Adv Biochem Engin/Biotechnol (2007) 108: 263–288
DOI 10.1007/10_2007_060

Published online: 24 May 2007

Zymomonas mobilis for Fuel Ethanol and Higher Value Products

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Abstract High oil prices, increasing focus on renewable carbohydrate-based feedstocks for fuels and chemicals, and the recent publication of its genome sequence, have provided continuing stimulus for studies on *Zymomonas mobilis*. However, despite its apparent advantages of higher yields and faster specific rates when compared to yeasts, no commercial scale fermentations currently exist which use *Z. mobilis* for the manufacture of fuel ethanol. This may change with the recent announcement of a Dupont/Broin partnership to develop a process for conversion of lignocellulosic residues, such as corn stover, to fuel ethanol using recombinant strains of *Z. mobilis*. The research leading to the construction of these strains, and their fermentation characteristics, are described in the present review. The review also addresses opportunities offered by *Z. mobilis* for higher value products through its metabolic engineering and use of specific high activity enzymes.

Keywords Ethanol production · Glycose/Xylose fermentations · Higher value products · Lignocellulosics · Metabolic engineering · *Zymomonas mobilis*

1 Introduction

Zymomonas mobilis has attracted considerable interest over the past decades as a result of its unique metabolism and ability to rapidly and efficiently produce ethanol from simple sugars. An early paper by Millis [1] characterized the role which *Zymomonas* sp. play in causing cider sickness and a comprehensive review by Swings and DeLey [2] provided much of the background for the subsequent stimulus in research activity in the early 1980s which followed the first of the "oil price shocks". Further reviews over the ensuing decades [3–9] included extensive data on genetic and kinetic characterization of strains of *Zymomonas mobilis* capable of growing on an increasingly wide range of sugars. In a fine example of metabolic (pathway) engineering, recombinant strains of *Z. mobilis* were reported in 1995/6 from the National Renewable Energy Laboratory (NREL) Golden, CO, USA, that were capable of the efficient conversion to ethanol of the C5 sugars, xylose and arabinose present in lignocellulosic hydrolysates [10, 11]. Most recently, the reporting of the complete genome sequence of *Z. mobilis* ZM4 (ATCC 31821) [12] has opened up further potential for strain enhancement and for its use for higher value products.

Table 1 provides an outline of the key research milestones which have occurred for *Z. mobilis* over the past three decades with the present review focusing particularly on those developments which have been reported over the past 5–10 years.

Table 1 Zymomonas research milestones

Activity	Period	Refs.
Review of ethanologenic potential of *Z. mobilis*	Late 1970s	Swings & DeLey [2]
Kinetic confirmation of high rate, high ethanol yields	Early 1980s	Rogers et al. [13] Lee et al. [14]
Batch, continuous and cell recycle evaluations of various strains	Early 1980s	Lavers et al. [15] Lawford et al. [16] Doelle et al. [17]
Development of genetic engineering techniques for *Z. mobilis*	Early 1980s	Skotnicki et al. [18] Dally et al. [19] Drainas et al. [20]

Table 1 (continued)

Activity	Period	Refs.
Cloning of individual heterologous genes to extend substrate range beyond glucose, fructose and sucrose	Mid 1980s	Carey et al. [21] Goodman et al. [22] Strzelecki et al. [23] Su et al. [24]
Characterization of enzymes in the Entner–Doudoroff Pathway	Mid 1980s	Scopes et al. [25] Neale et al. [26, 27]
Cloning of genes to complete pathways for xylose/arabinose utilization	Mid 1990s	Zhang et al. [10] Deanda et al. [11]
Kinetic evaluation of rec strains using glucose/xylose/arabinose media	Late 1990s/ early 2000s	Joachimsthal et al. [28] Joachimsthal & Rogers [29] Lawford et al. [30–38] Mohagheghi et al. [39]
Evaluation of industrial lignocellulosic hydrolysates	Early 2000s	Lawford et al. [38, 40] Mohagheghi et al. [41]
Publication of complete genome sequence of *Z. mobilis* ZM4	2005	Seo et al. [12]
Metabolic engineering for efficient succinate production	2006	Kim et al. [42]
Dupont/Broin Partnership announced to develop *Zymomonas*-based process for ethanol from corn stover	October 2006	Industry report [43]

2
Development of Recombinant Strains of *Z. Mobilis*

2.1
Increased Substrate Range Through Expression of a Single Heterologous Gene

One of the possible disadvantages of *Z. mobilis* is that it has a limited carbon substrate range as it can only use the simple C6 sugars glucose, fructose and sucrose. As a result early studies on its genetic manipulation focused on extending its substrate range for ethanol production. Skotnicki et al. [18] first reported high frequency conjugal transfer of plasmids from *Escherichia coli* and *Pseudomonas aeruginosa*, and this was followed by expression of the *lac Z* gene and production of β-galactosidase in strains of *Z. mobilis* [21, 22]. However, the strain ZM6100 (RP1:Tn 951) derived from this work was shown to progressively lose all plasmid markers in batch culture under non-selective conditions. Subsequently a new strain, ZM6306, was developed in continuous culture which showed 100% stability for all plasmid markers when grown without selection pressure. Synthesis of β-galactosidase was induced

in continuous culture by addition of lactose resulting in increased ethanol production and unutilized galactose [23].

Further studies to extend the substrate range were reported which involved the cloning and expression of a *β-glucosidase* gene from *Xanthomonas albineans* [24] and *α-glucosidase* gene from a *Bacillus* sp [44], however enzyme expression levels were low.

2.2 Strain Construction for Utilization of C5 Sugars

An early attempt was made to construct a xylose-utilizing strain of *Z. mobilis* by Liu et al. [45, 46] involving expression of genes for xylose isomerase (XI), xylulokinase (XK) and the xylose transport protein from *X. albilineans* XA1-1. Although the recombinant strain was shown to possess both XI and XK activity, it was unable to grow on xylose as the sole carbon source. Subsequently, Feldmann et al. [47] constructed a recombinant strain of *Z. mobilis* ZM4 (pZY228) that expressed the *xyl*A and *xyl*B genes from *Klebsiella pneumoniae* for XI and XK, respectively, and the *tkt*A gene for transketolase (TKT) activity from *Escherischia coli*. However, this recombinant strain was also unable to grow on xylose.

On the basis of these earlier studies, Zhang et al. [10] constructed a recombinant strain that successfully converted xylose to ethanol by expression of a transaldolase (*talB*) gene from *E. coli* in addition to those expressing XI, XK and TKT activity. This recombinant strain encoded genes for enzymes both for xylose assimilation (XI, XK) and for completion of the pentose phosphate pathway (TKT, TAL) in *Z. mobilis*. The transformation of wild-type strains of *Z. mobilis* with the 14.4 kb expression vector (pZB5) was then shown to facilitate the efficient conversion of xylose to ethanol via a completed pentose phosphate pathway (Fig. 1).

This research at NREL was continued further by Deanda et al. [11] who successfully developed a strain capable of arabinose utilization. This recombinant strain harbored a plasmid (pZB206) expressing five heterologous genes from *E. coli* encoding L-arabinose isomerase (*ara*A), L-ribulokinase (*ara*B), L-ribulose-5-phosphate-4-epimerase (*ara*D), transaldolase (*tal*B) and transketolase (*tkt*A).

In related studies on the development of a xylose utilizing strain of *Z. mobilis*, De Graaf et al. [48] built on the earlier research by Feldmann et al. [47] and introduced a further plasmid (pZY228) into strain ZM4 (pXY228). This former plasmid harbored *tal*B from *E. coli* thereby facilitating expression of all the requisite additional enzymes in *Z. mobilis* for xylose assimilation and metabolism.

