Ethanol fermentation technology – *Zymomonas mobilis*

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Due to dwindling of fossil fuel, microbial production of bio-fuel from organic byproducts has acquired significance in recent years. Ethanol has been trusted as an alternate fuel for the future. Even though several microorganisms, including *Clostridium* sp., have been considered as ethanologenic microbes, the yeast *Saccharomyces cerevisiae* and facultative bacterium *Zymomonas mobilis* are better candidates for industrial alcohol production. *Z. mobilis* possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance, thus encouraging researchers for exploiting *Z. mobilis* ability to utilize sucrose, glucose, and fructose by Entner–Deudoroff pathway. The bottlenecks in *Z. mobilis* are: (i) its inability to convert complex carbohydrate polymers like cellulose, hemicellulose, and starch to ethanol, (ii) its resulting in byproducts such as sorbitol, acetoin, glycerol, and acetic acid, and (iii) formation of extracellular levan polymer. To circumvent these problems, genetic manipulation of *Z. mobilis* has been attempted for broadening the utilizable range of *Z. mobilis*, i.e. genes encoding several hydrolytic enzymes from related bacterial species have been cloned, and transferred into *Z. mobilis*. Interestingly, a *pet* operon (*p*roduction of *e*thanol) was constructed by combining *pdc* (pyruvate decarboxylase) and *adhII* (alcohol dehydrogenase) genes of *Z. mobilis,* and transferred to other bacterial strains to make them ethanologenic novel strains. Through classical mutation and selection approaches, mutants of *Z. mobilis* with improved fermentation characteristics and without byproduct formation have been obtained. In addition to ethanol, *Z. mobilis* has also been metabolically engineered to produce L-alanine and L-lactic acid. Genes encoding β-carotene synthesis have also been cloned and successfully expressed in *Z. mobilis* to enrich the fermented nutrients of farm animals. Several applications of levan in food and pharmaceutical industries provide an opportunity to exploit *Z. mobilis* for large-scale production of levan. The merits of *Z. mobilis* suggest the potential use of this organism in industrial production of various fermentation products.

THE natural energy resources such as fossil fuel, petroleum and coal are being utilized at a rapid rate and these resources have been estimated to last over a few years. Therefore, alternative energy sources such as ethanol, methane, and hydrogen are being considered. Some biological processes have rendered possible routes for producing ethanol and methane in large volumes. A worldwide interest in the utilization of bio-ethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production[1]. Intense research has been carried out for obtaining efficient fermentative organisms, low-cost fermentation substrates, and optimum environmental conditions for fermentation to occur. Traditionally, ethanol has been produced in batch fermentation with yeast strains that cannot tolerate high concentration of ethanol. This necessitated the strain improvement programme for obtaining alcohol-tolerant strains for fermentation process. *Zymomonas mobilis*, a gram-negative bacterium, is considered as an alternative organism in large-scale fuel ethanol production. Comparative laboratory- and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast have indicated the suitability of *Z. mobilis* over yeasts due to the following advantages:

(i) its higher sugar uptake and ethanol yield,
(ii) its lower biomass production,
(iii) its higher ethanol tolerance,
(iv) it does not require controlled addition of oxygen during the fermentation, and
(v) its amenability to genetic manipulations.

The only limitation of *Z. mobilis* compared to the yeast is that its utilizable substrate range is restricted to glucose, fructose, and sucrose. *Z. mobilis* was originally isolated from alcoholic beverages like the African palm wine, the Mexican '*pulque*', and also as a contaminant of cider and beer in European countries. On the basis of evaluation using the modern taxonomic approaches, the genus *Zymomonas*[2] has only one species with two subspecies, *Z. mobilis* subsp. *mobilis* and *Z. mobilis* subsp. *pomaceae*. The ability to utilize sucrose as a carbon source distinguishes *Z. mobilis* from *Z. anaerobia*[3]. It is one of the few facultative anaerobic bacteria which metabolizes glucose and fructose via the Entner–Deudoroff (E–D) pathway, which is usually present in aerobic microorganisms[4]. Under anaerobic

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conditions, *Z. mobilis* produces byproducts such as acetoin, glycerol, acetate, and lactate, which result in reduced production of ethanol from glucose. During growth of *Z. mobilis* in fructose, the formation of acetoin, acetic acid, and acetaldehyde was clearly more pronounced than when grown in glucose. However the cell yield was low during its growth in fructose.

In addition to ethanol fermentation, *Z. mobilis* has potential application in polymer production. Levan, a polymer of fructose units linked by β-2,6-fructosyl bond, is produced by *Z. mobilis* during its growth on sucrose medium. Microbial levan is of commercial importance and is used as a thickening, gelling, and suspending agent. In recent years, strategies to improve the yield of levan production by microorganisms attracted greater attention. In this review, the recent developments in the potential applications of *Z. mobilis* are discussed.

Biochemistry of *Zymomonas mobilis*

In *Z. mobilis*, D-glucose and D-fructose are transported by facilitated diffusion[5]. Concentrated glucose solutions are not inhibitory to the E–D pathway enzymes, since conversion of glucose to ethanol by this organism proceeds rapidly[6]. Thus, the extracellular osmotic pressure of the glucose solution may rapidly be balanced by corresponding intracellular sugar concentrations. High sugar concentrations decrease the total water potential, and exert osmotic pressures which are comparable to those of relatively strong salt solutions. The low-salt tolerance of *Z. mobilis* poses problems for the fermentation of molasses which usually contains a high-salt content[4].

Study of metabolic intermediates showed that glucose-6-phosphate dehydrogenase and phosphoglycerolmutase are the limiting enzymes, and that phosphofructokinase is not present in the E–D pathway of *Z. mobilis*[7]. The pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) are the key enzymes in ethanol formation. The Pdc is an unique enzyme in *Z. mobilis* which requires thiamin pyrophosphate for its activity[8]. While Adh I of *Z. mobilis* is zinc-dependent and is similar to Adh IV of yeast, Adh II is very unusual in containing iron and not zinc[9]. The Adh II appears to facilitate continuation of fermentation at high concentration of ethanol.

In *Z. mobilis*, the E–D pathway enzymes are more tolerant to ethanol, as the cell-free system of *Z. mobilis* can rapidly consume glucose and produce ethanol more than 15% w/v (ref. 10). The cell membrane of *Z. mobilis* has acquired altered fatty acid content to counteract the adverse effects of ethanol. The major fatty acids occurring in *Z. mobilis* are myristic acid, palmitic acid, and *cis*-vaccenic acid. Among the phospholipids, phosphotidyl ethanolamine is the most abundantly present. The high concentration of *cis*-vaccenic acid and unusual hopanoids in the membrane are responsible for the high ethanol tolerance[11].

