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Laboratory of Bioprocess Engineering
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PROCESS DEVELOPMENT FOR MANNITOL PRODUCTION BY LACTIC ACID BACTERIA

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ABSTRACT

D-Mannitol (here: mannitol) is a naturally occurring sugar alcohol with six carbon atoms. It is only half as sweet as sucrose. However, mannitol and other sugar alcohols exhibit reduced caloric values compared to the respective value of most sugars, which make them applicable as sweeteners in so-called “light” foods. Moreover, sugar alcohols are metabolized independently of insulin and are thus also applicable in diabetic food products. Besides applications in the food industry, mannitol is also used in the pharmaceutical industry. In medicine, mannitol is used to decrease cellular edema (excessive accumulation of fluid) and increases the urinary output.

In this doctoral thesis, the development of a new bioprocess for the production of mannitol is described. For this purpose, aspects such as strain selection, choice of process method, optimization of process parameters, scale-up, and metabolic engineering were studied. At present, mannitol is produced commercially by catalytic hydrogenation of fructose-containing syrups. The existing chemical production methods are, however, characterized by several drawbacks. The uppermost being that when fructose is catalytically hydrogenated only about 50% of it is converted into mannitol, whereas the rest is converted into another sugar alcohol, sorbitol. In addition, ultra-pure (expensive) raw materials (fructose and hydrogen gas) are required for efficient conversion. When more cost-effective raw materials, such as glucose-fructose syrups are used as starting material for catalytic hydrogenation, the main product is sorbitol and mannitol is formed as a by-product. Hence, mannitol production becomes very dependent on the market demand of sorbitol. Furthermore, mannitol is relatively difficult to purify from sorbitol. In addition, ion exchange is required for removal of the metal catalyst from the production solution. This results in even higher production costs and decreased yields.

The microbial mannitol production process described in this thesis is based on high cell density cultures of slowly growing heterofermentative lactic acid bacteria. The bioconversion of fructose to mannitol was performed in a slowly agitated membrane cell-recycle bioreactor equipped with pH and temperature control. Neither aeration nor nitrogen flushing of the bioconversion medium was required, which drastically lowers the investment costs of such a plant. An important detail in the new bioprocess was the re-use of cell biomass in successive bioconversions. In a semi-continuous production experiment, the initial cell biomass provided stable mannitol productivities and yields for at least 14 successive batches. Moreover, using a simple

purification protocol comprising cooling crystallization of a supersaturated solution and crystal recovery by means of drum centrifugation, high yields of high-purity (>98%) mannitol crystals were obtained. Moreover, in scale-up trials the microbial mannitol production process was successfully run at a small pilot-scale (100 L).

The yield of crystalline mannitol from the initial sugar consumed in the bioprocess was about 52% (w/w). This compares favorably to a commercial chemical process with a yield of about 39%. Hence, under optimized conditions the best production strain (*Leuconostoc mesenteroides*) converted up to 95% (mol/mol) of fructose consumed into mannitol. Unfortunately, this is only achieved when a significant amount of glucose is co-metabolized by the cells. The catabolism of glucose enables cofactor regeneration in the cells and is thus, essential for the bioconversion of fructose to mannitol. Moreover, some mannitol is also lost in the purification steps. Another significant improvement brought about by the new bioprocess was a reduced by-product burden. In the commercial chemical process, a total of 1.58 kg by-products are formed for each kilogram of mannitol crystals produced. In the bio-process, only 0.67 kg by-products are produced per kilogram crystalline mannitol.

Using tools of genetic engineering, two key enzymes involved in the primary metabolism of another efficient mannitol-producer, *Lactobacillus fermentum*, were inactivated. A mutant deficient in D-lactate dehydrogenase and grown in a fructose-glucose medium produced high levels of both mannitol and pure L-lactate. Inactivation of both lactate dehydrogenases resulted in major rerouting of glucose catabolism, which led to the accumulation of pyruvate and production of 2,3-butanediol. Moreover, mutants with lowered fructokinase activity and deficient in acetate kinase were constructed and studied.

PREFACE

The work was carried out in the Laboratory of Bioprocess Engineering, Helsinki University of Technology during the years of 1999-2002. First and foremost, I would like to express my warmest thanks to Professor Matti Leisola for supervising my work and encouraging me to study the fascinating field of biotechnology. The two pre-examiners, Professor Seppo Salminen and Dr. Heikki Ojamo, are warmly thanked for their constructive feedback and good suggestions.

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Finally, I thank my family and friends for their support and understanding.

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Mummon (1914-99) ja Tattan (1911-98) muistolle.

Till minnet av Farfar (1917-99).

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
C_i	concentration (g/L)
cdw	cell dry weight (g)
DOT	dissolved oxygen tension
HPLC	high-performance liquid chromatography
LAB	lactic acid bacteria
m_i	mass (g)
M_i	molecular mass (g/mol)
MCRB	membrane cell-recycle bioreactor
n_i	number of moles (mol)
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
P	inorganic orthophosphate ($-PO_4$)
PCR	polymerase chain reaction
r_i	volumetric productivity (g/L/h or mol/L/h)
q_i	specific productivity or consumption rate (g/g cdw/h)
Q_{fru}	fructose feeding rate per biomass (g/g cdw/h)
t	time (h)
V	volume (L)
v/v	volume per volume (L/L × 100%)
vvm	volume of air per minute per working volume in reactor (L/L/min)
w/v	weight per volume (g/mL × 100%)
w/w	weight per weight (g/g × 100%)
x_i	conversion (mol/mol)
X	biomass concentration (g cdw/L)
X’	logarithmic mean of the biomass (g cdw/L)
Y_i	yield (mol/mol × 100%)
μ_{max}	maximum specific growth rate (1/h)

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1. INTRODUCTION

1.1 D-Mannitol

Sugar alcohols, also known as polyols, are the hydrogenated form of sugars. Hydrogenation reactions involve the addition of hydrogen atoms. In the case of monosaccharide hydrogenation, free hydrogen atoms attack the carbonyl group, resulting in the breakdown of the C=O double bond. Monosaccharides are polyhydroxy ketones and aldehydes and thus, the location of the carbonyl group varies among the different sugars. In D-fructose, also known as fruit sugar, the second carbon atom forms the carbonyl group and when hydrogenated, D-mannitol and D-sorbitol are formed (Figure 1). Hence, the two hydrogen atoms added in the reaction are bound to carbon number two and to the oxygen atom of the same carbon atom. Other common sugar alcohols derived from monosaccharides are e.g. xylitol (from xylose) and ribitol (from ribose). Sorbitol is usually derived by hydrogenation of glucose.

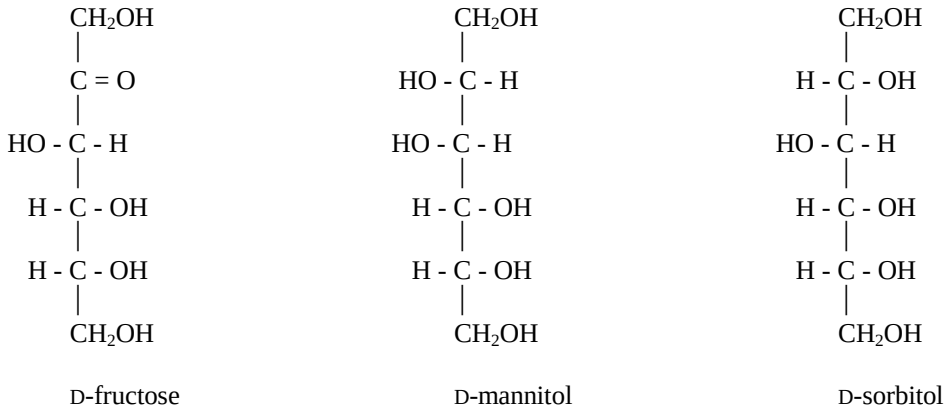


Figure 1. Chemical structures of D-fructose, D-mannitol, and D-sorbitol.

1.2 Natural occurrence of mannitol

D-Mannitol is a naturally occurring sugar alcohol found in animals and plants. It is present in small quantities in most fruits and vegetables (Ikawa *et al*., 1972). Typically, it can be found in such plants as pumpkins, celery, onions, grasses, olives, mistletoe, and lichens. Mannitol is also found in manna, the dried exudate of the manna ash tree (*Fraxinus ornus*) (Schwarz, 1994). Manna is obtained by heating the bark of the tree and it can contain up to 50% mannitol. Hence, manna has been a commercial source of mannitol in Sicily, Italy (Soetaert *et al*., 1999).

Marine algae, especially brown algae, are also rich in mannitol (10-20% depending on the time of harvest) (Schwarz, 1994). Furthermore, mannitol is commonly found in the mycelium of various fungi and it is present in fresh mushrooms at about 1%.

Also some fungi and bacteria produce mannitol. Moreover, small quantities of mannitol are found in wine (Benson, 1978).

The optical isomer, L-mannitol, does not occur naturally, but is obtained e.g. by the reduction of L-mannose or L-mannonic acid lactone (Benson, 1978). This doctoral thesis is focused on the production of the D-form and hence, the use of "mannitol" refers exclusively to D-mannitol.

1.3 General properties of mannitol

The properties of mannitol (mannite, D-mannohexane-1,2,3,4,5,6-hexaol, mannitolum, mannitolo, or manna sugar) are fairly similar to those of its stereoisomer, sorbitol. However, the solubility of mannitol in water is significantly lower than that of sorbitol and most of the other sugar alcohols. At 14°C the solubility of mannitol in water is only approximately 13% (w/v) (Perry *et al*., 1997). At 25°C the solubility of mannitol in water is approximately 18% (w/v) (Soetaert *et al*., 1999). Mannitol is sparingly soluble in organic solvents, like ethanol and glycerol, and practically insoluble in ether, ketones, and hydrocarbons (Schwarz, 1994).

The relative sweetness (to sucrose) varies among different sugar alcohols. The relative sweetness of xylitol is 100%, whereas the relative sweetness of mannitol is only 40-50% (Schiweck *et al*., 1994; Anon., 2001a). Mannitol forms white orthorhombic needles and the crystals have a melting point of 165-168°C (Schwarz, 1994). Owing to the high positive heat (or enthalpy) of solution in water, 120.9 kJ/kg (Schiweck *et al*., 1994; Lawson, 1997), a cooling sensation occurs when mannitol crystals dissolve in the mouth. This effect is commercially used in e.g. chewing gums, but is less pronounced than that observed with xylitol (153.1 kJ/kg) (Schiweck *et al*., 1994; Lawson, 1997). Crystalline mannitol exhibits a very low hygroscopicity (it does not add moisture or contribute to moisture pick-up). Moreover, it is also chemically inert. These properties make mannitol very useful in production of tablets and granulated powders.

1.4 Applications of mannitol

The chemical structure of sugar alcohols allows them to be absorbed more slowly by the body than regular sugars. Therefore, they have a smaller impact on blood insulin levels. Due to the sweet taste of sugar alcohols combined with the independence of insulin when metabolized by the body, individuals who should not eat sugar (due to e.g. diabetes) now consume products in which common sugars are replaced with sugar alcohols. As mentioned earlier the sweetness of mannitol is somewhat lower than that of sucrose and thus, artificial sweeteners, such as e.g. Saccharin or Aspartame, are needed to enhance the final sweetness of mannitol-based products. In the production of such foods, mannitol usually functions as a bulking agent giving the final product a mouth-feel similar to the sugar-based foods.

Sugar alcohols also exhibit reduced physiological caloric values compared to sugars. For example, the caloric contents of mannitol and sucrose are 1.6 kcal/g and 4

kcal/g, respectively (Anon., 2001a). The reduced caloric values are due to the fact that sugar alcohols are only partially absorbed in the upper intestine (see above). Thus, a large part of the ingested sugar alcohols reaches the large intestine, where bacteria degrade it (Schiweck *et al*., 1994). Because of the reduced caloric value compared to most sugars, sugar alcohols are commonly used for the production of reduced-calorie (light) products. On the other hand, incomplete absorption can result in gastrointestinal side effects, like gas, flatulence, and diarrhea (Schiweck *et al*., 1994). Mannitol has the lowest laxatation threshold observed for sugar alcohols and hence, a maximum daily intake should not exceed 20 g (Anon., 2001a). Moreover, when sugar alcohols are used as a sweetening agent in diabetic foods, the calories should always be accounted.

At present, the main application for mannitol in the food industry is as a sweetener in sugar-free chewing gums and for dusting chewing gum sticks. In addition, mannitol is used as a bodying and texturizing agent, anticaking agent, and humectant (Salminen *et al*., 1998). Furthermore, mannitol is also used to increase the shelf life of various foodstuffs (Soetaert *et al*., 1999).

Toxicity studies have not indicated any mannitol caused adverse effects other than diarrhea (Anon., 2002a). Therefore, mannitol is considered safe for use in foods and it has a food additive status (E421). Mannitol is presently on the U.S. FDA GRAS-/INTERIM (*Generally Recognized As Safe*) list (Anon., 2002b). Hence, large-scale use of mannitol in the food industry is mostly inhibited by properties like the laxative side effects, a higher price compared to e.g. sorbitol, and a high tendency to crystallize in water-containing products.

In the pharmaceutical industry mannitol is used in various applications. Mannitol exhibits low chemical reactivity, excellent mechanical compressing properties, and low hygroscopicity and is thus commonly used as a constituent in chewable tablets and granulated powders. Furthermore, its sweet cool taste is used to mask the unpleasant taste of many drugs (Schwarz, 1994; Soetaert *et al*., 1999).

In medicine, mannitol (“Osmitrol”) is used to increase the formation of urine in order to prevent and treat acute renal failure, and also in removal of toxic substances from the body. Hence, mannitol belongs to a group of drugs referred to as *osmotic diuretics*. Due to its large size, mannitol molecules are kept in the vascular space of the body. This creates an osmotic gradient between the tissue and the intravascular systems, resulting in movement of fluid from the former space to the latter. Thus, mannitol is also used to reduce both cerebral edema (increased brain water content) and intraocular pressure. Furthermore, it is used to alter the osmolarity of the glomerular filtrate in treating kidney failures (Anon., 2001b).

In addition, mannitol is useful in making artificial resins and plasticizers and as an intermediate in the manufacture of the vasodilator D-mannitol hexanitrate (Johnson, 1976). The complex of boric acid with mannitol is used in the production of dry electrolytic capacitors (Soetaert *et al*., 1999).

1.5 Mannitol market

The global market for sugar alcohols (polyols) in 2000 was $1.3 billion (Anon., 2001c). The largest sugar alcohol in terms of volume and dollar sales was sorbitol. Sorbitol, which was developed in 1950s, is mostly sold as a 70% (w/v) aqueous solution. More than one million tons of sorbitol (liquid and crystalline) was sold in 2000, mainly in the U.S., Europe and Asia. The bulk prices for liquid and crystalline sorbitol are about $0.55-0.65 per kg and $1.61-2.26 per kg, respectively (Anon., 2002c).

All other sugar alcohols, typically developed and introduced into the market in the 1990s, are relatively small in volume. The annual mannitol market was estimated at about 30 000 tons (Soetaert *et al*., 1995). In 1999 (1.1.-31.9.), about 33 tons of crystalline mannitol was imported to Finland, with an average price of $4.34 per kg (Anon., 2000a). In 2001 (1.1.-31.12.) the respective values were 44 tons and $4.11 per kg (Anon., 2002c). According to a recent issue of the *Chemical Market Reporter*, however, the bulk price of mannitol (powdered) is $7.32 per kg (Anon., 2002d).

For most of the sugar alcohols the market is mature and volume growths are expected to follow the trends of the large-scale consumer products in which sugar alcohols are used (Anon., 2001c). For the next five years, the annual volume growth is estimated at 2-3%, while the pricing is expected to decline by 1-2%. Sorbitol is facing growing competition from other commercial sugar alcohols, like e.g. mannitol and xylitol. Also, the largest application of sorbitol in toothpaste formulation is mature and is expected to grow only with population growth, i.e. annually 1-2% (Anon. 2001d).

2. PROCESSES FOR THE PRODUCTION OF MANNITOL

2.1 Chemical hydrogenation

Production of mannitol by extraction of plant raw material (e.g. manna, seaweed, or algae) is no longer economically relevant (Schwarz, 1994). Today, mannitol is commercially produced by catalytic hydrogenation of fructose, sucrose (invert sugar), or glucose-fructose syrups (e.g. HFCS, high-fructose corn syrup) (Schwarz, 1994; Ojamo *et al*., 2000).

The hydrogenation process is performed at high pressure and temperature applying a metal catalyst (e.g. Raney nickel) and hydrogen gas. In this catalytic hydrogenation reaction, only the β-fructose molecules are hydrogenated into mannitol, whereas the α-fructose molecules are hydrogenated into sorbitol (Soetaert *et al*., 1999). Therefore, when a 50/50 glucose-fructose mixture (e.g. invert sugar) is used as the starting material, at neutral pH, only approximately 25% (w/w) of the initial sugar is hydrogenated into mannitol, whereas 75% is hydrogenated into sorbitol. If sucrose is used as the starting material and the hydrogenation is performed at alkaline pH, mannitol yields up to 31% can be obtained (Schwarz, 1994).

Even higher mannitol yields from initial sugar raw material are obtained, when syrups with high fructose content or pure fructose are used. The hydrogenation of such solutions result in mannitol yields of 48 and 50% (w/w), respectively (Devos, 1995). However these raw materials are expensive and therefore seldom used for commercial scale production.

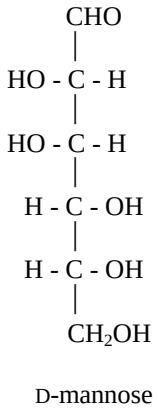


Figure 2. Chemical structure of D-mannose.

Mannitol production by chemical one-step hydrogenation is always linked to the simultaneous production of sorbitol and mannitol is thus, justifiably considered as the side-product of the process. The separation of mannitol from sorbitol relies on the differences in solubility of these compounds and the purification steps usually

comprise concentration, cooling crystallization, crystal separation, and drying. The yield of crystalline mannitol gained from a typical reaction solution is normally in the range of 65...75% (w/w). Hence, the cumulative yield of crystalline mannitol from initial sugar raw material (50/50 glucose/fructose) is reported to be as low as 17% (w/w) (Takemura *et al*., 1978).

In another hydrogenation process, D-mannitol is produced from D-mannose (see Figure 2) (Takemura *et al*., 1978). According to this invention pure D-glucose is first chemically epimerized to yield 30-36% (w/w) D-mannose. Mannose is then hydrogenated directly to mannitol. The theoretical yield of mannitol from mannose is 101% (w/w), whereby the combined mannitol yield from the initial sugar can be as high as 36% (w/w). Pure mannose can also be used as the starting material in hydrogenation, but is not economically feasible due to the high price of mannose. Takemura *et al*. (1978) suggested that the non-epimerized glucose could be enzymatically isomerized (by glucose isomerase) to D-fructose and further hydrogenated into mannitol. For such a process, the final mannitol yield from the initial sugar would rise up to 50% (w/w). However, the production costs arising for this four-step process are most likely too high compared to the selling price of mannitol.

In a recent patent, Devos (1995) suggests a process where a fructose syrup, containing less than 15% glucose, is first isomerized enzymatically with mannose isomerase to yield a fructose/mannose/glucose syrup. After chromatographic separation the fraction rich in mannose (X1), but also containing high levels of fructose, is subjected to hydrogenation. Mannose is hydrogenated in stoichiometric yields to mannitol, whereas fructose is hydrogenated to mannitol and sorbitol. The fructose-rich fraction (X2) is recycled to the top of the isomerization column and re-processed. Using pure fructose as starting material, yields of crystalline mannitol from initial sugar is claimed to be as high as 70%. This, however, requires the fructose-rich fraction to be recycled several times, as well as, several successive crystallizations of the mother liquors. Moreover, mannose isomerase is not commercially available. The isomerization step thus requires the production of mannose isomerase-rich cells, cross-linking of the cells to a carrier and packing the material into a column.

2.2 Enzymatic hydrogenation

Mannitol can be produced enzymatically. The reduction of D-fructose to D-mannitol requires an NAD(P)H-dependent mannitol dehydrogenase (MDH) (see Figure 3). An NADH-dependent MDH (EC 1.1.1.67) has been purified from e.g. *Lactobacillus brevis* (Martinez *et al*., 1963), *Leuconostoc mesenteroides* (Sakai, 1967; Sakai and Yamanaka, 1968), *Saccharomyces cerevisiae* (Quain and Boulton, 1987), *Rhodobacter sphaeroides* (Schneider and Giffhorn, 1989; Schneider *et al*., 1993), *Torulaspora delbrückii* (Nidetzky *et al*., 1996), *Pseudomonas fluorescens* (Brünker *et al*., 1997), and the mangrove red algae (*Caloglossa leprieurii*) (Karsten *et al*., 1997). Furthermore, an NADPH-dependent MDH (EC 1.1.1.138) has been purified from e.g. *Aspergillus parasitius* (Niehaus and Dilts, 1982), *Zymomonas mobilis* (Viikari and Korhola, 1986), and *Gluconobacter suboxydans* (Adachi *et al*., 1999).

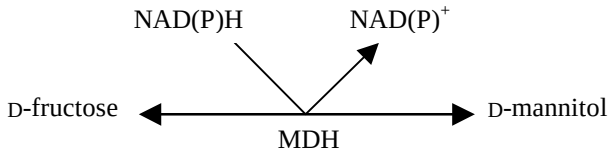


Figure 3. Mannitol dehydrogenase (MDH) catalyzed reaction.

The cofactor dependency of MDH is the major limitation of an enzymatic process. NADH, and especially NADPH, are very expensive. This makes the enzymatic process unfeasible. The common way to circumvent this problem is by so-called cofactor regeneration. Wichmann *et al*. (1981) suggested the simultaneous conversion of fructose and formate using mannitol dehydrogenase and formate dehydrogenase as shown in Figure 4. Formate (e.g. Na-formate) is cheap and the CO_2 formed is easily separated from mannitol. Using recombinant *P*. *fluorescens* MDH in the set-up described above, a volumetric mannitol productivity of 2.2 g/L/h was achieved (Slatner *et al*., 1998).

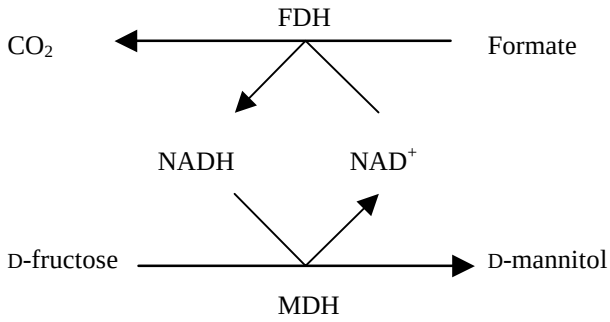


Figure 4. Cofactor regeneration using mannitol dehydrogenase (MDH) and formate dehydrogenase (FDH).

The cofactor can also be regenerated in a system were a glucose/fructose mixture is converted into gluconate and mannitol using glucose dehydrogenase and mannitol dehydrogenase (see Figure 5) (Howaldt *et al*., 1988).

As mentioned earlier, mannose can also be reduced to mannitol enzymatically. However, the reversible reaction favors mannitol oxidation rather than mannose reduction and is thus not suitable (Stoop *et al*., 1998). In practice, several problems still remain to be addressed for these process alternatives. Hence, the use of enzymatic hydrogenation for the production of mannitol is affected by factors such as retention of cofactors in the reactor with special membranes, the strong product inhibition of mannitol dehydrogenase, and the high K_m value of mannitol dehydro-

genase for fructose (Soetaert *et al*., 1999). Moreover, the increased costs of a two-enzyme system have to be considered (Röper and Koch, 1988).

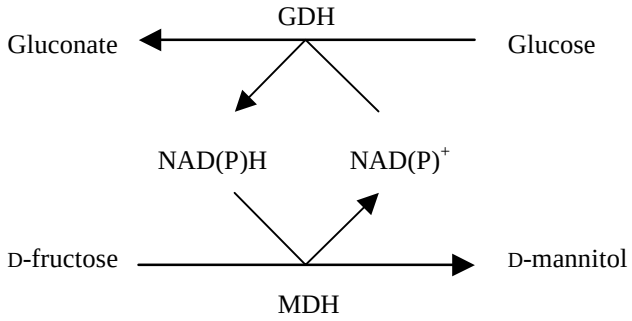


Figure 5. Cofactor regeneration using mannitol dehydrogenase (MDH) and glucose dehydrogenase (GDH).

2.3 Microbial production

In order to improve the total yield of mannitol and to avoid the difficulties relating to mannitol separation from sorbitol it would be advantageous to develop a process with mannitol as the main product and with no sorbitol formation. In the previous chapter enzymatic hydrogenation was discussed. Although no sorbitol is formed in the enzymatic processes, other problems still persist (see chapter 2.2). Some alternative mannitol production processes based on the use of microbes have been suggested in the literature. Yeast, filamentous fungi, and lactic acid bacteria (LAB) especially, have been shown to effectively produce mannitol without the co-formation of sorbitol (Itoh *et al*., 1992). Microbes have the further advantage of a "built-in" cofactor regeneration machinery, i.e. there is no need to add cofactors to the reaction broth. Instead the cells produce cofactors as a result of sugar catabolism. Based on studies described in the scientific literature, bacteria, namely LAB, seem to be the most potent producers of mannitol.

Most of the microbial mannitol production studies described in the scientific literature are based on batch cultivation protocols. However, some more severe attempts have been made using fed-batch and cell immobilization techniques. Soetaert and co-workers have studied the bioconversion of fructose into mannitol with *Leuconostoc pseudomesenteroides* (e.g. Soetaert, 1990; Soetaert *et al*., 1994). Using a fed-batch cultivation protocol they reached a maximum volumetric productivity of 11 g mannitol/L/h and a mannitol yield from fructose of approximately 94 mol-%. Using the same microbe and applying cell immobilization, Ojamo *et al*. (2000) reported average volumetric productivities of up to 30 g/L/h and mannitol yields around 85 mol-%. In addition to fructose, glucose (ratio 2:1 fructose/glucose) was also needed as a raw material in these processes.

For a more detailed literature review on microbial production of mannitol see chapter 3.

3. PRODUCTION OF MANNITOL BY MICROBES

3.1 Filamentous fungi

Several filamentous fungi (i.e. molds) produce mannitol from carbohydrates. In studies reported by Smiley *et al*. (1967) *Aspergillus candidus* was found to produce mannitol from glucose. Enzymatic studies of the metabolic route have confirmed that mannitol is formed from glucose via fructose 6-phosphate and mannitol 1-phosphate (Strandberg, 1969). Consequently, a process was developed and a volumetric productivity of 0.15 g mannitol/L/h was reported (Smiley *et al*., 1969). The yield of mannitol from glucose was 31.0 mol-%. Moreover, using sodium acetate as the sole carbon source, *Aspergillus niger* forms mannitol (Barker *et al*., 1958).

The same metabolic route (glucose to mannitol) seems to be active in species of *Penicillium* (Boonsaeng *et al*., 1976). Hendriksen *et al*. (1988) found *Penicillium scabrosum* to produce high concentration of mannitol and glycerol from sucrose. The volumetric mannitol productivity (0.14 g/L/h) was similar to that reported for *A. candidus*. However, the yield of mannitol (56.7 mol-%) was now clearly better. Both Smiley *et al*. (1969) and Hendriksen *et al*. (1988) noted that when the initial sugar was consumed the cells started to utilize the sugar alcohols produced. Glycerol was favored over mannitol as the carbon source.

In a study screening 500 fungal isolates high mannitol producers were identified to be species of *Aspergillus*, *Eurotium*, and *Fennellia* (El-Kady *et al*., 1995). Furthermore, *Alternaria alternata, Cladosporium herbarum, Epicoccum purpurascens*, and *Fusarium pallidoroseum* isolated from cotton leaf dust were suggested to be the source of mannitol found in cotton dust (Domelsmith *et al*., 1988).

3.2 Yeasts

Production of sugar alcohols is also common among yeasts. Japanese scientists have been especially active in this field. Species belonging to a soy sauce-isolated *Torulopsis* -genus have been the focus of H. Onishi and T. Suzuki at Noda Institute for Scientific Research in Japan. Using various simple carbon sources (glucose, fructose, mannose, galactose, maltose, glycerol, and xylitol) they found *Torulopsis versatilis* and *T. anomala* to be good mannitol producers (Onishi and Suzuki, 1968). A few years later they reported that another species, *T. mannitofaciens*, exclusively produces mannitol from both glucose and glycerol (Onishi and Suzuki, 1970). The yield of mannitol from glycerol with *T. mannitofaciens* was about 31 mol-%. At this point, their aim was, however, to use a species of *Acetobacter* to convert the mannitol produced by *T. mannitofaciens* further into D-fructose.

Consequently, the invention describing the use of the three *Torulopsis* -species for the production of mannitol was patented (Onishi and Suzuki, 1971). Using glucose as the carbon source a volumetric mannitol productivity of 0.23 g/L/h was obtained. The yield of crystalline mannitol from glucose was approximately 28 mol-%. They

concluded their studies with *Torulopsis* by showing that mannitol in these species was formed by a mannitol dehydrogenase-catalyzed reduction of fructose (Onishi and Suzuki, 1972).

Also, *T. bombicola* (Inoue and Kimura, 1986) and *T. apicola* (Zalashko *et al*., 1987) have been shown to be good mannitol producers. The volumetric productivities and yields were, however, not improved compared to that obtained with *T. mannitofaciens*.