Although there were differences in their construction of these two recombinant strains, the metabolic pathway for both recombinant strains resulting from expression of *xyl*A, *xyl*B, *tkt*A and *tal*B was the same as shown pre-

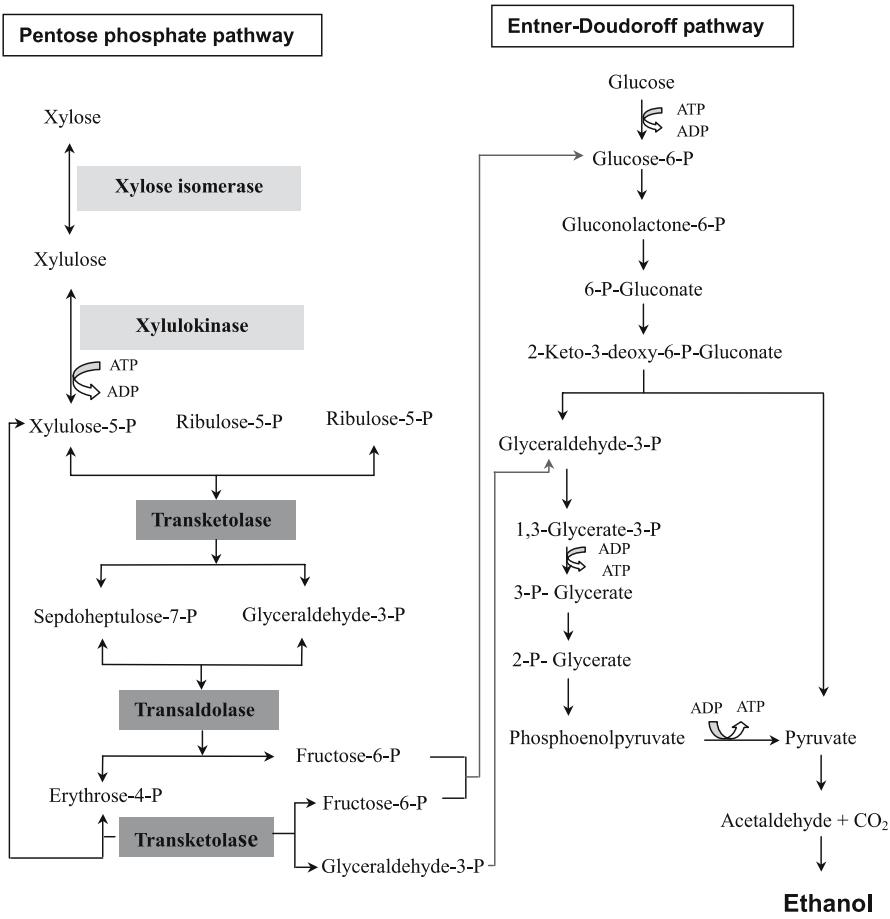

Fig. 1 Pathways for pentose and glucose metabolism (Entner–Doudoroff pathway) in genetically engineered *Z. mobilis* (after Zhang et al. [10]). The shaded enzymes indicate those which have been cloned into *Z. mobilis* from *E. coli*

viously in Fig. 1. Xylose enters the Entner–Doudoroff pathway via fructose-6-phosphate and glyceraldehyde-3-phosphate and is converted into ethanol. The following balance equations represent the metabolism of glucose and xylose by these recombinant xylose-metabolizing strains of *Z. mobilis*.

$$\text{Glucose} + \text{ADP} + \text{Pi} \rightarrow 2\text{Ethanol} + 2\text{CO}_2 + \text{ATP},$$

$$3\text{Xylose} + 3\text{ADP} + 3\text{Pi} \rightarrow 5\text{Ethanol} + 5\text{CO}_2 + 3\text{ATP},$$

Theoretical ethanol yield = 0.51 g ethanol/g sugar (glucose or xylose).

Further studies on recombinant strains created at NREL have involved the construction of integrant xylose-utilizing strains [36–38] and additionally an

integrant xylose/arabinose-utilizing strain designated AX101 [39]. This strain was produced using random insertion and site-specific insertion via homologous recombination.

The specific enzyme activities of the various xylose-utilizing recombinant strains have been determined as a means of identifying possible rate limitations. For the strains developed at NREL, the specific activity associated with XI was the lowest [49, 50]. However, based on calculation of the metabolic fluxes associated with the each enzyme introduced for xylose metabolism, De Graaf et al. [48] concluded for their strain that the flux associated with XK was significantly lower than that of others. This suggested that a metabolic bottleneck may exist in their strain, ZM4 (pZY228) (pZY557 *tal*), due to the low expression of xylulokinase.

Subsequent kinetic studies involving the over-expression of XK (Fig. 2) in an acetate-resistant mutant of the NREL-derived strain ZM4 (pZB5) showed no increase in the maximum specific growth rate or specific rate of xylose metabolism, although there was evidence of a small increase ($0.4\,g\,L^{-1}$) in production of xylitol for the over-expressing strain [51]. Further research on

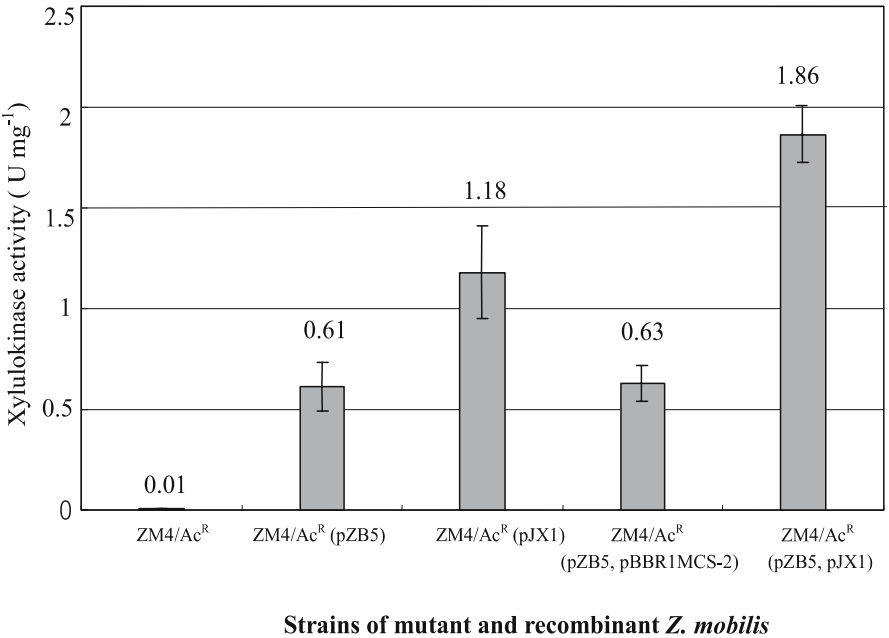

Fig. 2 Xylulokinase (XK) over-expression in acetate-resistant recombinant strains of *Z. mobilis* ZM4/Ac^R (pZB5). Both pZB5 and pJX1 carried genes from *E. coli* for XK expression in *Z. mobilis*. The plasmid pBBR1MCS-2 was based on a broad host range vector suitable for transformation of *Z. mobilis* and used to construct pJX1. Error bars show mean and standard deviation values from triplicate experiments

these recombinant strains is likely to focus on potential rate-limiting sites, as well as expression of heterologous enzymes from other microbial sources for increased ethanol tolerance.

2.3 NMR Analysis of Metabolic Characteristics of Recombinant Strains

The application of ^{13}C and ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy can provide information on both metabolic and energy status during cell growth through determination of the levels of various phosphorylated intermediates and energy rich compounds as shown in earlier studies on wild-type strains of *Z. mobilis* [48, 52–55].

More recent research with ^{31}P NMR has identified a less energized state of ZM4 (pZB5) when grown on xylose media [56, 57]. ^{31}P NMR studies have established that levels of nucleoside tri-phosphates (mostly ATP) and sugar phosphates were lower for growth on xylose compared to that on glucose, with this energy limitation resulting in a potential growth restriction. The presence of by-products identified as xylitol, acetate, lactate, acetoin and dihydroxyacetone by ^{13}C NMR spectroscopy and high-performance liquid chromatography may also result in some inhibition of growth. Further ^{31}P NMR studies [58] have shown that the addition of inhibitory concentrations of sodium acetate caused decreased levels of nucleotide tri-phosphates and sugar phosphates, together with increased cytoplasm acidification.

