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Enzymes of the Entner–Doudoroff and pyruvate decarboxylation pathways in *Zymomonas mobilis* wild-type CP4 and mutant strains grown in continuous culture

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Abstract

The osmotolerant *Zymomonas mobilis* strain $suc_{40,}$ (containing plasmid pDS3154-*inaZ*), which is capable of producing simultaneously ethanol and ice nuclei protein, was cultivated in a chemically defined complete sucrose medium, as well as in a sugar beet molasses medium in continuous culture. The strain exhibited the normal Monod's relationship between biomass and dilution rate, and between growth substrate concentration and dilution rate. Specific activities of a number of enzymes that appear to control important steps in the metabolic flux of the Entner–Doudoroff and pyruvate decarboxylation pathways were investigated over a range of growth rates in steady state cultures. With the exception of glucose-6-phosphate dehydrogenase and gluconate kinase, all of the enzymes exhibited a very similar pattern for the wild type *Z. mobilis* CP4 and for the osmotolerant mutants, independent of the media used; the enzyme patterns remained relatively constant over the studied growth range. The specific activity of glucose-6-phosphate dehydrogenase was increased 2-fold by decreasing dilution rate in sugar beet molasses. The specific activity of gluconate kinase was 2-fold lower at medium growth rates compared with that at either low or high growth rates. Pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase activities were significantly higher compared with those of the enzymes governing the early steps of the Entner–Doudoroff pathway. The present study, which was designed to determine the behaviour of important enzymes in sucrose metabolism of *Z. mobilis* suc_{40}/pDS3154-*inaZ* grown in continuous culture showed that the microorganism required regulation of specific enzyme activities at the transcriptional level when sugar beet molasses were used as the growth medium.

Introduction

Zymomonas mobilis ferments glucose, fructose and sucrose exclusively via the Entner–Doudoroff pathway, yielding one mole of ATP per mole of sugar with 96% efficiency followed by pyruvate decarboxylation (Gibbs & DeMoss 1954; Buchholz et al. 1987). In this way, it competes favourably with yeasts for ethanol production, as it displays significantly higher specific rates of sugar uptake, higher ethanol productivities in continuous cell recycle systems and lower biomass production (Rogers et al. 1980; Barrow et al. 1984). The interest in enzyme synthesis and activity has been focussed for the past 15 years on the glycolytic flux and on enzymes in the lower part of the fermentative pathway (Osman et al. 1987). During this period all of the enzymes involved have been purified and characterised (for review see Dawes et al. 1966; Bringer-Meyer & Sahm 1988; Doelle et al. 1993).

The ethanol yield obtained from sucrose in batch and continuous cultures is generally lower than that from glucose. This is due to the production of levan and other byproducts, such as sorbitol and fructo-oligomers at higher levels during sucrose metabolism. In contrast, the optimization of fermentation conditions led to an inhibition in the formation of these

byproducts. The ethanol yields from glucose are approximatelly 0.45 – 0.48 g ethanol per g of glucose consumed and of sucrose 0.42 – 0,47 g ethanol per g of sucrose consumed (Viikari 1984; Doelle & Greenfield 1985).

The only data obtained from continuous culture suggested strong depression of glucokinase activity when cells were grown under glucose or nitrogen limitation (Jones & Doelle 1991). In batch cultures, glucose-6-phosphate dehydrogenase, which catalyses the conversion of glucose-6-phosphate to 6-phosphogluconate, appears to be the limiting enzyme in the *Z. mobilis* Entner–Doudoroff pathway (Anderson & Dawes 1984; Barrow et al. 1984; Scopes 1997). The activities of all of the enzymes involved in the formation of pyruvate and ethanol in *Z. mobilis* are far higher than those in yeasts (Buchholz et al. 1987). Pyruvate kinase has some unusual features, including the absence of obvious allosteric effectors such as glucose-6-phosphate and 6-phosphogluconate (Pawluk et al. 1986). Although pyruvate decarboxylase and alcohol dehydrogenase represent 10% of the soluble protein of the cell, their activity enables *Z. mobilis* to maintain a high conversion efficiency to ethanol and carbon dioxide (Buchholz et al. 1987; Hoppner & Doelle 1983).