Some *Candida* species are able to produce mannitol from different hydrocarbons. De Zeeuw and Tynan (1973) showed that *C. lipolytica* produces mannitol as the main sugar alcohol. In flask experiments a volumetric mannitol productivity of 0.16 g/L/h was obtained (Johnson, 1976). Moreover, also *C. petrovorus* (Iwamoto and Ozawa, 1973), *C. aliphatica* (Iwamoto *et al*., 1973), and *C. zeylanoides* (Hattori and Suzuki, 1974) have been described to be potential mannitol producers.

The most promising result reported for yeasts was achieved with *Zygosaccharomyces rouxii* (Looten *et al*., 1992). When grown on glucose, a volumetric mannitol productivity and mannitol yield of 0.68 g/L/h and 51 mol-%, respectively, were obtained. Furthermore, in an earlier study *Rhodotorula minuta* was found to produce mannitol from various D-aldopentoses (Stankovic *et al*., 1989). Ribose was the best choice, whereas growth on xylose, arabinose, and lyxose resulted in lower yields of mannitol.

In conclusion, although possessing the ability to produce mannitol from glucose, the volumetric productivities achieved with yeasts and filamentous fungi are simply too low for economical production. Most of the microbes described above utilize readily mannitol, which makes a process applying yeast or filamentous fungi difficult to control. Purification, especially with yeasts, is complicated by the high concentrations of glycerol present in the culture media. Furthermore, the suitability of these microbes for food industry is questionable.

3.3 Bacteria

Various bacterial mannitol dehydrogenases (MDHs) have been used for enzymatic hydrogenation of fructose to mannitol (chapter 2.2). On the other hand, very few attempts have been made to produce mannitol with bacteria other than lactic acid bacteria (LAB). In one such case, using an MDH-overproducing strain of nitrogen-fixing *Rhodobacter sphaeroides* Schneider and Giffhorn (1994) achieved a volumetric mannitol productivity of 1.5 g/L/h. However, the main goal of that study was not the production of mannitol, but the production of MDH.

The knowledge of mannitol formation in LAB cultures dates back to the late 1930's [*J. Agr. Chem. Soc. Japan* **14** (1938) 1449-] (Onishi and Suzuki, 1971). Research relating to the mannitol-producing ability of these bacteria was re-awakened by studies with *Leuconostoc pseudomesenteroides* ATCC 12291 (at that point classified as *L. mesenteroides*) (Vandamme *et al*., 1987). Trying to produce sucrose phosphorylase with this species, the group at Ghent University, Belgium, observed

that the fructose moiety was efficiently converted into mannitol. Moreover, they found that low pH (4.5) seemed to increase the yield of mannitol from fructose. Encouraged by these results a doctoral thesis was completed at Ghent University (Soetaert, 1992).

The results were promising. Using a fed-batch cultivation protocol and *L. pseudomesenteroides*, Soetaert (1990) reported an average volumetric mannitol productivity and a mannitol yield of 6.3 g/L/h and 94 mol-%, respectively. Based on continuous cultivation studies it was also observed that the yield of mannitol from fructose was strongly correlated to the substrate concentration in the growth medium. At a low fructose concentration (3 g/L) only a 48 mol-% yield was obtained, while 99 mol-% was obtained at 120 g/L. This was most likely due to the kinetic characteristics of MDH in *L. pseudomesenteroides*. The enzyme is reported to have a relatively high K_m -value for fructose, ~10 g/L, which results in a drastic slow down of the reaction at low fructose concentrations (Soetaert, 1990).

Soetaert *et al*. (1990) also tested a production protocol, in which the *L. pseudomesenteroides* cells were immobilized to reticulate polyurethane foam. In this once-through continuous process a small improvement in volumetric productivity was seen (8.9 g/L/h). However, the change from fed-batch cultivation to immobilization was found to have a negative effect on the yield, which was decreased to 60 mol-%. In comparison batch cultivations were also run. The highest yield (85 mol-%) was obtained at low pH (4.5) and low growth temperature (20ºC). The volumetric productivities in these trials were about 1 g/L/h. In contrast to earlier results, they did not see any notable effect of the initial sugar concentrations on the yields.

A few years later the same group reported minor productivity improvements to the processes run in batch and fed-batch mode (Soetaert *et al*., 1994). When *L. pseudomesenteroides* was grown on partially isomerized hydrolyzed starch containing fructose and glucose, a productivity of 3.8 g/L/h and a yield of 92 mol-% were achieved in batch cultures. Using a random mutant in a fed-batch culture the volumetric productivity was increased to 7.7 g/L/h (compared to 6.3 g/L/h before). Furthermore, the culture broth was separated by electrodialysis into a mannitol-containing and an acid-containing fraction. Mannitol was crystallized yielding 85 mol-% of mannitol present in the culture broth. The purity of the crystals was 99.1%.

Growing wine-isolated *Oenococcus oeni* (earlier *Leuconostoc oenos*) in medium containing equal amounts of fructose and glucose, Salou *et al*. (1994) observed that 83 mol-% of the fructose consumed was reduced to mannitol. However, *O. oeni* is a very slow-growing species and thus, the volumetric mannitol productivities were very low (about 0.2 g/L/h). Yun and Kim (1998) cultivated two food-isolated LAB strains in Erlenmeyer flasks. From a variety of carbohydrate substrates tested, notable mannitol formation was detected only when either fructose or sucrose were used as the substrate. Under optimal growth conditions they found that the more effective strain (*Lactobacillus* sp.) converted 0.72 mol/mol of the initial fructose into mannitol (yield of mannitol from fructose = 86 mol-%), whereas the other strain (*Leuconostoc* sp.) had a conversion of only 0.26 mol/mol (yield = 65 mol-%). The volumetric productivities for the both strains were less than 1 g/L/h.

Recently, Korakli *et al*. (2000) reported a 100% yield of mannitol from fructose with sourdough-isolated *Lb. sanfranciscensis* grown in a fructose-glucose medium. However, the volumetric productivity in a fed-batch culture was only 0.5 g/L/h. In other studies, also *L. mesenteroides* (Erten, 1998), *Lb. buchneri* (Soetaert *et al*., 1999), and *Lb. fermentum* (Itoh *et al*., 1992) were reported to efficiently produce mannitol from fructose or sucrose.

Moreover, a few patents relevant to this field are found. In JP 62239995, Shirae *et al*. (1987) suggest the use of *Lb. brevis* for the production of mannitol. In batch cultures they achieved a volumetric mannitol productivity of 2.4 g/L/h. EP 486024 and EP 683152 describe a strain named *Lb*. sp. B001 with volumetric productivities up to 6.4 g/L/h in batch cultures (Itoh *et al*., 1992; Itoh *et al*., 1995). More recently, Ojamo *et al*. (2000) submitted a patent application for a process for the production of mannitol by high-densities of immobilized cells (e.g. LAB). In this process the average volumetric mannitol productivity and mannitol yield achieved with *L. pseudomesenteroides* ATCC 12291 were approximately 30 g/L/h and 85 mol-%. A low-nutrient bioconversion medium was used which considerably lowers the production costs. Immobilization also enables the re-use of the cell biomass in successive batches.

Comparing fungal and bacterial processes for the production of mannitol, it can be concluded that the production times change from days with fungi to hours with bacteria. Hence, it is obvious that bacteria-based processes are more attractive. Within bacteria LAB seem to be the most potent producers of mannitol. Furthermore, looking at the review above, it can be concluded that the ability to convert fructose into mannitol in LAB is most likely limited to only heterofermentative species.

In conclusion, from a bioprocess engineers' point of view, high-level process development work is recognizable only in the studies conducted by Korakli, Ojamo and Soetaert and co-workers. Most of the other results described in the current literature are based on simple Erlenmeyer flask experiments and are limited to batch cultures.

4. PRIMARY SUGAR METABOLISM OF LACTIC ACID BACTERIA

The lactic acid bacteria (LAB) are a group of Eubacteria, which were originally identified as the organisms responsible for milk souring. The LAB comprise of a wide variety of microbes and revisions of the classification and taxonomy still appear regularly. This has caused much confusion over the years in this particular field of microbiology. Recent advances in genetic classification methods (e.g. DNA homology testing and rRNA sequencing) are, however, finally bringing some improvement to this dilemma. Based on extensive work by M.D. Collins and co-workers, the LAB group is divided into 11 major eubacterial phyla: *Aerococcus*, *Carnobacterium*, *Enterobacterium*, *Vagococcus*, *Tetragenococcus*, *Oenococcus*, *Leuconostoc*, *Weissella*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* (Axelsson, 1998). Furthermore, the phylum of *Lactobacillus* is sub-divided into a *Lb. delbrückii* and a *Lb. casei-Pediococcus* group. A schematic phylogenetic tree of LAB as a group is shown in Figure 6. The genera *Bacillus*, *Listeria*, and *Staphylococcus* seem to overlap with some LAB, but these aerobic microbes are not included in the group.

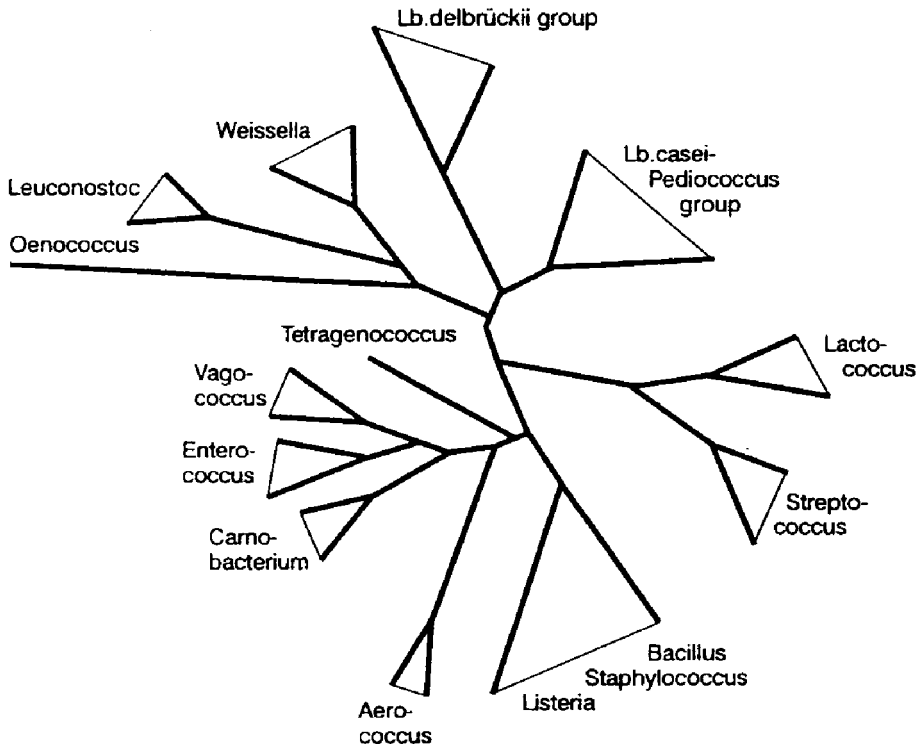


Figure 6. Schematic, unrooted phylogenetic tree of lactic acid bacteria (Axelsson, 1998).

A more traditional way of classification is presented by Axelsson (1998). According to him a typical LAB can be described as Gram-positive, nonsporing, catalase-negative, devoid of cytochromes, facultative anaerobe, fastidious, acid-tolerant, and strictly fermentative with lactic acid as the major end product during sugar fermentation. Gram-positive bacteria possess peptidoglycan-rich cell walls, making them resistant to mechanical stress and difficult to disrupt. Mechanical strength of the cells is an advantageous property, e.g. when the process conditions generate significant pressure changes and shear stress. Typically, LAB also lack porphyrin groups and are thus unable to build catalase or cytochromes needed for oxygen breakdown and oxidative phosphorylation (Axelsson, 1998). Therefore, in laboratory conditions, LAB carry out strictly fermentative metabolism of sugars, regardless of the presence or absence of air (Glazer and Nikaido, 1995). The oxygen present in the growth environment of LAB is usually eliminated by the action of NADH oxidase resulting in the re-oxidation of NADH and the production of either water or hydrogen peroxide. In nature, on the other hand, LAB can find hematin or related hemoglobin compounds enabling these microbes to respire (Axelsson, 1998).

LAB inhabit partially all mucous membranes of mammals (e.g. mouth, intestine and vagina) and are found in e.g. dairy products, meat, beverages and vegetables (Salminen *et al*., 1998). These natural nutrient-rich environments of LAB have resulted in loss of ability to produce many of the essential factors needed for cellular growth (amino acids, vitamins etc.) and thus, grow poorly on mineral media. Moreover, the acidic conditions predominant in the stomach and intestine of mammals denote that these bacteria are exceptionally tolerant to low pH and high concentrations of salts.

4.1 Hexose (glucose) metabolism

The group of LAB is traditionally classified into two metabolic sub-groups according to which pathway is active in catabolism of hexose sugars: homo- or heterofermentative LAB. Although this classification is still commonly used, it is important to realize that some heterofermentative microbes possess enzymes of the homofermentative pathway and *vice versa*. In future, the classification of LAB into either pure homo- or heterofermenters will be revised and more accurate means of classification can be implemented.

4.1.1 Homolactic fermentation

The homofermentative (or homolactic) pathway in LAB is presented in detail in Figure 7. Hexose sugars, like glucose, are transported into LAB either actively as free sugar molecules or with the aid of simultaneous phosphorylation. In the former case, the first reaction step inside the cell is the phosphorylation of glucose and thus, the first intermediate, in both cases, is glucose-6-P. The homolactic fermentation then follows the well-established Embden-Meyerhof-Parnas (EMP) pathway to pyruvate. This pathway is characterized by the formation of fructose-1,6-diP (FDP), which is split by FDP aldolase into dihydroxyacetone-P (DHAP) and glyceraldehyde-3-P (GAP). Thus far 2 moles of ATP are consumed per mole initial

glucose (active glucose transport). GAP is then converted into pyruvate in a series of reactions resulting in the net formation of 2 moles of pyruvate, ATP and NADH. Under feasible conditions (anaerobisis, excess glucose and excess growth factors), pyruvate is reduced fully into lactate by the action of lactate dehydrogenase (LDH). In theory, the complete homolactic fermentation of 1 mole glucose results in 2 moles of lactate and a net gain of 2 moles of ATP.

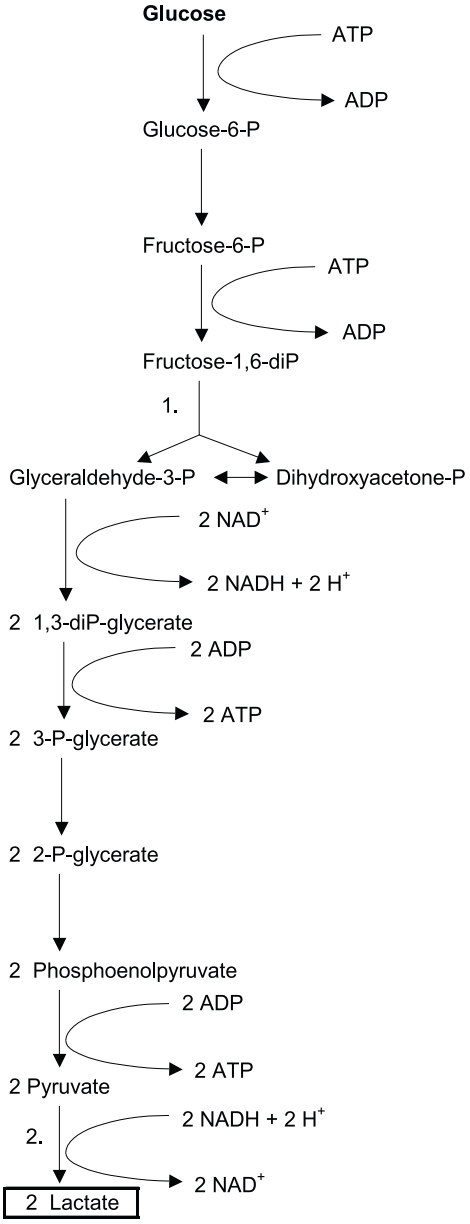


Figure 7. Homolactic fermentation in LAB. Enzymes shown: (1) fructose-1,6-diP aldolase; (2) lactate dehydrogenase.

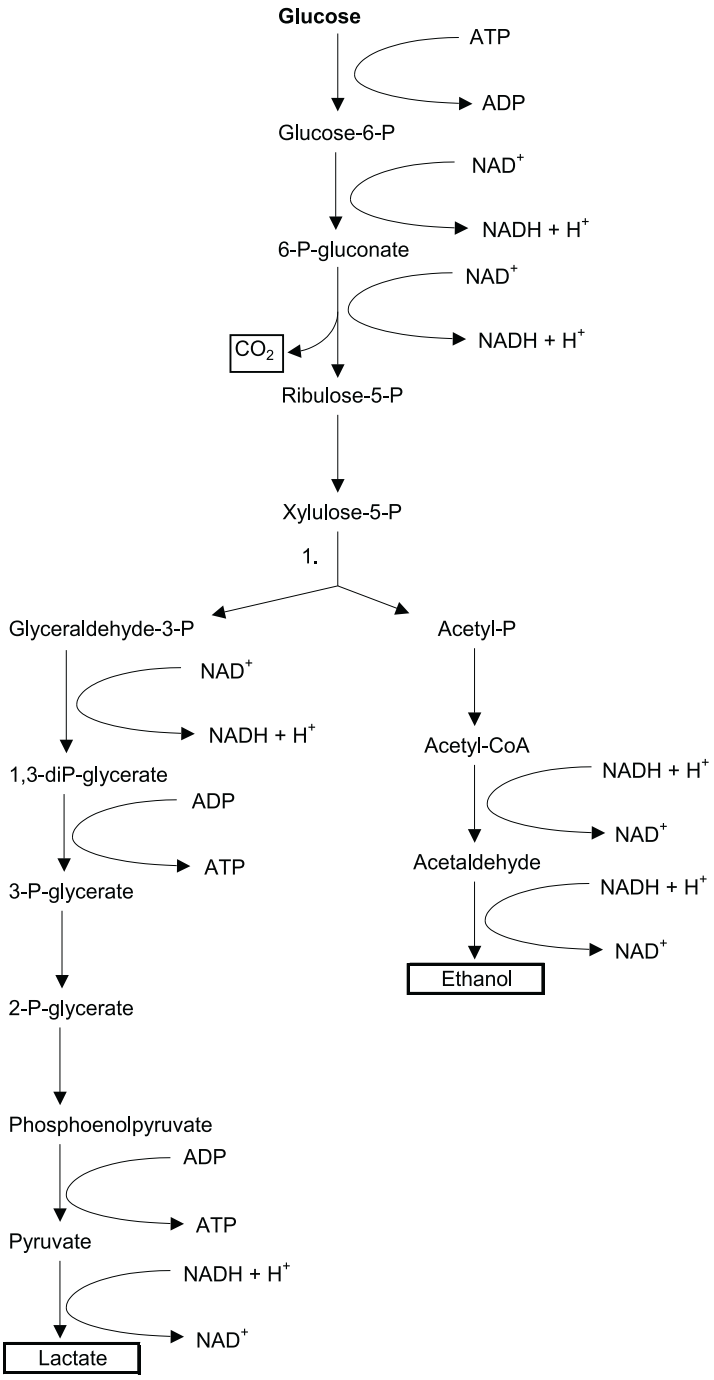


Figure 8. Heterolactic fermentation in LAB. Enzyme shown: (1) phosphoketolase.

4.1.2 Heterolactic fermentation

In contrast to homofermentative LAB, the heterofermentative LAB lack the enzyme FDP aldolase and are unable to split FDP (see Figure 8). Instead, the pathway proceeds from glucose-6-P by two successive oxidations and one epimerization to yield xylulose-5-P. The heterofermentative pathway is strongly characterized by the next step, where xylulose-5-P is split by a phosphoketolase to GAP and acetyl-P.

This pathway is therefore commonly referred to as the phosphoketolase (PK) or the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway. While GAP proceeds as described above with homolactic fermentation, finally yielding 1 mole lactate, acetyl-P is reduced to ethanol. The latter reaction series is driven by a redox imbalance present in the cells. The two oxidation steps from glucose-6-P to ribulose-5-P result in excess of 2 moles of NAD(P)H per mole of glucose. Hence, this surplus drives the formation of acetyl-CoA, which in turn is reduced to acetaldehyde and further into ethanol, restoring the redox balance in these cells.

In theory, homolactic fermentation of 1 mole glucose results in the formation of 2 moles of both lactate and ATP, while heterolactic fermentation of 1 mole glucose results in the formation of 1 mole of each lactate, carbon dioxide, ethanol and ATP. In practice, these theoretical values are seldom achieved, because a variable fraction of the glucose transported into the cell is consumed for the production of biomass. However, the conversion factor of glucose into metabolites in LAB can commonly be as high as 0.95. The respective value for aerobic bacteria and yeast is closer to 0.5.

4.2 Co-metabolism

4.2.1 Role of oxygen

As mentioned earlier LAB are able to grow in the presence of air although they are lacking the typical oxygen removal mechanisms (cytochromes, hemes, catalase etc.). In LAB, oxygen can act as an external electron acceptor in a reaction catalyzed by NADH oxidases (NOXs). There are two kinds of NADH oxidases in LAB (Higuchi *et al*., 2000). The reaction catalyzed by these enzymes results in the formation of either hydrogen peroxide or water, depending on whether two or four electrons are transferred to the oxygen molecule: $O_2 + NADH + H^+ \rightarrow H_2O_2 + NAD^+$; $O_2 + 2\ NADH + 2\ H^+ \rightarrow 2\ H_2O + 2\ NAD^+$. Many LAB also possess an NADH peroxidase, which reduces the hydrogen peroxide formed to water (Axelsson, 1998). However, depending on the strain, the lack of catalase, an enzyme vital in efficient hydrogen peroxide breakdown, can easily result in production of auto-inhibitory levels of hydrogen peroxide (Condon, 1987).

In homofermentative LAB, the NOXs compete with lactate dehydrogenase for the oxidation of NADH, resulting in decreased lactate production and formation of surplus pyruvate. Consequently, the cells are forced to change their primary metabolism in order to avoid pyruvate accumulation. The modified metabolite composition is strongly dependent on external factors, such as aeration conditions and substrate limitations. Typical end products in such cases, are diacetyl, acetoin, 2,3-butanediol, acetate and ethanol. Moreover, the excess pyruvate can also be directed into production of e.g. lipids and other biomass components.

On the other hand, in heterolactic fermentation it is believed that NOXs are competing with the ethanol-producing branch rather than LDH (Borch and Molin, 1989; Axelsson, 1998). This would cause no changes to the level of lactate

produced, but probably result in an increased level of acetate (see Figure 9). The role of the acetate branch is discussed in more detail below.

4.2.2 Fructose as an alternative electron acceptor

Most LAB are able to consume glucose and fructose simultaneously (see Figure 9). In homofermentative species, both glucose and fructose are used to produce lactate. On the other hand, in most heterofermentative species, fructose can act as an external electron acceptor in a reaction involving mannitol dehydrogenase (MDH). MDH, described in more detail in chapter 2.1, catalyzes the conversion of fructose to mannitol and *vice versa*. When the reaction proceeds from fructose to mannitol, NAD(P)H is oxidized to $NAD(P)^+$. This will affect the redox balance in the cells. A variable amount of NAD(P)H formed, when glucose-6-P is converted to ribulose-5-P, is here re-oxidized by MDH instead of the ethanol-producing branch. This will result in decreased ethanol production and hence, accumulation of the precursor acetyl-P. In a reaction catalyzed by an acetate kinase the excess acetyl-P is normally de-phosphorylated into acetate simultaneously producing an extra mole of ATP per mole of acetate produced.

When fructose is the sole sugar source in LAB fermentations, only 2/3 mole of ATP is produced per mole of fructose consumed. As mentioned earlier, the respective value for cultivations with glucose as the sole sugar source is 1 mole of ATP per mole glucose consumed. Surprisingly, when the growth rates on different sugars were studied, several heterofermentative LAB were found to grow faster on fructose than on glucose (Axelsson, 1998; von Weymarn, unpublished results). An even higher maximum specific growth rate was observed with some species of *Leuconostoc* and *Lactobacillus*, when glucose and fructose were co-fed (1:2 ratio) (von Weymarn, unpublished results). Using the co-feeding strategy, the net gain is still only 2/3 mole of ATP per mole of sugar. Hence, it seems that these cells are prioritizing a fast growth over efficient substrate utilization (Axelsson, 1998).

Because fructose is significantly more expensive than glucose, it is cost-effective to use glucose as the source of cofactors in bacterial mannitol production, while fructose added is strictly converted to mannitol. In theory, the bioconversion follows the equilibrium Glucose + 2 Fructose $\rightarrow$ 2 Mannitol + CO_2 + Lactate + Acetate. Hence, the theoretical maximum yield of mannitol from initial sugars is 66.7 mol-%. As a starting point for the practical experiments in this doctoral thesis, a 1:2 glucose to fructose ratio was used.

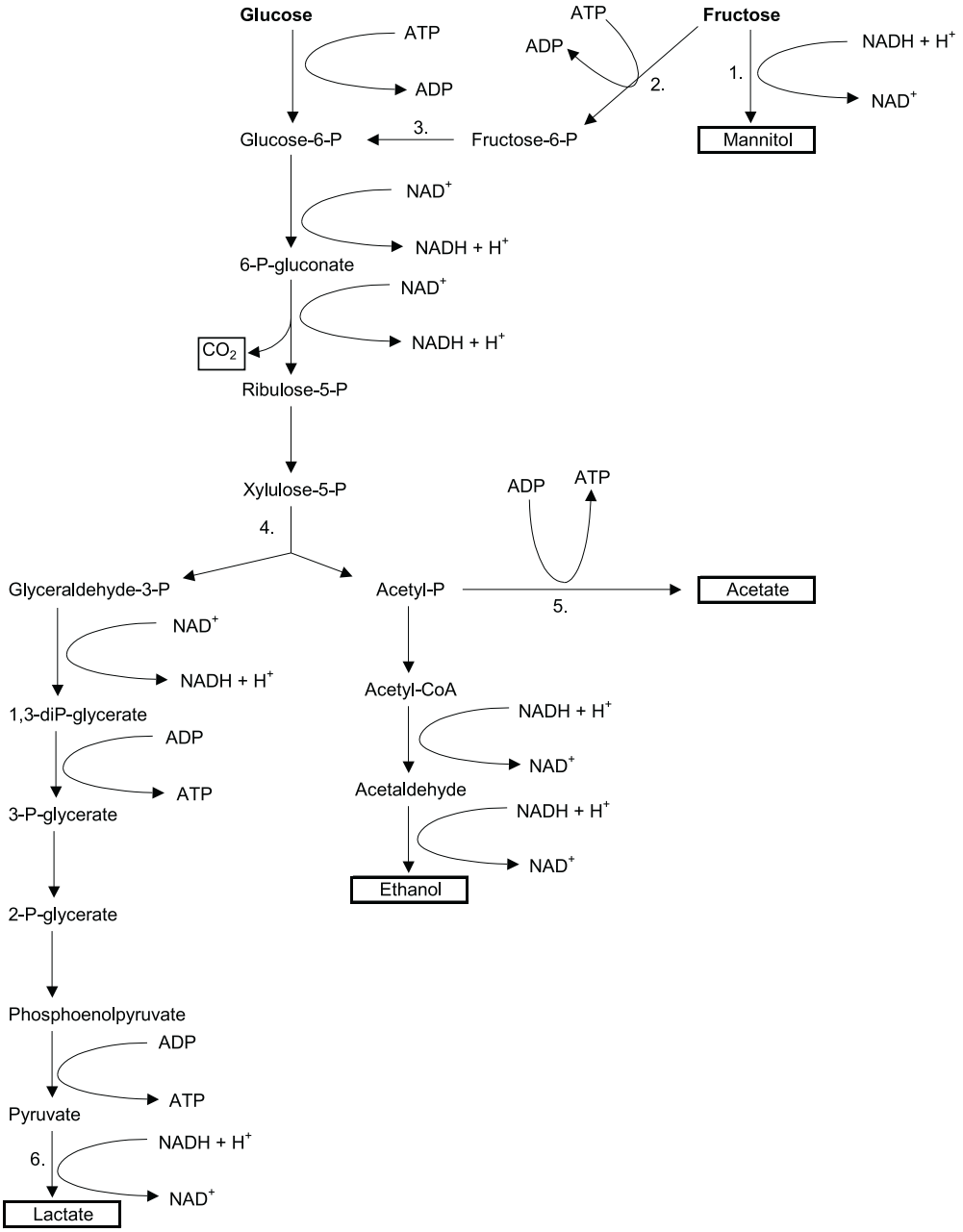


Figure 9. Co-metabolism of glucose and fructose in heterofermentative LAB. Enzymes shown: (1) mannitol dehydrogenase; (2) fructokinase; (3) phospho-glucoisomerase; (4) phosphoketolase; (5) acetate kinase; (6) lactate dehydrogenase. In the figure NAD/NADH is assumed to be only cofactor of primary metabolism.

4.2.3 Other alternative electron acceptors

In many heterofermentative LAB fructose can be replaced with glycerol in co-feeding with glucose. In these microbes glycerol is reduced first to 3-hydroxy-propionaldehyde (3-HPA) and then further into 1,3-propanediol. Each reaction step re-oxidizes one NAD(P)H. The co-metabolism of glucose and glycerol thus results

in the production of lactate and acetate, carbon dioxide, 1,3-propanediol and some ethanol.