2.4 Kinetic Characteristics of Recombinant Strains

Detailed kinetic studies have been reported in the literature for several recombinant strains of *Z. mobilis* from NREL capable of utilizing both glucose and xylose. The initial evaluation by Zhang et al. [10] involved the batch culture growth of the strain CP4 (pZB5) on medium containing $25\,g\,L^{-1}$ glucose and $25\,g\,L^{-1}$ xylose. Batch and continuous culture studies on strain 39676 (pZB4L) were reported subsequently by Lawford et al. [31, 33, 34]. This strain was derived from the host ATCC 39676 transformed with a plasmid derived from pZB4. Final product values for $40\,g\,L^{-1}$ glucose/$40\,g\,L^{-1}$ xylose medium included $4.04\,g\,L^{-1}$ xylitol as well as $36.6\,g\,L^{-1}$ ethanol [49] although it should be noted that xylitol levels with this particular recombinant strain were unusually high. Further studies reported by Lawford and Rousseau [35] focused on kinetic and energetic evaluations of strain CP4 (pZB5) in batch and fed-batch fermentations. Kinetic characterization of the chromosomally integrated xylose/arabinose strain AX101 (derived from ATCC 39676) was also reported [37, 38].

To determine which of the strains was likely to be most suitable for larger scale ethanol production, a comparative evaluation in batch and continuous

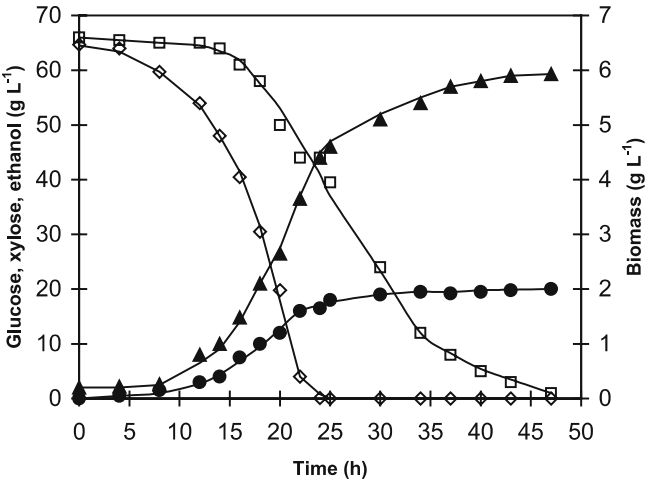

Fig. 3 Kinetics of ethanol production by *Z. mobilis* ZM4 (pZB5) in controlled batch culture on medium containing 65 g L^{-1} glucose and 65 g L^{-1} xylose (T = 30 °C, pH = 5.0). Symbols: ● biomass; ◊ glucose; □ xylose; ▲ ethanol

culture of strains CP4(pZB5) and ZM4(pZB5) was carried out by Joachimsthal et al. [28]. From the results it was found that ZM4(pZB5) was capable of converting a mixture of 65 g L^{-1} glucose and 65 g L^{-1} xylose to more than 60 g L^{-1} ethanol in 48 h in batch culture with an ethanol yield of 0.46 g g^{-1}, with this latter strain demonstrating superior specific sugar uptake and ethanol production rates. The results for ZM4(pZB5) are shown in Fig. 3 together with the values of comparative kinetic parameters in Table 2. Higher sugar concentrations (75 g L^{-1} each sugar) resulted in incomplete xylose utilization (80 h) presumably due to increasing ethanol inhibition of xylose assimilation/metabolism at ethanol concentrations of 65–70 g L^{-1}.

The results for continuous culture with ZM4 (pZB5) and medium containing 40 g L^{-1} glucose and 40 g L^{-1} xylose are shown in Fig. 4 [28]. While the concentration of glucose was close to zero at dilution rates up to D = 0.15 h^{-1}, increasing residual xylose at dilution rates higher than 0.08 h^{-1} indicated that the maximum volumetric rate of xylose uptake for the culture had been exceeded. The maintenance energy coefficient (m) under these conditions was estimated by extrapolation as 1.6 ± 0.2 g g^{-1} h^{-1} (within 95% confidence limits) based on linear regression analysis of the data from Fig. 4a for the maximum specific sugar uptake rate (glucose and xylose) vs. dilution rate (D) (Fig. 4b). A "true biomass yield" of 0.044 g g^{-1} was determined from the inverse of the gradient of this linear plot. For similar experimental conditions, closely related values were observed by Lawford and Rousseau for strain CP4 (pZB5) [34]. However, Lawford and Rousseau noted, when ob-

Table 2 Kinetic Comparison of *Z. mobilis* CP4 (pZB5) and ZM4 (pZB5) on glucose/xylose media ($T = 30\,^{\circ}C$, pH = 5.0). After Joachimsthal et al. [28]

	CP4(pZB5)		ZM4(pZB5)	
	Glucose/xylose (gL^{-1})			
Kinetic parameters	50/50	65/65	50/50	65/65
Max. specific rates				
Glucose/xylose				
μ_m (h^{-1})	0.28	0.27	0.26	0.20
$(q_s)_m$ ($gg^{-1}h^{-1}$)	8.4	6.5	9.5	9.0
$(q_p)_m$ ($gg^{-1}h^{-1}$)	3.1	3.0	4.5	3.8
Max. specific rates				
Xylose				
μ_m (h^{-1})	–	–	0.02	0.01
$(q_s)_m$ ($gg^{-1}h^{-1}$)	1.1	0.6	2.1	2.1
$(q_s)_m$ ($gg^{-1}h^{-1}$)	0.5	0.3	1.0	0.8
Residual xylose (48 h)	0	20	0	0
Overall yields				
$(Y_{x/s})$ (gg^{-1})	0.02	0.02	0.03	0.03
$(Y_{p/s})$ (gg^{-1})	0.46	0.46	0.48	0.46

μ_m: maximum specific growth rate (h^{-1})
$(q_s)_m$: maximum specific sugar uptake rate ($gg^{-1}h^{-1}$)
$(q_p)_m$: maximum specific ethanol production rate ($gg^{-1}h^{-1}$)
$(Y_{x/s})$: overall cell yield (based on total sugar utilized) (gg^{-1})
$(Y_{p/s})$: overall ethanol yield (based on total sugar utilized) (gg^{-1})

served over the lower dilution rate range of D = 0.04–0.08 h^{-1}, that both strain CP4 (pZB5) and a biomass hydrolysate adapted variant of 39676(pZB4L) exhibited values of m and "true biomass yield" that were significantly lower [35].

Results with a potentially high productivity cell recycle system using a membrane bioreactor are shown in Fig. 5 [29]. From Fig. 5(a), at sugar concentrations of 50 g L^{-1} glucose and 50 g L^{-1} xylose and D = 0.1 h^{-1}, an ethanol productivity of 5 g L^{-1} h^{-1} was achieved with an ethanol yield based on total sugars utilized ($Y_{p/s}$) = 0.50 g g^{-1}. No decline in specific ethanol productivity was evident up to 70 h, however as shown in Fig. 5(b), a decrease in total viable cells was observed after an initial steady state (40–50 h). This indicates that for effective longer term operation, high cell concentrations should be

