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# Osmotic adjustment of *Zymomonas mobilis* to concentrated glucose solutions*

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**Summary.** The physiological basis of the exceptionally high sugar tolerance of *Zymomonas mobilis* was investigated. Determinations of the internal metabolite concentrations of *Z. mobilis* showed that an increase in the extracellular glucose concentration was accompanied by a parallel rise in the intracellular glucose concentration, bringing about an almost complete osmotic balance between internal and external space. Studies of glucose transport confirmed that *Z. mobilis* has a facilitated diffusion system which enables a rapid equilibration between internal and external glucose concentrations. Studies using the non-metabolisable sugars maltose (impermeable) and xylose (permeable) revealed that these sugars were able to alter the osmotic pressure on the cytoplasmic membrane resulting in volume changes.

## Introduction

In general, growth of microorganisms is inhibited in media of high osmolarity. This is usually ascribed to the low water activity ($a_w$) of such media, often below 0.85 (Brown 1976; Jones and Greenfield 1986), and the phenomenon is widely exploited in food preservation (Duckworth 1975). Due to an enhanced external osmotic pressure, cell volume and turgor decrease until a new osmotic equilibrium is reached. Many cells treated in this way recover their initial turgor by accumulation of solutes, by transport or biosynthetic interconversion of metabolites (Cram 1976). In most organisms low-molecular-weight carbohydrates, particularly polyols (e.g. glycerol, arabitol, sorbitol) and disaccharides (e.g. trehalose) or quaternary ammonium compounds (e.g. betaines) are involved in adaptive responses to osmotic stress (Jennings 1984; Adler et al. 1985; Hocking 1988). Adaptation of microorganisms to high osmotic pressure has been reported for a wide range of organisms including bacteria (Measures 1975; Reed et al. 1984; Mackay et al. 1984), fungi (Jennings 1983) and algae (Hellebust 1976).

In the case of *Zymomonas mobilis* little is known about the basis of the observed high sugar tolerance. The ethanol-producing bacterium is able to grow in media containing up to 40% glucose (Swings and De Ley 1977). It has been shown that high concentrations of glucose (10–25%) can be efficiently and rapidly converted to ethanol via the Entner-Doudoroff pathway (Lee et al. 1979; Rogers et al. 1979). Both the specific glucose uptake rate and specific ethanol productivity are several times greater for *Z. mobilis* than for the yeasts *Saccharomyces cerevisiae* and *S. carlsbergensis*. The experimental data of fermentations with high sugar concentrations show decreases in cell yield and specific growth rate or extension of the lag-phase (Rogers et al. 1979). During batch fermentations the efficient conversion of glucose, fructose and sucrose to ethanol depends particularly on the tolerance to high initial substrate concentrations. As shown by Di Marco and Romano (1985), *Z. mobilis* uses a facilitated diffusion system for glucose transport into the cell.

This report focuses on the influence of various external glucose concentrations (2–16%) on the internal metabolite concentrations and on the catabolic rate of this substrate. Studies on the glucose transport system were performed to determine the kinetic parameters and rate-limiting step in glucose utilization. The objective of this research was the elucidation of the high-sugar-tolerance mechanism of *Z. mobilis*.

## Materials and methods

*Bacterial strain and culture conditions. Z. mobilis* subsp. *mobilis*, strain ATCC 29191, was grown anaerobically as described previously (Bringer et al. 1985). The following standard medium was used (per litre): 10 g Bacto yeast extract, 1 g $KH_2PO_4$; 1 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4 \cdot 7H_2O$; 20 g glucose (or as stated in the

* Dedicated to Professor R. K. Finn on the occasion of his 70th birthday

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text), pH 5.0. Glucose and fructose were sterilized separately and added to the final concentration as stated in the text.

Anaerobic and pH-regulated batch cultivations in 1.7-l laboratory fermentors were performed as decribed previously (Bringer et al. 1985). These cultures were inoculated with 10% of a preculture grown on standard medium with 50 g/l glucose.

For fermentations with gradually increasing sugar concentrations, cells in the exponential growth phase were harvested by centrifugation and suspended in standard medium without sugars. This suspension was used as inoculum for the standard medium containing various sugar concentrations (2–16% w/v). The cultures were incubated at 30° C. Samples of 0.1–0.2 ml for the determination of intracellular sugars were removed at an optical density measured at 550 nm ($OD_{550}$) of 3.5–4.0 corresponding to a cell mass of 0.6–0.7 mg dry weight/ml.