Some heterofermentative species (e.g. *Lb. brevis*) ferment glucose poorly anaerobically (Axelsson, 1998). However, when these strains are co-fed with glycerol, the glucose fermentation is drastically improved. A similar effect has been reported for the co-feeding of maltose and glycerol in *Lb. reuteri* (Ragout *et al*., 1996). In an earlier glucose-glycerol co-feeding experiment, cells of *Lb. reuteri* were kept in a resting state, resulting in accumulation of the intermediate 3-HPA rather than 1,3-propanediol (Schütz and Radler, 1984).

Co-metabolism of glucose and citrate is well established in e.g. the dairy industry. Adding citrate to cultures of some *Leuconostoc* and *Lactococcus* species (e.g. *L. mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*) results in the production of diacetyl, an important aroma compound in milk and cheese products. Citrate, as such, is not directly an electron acceptor. However, when transported into the cell, citrate is cleaved by citrate lyase to acetate and oxaloacetate. Oxaloacetate is further decarboxylated by many LAB to pyruvate resulting in an excess of pyruvate, if co-metabolized with hexose sugars.

Co-feeding *O. oeni* cells with glucose and citrate resulted in a shift from ethanol production to acetate production (Ramos and Santos, 1996). Also 2,3-butanediol was produced. Moreover, an increased growth rate was observed. Schmitt *et al*. (1992) reported increased growth rate, but decreased yield of biomass from glucose, when *L. mesenteroides* was co-fed with glucose and citrate. According to studies with radioactive markers, part of the acetate and lactate produced by the cells originated from citrate metabolism. Metabolites of citrate metabolism were also incorporated into cell material, primarily into lipids. In both studies, the addition of citrate to cells fermenting glucose resulted in an increased glucose catabolism. Other possible end products of glucose/citrate co-feeding are formate, succinate, and acetoin.

4.3 Bioenergetics and transport functions

In controlled laboratory conditions LAB do not possess an electron transport chain and hence, are not able to form ATP via oxidative phosphorylation. In respiring bacteria, like *Escherichia coli* and *Bacillus subtilis*, protons are extruded outside the cell by specific electron transport proteins situated in the cytoplasmic membrane. This exerts an inwardly directed force, the proton motive force (PMF). In such respiring cells the PMF and the action of H^+ ATPase (also known as ATP synthase) result in the phosphorylation of ADP to ATP.

In LAB, ATP is formed strictly by substrate-level phosphorylation (direct transfer of high-energy phosphate molecules from a phosphorylated organic compound to ADP). The difference in yield of ATP per glucose consumed between fermenting and aerobically respiring microbes is vast. As mentioned earlier, the fermentation of 1 mole glucose results in a net gain of either 2 moles (homolactic) or 1 mole (heterolactic) ATP. In contrast, in aerobic respiration a total of 38 moles of ATP can

be formed per mole of glucose consumed (Brock *et al*., 1994). Since much more energy (ATP) is gained by complete oxidation of the energy source, it is possible to obtain much higher biomass yields from the same carbohydrate source in an aerobic process compared to an anaerobic process.

An enzyme very similar to ATP synthase is found in LAB. However, this enzyme catalyses the reverse reaction, i.e. pumping protons out of the cell at the expense of ATP (Konings *et al*., 1989; Maloney, 1990). LAB (and fermentative bacteria in general) thus establish a PMF, which can drive energy-consuming reactions such as the uphill transport of metabolites and ions (Axelsson, 1998). The function of this enzyme in LAB is essential in maintaining the internal pH over a certain threshold level. LAB are well adapted to acidic environments and some species can tolerate environments with a pH as low as 2. However, among different LAB this tolerance level varies. As a general rule, species of *Lactobacillus* are significantly more tolerant to low pH than species of *Streptococcus* (Kashket, 1987). In a study by McDonald *et al*. (1990), the growth inhibitory internal pH of *L. mesenteroides* and *Lb. plantarum* was 5.4-5.7 and 4.6-4.8, respectively. *Lb. plantarum* maintained its pH gradient (ΔpH) in the presence of either acetate or lactate down to an external pH of 3, whereas ΔpH of *L. mesenteroides* was zero at pH 4 with acetate and at pH 5 with lactate.

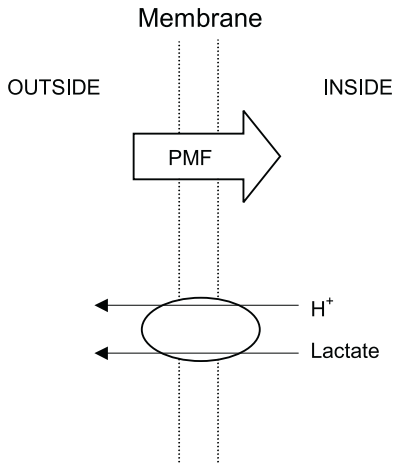
Keeping the internal pH within a feasible range requires the use of a substantial part of ATP generated by substrate-level phosphorylation and hence, less ATP is available for biosynthesis. Some LAB possess alternative systems, which can contribute to the generation of a PMF, thus sparing ATP required for transport functions. Such systems are proton/lactate symport, precursor/product exchange as in malolactic or citrate fermentation, and electrogenic uniport as in malate and citrate uptake in *O. oeni* (Konings *et al*., 1997). The two former systems are described in more detail below.

A proton/lactate symport is shown in Figure 10 a. The efflux of lactate molecules is accompanied by proton efflux generating an inwardly directed PMF. In *Lc. lactis* such symport system is known to operate only at the initial stages of batch fermentations (pH > 6.3 and external lactate concentration < 10 mM) (Axelsson, 1998). This feature is believed to be advantageous in competition with other microbes.

The precursor/product exchange system was first proposed for energy generation by malolactic fermentation in *Lc. lactis* (Poolman, 1993). Energy generation by citrate metabolism in *Lc. lactis* was also explained by the same mechanism (Hugenholtz *et al*., 1993). Citrate is a typical component in LAB growth media. For instance, citrate is found in MRS, the standard medium for culturing LAB. The uptake of these weak acids (citrate and malate) is meditated by transport proteins (Figure 10 b). When the cells take up citrate a net negative charge is translocated into the cell, thereby generating a membrane potential. In the cell, citrate is cleaved to acetate and oxaloacetate. Oxaloacetate is then decarboxylated to pyruvate, which can be further reduced to metabolic end products (e.g. lactate). Decarboxylation reactions in breakdown of weak acids consume cytoplasmic proton, thereby generating a pH gradient (PMF) across the membrane. Hence, the pH gradient is the driving force for citrate

and malate uptake and the generation of metabolic energy by citrate metabolism contributes to the growth advantages observed during e.g. co-metabolism of citrate and glucose (Marty-Teysset *et al*., 1996).

(A)



(B)

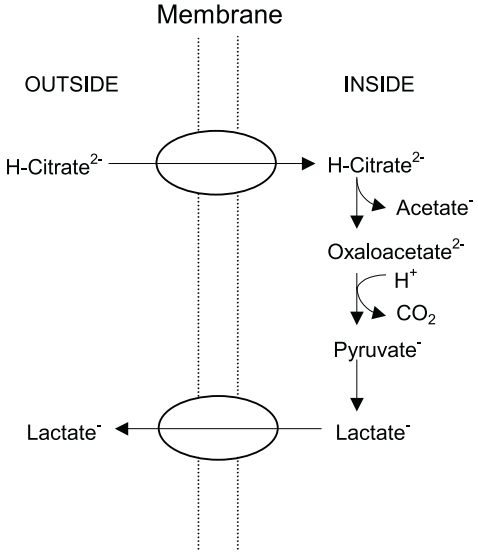


Figure 10. (A) Proton/lactate symport. (B) Precursor/product exchange in citrate metabolism. The metabolic product (e.g. pyruvate, acetate or lactate) is transported outside the cell by either a separate carrier (as shown in figure) or by the same carrier transporting citrate into the cell. *PMF* = proton motive force.

Sugar transport and phosphorylation are key factors in understanding the growth behavior of cells, but unfortunately precise knowledge for all microbes is not

available. It is generally believed that the transport of sugars into LAB cells occurs either by membrane-localized permeases (secondary active transport) or by the phosphotransferase systems (group translocation). Secondary active transport involves transport of a substance across the cytoplasmic membrane at the expense of a previously established gradient of another substance. Whether the transport process is *symport* (two substances are transported in the same direction), *antiport* (two substances are transported in opposite directions), or *uniport* (ion transport driven by an electrochemical potential), energy is required for the uphill transport event. As a general assumption, the uptake of 1 mole of glucose by permeases consumes energy equivalent to hydrolysis of 1 mole ATP to ADP. This assumption is based on two ratios: First, exporting 1 proton out of the cell requires the hydrolysis of 1 ATP. Second, the uptake of 1 sugar molecule is achieved by the simultaneous transport of 1 proton back into the cell. These ratios, however, are commonly not 1:1. For example, in *E. coli* it is believed that the export of 1 proton costs only 0.5 ATP (Stephanopoulos *et al*., 1998). The ratio of the glucose/H^+ symport in *E. coli* is 1:1 and the uptake cost is thus only 0.5 ATP per 1 glucose.

On the other hand, in a phosphotransferase system (PTS) the substance (sugar) is chemically modified upon uptake and thus no actual concentration gradient is produced across the cell membrane. When e.g. glucose is transported into the cell by means of a PTS, glucose is simultaneously phosphorylated to glucose-6-P. The phosphate group is donated from phosphoenolpyruvate (PEP), which is an intermediate in the EMP pathway. Even though a high-energy phosphate group originally present in PEP is used, the high-energy bond is conserved in the sugar phosphate and the PTS thus becomes more economical from an energy point of view compared to the permease-mediated transport (Nielsen and Villadsen, 1994).

Although transport events have been widely studied in some LAB species (e.g. *Lc. lactis* and *Lb. casei*), it is daring to generalize these findings to all LAB. A general assumption is, however, that homofermentative species prefer group translocation in sugar transport, whereas heterofermentative species prefer secondary active transport. The basis for this assumption is that the presence of a PTS is well correlated to the ability to ferment sugars by the EMP pathway and hence, to the ability to produce 2 moles of PEP per mole hexose consumed (Axelsson, 1998). As known, heterolactic fermentation only produces 1 mole of PEP per mole hexose consumed. This claim is supported by an early study by Romano *et al*. (1979). They surveyed a number of fermentative bacteria for the presence of a glucose-PTS. A glucose-PTS was found in all homofermentative LAB that fermented glucose via the EMP pathway, but in none of the heterofermentative species of *Lactobacillus* and *Leuconostoc*, which ferment glucose via the 6-PG/PK pathway.

Since the study by Romano and co-workers, only a few reports can be found describing sugar transport systems in species of *Leuconostoc* and obligately heterofermentative *Lactobacillus*. Huang *et al*. (1995) studied the uptake of lactose by 33 strains belonging to different species of *Leuconostoc*. Phospho-β-galactosidase activity was not detected in these cells, suggesting that a lactose-PTS is not functional in *Leuconostoc*. In another study, it was found that galactose does not accumulate in the media, when cells are grown on lactose, meaning that lactose transport does not occur via a lactose-galactose antiport (Huang *et al*., 1994). As a

consequence, it was shown that lactose uptake by species of *Leuconostoc* occurs via a proton symport (Cetutti de Guglielmone *et al*., 1996). Moreover, citrate uptake in *L. mesenteroides* was found to occur by a proton symport (Marty-Teysset, *et al*., 1995). No studies describing a fructose uptake system in species of *Leuconostoc* were found in the literature. It is, however assumed that both fructose and sucrose uptake in *L. mesenteroides* are controlled by secondary active transport systems (Dols *et al*., 1997).

Strains of *Lb. brevis*, *Lb. büchneri*, and *Lb. fermentum* (all obligate hetero-fermenters) were studied for the presence or absence of a sugar-PTS (Nagasaki *et al*., 1992). Contrary to common expectations, a sucrose-PTS was found in two strains of *Lb. fermentum*. In more detailed studies, *Lb. brevis* was shown to transport glucose and lactose via a proton symport (Ye *at al*., 1994), whereas fructose was transported by a PTS (Saier *et al*., 1996). Even more significant was the finding that under anaerobic growth fructose induces the synthesis of glycolytic enzymes in *Lb. brevis*, which allow fructose to be metabolized via the EMP pathway, i.e. homolactic fermentation.

5. RECOVERY OF MANNITOL FROM BIOCONVERSION MEDIUM

The recovery of mannitol from aqueous solutions is generally based on its low solubility (Figure 11). As mentioned earlier, the maximum solubility of mannitol in water is only about 180 g/L at 25°C. As a comparison, the maximum solubility of sorbitol at 25°C, is about 700 g/L (Schiweck *et al*., 1994). Whereas a low solubility is beneficial in the purification steps, it will limit the final mannitol concentration in process solutions, resulting in additional energy costs in the concentration step.

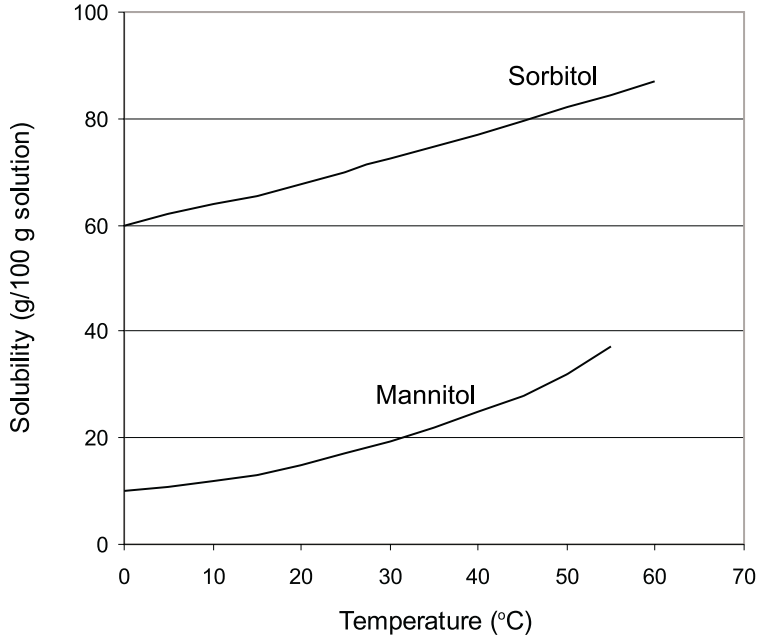


Figure 11. The solubility of mannitol and sorbitol in water. Adapted from Schiweck *et al*. (1994).

The mannitol production solutions used as a starting material for downstream processing usually contain significant amounts of various impurities. When mannitol is produced by chemical hydrogenation of a glucose/fructose mixture, mannitol is considered as the by-product, whereas sorbitol is the main product. The mannitol content in industrial production solutions is typically about 30-50% (w/w). In order to purify such a solution Pence (1956) described the following protocol. First, a concentrated product solution obtained by hydrogenation of invert sugar was acidified (pH < 3.5) by sulfuric acid. The solution was then cooled down under constant mixing and crystals were eventually formed. Finally, the crystals were separated from the mother liquor and dried. A similar process protocol is still used today and recently, using fructose syrups as the starting material, a yield of 39% crystalline mannitol from initial sugars was reported (Devos, 1995).

A 2-stage crystallization method is described by Lemay (1986). A supersaturated product stream containing about 400 g/L mannitol is fed to a first crystallization unit controlled at 68°C. After approximately 15 h the mannitol mixture is fed to a second crystallization unit, where the temperature is lowered from 68 to 20°C (1.3°C/h). In this process mannitol crystals are formed with a productivity of 189 g/L/h. Melaja and Hämäläinen (1975) suggested the use of chromatographic methods for the separation of mannitol from sorbitol. More recently, also Ojamo *et al*. (2000) have described a similar chromatographic technology for separation of mannitol, acetic and lactic acid.

On the other hand, a culture broth from a mannitol bioconversion process contains (besides mannitol) cells, organic acids and some residual raw materials. Obviously, the first downstream processing step in typical microbial processes is the separation of cells from the product solution. This is most commonly achieved by either centrifugation or filtration. Regardless of the method, separation of cells will result in some volume (and mannitol) losses. Vanecek (1962) purified mannitol obtained by fermentation of *L. mesenteroides* in sucrose-containing growth medium. Due to the formation of a highly viscous polymer (dextran), the first purification step was the precipitation of dextran with alcohol. Next, the precipitate formed was separated by centrifugation. Otherwise, a crystallization protocol, similar to the one described by Pence (1956), was used (see above).

Itoh *et al*. (1992) used filtration to separate the cells from the product solution. The cell-free solution was then evaporated, crystallized and the crystals separated by centrifugation. The mother liquor was further fractionated by chromatographic methods into an acetate and a lactate/mannitol fraction. Mannitol was separated from lactate by crystallization, whereas lactate was isolated by precipitation with calcium hydroxide. An alternative protocol was presented by Soetaert *et al*. (1995) and Deusing *et al*. (1996). Based on this protocol, the cell-free cultivation solution was first run through an electrodialysis equipment separating mannitol from the organic acids. Mannitol was isolated from the fraction by two subsequent crystallizations, yielding 86% of crystalline mannitol (from mannitol dissolved in the culture broth). In the crystallization steps a supersaturated solution containing about 250 g/L mannitol, at 60°C, was cooled down to 20 °C. Seed crystals were used to initiate crystal formation. Furthermore, from the acidic fraction D-lactic acid was obtained by acidification of the solution.

Although several effective methods for the recovery of mannitol are available, a significant question still remains: What to do with the side streams produced by the different process alternatives? When producing mannitol by chemical hydrogenation, the side stream contains mainly sorbitol and small amounts of mannitol. On the other hand, when LAB fermentation is applied, the side stream is a mixture of acetate and lactate with some residual mannitol. In both cases isolation of respective pure products is very laborious and costly and thus, alternative applications must be developed. Ojamo *et al*. (2000) suggested that the acidic side stream, obtained from a LAB fermentation process, could be applicable as feed preservative.

6. AIMS OF THIS STUDY

At present, commercial mannitol production is based on processes applying chemical hydrogenation techniques. These processes require ultra-pure raw materials and the mannitol yields from initial sugar substrates are low. Hence, the primary aim of this doctoral thesis was to develop an alternative microbiological process for the production of mannitol with improved characteristics compared to the existing ones. First, the aim was to identify a good mannitol-producing microbe, to study the strain using various bioprocess alternatives and consequently, to optimize the essential process parameters applying the best strain and the best bioprocess alternative. A further aim was to study the recovery of mannitol from the culture broth and thus, develop an efficient purification protocol. Third, the aim was to scale up the complete optimized process (bioconversion and recovery) from a laboratory scale to a small pilot factory scale, thus proving that the new bioprocess concept is most likely also suitable for industrial scale production.

An additional aim was to improve the metabolic characteristics of the cells by means of genetic engineering. Four separate goal were set for this part:

- to improve the yield of mannitol from fructose by blocking the leakage of fructose to the glucose pathway (i.e. inactivation of fructokinase),
- to study the metabolic effects of blocking the acetate-producing branch (i.e. inactivation of acetate kinase),
- to block the production of D-lactate, thus resulting in a two-product system with mannitol and isomerically pure L-lactate as end products (i.e. inactivation of D-lactate dehydrogenase),
- and to block the production of lactate (i.e. inactivation of both lactate dehydrogenases), resulting in excess reduction power (NADH) in the cell and simultaneous accumulation of pyruvate, a new by-product with potential commercial value.

7. MATERIALS AND METHODS

The materials and methods used in this thesis are presented in Chapter 7. More detailed information concerning specific experiments can, however, be found in Chapter 8 together with the pertinent results section.

7.1 Strains

Microbial strains used in this study are summarized in Table 1. They were maintained in standard MRS growth medium supplemented with glycerol at 15% (v/v) and stored at -80ºC. In this thesis the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Oenococcus* are abbreviated as *Lb.*, *Lc.*, *L.* and *O.*, respectively.

Table 1. Microbial strains used in this study. Mutants constructed in this study are marked in bold. The parent strains for mutant construction were *Lb. fermentum* NRRL-B-1932 and *L. pseudomesenteroides* ATCC-12291.

Species:	Strain:	Source:
Lb. brevis	ATCC-8287	American Type Culture Collection, USA
Lb. buchneri	TKK-1051	Prof. Simo Laakso [a]
Lb. fermentum	NRRL-B-1932	Agricultural Research Service Culture Collection, USA
	BPT-152	Prof. Matti Leisola [b]
	GRL-1030	Prof. Airi Palva [c]
	GRL-1032	Prof. Airi Palva [c]
Lb. sanfranciscensis	ATCC-27651	
Lb. sp. B001	BP-3158	Patent Microorganism Depository, Japan
Lc. lactis	GRS-71	Prof. Airi Palva [c]
L. mesenteroides	ATCC-8086 ATCC-8293 ATCC-9135 ATCC-10830	
L. pseudomesenteroides	ATCC-12291	
	DSM-14613	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany
O. oeni	E-97762	VTT Culture Collection, Finland

[a] Laboratory of Biochemistry and Microbiology, Helsinki University of Technology, Finland.
[b] Laboratory of Bioprocess Engineering, Helsinki University of Technology, Finland.
[c] Department of Microbiology, Faculty of Veterinary Medicine, University of Helsinki, Finland.

7.2 Culture and bioconversion media

Standard MRS growth medium (Pronadisa M.R.S. Broth, Hispanlab, S.A., Spain) contained (in grams per liter distilled water):

Bacteriological peptone	10
Meat extract	8
Yeast extract	4
Glucose	20
Tween 80	1
K_2HPO_4	2
Na-acetate	5
NH_4-citrate	2
$MgSO_4$	0.2
$MnSO_4$	0.05

Before sterilization (121°C, 13 min), the medium was adjusted to pH 6.2 with HCl.

The self-assembled MRS growth medium contained (in grams per liter distilled water):

Proteose peptone (Pronadisa, Hispanlab, S.A., Spain)	10
Beef extract (Pronadisa, Hispanlab, S.A., Spain)	10
Yeast extract (Difco, Becton Dickinson and Company, USA)	5
Sugar components (HPLC grade, total concentration)	30
Tween 80 (Fluka Chemie AG, Switzerland)	1
K_2HPO_4	2
Na-acetate	5
NH_4-citrate	2
$MgSO_4$	0.5
$MnSO_4$	0.25

Before sterilization (121°C, 13 min), the medium was adjusted to pH 6.2 with HCl, with the exception of cultures of *O. oeni*, in which the medium was pre-set to pH 5.0.

The simplified cell production (SCP) medium comprised (grams per liter distilled water):

Tryptone (Pronadisa, Hispanlab, S.A., Spain)	10
Yeast extract (Difco, Becton Dickinson and Company, USA)	5
Fructose	20
Glucose	10
K_2HPO_4	2
$MgSO_4$	0.2
$MnSO_4$	0.01
$FeCl_3$	0.01
$CaCl_2$	0.02
NaCl	0.01

The amounts of the variable components (Mg, Mn, Fe, Ca, and Na) were adapted from Dols *et al*. (1997).

The optimized simplified cell production (OSCP) medium contained (grams per liter distilled water):

Tryptone (Pronadisa, Hispanlab, S.A., Spain)	10
Yeast extract (Difco, Becton Dickinson and Company, USA)	5
Fructose	20
Glucose	10
K_2HPO_4	2
$MgSO_4$	0.2
$MnSO_4$	0.01

For cultures of *Lb*. *fermentum* double concentrations of $MgSO_4$ and $MnSO_4$ were used.

The simplified cell production (SCP2) medium used in semi-continuous bio-conversion experiments comprised (grams per liter distilled water):

Yeast extract (LAB M, International Diagnostics Group, England)	15
Glucose	30
K_2HPO_4	2
$MgSO_4$	0.2
$MnSO_4$	0.05

The feed solution (FS) for cell production in continuous mode contained (grams per liter distilled water):

Yeast extract (LAB M, International Diagnostics Group, England)	15
Glucose	50
K_2HPO_4	2
$MgSO_4$	0.2
$MnSO_4$	0.05

The bioconversion (BC1) medium used in experiments with resting cells at low sugar concentration (total sugar concentration 30 g/L) contained (grams per liter distilled water):

Tryptone (Pronadisa, Hispanlab, S.A., Spain)	0.5
Yeast extract (Difco, Becton Dickinson and Company, USA)	0.25
Fructose	20
Glucose	10
K_2HPO_4	2
$MgSO_4$	0.2
$MnSO_4$	0.01

For cultures of *Lb*. *fermentum* double concentrations of $MgSO_4$ and $MnSO_4$ were used.

The bioconversion (BC2) medium used in experiments with resting cells at high sugar concentration (total sugar concentration over 50 g/L) comprised (grams per liter distilled water):

Tryptone (Pronadisa, Hispanlab, S.A., Spain)	1
Yeast extract (LAB M, International Diagnostics Group, England)	0.5
Fructose	variable
Glucose	variable
K_2HPO_4	1
$MgSO_4$	0.2
$MnSO_4$	0.02

Any further medium distinctions are described under respective cultivation protocols. In general, sterilization of media was performed at 121°C (20 min). Sugar stock solutions were separately autoclaved and mineral stock solutions were sterilized by filtration (0.2 μm).

7.3 Lab-scale bioreactor equipment

The initial comparison of LAB strains and the development of the OSCP medium were conducted in a Bioscreen C analyzer (Labsystems Oy, Finland). A working volume of 400 μL was applied. The temperature was controlled at 30°C and the optical density (600 nm) of the cell suspensions was measured automatically at regular intervals. Before each measurement the culture wells were automatically shaken for 10 seconds. All Bioscreen experiments were carried out in quadruplicate.

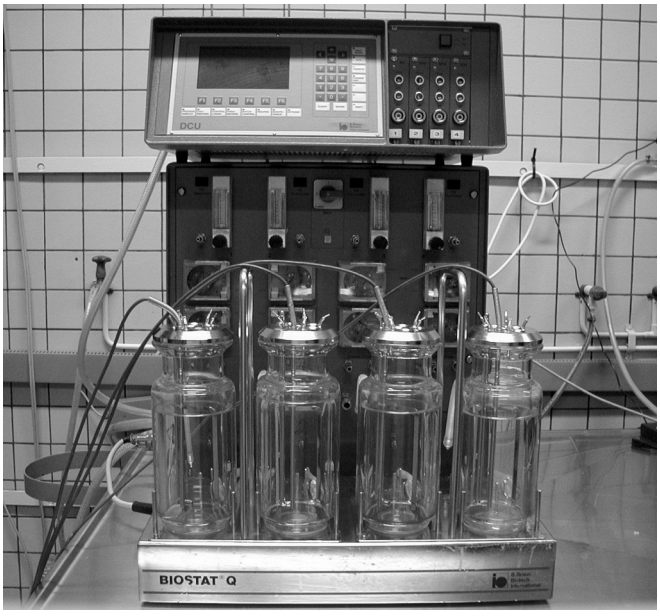


Figure 12. Biostat Q bioreactor system.

The Biostat Q multiple bioreactor system (B. Braun Biotech International, Germany) shown in Figure 12 consisted of four identical culture vessels. The working volume used in these vessels was in the range of 300-800 mL. The system was equipped with automatic probes for the measurement and control of temperature, pH, and dissolved oxygen tension (DOT). Magnetic bars and a magnetic drive unit were used for mixing. Air or nitrogen gas was added below the medium surface through a sparger pipe equipped with a frit to break down the bubbles.

The Biostat MD bioreactor system (B. Braun Biotech International, Germany) shown in Figure 13 was equipped with a M2 culture vessel (working volume 0.5-2.0 L). The system was equipped with automatic probes for the measurement and control of temperature, pH, foaming, and DOT. Two 6-blade Rushton turbines were used for mixing. Air or nitrogen gas was added below the medium surface through a ring sparger. The culture vessel was placed on a balance (Sartorius AG, Germany) connected to the control unit of the MD system. Feed and harvest pumps used were of type 101U/R from Watson Marlow, UK.

Figure 13. Biostat MD bioreactor system.

DOT probes were calibrated with air and nitrogen gas (purity 99.5%). During anaerobic experiments the medium was flushed with nitrogen gas. When flushed, the composition of the bioreactor exhaust gas was analyzed on-line with mass spectrometer (VG Prima 600, V.G. Gas Analysis Systems, Ltd., UK). Gas flows in and out of the Biostat MD bioreactor were controlled and measured with GFC17 mass flow controllers (Aalborg Instruments & Controls, Inc., USA). The pH of the medium was controlled with 3-5 M NaOH and 3 M H_2SO_4.

The membrane cell-recycle bioreactor (MCRB) consisted of the Biostat MD bioreactor system (working volume 2 L) attached to a tangential flow filtration module, Pellicon 2 Biomax 1000 (1000 kDa, 0.1 m^2 filtration area, V channel, Millipore Corp., USA), and a hose pump, Masterflex model no. 754944 (Cole-Parmer Instrument Company, USA) for cell-recycle with a flow rate of 1.5-2.0 L/min (Figure 14). The filtration unit of the MCRB was disinfected with 0.5 M NaOH and washed thoroughly with sterile water before use.