Located in the cell membrane of *Z. mobilis* are NADH- and NADPH-oxidases which catalyse the oxidation of NAD(P)H (ref. 12). In addition, enzymes catalase, superoxide dismutase, and peroxidase are also present. Here, the transfer of electrons via the respiratory chain is not coupled with oxidative phosphorylation. Both glucose-fructose oxido-reductase and glucose dehydrogenase lead to the formation of gluconic acid which after phosphorylation enters the E–D pathway after the NAD(P)H formation. A mole of reduced co-enzyme formed is directly consumed by mannitol dehydrogenase and NAD(P)H oxidase, resulting in accumulation of byproducts, namely, acetaldehyde, acetoin, and acetic acid[13]. Production of acetaldehyde by *Z. mobilis* in the presence of oxygen is due to increased NADH oxidase activity resulting in the decreased availability of NADH for the reduction of acetaldehyde to ethanol by Adh. It does not have an aldehyde dehydrogenase to oxidize acetaldehyde to acetic acid. Adh mutants of *Z. mobilis* showing increased levels of acetaldehyde production have been isolated by using allyl alcohol as a selective agent[14].

Z. mobilis possesses both acid and alkaline phosphatases. While acid phosphatase activity is highest in the presence of Mg^{2+}, the alkaline phosphatase activity is highest with Zn(II) (ref. 15). The alkaline phophatase of *Z. mobilis* is associated with membranes and it occurs in two isoforms[16].

The sucrose hydrolysing activity seems to be stimulated by sucrose and fructose[17]. The hydrolysis rate of sucrose and the rate of transfructosylation are shown to be higher than the sugar uptake rate of *Z. mobilis*. During fermentation of sucrose by *Z. mobilis*, three types of transfructosylation occur resulting in the formation of free fructose, oligosaccharides, and higher polymers of fructose and levan where water, sucrose, and levan of polyfructose respectively can act as an acceptor[18].

Levansucrase has been purified from cells[19] as well as from the culture broth[20]. Three different saccharolytic enzymes have been reported in *Z. mobilis*; an endocellular sucrase, an exocellular levansucrase, and an exocellular sucrase[21]. These enzymes are β-D-fructofuranosyl-transferases and are therefore able to hydrolyse sucrose. Moreover, levansucrase synthesizes levan and can in turn be partially hydrolysed. Sucrase and levansucrase do not hydrolyse inulin, a low molecular weight fructose polymer.

Molecular biology of *Z. mobilis*

The genome size of *Z. mobilis* strains is in the range of $1.53 \pm 0.19 \times 10^9$ Da, about 56% of the *E. coli* genome, and can accommodate about 1500 cistrons. The DNA

base composition of *Z. mobilis* (48.5 ± 1.0% G + C) was determined by thermal denaturation[22].

The pyruvate decarboxylase gene (*pdc*) has been cloned from *Z. mobilis* strains ATCC 31821 (ref. 23) and ATCC 29191 (refs 24, 25). The coding region is 1.7 kb-long and encodes a polypeptide of 567 amino acids with a subunit mass of 60.8 kDa (ref. 25). As the promoter region does not contain sequences homologous to the generalized promoter structure for *E. coli*, the promoter of *pdc* is not recognized in *E. coli*, although the cloned gene is expressed relatively at high levels under the control of alternative promoters. Comparison of the nucleotide sequence of the *pdc* gene from *Z. mobilis* strains ATCC 29191 and ATCC 31821 showed the existence of polymorphism in different isolates[26].

The *adh* gene from *Z. mobilis* has been cloned using a novel indicator plate technique where mixtures of *para* rosaniline and bisulphite are incorporated. The DNA sequence for this gene contains an open reading frame (ORF) that encodes a polypeptide of 383 amino acids with mol wt of 40 kDa (ref. 27). The *adhI* gene is transcribed at low levels in *E. coli* from the P2 promoter of *Z. mobilis*, but is expressed well in *E. coli* under the control of *lac* promoter. This Adh I is found to exhibit very little homology with other known products of *adh* genes, but exhibits strong homology with Adh IV of *S. cereviseae*[28].

The gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*gap*) has been isolated from *Z. mobilis* by complementing a deficient strain of *E. coli*. The ORF for this gene encodes 337 amino acids with a mol wt of 36.1 kDa (ref. 29). Although the primary amino acid sequence of Gap has considerable functional homology and amino acid identity with same enzyme from other eukaryotes and bacteria, it appears to be more closely related to that of the thermophilic bacteria and to chloroplast isoenzymes. Comparison of this gene with other glycolytic genes from *Z. mobilis* reveals several common features of gene structure including a conserved pattern of codon bias. Other glycolytic genes that have been cloned and characterized are given in Table 1.

The *Z. mobilis* gene encoding phosphoglycerate kinase (*pgk*) has been cloned in *E. coli* and was sequenced[34]. This promoter-less *pgk* gene is located 225-bp downstream from the *gap* gene, and is part of the *gap* operon. The deduced amino acid sequence from the *Z. mobilis pgk* gene is less conserved than those of other known sequences for *pgk*, and also shows a high degree of conservation of structure. In general, the amino acid positions at the boundaries of α-sheet and β-helical regions, and those connecting these regions are more conserved than the amino acid positions within regions of secondary structure.

The *phoC* gene of *Z. mobilis* encodes for an acid phosphatase with a mol wt of 29 kDa (ref. 15). Its promoter comprises a – 35 pho box region, similar to that of *E. coli* genes, as well as the regulatory sequences of *S. cerevisae* acid phosphatase (*pho5*). The *phoC* gene contains a 5′ terminus which is AT-rich, has a weak ribosome-binding site, and has less biased codon usage than the highly expressed *Z. mobilis* genes. A comparison of the genes reveals that promoters of all these genes are similar in degree of conservation of spacing and identity with proposed *Z. mobilis* consensus sequence.

Five *trp* genes of *Z. mobilis* were identified by their ability to complement the *trp* mutants[43]. The organization

Table 1. Glycolytic genes of *Zymomonas mobilis* cloned and characterized

Gene	Gene product (enzyme)	ORF (bp)	Mol wt (kDa)	Reference
glf	Glucose transporter	1422	50.2	30
glk	Glucokinase	984	35.4	30
frk	Fructokinase	906	32.6	31
pgi	Phosphoglucose isomerase	1524	55.4	32
zwf	Glucose-6-P-dehydrogenase	1458	53.9	30
edd	6-Phosphogluconate dehydratase	1752	62.9	30
eda	2-kdpg aldolase	627	21.5	33
gap	Glyceraldehyde-3-P-dehydrogenase	1011	36.1	29
pgk	Phosphoglycerate kinase	1191	41.4	34
pgm	Phosphoglycerate mutase	684	25.4	35
eno	Enolase	1293	45.8	36
pdc	Pyruvate decarboxylase	1677	60.0	24
adhA	Alcohol dehydrogenase I	1011	36.1	28
adhB	Alcohol dehydrogenase II	1149	40.1	27
sacA	Intracellular sucrase	1533	58.4	37
sacB	Extracellular levansucrase	1260	47.0	38
gfor	Glucose-Fructose oxidoreductase	1317	49.0	39
gl	Gluconolactonase	855	31.1	40
sacC	Extracellular sucrase	1239	46.0	41
	Pyruvate dehydrogenase complex			42
pdhAα		1065	38.6	
pdhAβ		1389	49.8	
pdhB		1323	46.8	
lpd		1401	49.8	

of *Z. mobilis trp* genes is similar to that found in species of *Rhizobium*, *Acinetobacter calcoaceticus*, and *Pseudomonas acidoovorans*. The *trpF*, *trpB*, and *trpA* genes appear to be linked but they are not closely associated with *trpD* or *trpC* genes in *Z. mobilis*.

