x_{av})\ (\triangle s/\Delta t)$ and $q_{pmax,} = (1/x_{av})\ (\Delta p/\Delta t)$, where $\triangle s$ and $\triangle p$ are the changes in the glucose and ethanol concentrations respectively, over the time period Δt, and x_{av} is the average biomass concentration over Δt, where x, s and p are the concentrations of biomass, glucose and ethanol respectively.

The overall yields for biomass ($Y_{x/s}$) and ethanol ($Y_{p/s}$) were based on the initial and final concentrations of biomass, glucose and ethanol.

Results

Strain construction for PDC inactivation

Insertion inactivation

The DNA of pNSW812 was transferred into ZM4 via electroporation to facilitate a homologous recombination event between the homologous regions in pNSW812 and its complementary region in the ZM4 genome. The construction strategy is shown in Fig. 1(A) and the strain isolated with this strategy was designated as ZM4812. Chloramphenicol resistant colonies were selected on RM agar plates containing 100 μg choramphenicol ml^{-1}. Genomic DNA extraction

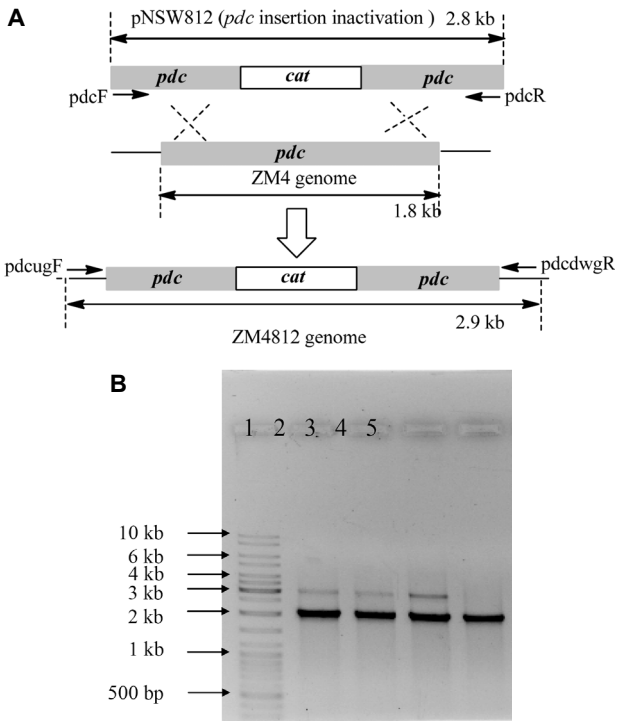

Fig. 1. Insertional inactivation of *pdc* in *Z. mobilis* ZM4. **A**: Schematic diagram of the insertional inactivation strategy for *pdc* in ZM4 by introduction of *cat* gene. **B**: PCR analysis for insertional inactivation for *pdc* using genomic DNA of ZM4812 with the primer set pdcugF and pdcdwgR . Lane 1, DNA size marker (Fermentas 1 kb Gene Ruler); lane 2, ZM4812-1, lane 3, ZM4812-2; lane 4, ZM4812-3, lane 5, ZM4 wild type.

from the selected strains and from ZM4 as a control was carried out to determine whether or not successful gene inactivation had occurred. For confirmation, PCR analysis was carried out using a primer set of pdcUPGR and pdcDWGR which targeted the outside homologous region on the genome of ZM4812 which was not present in the pNSW812 vector. The genotyping experiment identified a DNA fragment of approximately 1.8 kb in size in the parent strain ZM4, while in ZM4812 an additional band of 2.9 kb was present (see lanes 2-4 for ZM4812-1, 2, and -3 in Fig. 1(B)). DNA sequencing analysis was carried out with the two PCR products derived from the constructed strain. The results indicated that *cat* was present in the 2.9 kb DNA band as expected. The confirmed that successful insertion of the marker cat from pNSW812 had occurred via homologous recombination into the native *pdc* of the ZM4 genome. Unexpectedly however, a copy of an intact *pdc* gene, identified for the 1.8 kb fragment, still remained in ZM4812 (see Fig. 1 (B)). The sequence of this residual *pdc* in ZM44812 was compared with the previously reported *pdc* sequence for ZM4 [22], and its sequence similarity at nucleotide level was 100% to that for ZM4.