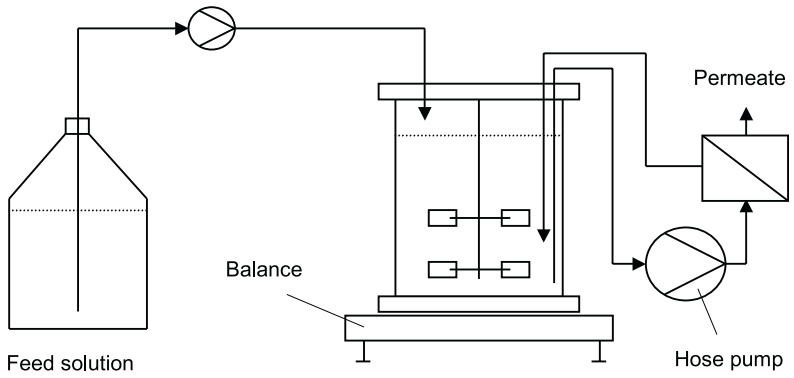


Figure 14. Schematic diagram of the membrane cell-recycle bioreactor.

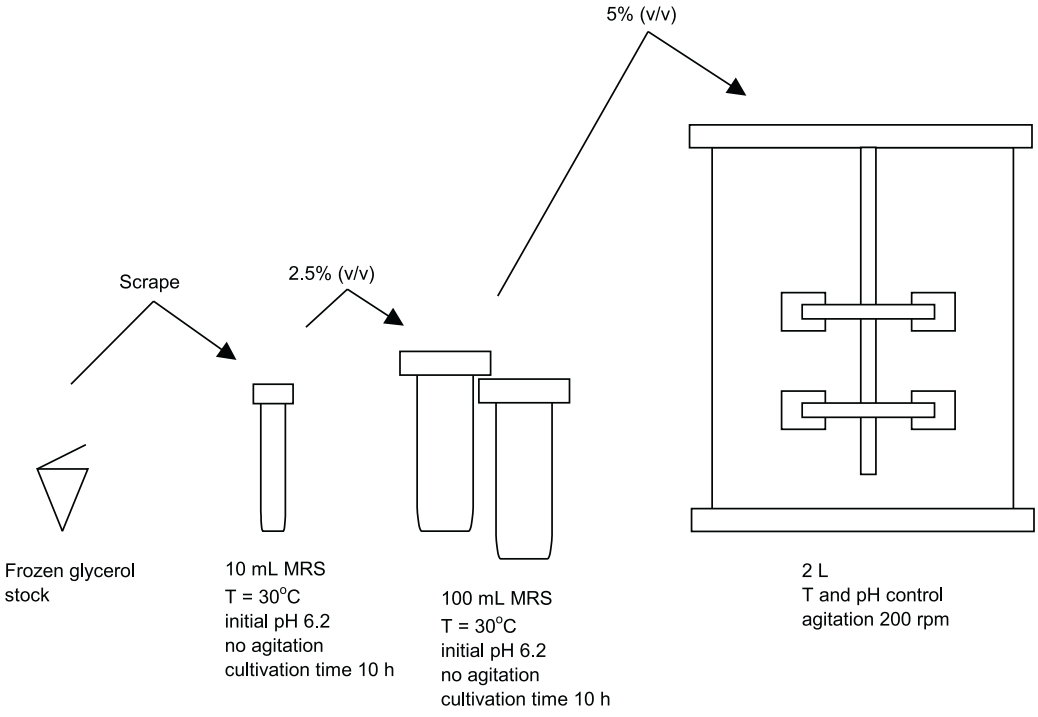


Figure 15. A typical pre-culture protocol for experiments with resting cells.

All data collected by the DCUs and the mass spectrometer were transferred to a PC with MFCS/win software (B. Braun Biotech International, Germany) for data handling.

7.4 Pre-cultures and cell production in lab-scale

For the first pre-culture, 10 mL of standard MRS growth medium (pH 6.2) in a test tube was inoculated from a frozen glycerol stock and grown for 10 h at 30°C without mixing. As a general rule, the subsequent pre-cultures were inoculated with 5-10% (v/v) of the first pre-culture grown to late exponential phase. Due to high-level formation of carbon dioxide, the test tube plugs were not fully sealed. A typical pre-culture protocol for resting cell experiments is shown in Figure 15. For resting cell experiments in Biostat Q bioreactors, cells from a 2-L standard MRS culture were collected at early stationary growth phase by centrifugation (6000 g, 10 min), resuspended in phosphate buffer (pH 6.2) containing small amounts of nutrients and sugars, and transferred to the Biostat Q bioreactors. For resting cell experiments in Biostat MD bioreactors, the cell biomass was produced in a 2-phase (batch + continuous cell-recycle) Biostat MD cultivation. Feeding of fresh growth medium to the MD bioreactor was started when the cells reached late exponential growth phase indicated by a slow-down in base consumption rate.

7.5 Crystallization in lab-scale

The laboratory-scale downstream processing equipment comprised the following: a basic Rotavapor evaporator (RE 111, Büchi, Switzerland); a 400-mL decanter glass placed in a temperature controlled water bath and equipped with external mixing (Figure 16); crystal separation by suction filtration in a Büchner funnel; and an oven (60°C). The purity of the crystals was analyzed by HPLC and spectrophotometer (spectra: 220-820 nm).

The optimized purification protocol was as follows: The cell-free product solution was concentrated by evaporation at 35°C to 250 g mannitol/L. The concentrate was poured into the decanter glass and the temperature was cooled down under slow mixing to 5°C (cooling rate 2°C/h). The crystals were separated by filtration and the mother liquor from the first crystallization was crystallized using the same protocol. After combining the wet crystals from the primary and mother liquor crystallization, these were re-crystallized. The final mannitol concentration was 300 g/L (at 45°C) and the cooling rate 3°C/h. The white crystals were dried over night at 60°C and finally homogenized in a porcelain mortar.

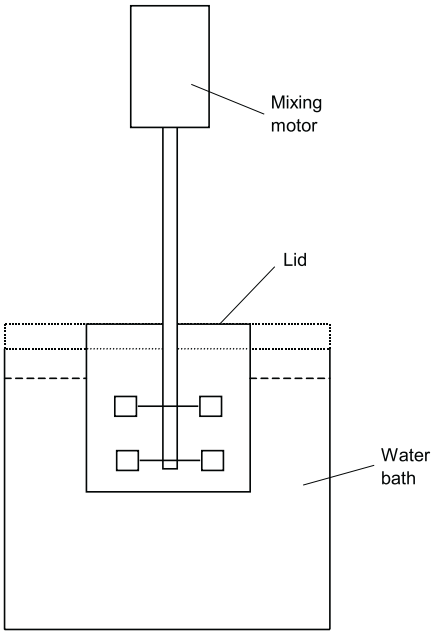


Figure 16. Laboratory-scale cooling crystallizer.

7.6 Pilot plant

A schematic diagram of the pilot plant is shown in Figure 17. The following raw materials were used in the pilot-scale experiments: fructose (Fructofin, Xyrofin Oy, Finland); glucose (Dextrose anhydrous, Xyrofin Oy, Finland); yeast extract powder (MC1, Lab M, International Diagnostics Group, UK); tryptone (MC5, Lab M, UK). All dry raw materials were suspended in tap water and pumped (KM80-40 diaphragm pump, Groschopp u. Co, Germany) into a mixing reactor. The mixing reactor was a stainless steel 200-L tank equipped with a jacket for temperature control, two 6-blade Rushton turbines for mixing and external surface level meter. Temperature control was implemented with 1.0-2.5 bar steam and 12°C tap water. The steam was produced with SteamElmo (Steamrator Oy, Finland).

The pilot-scale membrane cell-recycle bioreactor (MCRB) comprised a 200-L Marubishi MPF-U bioreactor (B.E. Marubishi Co, Japan, working volume used 100 L) attached to a tangential flow filtration module, Pellicon 2 Biomax 1000 (1000 kDa, maximum filtration area 2.0 m^2, V channel, Millipore Corp., USA), and a hose pump, SP/32 (Bredd Hose Pumps, Holland) for cell-recycle with a flow rate of 15-30 L/min. The Marubishi bioreactor was equipped with an automatic probe for the measurement and control of temperature. The temperature was adjusted with steam and 12°C water. The pH was controlled with an external pH regulator (EH controller, Iwaki Co, Japan) connected to the standard pH electrode (Type ML0248, L.E. Marubishi Co, Japan). 6 M NaOH was used as base and no acid was required. The external base reservoir was placed on a balance. Two Rushton turbines were used for mixing. The bioreactor was connected to a MPF-U control unit (B.E. Marubishi Co, Japan) and data was collected by FermExpert software (Version 2.10.175, BioExpert Ltd., Estonia). Solutions of raw material were pumped from the

mixing reactor to the bioreactor through an Opticap 10'' sterile filter (Millipore Corp., USA) using a hose pump, SP/15 (0.5-5 L/min, Bredd Hose Pumps, Holland).

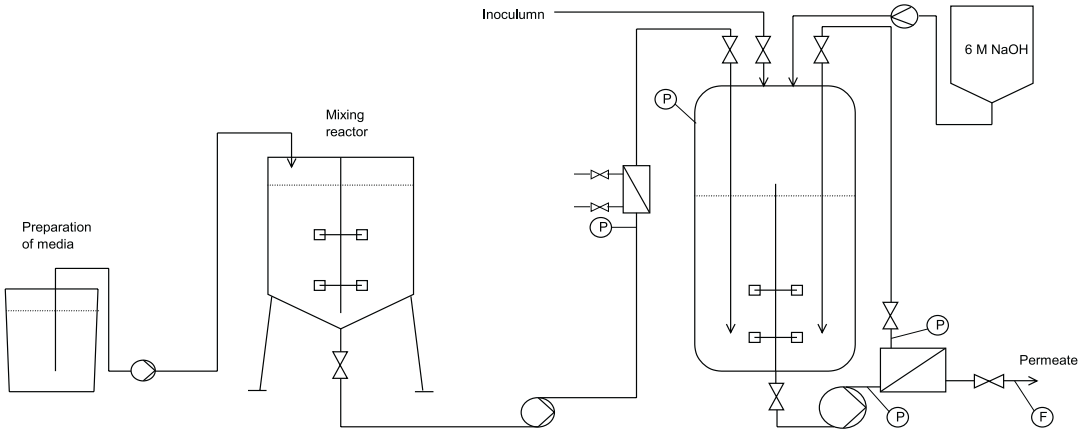


Figure 17. A schematic diagram of the pilot plant for mannitol production.

The main bioreactor was inoculated with a 6-L culture broth at late exponential growth phase, grown in SCP2 medium in a 10-L Biostat E bioreactor (B. Braun, Germany). The earlier pre-cultures were grown as follows: First, 10 mL of standard MRS growth medium was inoculated from a frozen glycerol stock and grown in a test tube at 30°C for about 10 hours without mixing. Next, 6.25 mL of this broth was added to 243.75 mL of fresh MRS medium in five 50-mL test tubes and grown at 30°C for about 10 hours without mixing. The broth of this final pre-culture was then used to inoculate the SCP2 medium in the Biostat E bioreactor. The temperature, pH and agitation in the Biostat E bioreactor were controlled at 30°C, 6.0, and 50 rpm, respectively. No gases were added to the system and 6 M NaOH was used as base.

Each bioconversion batch from the semi-continuous experiment resulted in approximately 75 L of cell-free mannitol-rich solution. Besides mannitol this solution contained mainly acids, some ethanol, and residual amounts of glucose and fructose. The cell-free product solution was concentrated with a Centri-Therm CT-1B vacuum evaporator (Alfa-Laval Ab, Sweden). The solution was fed into the cone of the evaporator at 0.7 L/min. The primary and secondary temperatures were 95°C and 61°C, respectively, and the secondary pressure was 0.25 bar.

The supersaturated mannitol solution (35°C) was transferred into a cooling crystallizer. The crystallizer was constructed in-house and consisted of a closed vertical stainless steel mixing vessel with a water jacket for temperature control. The maximum volume of the vessel was 25 L and the agitation was set at 3 rpm with an external controller (OPM2, Siemens, Germany). Inclined disc impellers were connected to the mixing shaft at three different levels. The water jacket was connected to a K40 cryostat (Haake, Germany), which received its temperature signal from a probe positioned inside the reactor. This cryostat was controlled by a

PC with ThermStar 95 plus software (Version 2.0, Haake, Germany). The temperature of the solution decreased linearly from 35 to 5°C within 15 hours.

The crystallizer was emptied through a bottom valve and the mother liquor separated from the crystals with an Esteri C61 drum centrifuge (Podab Ab, Sweden, 1480 rpm, G-factor 622 kp/kg, maximum capacity 12 kg wet crystal mass) using a filtration bag (pore size unknown). The filtration time was 30 minutes. The primary crystals were dried with a fluidized bed dryer (TG1, Retsch GmbH, Germany). The temperature of the air in the dryer was 45°C and the blower was used at full speed for 45 minutes. Before purity control a sample of the dried crystals was kept over night in an oven (60°C) and thereafter homogenized in a porcelain mortar. A schematic diagram of the downstream processing equipment is shown in Figure 18.

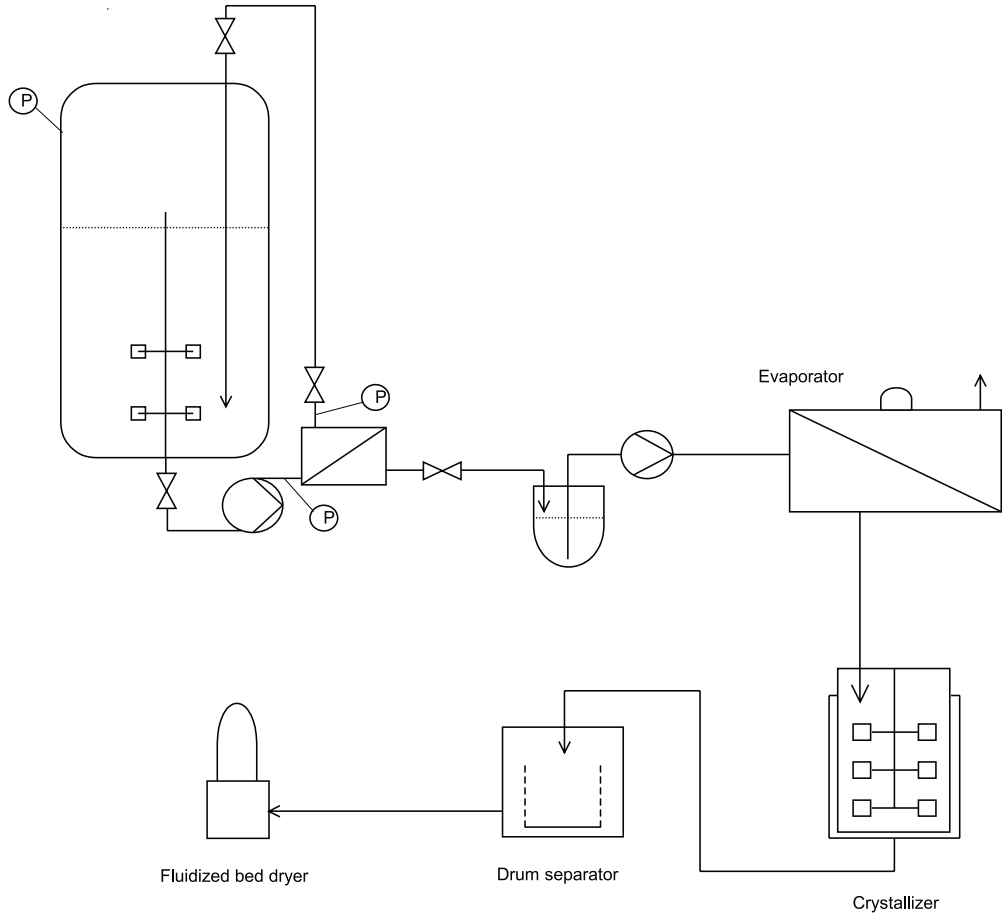


Figure 18. A schematic diagram of the downstream processing equipment.

7.7 Metabolic engineering of mannitol-producing LAB

The work involving genetic modification of the primary sugar metabolism of two mannitol-producing LAB, *Lb. fermentum* NRRL-B-1932 and *L. pseudomesente-*

roides ATCC-12291, was a collaboration between the Laboratory of Bioprocess Engineering, Helsinki University of Technology and the research group of prof. Airi Palva, University of Helsinki, Finland. Ulla Airaksinen and Miia Helanto at Helsinki University of Technology constructed the fructokinase and acetate kinase mutants; Johannes Aarnikunnas at the University of Helsinki constructed the lactate dehydrogenase mutants. The following procedures were used:

7.7.1 Fructokinase inactivation

Chemical mutagenesis of *L. pseudomesenteroides* ATCC-12291 was performed using cells at exponential growth phase (optical density at 600 nm = 1.0) grown in M17 growth medium (Difco, Becton Dickinson and Company, USA) supplemented with 1% (w/v) glucose (GM17). The initial pH was 6.9. Cells washed with 50 mM sodium phosphate buffer (pH 7) were treated with 1-methyl-3-nitro-1-nitroso-guanidine (0.5 mg/ml) for 40–50 min, at room temperature. The washing procedure was repeated three times. Washed cells were incubated in GM17 (1 h and 30°C), plated on GM17 agarose growth medium and incubated for 2 days at 30°C. Colonies on GM17 plates were replica-plated on a chemically defined medium (CDM; Anon., 2000b) supplemented with either 1% glucose or 1% fructose. After 2 days of incubation at 30°C colonies growing on glucose, but not on fructose, were selected. Conversion of fructose to mannitol by these colonies was tested to ensure that the fructose permease was not affected by the mutagen. The production strain, with reduced fructokinase activity, but able to convert fructose to mannitol, was named BPT-143. The strain was deposited at the Deutsche Sammlung von Mikro-organismen und Zellkulturen, GmbH, Mascheroder Weg 1b, D-34124 Braun-schweig, Germany on 13 November 2001 with the accession number DSM-14613.

7.7.2 Acetate kinase inactivation

The inactivation plasmid for disrupting acetate kinase in *Lb. fermentum* NRRL-B-1932 was constructed by ligating an internal fragment of the acetate kinase gene (413 bp) between suitable restriction sites (*Bam*HI and *Hind*III) of pGhost4 (prof. Airi Palva, Helsinki University, Finland). The ligation mixture was electroporated into *Lactococcus lactis* GRS-71 and transformants were incubated for 1 day at permissive temperature (30°C) on M17 plates (Difco, Becton Dickinson and Company, USA) supplemented with erythromycin (Em, 5 μg/ml). The *Lc. lactis* transformants were screened by PCR with pGhost4-specific primers. Recombinant plasmids, containing the internal fragment of an acetate kinase gene, were isolated and electroporated into *Lb. fermentum*. Transformants were incubated anaerobically on MRS plates (Em, 5 μg/ml) for 1 day at 30°C, and verified by PCR with the previously mentioned primers. Positive clones, carrying the recombinant plasmids, were grown over night at 30°C in standard MRS growth medium supplemented with 5 μg/ml Em. A new culture was inoculated using the overnight cell culture and grown for 5 hours at 42°C in MRS (Em, 5 μg/ml). Next, the culture broth was diluted 1:100 000 in saline, plated on MRS-Em and incubated for 2 days at 42°C. As a result of this strategy, colonies growing in the presence of Em at 42°C were likely

to have a disruption plasmid integrated into the chromosome at the acetate kinase locus. Disruption of the acetate kinase gene in this way resulted in the acetate kinase activity of the positive transformants being reduced compared to that of *Lb. fermentum* parent cells. Disruption of the acetate kinase gene was confirmed by PCR analysis using as template the chromosomal DNA isolated from clones with reduced acetate kinase activity. Primers for PCR analysis were chosen to lie outside the site of integration into the chromosomal acetate kinase gene.

7.7.3 D-lactate dehydrogenase inactivation

A 456 bp deletion was made to a *Lb. fermentum* NRRL-B-1932 gene sequence (1.7 kb) containing the D-lactate dehydrogenase gene. The deletion comprised a promoter area (-247) and part of the structural gene (209). The remaining fragments were joined together (*Hind*III) and ligated to pGhost4 (*Xba*I and *Eco*RI). The ligation mixture was electroporated into *Lc. lactis* GRS-71 and incubated 1 day on M17-Em plates at 30°C. The recombinant plasmids were screened by PCR using pGhost4-specific primers. Plasmids containing the cloned fragments were isolated and electroporated into *Lb. fermentum*. Transformants were incubated anaerobically on MRS-Em plates for 1 day at 30°C. pGhost4-specific primers were used to ensure the presence of the recombinant plasmids and correct insert sizes in the resultant colonies. Positive clones were grown overnight in MRS-Em medium at 30°C. The overnight cultures were used to inoculated fresh MRS-Em medium and the cultures were grown for 5 h at 42°C. Next, the cultures were diluted 1:100 000 in saline, plated on MRS-Em and incubated for 2 days at 42°C. Raising the temperature as described for the disruption plasmids resulted in single crossover recombination of the recombinant plasmid to the chromosome. Sites of the integration were confirmed by Southern blotting of chromosomal DNA isolated from the integrant strains. The *Lb. fermentum* clones carrying an integrated recombinant plasmid at the D-lactate dehydrogenase locus were then grown in MRS medium without Em for 50 generations at 30°C and finally plated on MRS (no Em). Omission of the antibiotic resulted in dissociation of the integrated plasmid from the chromosome. Depending on the recombination site either restoration of the parent cells or deletion of the D-lactate dehydrogenase gene occurred. Em-sensitive clones were detected by replica plating on MRS with and without Em. Only 10-15% of the colonies were Em-sensitive. Among the Em-sensitive clones those with deficient D-lactate dehydrogenase activity were selected (2/42). Deletion of the gene was confirmed by PCR using primers both outside (correct bands) and inside (no bands) the deletion site and also with plasmid-specific primers (no bands).

7.7.4 D/L-lactate dehydrogenase inactivation

Two fragments amplified by PCR from *Lb. fermentum* chromosome, surrounding the target deletion site (L-lactate dehydrogenase, -320 and 94), were ligated to pGhost4. The recombinant plasmid containing the deletion insert was constructed as described in Chapter 7.7.3 and transformed into a D-lactate dehydrogenase negative *Lb. fermentum* by electroporation. The integration steps were performed as described in Chapter 7.7.3, but the integrants were grown without Em at 30°C for 100

generations instead of 50. Of the integrants 85-90% were Em-sensitive and the deletion was found in 2/250 colonies. Deletion of genes was confirmed by PCR as described in Chapter 7.3.3. Moreover, the presence of either D- or L-lactate dehydrogenase messenger-RNA in the cells was confirmed by Northern hybridization. The hybridization probes (526 bp, L-ldh and 625 bp, D-ldh) were complementary to sites downstream of the deletion sites.

7.7.5 Cultivation conditions

The effects of the inactivation on the primary metabolism of the cells were studied in batch bioreactor experiments. Mutants were maintained in standard MRS growth medium supplemented with 15% (v/v) glycerol and stored at -80ºC. For the first pre-culture, 10 mL of standard MRS growth medium supplemented with 20 g/L fructose was inoculated from frozen glycerol stocks and grown for 10 h at 30°C without mixing. For the second pre-culture, 100 mL of standard MRS growth medium supplemented with 20 g/L fructose was inoculated with 5% (v/v) of the broth from first pre-culture and grown at 30°C without mixing to late exponential growth phase. The initial pH in standard MRS growth medium was 6.2.

Complex OSCP growth medium was used in bioreactor experiments and contained: 10 g/L, tryptone (LAB M, International Diagnostics Group, England); 5 g/L, yeast extract (LAB M); 20 g/L, fructose; 10 g/L, glucose; 2 g/L, K_2HPO_4; 400 mg/L, $MgSO_4$; 20 mg/L, $MnSO_4$. Tryptone, yeast extract and K_2HPO_4 were autoclaved with the bioreactor (121°C, 13 min). Stock solutions of fructose and glucose were autoclaved separately (121°C, 13 min) and stock solutions of $MgSO_4$ and $MnSO_4$ were sterilized by filtration (0.22 μm).

The bioreactor cultivations were performed in 2-L glass-vessel bioreactors (Biostat MD, B. Braun Biotech International, Germany) (Figure 13). The bioreactors were equipped with three 6-blade Rushton turbines and the agitation rate was 200 rpm. The temperature was set at 30°C (*L. pseudomesenteroides*) or 37°C (*Lb. fermentum*). The pH was controlled with 3 M NaOH and 2 M H_2SO_4. The growth media were sparged with pure nitrogen for 30 min prior to inoculation and flushing of the media was continued throughout the experiments, at a rate of 0.2 L/min. Adding the second pre-culture into the bioreactor started an experiment. Samples were taken hourly.

7.8 Assay methods

The optical density of the bioconversion broths was measured at 600 nm against distilled water. The samples were diluted in such a manner that the absorbance values were in the range of 0.1 to 0.6. The cell dry weight (cdw) were measured as follows: a sample of bioconversion broth was pipetted into a pre-weighted centrifuge tube followed by centrifugation at 6000 g for 5 min. The cell pellet was washed with sterile saline (0.9% (w/v) NaCl), the centrifugation was repeated, after which the centrifuge tube was dried at 80°C until a constant cdw was achieved. Frequently, a pre-determined linear correlation factor was used to convert the optical density values into cdw's. The viable cell counts were measured as follows: a

sample of cultivation broth was diluted in sterile saline and 100 µL of diluted suspensions were spread over Petri dishes with MRS agar growth medium (pH 6.2). The Petri dishes were incubated at 30°C for 24 h, and subsequently, the colonies were counted. The dilutions were chosen so that the 100 µL spread onto the Petri dishes contained approximately 20-300 colonies.

The cell free extracts for enzyme activity measurements were prepared as follows: When grown in test tubes, the standard MRS medium was supplemented with 0.75 g/L glycine. When grown in bioreactors, no glycine was added to the growth media. At late exponential growth phase the cells were harvested and washed twice in a cold enzyme-specific buffer (see below):

Enzyme:	**Washing buffer:**
Mannitol dehydrogenase (MDH)	25 mM K_2HPO_4, pH 7.0
NADH oxidase (NOX)	50 mM phosphate buffer, pH 6.5
Acetate kinase (AcK)	50 mM Tris-HCl, pH 7.0
Fructokinase (FK)	50 mM Tris-HCl, pH 7.0
Lactate dehydrogenase (LDH)	10 mM phosphate buffer, pH 6.8

After the second wash the cells were suspended in 4 mL of cold sonication buffer (see below):

Sonication buffers:

MDH

1.25 mL	2 M Tris HCl, pH 7.0
0.5 mL	1 M $MgCl_2$
0.1 mL	0.5 M EDTA
30 mL	50% (v/v) glycerol
2 tablets	Protease inhibitor (EDTA-free, Roche Diagnostics GmbH, Germany)
0.5 mL	100 mM DTT
17.65 mL	Distilled water
50 mL	

NOX

50 mM phosphate buffer, pH 6.5

AcK and FK

0.5 mL	2 M Tris HCl, pH 7.5
0.04 mL	0.5 M EDTA
4 mL	50% (v/v) glycerol
1 tablet	Protease inhibitor (EDTA-free, Roche Diagnostics GmbH, Germany)
0.2 mL	100 mM DTT
15.26 mL	Distilled water
20 mL	

LDH
10 mM phosphate buffer, pH 6.8

A micro spoon of glass beads was added to the suspension. The cells were then sonicated for 8 × 15 s with 30 s cooling on ice between pulses. The cell debris was separated by centrifugation (4500 g, 10 min, 5°C) and the supernatant (CFE) was kept on ice.

The MDH activities were assayed at 30°C in 20 mM acetate buffer (pH 5.35) containing 0.15 mM NADH and variable amounts of D-fructose. For estimation of the K_i -factor (product), the reaction solution was supplemented with 5 g/L D-mannitol. The NADH oxidase activities were assayed at 30°C in 50 mM sodium phosphate buffer (pH 6.5) containing 0.15 mM NADH. The AcK assays were performed at 25°C in a solution containing 70 mM Tris-HCl (pH 7.5), 0.2 mM NADH, 50 mM $MgCl_2$, 3 mM phosphoenolpyruvate, 3 mM ATP, 340 mM potassium acetate and 10 U/mL L-Lactic dehydrogenase (catalogue no. L-2375, Sigma Chemical Co., USA). The FK assays were performed at 25°C in a solution containing 125 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, 1.25 mM ATP and 1.25 mM NAD^+, 100 mM D-fructose, 20 U/mL Glucose-6-Phosphate Dehydrogenase (catalogue no. 165 875, Roche Diagnostics GmbH, Germany) and 20 U/mL Phosphoglucose-isomerase (catalogue no. 127 396, Boehringer Mannheim GmbH, Germany). The L-LDH activities were assayed at 30°C in 40 mM Hepes buffer (pH 8.0) containing 10 mM NAD^+ and 150 mM L-lactate, and the D-LDH activities at 30°C in 40 mM Tris-HCl buffer (pH 9.0) containing 10 mM NAD^+ and 100 mM D-lactate. In all enzyme assays the oxidation or reduction was followed at 340 nm.

Protein concentrations were measured by the Bradford method using Bio-Rad Protein Assay (Bio-Rad Laboratories, USA). Kinetic parameters (K_m and K_i) were determined using DynaFit software to perform non-linear least-squares regression analysis of enzymatic data (Kuzmic, 1996). The D- and L-lactate concentrations were analyzed enzymatically using the kit by Boehringer Mannheim GmbH, Germany (catalogue no. 1 112 821).