The *sacA* gene encoding an intracellular sucrase from *Z. mobilis* has been cloned, sequenced, and characterized[44,45]. The SacA protein is a monomer with a molecular weight of 58 kDa and its deduced amino acid sequence shows strong homology with the intracellular sucrase of *B. subtilis* and yeast invertase. The *sacB* encoding extracellular levansucrase and *sacC* encoding extracellular sucrase were cloned and expressed in *E. coli*[38,41]. Nucleotide sequence analysis of *sacB* gene revealed an ORF 1269-bp long encoding for a protein with mol wt of 46.7 kDa. The deduced amino acid sequence was identical to the N-terminal sequence of that deduced from the *sacB* gene of *Z. mobilis* Z6C (ref. 46.) The amino acid sequence of SacB showed very little similarity to those of other known sucrases, but was very similar to the levansucrase of *Z. mobilis* (61.5%), *Erwinia amylovora* (40.2%), and *Bacillus subtilis* (25.6%). The nucleotide sequence analysis of *sacC* revealed an ORF 1239-bp long encoding a 46-kDa protein. The first 30 deduced amino acids from this ORF were identical with those from the N-terminal sequence of the extracellular sucrase of *Z. mobilis* strain ZM4. This *sacC* gene is located 155-bp downstream of *sacB* gene forming a sucrase gene cluster. The SacB and SacC are the two extracellular enzymes of *Z. mobilis* sequenced which do not possess signal sequences[38].

Studies have demonstrated the sequence requirements for membrane localization or protein transport in *Z. mobilis*. The *lacZ* fusion, which contains anchor sequences conferring membrane association, is used to isolate DNA from *Z. mobilis* containing promoter activity and amino terminal sequences[47]. Comparison of the sequences and transcription initiation sites showed that both *E. coli* and *Z. mobilis* recognize similar regions of DNA for transcription initiation. Five to eight consecutive hydrophobic amino acids in the amino terminus serve to anchor these hybrid proteins to the membrane in both *E. coli* and *Z. mobilis*.

Bioprocess potentials: Ethanol production

Ethanol production by *Z. mobilis* has been restricted to glucose, fructose, and sucrose substrates. Alternatively, crude sucrose substrates such as sugar beet, bagasse and molasses have also shown promise as substrates for direct fermentation to ethanol. Recombinant DNA technology could be exploited to construct microbial strains to produce ethanol from these low-cost agricultural substrates. To obtain ethanologenic strains utilizing the above-mentioned substrates, either *Z. mobilis* was transformed with genes of interest acquired from other organisms or gene of *Z. mobilis* involved in ethanol synthesis was transferred to other organisms with required characteristics. Ethanol production potential of *Z. mobilis* from a variety of substrates is discussed.

Genetic manipulation of Z. mobilis

By genetic manipulation, *Z. mobilis* substrate range could be extended to industrially attractive agricultural byproducts such as whey, starch, and cellulose. This could be achieved by transfer of genes encoding appropriate hydrolases. Various genes encoding enzymes required for utilization of wide range of carbon sources transferred into *Z. mobilis*, are listed in Table 2.

Gene cloning in *Z. mobilis* is carried out using three kinds of vectors, viz. broad-host range plasmids, shuttle vectors, and modified broad-host range vectors. Though *Z. mobilis* strains can act as recipient for a number of broad-host range plasmids such as RP1, RP4, R68, etc.[4,59,60] commonly used cloning vectors and phage vectors cannot be transferred or maintained in *Z. mobilis*. The most commonly used high-copy-number cloning vectors, derived from RSF1010, have been found to be more stable in *Z. mobilis* than in RP4-derived plasmids[60]. The occurrence of natural plasmids in *Z. mobilis* has been described and a high degree of homology has been demonstrated between them[61,62]. The native plasmids of low molecular weight present in *Z. mobilis* are used to develop cloning vehicles for *Z. mobilis*. They have been fused with small *E. coli* plasmids, such as pACYC184 and pBR325, to produce novel shuttle vectors[63–65]. The well-defined *E. coli* segments facilitate further plasmid maintenance, while the *Z. mobilis* portion which includes presently undefined sequences yields stability in *Z. mobilis* via the origin of replication or native plasmid[66]. Integrative shuttle vector plasmid, pZMOCP1, was constructed by ligating *Eco*RV digests of the cloning vector plasmid, pZMP1, with *Eco*RV-digested plasmid, pBluescript DNA (ref. 67).

Transfer of R-plasmids into *Z. mobilis* from either *E. coli* or *Pseudomonas* is usually achieved by conjugation. High conjugation rates have been reported for plasmid transferred in an antibiotic-sensitive mutant strain, CP4.45 (ref. 68). Efficient conjugation is routinely achieved by either a self-mobilizable or helper plasmid, such as RP4, pRK2013, etc., as the transformation system of *Z. mobilis* is in its rudimentary stage. Plasmid transfer mediated by donor cell chromosome mobilization, such as by *E. coli* S17.1, is very inefficient. To improve sucrose hydrolysis, extracellular sucrase genes, *sacB* and *sacC*, were subcloned in *E. coli* and *Z. mobilis* shuttle vector pZA22. This construct was transferred into *Z. mobilis* by conjugation using the helper plasmid pRK2013 (Gunasekaran *et al.*, unpublished result). An alternate

method for gene transfer in *Z. mobilis* is by spheroplast fusion. Yanase *et al.*[69] isolated fusants on a raffinose medium by crossing fructose-assimilation negative strains, but the fusants could not assimilate fructose.

A lactose operon has been introduced into *Z. mobilis*, using plasmid pGC91.14 carrying the transposon Tn*951* encoding the operon[70,71]. *Z. mobilis* containing the lactose operon, Tn*951*, produced 0.4% ethanol from 4% lactose in 40 h (ref. 72). Since *Z. mobilis* can metabolize only the glucose portion of lactose and also generate only one mole of ATP per mole of glucose metabolized, there is no net gain of energy for cell growth as the same ATP is expended for each lactose molecule transported across the membrane via proton symport. To circumvent this problem, Buchhol *et al.*[11] suggested the transfer of β-galatosidase gene into a leaky strain of *Z. mobilis* together with the galactose operon, so that the genes encoding for both uptake and complete utilization of lactose would be available.

A gal$^+$ recombinant plasmid, pZG13, was constructed by the insertion of the *galETK* genes of *E. coli*, downstream to the *Z. mobilis* promoter in pZA22 and the plasmid was introduced into a *Z. mobilis* strain IFO133756 (ref. 44). The recombinant *Z. mobilis* could take up galactose and produce a small amount of ethanol.

In order to introduce the ability to catabolize raffinose, a plasmid, pRRL1, a resultant from the cointegration between pOD118 and R68.45 was introduced into *Z. mobilis*, but it was unstable[73]. Two recombinant plasmids; pZER193 containing α-galactosidase gene of *E. coli*, and pZY1 containing the lactose permease gene of *E. coli* were introduced into the strain Z6C of *Z. mobilis* by spheroplast transformation. Cells of the strain carrying both the plasmids could ferment raffinose and melibiose to ethanol[54].

