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STUDIES CONCERNING ETHANOL PRODUCTION FROM SWEET SORGHUM EXTRACT USING A *Z. MOBILIS* HIGH PRODUCTIVE MUTANT STRAIN

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ABSTRACT. Fuel ethanol is currently made by large-scale yeast fermentation of sugars, that are extracted or prepared from crops, followed by distillation. Traditionally, sugar cane (Saccharum officinarum) juice or beet (Beta vulgaris) molasses are used in ethanol production. The use of sweet sorghum to provide liquid fuels for the transport sector represent a new challenge, mainly due to its high yield in comparison with grains, sugar, lignocellulosic biomass. The aim of our study was to find a strain high ethanol producing, and to identify the biochemical mechanism involved in Z. mobilis culture on the sweet sorghum extract. Using growth curves, sucrose consumption and electrophoresis studies we demonstrate that $CP4^{Suc40Preventol\ Resistant}$ Z. mobilis strain is able to grow and to metabolise sucrose from sweet sorghum extract. The metabolic pathways involved in sorghum fermentation are discussed.

KEYWORDS. *sweet sorghum, Z. mobilis, ZADH, GFOR, NAD(P)H dehydrogenase, SOD*

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INTRODUCTION

Bioethanol, as a fuel, is currently made by large-scale yeast fermentation of sugars [Laluce, 1991]. Although many other countries produce ethanol for fuel and other purposes, major production has only occurred in those countries with especially favourable agricultural and economic conditions [Wheals *et al.*, 1999]. Costs and benefits are presented in **table 1**.

Table 1

Different biomass feedstocks can be used for fuels and chemicals production. These include various agricultural residues (corn stalks, wheat straws, potato or beet waste), wood residues (leftovers from harvested wood, and unharvested dead and diseased trees), specifically grown crops (hybrid poplar, black locust, willow, silver maple, sugarcane, sugar beet, corn, and sweet sorghum), and waste streams (municipal solid waste, recycled paper, baggasse from sugar manufacture, corn fibber, and sulphite waste) [Aristidou & Penttila, 2000].

Traditionally, sugar cane juice or beet molasses are used in ethanol production. Both sugar cane and molasses (after adjusting the sugar concentration) normally contain sufficient minerals and organic nutrients to be immediately suitable for ethanol production by yeast fermentation, like *Saccharomyces cerevisiae* [Atiyeh & Duvnjak, 2002]. For biotechnological reasons, the ethanologenic bacteria (*Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis*) are the most promising for industrial exploitation [Dien *et al.*, 2003]. Genomics technology promises to rapidly develop and bring to market new “energy traits” in major crops like corn and sorghum, improving feedstocks and greatly reducing the cost of bioenergy production.

The use of sweet-stemmed crops (including sweet sorghum) to provide liquid fuels for the transport sector is not a new concept [Woods, 2001]. Sweet sorghum can be grown in temperate and tropical region and it has a very high yield comparable to grains, sugar, lignocellulosic biomass (on average a total of 30 dry tons/ha per year) In addition, sorghum plantations need less seed than other crops, 15 kg/ha compared with 40kg/ha for corn, or 150 kg/ha for wheat.

Many efforts try to improve the fermentative systems; additional growth of sweet sorghum outside the corn estates by smallholder farmers for processing by sugar would allow these farmers to access the infrastructure and markets of this huge agro-industry, so aiding rural development [Roman, 1995].

Ethanol-producing bacteria are very important because their growth rate is higher than that of the *Saccharomyces cerevisiae*, presently used for commercial production of fuel alcohol. Recent advances in biotechnology are expected to make industrial ethanol production more economical [Yanase *et al.*, 2005]. Among such ethanol-producing bacteria is *Zymomonas mobilis*, which has historically been used in tropical areas to make alcoholic beverages from plant sap [Swings & De Ley, 1977]. *Z. mobilis* is noteworthy for its high growth rate and high specific ethanol production, which would seem to make it an attractive candidate for industrial ethanol production, but its narrow spectrum of fermentable carbohydrates has limited

its use, especially for fuel ethanol production from varied sources [Skotnicki *et al.* 1982].

The Gram-negative anaerobic aerotolerant bacterium *Zymomonas mobilis* is unique among bacteria because it ferments sugars (D-glucose, D-fructose, and sucrose) to ethanol and carbon dioxide via the Entner-Doudoroff pathway (2-keto-3-deoxy-6-phosphogluconate, KDPG) [Swings & DeLey, 1977] pathway in conjunction with the enzymes pyruvate decarboxylase et alcohol dehydrogenase and ethanol and carbon dioxide is the sole means for energy generation (figure 1). This pathway yields only a single mole of ATP per mole of sugar fermented, giving Z. mobilis the lowest molar growth yield reported for a bacterium. In order to cope with this low energy yield, glucose is metabolised at a remarkably high rate [Sprenger, 1996]. The organism tolerates high concentrations of substrate (up to 30% glucose) and product (13% ethanol) [Swings & DeLey, 1977; Bringer-Meyer & Sahm, 1988] and may be used for large-scale ethanol production [Rogers *et al*., 1984].

Figure 1

There are many studies concerning genetically, biochemical and biotechnological aspects in *Z. mobilis*, however, a single study takes into account the cultivation of this bacterium on Milo flower containing medium [Millichip & Doelle, 1989].

The aim of this work was to elucidate biochemical mechanisms involved in sweet sorghum fermentation using the Gram-negative bacteria *Z. mobilis*.

MATERIAL AND METHODS

Strains and growing conditions. Cell culture of **CP4** $^{\text{Suc 40 Preventol Resistant}}$ mutant *Z. mobilis* (CP4PR) has been growth in complete medium [Galani *et al*., 1985] and .subjected to UV mutagenesis. After 20 minutes irradiation, the cell culture aliquots have been plated on sweet sorghum (2,5%) containing 2% agar and the clones has been selected after 24-36 hours incubation at 30° C. The new mutants strains have been grown in complete medium with glucose (20g l^{-1}), sucrose (100g l^{-1}), or with sweet sorghum extract semiaerobically at 30°C without shaking. Exponentially growing cells were used as inocula to yield a starting liquid culture of approximately 10^7 cells per ml. Growth were monitored turbidimetrically at a wavelength of 600 nm. An optical density at 600 nm (OD_{600}) of 0.9corresponds to 0.35 mg of dry cell weight·ml^{-1}. Sweet sorghum extract has been obtained from sorghum crop after two times boiling (2 hours) in distilled water following filtration using 0.45 µm membrane filter. The yellow filtrate was been used to monitored turbidimetrically the growing of cells at 600 nm. Cells being in exponentially faze have been centrifuged at 12,000 g and the pellet has been wash two times with 0.9% NaCl. Finally, the cells were resuspended in 1 ml same solution and used as inoculums in the aim to monitored sugar consumption.

Biochemical methods. Cells from 40-ml cultures were harvested by centrifugation at 5000g for 10 min at 4°C, resuspended in 3 ml of distilled water, and

disrupted by ultrasonication with a sonifier (Sanyo, UK). Ten sonication rounds were performed for 60 s on ice, with 1 min intervals on ice. The cell debris was removed by centrifugation at 12,000 g for 15 min at 4°C, and the supernatant fraction, designated soluble extract, was assayed for glucose-fructose oxidoreductase and alcohol dehydrogenases activities [Menendez *et al.*, 2002]. In the aim to study NADH and NADPH dehydrogenase activities, 100 µl of crude extract have been treated for 90 minutes with 0.5 M $NaClO_4$ in presence of 1 mM dithiotreitol. Chaotropic extraction at 30°C with shaking was done. After that, the treated extract was subject to dialysis overnight at 4°C [Galante & Hatefi, 1978].