Deletion inactivation

The strategy for deletion inactivation is shown in Fig. 2A. The DNA of pNSW813 was transferred into ZM4 with a similar manner for ZM4812 and form this experiment the strains of ZM4813-1 and -2 were isolated. Genotypic analysis for the knockout strains was carried out with a primer set of pdc5cForand pdc6cRev. For the chloramphenicol resistant colonies derived from letter strategy, the same procedure was followed for their genotype confirmation using a primer setpdc5cFor and pdc6cRev. A PCR product of approximately

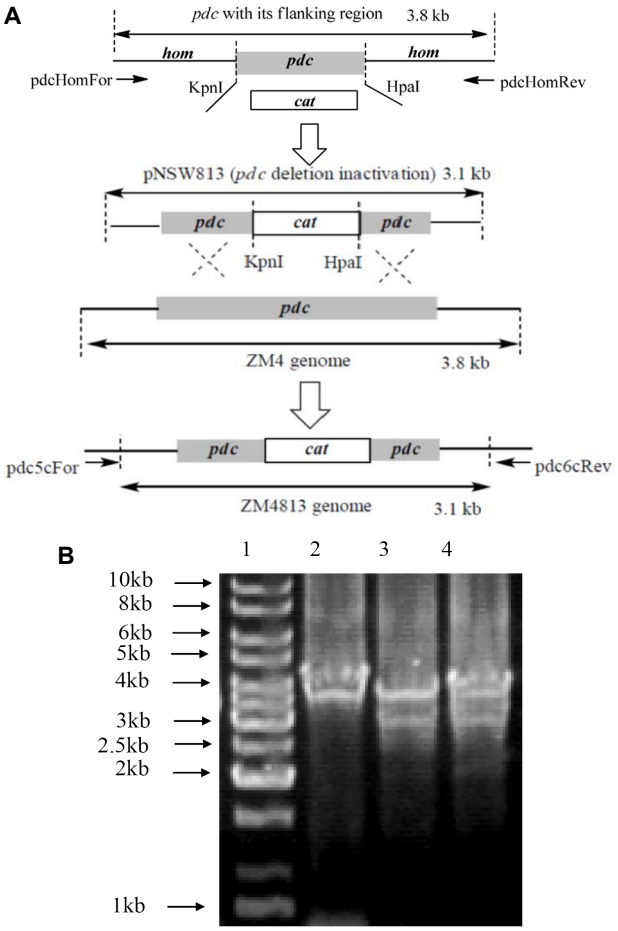

Fig. 2. Deletion inactivation of *pdc* in *Z. mobilis* ZM4. **A**: Schematic diagram of the deletion inactivation strategy for *pdc* in ZM4 by replacement of *cat* gene. **B**: PCR analysis for deletion inactivation for *pdc* using genomic DNA of ZM4813 with the primer set pdc5cFor and pdc6cRev. Lane 1, DNA size marker (Fermentas 1kb Gene Ruler); lane 2, ZM4 the wild type l strain, lane 3, ZM4813-1; lane 4, ZM4813-2.

3.8 kb in size appeared in the parent strain ZM4 (Fig. 2B, lane 2), while ZM4813 also similar results as appeared in ZM4812 were found, two bands with sizes of approximately 3.8 kb and 3.1 kb, respectively were observed (Fig. 2B). Similar results to those for ZM4812 were found with both a deleted pdc and an intact pdc present in ZM4813. The sequence of this extant pdc in ZM4813 was compared with the native pdc sequence for ZM4 and 100% similariy at nucleotide levels was confirmed . and the wild-type *pdc* gene (Fig. 2B, lane 3 and 4, upper band, 3.8 kb). The results were similar to those for ZM4812 showing both a deleted *pdc* and another copy of intact *pdc* in the genome.

As a further investigation, we attemped to inactivate the existing intact *pdc* in ZM4813 by introducing another homologues this time containing a tetracycline resistance gene as the selection maker. However, no transformants expression both tetracycline and chloramphenicol resistance were found following repeated attempts.