α-Amylase gene from *Bacillus licheniformis* was subcloned into pKT210 and the recombinant plasmid, pGNB6, was transferred into *Z. mobilis*[50]. Here, the enzyme was released by secretion than by cell lysis. It is also necessary to clone and transfer an amyloglucosidase so that the strain can grow directly on starch. Cloning of glucoamylase gene from *Aspergillus niger* was also attempted but stable transconjugants were not obtained in *Z. mobilis*[49].

In order to convert cellulose directly to ethanol, cellulase genes have to be transferred into *Z. mobilis*. A CMCase gene of *Cellulomonas uda*, CB4, was cloned on pZA22, a cloning vector for *Z. mobilis*[74]. *Z. mobilis* carrying this gene synthesized cellulase immunologically identical with that of *C. uda*. Endoglucanase gene from *B. subtilis*[51] and *P. fluorescens*[52] were subcloned and introduced into *Z. mobilis*. In all these cases, no endoglucanase activity was detected in the culture supernatant, and poor expression in *Z. mobilis* was obtained compared to *E. coli*. However, the cellulase gene from *E. chrysanthemi* coding for endoglucanase was subcloned into a broad-host-range vector pGSS33, and was conjugally transferred into *Z. mobilis* with the help of RP4 (ref. 50). In this case, most of the endoglucanase accumulated in the periplasmic space, suggesting an efficient export of this foreign

Table 2. Heterologous genes expressed in *Z. mobilis*

Substrate	Gene coding	Source	Level of expression (U/mg)	Reference
Starch	α-Amylase	*Bacillus licheniformis*	70.8	48
	Amyloglucosidase	*Aspergillus niger*	ND	49
Cellulose	Endo-β-1-4-gluconase	*Erwinia chrysanthemi*	4.2	50
	Endo-β-1-4-gluconase	*Bacillus subtilis*	0.025	51
	Endo-β-1-4-gluconase	*Pseudomonas fluorescens*	0.35	52
	β-glucosidase	*Xanthomonas albilineans*	1.6	53
Raffinose	α-galactosidase	*Escherichia coli*	2.9	54
Lactose	β-galactosidase	*E. coli*	0.39	44
	β-galactosidase	*E. coli*	1230	54
	Lacpermease	*E. coli*	16*	55
	β-galactosidase	*E. coli*	125	55
	β-galactosidase	*E. coli*	154	55
	β-galactosidase	*E. coli*	50*	56
	β-galactosidase	*E. coli*	45*	56
Xylose	Xyloseisomerase	*Xanthomonas campestris*	0.645	57
	Xylulokinase	*X. campestris*	0.145	57
	Xylosepermease	*X. campestris*	1.1*	57
	Xyloseisomerase	*E. coli*	0.11	58
	Xylulokinase	*E. coli*	1.5	58
	Transaldolase	*E. coli*	0.88	58
	Transketolase	*E. coli*	0.16	58
Mannose	Phosphomannose Isomerase	*E. coli*	3.3	58

ND, Not determined; *U/ml.

protein in *Z. mobilis*. Here, the CM-cellulase activity was cell bound during exponential growth phase, and after 28 h up to 40% of the activity was recovered from the culture medium. A CM cellulase gene of *Acetobacter xylinum* was subcloned into *Z. mobilis–E. coli* shuttle vector plasmid pZA22 (ref. 75). The resulting recombinant plasmid, pZAAC21, was introduced into *Z. mobilis* IFO 13756 by electroporation. Though the expression of CM cellulase in *Z. mobilis* increased 10 fold, no attempt was made to use this strain in ethanol production from cellulose.

Transfer of the β-glucosidase gene of *Xanthomonas albilineans* to *Z. mobilis* was achieved by subcloning the β-glucosidase gene into pKT404 followed by triparental mating involving the helper plasmid pRK2013. The amounts of β-glucosidase produced by the recombinant strains ZM6901 and ZM6902 were 7.5 and 10%, respectively, of those expressed in *E. coli*. *Z. mobilis* ZM6901 produced 13.3 mM ethanol from 5 mM cellobiose after 3 days, using the whole cells[53].

Hemicellulose, a major constituent of plant cell wall materials, makes up to 40% of many agricultural residues. Upon hydrolysis with acids and enzymes, hemicellulose is converted to a mixture of hexose sugars; D-xylose and D-arabinose. The microbial conversion of these pentose sugars to ethanol for use as a fuel additive has received considerable attention. Hence, D-xylose catabolic genes from *Xanthomonas* were also introduced into *Z. mobilis*[57]. *Z. mobilis* CP4 was incorporated together with two operons encoding xylose assimilation and pentose phosphate pathway enzymes such as xylose isomerase, xylulokinase, transketolase, and transaldolase[58]. This engineered strain yielded 0.44 g ethanol/g xylose corresponding to 86% of the theoretical yield. Lawford *et al.*[76] have transferred plasmid pZB5 carrying genes encoding enzymes for xylose metabolism into *Z. mobilis*, and used this recombinant strain for ethanol production.

A *Zymomonas* sp. transformed with a gene encoding L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, xylose-isomerase or xylulokinase under the Z. *mobilis* glyceraldehyde-3-phosphate-dehydrogenase promoter. The substrate fermentation range of *Z. mobilis* ATCC 39676 was expanded to include L-arabinose by introduction of genes encoding L-arabinose-isomerase, L-ribulokinase, L-ribulose-phosphate-4-epimerase, transaldolase and transketolase of *E. coli*[77]. The engineered strain with plasmid pZB206, grew on arabinose as sole C-source and produced ethanol at 98% of theoretical yield. Thus, arabinose was metabolized more or less completely to ethanol as the sole fermentation product with little byproduct formation. Weisser *et al.*[78] transferred *E. coli* K12 CA 8000 *pmi* gene encoding phosphomannose isomerase in *Z. mobilis* from a *lacI*^{q}*–ptac* system on plasmid pZY507. The recombinant *Z. mobilis* utilized mannose as the sole C-source with a growth rate of 0.07/h. This recombinant *Z. mobilis* is useful for production of ethanol from mannose.

As pyruvate is the major intermediate in E–D pathway, researchers attempted to shift this catabolic pathway towards the production of L-alanine[79]. NAD-dependent L-alanine dehydrogenase of *B. stearothermophilus* carrying vector pZY50 showed poor transfer rates in *Z. mobilis* strains.

Z. mobilis genes transfer into other organisms

An artificial operon, *pet* operon, for the production of ethanol was constructed by combining *pdc* and *adhII* genes, and was placed under the control of *lac* promoter[80]. This *pet* operon was introduced into *E. coli*, where these two genes expressed at high levels, resulting in ethanol production from a variety of sugars[81]. A decreased feed-flow rate was used to optimize fuel ethanol production in recombinant *E. coli* which produced 1.8 g/l/h ethanol under the optimized conditions of pH 6.4, 34°C and 125 rpm (ref. 82). However, cells carrying cloned *pdc* gene grew only one-fourth compared to the wild type and tolerated only 2% ethanol[83]. Saucedo *et al.*[84] demonstrated optimization of ethanol production by recombinant *E. coli* with plasmid pLOI297 containing *Z. mobilis* genes (*pdc* and *adhII*). Recombinant *E. coli* FBR1 and FBR2 were constructed by transformation of FMJ39 with *pet* operon plasmids pLOI295 and pLOI297, respectively, which produced 3.8% (FBR1) and 4.4% (FBR2) ethanol from 10% glucose in batch culture[84]. Both FBR1 and FBR2 strains showed no plasmid loss even after 60 generations. Introduction of *pdc* gene into *E. chrysanthemi* resulted in ethanol production from D-xylose and D-arabinose[85].