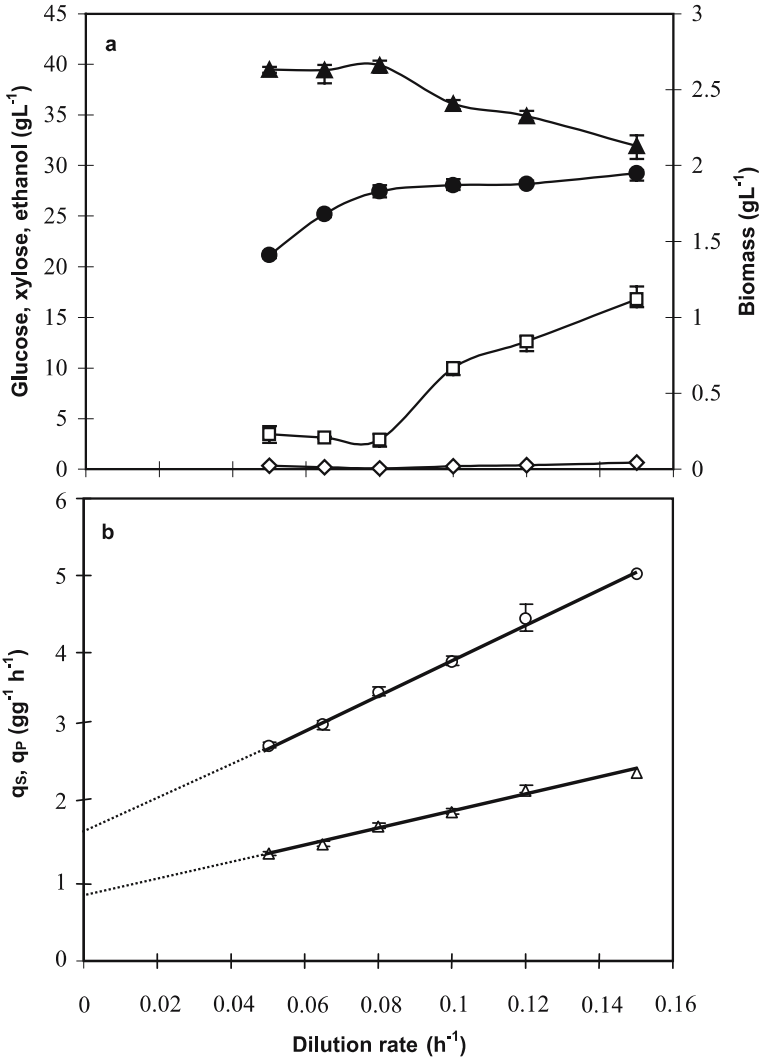

Fig. 4 (**a**) Kinetics of ethanol production by *Z. mobilis* ZM4 (pZB5) in continuous culture on medium containing 40 g L^{-1} glucose and 40 g L^{-1} xylose (T = 30 °C, pH = 5.0). Symbols: biomass •; glucose ◊; xylose □; ethanol ▲ (**b**) Effect of dilution rate on specific rates of total sugar uptake (q_s) and ethanol production (q_p). Estimation of maintenance energy (m) value at D = 0 by extrapolation. Symbols: q_s ○; q_p △

achieved by less stressful methods than membrane-based cell recycling (e.g., by use of flocculent cells and cell settling).

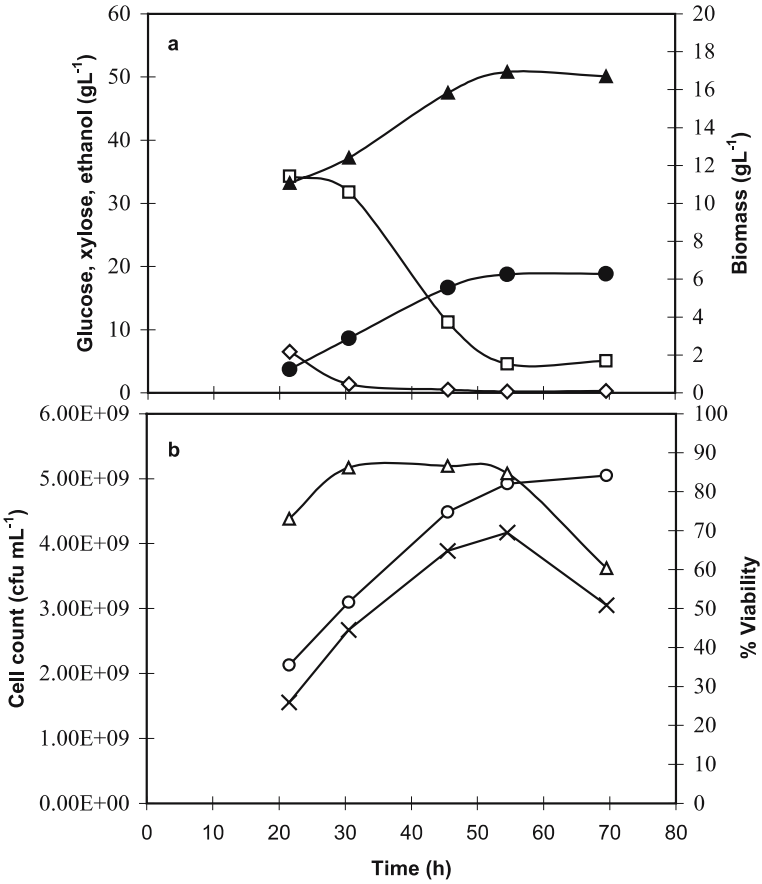

Fig. 5 **a** Time profile for *Z. mobilis* ZM4 (pZB5) for high productivity continuous system with total cell recycle using a membrane Filtron ultrasette and medium containing 50 g L^{-1} glucose and 50 g L^{-1} xylose ($D = 0.1\ h^{-1}$, $T = 30\ °C$, pH = 5.0). Symbols: • biomass; ◊ glucose; □ xylose; ▲ ethanol **b** Total and viable cell counts, and % viability, for continuous cell recycle system. Symbols: total cell count ○; viable cell count △; % viability *x*

2.5 Kinetic Model Development

On the basis of earlier kinetic modelling for the conversion of glucose to ethanol by wild-type *Z. mobilis* [59], a further model has been developed for the fermentation of glucose/xylose mixtures by ZM4 (pZB5) [60]. A two-substrate model was constructed based on Monod kinetics for substrate limitation, as well as functions for product (ethanol) inhibition and substrate inhibition at the higher glucose and xylose concentrations. The model simulation data for various glucose/xylose concentrations were compared with the

experimental results using a Microsoft Excel-based program and statistical analysis for error minimization. Using this approach, it was established that the model (with relevant values of the constants) provided good agreement with the experimental batch culture data for 25/25, 50/50 and 65/65 g L^{-1} glucose/xylose media. It should be noted that the model did not include any repression of xylose uptake by glucose as experimentally both glucose and xylose are taken up simultaneously even at the high initial glucose concentrations. However, this does not preclude the possibility that some glucose repression of xylose might be occurring. The results indicate that ethanol inhibition of xylose utilization is likely to be the more dominant factor in influencing its kinetics.

2.6 Effect of Inhibitors in Lignocellulosic Hydrolysates

A number of components in lignocellulosic hydrolysates can inhibit the growth and ethanol production of bacteria and yeasts, and acetic acid has been identified as a major potential inhibitor of *Z. mobilis* in such acid-produced hydrolysates [61–65]. Lawford and Rousseau [32] examined the role of glucose feeding as a means of improving fermentation performance in acetate-containing media. Another approach to solving this problem has been to use a hydrolysate-fed chemostat to produce adapted or mutant strains [33, 34]. Following chemical mutagenesis, Joachimsthal et al. [66] isolated a mutant strain, designated ZM4/Ac^R with a higher acetate resistance than the parent strain. This strain was then transformed by Jeon et al. [67] to the mutant recombinant ZM4/Ac^R (pZB5). Compared to ZM4 (pZB5), this strain showed enhanced kinetics in batch culture in the presence of 12 g L^{-1} sodium acetate (8.8 g L^{-1} acetic acid) at pH = 5.0 in batch culture on 40 g L^{-1} glucose, 40 g L^{-1} xylose medium. In continuous culture there was evidence of increased maintenance energy requirements/uncoupling of metabolism in the presence of acetate.

In more recent studies Saez-Miranda et al. [68] have determined ATP levels for growth on glucose/xylose media in the presence of different concentrations of acetic acid. From their results they have found that ATP production and accumulation rates are most sensitive to acetic acid at lower pH values—a result consistent with the earlier NMR studies by Kim et al. [57] which demonstrated increasing de-energization of the cells as the inhibitory effects of acetic acid increased. The greater toxicity of acetic acid at lower pH is related to its pK_a value as only unprotonated acid can be transported into the cells.