*Chemicals.* [U-$^{14}$C]Taurine, D-[U-$^{14}$C]glucose, D-[U-$^{14}$C]xylose, [U-$^{14}$C]maltose and $^3H_2O$ were purchased from Amersham International (Amersham, Buckinghamshire, UK).

Silicone oil PN 200 (density 1.03 g/cm$^3$) and PH 300 (density, 1.06 g/cm$^3$) were from Bayer (Leverkusen, FRG); silicone oil with a density of 1.05 g/cm$^3$ was obtained from Aldrich (Steinheim, FRG). Biochemicals were from Boehringer (Mannheim, FRG), other chemicals of analytical grade were obtained from Merck (Darmstadt, FRG) and Sigma (St. Louis, Mo., USA).

*Extraction and measurement of intracellular metabolites.* A 400-µl tube containing 30 µl of 20% $HClO_4$ as acid fixation layer, 65 µl silicone oil as separation layer and 100 µl of a cell culture grown as described above was used for centrifugation (Klingenberg and Pfaff 1967). Depending on the density of the medium, the density of the silicone oil was varied from 1.03 g/cm$^3$ (sugar concentrations up to 80 g/l) to 1.04 g/cm$^3$ (80–120 g/l) and to 1.06 g/cm$^3$ (sugar concentration 120–160 g/l).

After silicone oil centrifugation in a Beckman Microfuge E (Beckmann Instruments, Waldwich, NJ, USA) for 30 s, the sedimentated cells were ruptured by sonication (Bransonic B 2200 E-1, Branson Europa B.V., AA Soest, The Netherlands) for 5 min and neutralized by adding 24 µl of 5 M KOH in 1 M triethanolamine. Neutralization was followed by centrifugation in the cold to separate the precipitated $KClO_4$, denaturated protein and cell fragments from the extract. For calculation of the intracellular metabolite concentrations, the amount of extracellular sugar cosedimented with the cells in the intercellular space was subtracted from the total sugar concentration. Knowledge of the internal volume enabled calculation of the internal metabolite concentrations (Klingenberg and Pfaff 1967). The supernatant was used for determination of extracellular sugars and related compounds.

*Determination of cytoplasmic volume and internal metabolite concentrations.* According to Rottenberg (1979) the cytoplasmic volume was determined from the distribution of solutes in cell suspensions using [U-$^{14}$C]taurine as an impermeable marker for the extracellular space and [$^3$H]water for the total volume. Determination of the volumes occupied by these compounds allows the calculation of the total cytoplasmic volume of the cell. For measurement of the cytoplasmic volume an aliquot (500 µl) of the cell culture (standard medium) with an $OD_{550}$ between 5 and 7 (0.9–1.3 mg dry weight/ml) was added to a reaction mixture containing [U-$^{14}$C]taurine (0.2 µCi), $^3H_2O$ (0.2 µCi) and 0.38 mM taurine (final concentration). After 2–10 min, samples (0.1 ml) were treated by silicone oil centrifugation as described above. In order to determine the radioactivity of the sedimented cells in the perchloric acid layer, the tips of the tubes were cut at the silicone oil layer and homogenized by sonication. The extracts were centrifuged to remove the denaturated protein. Radioactivity was measured by liquid scintillation counting. Because of cosedimented radioactivity from the external medium, the total values were corrected by subtracting the amount of radioactivity of the taurine space.

*Determination of glucose uptake.* Cells in the late exponential phase were harvested by centrifugation followed by two wash cycles in a 0.05 M potassium phosphate buffer, pH 6.5, containing 0.2 g/l $MgSO_4 \cdot 7H_2O$ (=suspension buffer). Cell suspensions with an $OD_{550}$ between 5 and 7 were incubated in a water bath at 30° C for 15 min. Cells were added to test tubes containing [U-$^{14}$C]glucose (0.2 µCi) from to 50 mM (final concentration). The incubation time (3–30 s) was terminated by silicone oil centrifugation as described above.

*Determination of the intracellular osmolality.* Cell suspensions (≥ 50 mg/ml dry weight) were prepared as described above except that they were washed twice with water and resuspended in water. The intracellular osmolality was estimated by measuring changes in the osmolality in response to the presence of the detergent cetyltrimethylammonium bromide (1%). The osmolality was measured with a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, FRG).