Continuous culture techniques have been extensively used to examine the influence of growth environment and growth rate on enzyme activity in microorganisms (Karagouni & Slater 1979; Toran-Diaz et al. 1984; Tsai et al. 1992). So far, all published work on *Z. mobilis* has been concerned with the individual enzymes involved in glucose, fructose and sucrose fermentation; have been enzyme activities compared with uptake values in order to determine the rate-limiting steps. However, as the measuring conditions (e.g. batch or continuous cultivation, pH, temperature, carbon source), as well as the background for calculations (cells, crude extracts or purified enzymes) vary greatly, it is difficult to compare values. It is clear therefore, that studies on the key enzymes involved in carbohydrate metabolism in *Z. mobilis* ought to be independent of the physicochemical parameters in order to compare and draw convincing conclusions. Thus, in order to correlate growth rates and enzyme activities we investigated the influence of growth rate on selected enzymes of the Entner–Doudoroff and ethanol production pathways in continuous cultures of a wild type and an osmotolerant mutant *Z. mobilis* strain (Savvides et al. 2000), under the same steady-state growth conditions, using chemically defined and sugar beet molasses media.

Materials and methods

Strains and culture conditions

The wild-type *Zymomonas mobilis* strain CP4 and the osmotolerant mutant suc_{40}, with or without plasmid pDS3154-*inaZ*, were used throughout this work. The mutant strain was derived from the wild-type CP4 following *N*-nitro-N′-nitrosoguanidine treatment by using methods described previously (Savvides et al. 2000). Similarly, the media used throughout this study, i.e., complete sucrose medium (CSM) and sugar beet molasses medium (SBM), and inoculum preparation and continuous culture conditions were as described by Savvides et al. (2000). CSM contained (g l^{-1}): sucrose 100; yeast extract 2; KH_2PO_4 2; $(NH_4)_2SO_4$ 2; $MgSO_4$ 1. SBM contained a 20% (w/v) solution of sugar beet molasses in water supplemented with 2 g of KH_2PO_4 per litre. The continuous cultures were established in a water-jacketed culture vessel (New Brunswick Scientific, USA) with a working volume of 5 l. The sugar beet molasses were kindly provided by the Hellenic Sugar Industry.

Analytical methods

Biomass, ethanol and total sugar concentrations were estimated by using previously described methods (Savvides et al. 2000). Levan concentration was estimated according to Viikari (1984). All points on graphs are the mean of four independent experiments.

Preparation of cell-free extract

The cells were harvested from steady state cultures by centrifuging at 21 250 × *g*, for 20 min, at 4°C. Washed pellets were re-suspended in approximately 5 ml of 0.015 mol l^{-1} ice-cold Tris–HCl buffer, pH 7.0, and cells disrupted by two passages through a French Press, at 83 MPa and 4°C. Remaining intact cells and cell debris were removed by centrifuging at 21 250 × *g*, for 1 h at 4°C; the extracts were used immediately for enzyme assays. Cell disruption efficiency was estimated by determining cell numbers before and after disruption. Protein was estimated by the Lowry method (Lowry et al. 1951), using bovine serum albumin as a standard.

Assays of enzyme activities

Assays were carried out in a Hitachi spectophotometer (model U-1100) at room temperature (25°C). Reactions were initiated by the addition of cell-free extracts. Rates of $NAD(P)^+$ reduction or NAD(P)H oxidation were followed spectrophotometrically at 340 nm (ϵ_{340} = 6.22 cm^{-1} mM^{-1}).

The activities of the following enzymes were measured: glucokinase (E.C. 2.7.1.2, GK; Scopes et al. 1985); glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, G6PDH; Kuby & Noltmann 1966); pyruvate kinase (E.C. 2.7.1.40, PK; Hunsley & Suelter 1969); pyruvate decarboxylases (E.C. 4.1.1.1, PDC) gluconate kinase (E.C. 2.7.1.12, GNK; Zachariou & Scopes 1985); fructokinase (E.C. 2.7.1.4, FK; Scopes et al. 1985) and phosphoglucose isomerase (E.C. 5.3.1.9, G6PI; Maitra & Lobo 1971) and alcohol dehydrogenases (E.C. 1.1.1.1, ADH) were assayed according to Hoppner & Doelle (1983). Enzyme activities were expressed in units (μmol/min) per mg of protein. All points on graphs are the mean of four independent experiments.