Analytical methods. Sugar concentration from sweet sorghum extract has been determinate using polarimetric method. Native PAGE was carried out at 4°C in the same way as SDS–PAGE [Laemli, 1970] in an omogen polyacrylamide gel (T = 9%), except that gels contained 0.1% Nonidet P40 instead of Triton X-100. The mutant alcohol dehydrogenase (ZADH) activities were characterized from cell extracts followed by incubation of the gel with ethanol, phenazine methosulfate, NAD+, and nitrobluetetrazolium to observe the location of ADH by production of insoluble formazan by nondenaturating as detected by overnight incubation of gel slices at room temperature in darkness in 50 mM MES buffer (pH 6.5) [O'Mullan *et al.*, 1995]. The NAD(P)H-NBT oxidoreductase activity was detected by overnight incubation of gel slices at room temperature in darkness in 50 mM MES buffer (pH 6.5), 1 mM Na_2-EDTA, 0.2 mM NAD(P)H and 0.35 mg ml^{-1} NBT. No colour developed in controls without NADH. [Quiles *et al.*, 1999; Casano *et al.*, 2000]. Protein concentration was determined by the Lowry method [Lowry *et al.*, 1951].

RESULTS AND DISCUSSIONS

The interest in studying sorghum was generated by the researches of Doelle's group, that have already been used in industrial scaled trails (up to 586,000 l) for fermenting ground milo (sorghum), corn or wheat [Doelle *et al.* 1989; Millichip & Doelle, 1989].

We have isolated several strains of *Z. mobilis* subspecies able to growth at high concentrations of sorghum extract, attempting to transform *Z. mobilis* into an efficient ethanol producer from an abundant and renewable carbon sources. Thus, we studied the substrate degradation using different strains isolated in our laboratory. Figure 2 shows growth curves of the best isolated strains.

Figure 2

Our results show that UV treatment following growth on sorghum extract containing medium induced different effect on mutant strains isolated from CP4PR. These strains were been subjected to sugars counting test in the same sucrose concentration (**Figure 3a and 3b**).

Figure 3, 4, 5, 6, 7, 8

Z.mobilis mutants were isolated after 20 min of UV treatment, followed by platting on 2.5% sorghum - 2% agar solid medium, in the course of screening for sweet sorghum growing tests. Some of the strains appeared to have different

phenotypical mode of growing in glucose, sucrose at two sweet sorghum different concentration of sucrose. The wild type strain of NCIB 11163 appears does not mechanism of sucrose hydrolyzing enzymes as well as it grow on glucose. On the other hand, the methotrexate mutant posses the mechanism(s) involved in sorghum fermentation. Consumption curves show an apparent direct relation between sweet sorghum fermentation and growing curves. Millichip &Doelle (1989) reported a laboratory *Z. mobilis* strain developed from *Z. mobilis* NCIB 11163 (UQM 2716, deposited as ATCC 39767) which has grown at 32-37°C in a culture medium containing 7% glucose, 0.2% of each of yeast extract and casein hydrolysate (peptone), KH_2PO_4, $MgSO_4$ and $(NH4)_2SO_4$. This strain was grew on milo flower with/without corn extract as substrate(s) and the results demonstrated that the milo flower extract and milo flower and corn mixture extract gave, after 36-45 hours of fermentation higher yield comparative with yeast's' fermentation. However, the authors don't discuss the biochemical mechanism of this *Z. mobilis* strain feature.

In order to elucidate the mechanism(s) engaged in sweet sorghum we studied 5 different enzyme involved in biochemical mechanism of sucrose/glucose metabolism on 4 different strains (CP4PR, CP4PR2A, NCIB 11163/76 and CP4PR2) which were grown on glucose, 2% and sweet sorghum extract (2.5%). Figure 9 depicted alcohol dehydrogenases profile.

Figure 9

ADH activities play a key role in both ethanol production and the concomitant regeneration of NAD^+ in the fermentative pathway. *Z. mobilis* has two ADH dehydrogenases designated ZADH-1 and ZADH-2 [Conway *et al*., 1987; Kinoshita *et al*., 1985].

The two alcohol dehydrogenases, with different properties, have been purified from *Zymomonas mobilis*; the isoenzyme with lower electrophoretic mobility (ZADH-1) is a zinc enzyme and the faster isoenzyme (ZADH-2) accounted for some 90% of the ethanol-oxidising activity in freshly prepared extracts and corresponded to the iron-activated enzyme. The former enzyme was inactivated by zinc; activity could only be retained during purification by including either ferrous ions or cobalt ions in the buffers. ZADH-2 has relatively low acetaldehyde reductase activity; consequently, ZADH-1 is responsible for about half of the physiological activity (acetaldehyde reduction) in *Zymomonas* cells. Kinetic studies showed that ZADH-2 is activated by ethanol in both reaction directions and metal ion analyses of ZADH-2 prepared in the presence of iron or cobalt indicated one atom of the relevant metal per subunit, with no significant zinc content. N-terminal sequence analyses showed that the ZADH-1 has some homology with the *Bacillus stearothermophilus* enzyme, whereas ZADH-2 resembles the yeast enzyme more closely [Neale *et al.,* 1986].

According to Kalnenieks *et al.* (2002), ZADH-2 participates in *Z. mobilis* respiration rather than in ethanologenesis and may be considered in aerobic and semi-aerobic growth conditions as a cytoplasmic respiratory component. ZADH-2 deficient mutant strain showed a reduced respiration rate in glucose containing medium [Kalnenieks *et al.,* 2002]. Direct channelling of the reducing equivalents

from glyceraldehyde-3-phosphate dehydrogenase to ZADH-2 has proposed to be the essential driving force for the cycle: different supply of the active centres of ZADH-1and ZADH 2 with NADH would enable both ZADH isoenzymes to catalyse opposite reactions at the same time [Kalnenieks *et al.,* 2002; Kalnenieks *et al.,* 2003].

According to figure 9, ZADH-2 activities of all strain used in this experiment are higher when the four strains have been grown on sweet sorghum comparative with glucose complete medium, which appears to be less stressed than sweet sorghum extract. However, the CP4PR *Z. mobilis* strain has an increased ZADH-2 activity also in glucose complete medium, and this feature maybe explained by *adhII* gene alteration. The presence of a bigger numbers of bands can be explained by dimerization phenomenon, which is also present in the case of this activity [Neale *et al.,* 1986]. On the other hand, high level of sorghum fermentation appear to be related with the role of ZADH-2 activity in ethanol production uncoupled with bacteria growth [O'Mullan *et al.*, 1995]. Notably, the mineral analysis of sorghum flower extract [Millichip & Doelle, 1989] shows a high concentration of iron ions and this fact contributes to ZADH 2 activation, and this activation produces NADH into the respiratory chain by the "ethanol cycle" [Kalnenieks *et al.,* 2002].

The only sugars that can support the growth of wild-type Z. mobilis strains are glucose, fructose, and sucrose, the last of these carbon sources being cleaved outside the cell by various sucrase enzymes to provide free glucose and fructose [Preziosi *et a*l., 1990] and the preferential catabolism of glucose from sucrose or mixtures of glucose and fructose have been attributed to inhibition of fructokinase by glucose [Rogers *et al*., 1982].

As by-products of *Z. mobilis* fermentations on substrate mixtures (e.g., glucose plus fructose, or on sucrose, which can be split into the two sugars), gluconic acid and sorbitol were discovered [Leigh *et al*., 1984; Viikari, 1984]. The two products are formed by the homotetrameric enzyme glucose-fructose oxidoreductase (GFOR, E.C.1.1.99.-), that uses NADP(H) as a tightly, however noncovalently, bound cofactor. GFOR oxidises glucose to gluconolactone and reduces fructose to sorbitol figure 14 [Zachariou & Scopes, 1986; Hardman & Scopes, 1988]. Gluconolactone, which is hydrolyzed to gluconic acid (by a gluconolactonase) can be phosphorylated to the Entner-Doudoroff pathway to intermediate 6-phosphogluconate into a subsequent degradation (figure 1). GFOR is located in the periplasmic space of the bacterial cells [Aldrich *et al*., 1992], where its proposed biological function is to protect the bacterium against osmotic stress caused by high external sugar concentrations [Loos *et al*., 1994]. The protective mechanism arises from the conversion of fructose into sorbitol, which is a compatible solute for the bacterium (i.e. can be accumulated in the cell without harm to the organism) [Kingston *et al*., 1996]. Notably, the profiles of fermentation side products for fructose and glucose are very different [Viikari, 1984] and a significant amount of fructose is converted to the extracellular polymer levan by the enzyme levansucrase when *Z. mobilis* is grown on sucrose [Viikari, 1984.].