*Analytical methods.* Cell densities were measured at 550 nm with a Shimadzu (Shimadzu Corp., Kyoto, Japan) photometer (UV-160). Glucose, fructose, sucrose, glycerol and sorbitol were analysed by HPLC (Merck Hitachi, Tokyo, Japan; L 6200) using an Aminex HPX-87C column (Biorad Labs, Richmond, Calif., USA) at 85° C with water as eluent (0.6 ml/min) and a refractometer (ERC 7512) as detector. Glucose, fructose, and sorbitol were also determined enzymatically as reported by Strohdeicher et al. (1988). Xylose and the contamination of the glucose solution with small amounts of fructose was determined by carbohydrate borate complex chromatography (Schimz et al. 1985). Ethanol was analysed by gas chromatography as described by Finn et al. (1984).

## Results

### *Influence of the osmolality of the medium on kinetic parameters*

As shown in Table 1 the growth rate and the cell yield of *Z. mobilis* in batch cultures decreased as the glucose concentration of the medium increased, whereas the specific ethanol productivity and specific glucose uptake rates were almost unaffected by high initial glucose concentrations. Thus the osmolality of the medium had no influence on the cell-specific rate of glucose breakdown. However, a pronounced extension of lag-phase times was observed with increasing glucose concentration.

### *Determination of internal metabolite concentrations of Z. mobilis*

For the measurement of internal glucose concentrations of *Z. mobilis* a series of batch cultures was grown with initial external glucose concentrations in the range 111–999 mM. In order to obtain cells with comparable rates of glucose uptake at still high sugar but low ethanol concentrations at the time of sampling, the specific glucose uptake rates of the individual cultures were kept at approx. 4.3 $g \cdot g^{-1} \cdot h^{-1}$ by varying the cultivation temperatures (see Fig. 1 legend). An increase in the extracellular sugar concentration was correlated with a parallel rise in intracellular non-phosphorylated glucose concentration (Fig. 1). However, external and internal glucose concentrations were not completely balanced,

**Table 1.** Kinetic parameters of *Zymomonas mobilis* batch fermentations with different initial glucose concentrations

| Initial osmolality of the medium [Os/kg] | 0.225 | 0.405 | 0.737 | 1.121 | 1.559 |
|---|---|---|---|---|---|
| Initial glucose concentration [g/l] | 20 | 50 | 100 | 150 | 200 |
| Specific growth rate $\mu$ [$h^{-1}$] | 0.462 | 0.347 | 0.276 | 0.267 | 0.168 |
| Lag-time of growth [h] | 1 | 1.5–2 | 6 | 8.5–9 | $\geq 10$ |
| Specific ethanol productivity $q_p$ [g/g/h] | 3.74 | 4.31 | 3.63 | 3.71 | 3.91 |
| Specific glucose uptake rate $q_s$ [g/g/h] | 7.40 | 8.13 | 7.68 | 7.91 | 7.56 |
| Cell yield $Y_{x/s}$ [g/g] | 0.075 | 0.063 | 0.062 | 0.056 | 0.043 |

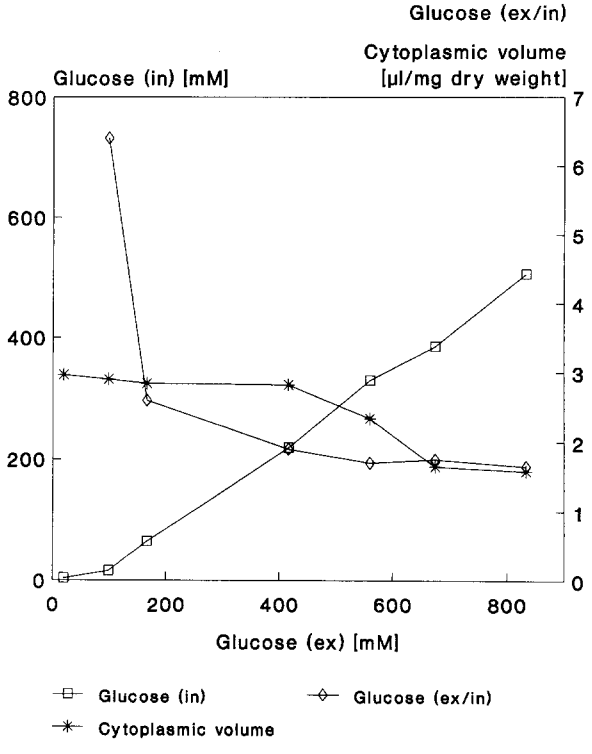


**Fig. 1.** Determination of the internal (in) glucose concentrations of *Zymomonas mobilis* cells growing at a constant glucose uptake rate in relation to the glucose concentration of the medium (ex). The almost constant glucose uptake rate was reached by shifting the incubation temperature from 20° C to 30° C when the initial glucose concentration was higher than 120 g/l. Cytoplasmic volumes were determined with 0.2 µCi [U-$^{14}$C]taurine (115 mCi/mmol) and 0.2 µCi $^3H_2O$

i.e. the internal glucose concentrations were always lower than the external.