Statistical analysis

Statistical analyses were accomplished using the MINITAB statistical package (Minitab Statistical Software, State College, PA.). Minimum significant differences (MSD) were calculated from analysis of variance using the Tukey–Kramer method (Petersen 1985; Fry 1989).

Results

Effect of dilution rate on biomass, residual sugar concentration and ethanol production of Z. mobilis *strains in steady-state continuous culture*

Steady-state cultures of the *Z. mobilis* strains were obtained with dilution rates between 0.04 and 0.14 h^{-1} for the wild-type CP4 (Fig. 1a) and 0.04 and 0.13 h^{-1} for the mutant strains suc_{40} and the plasmid containing suc_{40}/pDS3154-*inaZ* (Fig. 1b). As biomass, residual sugar concentration and ethanol production were identical for the two mutant strains in all of the media tested, only values of suc_{40}/pDS3154-*inaZ* are presented for clarity (Fig. 1b). Culture biomass, measured as dry weight, showed the normal nutrient-limited chemostat pattern with the biomass concentration remaining relatively constant at dilution rates up to 0.10 h^{-1} for CP4 and 0.07 h^{-1} for the mutant strains, thereafter the biomass concentration declined as the dilution rate was increased towards the critical value. Under the same conditions the steady-state biomass concentration of the mutant strains was approximately 19% lower than for strain CP4. Cell density was low above the dilution rate 0.13 h^{-1} and culture washout occurred at dilution rates above 0.14 h^{-1}. Residual sugar concentration showed the normal nutrient limited chemostat pattern for both media, i.e., CSM and SBM, and was very low at dilution rates below 0.10 h^{-1} and, as expected, a sharp increase was observed at dilution rates above 0.12 h^{-1}. Ethanol production followed the pattern of steady state biomass for all strains, and was constant (42 g l^{-1}) between dilution rates of 0.04 and 0.12 h^{-1} for the wild-type CP4 (Fig. 1a) and 0.04 and 0.10 h^{-1} for the mutant strains (Fig. 1b). Thereafter, an 80% decrease in ethanol occurred as a result of the high dilution rate employed.

The levan concentration, which is the main byproduct during growth in CSM and SBM, was also estimated over the studied dilution rate range for both strains. Higher levan concentration was obtained at dilution rate 0.10 h^{-1} for the mutant strain (5 g l^{-1}) and at dilution rate 0.12 h^{-1} for the wild-type strain (5.4 g l^{-1}) (data not presented). At these dilution rates, under the studied conditions, levan concentration did not seem to affect ethanol production.

The wild-type strain CP4 failed to grow in a 20% (w/v) solution of sugar beet molasses over the range D=0.04 h^{-1} to D=0.14 h^{-1} and it was not possible to achieve steady-state conditions. On the contrary, the osmotolerant mutant suc_{40}/pDS3154-*inaZ* produced a simultaneous ethanol (56 g l^{-1}) and ice nuclei protein (−2.1 log ice nuclei × $cell^{-1}$) in SBM (25% w/v) under the continuous growth parameters (data not presented).

Influence of dilution rate on the steady-state activities of the Entner–Doudoroff pathway enzymes

The specific activities of six selected enzymes of the Entner–Doudoroff pathway were examined in steady-state cultures grown under a constant growth environment over a range of dilution rates from 0.04 to 0.13 h^{-1}. In general, the changing patterns enzyme specific activity with dilution rate differed only slightly for all of the strains examined. Here again, values for suc_{40} and suc_{40}/pDS3154-*inaZ* were identical hence only those of the latter are presented for clarity. In particular, fructokinase did not show any detectable