The effects of a range of inhibitory compounds at levels reported previously for a pre-treated hardwood hydrolysate [65] on specific rates of xylose utilization and ethanol production for ZM4 (pZB5) have been analyzed by Kim et al. [57]. From the results, sodium acetate was found to have the

greatest inhibitory effect at the concentration tested (10.9 g L^{-1} at pH = 6.0), followed by vanillin (0.04 g L^{-1}), syringaldehyde (0.13 g L^{-1}) hydroxymethylfurfural (0.9 g L^{-1}) and furfural (0.3 g L^{-1}). Vanillic acid (0.08 g L^{-1}) did not show any inhibitory effects at this experimental concentration. At the levels tested, these inhibitory compounds did not affect ethanol yields on xylose. Volumetric rates of xylose utilization and ethanol production were reduced by up to 20% by addition of the individual inhibitory components.

2.7 Application to Industrial Raw Materials

Several studies on ethanol production by wild-type strains of *Z. mobilis* on industrial starch-based raw materials have been reported. Bringer et al. [69] investigated an industrial-scale process and Poosaran et al. [70] evaluated a cassava-derived starch hydrolysate. In the latter case in a batch culture at controlled $T = 30\,^{\circ}C$ and pH = 5.0, fermentation using *Z. mobilis* ZM4 gave an ethanol yield of 95% theoretical, a productivity of 6 g L^{-1} h^{-1} and a final ethanol concentration of 114 g L^{-1}. Under the same conditions, a strain of *Saccharomyces uvarum* gave an ethanol yield of 90% theoretical, a productivity of 4 g L^{-1} h^{-1} and a final ethanol concentration of 106 g L^{-1} for a cassava starch suspension (23% glucose equivalent). A comparative batch and continuous culture study with starch hydrolysate using yeast and *Z. mobilis* 29191 has also been reported by Beavan et al. [71].

Extensive studies with various strains of *Z. mobilis* have been reported by using sugar cane syrup and molasses [72–76] and for sugar beet molasses [77, 78] with evidence of yield reductions on sucrose based media due to production of the fructose polymer levan as by-product [3, 6] and rate reductions due to high salt concentrations in the molasses. Improved productivities were reported following membrane desalting of high salt-containing sugar cane molasses [72].

Most recently, Davis et al. [79] studied the fermentation of a hydrolyzed waste starch stream from flour wet milling using both *Z. mobilis* ZM4 and an industrial ethanol-producing strain of *S. cerevisiae*. With glucose concentrations in the range 80–110 g L^{-1}, *Z. mobilis* ZM4 demonstrated superior fermentation characteristics. In a repeated batch process (five cycles), rapid concentration of the cells and increased productivities were achieved by cell settling between batches using the flocculent strain *Z. mobilis* ZM401 (ATCC 31822) as characterized by Skotnicki et al. [80]—see Fig. 6.

Similar flocculent mutants of wild-type *Z. mobilis* strains CP4 and ATCC 29191 have been isolated by Lawford et al. [16] and Fein et al. [81] using a specially designed chemostat. These strains were deposited with the ATCC as strains 35 000 and 35 001, respectively. The use of such flocculent cultures was demonstrated to increase volumetric productivity by as much as ten-fold [82]

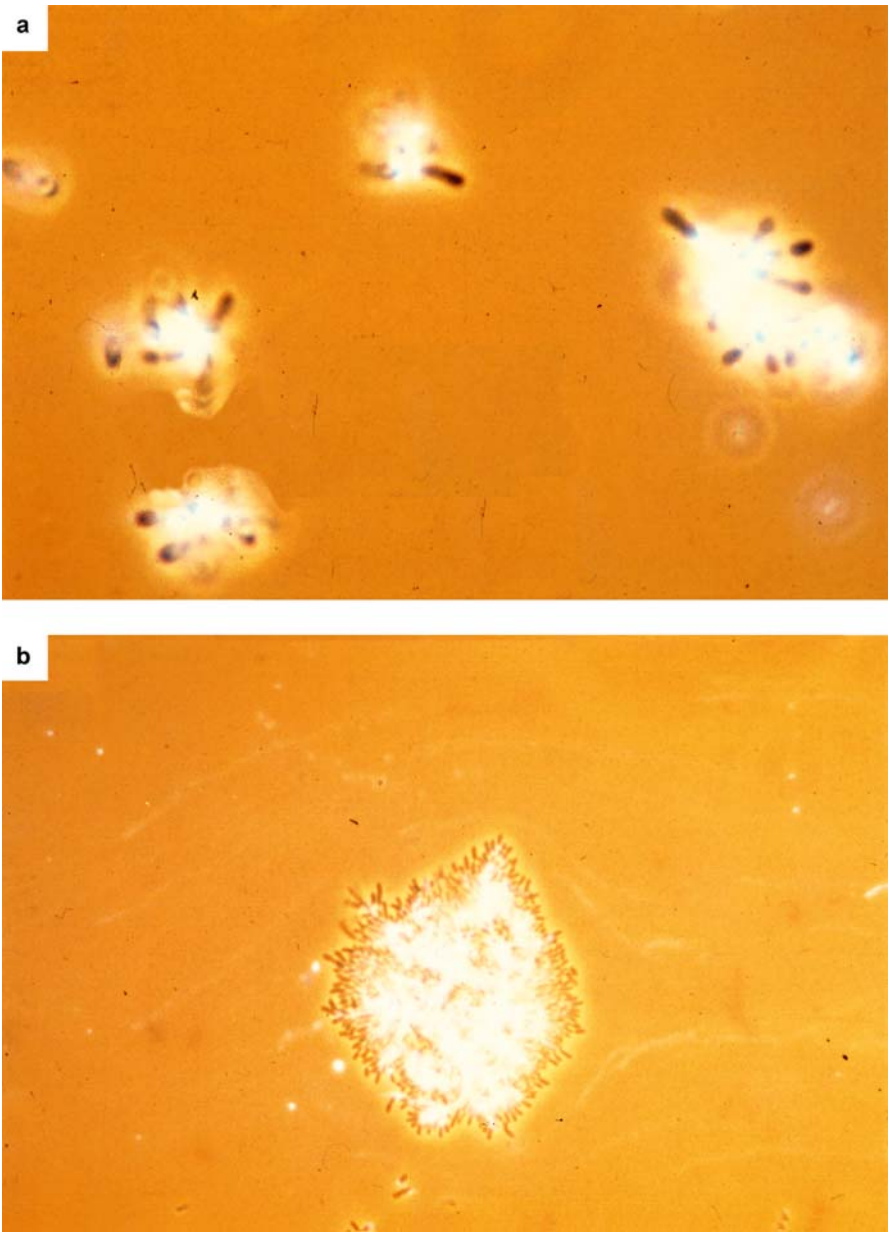

Fig. 6 **a** Photograph showing initial floc formation by a mutant strain of *Z. mobilis* ZM401. This is indicated by cell/cell attachment and fluorescence under UV light following addition of calcafluor which is known to bind to cellulose. **b** Photograph showing formation stable floc of ZM401 and its fluorescence following addition of calcafluor. Floc diameter is approx. 130 microns

and may have considerable potential in future large-scale processes for more stable fermentations.

Recombinant strains of *Z. mobilis* developed for xylose utilization have been evaluated on various agricultural residues including oat hull hydrolysate produced by the Iogen process [40]. Oat hull hydrolysate contains glucose, xylose and arabinose in a mass ratio of 8 : 3 : 0.5. Synthetic hydrolysate (6% w/v glucose; 3% w/v xylose; 0.75% w/v acetic acid) at initial pH 5.75 was mixed with either 2 ml L^{-1} corn steep liquor (CSL) or 1.2 g L^{-1} di-ammonium phosphate as N source and used for evaluation of ethanol production. From the results it was concluded that the highest productivity was achieved with *Z. mobilis* ZM4 (pZB5). In this and other studies, CSL was also found to be an effective nutrient source to replace yeast extract in the fermentation media for *Z. mobilis* [83–85].