GFOR-protein posses a twin-arginine motif which is similar with a signal sequences of a class of proteins that are imported into the thylakoid lumen of plant

chloroplasts via a Sec-independent pathway, the so-called ΔpH import pathway [Chaddock *et al.*, 1995; Robinson & Mant, 1997] and it has been demonstrated that there are structural similarity of the targeting signals which suggests that the two twin-R transport pathways in the prokaryote and the prokaryote-derived organelle are of common phylogenetic origin [Halbig *et al.*, 1999]. Successful translocation by the Sec preprotein translocase requires that the respective precursor proteins are kept in a loosely folded, export-competent state, whereas tight folding of the precursor proteins is inhibitory to translocation [Blaudeck *et al.*, 2001]. On the other hand, GFOR, which contain redox cofactors needs a separate translocation of its with subsequent periplasmic assembly to the holoenzyme or translocation of the complete holoenzyme in the fully folded form by a particular and novel, Sec independent export apparatus [Weiner *et al.,* 1998; Bogsch *et al.*, 1998].

Glucose-fructose oxidoreductase from the Z. mobilis catalyzes the oxidation of glucose to gluconolactone and the reduction of fructose to sorbitol in a ping-pong type mechanism [Zachariou & Scopes, 1986; Hardman & Scopes, 1988]. The apparent physiological role of GFOR is the production of sorbitol from the two sugar moieties of sucrose, a natural carbon source of the bacterium which dwells in sugar-rich habitats [Swings & DeLey, 1977].

Electrophoretically studies of the four strains GFOR demonstrated the existence of mechanisms for adaptation after since has been shown by Bekers *et al.* (2000) using adapted cells inoculums in sugar beet juice fermentation by Z. mobilis. Activation of glucose-fructose-oxidoreductase and increased synthesis of gluconic acid can be the reasons of decreased levan formation [Bekers et al., 2000]. Interesting that Z. mobilis CP4PR is already adapted as well as the GFOR activity is also present in the glucose complete medium fermentation (figura 10). On the other hand, as well as view, it can observe also two molecular forms with different electrophoretic motilities perhaps due by monomer-tetramer aggregates [Zachariou and Scopes, 1986; Furlinger et al., 1998] or, different isoforms (active form and an active precursor (pre-GFOR) with catalytic activity. Electrophoretic separation of GFOR activities can be explain the fact of alterations in the GFOR apoprotein's ability to bind its cofactor can be affect membrane translocation. Another speculation is the fact that some mutants can have altered GFOR with glucose dehydrogenase activity which as demonstrated by Furlinger and co-workers (1998) can be reversed through a prolonged incubation of the Ser116-Asp mutant with $NADP^+$ and a restored Glc-Fru oxidoreductase activity with a concomitant decrease in glucose dehydrogenase activity.

The closed relationship between protein export into the periplasmic space of *Z. mobilis* (GFOR for instance) and higher plant chloroplasts proteins transport pathways across the thylakoid membranes [Stanley *et al.*, 2002] leaded our studies to membrane bound NAD(P)H dehydrogenase [Quiles *et al.*, 1999]. As well as shown the figures 11 and 12, NAD(P)H dehydrogenases activities are increased in the presence of sweet sorghum extract, and both NADPH and NADH activities are dramatically raised in the CP4PR mutant, maybe to supply NAD^+, needed for ZADH activities.

We can speculate also that the high level of NAD(P)H dehydrogenases activities can be related with GFOR activities, perhaps these two activities are involved in proton gradient needed in the targeting of GFOR passengers protein into periplasmic space (NADH dehydrogenase activity) [Wexler *et al.*, 1998; Battchikova *et al.*, 2005] and in the supplying of $NADP^+$ for activation of GFOR [Kalnenieks *et al.*, 2000].

Figure 13

The presence of multiple bands with NAD(P)H dehydrogenase activities can be related with aggregation phenomenon and also, perhaps with different isoenzymes with NAD(P)H activities. Recent genome elucidation of two Z. mobilis strains has been revealed at least two genes that codified NADH activities [Seo et al., 2005].

Studies of superoxide dismutase activities relived on GFOR activities gel (figure 10) as formazan product inhibition shows the existence of the superoxide ions production when *Z. mobilis* cells are grew on sweet sorghum extract. We can suppose that one of NADH or NADPH activity is an superoxide generating enzyme, or as well as probably, sweet sorghum extract contains different polyphenols which are not directly involved in any metabolic process and are therefore considered secondary metabolites. It has been shown that sorghum extract contains some polyphenolic compounds (phenolic acids, flavonoids and condensed polymeric phenols) that have a role as defence chemicals, protecting the plant from predatory attacks of herbivores, pathogenic fungi and parasitic weeds. (Harris & Burns, 1970, 1973; Dreyer *et al.*, 1981]. They inhibit growth of microorganisms and probably impart resistance against grain mould [Bate-Smith, 1969].

Other studies suggest that changes in the rate of mitochondrial ROS production may play a major role in oxygen sensing by the carotid body [Bunn & Poyton, 1996]. Furthermore, it was found that the β-subunit of the potassium channel resembles the structure of NADPH-oxidoreductase [Gulbis *et al.*, 1999: Droge, 2002] and is very possible that ROS activation to be in relation with K^+ channels activation, as well as sorghum extract appears to contain.

CONCLUSIONS

In our knowledge, is the first time when *Z. mobilis* enzymes activities (GFOR, SOD and NAD(P)H dehydrogenase) are visualized on native PAGE. Our results represent an important confirmation of NADH channelling phenomenon in the case of *Z. mobilis* process supported also by immuno cytochemical data. Sweet sorghum extract represents an excellent medium for the selection and culture of *Z. mobilis*, a high ethanol producing bacterial strain.

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LEGEND OF FIGURES AND TABLES

Table 1. Costs and benefits of fuel-ethanol production [Wheals et al., 1999].

Direct costs	**Direct benefits**
Grain, sugar-cane and beet feedstock agricultural industry	Sale of ethanol
Electricity and water use	Sale of coproducts
Transport, processing, fermentation and distillation	Major national agricultural industry
Distribution and supply	Cogeneration of electricity
Tax subsidy or exemption	Technological development
Indirect costs	**Indirect benefits**
Land degradation	Reduced emissions of “green pollution house” gases
Increased acetaldehyde	Reduced pollution by CO and NOx
Foreign currency payments and interest	Rural employment and social stability
Military protection of gasoline supplies	Renewable resource
	Reduction in fossil-fuel use
	Reduced dependency on imported oil