The ratio of external to internal glucose concentrations was nearly constant above 200 mM extracellular glucose; it increased at lower extracellular glucose concentrations. This gradient between internal and external glucose concentrations, the driving force for the net influx, was maintained by the catabolism of glucose, which proceeded at an identical rate in all cultures examined. These results are in accordance with the characteristics of the glucose transport system of *Z. mobilis* reported recently by Romano et al. (1979) and Di Marco and Romano (1985). These authors demonstrated substrate specificity for and counterflow of glucose and the absence of an energy-requiring step for glucose transport. They concluded that glucose is transported via a facilitated diffusion system in *Z. mobilis*. As a consequence of the concentration gradient between external and internal glucose, cell shrinkage was observed (Fig. 1). However, the degree of cell shrinkage was not strictly correlated with gradient magnitude; the largest discrepancies were observed at external glucose concentrations below 200 mM.

At external glucose concentrations above 450 mM, *Z. mobilis* cells also contained the sugar alcohol sorbitol (data not shown). Since the internal sorbitol concentrations reached a maximum of only 50 mM (at an external glucose concentration of 650 mM) a significant contribution to the overall osmotic balance could not be attributed to sorbitol. Other cytoplasmic solutes, however, should contribute to the intracellular osmotic pressure. The cytoplasmic osmolality of sugar-depleted cells of *Z. mobilis* was 310 mOs/kg whereas the osmolality of the medium without carbon source was 110 mOs/kg. Comparable values for the intracellular osmolalities of *Salmonella typhimurium* and *Escherichia coli* of approximately 300 mOs have been reported when the cells were suspended in mineral salts medium (Stock et al. 1977).

The membrane carrier mediating a facilitated diffusion of glucose in *Z. mobilis* implies that the net rate of transport across the cell membrane is the difference between influx and efflux and that it depends on the ratio of external to internal glucose concentrations. To support this assumption we determined the internal concentration of xylose, a sugar which is transported –

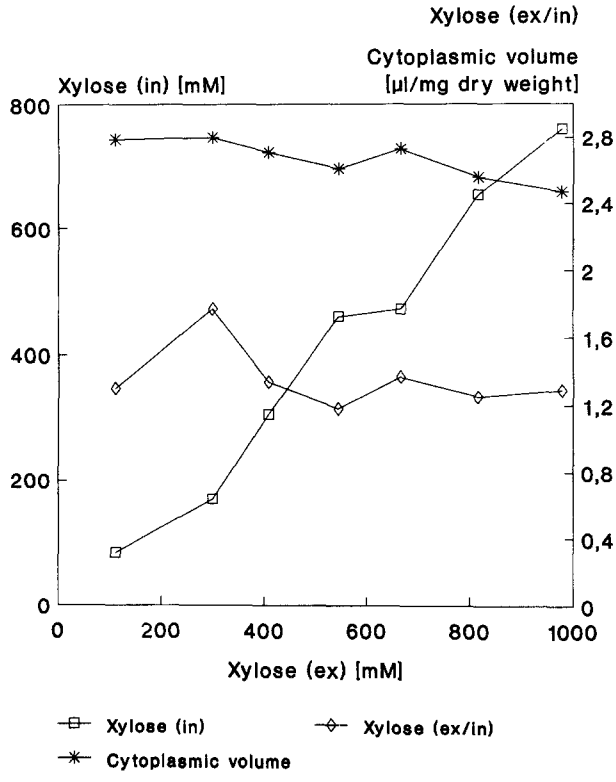


**Fig. 2.** Relationship between the internal xylose concentration and the ratio (external/internal xylose) to the external xylose concentration. *Z. mobilis* cells were grown in a medium with 5% (w/v) glucose as carbon source, washed and incubated in a glucose-free medium with various xylose concentrations (2–14% w/v) for 20 min at 30° C

most probably by the glucose carrier (Di Marco and Romano 1985) – but not further metabolized by *Z. mobilis*. Therefore, *Z. mobilis* was incubated with xylose concentrations (2–14%) that resulted in high intracellular xylose concentrations (Fig. 2). As the external xylose increased, the quotient of external to internal xylose concentration remained almost constant at 1.3 compared to 1.7 when the cells were incubated with glucose, i.e. a more complete balance was approached with the non-metabolizable sugar xylose.