Concentrations of organic acids, sugars, ethanol, acetoin, 2,3-butanediol and mannitol were determined by high-performance liquid chromatography (HPLC). The following equipments was used:

System 1:
Autosampler: 717 plus (Waters Corp., USA)
Pump: 510 (Waters Corp., USA)
Detectors: 410 refractive index (Waters Corp., USA)
486 UV (Waters Corp., USA)

System 2:
Autosampler: SIL-6B (Shimadzu Corp., Japan)
Pump: LC-6A (Shimadzu Corp., Japan)
Detector: RID-10A refractive index (Shimadzu Corp., Japan)

System 3:
Autosampler: PE Series 200 (Perkin-Elmer Corp., USA)
Pump: LC (Perkin-Elmer Corp., USA)
Detector: HP 1047A refractive index (Hewlett-Packard Comp., USA)

Mannitol, acetoin and 2,3-butanediol concentrations were analyzed using an Aminex HPX-87P column (Bio-Rad Laboratories, USA) at 70°C with distilled water as the mobile phase. Glucose, fructose, and sucrose concentrations were measured using an Aminex HPX-87C column at 70°C with distilled water as the mobile phase. Consequently, organic acids and ethanol concentrations were measured using Aminex HPX-87H ion exclusion column at 60°C with 5 mM H_2SO_4 as the mobile phase. Pyruvate was analyzed with a ultraviolet (UV) detector, while all the other components were analyzed with a refractive index (RI) detector.

A Deashing Micro-Guard pre-column (Bio-Rad Laboratories, USA) was used in analysis of mannitol, acetoin, 2,3-butanediol, and all sugars, while a Cation H Micro-Guard pre-column (Bio-Rad Laboratories, USA) was used in analysis of organic acids and ethanol. The elution rate in all systems was 0.6 mL/min.

Concentrations of Mn^{2+} and Mg^{2+} in the permeates from the semi-continuous production experiments were analyzed with atomic absorption spectrometry (AAS) by the Centre for Chemical Analysis at Helsinki University of Technology, Finland. Mn^{2+} was measured by means of graphite furnace AAS (Varian 400 P, Varian Inc., USA) with GTA 96 graphite furnace and deuterium background correction. Mg^{2+} measurements were performed using flame AAS (Varian 600, USA) with an N_2O-acetylene flame. Wavelengths used: 279.5 nm (Mn^{2+}) and 285.2 nm (Mg^{2+}).

Viscosity of the bioconversion broths was measured with a DV II plus viscometer (Brookfield Engineering Laboratories Inc., USA).

7.9 Calculations

The maximum specific growth rates (μ_{max}) were calculated with Microsoft Excel. A chart for the natural logarithm of cell dry weights versus time was plotted. The maximum specific growth rate was the steepest slope of a linear trendline (3-5 successive values) in the exponential growth phase.

The following substrate and product abbreviations are used in the equations below:

fru	fructose
glu	glucose
mtol	mannitol
HAc	acetic acid
HLac	lactic acid
CO2	carbon dioxide
EtOH	ethanol
BD	2,3-butanediol
X	biomass

The efficiency of the fructose to mannitol reaction at t = t was characterized either by yield (Y_{mtol}) or by conversion (x_{mtol}), and calculated as presented below:

$$Y_{mtol} = (n_{mtol,\ t=t} - n_{mtol,\ t=0}) \div (n_{fru,\ t=0} - n_{fru,\ t=t}) \times 100\ \%,$$

where n_{mtol} = moles of mannitol (mol)
n_{fru} = moles of fructose (mol).

$$x_{mtol} = n_{mtol,\ t=t} \div n_{fru,\ t=0}.$$

The latter equation assumes that no mannitol was present at t = 0. If present, the amount was subtracted from the concentration of mannitol at t = t.

The specific fructose consumption rates (q_{fru}) were calculated as presented below:

$$q_{fru} = (C_{t1} - C_{t2}) / (X' \times t),$$

where C = concentration (g/L)
t = cultivation time (h).

The logarithmic mean of the biomass (X') was calculated as presented below:

$$X' = (X_{t2} - X_{t1}) / (\ln X_{t2} - \ln X_{t1}),$$

where X = biomass concentration (g cdw/L).

The carbon-balances were calculated on a C-molar basis as the ratio between the sum of the end products and consumption of sugars as described by Curie *at al*. (1999). The consumption (in moles) of fructose and glucose and the formation of mannitol, ethanol, 2,3-butanediol, acetoin, acetate, lactate, pyruvate and formate were determined by HPLC. The formation of carbon dioxide was calculated using the following equation (see Appendix 1):

$$n_{CO2} = n_{HAc} + n_{EtOH} + 2 \times n_{BD},$$

where n_{CO2} = moles of carbon dioxide produced (mol)
n_{HAc} = moles of acetic acid produced (mol)
n_{EtOH} = moles of ethanol produced (mol)
n_{BD} = moles of 2,3-butanediol produced (mol).

The elemental composition of the biomass, $C_{4.63}H_{7.89}O_{2.35}N_{1.0}$, was taken from Novak *et al*. (1997). The fraction of glucose-6-phosphate channeled into formation of biomass was calculated with the following equation (see Appendix 1):

$$n_X = 6 \div 4.63 \times (n_{fru} - n_{mtol} + n_{glu} - n_{HAc} - n_{EtOH}),$$

where n_X = moles of biomass produced (mol)
n_{fru} = moles of fructose consumed (mol)

n_{mtol} = moles of mannitol produced (mol)
n_{glu} = moles of glucose consumed (mol).

The redox balances were as follows (see Appendix 1):

$$NAD/NADH = (3 \times n_{HAc} + 3 \times n_{EtOH}) / (r_{mtol} + 2 \times n_{EtOH} + n_{HLac} + n_{BD}).$$

If otherwise not stated, all data are given as mean values and standard deviations of two independent experiments.

8. RESULTS AND DISCUSSION

8.1 Production of mannitol with growing cells

8.1.1 Comparison of different heterofermentative LAB

Eight species of obligately heterofermentative lactic acid bacteria were compared in their ability to convert D-fructose into D-mannitol during growth. Initial studies were conducted with a Bioscreen C analyzer. The strains were grown in a self-assembled MRS medium containing 20 g/L fructose and 10 g/L glucose. Samples from four parallel cultivations were collected at early stationary growth phase, centrifuged, combined and analyzed with HPLC. The results are presented in Table 2.

Table 2. Volumetric (r_{mtol}) and specific mannitol productivities (q_{mtol}) and mannitol yields from fructose (Y_{mtol}) in initial comparison experiments. The temperature was set at 30°C and the initial pH of the self-assembled MRS growth medium was 6.2.

Strain:	r_{mtol} (g/L/h)	q_{mtol} (g/L/h) [a]	Y_{mtol} (mol-%)
L. mesenteroides ATCC-9135	1.53	0.96	91.5
Lb. brevis ATCC-8287	1.47	0.72	85.2
Lb. fermentum NRRL-B-1932	1.45	0.74	79.0
L. pseudomesenteroides ATCC-12291	1.44	0.87	85.7
Lb. sp. B001 BP-3158	0.86	0.44	90.0
Lb. sanfranciscensis ATCC-27651	0.78	0.44	97.5
Lb. buchneri TKK-1051	0.76	0.37	84.0
O. oeni E-97762	0.11	0.12	93.0

[a)] Here: volumetric productivity divided by the optical density.

L. mesenteroides (μ_{max} = 0.57 h^{-1}), *L. pseudomesenteroides* (0.46 h^{-1}), *Lb. brevis* (0.45 h^{-1}) and *Lb. fermentum* (0.55 h^{-1}) grew significantly faster than the other four species and thus, the former were also superior in volumetric mannitol productivity. With most of the strains tested a significant fraction of fructose consumed was found to escape, probably into the phosphoketolase pathway and thereby into formation of excess acetic and lactic acid, ethanol and carbon dioxide. However, in agreement with Korakli *et al*. (2000) it was found that *Lb. sanfranciscensis* converted almost 100% of fructose consumed into mannitol. Based on the productivities shown in Table 2, *L. mesenteroides*, *L. pseudomesenteroides*, *Lb. brevis*, and *Lb. fermentum* were chosen for the bioreactor studies.

The complex MRS medium was not applicable for production studies in bioreactor-scale. Hence, a simplified medium (called OSCP) was developed using the Bioscreen analyzer and the SCP medium. In this experiment the variable metal components (Mg, Fe, Ca, Mn, and Na) were omitted on at a time from a basic SCP medium (tryptone, yeast extract, K_2HPO_4, and sugars). The removal of the variable

components resulted in only minor changes in maximum specific growth rates (data not shown). The volumetric mannitol productivities, however, were significantly affected by the removal of Mn^{2+} from the growth media of all four strains. In comparison to the respective values in the complete SP medium, the volumetric productivities of *Lb. brevis*, *Lb. fermentum*, *L. mesenteroides* and *L. pseudo-mesenteroides* in medium without Mn^{2+} decreased 6, 32, 9, and 17%, respectively. To a lesser degree the removal of Mg^{2+} was also found to decrease the volumetric mannitol productivities (2-4%). Furthermore, a significant decrease in volumetric mannitol productivity was only seen with *Lb. brevis*, when using the simple SCP medium instead of the nutrient-rich MRS medium (32%). In an additional experiment, the basic SCP medium was supplemented with variable concentrations of Mg^{2+} and Mn^{2+}. The results indicated that the volumetric mannitol productivity was improved even further when doubled amounts of both Mg^{2+} and Mn^{2+} were used in cultivations of *Lb. fermentum*.

Obviously, the volumetric mannitol productivity is strongly influenced by the growth rate of the cells. However, species with similar growth rates are still likely to differ in mannitol production capabilities. Mannitol dehydrogenase (EC 1.1.1.67) is the key enzyme responsible for converting D-fructose into D-mannitol. Typically, among heterofermentative LAB, a varying fraction of the fructose that has been actively transported into the cell is phosphorylated by fructokinase to form fructose-6-P and thus, channeled into the phosphoketolase pathway. The "leaking" carbon skeleton is then converted stepwise into end products such as acetic and lactic acid, ethanol, and carbon dioxide. The leakage of fructose to the phosphoketolase pathway is a serious consideration in mannitol production, mostly because fructose is rather expensive in comparison to the final selling price of mannitol.

Manganese and magnesium ions are essential cofactors for enzymes in the primary sugar metabolism of LAB. Magnesium functions as a cofactor for e.g. fructokinase, phosphoketolase and acetate kinase, whereas manganese functions as a cofactor for some enzymes in the pathway from GAP to pyruvate, and for lactate dehydrogenase. Clearly these metal ions play a central role in the production of reducing power (NAD(P)H) and ATP and are thus essential for many cellular functions and more importantly, for transport and reduction of fructose. Mannitol dehydrogenase, on the other hand, does not require any cofactors.

8.1.2 Bioreactor cultivations

In the previous chapter the identification of four promising mannitol producers was described. Also a simplified growth medium was developed (OSCP). Next, the effects of growth temperature, pH and nitrogen flushing on mannitol production of these strains were studied in batch bioreactor cultivations (Biostat Q). If otherwise not stated, the temperature was 30°C and pH 5.0. During anaerobic experiments, the growth media were constantly flushed with nitrogen gas, whereas during semi-anaerobic experiments no gases were added to the bioreactors. Two independent experiments were conducted for each set of parameters and the results are given as mean values.

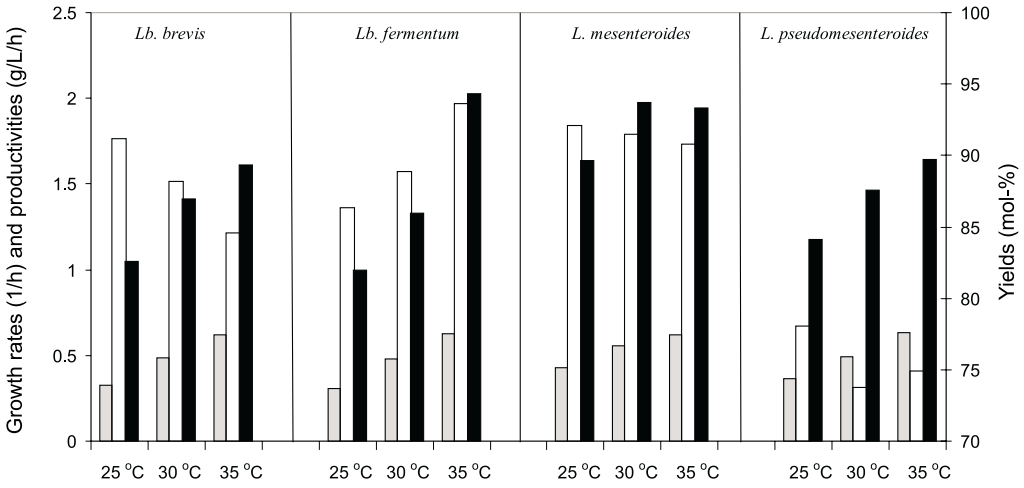


Figure 19. Effect of temperature on maximum specific growth rate, volumetric mannitol productivity and mannitol yield of four different LAB species. The standard deviations were found to be significantly smaller than the changes in the actual results and are therefore not shown in the figure. Columns: *grey*, maximum specific growth rates (1/h); *white*, yields of mannitol produced from fructose consumed (mol-%); *black*, volumetric mannitol productivities (g/L/h).

The maximum specific growth rates of all four strains were clearly improved when the growth temperature was increased from 25 to 35°C (Figure 19). The growth temperature was also observed to have a strong influence on the volumetric mannitol productivity of the cells. The effect of growth temperature on productivity was particularly evident with *Lb. fermentum*, where a change from 25 to 35°C brought about an approximately two-fold increase in the volumetric mannitol productivity (1.00 ± 0.02 to 2.03 ± 0.04 g/L/h). Of the four strains tested, *L. mesenteroides* was least affected by changes in the growth temperature. In fact, increasing the growth temperature from 30 to 35 °C with *L. mesenteroides* resulted in a small decrease of productivity (1.97 ± 0.00 to 1.94 ± 0.00 g/L/h).

The specific mannitol productivities (here: volumetric productivity divided by the optical density) were also clearly higher at 35°C than at 25°C. In cultivations with *Lb. brevis*, *Lb. fermentum*, *L. mesenteroides*, and *L. pseudomesenteroides* the specific mannitol productivities were improved from 0.18 to 0.22, 0.16 to 0.30, 0.37 to 0.50, and 0.34 to 0.45 g/L/h, respectively, when grown at 35°C instead of 25°C.

A low temperature, on the other hand, enhanced mannitol yields with *Lb. brevis*, *L. mesenteroides*, and *L. pseudomesenteroides*. An entirely opposite finding was made with *Lb. fermentum*, where higher temperatures resulted in increased yields. When the temperature was controlled at 25°C, the yield with *Lb. fermentum* was 86.4 ± 0.8 mol-%. Respectively, at 35°C *Lb. fermentum* converted up to 93.6 ± 0.6 mol-% of fructose consumed into mannitol.

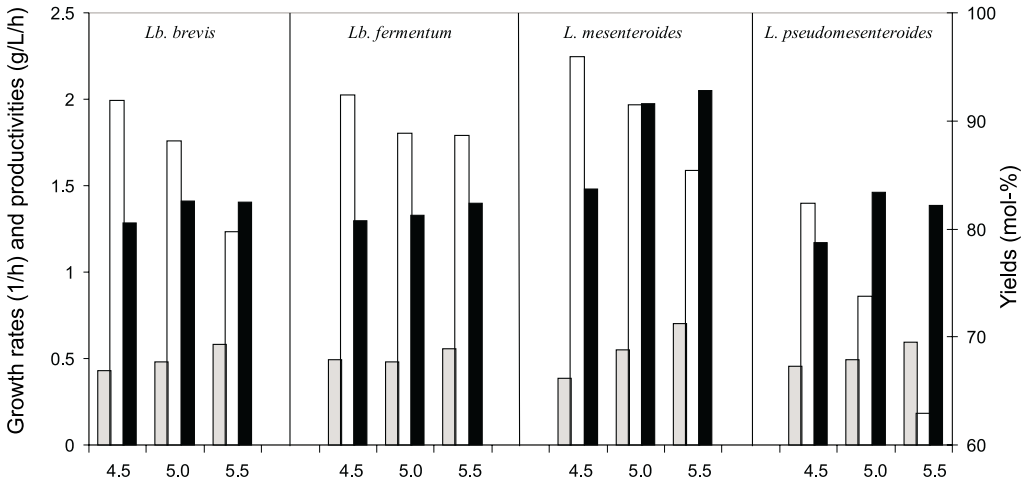


Figure 20. Effect of pH on maximum specific growth rate, volumetric mannitol productivity, and mannitol yield of four different LAB species. The standard deviations were found to be significantly smaller than the changes in the actual results and are therefore not shown in the figure. Columns: *grey*, maximum specific growth rates (1/h); *white*, yields of mannitol produced from fructose consumed (mol-%); *black*, volumetric mannitol productivities (g/L/h).

The highest maximum specific growth rates were achieved when the pH was 5.5 (Figure 20). Lower pH values (5.0 and 4.5) decreased the maximum specific growth rates of all four strains. The maximum specific growth rate of *L. mesenteroides* was especially affected by a low pH. The maximum specific growth rate of *L. mesenteroides* at pH 4.5 was approximately only half of the respective value at pH 5.5. A high pH value was also found to improve the volumetric mannitol productivities, whereas better mannitol yields were observed at low pH values. The effect of pH on the specific mannitol productivities was found to be small (data not shown).

Earlier Soetaert (1990) observed in studies done with *L. pseudomesenteroides* that decreasing the growth temperature and pH results in more efficient conversion of fructose into mannitol, i.e. a better yield. On the other hand, he also observed that a low temperature and a low pH led to decreased volumetric mannitol productivities. Similar observations are reported here. For example, in cultivations with *Lb. fermentum* a change in growth temperature from 35 to 25°C resulted in a 50% decrease in volumetric mannitol productivity. However, the present study also revealed that this behavior does not apply to all heterofermentative LAB. With *L. mesenteroides* a change from 35 to 30°C resulted in a small improvement of volumetric mannitol productivity. Even more divergent from earlier findings is the observation made with *Lb. fermentum*, where high temperatures resulted, in addition to increased productivities, also in better mannitol yields.

In general, the pH of the growth medium had a small influence on the specific mannitol productivities of the four strains studied here. The specific mannitol productivities of *Lb. brevis*, *Lb. fermentum*, and *L. pseudomesenteroides* were

favored by a high pH (5.5), whereas the specific mannitol productivity of *L. mesenteroides* was favored by a low pH (4.5). Typically, a high growth temperature resulted in better specific mannitol productivities. Based on these findings it is evident that possible optimization experiments for mannitol production with growing cells should definitely include higher growth temperatures than 35°C, especially in the case of mannitol production with *Lb. fermentum*.

The growth of all four strains was considerably more rapid under semi-anaerobic conditions (i.e. no gassing of the growth media) than under strict anaerobic conditions (i.e. constant nitrogen gas flushing of the growth media). When comparing maximum specific growth rates, *Lb. brevis* was least affected (up approximately 6%) by the change of anaerobic to semi-anaerobic conditions, whereas the maximum specific growth rates of the other three strains increased in the range of 15 to 19%. On the other hand, under anaerobic conditions slightly higher final cell densities were obtained with all four strains than under semi-anaerobic conditions.

The volumetric mannitol productivity of *Lb. fermentum* increased from 1.33 ± 0.02 to 1.65 ± 0.06 g/L/h, when the cells were grown under semi-anaerobic conditions rather than under anaerobic conditions. A more subtle increase was obtained with the other strains, where the volumetric mannitol productivities of *Lb. brevis*, *L. mesenteroides*, and *L. pseudomesenteroides* improved with 0.9, 2.9, and 3.7%, respectively. The differences in specific mannitol productivities observed under anaerobic and semi-anaerobic conditions were very small (data not shown). On the other hand, the yield of mannitol from fructose was higher under anaerobic conditions with three of the strains (*Lb. brevis*, *Lb. fermentum*, and *L. mesenteroides*). The respective behaviour of *L. pseudomesenteroides* did, however, deviate from this pattern. Under anaerobic conditions the mannitol yield of *L. pseudomesenteroides* was 73.8 ± 0.1 mol-%, whereas under semi-anaerobic conditions it was up to 77.1 ± 1.7 mol-%.

Hence, nitrogen gas flushing of the growth media seems to be ineffective as a way to assure high volumetric and specific mannitol productivities. In fact, a clear enhancement in volumetric productivities was seen with all four strains, when grown under semi-anaerobic conditions rather than under strict anaerobic conditions. The most significant change was again seen with *Lb. fermentum*, where an almost 25% increase in volumetric mannitol productivity was obtained under semi-anaerobic conditions. This observation is naturally at least partly a direct correlation with the improved growth rate of this strain under semi-anaerobic conditions compared to the respective rate under anaerobic conditions. Although the yield of mannitol from fructose was higher in cultivations with constant nitrogen gas flushing (exception: *L. pseudomesenteroides*), it would be more cost-effective to build and run an industrial-scale production facility without the need to invest in expensive bioreactor gassing systems.

Surprisingly, during the semi-anaerobic experiments oxygen depletion in the growth medium differed notably among the studied strains. At the beginning of these experiments the dissolved oxygen tension (DOT) in the growth media was approximately 90 ± 5%. During the experiments the *L. mesenteroides* culture was

observed to run out of oxygen (DOT = 0%) after 2.0 ± 0.1 hours, whereas the *Lb. brevis*, *Lb. fermentum*, and *L. pseudomesenteroides* cultures ran out of oxygen at 5.4 ± 0.5, 6.7 ± 1.0, and 7.0 ± 0.4 hours, respectively. The NADH oxidase activities at t = 7 h in the semi-anaerobic experiments were as follows: *Lb. brevis*, 0.57 U/mg protein, *Lb. fermentum*, 0.20 U/mg, and *L. mesenteroides*, 0.49 U/mg. No activity was detected in *L. pseudomesenteroides*. The specific activity measured for *L. mesenteroides* is similar to earlier reports, 0.6 and 0.44 U/mg (Schmitt and Diviès, 1992; Schmitt *et al*., 1997). The lack of NADH oxidase activity in *L. pseudomesenteroides* is supported by two related observations. First, the yield of mannitol from fructose with *L. pseudomesenteroides* was not improved, when the cells were grown under anaerobic conditions compared to semi-anaerobic conditions. If an NADH oxidase activity was present, it would most likely be competing with mannitol dehydrogenase for the reducing equivalents (NAD(P)H) in the cells and thus, negatively affecting the fructose-to-mannitol yield. Second, the disappearance of dissolved oxygen from the growth medium was notably slower with *L. pseudomesenteroides* (no activity) than with *L. mesenteroides* (detectable activity). In the case of *L. pseudomesenteroides*, the oxygen dissolved in the growth medium was slowly replaced by carbon dioxide produced by the cells. Hence, it is speculated that the rapid decrease in dissolved oxygen, seen with *L. mesenteroides*, is due both to formation of carbon dioxide and the presence of a significant NADH oxidase activity.

Furthermore, glucose and fructose were used up approximately simultaneously in cultivations with the *Leuconostoc* species. In contrast, the two *Lactobacillus* species were found to consume less glucose, while these strains still consumed all the initial fructose. When the initial fructose (20 g/L) was depleted in cultivations with either *Lb. brevis* or *Lb. fermentum* the residual concentrations of glucose (initially 10 g/L) varied from 1.23 ± 0.17 up to 4.56 ± 0.09 g/L. In general, the conversion of fructose into mannitol was notably less efficient with *L. pseudomesenteroides* than with the other three strains. Consequently, clearly higher concentrations of ethanol and lactic acid were measured with *L. pseudomesenteroides*.

8.1.3 Production of mannitol in a batch system

Lb. fermentum was grown in the Biostat MD bioreactor containing OSCP medium with 10 g/L yeast extract and initial fructose and glucose concentrations of 100 g/L and 50 g/L, respectively. The cultivation was performed semi-anaerobically at 40°C under slow agitation (200 rpm) and at controlled pH (5.0). The initial pH was 5.9, but it decreased in about 2.5 h to the control value. The medium was inoculated 10% (v/v) with a cell culture at late exponential growth phase, grown in standard MRS medium.

When *Lb. fermentum* was cultivated at high initial fructose and glucose concentrations, efficient bioconversion of fructose to mannitol was achieved (Figure 21). After 11 hours, 193.6 g fructose was consumed by the cells and resulted in production of 175.3 g mannitol. Hence, the volumetric mannitol productivity, mannitol yield and conversion were 7.6 g/L/h, 89.6 mol-%, and 0.88 mol/mol, respectively (final volume = 2.11 L). A maximal volumetric productivity of 16.0

g/L/h was achieved between t = 8 h and t = 9 h. No residual glucose was detected when the initial fructose was depleted, as seen in experiments with low initial sugar concentrations. Although an increased growth temperature (40°C) was used, the yield of mannitol from fructose was not as high as expected based on earlier comparison studies (see Figure 19). It is speculated that the high initial sugar concentrations used in this experiment most likely altered the metabolism of the cells in an unfavourable direction. In earlier studies with growing LAB cells, volumetric productivities of 6.4 and 3.8 g/L/h were reported for *Lactobacillus* sp. B001 (Itoh *et al.*, 1992) and *Leuconostoc pseudomesenteroides* (Soetaert *et al.*, 1995), respectively. Hence, the results reported here represent an improvement on the production levels described previously.

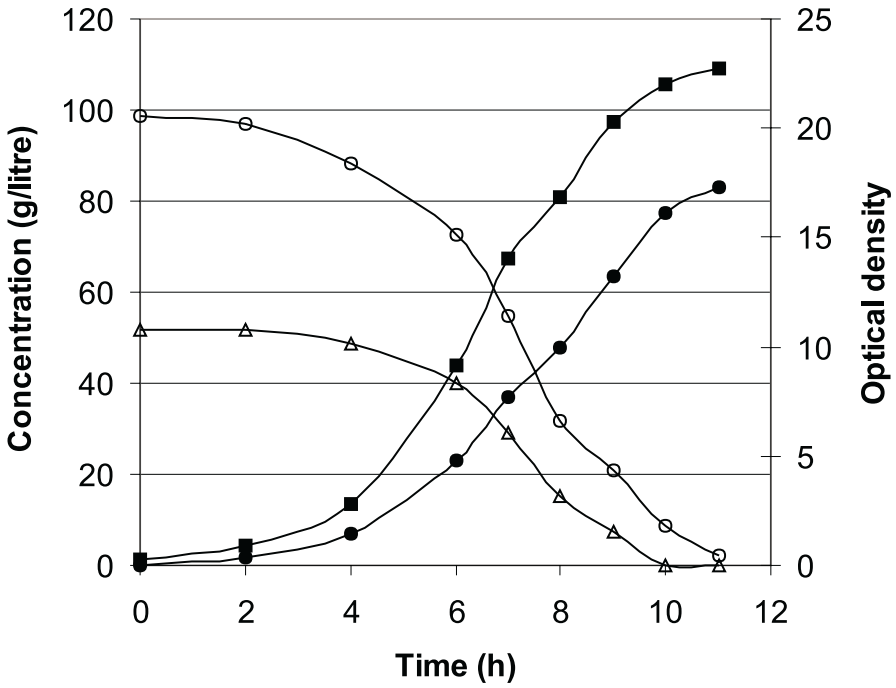


Figure 21. Mannitol production with *Lactobacillus fermentum* NRRL-B-1932 in a 2-L batch bioreactor. Legends: *open circles*, fructose (g/L); *open triangles*, glucose (g/L); *closed circles*, mannitol (g/L); *closed rectangles*, optical density at 600 nm.

In a pilot-scale experiment, *L. mesenteroides* ATCC-9135 was grown in the Marubishi bioreactor (working volume 100 L) containing SCP2 medium with initial fructose and glucose concentrations of 100 g/L and 50 g/L, respectively. The cultivation was performed semi-anaerobically at 32.5°C and pH 5.0 under slow agitation (50 rpm). The growth medium was inoculated 6% (v/v) with cells at late exponential phase, grown in SCP2 medium lacking fructose (Biostat E). In 9 hours, 10 kg fructose was converted into 9.45 kg mannitol. The volumetric mannitol productivity was now even higher (9.7 g/L/h) than with *Lb. fermentum*, whereas the yield and conversion were 94.7 mol-% and 0.934 mol/mol, respectively.

In conclusion, the production of LAB biomass is relatively expensive because these cells are typically incapable of producing most of the amino acids *de novo* (from e.g. glucose) needed as cellular building blocks. As a consequence the LAB growth media are frequently supplemented with various amino acids, which adds to the cost of a batch process with growing cells. The amino acid supplements are usually added to the media in the form of various protein hydrolysates. On the other hand, low-cost raw materials such as milk and whey (or whey permeate) are commonly used as cell production media in the starter culture industry. However, neither of these sources is ideal for LAB cell growth. For instance, milk and whey usually lack many of the important vitamins and amino acids needed for growth (Mäyrä-Mäkinen and Birget, 1998). They also contain many growth inhibitory compounds, such as salts, antimicrobials, and antibiotic residues.

8.2 Production of mannitol with resting cells

8.2.1 Comparison of different heterofermentative LAB

In Chapter 8.1 eight species of obligately heterofermentative LAB were studied in respect to the production of mannitol during cell growth. Seven of these, not including *O. oeni*, were compared in their ability to produce mannitol in a resting or slowly growing state. An adequate biomass was produced using standard MRS growth medium. The cells were collected by centrifugation (3500 g, 10 min), washed in 0.2 M phosphate buffer (pH 6.2), re-collected by centrifugation and finally, suspended in 50 mL BC1 medium (initial pH 5.0), and incubated in test tubes for 8 hours at 30°C. Based on the specific mannitol productivities at t = 8 h (data not shown), *L. mesenteroides*, *L. pseudomesenteroides* and *Lb. fermentum* were selected for more thorough bioreactor studies.