Inactivation of the second intact *pdc* gene present in strain ZM4813was also attempted by introducing another *pdc* homolog. However, no transformants were generated.

Pyruvate decarboxylase (PDC) activity on the constructed strains

As genotype analyses for the mutant strains showed inactivation of *pdc* in both ZM4812 and ZM4813, their PDC activities were determined for comparison with that of the parent ZM4 strain. As shown in Fig. 3, the specific PDC activities of both ZM4812 and ZM4813 were both approximately 50% less than that of ZM4, with values of 1.1 and 1.2 U mg total protein $^{-1}$ compared to 2.1 U mg total protein $^{-1}$ for the parent strain. The partial PDC inactivation was maintained by the presence of 100 μg chloramphenicol ml^{-1}. However, it was found after growth on the RM medium in the absence of chloramphenicol for 24 generations that the PDC activities of both strains increased to approximately

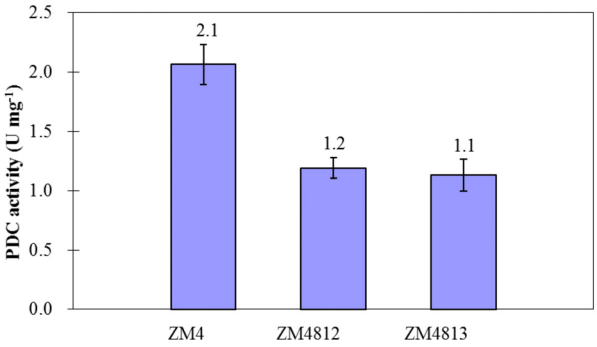

Fig. 3. Comparison of the PDC activity of the parental strain ZM4, the mutant strain ZM4812 (PDC insertional inactivation) and the mutant strain ZM4813 (PDC deletion inactivation). Data is average of three sets of analyses each carried out in duplicate. The error bars show standard deviations.

2.0 U mg total protein^{-1}, approximately the same as for ZM4 grown under same conditions.

Fermentation studies

Fermentation studies with ZM4813 and ZM4 as a control were carried out with the medium containing 50 g glucose l^{-1} as described previously. The medium also contained 100 μg chloramphenicol ml^{-1} for strain stability. As shown in Fig. 4, ZM4813 required approximately twice the time to fully utilize 50 g glucose l^{-1} compared to ZM4, and its growth resulted in lower biomass production although inoculums concentrations were similar. Final ethanol concentrations were similar although transient pyruvate accumulation up to 2.5 g l^{-1} occurred in ZM4813 presumably reflecting its decreased PDC activity. Lactate and succinate reached similar concentrations when compared to those of ZM4, although their accumulation occurred at slower rates for ZM4813.

Kinetic analyses of the fermentation results were performed for both strains and the results are summarized in Table 1. Lower biomass yield and lower specific rates of

Table 1. Kinetic analysis of parent ZM4 and PDC mutant strains

Kinetic parameters	Strains		
	ZM4812	ZM4813	ZM4
Maximum specific growth rate O_{max} (hr^{-1})	0.23[a]	0.24[a]	0.48
Maximum specific glucose uptake rate $q_{smax,\ glucose}$ (g g^{-1} hr^{-1})	6.6	6.3	9.7
Maximum specific EtOH rate q_{pmax} (g g^{-1} hr^{-1})	2.7	2.7	4.5
Yx/s (g g^{-1})	0.025	0.0027	0.036
Yp/s EtOH (g g^{-1})	0.43	0.43	0.48

[a]Values are averages from duplicate experiments for both ZM4812, ZM4813 and the wild type strain ZM4. The results from the kinetic analysis of ZM4813 were very similar to those for ZM4812.