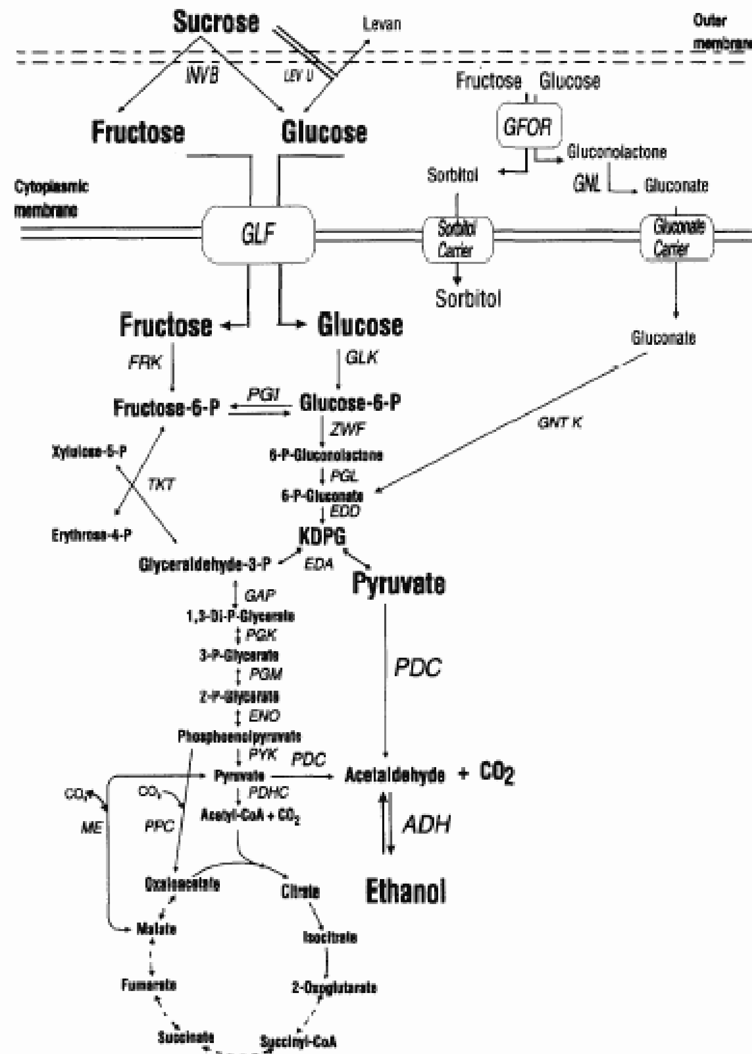


Figure 1. *Major carbohydrate pathways in Zymomonas mobilis. Sucrose is extracellular spitted by the action of invertases (InvB and LevU) into glucose and fructose (or levan). Glucose and fructose enter into cell and they are converted by the periplasmic GFOR into sorbitol and gluconolactone. Sorbitol is then transported into the cell and it participates to counteract osmotic effects. The enzymes of the KDPG pathway are written in three-letter code next to the arrows that symbolize the reaction steps. Two arrows indicate reactions that are, in principle, reversible. Dashed lines in the TCA cycle indicate missing reactions, the transketolase (TKT) reaction is the entrance to the pentose-phosphate pathway, which is incomplete. Abbreviations: ADH, alcohol dehydrogenases I and II; EDA, 2-keto-3-deoxy-gluconate aldolase; EDD, 6-phosphogluconate dehydratase; ENO, enolase; FRK, fructokinase; GAP, glyceraldehydes 3-phosphate dehydrogenase; GFOR, glucose-fructose oxidoreductase; GLF, glucose facilitator; GLK, glucokinase; GNL, gluconolactonase; GNT K, gluconate kinase; INV B, invertase B; KDPG, 2-keto-3-deoxy-6-phosphogluconate; LEV U, levansucrase; ME, malic enzyme; PDC, pyruvate decarboxylase; PDHC, pyruvate dehydrogenase complex; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PGL, phosphogluconolactonase; PGM, phosphoglyceromutase; PPC, phosphoenolpyruvate carboxylase; PYK, pyruvate kinase; TKT, transketolase; ZWF, glucose 6-phosphate dehydrogenase [from Sprenger, 1996].*

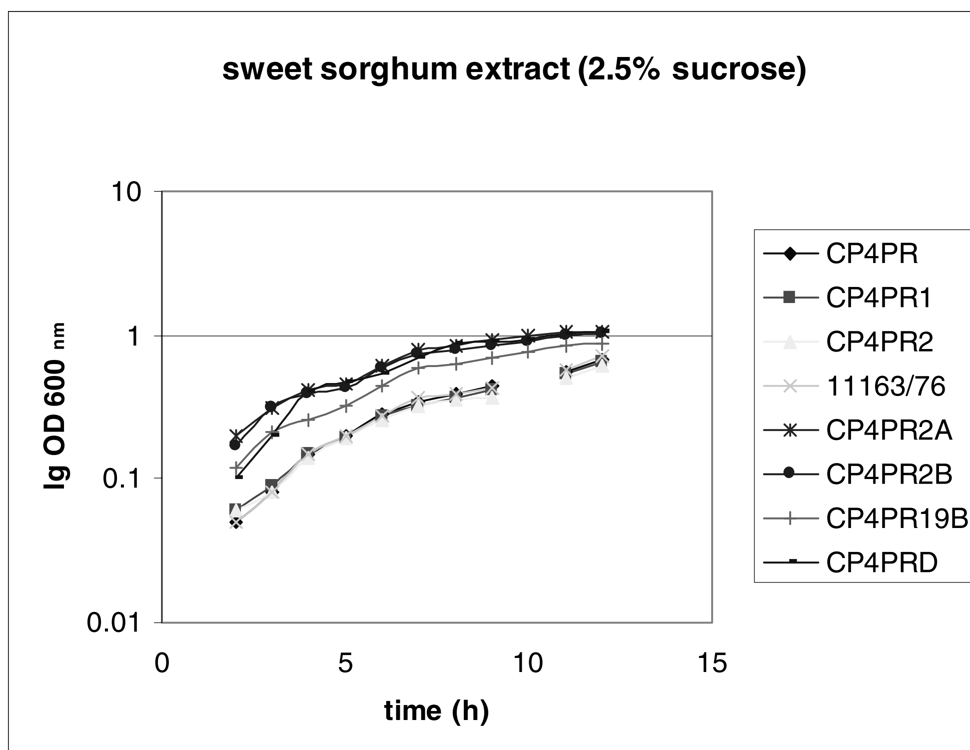
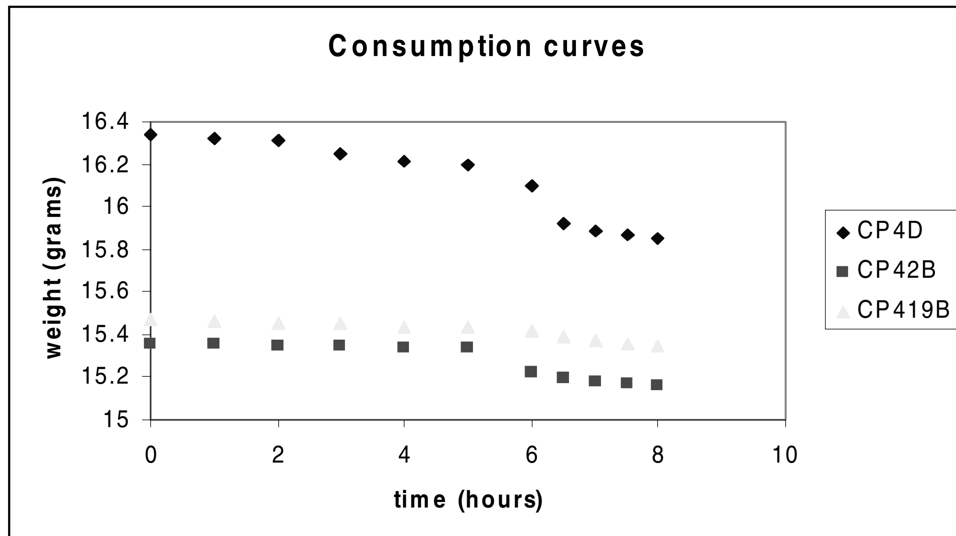


Figure 2. *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum, 2.5% sucrose. The strain 1163/76 is a methotrexate isolated strain.*