Cells at early stationary growth phase (MRS) were collected by centrifugation (4225 g, 10 min), washed in 0.2 M phosphate buffer (pH 6.2), centrifuged and re-suspended in 50 mL of 0.2 M phosphate buffer (pH 5.8). Next, this cell concentrate was added to Biostat Q bioreactors containing 450 mL BC1 resulting in an average cell dry weight of 0.5-1 g/L. A typical bioconversion plot from these experiments is shown in Figure 22 and the main results are summarized in Table 3. Based on these results, *L. mesenteroides* was identified as the best alternative for use in further process development studies.

Table 3. Volumetric (r_{mtol}) and specific mannitol productivities (q_{mtol}) and yields of mannitol from fructose consumed (Y_{mtol}) in bioreactor comparison experiments. The temperature, pH and agitation were set at 30°C, 5.0 and 200 rpm, respectively.

Strain:	r_{mtol} (g/L/h)	q_{mtol} (g/g/h)	Y_{mtol} (mol-%)
L. mesenteroides ATCC-9135	2.3	2.6	97.8
L. pseudomesenteroides ATCC-12291	1.6	1.5	79.6
Lb. fermentum NRRL-B-1932	0.8	1.0	86.1

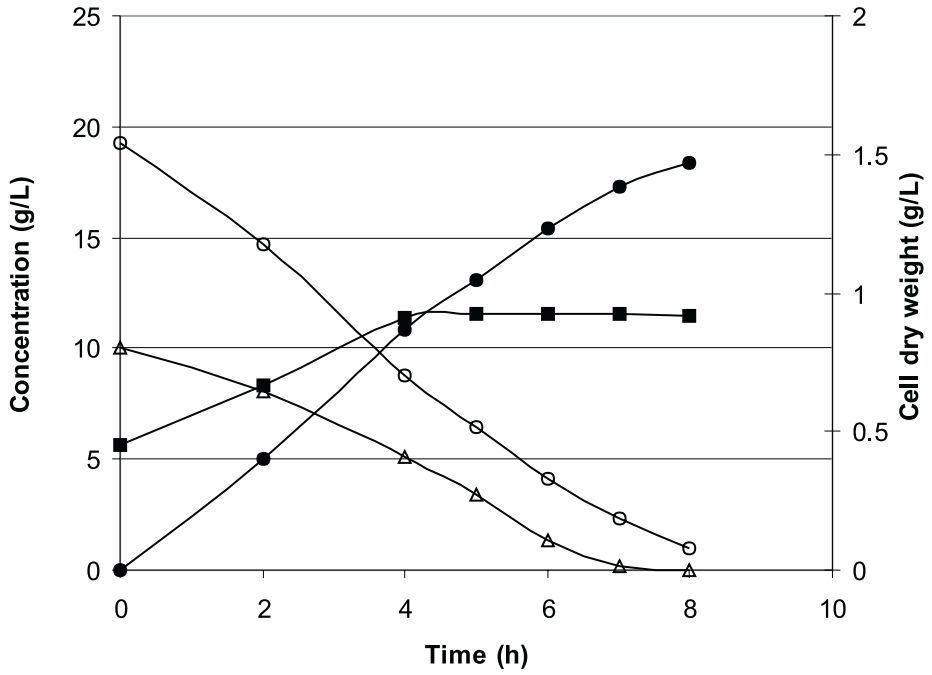


Figure 22. Mannitol production with resting or slowly growing cells of *L. mesenteroides* ATCC-9135 in a 500-mL batch bioreactor culture. The temperature, pH and agitation were set at 30°C, 5.0 and 200 rpm, respectively. Legends: *open circles*, fructose (g/L); *open triangles*, glucose (g/L); *closed circles*, mannitol (g/L); *closed rectangles*, cell dry weight (g/L).

8.2.2 High-cell density batch bioconversion

The batch bioreactor bioconversion set-up described above was repeated but now the biomass was raised to about 10 g cdw/L (previously appr. 1 g cdw/L). Furthermore, the biomass was produced as described in Chapter 7.4 and BC2 medium was used (initial fructose concentration 100 g/L and glucose concentration 50 g/L). In Figure 23 the concentrations of fructose, glucose and mannitol are plotted as a function of time. Both fructose and glucose was depleted at t = 4 h resulting in a final mannitol concentration of about 83 g/L. The volumetric and specific mannitol productivities were 20.7 g/L/h and 2.0 g/g/h, respectively. Neither the mannitol yield nor the conversion is given here due to a failure to collect a representative sample at t = 0 h, i.e. the reaction simply proceeded too fast for accurate measurement.

The volumetric mannitol productivity achieved in this experiment is clearly higher than reported to date in the scientific literature. The only study with productivity levels comparable to results presented here was described in a patent application by Ojamo *et al*. (2000) using resting cells immobilized to a solid carrier. They also report productivity levels over 20 g/L/h. However, although no accurate conversion could be calculated in this experiment, it is clear that the conversion level achieved with *L. mesenteroides* ATCC-9135 is significantly higher than for the strain (*L. pseudomesenteroides* ATCC-12291) used in the patent application. The initial

studies described in chapter 8.2.2 also show that *L. mesenteroides* has an improved specific mannitol productivity compared to *L. pseudomesenteroides*.

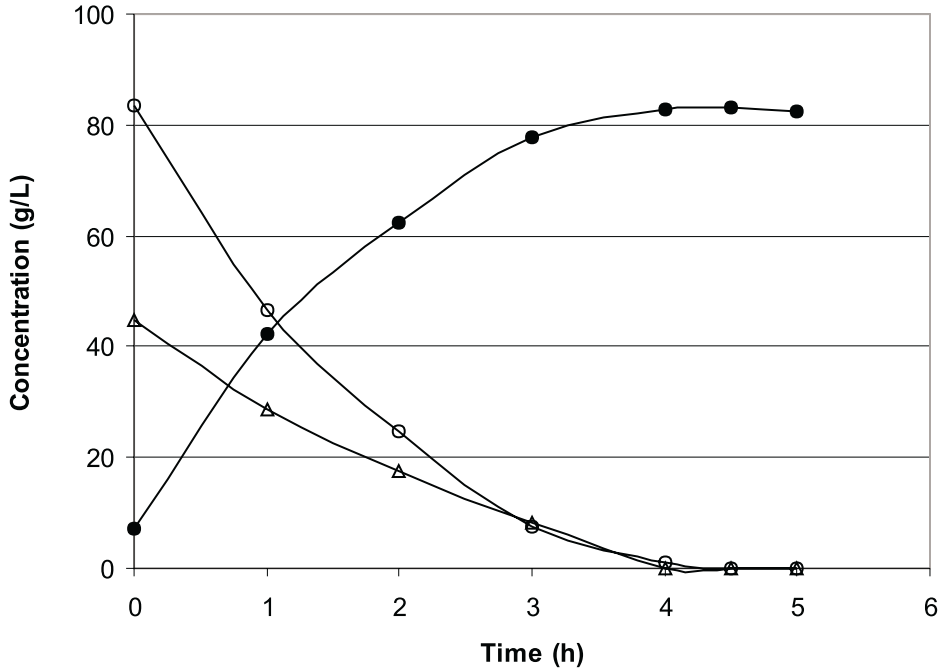


Figure 23. Mannitol production with resting or slowly growing cells of *L. mesenteroides* ATCC-9135 in a 400-mL batch bioreactor culture. The temperature, pH and agitation were set at 30°C, 5.0 and 200 rpm, respectively. The cell dry weight was about 10 g/L. Legends: *open circles*, fructose (g/L); *open triangles*, glucose (g/L); *closed circles*, mannitol (g/L).

8.2.3 Membrane cell-recycle bioreactor with medium circulation

In this experiment, the process described in the patent application by Ojamo *et al.* (2000) was copied with the exception that the column packed with cells immobilized to a solid carrier was now replaced with a membrane cell-recycle bioreactor (MCRB). In the system presented here, the cell suspension was contained in a Biostat Q vessel and in the retentate of the filtration unit (combined initial volume 500 mL). The basic concept of the MCRB is shown in Figure 14 (without circulation). In this experiment, however, the permeate from the filtration unit was directed to a circulation reactor (Biostat MD; initial volume 1 L) containing BC2 medium and placed on a balance. The volume in the circulation reactor was kept constant by pumping medium back to the bioconversion bioreactor (Biostat Q). Both Biostat Q and MD reactors were temperature controlled (30°C). The pH of the bioconversion solution (5.0) was only controlled in the Q bioreactor.

In contrast to the behavior seen in a batch bioconversion (Figure 23), the consumption rate of fructose and glucose in a circulation system was drastically slowed towards the end of the bioconversion (Figure 24). Consequently, fructose and

glucose added to the system were not depleted in a reasonable time. At t = 9 h, however, the volumetric and specific mannitol productivities were 21.6 g/L/h and 2.5 g/g/h, respectively. The residual fructose and glucose concentrations were about 12 and 10 g/L, respectively (initially appr. 100 and 50 g/L). Although the mannitol yield was almost stoichiometric (98 mol-%), the conversion was only 0.73 mol/mol. The biomass in this experiment was slightly lower than in the batch experiments, 8.7 g cdw/L.

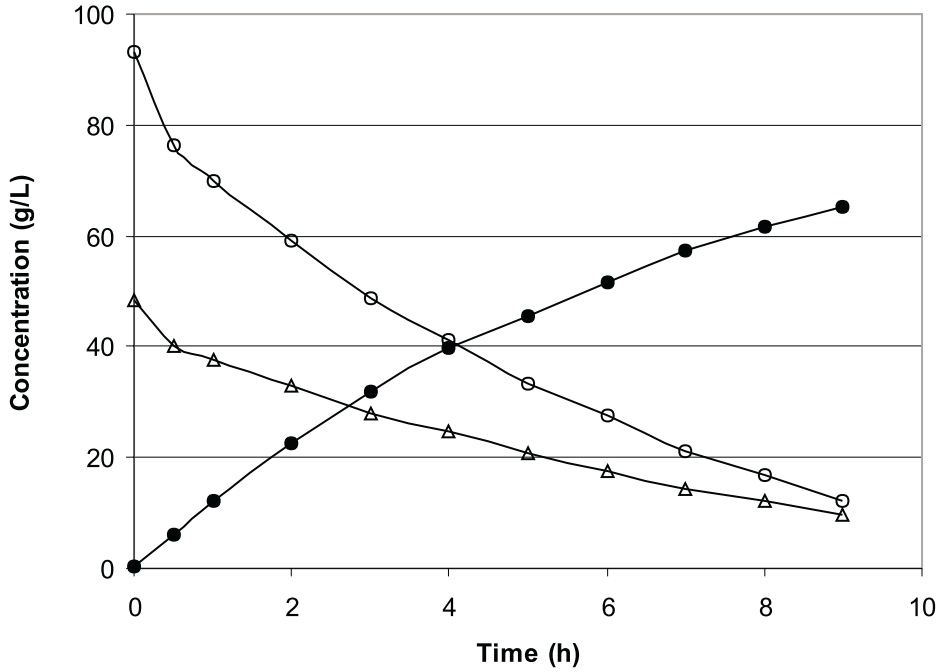


Figure 24. Mannitol production with resting or slowly growing cells of *L. mesenteroides* ATCC-9135 in a MCRB with medium circulation. The temperature, pH and agitation were set at 30°C, 5.0 and 200 rpm, respectively. The cell dry weight was about 8.7 g/L. Legends: *open circles*, fructose (g/L); *open triangles*, glucose (g/L); *closed circles*, mannitol (g/L).

The fructose concentrations at t = 8 h in the Biostat Q (cells suspension) and Biostat MD (cell-free solution) vessels were 13.6 and 16.9 g/L, respectively. Hence, it was concluded that the slowdown in conversion was not due to fructose limitation in the Q vessel. Moreover, the solution in the MD vessel was clear of cells for the duration of the experiment. In an attempt to copy the process described in the patent application by Ojamo *et al*. (2000), it was observed that immobilization of the cells to a carrier did not keep all the cells in the column reactor. Hence, the cells were also present in the circulation reactor (data not shown).

8.2.4 Continuous membrane cell-recycle bioreactor

Next, a continuous MCRB system was applied for production of mannitol (Figure 14). In this experiment, permeate was constantly removed from the system and fresh medium was fed to the bioreactor (Biostat MD) to maintain a constant volume. The dilution rate was adjusted with a harvest pump on the permeate side of the filtration unit. The feeding solution was a BC2 medium with either 25 and 12.5 g/L or 40 and 20 g/L initial fructose and glucose, respectively. The temperature and pH were controlled in the Biostat MD vessel. The same dilution rate was applied until constant biomass and mannitol concentrations were achieved. Table 4 summarizes these experiments. Mannitol yields from fructose consumed were in the range of 92-94 mol-%.

Table 4. Fructose concentrations in feed solution ($C_{fru,\ in}$), dilution rates (D), fructose feeding rates (g/h) per gram cell dry weight (Q_{fru}), cell dry weights (cdw), volumetric mannitol productivities (r_{mtol}), and fructose concentrations in permeates ($C_{fru,\ out}$) in continuous MCRB experiments with *L. mesenteroides* ATCC-9135 at 30°C and pH 5.0. Agitation was set at 200 rpm.

$C_{fru,\ in}$ (g/L)	D (h^{-1})	Q_{fru} (g/g/h)	cdw (g/L)	r_{mtol} (g/l/h)	$C_{fru,\ out}$ g/L
22.7	0.34	1.3	5.8	4.2	1.5
22.7	0.68	2.2	6.9	12.5	3.2
41.3	0.61	4.0	6.3	15.9	13.2

8.3 Comparison of mannitol bioconversion systems

Traditionally, studies on the microbial production of mannitol have been based on bioprocesses using growing cells. As shown in Chapter 8.1.3, productivities up to 9 g/L/h can be achieved in a simple batch cultivation. Similar productivity levels were achieved by e.g. Itoh *et al*. (1992) (6.4 g/L/h with *Lactobacillus* sp. B001) and by Hideyuki *et al*. (1987) (2.4 g/L/h with *Lb. brevis* IFO 3960). Due to some level of substrate inhibition of the mannitol dehydrogenase, fed-batch protocols have commonly been employed. Using such systems, Korakli *et al*. (2000) reported a volumetric mannitol productivity of only 0.5 g/L/h with *Lb. sanfranciscensis* LTH 2590, whereas Soetaert *et al*. (1990) reported a productivity of approximately 6.3 g/L/h with *L. pseudomesenteroides* ATCC-12291. As discussed already in Chapter 8.1.3, the production of LAB biomass is relatively expensive and thus, batch processes based on the use of growing cells are most likely uneconomical.

The application of resting cells has found wide popularity in various studies with LAB. Such systems have been used for studying e.g. the production of lactic acid, acetoin and nisin. In this study, high-cell densities of resting or slowly growing LAB cells were applied in a process in which fructose was reduced to mannitol. This approach has several advantages:

- Simple process steps → lowers the threshold for understanding the process and makes the control of the process easy (applies to the batch bioconversion system, 8.2.2).
- No need for a gassing system → lowers the plant investment costs.
- Slow agitation → lowers the energy requirements, i.e. production costs.
- 100% cell-free product stream → reduces downstream processing steps.
- Product stream contains minimal amounts of residual nutrients → increases product purity.
- Complete consumption of fructose within a reasonable timeframe (batch bioconversion).
- Semi-continuous production (i.e. the use of the same initial biomass in successive batches) enables regular cleaning of the filtration unit without loss of bioconversion time (batch bioconversion).
- Low pH and high salt concentration (acetate and lactate) → lowers significantly the contamination risk → lowers the plant investment costs.

The three first features will play a major role in future process scale-up decisions. Agitation and gassing capacities are usually the most common problems a process developer is faced with, when she/he is taking a bioprocess from bench-top to pilot- or factory-scale. Hence, the agitation (rpm) and/or aeration (vvm) rates used in laboratory-scale are simply not applicable on a large scale (discussed in more detail in Chapter 8.6 "Scale-up of the optimized mannitol production process"). The implementation of a large ultrafiltration or a 0.22 μm microfiltration membrane ensures that the product stream (permeate) is 100% cell-free and can be used as such in downstream processing. As mentioned earlier, immobilization of the cells to a solid carrier is not a foolproof way to contain the cells in the bioreactor. In an attempt to copy the process described by Ojamo *et al*. (2000), we observed that a significant amount of the cells were not contained in the bioreactor, but did in fact circulate through the whole system (data not shown).

Production of mannitol in a MCRB with medium circulation, as well as the immobilization process, has a serious drawback in that the conversion rate of fructose to mannitol decreases and becomes very slow towards the end of the process. This could be by-passed by stopping the bioconversion halfway and circulating the purified residual sugars from downstream processing to the circulation reactor, as suggested by e.g. Ojamo *et al*. (2000). This however, would drastically complicate this otherwise simple process (including the downstream processing steps) and also increase the plant investment costs.

The continuous MCRB system (Chapter 8.2.4) provided promising productivity levels. The cell biomass in the bioconversion reactor was less than 10 g cdw/L and it can be speculated that further optimization studies with higher biomasses could make this alternative as attractive as the batch bioconversion protocol (Chapter 8.2.2). However, fouling and clogging of the continuously running filtration unit is most likely going to result in unexpected problems. Also the fate of dead cell debris build-up in the bioconversion reactor adds to the uncertainty of this alternative.

Cell immobilization has been suggested as a solution to processes with severe end-product inhibition. In an immobilization process it is also possible to use much

higher dilution rates than in a traditional continuous stirred tank reactor thus improving the productivity. Moreover, immobilization of whole cells (or enzymes) has been shown to improve the stability and increase the age of such systems. On the other hand, the bioprocess for the production of mannitol suggested by Ojamo *et al*. (2000) requires pressurization (0.5-1.0 bar) to avoid CO_2-created breakage of the packed bed. It is also claimed here that the difficult purification and regeneration of the resin material is a drawback of the immobilization process.

In conclusion, of the bioconversion systems reviewed here, the batch bioconversion protocol was evaluated to be the most potent alternative for microbial mannitol production and hence, it was chosen for further optimization (Chapter 8.4) and scale-up (Chapter 8.6) experiments.

8.4 Optimization of the batch system with resting cells

8.4.1 Effect of temperature and pH

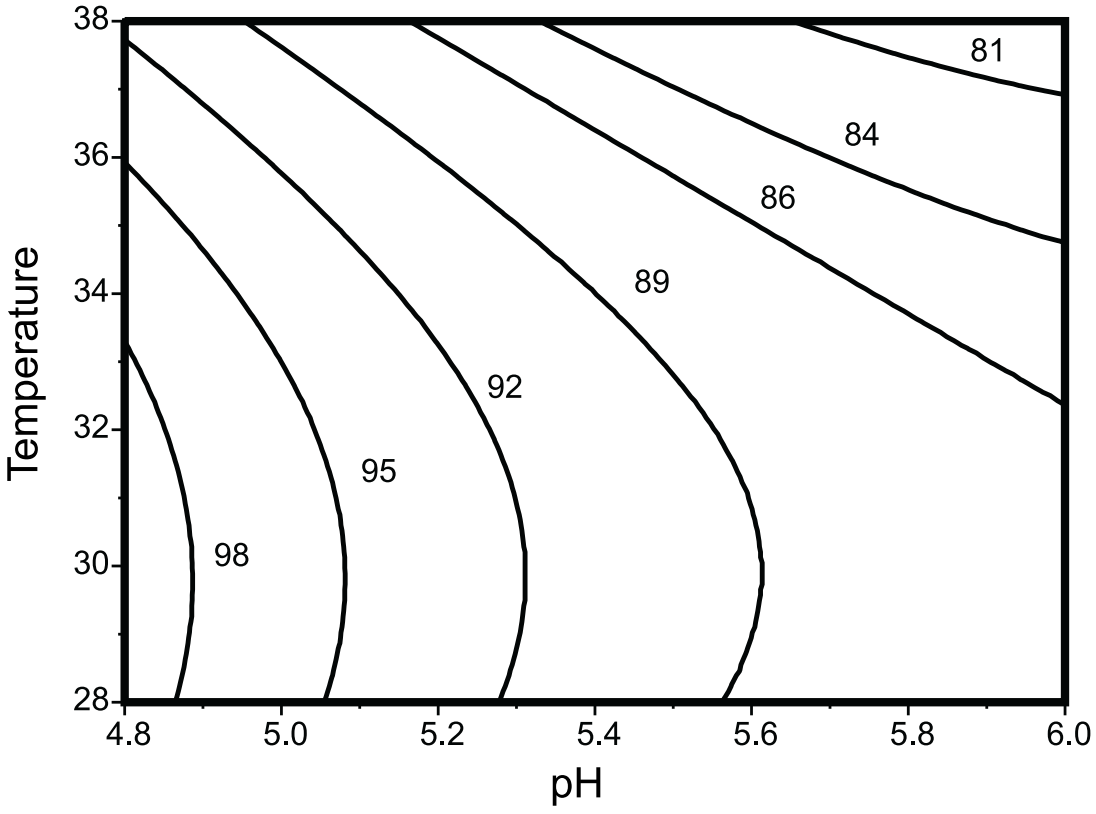


Figure 25. Contour plots showing the dependence of the yield of mannitol from fructose consumed (mol-%) by resting cells of *L. mesenteroides* ATCC-9135 in BC2 medium (100 g/liter fructose and 50 g/liter glucose) on the temperature and pH.

The combined effect of temperature and pH on mannitol production by resting cells of *L. mesenteroides* ATCC-9135 was studied using response surface methodology (multiple linear regression). A quadratic polynomial model with four center point runs was applied and the results were analyzed and plotted using Modde version 4.0 software (Umetri Ab, Sweden). The levels of the variables were as follows: temperature 28, 33, 38°C; and pH 4.8, 5.4, and 6.0. The experiments (12 in total) were conducted in random order in the Biostat Q bioreactor system (volume 500 mL, agitation 400 rpm). The biomass was produced as described in Chapter 7.4 and the concentration in the vessel was about 2.5 g cdw/L. BC2 medium was used (initial fructose 100 g/L and glucose 50 g/L). The responses (mannitol yield and specific mannitol productivity) are shown in Figures 25 and 26. The R^2 value for the model was 0.949.

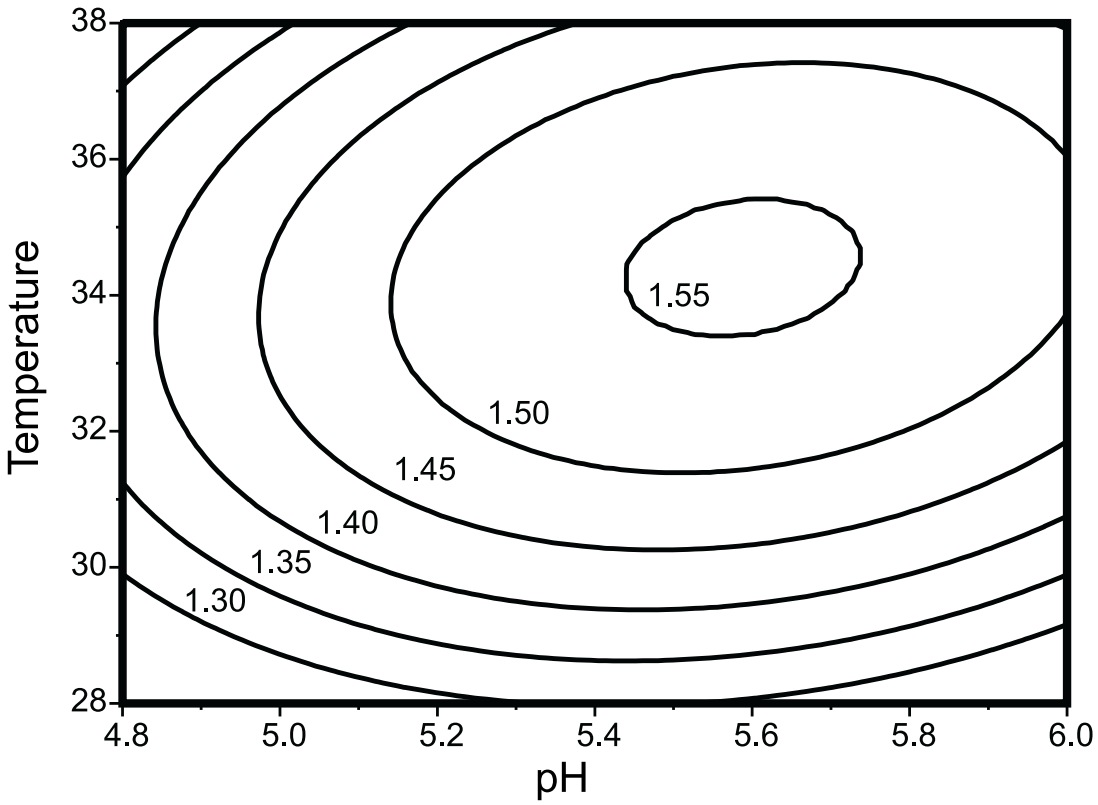


Figure 26. Contour plots showing the dependence of the specific mannitol productivity (g/g cdw/h) by resting cells of *L. mesenteroides* ATCC-9135 in BC2 medium (100 g/liter fructose and 50 g/liter glucose) on the temperature and pH.

The mannitol yield (mol-%) was strongly influenced by pH, but not so by temperature (Figure 25). The best yields were achieved at low pH (4.8) and low temperature (28°C) values. As expected, the specific mannitol productivity (g/g/h) was negatively influenced by these conditions (Figure 26). Hence, an optimum response area for the specific productivity was identified within a pH and temperature range of 5.4-5.8 and 33-35.5°C, respectively. As a compromise of the

responses in Figures 25 and 26, pH and temperature values of 5.2 and 32°C, respectively, were chosen for further studies. Using these values the model predicted the following mannitol yield and specific mannitol productivity values: 92.8 ± 1.9 mol-% and 1.49 ± 0.06 g/g/h.

8.4.2 Effect of biomass

Thus far the majority of the experiments were conducted with biomasses under 5 g cdw/L. In this chapter, the effect of increasing the biomass concentration on the process parameters was studied. The same equipment and process protocol described in chapter 8.4.1 was applied. All biomass concentrations were studied in two independent experiments.

Table 5. Cell dry weights (cdw), volumetric (r_{mtol}) and specific (q_{mtol}) mannitol productivities, mannitol yields (Y_{mtol}) and conversions (x_{mtol}) in batch bioconversions with resting cells of *L. mesenteroides* ATCC-9135 at 32°C and pH 5.2.

cdw (g/L)	r_{mtol} (g/L/h)	q_{mtol} (g/g/h)	Y_{mtol} (mol-%)	x_{mtol} (mol/mol)
5.9 ± 0.2	8.3 ± 0.1	1.4 ± 0.0	87.1 ± 2.8	0.84 ± 0.04
8.6 ± 0.5	13.4 ± 0.6	1.6 ± 0.0	94.1 ± 2.3	0.91 ± 0.02
12.1 ± 0.3	16.4 ± 0.1	1.4 ± 0.0	97.7 ± 0.8	0.95 ± 0.01
16.0 ± 1.4	26.2 ± 0.1	1.6 ± 0.1	96.6 ± 3.2	0.91 ± 0.02

Increasing the biomass concentration in a resting cell bioconversion did not alter the specific mannitol productivities (Table 5). When the biomass concentration was raised to about 16 g cdw/L, a volumetric mannitol productivity of 26.2 g/L/h was achieved. In addition, increasing the biomass concentration also improved the yield and conversion of fructose to mannitol. It should be noted that the yield for the experiment with the highest biomass concentration is probably close to 98 mol-%. As seen in the standard deviation value for that particular experiment, a very short bioconversion time (about 3.5 h) negatively affected the sample representability and thus, the result presented in the table.

The decrease in conversion seen when the biomass concentration was raised from 12 to 16 g/L, was a result of an increased glucose consumption rate (Figure 27). Typically, when the concentration was low (< 10 g cdw/L), fructose was depleted before glucose. The consumption rates, however, changed when the biomasses were increased and at concentrations over 10 g cdw/L, glucose was depleted first. Hence, efficient conversion was dependent on glucose being present in the bioconversion medium (see Chapter 8.4.4). Moreover, it seems tempting to further increase the biomass concentration, but this would result in difficulties with the overall control of the process, mainly the correct timing of the concentration phase.

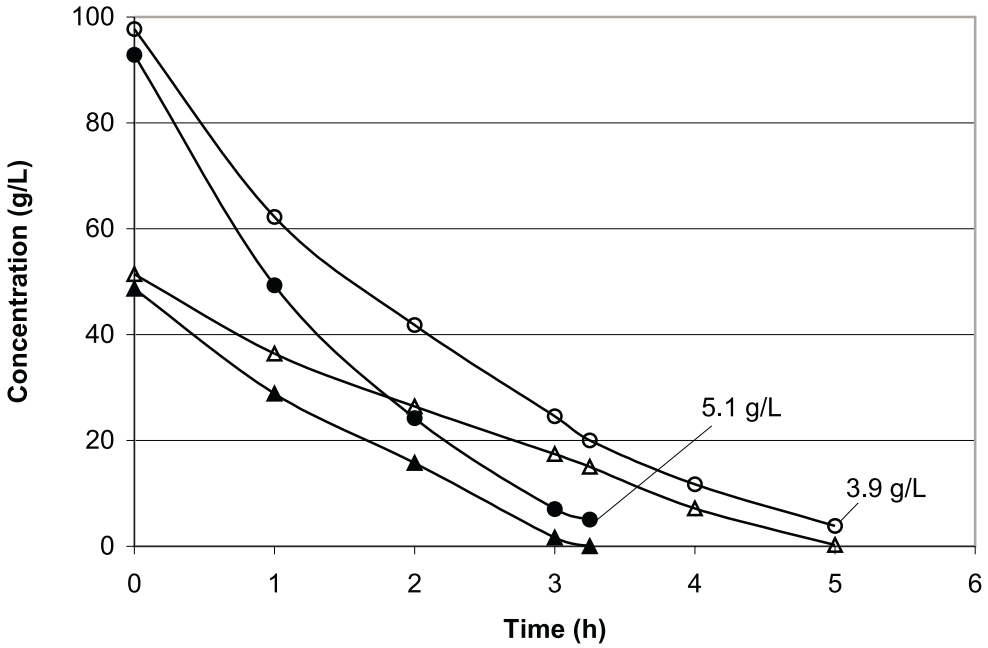


Figure 27. Sugar consumption by resting cells of *L. mesenteroides* ATCC-9135. The temperature, pH and agitation were set at 32°C, 5.2 and 200 rpm, respectively. Legends: *circles*, fructose (g/L); *triangles*, glucose (g/L); *open symbols*, 12 g cdw/L; *closed symbols*, 16 g cdw/L.