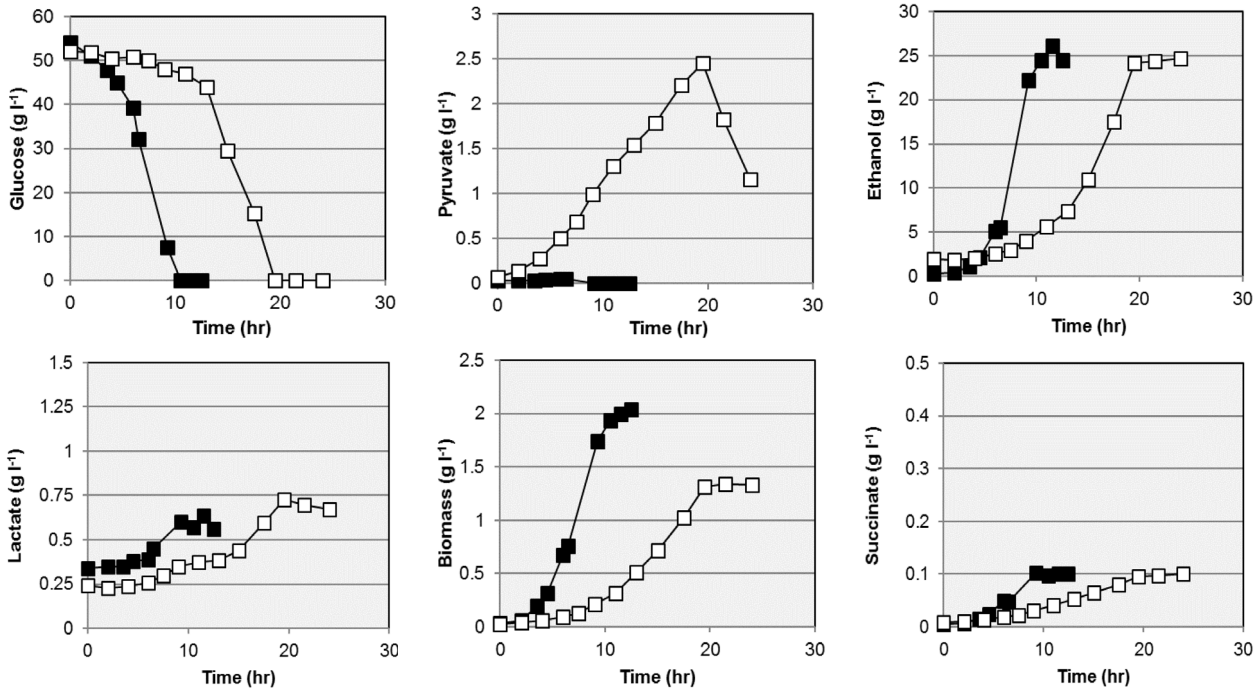

Fig. 4. Fermentation profiles for the wild type ZM4 (closed squares) and the insertion-inactivation mutant ZM4812 (open squares). A similar profile to the latter was found for ZM4813.

growth, glucose uptake and ethanol production were evident for both of the mutants constructed. Ethanol yields were similar, indicating that despite the reduced specific rates, only minimal redirection of metabolism had resulted from a 50% reduction in PDC activity.

Discussion

Despite numerous attempts, no strains were isolated in which complete *pdc* inactivation occurred and this may be a consequence of the energetic imbalance which would then exist in such strains. During the normal metabolism of *Z. mobilis* for the conversion of glucose to ethanol via the Enter-Doudoroff pathway, there is a net gain of 1 mole of ATP per mole of glucose metabolised, as well as reduction of 1 mole of each of NAD (P) [1]. These latter would normally be reoxidized in *Z. mobilis* by reduction of the acetaldehyde formed after decarboxylation of pyruvate to form 2 moles each of ethanol and carbon dioxide per mole of glucose. In the absence of any PDC activity in *Z. mobilis*, the reduction of pyruvate to acetaldehyde could not occur thereby preventing the reoxidation of NADH. Under these conditions such strains would be unable to grow [25].