The physiological influence of *pet* expression in *E. coli* ATCC11303 was investigated using glucose, xylose and mannose as the substrates[86]. Ethanol production was studied by introducing a plasmid containing the ethanol pathway from *Z. mobilis* into *E. coli* K12/FMJ39, the resulting strain grew well under anaerobic condition, and was genetically stable. This ethanologenic *E. coli* when grown in batch culture, an ethanol yield of 0.4–0.42 (w/w) was achieved. Two types of recombinants, one strain with *pet* expression via a multicopy plasmid, pLOI297, and another strain KO11 with chromosomal integration of *pet* operon were studied for ethanol production. Both the recombinants produced ethanol, while the parent strain produced exclusively lactic acid from glucose and mannose. Lawford *et al.*[87] studied the effect of oxygen on ethanol production by recombinant *E. coli* ATCC11303 bearing *pet* plasmid pLOI297. They suggested that under anaerobic conditions, the *pet* plasmid channeled the flow of carbon to ethanol as the predominant end product of hexose and pentose catabolism. Ethanol was produced by recombinant *E. coli* KO11 using crude yeast autolysate as a nutrient supplement[88]. The recombinant *E. coli* KO11 produced 0.51 g ethanol/g sugar when grown on hemicellulose hydrolysates of agricultural residues[89,90]. Fermentation of an enzymatic hydrolysate of ammonia

fibre explosion-pretreated corn fibre by recombinant *E. coli* strains, SL40 and KO11, and *Klebsiella oxytoca* strain P2 was examined[91]. Both *E. coli* strains efficiently utilized most of the sugars in the hydrolysate and produced a maximum of 26.6 and 27.1 g/l ethanol, respectively, equivalent to 90 and 92% of the theoretical yield. Fermentation of sugarcane bagasse or sugars by *K. oxytoca* P2-containing chromosomally integrated *Z. mobilis* ethanol pathway genes showed ethanol yield of 40 g/l and 33.3 g/l, respectively[92,93]. Gold *et al.*[94] transferred *pet* genes of *Z. mobilis* into *Lactobacillus casei* using vector pRSG02; the recombinant strain produced 0.314 g ethanol/g glucose. York *et al.*[95] have reported production of 44–45 g/l ethanol by an engineered *E. coli* KO11 grown on crude soybean hydrolysate from Spezyme FAN treatment.

Genetically engineered *K. planticola*, upon introduction of *pdc* gene of *Z. mobilis*, markedly increased the yield of ethanol to 1.3 mol/mol of xylose (25.1 g/l). Concurrently, the significant decrease in the yields of formate, acetate, lactate, and butanediol were observed[96]. The pyruvate-formate-lyase-defective strain of *K. planticola* harbouring the plasmid carrying *pdc* gene of *Z. mobilis* became an efficient ethanol producer[97]. This recombinant strain produced 387 mM ethanol from 275 mM xylose in 80 h, about 83% of the theoretical yield. Furthermore, this mutant consumed more than double the amount of xylose compared to the wild type, due to reduced production of inhibiting acids during growth[98]. An *E. coli* strain containing a recombinant plasmid encoding the *pdc* and *adh* genes from *Z. mobilis*, metabolized glucose and xylose to near theoretical yields of ethanol[99]. In aerobic condition, the natural expression of *adh* of *E. coli* resulted in less ethanol production from clones expressing only *Z. mobilis* *pdc* gene. The ethanol-producing genes of *Z. mobilis* were also incorporated into gram-positive bacteria, *B. subtilis*[100].

Fermentation: Process development

Batch fermentation

Generally polymeric carbohydrates are prehydrolysed with appropriate enzymes and the hydrolysate is used for fermentation in batch and continuous cultures. Mixed culture of different ethanologenic strains may be used in fermentation to obtain improved productivity. An ethanologenic strain is also mixed with ethanol nonproducer strain. The latter might hydrolyse carbohydrate polymer, facilitating the fermentation by *Z. mobilis*. In order to recycle the cells in fermentation, the cells are immobilized in a suitable matrix, and used in fermentation.

Starchy materials are attractive substrates for industrial production of alcohol and many reports are available on the production of ethanol from such materials. The different pathways for industrial ethanol production are depicted in Figure 1. Chay *et al.*[101] reported that the best strains for ethanol production from saccharified syrups were strains of *Z. mobilis* and *S. diastaticus*. Toran-Diaz *et al.*[102] investigated the effect of acid-hydrolysed substrate and enzyme-hydrolysed substrate on ethanol production by ZM4 and ZM4F strains of *Z. mobilis*. They obtained ethanol productivity of 4.8 g/g/h, with *Z. mobilis*