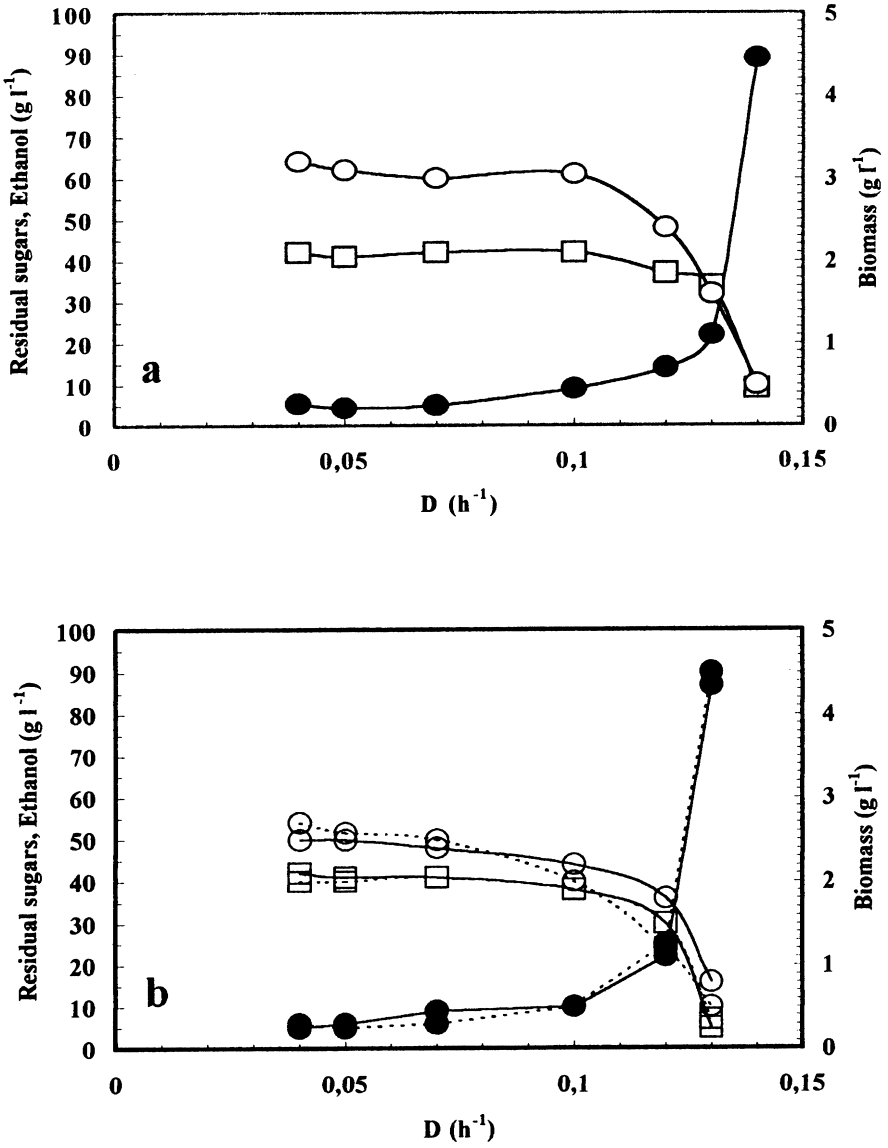

Figure 1. Effect of dilution rate on steady-state biomass concentration (◯), residual sugar concentration (•) and ethanol production (□) of *Z. mobilis* CP4 (a) and suc_{40}/pDS3154-*inaZ* (b) grown in continuous culture containing CSM (—-) and SBM (- - -). All points on the graphs are the mean of four independent experiments with an error probability below 0.05.

activity under all of the growth conditions with all of the strains (Fig. 2a–c). Similarly, the specific activity of phosphoglucose isomerase was relatively constant for all of the strains, in all of the media used over the studied growth range. A slight increase in activity occurred for the wild-type CP4 strain above the dilution rate 0.10 h^{-1} although these changes may not be significant (Fig. 2a). The specific activity of glucokinase showed a slight increase with increasing growth rate above 0.10 h^{-1} for *Z. mobilis* CP4 and displayed maximal activity of 2.5 U mg^{-1} at dilution rate 0.13 h^{-1} (Fig. 2a); whereas it increased slightly with increasing growth rate above D=0.07 h^{-1} for the mutant strains in CSM, whilst remaining constant for all dilution rates examined in SBM (Fig. 2b, c). The specific activity of glucose-6-phosphate dehydrogenase was relatively constant between dilution rates 0.04 and 0.10 h^{-1}, but doubled at the higher dilution rates for CP4 (Fig. 2a). It remained constant for the mutant strains at all values of growth rates ex-