In the present study, strains were isolated following insertion- and deletion-inactivation of *pdc* which had approximately 50% of their usual PDC activity. One possibility is the presence of multiple copies of *pdc* in the genome of *Z. mobilis* ZM4. However, this was not found in the earlier analysis of the sequence data of the chromosomal DNA by Seo et al. [22]. In previous studies on yeasts as reported by Schmitt and Zimmermann [21] it was found that a *pdc* gene inactivated strain of *S. cerevisiae* could maintain partial PDC activity due to the presence of a second structural gene. Other authors have proposed possible mechanisms for gene duplication in various microorganisms [3, 6, 14, 30,]. The occurrence of gene duplication has been focused in the genomes of a number of bacteria, archaea and eukaryotes, and in some cases of significant number of genes have been generated by gene duplication: for example, in *M. pneumonia* and *H. influenza* in bacteria, *A. flugidus* in archaea, and *S. crevisiae*, *C. elegans*, *D. melanogaster* in eukaryotes. The most obvious contribution of gene duplication to evolution is in providing additional genetic material following mutation or drift, and in compensating for specific gene knockout or knockdowns in the various microorganism [30]. Therefore, the present results would suggest an adaptive mechanism in *Z. mobilis* whereby *pdc* inactivation has only partially occurred in ZM4812 and ZM4813. As evident from the PCR analyses, both inactivated and complete *pdc* genes are present in the above strains, and without the latter, the cells

would be unable to generate sufficient energy for cell growth. PDC enzyme activities were markedly decreased in both ZM4812 and ZM4813, although in the absent of selection pressure (100 μg chloramphenicol ml^{-1}), the PDC activities returned to normal levels after approximately 24 generations with no selection pressure to retain inactivated *pdc*.

The present study provides an interesting insight into the metabolism of *Z. mobilis* with its higher ethanol yields and specific ethanol production rates compared to the traditional fermentation yeasts. As the potential exists for the redirection of metabolism in *Z. mobilis* from pyruvate to higher value fermentation products, both gene insertion-inactivation and gene-deletion strategies have been used in the present study to influence PDC activity. From the fermentation data with the resultant strains, it was evident that increased pyruvate accumulation occurred, although the effect was transitional and final ethanol concentrations were similar to those for the parent strain. The strain with trace amounts of lactate and succinate accumulation implicated that other enzymes such as pyruvate dehydrogenase (PDH) and lactate dehydrogenase and enzymes involved in production of succinic acid might serve as metabolic bottlenecks for their end-product production, and these enzyme may need to be heterologously expressed to increase the level of these higher value products.

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초록 : Pyruvate decarboxylase 돌연변이 *Zymomonas mobilis* 균주의 생장 특성 연구

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에탄올 생산 세균 *Zymomonas mobilis*에서 에탄올 생산 경로의 핵심으로 작용하는 효소인, pyruvate decarboxylase (*pdc*) 유전자의 불활성 실험을 통해, PDC 활성이 50% 감소된 PDC 활성 변형균주가 분리되었다. 이러한 균주들의 에탄올 탄소대사 흐름이 고부가가치 화합물인 피루브산, 숙신산 및 젖산 등으로 전환되는지를 발효실험을 통해 평가하였다. 하지만 *pdc*의 발현을 중지시키기 위해 cat-삽입형-pdc와 pdc-결손형 아형 유전자를 전기천공법을 이용해 야생형 균주 ZM4의 염색체에 이식하기 위한 다수의 시도에도 불구하고, 이러한 방법을 통해 분리된 균주들은 대부분 부분적 유전자 불활성 특성을 보였으며, PDC 활성이 완전히 손실된 삭제 돌연변이 균주를 획득할 수는 없었다. PDC활성이 변형된 돌연변이 균주의 발효 실험에서, 야생형 균주와 비교 시 감소된 PDC 효소 활성의 변화로 인해 기질 흡수율과 에탄올 생산율이 감소되어 피루브산 생산이 약 2.5 g l^{-1} 정도로 증가함을 확인하였으나, 젖산과 숙신산의 생산에 현저한 농도 변화를 보이지 못했다. 이러한 결과는 *Z. mobilis*의 산화환원 에너지가 PDC 효소 활성에 의한 에탄올 생산 경로에 전적으로 의존하여 발생한다는 것을 암시하였다. 상기 결과를 토대로 *pdc* 유전자의 완전한 불활성 유도와 산화환원 에너지의 균형은, 젖산 생산을 위한 lactate dehydrogenase, 숙신산 생산을 위한 pyruvate dehydrogenase와 malic enzyme과 같은 효소의 활성 증가를 통해, 세포내 NAD와 NADH 농도의 산화환원 균형이 이루어져야 발생할 수 있음을 시사하였다.