8.4.3 Effect of initial fructose concentration

Thus far, only moderate initial fructose concentrations (100 g/L) have been used. A maximum initial concentration is basically determined by the yield of mannitol from fructose, i.e. the function of the relevant enzymes, and by the solubility of mannitol in the medium used. Taking into consideration the downstream processing of mannitol, which usually involves concentration, a high final concentration is desirable. The process protocol for this experiment was as described in Chapter 8.4.1. The biomass concentration was ~15 g cdw/L and the glucose-to-fructose ratio 0.5:1. The data are given as mean values of two independent experiments.

Table 6. Initial fructose concentrations ($C_{fru,\ in}$), volumetric (r_{mtol}) and specific (q_{mtol}) mannitol productivities, mannitol yields (Y_{mtol}) and conversions of fructose to mannitol (x_{mtol}) in batch bioconversions with resting cells of *L. mesenteroides* ATCC-9135 at 32°C and pH 5.2.

$C_{fru,\ in}$ (g/L)	r_{mtol} (g/L/h)	q_{mtol} (g/g/h)	Y_{mtol} (mol-%)	x_{mtol} (mol/mol)
100	26.2 ± 0.1	1.6 ± 0.1	96.6 ± 3.2	0.91 ± 0.02
120	24.1 ± 1.0	1.5 ± 0.0	98.4 ± 0.1	0.95 ± 0.01
140	21.0 ± 0.1	1.4 ± 0.0	98.9 ± 0.1	0.96 ± 0.00

Increasing the initial fructose concentrations of the bioconversion medium had a negative effect on the productivities (Table 6). A clear decrease in volumetric mannitol productivity was seen, when the initial fructose concentration was increased from 100 g/L to 120 and further to 140 g/L. This was most likely due to either substrate or product inhibition or both. Also a small decrease in specific mannitol productivity was seen. In an additional experiment, the K_m and K_i (product) values for mannitol dehydrogenase (MDH) were measured from *L. mesenteroides* cell lysates. When assayed with fructose as the substrate, MDH showed Michaelis-Menten kinetics up to 100 g fructose/L (K_m = 13.1 ± 0.9 g/L). With 200 g fructose/L a small decrease in total activity was seen (8.4 to 7.8 U). This indicates some level of substrate inhibition. On the other hand, high osmotic pressure could also be the cause of productivity decline at higher initial fructose concentrations. The K_m measured for this enzyme was higher than reported previously for pure MDH from *L. mesenteroides*, 6.3 g/L (Sakai 1967). Moreover, the K_i value, measured with 5 g/L initial mannitol, was 66.4 g/L, indicating a strong end-product inhibition at high mannitol concentrations.

Both the mannitol yields and conversions benefited from a high initial fructose concentration. Similar observations were made by Soetaert (1990). He studied *L. pseudomesenteroides* in continuous cultivations and found that at an initial concentration of 3 g fructose/L the yield of mannitol from fructose was only 48 mol-%. The yield at an initial concentration of 120 g/L was 99 mol-%.

8.4.4 Effect of glucose-to-fructose ratio

In the previous chapter, it was found that increasing the initial fructose concentration was disadvantageous to mannitol productivity, but advantageous for conversion. Therefore, a bioconversion protocol with an initial fructose concentration of 120 g/L was used hereafter. The biomass concentration used was still about 15 g cdw/L. Earlier it was observed that when glucose was depleted from the bioconversion medium before fructose, the conversion was negatively affected (Chapter 8.4.2). This was mostly due to a severe slow-down of fructose consumption in medium without glucose. It was therefore suggested that the glucose-to-fructose ratio should be increased from 0.5:1. Subsequently, a bioconversion experiment was performed with a 0.6:1 glucose-to-fructose ratio (the initial fructose concentration was exceptionally 100 g/L). Applying this ratio, the conversion was raised from 0.91 ± 0.02 to 0.93 ± 0.01 mol/mol and more importantly, a total and rapid consumption of fructose was achieved. Furthermore, extending the bioconversion time by a further 0.5 hours resulted in over 0.95 mol/mol conversion. At the same time, the volumetric productivity decreased only slightly and was still above 22 g/L/h. No other important parameter changes were seen and a 0.55:1 ratio was chosen for further studies.

8.4.5 Comparison of different L. mesenteroides strains

In order to study the *L. mesenteroides* species in more detail, three additional strains were acquired. The strain ATCC-8086 was discarded from bioreactor-scale

experiments based on initial test tube results (data not shown). Although this strain was the fastest consumer of glucose, only slow consumption of fructose was detected. Moreover, this strain did not produce mannitol. The mannitol formation capabilities of the two other strains in comparison to strain ATCC-9135 are summarized in Table 7. The optimized experimental protocol presented in the previous chapters was used (initial fructose = 120 g/L, initial glucose = 66 g/L, T = 32°C, pH = 5.2, agitation 200 rpm, biomass concentration about 15 g cdw/L).

Table 7. Volumetric (r_{mtol}) and specific mannitol productivities (q_{mtol}), fructose to mannitol conversions (x_{mtol}) and mannitol yields (Y_{mtol}) in batch bioreactor bioconversions with resting cells of *L. mesenteroides* at 32°C and pH 5.2.

Strain:	r_{mtol} (g/L/h)	q_{mtol} (g/g/h)	x_{mtol} (mol/mol)	Y_{mtol} (mol-%)
ATCC-9135	23.5	1.6	0.93	97.6
ATCC-8293	23.2	1.5	0.96	97.0
ATCC-10830	15.0	0.9	0.94	96.1

In conclusion, the strain ATCC-8086 was found to diverge significantly from the other three *L. mesenteroides* strains. Strain 8086 did not produce mannitol, nor did it produce any novel end products. The ethanol concentration measured for strain 8086 was significantly higher than for the other strains, and, logically, it formed only small amounts of acetate. The HPLC spectra (Pb^{2+} and H^+ columns) of strains 8293, 9135 and 10830 were almost identical, i.e. the only major peaks were mannitol, lactate and acetate. Similar yields were obtained with all three strains, but strain 10830 was somewhat slower in conversion of fructose to mannitol compared to the other two (strains 9135 and 8293). Although strains 9135 and 8293 showed very similar mannitol production capabilities, further studies were done with strain 9135.

8.4.6 Effect of nitrogen gas flushing

The importance of nitrogen gas flushing of the bioconversion medium (i.e. anaerobic conditions) on mannitol production was assessed with two parallel experiments: one with constant nitrogen gas flushing of the medium and one where no gasses were added to the bioreactor. The bioconversion protocol was otherwise as described in the previous chapters. The changes in essential production parameters, brought about by nitrogen gas flushing were, however, of insignificant extent. Therefore, as a technical implication, it was concluded that nitrogen gas flushing of the bioconversion medium was unnecessary. Similar results were previously obtained with growing cells (chapter 8.1.2).

8.4.7 D-mannitol as the carbon source in fermentation

In this experiment, the aim was to learn if *L. mesenteroides* ATCC-9135 could use mannitol as a carbon source at the end of the bioconversions. Biostat Q bioreactors were used (initial mannitol = 10 g/L, initial glucose = 20 g/L, T = 32°C, pH = 5.2,

agitation 200 rpm, biomass = approximately 10 g cdw/L). The low-nutrient medium was alternatively flushed with either nitrogen gas or air and samples for HPLC were taken hourly for 4 hours.

Alarmingly high amounts of mannitol were consumed. Both with air and nitrogen gas flushing of the bioconversion medium the initial glucose was depleted in 1.5 hours. More importantly, simultaneous consumption of mannitol was detected in both cases. When the bioconversion medium was flushed with air and nitrogen gas, 32.5 ± 0.6 and 20.5 ± 0.6% (w/w) of initial mannitol, respectively, was consumed at t = 4 h. Thus, anaerobic conditions slow down this reaction. As concluded in chapter 8.4.6, the conditions in the reactor are practically anaerobic due to the strong formation of carbon dioxide, which in accordance with these results should minimize the mannitol utilization. In the bioprocess developed in this thesis, however, mannitol is only susceptible to fermentation for less than 30 minutes. Moreover, the residual levels of glucose and fructose present in the medium are assumed to prevent significant cellular consumption of mannitol.

8.4.8 Use of sucrose instead of fructose

As a consequence of the protectionism surrounding fructose production and sales in European Union countries, the price of fructose (e.g. pure fructose and fructose-glucose syrups) is significantly higher than the world market price. Therefore, from a mannitol production perspective in the EU countries, it would be desirable to find alternative sources of fructose. Sucrose, a disaccharide of glucose and fructose, is considerably less expensive than fructose and could be used in the bioconversion instead of pure fructose. *L. mesenteroides*, however, is renowned for utilizing the glucosyl moiety of sucrose for production of a viscous polymer, dextran, while fructose is liberated. On the other hand, the production/activity of the enzyme responsible for this reaction, dextransucrase, has only been detected in growing cells (Dols *et al*., 1997). Hence, only minor changes in medium viscosity due to dextran production were expected when resting (or slowly growing) cells are fed with sucrose.

Three parallel bioconversion experiments with differences in initial sugar composition were performed. In runs with either fructose + glucose or fructose + sucrose, the initial fructose and glucose concentrations were about 120 and 65 g/L, respectively. In all runs the initial total sugar concentration was 185 g/L.

The volumetric mannitol productivities, yields and conversions declined drastically, when the sugar raw materials were changed (Table 8). Applying the basic bioconversion protocol (fructose and glucose), 0.61 g mannitol was obtained per gram initial sugar. While the maximum theoretical conversion per initial sugar is about 0.65 g, the conversion of fructose to mannitol in the basic case was 0.93 mol/mol. When sucrose supplemented with pure fructose was used as raw material, the conversion was decreased to 0.43 g mannitol per initial sugar and an increased fraction of fructose is lost to other products. When pure sucrose was used as the single sugar raw material, an even lower sugar to mannitol conversion was achieved (0.31 g/g).

Table 8. Initial sugar concentrations (C_{in}), volumetric mannitol productivities (r_{mtol}), fructose to mannitol conversions (x_{mtol}), mannitol yields (Y_{mtol}), and residual fructose and/or sucrose concentrations (C_{out}) in batch bioconversions with resting cells of *L. mesenteroides* ATCC-9135 at 32°C and pH 5.2.

C_{in} (g/L)	r_{mtol} (g/L/h)	x_{mtol} (mol/mol)	Y_{mtol} (mol-%)	C_{out} (g/L)
Fru 120; Glu 66	23.5	0.93	97.6	Fru 3.9
Suc 130; Fru 55	12.4	0.65	77.9	Fru 2.6; Suc 32.0
Suc 185	7.7	0.61	82.1	Suc 42.0

The cells metabolized sucrose and fructose simultaneously, but the consumption rate was clearly higher for fructose. Both experiments with sucrose were interrupted before depletion of sugars. In the case of the sucrose/fructose experiment, it was stopped due to depletion of fructose. With no pure fructose in the medium, only minimal levels of mannitol were produced. In the case of the sucrose experiment, mannitol productivity was very low. The experiment was stopped at t = 6 h, when the volumetric mannitol productivity (between t = 5 and 6 h) was only about 2 g/L/h. Furthermore, as expected, only small increases in medium viscosity were observed when sucrose was used in the bioconversions.

8.4.9 Cell production

Next, a suitable nutrient source for the cell production phase of the final process protocol was selected. Using various catalogues, the nutrients were divided in two price categories. Bioscreen C analyzer was used to compare the growth of *L. mesenteroides* on the so-called low-price nutrients. The following low-price nutrients were examined: yeast extract powder (LAB M, International Diagnostics Group Plc, England), Balanced peptone no. 1 (LAB M), Bacteriological peptone (LAB M), Soytone (Bacto, Difco Laboratories, USA), fish protein hydrolysate (Primex S490 marine peptone, producer unknown, Norway), and corn steep liquor (Solulys L 48B, Roquette Frères, France). Standard MRS growth medium (Pronadisa, Hispanlab, S.A., Spain) was used as reference medium. Besides the variable complex nutrient sources (11 g/L), the medium contained 10 g/L glucose, 0.2 g/L $MgSO_4$, and 0.02 g/L $MnSO_4$. The temperature was controlled at 30°C and the initial pH was 6.2. The medium was buffered with 67 mM phosphate buffer (KH_2PO_4/Na_2HPO_4). Each complex nutrient source was examined in five parallel cultivations.

Yeast extract was both reasonably priced and provided good cellular growth (Table 9). Yeast extract was therefore chosen for further cell production experiments. A continuous membrane cell-recycle was seen as the most appropriate system for cell production. This decision was strongly influenced by the fact that in such a system the same bioreactor and tangential flow filtration unit could be used as in the actual bioconversion phase. Although some process time will be lost while the bioreactor(s) is occupied by the cell production phase, the savings in plant investment costs ought to be more significant. Using parallel bioreactors in such a system would

also add to the flexibility of the plant (capacity etc.), while one bioreactor entity could be used merely for cell production during low seasons.

Table 9. Optical densities at early stationary growth phase (Final OD_{600}), maximum specific growth rates (μ_{max}), and prices of the four most promising nutrient sources. Results were obtained from Bioscreen C cultures of *L. mesenteroides* ATCC-9135 at 30°C and at an initial pH of 6.2.

Nutrient:	Final OD_{600}	μ_{max} (1/h)	Price [a] (€/kg)
MRS	1.2 ± 0.1	0.38 ± 0.01	109 [b]
Yeast extract powder	1.1 ± 0.1	0.34 ± 0.01	36
Balanced peptone no. 1	0.9 ±0.1	0.39 ± 0.01	87
Soytone	0.8 ± 0.1	0.31 ± 0.02	67
Bacteriological peptone	0.6 ± 0.0	0.24 ± 0.02	-
Marine peptone	0.4 ± 0.0	0.10 ± 0.01	-
Corn steep liquor	no growth	-	-

[a)] Prices from Anu Rauhovirta, Labema Oy, Finland, 23.4.2001.
[b)] Price for MRS Broth (LAB M).

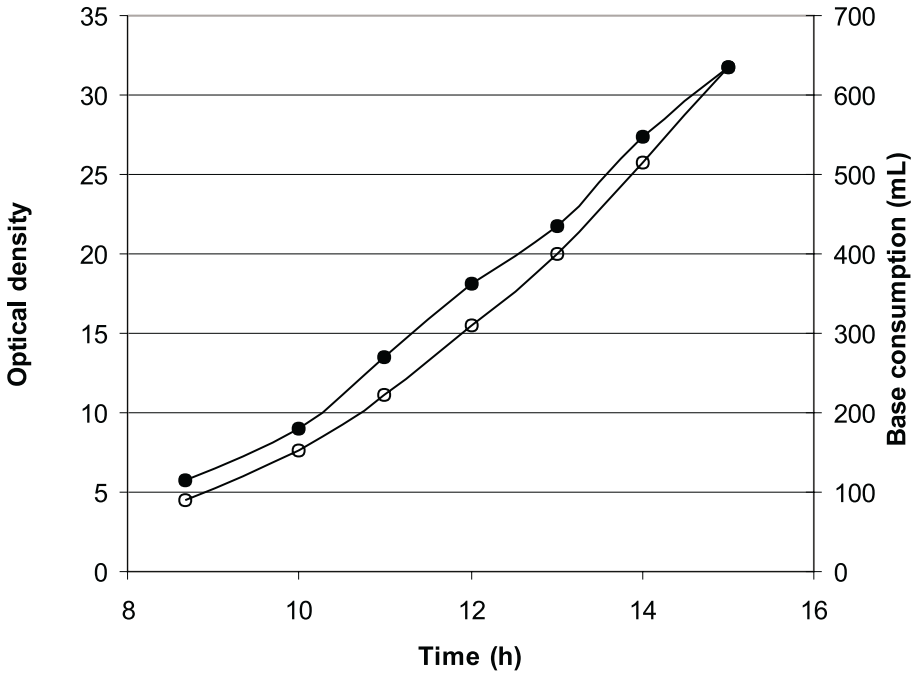


Figure 28. Optical densities (600 nm) and base consumption in a *L. mesenteroides* ATCC-9135 cell production experiment. This segment shows only the continuous production phase. The temperature, pH and agitation were set at 30°C, 6.0 and 200 rpm, respectively. Legends: *closed circles*, optical density at 600 nm; *open circles*, base consumption (mL).

The cell production phase was performed as follows: 2 L of SCP2 medium in the Biostat MD bioreactor was inoculated 5% (v/v) with a 10-h culture grown in standard MRS. The temperature, pH, and agitation were set at 30°C, 6.0, and 200 rpm, respectively. At the end of the batch cultivation, indicated by a slow-down in the base consumption rate, cell-recycling was started and fresh feed solution (FS) was pumped into the bioreactor (see Figure 14).

Figure 28 shows the main parameters from a typical cell production experiment. At the end of the batch phase (about 8.5 hours), the optical density was 5.7. This corresponded to cellular dry weight of about 2.9 g/L. Applying the continuous MCRB a final cell dry weight of 16.0 g/L was achieved in an additional 6.5 hours. The total feed volume was 6.8 L. As seen in Figure 28, the optical densities could reliably be estimated from the base consumption plot. Similarly, the exhaust carbon dioxide levels could also be used (not shown).

8.4.10 Successive batch cycles

In order to study the viability of the microbial cells and to identify any changes brought to essential process parameters in an on-going industrial process, the bioconversion was run semi-continuously. The MCRB equipment with a working volume of 2 L was used. The initial cell biomass was produced as described in chapters 7.4 and 8.4.9. When a cell concentration of about 15 g cdw/liter was achieved, the cells were concentrated to about 25% (v/v) and BC2 medium containing 220 g fructose and 121 g glucose was added to the bioreactor. When the first bioconversion batch was finished, the cells were again concentrated, fresh BC2 medium containing 160 g fructose and 88 g glucose was added to the bioreactor and a second bioconversion batch was started. This latter procedure was then repeated 13 times, using the same initial cell biomass. After the third bioconversion, the amount of glucose was reduced to 80 g. When the base consumption rate in a previous batch dropped under a threshold value, the cells were revived in the beginning of the next batch with an addition of extra yeast extract (10 g) and tryptone (20 g). Simultaneously, some cell concentrate was removed from the system. The permeates (approximately 1.5 liter/batch) were analyzed for mannitol concentration and thereafter used for downstream processing.

The semi-continuous bioconversion protocol resulted in stable production of mannitol during 14 batches (Figure 29). The average volumetric mannitol productivity of the 14 batches was lower than seen in a single batch bioconversion (17.1 ± 1.1 compared to 23.5 g/L/h). The decrease was primary due to dead volumes of the existing process equipment and some technical mistakes in specific batches. Consequently, a corresponding decrease was also seen in total mannitol conversion (from 0.93 to 0.85 mol-%). Proper process design would most likely result in even smaller dead volumes and hence, reduce the losses in volumetric productivity and conversion. We also believe that correct timing of the filtration is important, because mannitol can slowly be metabolized in the absence of fructose by the strain used in these studies (see chapter 8.4.7).

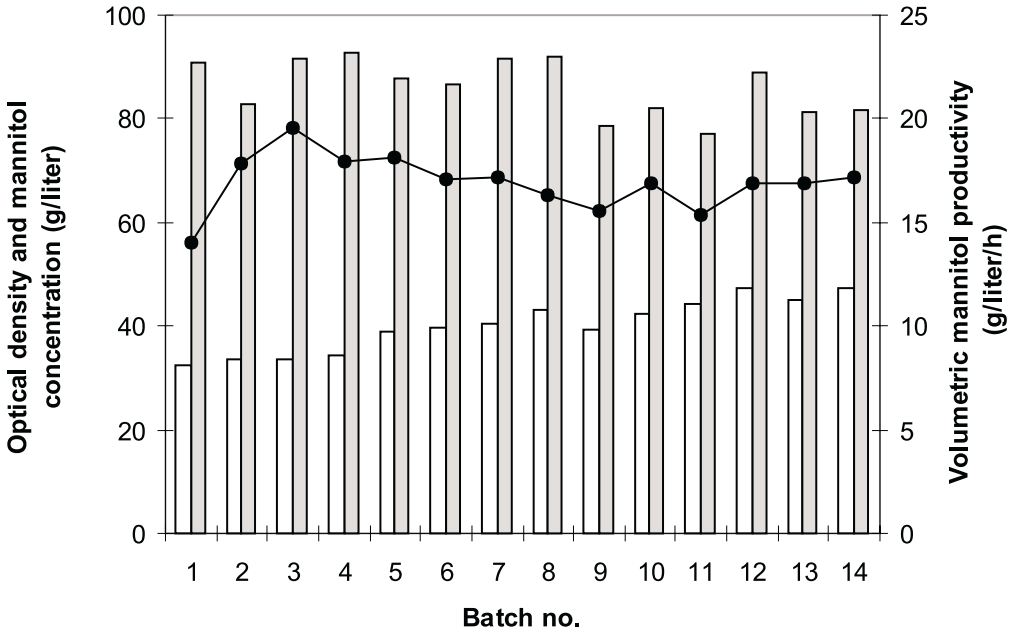


Figure 29. Optical densities (white columns), mannitol concentrations in permeates (g/L; gray columns), and volumetric mannitol productivities based on mannitol obtained in permeates (g/L/h; closed circles) in a semi-continuous bioconversion experiment with resting cells of *L. mesenteroides* ATCC-9135 in BC2 medium (T = 32°C, pH 5.2, and about 15 g cdw/L). Bleeding (100 mL) was performed in batches 9 and 13. Cells were revived in batches 5, 9 and 13.

The optical density measure at t = 3 h in each batch was constantly increasing. This was first assumed to be a consequence of dead cell debris corrupting the spectrophotometer analysis. However, when the viable cell counts were measured, very similar viable cell count-to-optical density ratios were observed throughout the batches. For example, the viable cell counts in batches 1 and 10 were 4.02×10^9 and 5.33×10^9 pmy/mL, respectively. Hence, the viable cell count-to-optical density ratios were 12.0×10^7 (batch 1) and 12.4×10^7 (batch 10).

In another set of experiments with successive bioconversions, both the mannitol yield and a possible accumulation of organic acids and metals were studied. The yield of the 7th batch was 98.7 mol-%, which is comparable to results gained in single batch bioconversions. It can thus be concluded that the performance of the microbial cells observed in single batch bioconversions, is not altered in a semi-continuous mode. In the same experiment the organic acid concentrations at the end of each batch were analyzed. Both lactate and acetate concentrations remained fairly stable for the duration of the experiment. The sodium lactate and sodium acetate concentrations were approximately 22 and 19 g/L, respectively. Stable final base consumption levels confirmed this observation.

Also, samples were taken from each batch permeate and they were analyzed with AAS to determine the concentrations of Mg^{2+} and Mn^{2+}. Moreover, triple amounts

of both metals were added to batches 8 (Mn) and 9 (Mg) to study the effect of these metals on the process. No significant accumulation of the metal ions was observed. The Mn^{2+} and Mg^{2+} concentration in the permeates varied in the range of approximately 2-10 and 40-50 mg/L, respectively. No effect on productivity was seen when the initial amounts were tripled.

8.5 Mannitol recovery

8.5.1 High-nutrient broth

L. pseudomesenteroides ATCC-12291 was grown in a 10-L Biostat E bioreactor according to the protocol described by Soetaert *et al*. (1999). The cells were separated using a tangential flow filtration system (Pellicon 2 Biomax 1000, 1000 kDa, 0.1 m^2 filtration area). The cell-free permeate (see Table 10) was fractionated into 0.5-L samples, which were then used for crystallization studies.

Table 10. Composition of the cell-free permeates obtained from a *L. pseudo-mesenteroides* ATCC-12291 batch cultivation. The cultivation was performed at 30°C, pH 5.0 and the agitation was 100 rpm.

Component:	Concentration (g/L)
Mannitol	112
Glucose	3
Fructose	31
Na-lactate	27
Na-acetate	35
Ethanol	6

Based on earlier studies described in the literature and on some preliminary experiments, the following purification steps were chosen: concentration by evaporation, cooling crystallization, crystal separation by drum centrifugation, vacuum drying and crystal homogenization. The laboratory-scale equipment used to mimic these unit operations and the details of the optimized purification protocol are described in Chapter 7.5.

The wet crystals from the main (primary crystals) and the mother liquor (secondary crystals) crystallizations were combined and re-crystallized. The yield (mass of dry crystalline mannitol per mass of mannitol dissolved in the culture broth) in such a process was 55% (w/w). However, re-use of the mother liquor (or washing solution) from the re-crystallizations in the next crystallization batch, increased the total yield to 71% (w/w). The purity of the combined re-crystallized dry crystals was over 99% (w/w) according to HPLC analysis.

Before the optimized purification protocol described in Materials and Methods, was obtained, some relevant downstream processing steps were studied in more detail.

Use of seed crystals resulted in a significant decrease in crystal purity. This was probably due to increased crystallization rate, which resulted in entrapment of impurities. Lowering the pH of the cell-free permeate before evaporation from about 5.1 to 2.3 resulted in a 40% increase in the acetate concentration in the condensate, but did not improve the final yield or purity of the crystals. In an additional test, $CaCO_3$ was added to the cell-free permeate before evaporation in order to precipitate the lactate present. This approach did not improve the crystal purity.

However, a clear correlation was observed between the cooling rate in crystallization and the purity of the crystals: the slower the cooling rate, the purer the final dry crystals. For instance, with cooling rates of 10 and 2°C/h, purities of 81.8 and 98.6%, respectively, were obtained. The improved purity was speculated to be partly a consequence of enlarged crystal size, which facilitated the separation of the mother liquor from the crystals in the subsequent filtration step. The change of cooling rate did not, however, affect the yield. Increasing the final mannitol concentration in the concentrate also affected the purification parameters. When the mannitol concentration in the concentrate was increased from 200 to 300 g/L, improvements in both yield (48 to 64 %) and purity (63.5 to 97.9 %) were achieved.

8.5.2 Low-nutrient broth

In the previous chapter the starting material for downstream processing originated from a bioconversion medium supporting cellular growth. This was, however, not the case for the process developed in this thesis. The bioconversion medium used in the bioprocess system described in this thesis contained low nutrient levels that supported only minimal growth. The difference between these two media was clearly perceived from the color of the cell-free permeates. The “high-nutrient” permeate was dark brown, while the “low-nutrient” permeate was light yellow.

The permeates from three successive batches (low-nutrient, see Chapter 8.4.10) were separately crystallized. The average mannitol yield of the main crystallizations was as high as 72% (w/w). However, the crystals were not pure enough and were thus re-crystallized. After re-crystallization the purity of the primary crystals was over 99% according to HPLC analysis. A minor impurity absorbance peak was detected at about 260 nm compared to a commercial sample (D-mannitol, ≥ 99.5%, Fluka Chemie AG, Germany). The mother liquors from the main crystallizations were combined and crystallized twice. The purity of these secondary crystals after two crystallizations was slightly lower (> 97.5%) compared to the primary crystals. Also, the impurity peak was now somewhat higher. Combining the primary and secondary crystals, the total crystallization yield was 56.4% (w/w). The mother liquor (or washing solution) from the re-crystallization of the primary crystals can be used to wash the next batch of crystals thus retaining the mannitol dissolved in this solution in the system. When this mannitol is added to the calculations, the total downstream processing yield was 85.5%.

In conclusion, a total downstream processing yield of about 86% was achieved, when a low-nutrient broth was used as the starting material, compared with 71%, when a high-nutrient broth was used. It is expected that downstream processing

specialists will be able to further improve the performance of the purification steps. Using regular downstream equipment the purities and perhaps the yields could further be improved (see Chapter 8.6). Moreover, the mannitol purification steps used here are very simple and are based on well-known technologies.

The starting material for downstream processing of a bioprocess generally differs from the starting material of a chemical process. Although chemical process streams typically contain metal catalysts, the other components are usually few and very pure. Bioprocess streams, on the other hand, often comprise variable materials including e.g. proteins and complex organic compounds. This situation can complicate the crystallization. In the bioprocess developed in this thesis, the level of protein and organic compounds is low and the major drawback probably is the slightly variable permeate composition. For instance, when the cells are periodically revived with the addition of nutrients like tryptone and yeast extract, the permeate from this batch differs from a permeate with very low level of residual nutrients.