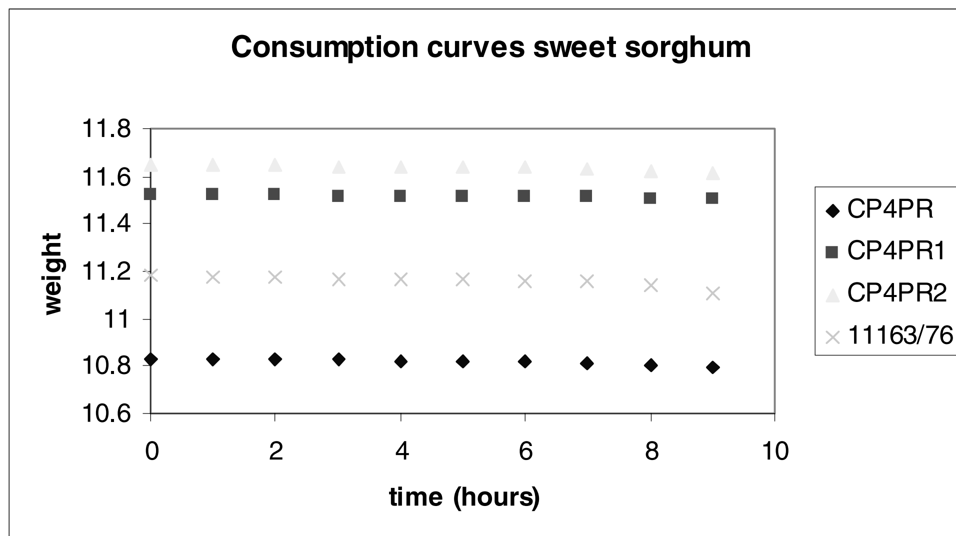


Figure 3. a. *Consumption curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum, 2.5% sucrose.* ***b.*** *Consumption curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum, 2.5% sucrose. The strain 1163/76 is a methotrexate isolated strain.*

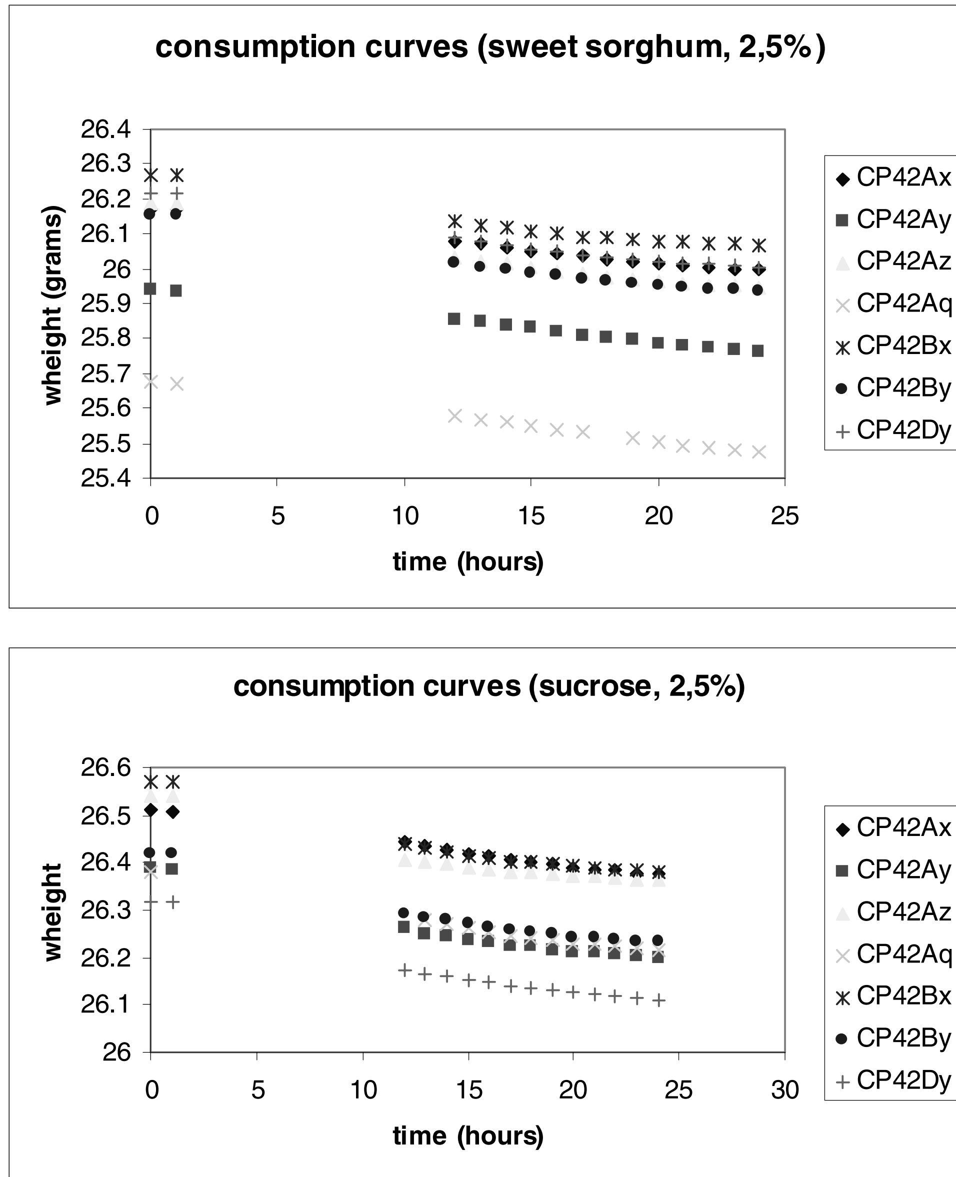


Figure 4. a. *Consumption curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum, 2.5% sucrose.* ***b.*** *Consumption curves of the Z. mobilis strains isolated throw UV mutagenesis on complete medium with 2.5% sucrose.*