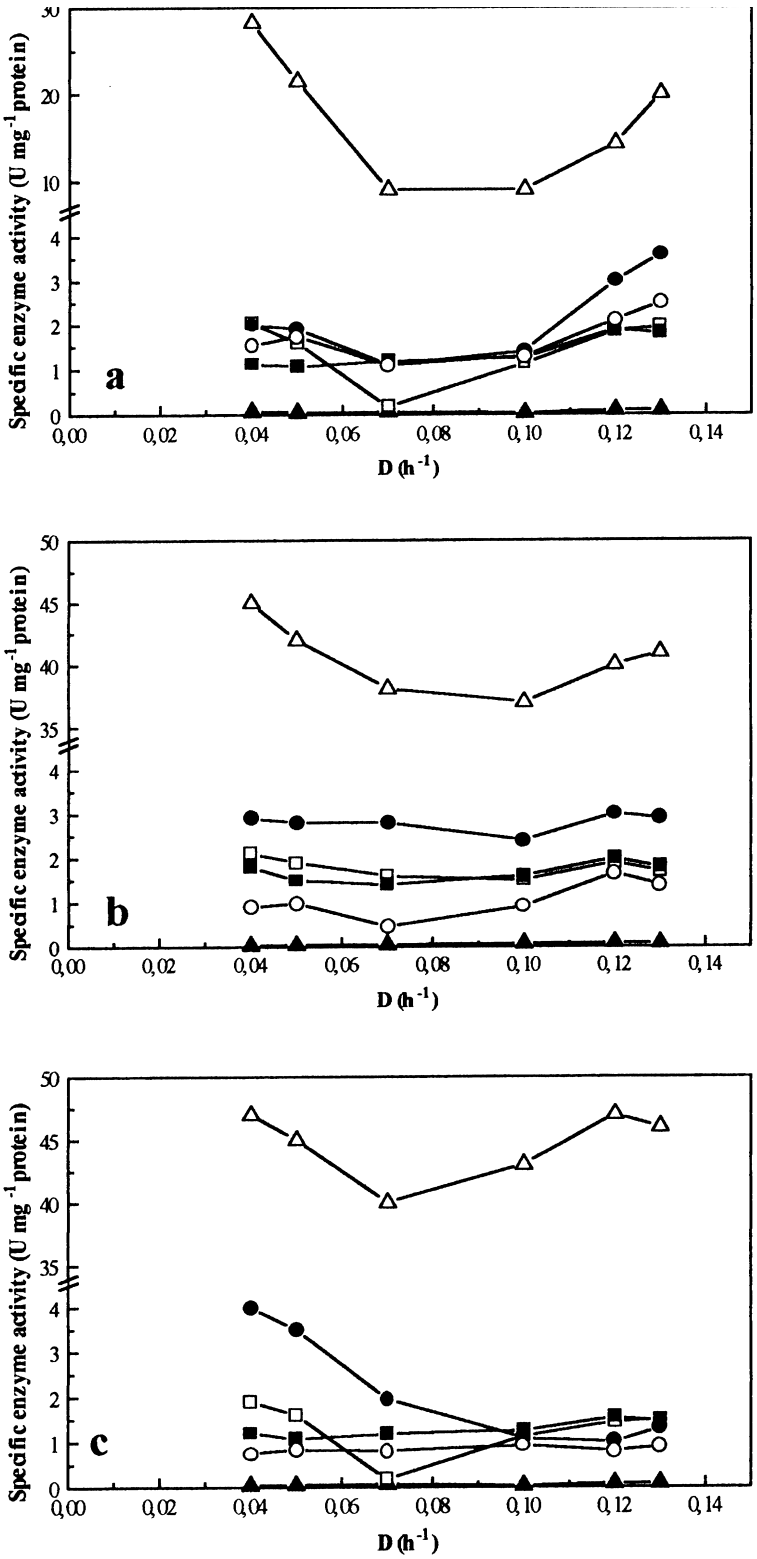

Figure 2. Effect of dilution rate on steady-state specific activity of glucokinase (○), glucose-6-phosphate dehydrogenase (●), gluconate kinase (□), phosphoglucose isomerase (■), pyruvate kinase (△) and fructokinase (▲) of *Z. mobilis* CP4 (a) and suc_{40}/pDS3154-*inaZ* (b) grown in continuous culture containing CSM, and suc_{40}/pDS3154-*inaZ* grown in SBM (c). All points on the graphs are the mean of four independent experiments with an error probability below 0.05.