Further studies were reported by Mohagheghi et al. [41] with an integrant strain (designated *Z. mobilis* Fig. 8b) derived from ZM4 (pZB5) using overlimed corn stover hydrolysate. The hydrolysate contained 16 g L^{-1} glucose, 69 g L^{-1} xylose and 11 g L^{-1} acetic acid at pH = 5.0. This medium was supplemented with 100 g L^{-1} glucose and diluted to various concentrations prior to fermentation. The authors found that up to 50 g L^{-1} ethanol was produced by the integrant strain with diluted 80% corn stover hydrolysate. Yields of 83–87% theoretical (based on sugars utilized) were reported.

One of the potential issues for large-scale *Z. mobilis* fermentations is whether or not contamination control is needed particularly in the presence of ethanol-tolerant strains of *Lactobacilli*. Such contamination constitutes a problem in many yeast-based processes and can reduce yields by an estimated 2–5%. However, its impact is reduced as pH decreases to 3.0–3.5 towards the end of batch fermentation (in the absence of pH control). "Acid washing" of the residual yeast at this pH or lower is often used to minimize contamination in yeast subsequently used in a repeated batch process. *Z. mobilis* is more sensitive to low pH than *S. cerevisiae* and contamination was identified as a problem by Bringer et al. [69] in their study on an industrial-scale process for conversion of starch to ethanol using *Z. mobilis* although Lawford and Rousseau [63] demonstrated that lactic acid in such circumstances is not likely to be inhibitory to *Z. mobilis*. Interestingly, although rarely observed in *Z. mobilis* fermentations due to the usual high metabolic flux rates in the ED pathway, conditions have been reported which can promote lactic acid synthesis in *Z. mobilis* [37, 85].

The issue of contamination control was addressed directly by Grote et al. [86] in which a continuous culture of *Z. mobilis* ZM4 was directly contaminated with a 10% (v/v) inoculum of *Lactobacillus* sp. isolated as an ethanol-tolerant contaminant from an industrial plant (Grain Processing Corporation, Muscatine, Iowa). It was found at $D = 0.1\ h^{-1}$ under conditions of glucose limitation, pH control at 5.0 and ethanol concentrations of 60–65 g L^{-1}, that the addition of the contaminant caused only a temporary

disturbance in the process. Steady state conditions with no evidence of sustained contamination were regained within five to six generations. These results suggest that contamination is not likely to be a significant problem once an active culture of *Z. mobilis* is established providing that the pH is maintained above 3.5–4.0. A similar conclusion was reached in a recent study [87] using an acid-tolerant strain of *Z. mobilis* under non-sterilized feed and operating conditions.

3 Genome Sequence of *Z. Mobilis*

As discussed earlier, the complete genome sequence of *Z. mobilis* ZM4 has been reported recently [12] following earlier related studies by Korean scientists [88–90]. It was found that the genome consists of 2 056 416 base pairs forming a circular chromosome with 1998 open reading frames (ORFs) and three ribosomal RNA transcription units. As reported by the authors, "the genome lacks recognizable genes for 6-phosphofructokinase, an essential enzyme in the Embden–Meyerhof–Parnas pathway, and for two enzymes in the tricarboxylic acid (TCA) pathway, the 2-oxoglutarate complex and malate dehydrogenase. Glucose can be metabolized therefore only by the Entner–Doudoroff pathway".

Comparison of whole genome microarray data for *Z. mobilis* ZM1 (ATCC10988) and ZM4 (ATCC 31821) revealed that the 54 ORFs present in ZM4 were absent for ZM1. Four of these ORFs that encode transport proteins or permeases, and two that encoded for specific enzymes—NAD(P)H:quinone oxidoreductase and an oxidoreductase related to short-chain alcohol dehydrogenases, were found to be highly expressed in *Z. mobilis* ZM4. The authors suggested that it is possible these genes relate to the higher specific rates of sugar uptake and ethanol production for ZM4 when compared to ZM1. They also reported that two genes encoding capsular carbohydrate synthesis enzymes were only actively expressed in ZM4 and may contribute to its relatively high resistance to increased osmotic pressure found in high sugar solutions (e.g. in 250–300 $g\,L^{-1}$ glucose media).

4 Applications for Higher Value Products

4.1 Metabolites and Related Products

The production of a range of byproducts from *Z. mobilis* is reviewed comprehensively by Johns et al. [6] and Panesar et al. [8] with the former authors

identifying potential commercial opportunities for the following products: fructose (using sucrose and a fructokinase negative mutant), sorbitol and gluconic acid, levan (a fructose polymer), fructo-oligosaccharides and various enzymes. As pointed out in a review by Scopes [91], *Z. mobilis* is a rich source (on an enzyme content per g cell basis) of many of the enzymes currently used in diagnostic analysis and research. Interestingly, in other studies by Park et al. [92], it was established that the activities of some of the key ED enzymes (e.g. glucokinase, G-6-phosphate dehydrogenase) were unaffected by the relatively high ethanol concentrations produced during fermentation, while the activity of an enzyme such as transketolase decreased appreciably above ethanol concentrations of 60 g L^{-1}. It has been estimated that for actively growing cells, as much as 30–50% of the cellular protein is comprised of ED enzymes [93]. However, the greatest difficulty for the commercial production of such enzymes is the low cell yield of *Z. mobilis* which is typically 0.02–0.03 g g^{-1} substrate sugar, compared to cell yields close to 0.5 g g^{-1} for many aerobically grown microorganisms.

4.2 Metabolic Engineering for Organic Acids and TCA Cycle Intermediates

There has recently been considerable interest in the redirection of metabolism in bacteria such as *E. coli* for the overproduction of specific metabolites and higher value products. At a commercial level, the large-scale production by Tate & Lyle/Dupont of 1,3-propandiol using a highly engineered strain of *E. coli* is indicative of an increasing trend towards such bio-based processes. The fast specific rates of sugar uptake by *Z. mobilis*, its highly efficient metabolism for a specific product (ethanol), and its relatively small genome size (facilitating genetic manipulation) may make it an ideal candidate for producing other metabolites via its genetic engineering.

As shown in Fig. 7, *Z. mobilis* has an incomplete TCA cycle and the potential exists via "knock out" mutation to redirect metabolism away from end-products such as lactate and ethanol, towards higher value products like succinic acid. As reported recently by Kim et al. [42], succinic acid overproducing *Z. mobilis* strains have been developed by disruption of the genes for pyruvate decarboxylase (*pdc*) and lactate dehydrogenase (*ldh*). Such strains can produce relatively high concentrations of succinic acid at yields of 1.73 mole/mole glucose (86% theoretical). The yield was reported to be more than 30% greater when compared to those of other succinic acid-producing bacteria such as *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* (about 1.34 mole/mole glucose). These strains of *Z. mobilis* were also reported to exhibit higher overall rates of succinic acid production (1.62 g L^{-1} h^{-1}) under Na bicarbonate supplemented conditions compared to those of other succinic acid producing bacteria (1.35 g L^{-1} h^{-1}).