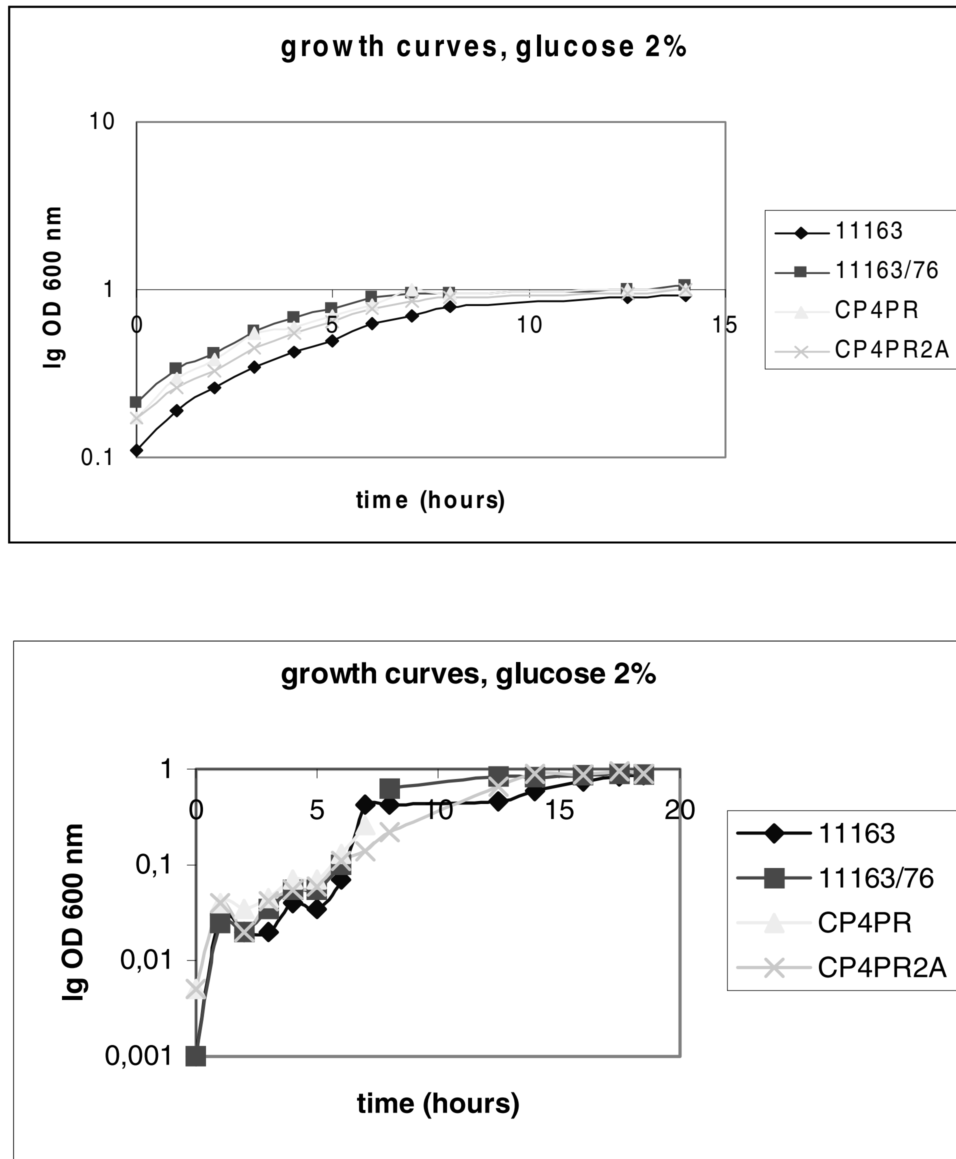


Figure 5. a. *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum, 2 % glucose. 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The cells were inoculated after washing with NaCl 0.9%.* ***b.*** *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on 2 % glucose. 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The culture was started using a preinoculum.*

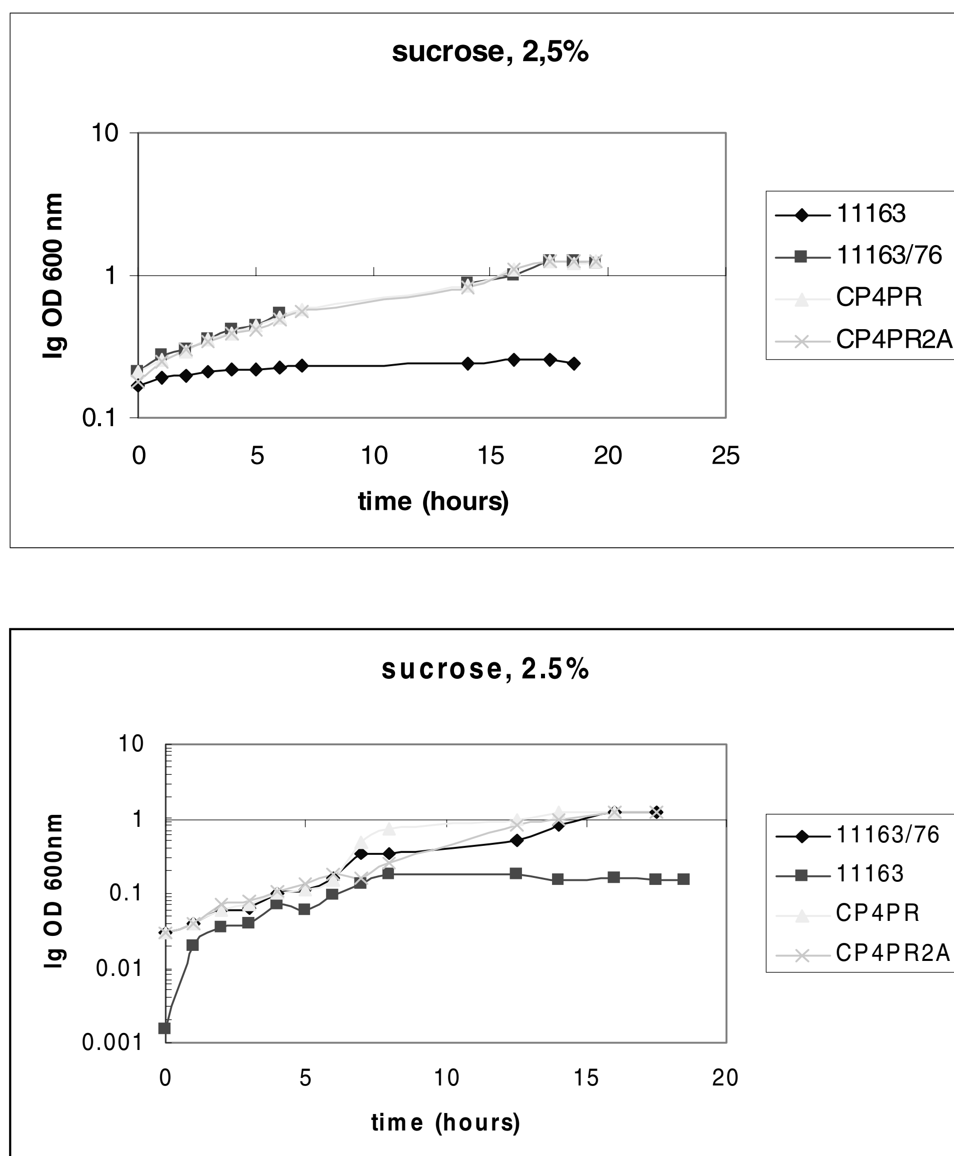


Figure 6. a. *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on 2.5 % sucrose 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The cells were inoculated after washing with NaCl 0.9%.* ***b.*** *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on 2.5 % sucrose. 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The culture was started using a preinoculum.*