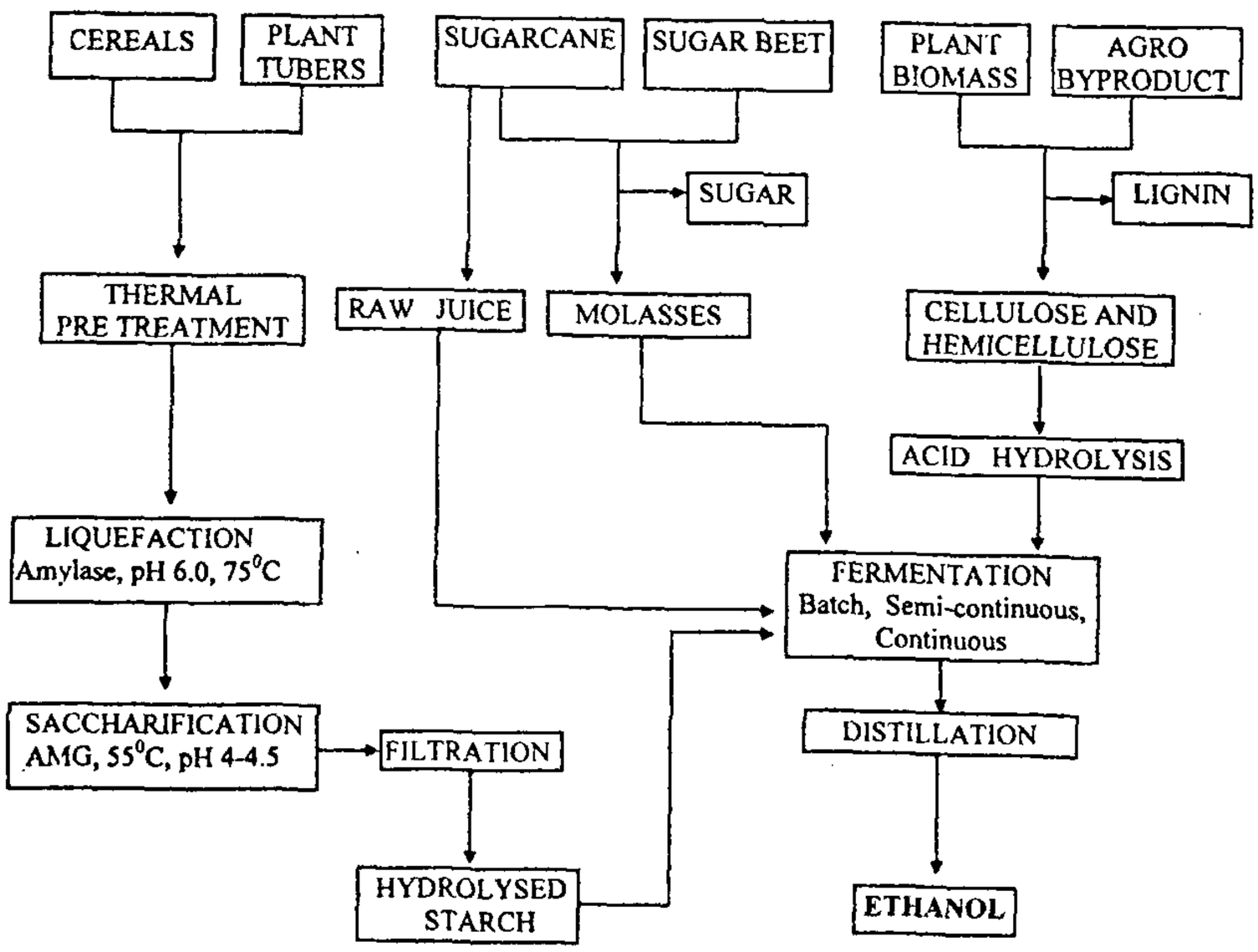

Figure 1. Industrial alcohol production from various substrates.

grown on Jerusalem artichoke juice, which was higher than that reported for the yeast *Kluyveromyces marxianus*, by Duvnjak *et al.*[103]. Further, they observed that the juice of Jerusalem artichoke could be fermented without the addition of any nutrients. Recently, Lawford *et al.*[76] demonstrated that corn steep liquor, a by-product of maize wet-milling, as a cost-effective substrate for production of ethanol by *Z. mobilis* CP4. Torres and Baratti[104] investigated the fermentation of wheat starch hydrolysate by *Z. mobilis*. They reported that in batch fermentation, sugar concentrations as high as 223 g/l could be fermented to 105 g/l ethanol in 70 h. The percentage theoretical yield was 92%. The fermentation pattern of *Z. mobilis* strains ATCC10988, ATCC12526 and NRRL B4286 on synthetic medium, cane juice and molasses showed that the strain NRRL B4286 produced maximum ethanol on synthetic medium, while the strains ATCC12526 and ATCC10988 performed well on cane juice. However, all the strains fermented molasses poorly[105].

We have already reported the ethanol fermentation of cassava starch hydrolysate (CSH) by *Z. mobilis*[106]. Studies by Nellaiah *et al.*[107] revealed the strain NRRL B-4286 of *Z. mobilis* to be superior to the already-established efficient strain, ZM4, in the fermentation of glucose, fructose, and sucrose up to a concentration of 200 g/l. NRRL B4286 also proved to be the best strain for fermentation of cassava starch hydrolysate. Our results showed that adaptation of the cells to the higher concentration of sugars in CSH could help to achieve maximal ethanol concentrations in relatively shorter period of time. With the culture adapted to the concentration of sugars in CSH, fermentation was completed in 28 h with a maximum concentration of 80.1 g/l ethanol. In contrast to this, a maximum concentration of alcohol of 78.5 g/l after 40 h of fermentation was obtained with the non-adapted culture. Supplementation of the CSH with various additives did not result in higher concentration of ethanol[107].

When *Z. mobilis* and *S. cerevisiae* were compared for their efficiency to produce ethanol from glucose and starch hydrolysate, higher yield was observed for *Z. mobilis* (Table 3). *Z. mobilis* distillates showed 5-fold lower non-ethanol byproducts than *S. cerevisiae* when quality ethanol was produced from rye grain by high temperature extrusion cooking. Ethanol production using a hollow-fibre membrane to recycle amyloglucosidase (AMG) and *Z. mobilis* (ZM4) in a single-stage continuous fermentor was described by Lee *et al.*[108]. Their findings showed that by using liquefied starch substrate, it was possible to obtain volumetric productivity of up to 60 g/l/h at ethanol concentrations of 60–65 g/l. A new method for converting cellulosic material to ethanol by cellulose hydrolysis using commercial cellulase and β-glucosidase, and glucose fermentation with *Z. mobilis* in a fluidized bed fermentor containing an ethanol adsorbant is reported[109].

Continuous fermentation

Among various kinds of fermentation processes studied, a continuous process using co-immobilized AMG and cell was most favorable, with operational stability for over 40 days. Continuous production of ethanol from Jerusalem artichoke Juice using ZM4F of *Z. mobilis* was studied by Allais *et al.*[111]. Their results showed the volumetric productivity to be 67.2 g/l/h with a final ethanol concentration of 42 g/l from 100 g/l initial sugars. Doelle[112] described a process for the continuous production of ethanol from hydrolysates of starch. This process made use of *Z. mobilis* in a single-stage fermentation. The author maintained that the quality of the starch hydrolysate was not crucial to the success of the fermentation, and reported a conversion efficiency of 92%. A process for the continuous production of ethanol on an industrial scale from hydrolysed wheat starch using *Z. mobilis* was described by Sahm and Bringer-Meyer[113]. These investigators reported that a strain of *Z. mobilis* that produced 60 g ethanol/l over a test period of 39 days was used for the industrial-scale fermentation. Hillary *et al.*[114] studied continuous culture using single- and double-fermentor systems, and reported that this system had higher productivity than immobilized enzyme systems. Biofilm reactors with polypropylene or plastic support were used for ethanol production[115]. Results showed that the ethanol production rate and concentration were greater in biofilm reactors than in suspension cultures[116]. In continuous fermentation using mixed cultures of *Z. mobilis* and *S. cerevisiae*, production of 54.3 g/l of ethanol was observed within 3 days. These authors reported that a high ethanol productivity of 70.7 g/l/h was obtained with a final ethanol concentration of 49.5 g/l and yield of 0.5 g/g. This amounted to 98% of the theoretical yield and 99% substrate conversion. Therefore this might

Table 3. Comparison of ethanol production capability of *Z. mobilis* and yeast[107,110]

	Z. mobilis		Yeast	
Parameter	Glucose	Starch*	Glucose	Starch
S	100.00	150	100.00	150.00
P	41.60	66.00	35.20	49.70
X	1.75	1.66	5.00	3.34
C	–	88.40	–	88.30
Yp/s	0.43	0.48	0.38	0.36
qp	1.55	5.15	0.52	1.19
Qp	0.87	2.75	0.73	1.38
μ	0.03	0.079	0.04	0.098
Yx/s	0.02	0.012	0.05	0.024
qs	3.23	11.88	1.27	3.91
E	81.20	–	68.70	–

*In co-culture fermentation with yeast.
S, initial sugar (%); P, final ethanol concentration (g/l); X, final biomass (g/l); C, conversion of sugar to ethanol (%); Yp/s, ethanol yield (g/g); qp, specific ethanol productivity rate (g/g/h); Qp, volumetric ethanol productivity (g/g/h or g/l/h); μ, specific growth rate (h^{-1}); Yx/s, biomass yield; qs, specific substrate uptake (g/g/h); E, theoretical yield (%).

be considered as right candidate for increasing the rate of the ethanol production in the existing industries.