amined with CSM as medium, but was higher (2.9 U mg^{-1}) than that obtained for the wild-type CP4 strain (Fig. 2b). The activity increased about 2-fold for the mutant strains with decreasing dilution rate in SBM (Fig. 2c). As it is known that the chemical composition of sugar beet molasses may vary significantly from one year's sample to the next, samples from two consecutive years were used and the specific activity of glucose-6-phosphate dehydrogenase in SBM was examined at dilution rate 0.04 h^{-1}. The activity was 1-fold higher when the strain was grown in SBM derived from 1998 molasses compared with the activity in SBM derived from 1999 molasses. It should also be noted that the presence of chloramphenicol, which is used as a selective agent in media associated with the suc_{40}/pDS3154-*inaZ* strain, affected both the activity and percentage of the enzyme protein (data not shown). These results clearly indicate that the composition of sugar beet molasses in combination with low growth rate affected the specific activity of the enzyme.

Dilution rates did not affect the levels of gluconate kinase for all the values of growth rate examined, neither for CP4 nor for the mutant strains grown in the chemically defined CSM, whilst it varied when the mutant strains were grown in SBM (Fig. 2b, c). A decrease in specific activity was observed for strain CP4 between dilution rates 0.05 and 0.10 h^{-1} being limited at dilution rate 0.07 h^{-1}.

The last enzyme of the Entner–Doudoroff pathway, pyruvate kinase, exhibited levels of activity higher than the enzymes mentioned above. Its activity increased 3-fold at low (D=0.04 h^{-1}) and 1-fold at high (D=0.13 h^{-1}) dilution rate for strain CP4, but was relatively low and constant between D=0.07 h^{-1} and D=0.10 h^{-1}. The mutant strains — again identical values were obtained — showed similar pyruvate kinase patterns with the wild-type CP4 when grown in CSM, but the activity level increased only by 1-fold (Fig. 2b). Enzyme activity showed a limit at dilution rate 0.07 h^{-1} when grown in SBM. The activity increased rather sharply below and above this dilution rate (Fig. 2c).

Influence of dilution rate on steady-state enzyme activities in the pyruvate decarboxylation pathway

Pyruvate decarboxylase and alcohol dehydrogenase were the two enzymes from the lower part of the pyruvate decarboxylation pathway that were examined. Both enzymes exhibited levels of activity 10 times higher than the enzymes of the early steps of the Entner–Doudoroff pathway. The wild type CP4 strain only grew in CSM, only values from this medium were compared. The specific pyruvate decarboxylase activities of all of the strains remained constant over the studied growth ranges, with the exception of a 1-fold increase in CP4 at dilution rate 0.04 h^{-1} (Fig. 3a) and a slight increase in the mutant strains below D=0.07 h^{-1} (Fig. 3b). The alcohol dehydrogenase specific activity remained constant for the wild-type strain over the studied growth range, except for the 1-fold increase in activity shown at dilution rate 0.04 h^{-1} (Fig. 3a). Growth rates of mutant strains in either CSM or SBM did not seem to significantly affect enzyme activity, except the 1-fold increased activity of alcohol dehydrogenase (Fig. 3b, c).

Discussion

In terms of biosynthetic capabilities, *Z. mobilis* is the 'phantom' organism for ethanol production (Buchholz et al. 1987), as it exclusively uses the Entner–Doudoroff and pyruvate decarboxylation pathways for ethanol production; the enzymes responsible for this fermentation form as much as 50% of the total soluble protein (Barnell et al. 1990). Several reports on the growth of *Z. mobilis* on glucose have been published, but its growth on sucrose is not well established. Moreover, its growth on complex media containing molasses appears to be very slow and desalting molasses by ultrafiltration (Rhee et al. 1984) or by the construction of salt resistant mutants (Park & Baratti 1991, 1993) were necessary steps to increase growth rates and ethanol productivities. In this context, our study of the wild-type CP4 and its osmotolerant mutant suc_{40} *Z. mobilis* strains in continuous fermentations with complex media containing sucrose from sugar beet molasses provides useful information towards understanding the fermentation of molasses by *Z. mobilis*.