### *Determination of the kinetic parameters of glucose transport*

The rate of uptake of glucose was determined for concentrations ranging from 1 to 50 mM glucose. The Lineweaver-Burk plot (data not shown) of initial glucose uptake rate versus glucose concentration indicated a glucose transport system with low affinity for the substrate glucose ($K_M = 11$ mM) and high uptake velocity ($V_{max} = 220$ nmol·min$^{-1}$·mg$^{-1}$ dry weight). These kinetic data are in good agreement with those of Di Marco and Romano (1985). However, the apparent $V_{max}$ value (220 nmol·min$^{-1}$·mg$^{-1}$ dry weight) is too low when compared to the in-vivo-glucose uptake rate (Table 1) of about 7.7 g·g$^{-1}$·h$^{-1}$ (≈712 nmol·min$^{-1}$·mg$^{-1}$ dry weight). For an explanation of this discrepancy it must be noted that in the assay for the determination of maximum rates of glucose transport ($V_{max}$) a self-adjusting net transport was measured instead of the initial maximum transport velocities. The net flux is the difference between influx and efflux of sugar obeying Michaelis-Menten-type kinetics.

Measurements of the initial velocity of sugar uptake under conditions of negligible efflux are not possible in practice. Due to the efficient transport system the internal concentration rises rapidly in a few seconds to high values (e.g. 2–3 s are sufficient to enhance the glucose concentration at 10 mM). Under these circumstances the efflux is not negligible and values calculated from the influx equation are erroneous. In addition, it was observed that cells prepared for the transport assay (two wash cycles, three centrifugation steps) showed a lower glucose uptake rate (3.05 g·g$^{-1}$·h$^{-1}$ = 282 nmol·min$^{-1}$·mg$^{-1}$ dry weight) and a lower productivity than cells grown in a fermentor and not submitted to washing and centrifugation. Therefore, the conclusion must be drawn that the apparent $V_{max}$ and $K_M$ values obtained from the transport assays reflect the catabolism of glucose under the special conditions used, rather than the maximum velocity of transport.

### *Effects of xylose and maltose on the cytoplasmic volume*

The presence of a non-transportable sugar in the medium should lead to a stronger cell shrinkage than that observed with glucose or other transportable sugars. Results with the non-transportable disaccaride maltose are shown in Table 2. In comparison with isoosmolal concentrations of the permeable sugars glucose and xylose, only maltose, which is not taken up (data not shown), caused a reduction in the cytoplasmic space of about 30%. Thus, the incapability of *Z. mobilis* cells to transport maltose into the cytoplasm resulted in an imbalanced osmotic pressure on the cytoplasmic membrane and a consequent decrease in cell volume. In the presence of maltose and low concentrations of glucose in the medium no cell shrinkage was observed, i.e. the osmotic pressure drop inside the cells was diminished since the osmotic pressure gradient exerted by maltose

**Table 2.** Determination of the cytoplasmic volume with 0.2 µCi [U-$^{14}$C]taurine (115 µCi/µmol) using isoosmolal concentrations of glucose, xylose or maltose

| Carbon source of the incubation medium | Concentration of the carbon source [mM] | Cytoplasmic volume [µl/mg dry weight] |
|---|---|---|
| glucose | 428 | 3.0 |
| xylose | 428 | 2.8 |
| maltose | 400 | 2.2 |
| glucose and maltose | 55<br>400 | 2.7 |

could be partially compensated by glucose uptake (Table 2).

## Discussion

The strategy employed by *Z. mobilis* to survive and grow in media with reduced water activities ($a_w$) due to the presence of high sugar concentrations essentially comprises two mechanisms. One of these is the transport of glucose into the cell to balance the osmotic pressure inside with the external osmotic pressure, thus preventing excessive movement of water out of the cell. Glucose contributes directly to an increase in the cytoplasmic osmotic pressure by rapidly permeating from the extracellular medium into the cell. Intracellular glucose together with other osmotically active cytoplasmic solutes (e.g. ions, ≈310 mOs/kg) are sufficiently concentrated to render the cell and its environment isoosmotic. The second response is an increase in the internal osmotic pressure by losses of water from the cytoplasm, i.e. cell shrinkage, until its osmotically active contents are sufficiently concentrated to balance the extracellular osmolarity. This mechanism for an osmotic equilibration of the cells is particularly evident when glucose or another transportable sugar is absent from the medium. Under these conditions the osmotic pressure on the cell wall exerted by non-transportable sugars (e.g. maltose) exceeds the intracellular osmotic pressure. As the cell wall is unable to resist compression, the cell loses water resulting in cell shrinkage (cf. Table 2) until the osmolality of the material inside the cell is equivalent to the osmolality of the surrounding fluid.