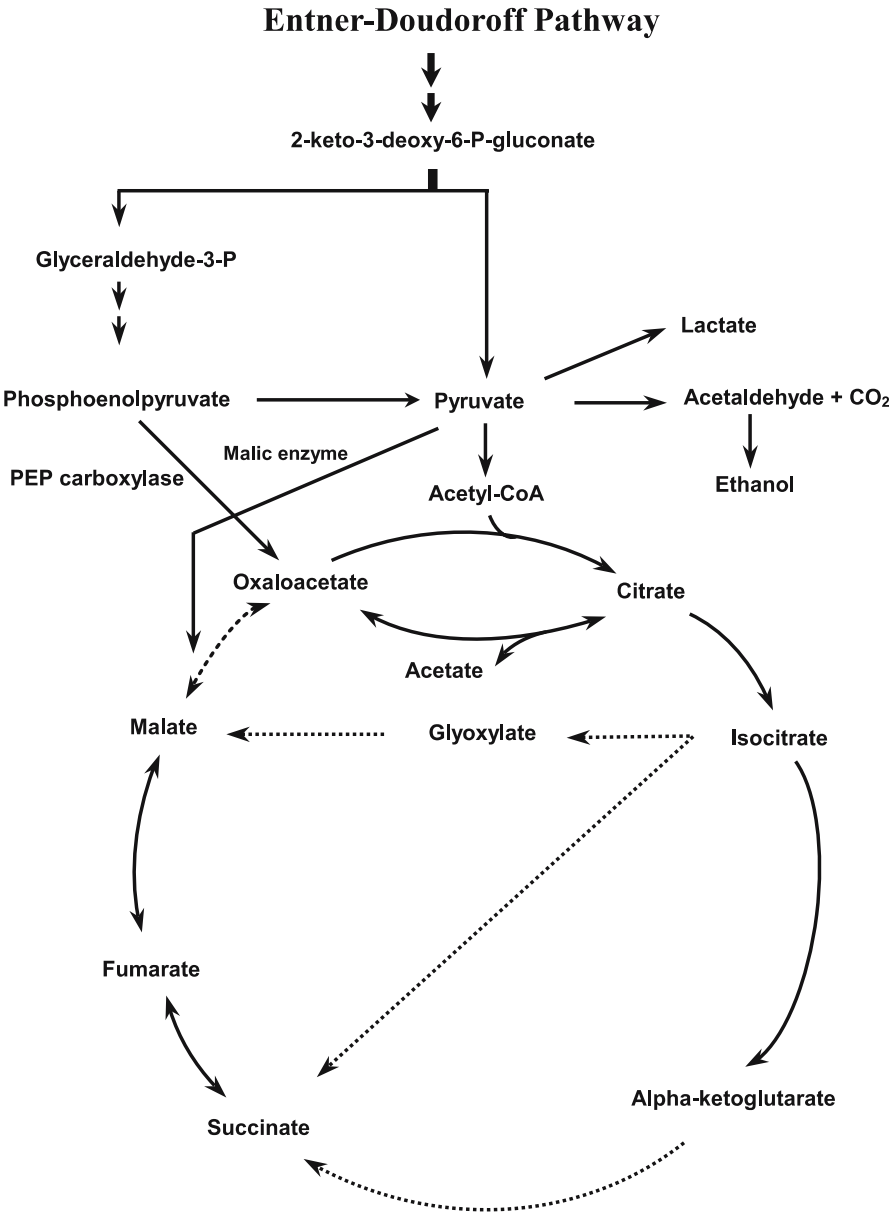

Fig. 7 Incomplete TCA cycle in *Z. mobilis* (after Seo et al. [12]) showing the potential for redirection of its rapid and efficient glucose metabolism to TCA intermediates by the use of "knockout mutants". Missing reactions are shown by *dotted lines*

4.3
Enzyme Based Biotransformations

4.3.1
Sorbitol/Gluconate Production

The production of sorbitol by *Z. mobilis* when grown on sucrose or a mixture of glucose and fructose has been reported earlier by several groups [94, 95]. In subsequent studies on the mechanism of sorbitol production, an enzyme complex was identified by Leigh et al. [96] which was capable of oxidizing glucose to gluconic acid concomitant with the reduction of fructose to sorbitol. This enzyme was described as a glucose-fructose oxidoreductase with a tightly coupled (non-dialyzable) co-factor identified as NADP [97]. The mechanism for sorbitol/gluconic acid production and the associated enzymes are shown in Fig. 8 with the pathway from gluconate to ethanol not being functional if cells of *Z. mobilis* are fully permeabilized. As shown in Fig. 8, the possibility exists also of producing a mixture of sorbitol and gluconolactone if gluconolactonase activity is deleted.

Kinetic studies have been reported for a 60% sugar solution (300 g L^{-1} glucose and 300 g L^{-1} fructose) using toluene-treated permeabilized cells of *Z. mobilis* in which a sorbitol concentration of 290 g L^{-1} and a gluconic acid concentration of 283 g L^{-1} were achieved after 15 h in a batch process [98]. A continuous process with immobilized cells was developed with only a small loss of enzyme activity (less than 5%) evident after 120 h. With a strongly basic anion exchange resin and a buffer system at pH = 9.0, good separation of sorbitol and gluconic acid was achieved. Subsequent studies using immo-

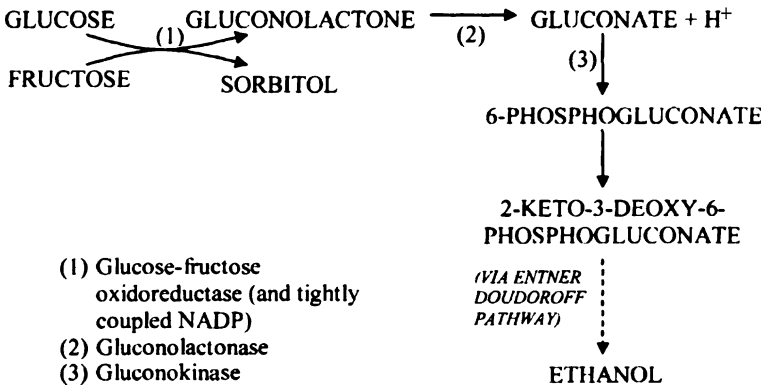

Fig. 8 Mechanism for rapid and efficient conversion of glucose to gluconic acid and fructose to sorbitol via action of glucose-fructose oxidoreductase (and tightly coupled NADP) and gluconolactonase in toluene treated cells of *Z. mobilis* ZM4

bilized (permeabilized) cells in a membrane bioreactor have confirmed the potential of this process for rapid and efficient product formation [99].

4.3.2 Pharmaceutical Intermediates and Fine Chemicals

R-phenylacetylcarbinol (PAC), an intermediate in the production of ephedrine and pseudoephedrine, is currently produced by the controlled addition of benzaldehyde to an actively growing culture of yeast (usually *Saccharomyces cerevisiae*). A decarboxylation/condensation biotransformation is effected by pyruvate decarboxylase (PDC) between pyruvate produced by the yeast and added benzaldehyde (see Fig. 9). Using this traditional process, 12–15 g L^{-1} PAC is usually produced in 10–12 h with a yield of 70% theoretical based on benzaldehyde [100].

Confirmation of PAC production from benzaldehyde and pyruvate using purified PDC from various sources, including *Z. mobilis*, *S. carlsbergenis*, *S. cerevisiae*, *S. fermentati* and *S. delbrueckii*, was demonstrated by several groups during the late 1980s to mid 1990s [101–105]. Bringer-Meyer et al. [106] isolated and characterized PDC obtained from *Z. mobilis*. By comparison with yeast PDC (*Saccharomyces* sp., *Candida* sp.), bacterial PDC (*Zymomonas* sp.) had a lower benzaldehyde affinity and was inhibited more strongly by benzaldehyde, even though its affinity for pyruvate was similar or higher than that of yeast PDC.