All three *Z. mobilis* strains exhibited a normal Monod relationship between biomass and dilution rate, as well as between growth substrate concentration and dilution rate, when grown in continuous culture under controlled conditions. The range of dilution rates studied was adequately detailed to provide a clear picture of the influence of growth rate on enzyme activities involved in ethanol production. With the exception of pyruvate kinase and glucose-6-phosphate dehydrogenase, all of the other enzymes involved in

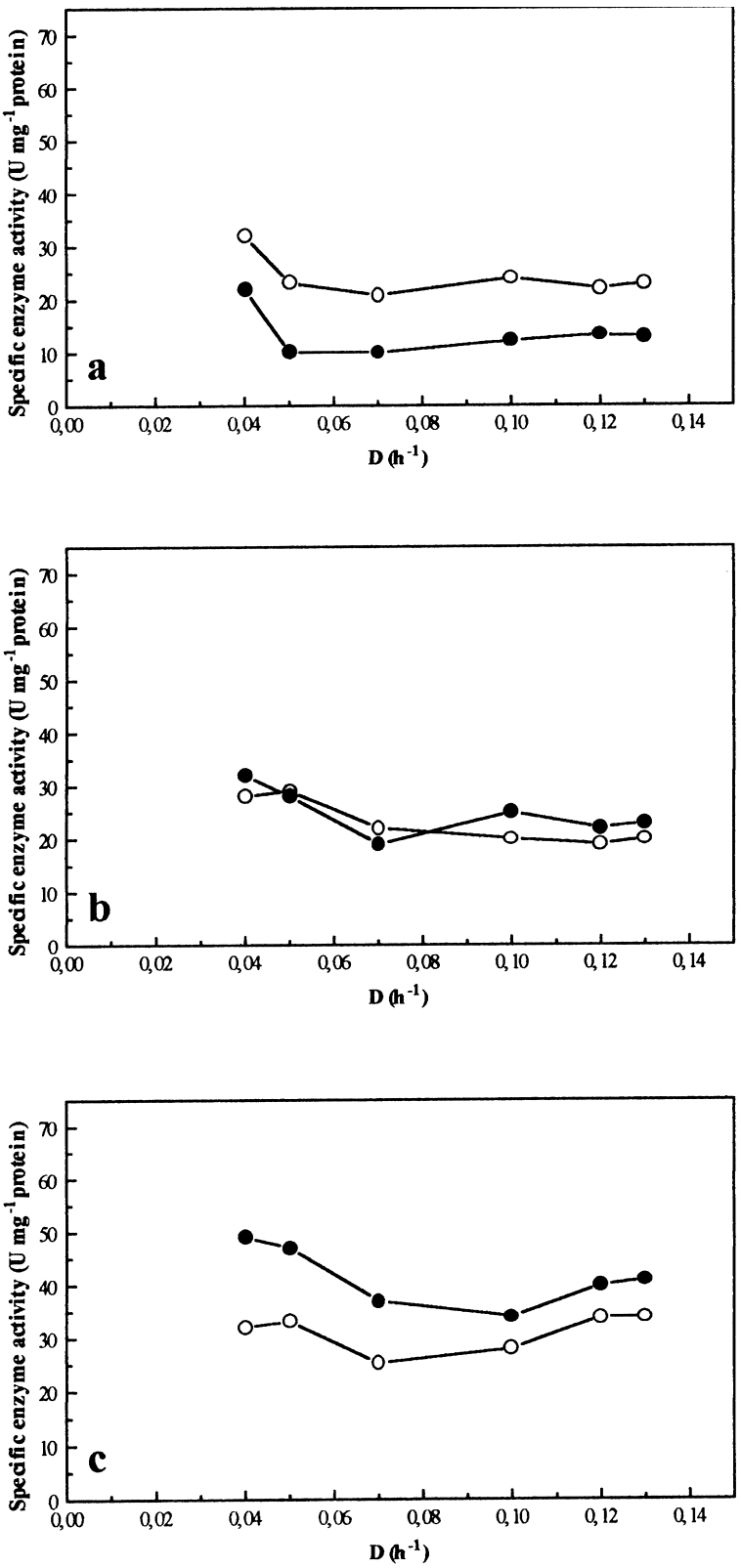

Figure 3. Effect of dilution rate on steady-state specific activity of pyruvate decarboxylase (◯) and alcohol dehydrogenase (•) of *Z. mobilis* CP4 (a) and suc_{40}/pDS3154-*inaZ* (b) grown in continuous culture containing CSM, and suc_{40}/pDS3154-*inaZ* grown in SBM (c). All points on the graphs are the mean of four independent experiments with an error probability below 0.05.