Despite the almost complete osmotic balance between cytoplasm and medium the cytoplasmic volume decreased when the osmolality of the medium was increased. This phenomenon, however, was not strictly related to the cell metabolism, which requires an imbalance of extracellular and intracellular glucose to create a chemical gradient, which in turn is a prerequisite of a net glucose influx. Shrinkage of the cells could also be observed with cells that had only a low metabolic rate (data not shown). A similar phenomenon has been reported for the sugar-tolerant yeast *Hansenula anomala* during growth with glucose at an $a_w$ of 0.95 (van Eck et al. 1989). The maximum cell volume and specific growth rate was measured at an $a_w$ of 0.998 and decreased linearly when the $a_w$ was reduced to 0.925 by addition of glucose. Concomitantly, this yeast produced glycerol and arabitol as compatible solutes, which accumulated to a concentration ratio (intracellular/extracellular) of as high as 10000-fold. The likely explanation for this behaviour is that cells cultivated under conditions covering a wide range of medium osmolalities were at different stages of growth. It is well known that the cell volumes of slow-growing bacteria are significantly smaller than the volumes of fast-growing bacteria. Changes in the cytoplasmic volume related to the growth phase of cells as reported for *E. coli* B/r A, *E. coli* B/r H266 and *E. coli* K-12 CR34 (Donachie and Robinson 1987) may also have contributed to the volume changes observed with the cells of *Z. mobilis*.

The occurrence of large amounts of glucose inside the cells of *Z. mobilis* is brought about by a carrier-mediated transport system with high velocity and high capacity (Di Marco and Romano 1985). Among microorganisms the transport of glucose via facilitated diffusion occurs only rarely, for example in *Saccharomyces cerevisiae* with a low-affinity system (Bisson and Fraenkel 1983; Bisson et al. 1987; Lang and Cirillo 1987; Romano 1982), in *Candida utilis* (Postma et al. 1988) *C. intermedia* (Loureiro-Días 1987), and in *C. shehatae* (Lucas and van Uden 1988). To our knowledge *Z. mobilis* is the only bacterium using this type of glucose transport system. In contrast, the uptake of glucose into most plant and animal cells cells is accomplished by facilitated diffusion, mediated by a family of structurally related proteins (Gould and Bell 1990). One of the most extensively studied facilitated transport systems is the glucose carrier of human erythrocytes (Wheeler 1989; Carruthers and Helgerson 1989). Glycerol as a widespread osmoadaptive substance is transported by many species of bacteria via facilitated diffusion (Dills et al. 1980; Sweet et al. 1990). Facilitated diffusion is a passive process driven by gradients in the chemical or electrochemical potential of the solute (Friedman 1986). The maximum transport capacity of a facilitated diffusion process is difficult to quantify, because the velocity of equilibration between the extracellular and intracellular space is extremely rapid in comparison to the time resolution of the experimental method.

The ability of *Z. mobilis* to grow in concentrated sugar solutions reflects the organism's natural abundance in saccharated environments. *Z. mobilis* strains have been isolated from fermenting agave and palm saps, sugar cane juice, ciders, beers, perries and from other alcoholic fermentations with high concentrations of glucose, fructose and sucrose (Swings and De Ley 1977). Catabolism of these carbon sources in the natural habitat can lead to sorbitol concentrations of up to 11% (w/v) when glucose and fructose as a mixture or as hydrolysis products of sucrose are present (Zachariou and Scopes 1986). Sorbitol is also formed when glucose is the sole carbon source (Viikari 1988), but at very low external concentrations. Although the internal sorbitol concentrations were higher than the external ones, sorbitol contributes only to a minor extent to the osmotic equilibrium between cytoplasm and medium.

Generally, the sugar tolerance of *Z. mobilis* is brought about by almost isoosmotic concentrations of internal and external solutes. Besides other osmotically active cytoplasmic solutes glucose is the main internal solute that is provided by an efficient glucose transport system. This is consistent with the natural occurrence of *Z. mobilis* in environments with high sugar concentrations and the rapid fermentation of sugar with low energetic yield.

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