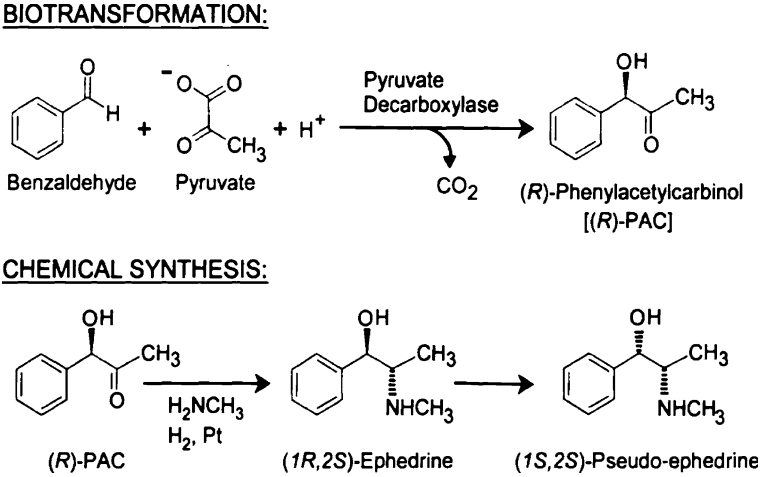

Fig. 9 Mechanism for production of pharmaceutical intermediate R-PAC from benzaldehyde and pyruvate via decarboxylation and condensation using an enzymatic process based on pyruvate decarboxylase present in fungi, yeasts and bacteria (including *Z. mobilis*)

However, interest in PDC from *Z. mobilis* continued due to its greater stability than yeast PDC at room temperature with an enzyme half-life in the absence of benzaldehyde of over 100 h [107, 108]. Unlike yeast PDC, it is also able to utilize the lower cost acetaldehyde as an alternative substrate to pyruvate for production of PAC [109]. Advances in site-directed mutagenesis techniques have facilitated the production of mutant PDC from *Z. mobilis* with greater carboligase activity and higher stability towards acetaldehyde [110]. This mutant enzyme, designated PDCW392M, resulted from replacement of the bulky tryptophan residue 392 with methionine. A continuous process with PDCW392M was used in a biotransformation process for conversion of acetaldehyde and benzaldehyde to PAC in an enzyme membrane reactor. A volumetric productivity (space-time yield) of 81 $g\,l^{-1}\,day^{-1}$ was reported with final PAC concentration of 22 mM and molar yields of 45% (initial substrates), based on 50 mM reaction mixture of both aldehydes [111, 112].

In further studies by Rosche et al. [113], a biphasic enzymatic biotransformation system for production of PAC from acetaldehyde and benzaldehyde with *Z. mobilis* PDCW392 was evaluated. Higher concentrations of benzaldehyde and PAC in the organic phase (octanol) provided protection for the aqueous phase PDC. As a result, a specific PAC production of 11 mg PAC U PDC^{-1} was achieved compared with 1.2 mg PAC U PDC^{-1} in the absence of an organic phase. A similar two-phase system has been developed subsequently for conversion of pyruvate and benzaldehyde to PAC using PDC from yeast (*C. utilis*) with higher concentrations and productivities being attained [114, 115].

A similar aqueous/organic two-phase system has been used also to screen a number of yeasts and bacteria for the enantio-specific reduction of the alpha, beta-unsaturated carbon bond in citral to produce citronellal [116]. In comparison to the bacteria tested, the eukaryotes showed at least 5-fold lower citral reductase activities. Bacterial strains were found to produce the (S)-enantiomer of citronellal preferentially with ee values > 99% for *Z. mobilis* and 75% for *Citronella freundii*. The possible use of a *Z. mobilis* biofilm bioreactor for production of other fine chemicals has been proposed also [117] as it has been demonstrated that increased tolerance to aromatic substrates such as benzaldehyde can occur with such a bioreactor.

5 Discussion and Conclusions

As outlined in the earlier reviews and summarized in Table 3, wild-type strains of *Z. mobilis* (and their mutants) can convert simple sugars to ethanol at faster rates and higher yields compared to yeasts. However, the ethanol industry has traditionally used yeasts, and despite the apparent advantages of *Z. mobilis*, there appears to be little incentive for change with sugar and

Table 3 Characteristics of *Z. mobilis* for production of fuel ethanol and higher value products

1. Considerably faster specific rates of sugar uptake and ethanol production (specific rates 2–3 times faster than yeasts).
2. Higher ethanol and lower biomass yields compared to yeasts due to different carbohydrate metabolism (Entner–Doudoroff vs. glycolytic pathway).
3. Higher reported productivities (120–200 $g\,L^{-1}h^{-1}$) in continuous processes with cell recycle (maximum reported values for yeasts are 30–40 $g\,L^{-1}\,h^{-1}$).
4. Simpler growth conditions. *Z. mobilis* grows anaerobically (not strict anaerobe) and does not require the controlled addition of oxygen to maintain cell viability at high ethanol concentrations.
5. Ethanol tolerance comparable if not better than yeasts. Ethanol concentrations of 85 $g\,L^{-1}$ (11% v/v) reported for continuous culture and up to 127 $g\,L^{-1}$ (16% v/v) in batch culture.
6. Laboratory scale studies with strains of *Z. mobilis* over many years in controlled fermentations (pH = 5.0, $T = 30\,°C$) have not revealed any significant contamination or bacteriophage infection problems.
7. The wide range of techniques developed for the genetic manipulation of bacteria (such as *Escherichia coli*) can be applied to developing recombinant strains of *Z. mobilis* and/or their metabolic engineering.
8. Integrant rec strains of *Z. mobilis* available for efficient ethanol production from glucose, xylose and arabinose. Ethanol concentrations above 60 $g\,L^{-1}$ in 48 h reported for medium containing 65 $g\,L^{-1}$ glucose, 65 $g\,L^{-1}$ xylose.
9. Sequencing of ZM4 genome now provides information for its metabolic engineering for additional higher value products (e.g., succinic acid).
10. Potential for use of its enzymes for fine chemical biotransformations.

starch-based raw materials. Some of the reasons lie in the concerns that *Z. mobilis* may be less robust than yeast and more susceptible to contamination in large-scale processes, as well as the lack of ethanol industry experience with large-scale bacterial fermentations. In addition, an established feed market exists for the high protein yeast by-product (as dried distiller's grains) and any new market for a high protein by-product from a Zymomonas process would need to be established. The key issues and alternative capabilities are summarized in Table 4.

The construction of recombinant strains of *Z. mobilis* able to use the additional C5 sugars xylose and arabinose have now opened up new opportunities as illustrated by the recently announced Dupont/Broin partnership to develop a Zymomonas-based process for conversion of corn stover to ethanol [43]. In an Integrated Corn Biorefinery (ICBR), this would be associated with conversion also of the corn starch to higher value products (e.g. to 1,3-propandiol using recombinant strains of *Escherichia coli*). Experience with large-scale recombinant bacterial fermentations could provide a future platform as well for an increased range of higher value products generated via the metabolic engineering of micro-organisms such as *Z. mobilis* which are capable of both rapid and highly efficient sugar metabolism.

Table 4 Possible reasons for non-commercialization of Zymomonas process for ethanol production from sugar and starch-based raw materials

	Issue	Comments
1.	Yeast is established ethanol producer for sugar and starch-based fermentations.	Established industry practice outweighs potential advantages of higher productivities and yields with Zymomonas-based process. No glycerol by-product with latter process may be potential advantage in minimizing 'fouling' problems during distillation.
2.	Yeast is more robust in terms of tolerance to inhibitors, salts and low pH conditions.	Both yeast and *Z. mobilis* influenced by inhibitors although *Z. mobilis* is more sensitive to high salt concentrations (e.g. in molasses) and low pH (less than 3.5). Inhibitor resistant strains of *Z. mobilis* have been isolated from lignocellulosic hydrolysates.
3.	Yeast has high protein by-product value in dried distiller's grain (DDG) for animal feed supplementation.	*Z. mobilis* is a GRAS organism and has higher crude protein content (65–70%) than yeast (50–55%). Niche nutritional/high protein feed market would need to be developed for *Z. mobilis* following animal feeding trials.
4.	Contamination control (e.g., with lactic acid bacteria) is easier to achieve with yeast using low pH conditions.	Laboratory experiments in continuous culture (pH = 5.0) have shown that *Z. mobilis* is very resistant to contamination once fermentation established (presumably due to much higher competitive rates of sugar uptake). High density cultures (e.g. use of flocs) would further minimize any contamination problems and facilitate high productivity fermentations. No evidence reported of any bacteriophage contamination with *Z. mobilis*. Evidence of high restriction enzyme activity in some strains.
5.	Lack of industry experience as well as regulatory issues with large-scale bacterial fermentations including those with recombinant bacteria.	Experience now being gained by industry with large-scale bacterial fermentations (e.g. production of 1,3-propandiol using rec *E. coli*). Similar regulatory issues would apply for use of recombinant yeasts with lignocellulosic hydrolysates.

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