Simultaneous saccharification and fermentation

In this process, along with *Z. mobilis* another organism capable of producing carbohydrate hydrolase is used to saccharify the polymeric substrate. The saccharified products are simultaneously utilized by *Z. mobilis* for ethanol production. Simultaneous saccharification and fermentation (SSF) of cassava starch using *Z. mobilis* or *S. uvarum* ATCC 26602 was investigated by Poosaran *et al.*[117]. They reported that *Z. mobilis* fermented considerably faster than *S. uvarum*, completing the fermentation in 20 h resulting in a yield 95% of the theoretical yield, while *S. uvarum* required a period of 33 h to complete fermentation resulting in a yield of 90% of the theoretical value. Rhee *et al.*[118] investigated various SSF processes with sago starch using free enzyme and free cells of *Z. mobilis*, free enzyme and immobilized cells or co-immobilized enzyme and cells. They compared the results obtained in each of the above processes with those obtained with a system using pre-saccharified sago starch. Improvement of ethanol production from sweet sorghum was achieved to 29.7 g ethanol/100 g dry sorghum stalks by using *Fusarium oxysporum* mixed culture with *Z. mobilis*[119].

Use of a mixed culture of *Saccharomycopsis fibuligera* and *Z. mobilis*, for simultaneous saccharification and fermentation process resulted in 29 g/l ethanol. Ethanol yield could be improved by growing *S. fibuligera* on liquefied starch for 12 h, followed by addition of *Z. mobilis* for fermentation. Recently, by this process, steam-pretreated willow was fermented using *Z. mobilis*, *S. cerevisiae*, and cellulase[119]. They could achieve over 85% of the theoretical ethanol yield based on the glucan available in the raw material in three days. *Z. mobilis* was also co-immobilized with an industrial glucoamylase within beads of kappa-carrageenan, and fermentation of maltodextrin was carried out to produce ethanol[120]. Co-immobilized *Z. mobilis* and glucoamylase were also used as a biocatalyst for fuel ethanol production in a three-phase fluidized bed reactor. Various antimicrobial agents were supplemented to SSF of paddy malt mash using a mixed culture of *Z. mobilis* and *S. cerevisiae*[121] that resulted in 10.1% v/v ethanol which was more compared to the ethanol produced by using boiled and fermented mash (9.3%).

Studies on co-immobilization of *Z. mobilis* and AMG in k-carrageenan for this process were carried out by Kim *et al.*[122] using sago starch. These workers observed the conversion of 10–20% sago starch to ethanol with 93–97% of theoretical yields. The authors stated that the combined co-immobilized system showed higher ethanol-producing activity compared to free enzyme and cells or separately immobilized cells and enzyme. Batch fermentation of Cassava starch hydrolysate by immobilized cells of *Z. mobilis* showed that while a maximum ethanol concentration of 59 g/l and productivity of 3.57 g/l/h could be obtained, the final ethanol concentration obtained with free cells was 66 g/l with a productivity of 2.75 g/l/h. The immobilized cells were reported to be stable for seven cycles.

Since the fermentation of lactose to ethanol is slower than glucose fermentation, considerable interest has been directed towards improving the rate of ethanol production from lactose. Gunasekaran *et al.*[123] have described the increased ethanol production to 72 g/l from lactose using co-immobilized yeast and *Z. mobilis* in alginate gel. Tanaka *et al.*[124] reported ethanol production by a co-immobilized mixed-culture system of *A. awamori* and *Z. mobilis*. The production of ethanol from Manioc (cassava) flour by strains of *Z. mobilis* was investigated by De Franca *et al.*[125]. According to these investigators, strain CP3 proved to be the best in their studies with an ethanol yield of 0.48 g/g and ethanol productivity of 4.14 g/l/h. Ho *et al.*[126] reported the production of ethanol from cassava starch by this process using co-immobilized *Z. mobilis* and immobilized glucoamylase. John *et al.*[127] improved ethanol production in co-immobilized cultivation of *Z. mobilis* and *A. awamori* by increasing oxygen concentration for 24 h, followed by using a mixture of 20% air + 80% nitrogen. *Z. mobilis* was entrapped into PVA-cryogel carrier in the presence of polyol cryoprotectants and the resultant biocatalyst was examined for fermentation of glucose to ethanol[128].

Flocculant strains of *Z. mobilis* can aid cell recycle in continuous fermentation[129,130] or increase the cell density of immobilized cell system and thus increase ethanol production[131]. Varieties of immobilization cell techniques, including attaching a flocculant strain to glass fibre pad, have been investigated to overcome the cost and complexities associated with maintaining high cell concentrations (70–80 g/l) (refs 132,133), and to reduce ethanol toxicity associated with cell recycle in CSTR. An economic analysis shows that a reduction in the production costs of ethanol[134] is expected with immobilized cells of *Z. mobilis* and *Saccharomyces bayanus*[135], *Zymomonas* produced ethanol at more than twice the rate of *Saccharomyces* from glucose with essentially no residual sugar present in the effluent system. Since *Z. mobilis* forms levan in sucrose medium, it poses instability to the cells during fermentation by immobilization. A mutant lacking the property of levan synthesis has been shown to work well in immobilized fermentation by Kannan *et al.*[136]. They reported alcohol production of 73.5 g/l from 150 g sucrose/l. Lee *et al.*[137] demonstrated that addition of immobilized invertase to *Z. mobilis* culture improved the ethanol production and reduced the byproduct formation. When they added 2,100 U/g of β-fructofuranosidase immobilized on a methacrylamide base polymer (0.2 g), the ethanol yield was improved from 0.29 to 0.40 g/g. The coupled microorganism-immobilized system was reported to be better than the

two-stage (hydrolysis and fermentation) system. Co-immobilization of fermentative cells and hydrolytic enzyme was also demonstrated in agar[138], pectin bead[139], and loofa sponge[140]. The effect of these on various parameters of ethanol fermentation using various processes is given in Table 4.

Coculture fermentation

Substrates like cellulose require more complex co-culture or extensive pre-treatment to permit efficient ethanol production by *Z. mobilis*. Ethanol production from lactose was studied using an adapted initial inoculum of *K. fragilis* NRRL 665 in monoculture and in co-culture with strains NRRL B 4286 and NRRL B 1960 of *Z. mobilis*[145]. The monoculture of adapted *K. fragilis* produced more ethanol than the non-adapted cultures. In co-culture with *Z. mobilis* NRRL B 4286, *K. fragilis* produced 61.3 g/l ethanol. After 72 h, *K. fragilis* 665 produced 53.5 and 55.1 g/l ethanol from 200 and 250 g/l lactose, respectively, and in co-culture with *Z. mobilis* 4286, produced 64.4 and 66.0 g/l ethanol. However on increasing the substrates concentration from 100 to 250 g/l, parameters related to growth and sugar uptake were affected in both the cases[146]. When *Z. mobilis* was co-cultured with yeast, both the specific ethanol productivity and volumetric productivity increased since both the strains produced ethanol. Though *K. fragilis* utilized glucose more rapidly than galactose, parameters relating to growth and ethanol productivity were more affected when galactose alone was supplied, than was glucose. Moreover at high ethanol concentrations, growth and ethanol productivity of *K. fragilis* 665 were greatly affected. Direct fermentation of cassava starch to ethanol using mixed cultures of *Endomycopsis fibuligera* and *Z. mobilis*, resulted in more ethanol (10.5%) production compared to other mixed cultures tested as well as monocultures[147]. When glucoamylase was supplemented to mixed culture, ethanol yield increased from 88% to 98% of the theoretical yield. A mixed culture of *Z. mobilis* and *S. cerevisiae* produced 0.5 g ethanol/g sugar consumed with a volumetric productivity of 15 g ethanol/h.