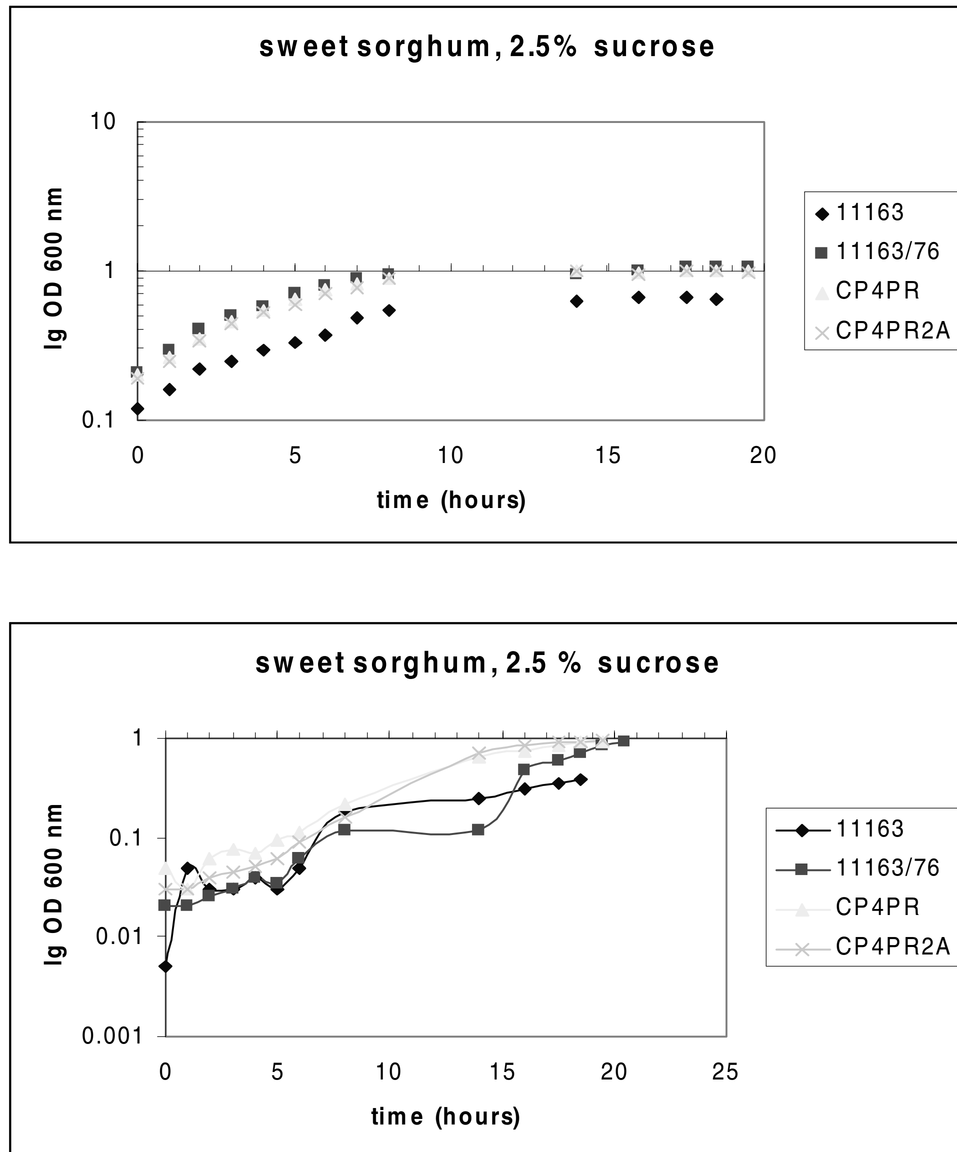


Figure 7. a. *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum (2.5 % sucrose) 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The cells were inoculated after washing with NaCl 0.9%.* ***b.*** *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on swet sorghum (2.5 % sucrose). 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The culture was started using a preinoculum.*

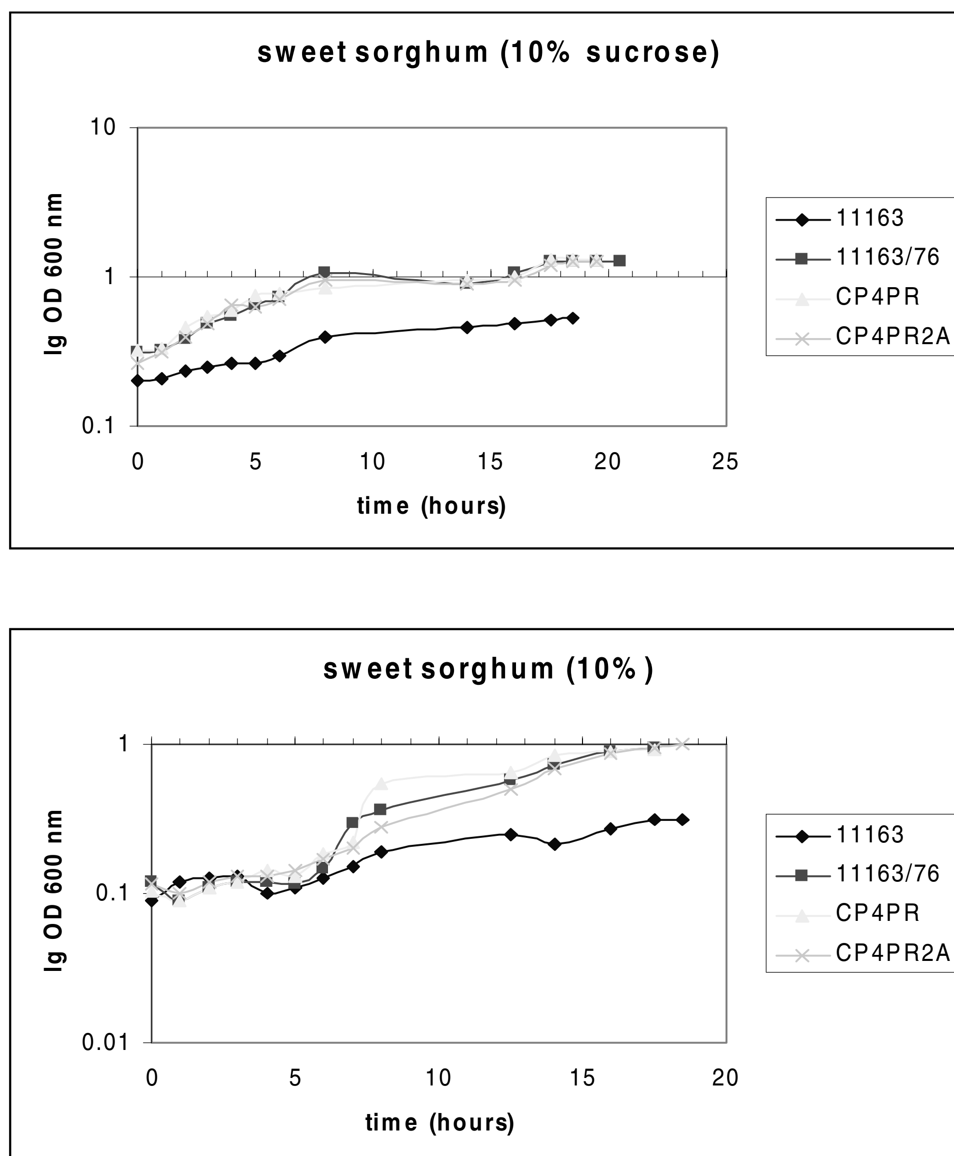


Figure 8. a. *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum (10 % sucrose) 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The cells were inoculated after washing with NaCl 0.9%.* ***b.*** *Growth curves of the Z. mobilis strains isolated throw UV mutagenesis on sweet sorghum (10 % sucrose). 11163 is a wild strain and 1163/76 is a methotrexate isolated strain. The culture was started using a preinoculum.*

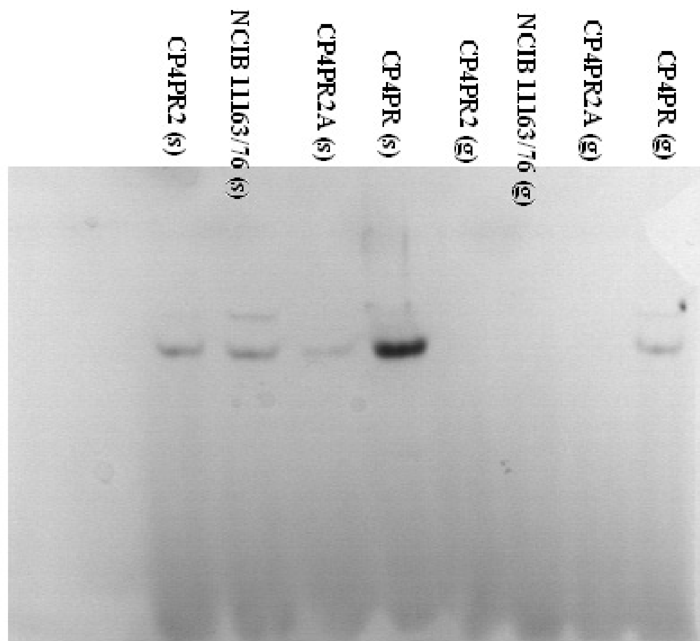


Figure 9. *Alcohol dehydrogenases profiles of CP4PR, CP4PR2A, NCIB 11163/76 and CP4PR2 strains grown on glucose (g) and sweet sorghum respectively (s).*