sucrose metabolism in *Z. mobilis* exhibited the same specific activity patterns. A notable result was the low activity of fructokinase which was observed in all cases; this result can be attributed to the concentration of glucose in the cell-free extract which strongly inhibits fructokinase activity (Doelle 1982; Viikari 1984). However, it should be noted that the wild-type strain CP4 failed to grow on the molasses-containing medium SBM, and that small differences in the enzyme activity patterns of glucose-6-phosphate dehydrogenase, gluconate kinase and pyruvate kinase were recorded for the mutant strains grown on CSM and SBM. These results obviously indicate the need of the organisms for enzyme regulation in order to sustain a constant metabolic rate for higher ethanol production. This phenomenon was observed under both low and high growth rates, where the cells are usually under substrate limitation, or washout stress, respectively. The growth rate of 0.07 h^{-1} seemed to be the most favourable for the cells since all of the enzymes showed the lowest activities for the same production result.

Glucose-6-phosphate dehydrogenase is responsible for one of the most crucial steps in the metabolic flux, i.e., the oxidation of glucose-6-phosphate (Barrow et al. 1984; Algar & Scopes 1985), the intracellular accumulation of the latter activates glucose-6-phosphate dehydrogenase to catalyze a flux-generating step (Barrow et al. 1984). In cell free extracts, the level of the glucose-6-phosphate dehydrogenase is very well balanced in order to keep a low concentration of the metabolic intermediate 2-keto-3-deoxy-6-phospho-gluconate, which is very toxic to the cell, (Algar & Scopes 1985). Under the conditions studied, glucose-6-phosphate dehydrogenase the specific activity patterns suggested transcriptional regulation, depending on growth media and growth rate. It is therefore anticipated that *Z. mobilis* elevates its specific enzyme activity to support high ethanol productivity. This deduction is in agreement with results from batch cultures (Osman et al. 1987) which show higher activity of the enzyme during high growth rates (exponential phase) and a 50% decrease in activity at lower growth rates (stationary phase). The specific activity of the enzyme was 2-fold higher at low dilution rates when SBM was used as a growth medium, compared with the CSM medium and high dilution rates. Such an elevation in specific activity has been reported to occur in enzymes that are involved in the initial stages of sucrose metabolism during growth in a limiting nutrient (Dean 1972). As our results show (Fig. 2), both the growth rate of the microorganism and the composition of the molasses affect the enzyme activities. Similar effects of molasses media have been reported on the physiology and morphology of *Z. mobilis* with changes in the protein profile and filament formation of the organism (Fein et al. 1984; Park & Baratti 1993).

The difference in the gluconate kinase specific activity patterns observed for the mutant strains, when grown in CSM and SBM, indicated that their enzymatic activity was substrate dependent. Zachariou and Scopes (1985) reported that the activity of this enzyme was possibly stimulated by the presence of fructose or other effector(s) in the cell during growth in SBM. In addition, the decrease of its activity observed at medium dilution rates suggests that *Z. mobilis* exclusively uses the major route from glucose to gluconate-6-phosphate and not gluconate kinase activity for the production of gluconate-6-phosphate during growth in SBM.

Finally, the wild-type strain CP4 showed a slightly different pattern of pyruvate kinase specific activity than that of the mutant strains, being lower at all dilution rates examined for the former. As this enzyme governs the last step of the Entner–Doudoroff pathway and has been reported to operate near its maximal capacity (Algar & Scopes 1985), it seems likely that it is not regulated. The complete absence of any allosteric effectors for this enzyme support this hypothesis (Pawluk et al. 1986). As all eight enzymes examined in this work operate at maximum capacity in the molasses complex media, we may conclude that the osmotolerant *Z. mobilis* strains, with or without the plasmid pDS3154-*inaZ*, are an ideal tool for ethanol production.

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