Table 4. Ethanol fermentation process performance of *Z. mobilis*[141–144]

Parameter	Batch	Continuous	Immobilized
Substrate: glucose			
Initial sugar concentration, g/l	250.00	150.0	150.00
Concentration, g/l	117.00	65.0	63.00
Yield, g/g	0.48	0.5	0.50
Volumetric productivity, g/l/h	5.00	12.0	50.00
Substrate: sucrose			
Initial sugar concentration, g/l	100.00	100.0	150.00
Concentration, g/l	37.50	50.0	65.20
Yield, g/g	0.46	0.46	0.47
Volumetric productivity, g/l/h	4.56	10.0	3.62
Substrate: starch hydrolysate			
Initial sugar concentration g/l	150.00	154.0	150.00
Productivity, g/l	29.03	62.6	58.90
Yield, g/g	0.36	0.44	0.41
Volumetric productivity, g/l/h	0.48	7.32	3.57

Solid state fermentation

In recent years, considerable interest has been shown in using agricultural byproducts such as sweet sorghum, corn, apple, grape, sugar cane, sugar beets, fodder beets, and Jerusalem artichoke tubers for fuel ethanol production. Due to the complex composition and insolubility of these agro-substrates, solid-state fermentation of these sources would be economical. Very few reports are available regarding the production of ethanol by solid state fermentation. Amin[148] has described ethanol fermentation in solid state by *Z. mobilis* grown on sugar-beet. Ethanol yield of 0.48 g/g sugar, volumetric productivity of 12 g/l/h and final ethanol concentration of 130 g/l showed good performance of *Z. mobilis* in a solid-state fermentation. Furthermore, Amin[149] reported that during solid-state fermentation fewer by-products were produced, compared to conventional submerged fermentation. At optimum fermentation temperature of 35°C, an ethanol yield of up to 95% of the theoretical value with final ethanol concentration of 142 g/l were obtained.

Improved strains for fermentation

Mutagenesis is particularly useful for manipulation of an organism to improve its tolerance to toxic compounds. Many mutagens have been used to develop high level of ethanol-yielding mutants of *Z. mobilis*[150]. Nitrosoguanidine (NTG) was found to be effective for *Z. mobilis*[151] and thus mutants tolerant to 15% ethanol and high temperature were isolated. Mutants have been selected to improve the utilization of feedstock; for example a strain that could efficiently produce ethanol from 25% sugar cane juice or sugar cane syrup without the need for nutritional supplementation or sterilization of the medium has been reported. The growth and ethanol production of *Z. mobilis* are poor on molasses due to the high concentration of salts. By mutation, the ethanol production from molasses was enhanced by two-fold[152]. Mutants showing tolerance to salt, low pH (ref. 153) and ability to grow on mannitol[154], flocculent mutants[155,156], and sucrose or fructose non-utilizers[157] have also been reported. A stable thermotolerant mutant of *Z. mobilis* ZM4 was isolated that showed better ethanol tolerance at 42°C. Growth of *Z. mobilis* in continuous culture for extended periods results in the selection of spontaneous flocculant variants, and selection for or against depends both on the nutrition and fermenter design[158]. Skotnicki *et al.*[159] have developed a system for transposon mutagenesis of *Z. mobilis*. Kannan *et al.*[160] isolated mutants Ls1 and Ls2 of *Z. mobilis* B-806 lacking extracellular levansucrase. The ethanol yield of the mutants increased from 0.48 g/g to 0.50 g/g on sucrose medium. Lindsay *et al.*[161]

isolated glucose-negative fosfomycin-resistant mutants of recombinant *E. coli* KO11 containing ethanol pathway of *Z. mobilis*. These mutants (SL31 and SL142) retained the ability to ferment sugars and were used to ferment pentose sugars to ethanol selectively in the presence of high concentrations of glucose. Some hyper-productive strains (SL28 and SL40) were also isolated which completed fermentation rapidly and produced 60 g/l ethanol from 120 g/l xylose in 60 h, 20% more ethanol than KO11 under identical conditions. Glucose-negative and fructose-negative mutants of *Z. mobilis* were isolated by D-cycloserine-mediated enrichment method[162]. Recently, a mutant of *Z. mobilis* was isolated which could use mannitol as the sole carbon source due to the presence of a newly evolved mannitol dehydrogenase. This was shown to have evolved from a mutation in the *adhA* gene (ZADH-1), thus expanding the substrate range of the wild type to include mannitol[163].

Other applications

Misawa *et al.*[164] reported the successful cloning of the β-carotene biosynthesis gene *crtB*, *crtE*, *crtI*, and *crtR* in pZA22 and transferring them by conjugation into *Z. mobilis*. The transconjugants of *Z. mobilis* strain accumulating β-carotene after fermentation process may be utilized as a nutrient for farm animals. *Z. mobilis* was also used to produce sorbitol and gluconic acid by control of ethanol production[165]. Belay *et al.*[166] demonstrated ethane production from glucose or starch by co-cultures of methanogens and ethanol producers. Cultures of *Methanosarcina barkeri* or *Thermoanaerobater ethanolica* or *Candida psuedotropicalis* in combination with *Z. mobilis*, produced ethane on glucose medium. A lactate dehydrogenase gene was introduced into recombinant *E. coli* K12/FMJ39 carrying plasmid containing genes of ethanol pathway of *Z. mobilis*. The resulting strain is expected to produce L-lactic acid from mixed sugars.

Conclusion

In recent years, attention has been focussed on effective utilization of agro-byproducts to produce fuel using *Zymomonas mobilis*. As seen from previous reviews, a thorough investigation of molecular biology and biochemistry of ethanol production by *Z. mobilis* has been accomplished. Transformation of *E. coli* with genes (*pet*) from *Z. mobilis* for alcohol production has been successfully carried out. However, *Z. mobilis* only utilizes glucose or fructose or sucrose for ethanol production. In order to broaden the capability of *Z. mobilis* to utilize other sugars, hydrolytic and isomerase genes from recombinant *E. coli* have been transferred to *Z. mobilis*, resulting in utilization of xylose, mannose, lactose and arabinose as the carbon source by *Z. mobilis*. At present, there exists a considerable literature on transformation of *Z. mobilis* to produce ethanol from lignocellulose. In addition to this, researchers are also interested in using mixed cultures to convert complex sugars to ethanol. Owing to the genetic amenability of *Z. mobilis*, it is possible to make use of this organism in industrial production of ethanol. Thus, it is time that the industrialists collaborate with academicians to translate the laboratory findings in science for the benefit of society.

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