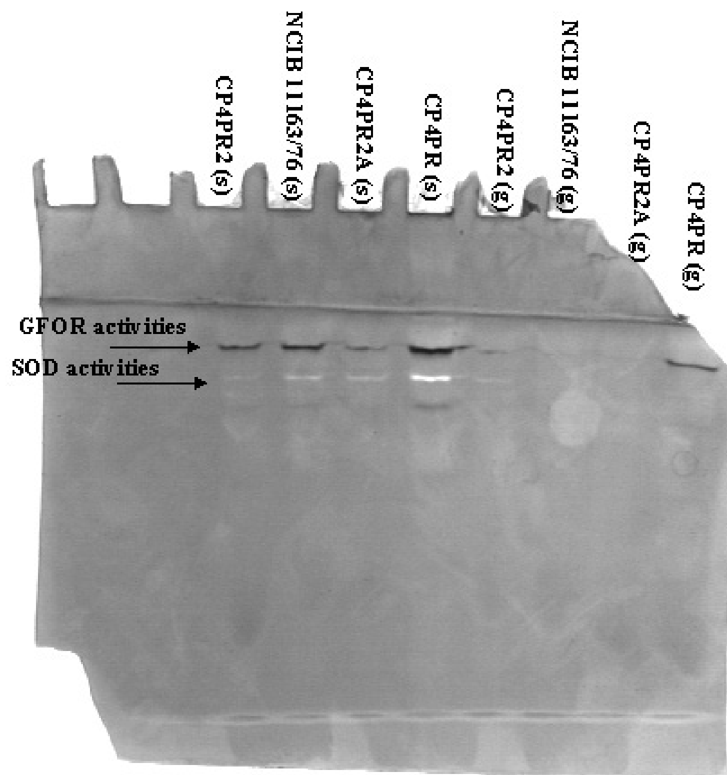


Figure 10. *GFOR and SOD activities of CP4PR, CP4PR2A, NCIB 11163/76 and CP4PR2 strains grown on glucose (g) and sweet sorghum respectively (s).*

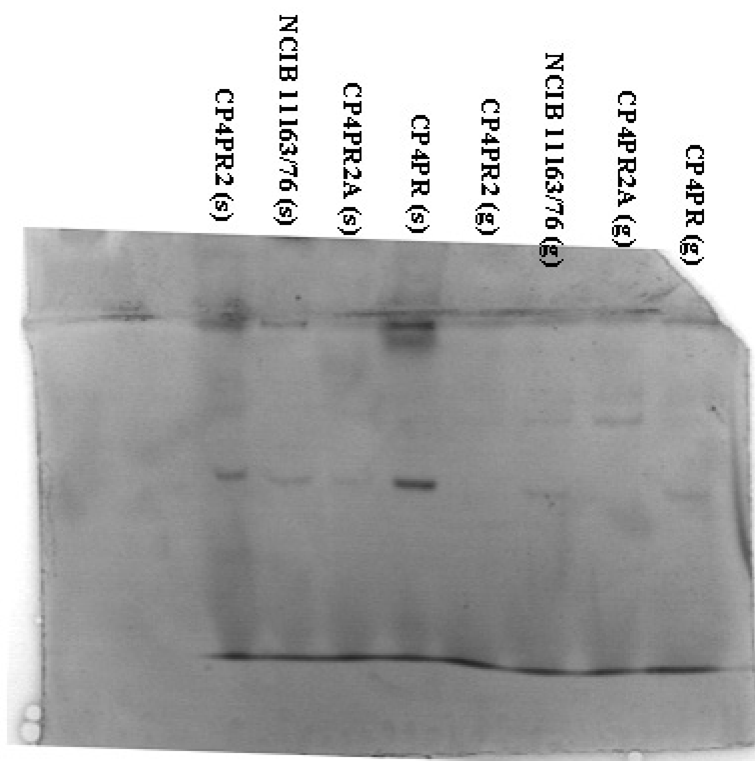


Figure 11. *NAD(P)H dehydrogenase profile of CP4PR, CP4PR2A, NCIB 11163/76 and CP4PR2 strains grown on glucose (g) and sweet sorghum respectively (s).*

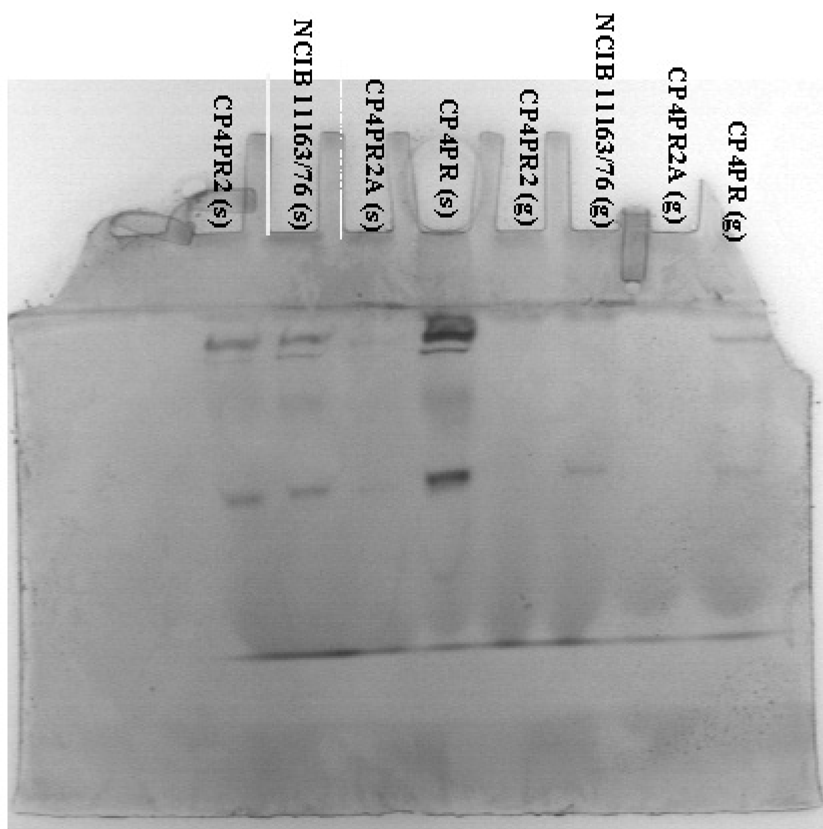


Figure 12. *NADH profile of CP4PR, CP4PR2A, NCIB 11163/76 and CP4PR2 strains grown on glucose (g) and sweet sorghum respectively (s).*

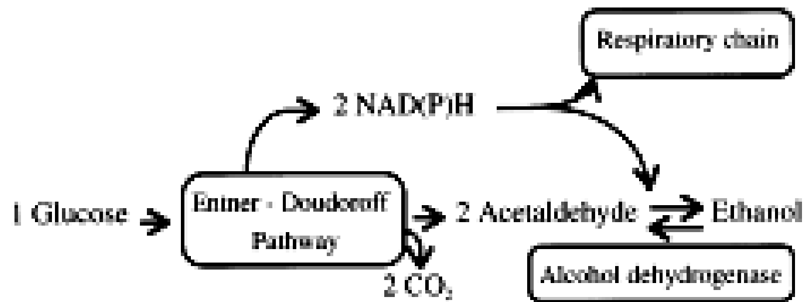


Figure 13. *Competition for reducing equivalents between the respiratory chain and alcohol dehydrogenase during aerobic glucose catabolism in Z. mobilis. Respiratory consumption of NAD(P)H limits reduction of acetaldehyde to ethanol [Kalnenieks et al., 2000].*

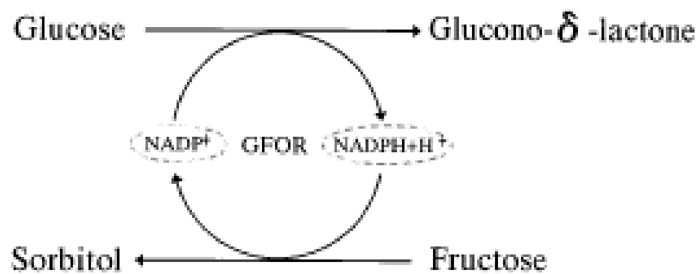


Figure 14. *Scheme of the ping-pong type reaction catalyzed by glucose-fructose oxidoreductase. NADP(H) as a redox carrier remains tightly bound to the enzyme. Gluconolactone is subsequently hydrolyzed to gluconic acid either spontaneously or by a specific lactonase.*