Furthermore, the fate of the side-stream remains unsolved. A typical chemical mannitol production process (hydrogenation) generates a total of 1.58 kg of by-products (85% sorbitol and 15% mannitol) for each kilogram of pure crystalline mannitol produced (Devos, 1995). The bioprocess developed in this thesis, on the other hand, generates only 0.66 kg of by-products for each kilogram of pure crystalline mannitol produced. The main by-products are sodium acetate and sodium lactate. Mannitol, some residual sugars and ethanol are usually also found in the side-stream. Hence, to improve the economy of this process, applications for this side-stream should be found. If e.g. lactic acid could be efficiently isolated from the side-stream as either pure D- or L-form, the economics of the process would be improved. Using tools of genetic engineering new pathways for glucose catabolism could be introduced to mannitol producing cells and hence, new valuable by-products could be produced (see Chapter 8.7).

8.6 Scale-up of the optimized mannitol production process

The process (including purification) developed and optimized on a 2-L laboratory-scale was next scaled-up to a 100-L pilot-scale. It is well established that a process, which works well on laboratory-scale may work poorly or not at all when, first attempted on a larger scale (Crueger and Crueger, 1990). First, it is generally not possible to take process conditions that function in the laboratory and blindly apply them to industrial-scale equipment. Second, scale-up is seldom done with geometrically identical bioreactors on laboratory, pilot plant and production-scale.

Typically, in laboratory studies process conditions are used that are impossible to implement on factory-scale. Problems can arise with e.g.:

- agitation; engines used for mixing on laboratory-scale are usually over-scaled in comparison to the vessel volume, which means that the agitation rates achieved in laboratory-scale are impossible to use on factory-scale. This results in e.g. lowered oxygen transfer rates resulting in lowered yields and

productivities. In the worst case the metabolism of the microbe is completely changed.

- aeration (or nitrogen flushing); on laboratory-scale growth media can be aerated at rates up to 2-3 vvm. On factory-scale (e.g. 100 m^3), 2 vvm would mean that 200 m^3/min of air must be pumped into the bioreactor. This is obviously impossible due to e.g. pump and tube sizes. The over pressure arising inside the bioreactor due to aeration is also easier to control on a laboratory-scale. Similar disadvantages to those associated with decreased agitation rate arise when the aeration is lower than expected. Also, aeration can be an essential part of mixing in some reactor types, thus a lowered aeration would also alter the mixing properties in the vessel.
- transparent glass-vessels; bench-top laboratory-scale bioreactor are typically made of glass and during an experiment it is thus easy to follow the events inside the reactor, whilst factory-scale bioreactors are usually stainless-steel vessels with only a small glass panel to allow inspection of the interior of the reactor.
- geometrically identical vessels; as mentioned above the vessels used on different scales (laboratory, pilot and production) are usually not geometrically identical, which results in altered mixing properties.
- sterilization and asepsis; on laboratory-scale medium components are typically sterilized separately thereby avoiding unwanted chemical reactions. For instance, stock solutions of sugars and some easily precipitating minerals are often sterilized separately, either by heat sterilization or filtration, where after they are added to a sterile bioreactor containing the other medium components. This is difficult to implement on factory-scale, which can lead to changes in the initial medium composition.
- fluid transport; in factory-scale processes, most if not all fluids are transported by pumps, which is not always the case on laboratory-scale.
- automation; a laboratory-scale experiment is usually easy to control and all control equipment is easily reachable. On the other hand, on factory-scale, this is not possible and comprehensive automation systems are needed.
- technical difficulties with large-scale equipment; for instance, a laboratory-scale pump or filtration unit is usually easy to use, but the respective factory-scale equipment can be much more difficult to manage.

The process studied in this thesis was, however, in principle easy to scale-up since no vigorous agitation was needed due to semi-anaerobic process conditions. The upstream, process and downstream protocols used in the pilot plant runs are described in Chapter 7.6. Schematic diagrams of the pilot-plant equipment are shown in Figures 17 and 18.

Some modifications to the laboratory-scale process were made: The last pre-culture in the pilot-scale experiment was cultivated in a bioreactor, i.e. under controlled agitation and pH conditions. This led to a shortened cultivation time compared to the laboratory-scale test tube cultivations. The main bioreactor (100 L) was inoculated 6% (v/v) compared to 5% at laboratory-scale and thus, the cultivation time of the first batch phase in cell production was also shortened. On laboratory-scale, sugar and mineral stock solutions were separately sterilized. For technical reasons this was not possible in the pilot plant. All solutions fed into the 100-L bioreactor were

instead pumped through a sterile filter (0.22 μm) and heat sterilization was thus avoided. Only the first medium (yeast extract, glucose, manganese and magnesium) used as the starting point for the batch cell production phase was heat sterilized in the bioreactor. Some glucose was believed to be lost in the sterilization due to e.g. Maillard reactions.

The initial pH of the growth medium in the cell production phase (100 L) was not adjusted (initially about 6.4). It dropped rapidly to the control-value (6.0), where after only base was added to the bioreactor. The total volume of base needed was so high that the bioreactor base reservoir (2 L) was replaced by an external reservoir (30 L). Furthermore, on laboratory-scale, the bioreactor working volume-to-filtration area ratio was 2 L:0.1 m^2, but in the pilot plant the respective ratio was 100 L:2 m^2, i.e. a proportionally smaller filter was used.

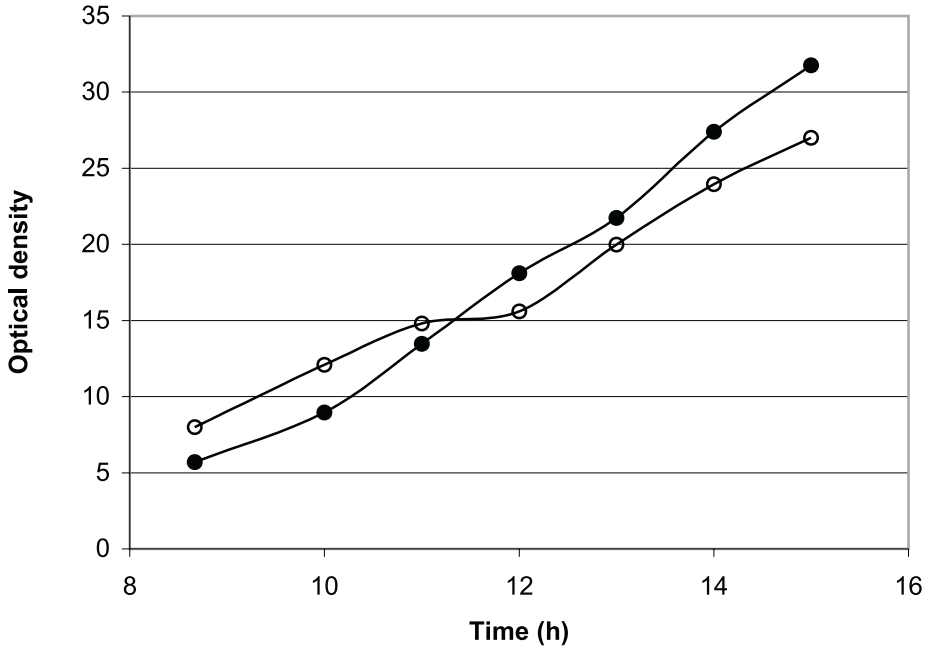


Figure 30. Optical densities (600 nm) in cell production experiments with *L. mesenteroides* ATCC-9135. This segment shows only the continuous production phase. The temperature and pH were set at 30°C and 6.0, respectively. Legends: *closed circles*, laboratory-scale experiment; *open circles*, pilot-plant experiment.

The last pre-culture cultivation in the Biostat E bioreactor was stopped at t = 8 h, when the base consumption clearly decelerated. This was 2 hours earlier than in the laboratory-scale experiment. Moreover, the sterilization of the 100-L bioreactor was for practical reasons performed one day before inoculation. To minimize the risk of contamination in pilot-scale, a pro-longed sterilization time (20 min) of the initial batch and over night vessel overpressure (0.2-0.4 bar) were applied. Media containing both yeast extract and glucose are normally autoclaved only around 12-

14 minutes (laboratory-scale) to avoid loss of glucose and other nutrients. Consequently, the cells grew faster in the initial batch phase of the pilot-scale experiment than in the initial batch phase of the laboratory-scale experiments. The pilot-scale batch phase ended after 7 hours (OD = 6.8), whilst the laboratory-scale batch phase lasted one hour longer (OD = 5.0). However, the continuous cell production phase was easier to control on laboratory-scale and a better cell growth in the continuous phase was achieved on the smaller scale (Figure 30). Hence, a higher final cell density was achieved in the laboratory-scale experiment.

The total cell production time (batch and continuous phases) on both scales was about 15 h. The first subsequent cell concentration on pilot-scale, however, did not go as planned. After about 40 minutes of filtration and with less than 30 L cell concentrate left in the bioreactor, cells were detected in the permeate. The filtration was followed through until about 25 L cell concentrate was left in the bioreactor (another 5 min). It was estimated that 1-2% of the cells were lost due to the leaking membrane. Moreover, the filtration time was not significantly increased compared to the laboratory-scale experiment, although a proportionally smaller membrane was used.

Table 11. Scale-up of the optimized mannitol production process applying *L. mesenteroides* ATCC-9135 and a membrane cell-recycle reactor. The values shown in the table represent results from laboratory-scale (2 L) and the pilot plant (100 L) experiments. The values are from the first bioconversion batch. The downstream yield is from the primary crystallization.

	Laboratory-scale	Pilot plant
Biomass concentration at t = 3 h (g cdw/L)	14.3	12.5
Bioconversion time (h:min)	4:45	5:00
Filtration time after batch (min)	25	-
Final mannitol concentration (g/L)	90.9	87.1
Conversion (fructose to mannitol) (mol/mol)	0.88	0.87
Volumetric mannitol productivity (g/L/h)	20.6	18.8
Specific mannitol productivity (g/g cdw/h)	1.40	1.43
Downstream yield (mol/mol or g/g)	0.72	0.75
Total conversion (mol mannitol/mol fructose)	0.63	0.65

Feeding solution (FS) was added, as planned, to the bioreactor and the first bioconversion batch was run. The specific productivity was not influenced by the scale-up and the other parameters also indicated that the performance at 100-L scale was similar to the performance at 2-L scale (Table 11). A second batch was not started because a significant amount of the cells were lost in the second concentration step. The 2 m^2 Maxi membrane module was changed to two 0.5 m^2 Cassette modules (otherwise the same membrane) and the permeate was re-filtered resulting in 55-L of cell-free production solution containing 4.53 kg mannitol. This solution was concentrated to about 1/3 yielding 17.5 L of concentrate (258.8 g/L mannitol) and 37.5 L of condensate (0.3 g/L mannitol). The supersaturated solution was cooled down to 5°C and the crystals separated by drum centrifugation. The crystals were dried yielding 3.42 kg of crystalline mannitol (purity 98.6%). Hence,

the downstream yield after one crystallization was 75.5% compared to 72% in laboratory-scale experiments. The mother liquor contained 1.11 kg mannitol, but no attempts were made to recover it.

In conclusion, the following scale-up related issues were noticed in this experiment: It was necessary to pre-heat all nutrient solutions fed into the bioreactor, because the heating capacity of the Marubishi bioreactor did not suffice (components were dissolved in tap water, 10-12°C). Although clamp connections were used, the high pressures used on pilot-scale resulted in leakage problems. Similar problems did not occur on laboratory-scale, although similar pressures were often used. Moreover, on pilot-scale a more concentrated base was used, which affected the volume changes and thus the concentrations. On the other hand, the total sample volume removed from bioreactor was insignificant on pilot-scale, whereas the amounts removed in laboratory-scale were proportionally much larger. Hence, in laboratory-scale significant amount of raw material, cells and mannitol were lost as samples. Furthermore, sterile filtration of solutions fed into the bioreactor had an impact on the color of the culture broth, which then consequently also affected the purification of mannitol. A significant improvement in final crystal purity was obtained by the use of a drum centrifuge in crystal separation (on laboratory-scale suction filtration was used). The centrifugation time also affected the purity of the primary crystals. When only 6 minutes of centrifugation was applied, the purity of the resulting dried crystals was 91.5%, while the height of impurity absorbance peak at 260 nm was 0.47. On the other hand, a 30-minute centrifugation resulted in purity and height of 98.6% and 0.34. On laboratory-scale, after two crystallization steps, the corresponding impurity peak height was 0.1-0.15. No extra peaks were detected in commercial HPLC grade D-mannitol.

8.7 Effect of gene inactivations

8.7.1 Inactivation of fructokinase in L. pseudomesenteroides

As shown with the LAB strains studied in this thesis, a 100% yield of mannitol from fructose was difficult to achieve (discussed in Chapter 8.1.1). Experiments with *Lb. sanfranciscensis*, however, indicated that this species might lack alternative pathways for fructose catabolism or these pathways might be strongly repressed by the presence of glucose. Hence, the yield of mannitol from fructose with *Lb. sanfranciscensis* was almost 100%. However, due to slow growth and fructose consumption, *Lb. sanfranciscensis* was not ideal for the development of a mannitol production process.

With some fast-growing species (*L. mesenteroides* and *Lb. fermentum*), yields over 90% were achieved by optimizing the temperature and pH. On the other hand, with another fast-growing species (*L. pseudomesenteroides*) typically over 20% of fructose consumed was lost to end products other than mannitol. It was also observed that the leakage of fructose only appeared towards the end of the bioconversions, i.e. when the fructose concentration was low and the mannitol concentration was high. For instance, with *L. mesenteroides* ATCC-9135, in the

presence of glucose, the yield of mannitol from fructose was 100% as long as the fructose concentration was kept over ~15 g/L. Substrate and end-product inhibition effects of mannitol dehydrogenase were discussed in Chapter 8.4.3.

It is commonly assumed that the leaking fructose in these species is phosphorylated by a fructokinase into fructose-6-P, and thus metabolized to other end products (see Figure 9). Using random mutagenesis, a mutant of *L. pseudomesenteroides* unable to grow on fructose (as sole carbon source) was produced. The sugar intake of this mutant was, however, not disrupted and fructose was still consumed by the mutant cells, if glucose was present in the growth medium. Further analysis revealed that fructose was still efficiently converted into mannitol. The primary sugar metabolism of the random mutant and its parent strain was studied in batch cultivations.

The mutant grew and consumed fructose faster than the parent strain (Table 12). The specific fructokinase activity of the mutant was about 10% of that of the parent strain, which led to a reduced leakage of fructose. Hence, the yield of mannitol from fructose was improved from about 74 mol-% (parent) to 86 mol-% (mutant). A faster fructose consumption and an improved yield subsequently also resulted in a better volumetric mannitol productivity for the mutant.

Table 12. *L. pseudomesenteroides* ATCC-12291 compared to a random mutant (DSM-14613) with lowered fructokinase activity in growth medium containing both fructose and glucose at 30°C and pH 5.0.

	Parent	Mutant
Time to fructose depletion (h)	8.5	8
Final cell dry weight (g/L)	1.3 ± 0.0	1.2 ± 0.0
Maximum specific growth rate (1/h)	0.59 ± 0.04	0.69 ± 0.01
Volumetric fructose consumption rate (g/L/h) [a]	2.1 ± 0.3	2.8 ± 0.3
Specific fructose consumption rate (g/g cdw/h) [a]	6.0 ± 0.2	6.5 ± 0.1
Yield of mannitol from fructose (mol-%)	73.7 ± 0.6	85.7 ± 0.4
Volumetric mannitol productivity (g/L/h)	1.6 ± 0.0	2.0 ± 0.0
Specific fructokinase activity (U/mg)	0.49 ± 0.11	0.06 ± 0.00
Mannitol (C-mol/C-mol sugar)	0.465 ± 0.002	0.537 ± 0.001
Lactic acid (C-mol/C-mol sugar)	0.256 ± 0.004	0.218 ± 0.003
Acetic acid (C-mol/C-mol sugar)	0.071 ± 0.001	0.095 ± 0.002
Ethanol (C-mol/C-mol sugar)	0.103 ± 0.001	0.054 ± 0.002
Carbon dioxide (C-mol/C-mol sugar)	0.087 ± 0.000	0.074 ± 0.000
Biomass (C-mol/C-mol sugar)	0.015 ± 0.004	0.023 ± 0.001
Carbon balance	**0.998 ± 0.003**	**1.000 ± 0.003**
NAD/NADH balance	**0.98 ± 0.00**	**1.03 ± 0.00**

[a] Between t = 2 and t = 6 hours. The specific consumption rates were calculated using a logarithmic mean of the biomass (see Chapter 7.9).

Both the parent strain and the mutant consumed approximately the same amount of glucose in relation to fructose (Figure 31). But due to an almost two-fold leakage of fructose to the glucose catabolism pathway, the parent cells produced more carbon dioxide, lactic acid and ethanol than the mutant cells. To balance the increased NADH oxidation due to increased mannitol production, the mutant cells produced less ethanol and more acetic acid. However, the yield of ATP per mole fructose consumed was still approximately the same with both strains: 111.7 mol/mol for the parent strain and 110.2 mol/mol for the mutant (ATP produced and consumed was calculated according to Figure 9). Hence, the yields of ATP correlated well with the similar final cell dry weights of both strains (Table 12).

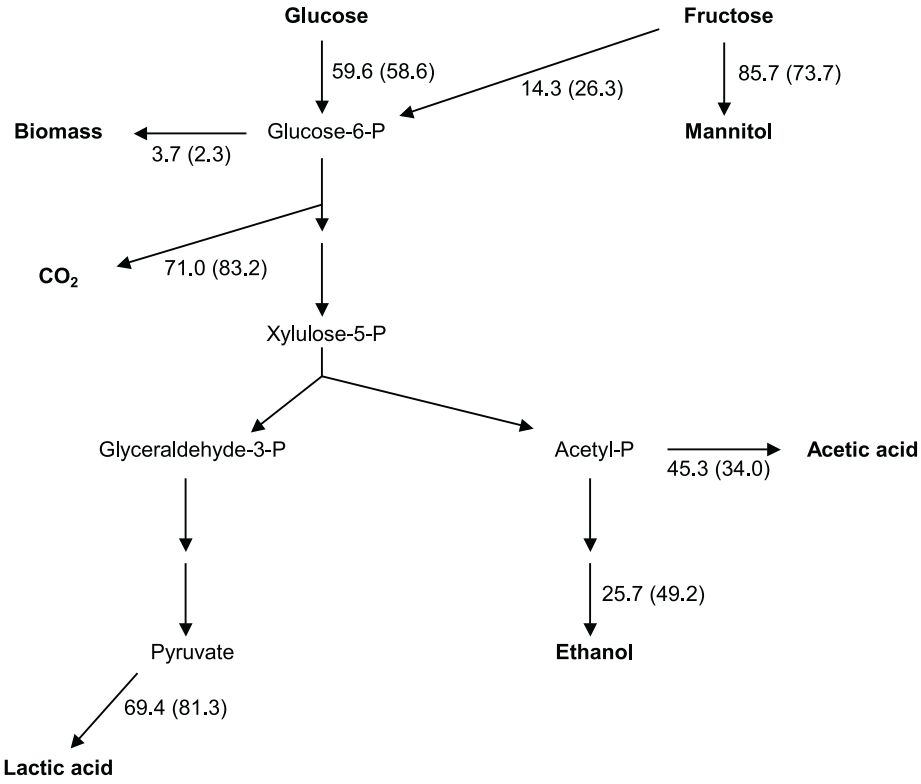


Figure 31. The primary metabolism of *L. pseudomesenteroides* ATCC-12291 and a mutant with lowered fructokinase activity (DSM-14613) at 30°C and pH 5.0. The values represent yields on fructose (mol/mol×100). Fructose, glucose, mannitol, lactic acid, acetic acid and ethanol were measured with HPLC, whilst the biomass and carbon dioxide were calculated as described in Appendix 1. The respective values for the parent strain are shown in brackets.

Complete removal of the fructokinase activity was not achieved. The plasmid DNA of the parent strain was isolated and fructokinase-specific primers (PCR) were used to test if a fructokinase gene was situated on a plasmid. The test was positive, but the presence of some chromosomal DNA in the isolated samples could not be ruled out. The total DNA was later isolated from the mutant strain and sequenced. A point mutation was identified in the mutant sequence and this was thought to be the reason for reduced activity.

The carbon and redox balances (Table 12) were within the accuracy of the experimental data. This ruled out the possibility of further routes for fructose leakage and it could be assumed that the major pathways for co-metabolism of fructose and glucose in *L. pseudomesenteroides* are as presented in Figure 31. Moreover, no new peaks were detected in HPLC spectra of samples from the mutant strain cultivations.

8.7.2 Inactivation of D-lactate dehydrogenase in Lb. fermentum

Mannitol production by co-metabolism of fructose and glucose with obligately heterofermentative LAB result in an acidic side-stream comprising mainly of lactate, acetate, ethanol and substrate residuals. Applications for this solution are few. For instance, Ojamo *et al*. (2000) suggested its use as a feed preservative. However, the price for such a product would most likely be very low. In an attempt to improve the economics of the bacterial mannitol production process, alternative two-product processes were developed. These are presented below and in Chapter 8.7.3.

The lactate produced by bacterial cells is usually optically impure, i.e. both D- and L-lactate are formed. From an economical point of view, production of optically pure lactate instead of the racemic solution is preferred. LAB deficient in D-lactate dehydrogenase are known to produce pure L-lactate from pyruvate (e.g. Bhowmik and Steele, 1994). Optically pure L-lactate is used e.g. in the production of valuable synthetic biopolymers.

Hence, the D-lactate dehydrogenase gene in an efficient mannitol-producer, *Lb. fermentum*, was inactivated using site-directed mutagenesis. The lack of the D-lactate dehydrogenase activity in *Lb. fermentum* resulted in minor changes of the primary sugar metabolism (Table 13). The mutant cells, however, grew and consumed fructose slightly slower than the parent cells. The effect of the mutation on the yield of mannitol from fructose was small. Furthermore, the primary fluxes in the mutant, shown in Figure 32, did not differ significantly from the respective fluxes of the parent strain. Surprisingly, the L-lactate dehydrogenase activity of the mutant cells was decreased to about 1/3 of that of the parent strain. However, the mutant produced high levels of pure L-lactate without a slow-down in mannitol production. No novel end products were detected.

In 5 hours about 40 g fructose (220.3 mmol) and 20 g glucose (108.6 mmol) were metabolized into about 36 g D-mannitol (197.1 mmol) and 10.5 g pure L-lactic acid (116.4 mmol). Hence, about 77.6% (C-mol/C-mol sugar) of the total carbon present in the sugars was now recovered in valuable end products (D-mannitol and L-lactic acid). The respective value with the parent strain was 59.5 C-mol-% (D-mannitol).

Table 13. *Lb. fermentum* NRRL-B-1932 compared to a D-lactate dehydrogenase negative mutant (GRL-1030) in a growth medium containing both fructose and glucose at 37°C and pH 5.0.

	Parent	Mutant
Time to fructose depletion (h)	4.75	5
Final cell dry weight (g/L)	4.7 ± 0.2	2.5 ± 0.1
Maximum specific growth rate (1/h)	0.91 ± 0.02	0.82 ± 0.02
Volumetric fructose consumption rate (g/L/h) [a]	3.6 ± 0.0	2.5 ± 0.0
Specific fructose consumption rate (g/g cdw/h) [a]	3.9 ± 0.1	2.3 ± 0.1
Yield of mannitol from fructose (mol-%)	90.6 ± 1.2	89.5 ± 0.5
Volumetric mannitol productivity (g/L/h)	3.7 ± 0.1	3.7 ± 0.0
Fraction of D-lactate (%)	56.7 ± 0.5	nd
Fraction of L-lactate (%)	43.3 ± 0.5	100
Specific D-lactate dehydrogenase activity (U/mg)	4.9 ± 0.4	nd
Specific L-lactate dehydrogenase activity (U/mg)	1.0 ± 0.0	0.3 ± 0.0
Mannitol (C-mol/C-mol sugar)	0.595 ± 0.000	0.599 ± 0.006
Lactic acid (C-mol/C-mol sugar)	0.185 ± 0.003	0.177 ± 0.006
Acetic acid (C-mol/C-mol sugar)	0.100 ± 0.001	0.091 ± 0.001
Ethanol (C-mol/C-mol sugar)	0.027 ± 0.001	0.033 ± 0.000
Carbon dioxide (C-mol/C-mol sugar)	0.063 ± 0.001	0.062 ± 0.001
Biomass (C-mol/C-mol sugar)	0.026 ± 0.005	0.027 ± 0.010
Carbon balance	**0.996 ± 0.005**	**0.990 ± 0.005**
NAD/NADH balance	**1.01 ± 0.01**	**0.97 ± 0.01**

[a] Between t = 1 and t = 3 hours. The specific consumption rates were calculated using a logarithmic mean of the biomass (see Chapter 7.9). *nd* = not detected.

The mutation resulted in small changes of the NADH oxidation patterns. While slightly less mannitol was produced per fructose consumed by the mutant, a small increase in ethanol production was observed. The yields of ATP per mole fructose consumed in *Lb. fermentum* were considerably lower compared to the respective values calculated for *L. pseudomesenteroides* (see Chapter 8.7.1). This was likely a consequence of the decreased flux of sugar carbon in the glucose catabolism pathway, i.e. a consequence of the improved yield of mannitol from fructose. Moreover, the yield of ATP per fructose consumed was lower in the D-lactate dehydrogenase negative mutant than in the parent strain (86.7 mol/mol compared to 96.3 mol/mol). This corresponded well with the lowered growth rate and final biomass of the mutant.

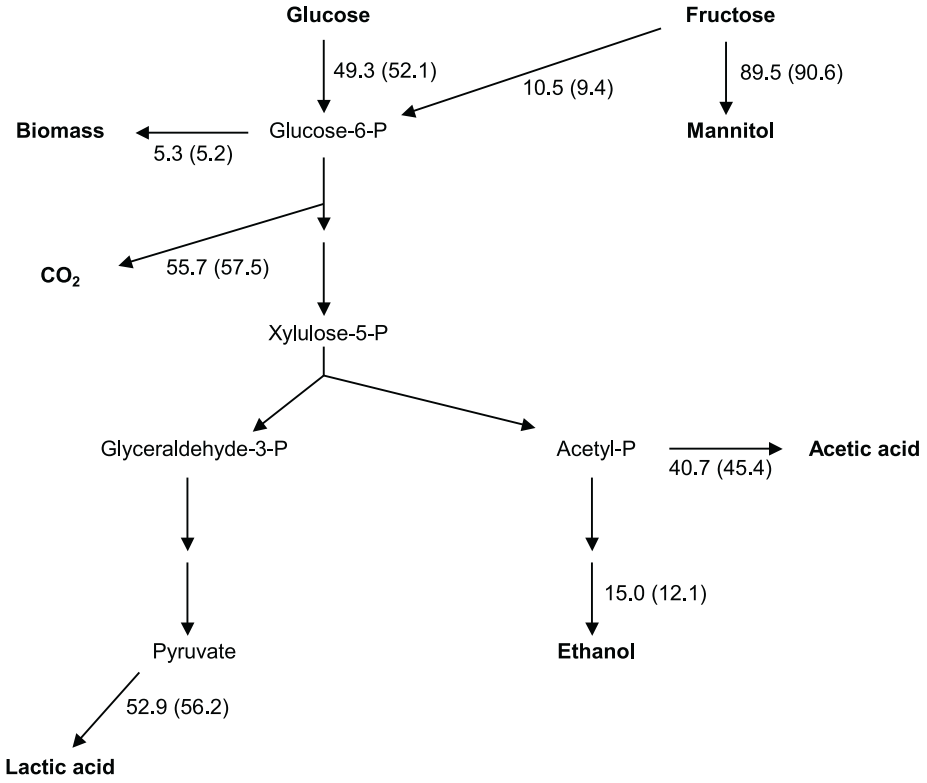


Figure 32. The primary metabolism of *Lb. fermentum* NRRL-B-1932 and a D-lactate dehydrogenase negative mutant (GRL-1030) at 37°C and pH 5.0. The values represent yields on fructose (mol/mol×100). Fructose, glucose, mannitol, lactic acid, acetic acid and ethanol were measured with HPLC, whilst the biomass and carbon dioxide were calculated as described in Appendix 1. The respective values of the parent strain are shown in brackets.

Use of metabolic engineering for the production of pure L-lactic acid has previously been studied in homofermentative LAB. For instance, using *Lb. helveticus*, Bhowmik and Steele (1994) constructed a D-lactate dehydrogenase negative mutant, which produced only pure L-lactate. The metabolism of the mutant cells was otherwise not changed. D-lactate dehydrogenase deficient *Lb. helveticus* mutants have also been studied by Kylä-Nikkilä *et al.* (2000), who reported similar results. Applying the same target of inactivation in *Lb. johnsonii* resulted in the production of pure L-lactate, but now some pyruvate was lost to other end products (e.g. diacetyl and acetoin) (Lapierre *et al.*, 1999). Lapierre and co-workers also detected a decrease in the remaining L-lactate dehydrogenase activity.

8.7.3 Inactivation of D- and L-lactate dehydrogenases in Lb. fermentum

Using the D-lactate dehydrogenase negative mutant (GRL-1030) as the starting point, a mutant deficient in both lactate dehydrogenases was constructed (GRL-1032). The hypothesis was that such a mutant would export pyruvate into the growth medium, thus giving rise to a microbial two-product process (D-mannitol and pyruvate). Industrially, pyruvate is used e.g. in the biosynthesis of pharmaceuticals,

such as L-tryptophan, L-tyrosine and alanine (Li *et al*., 2001). It is also used as an antioxidant, a fat reducing agent and in the production of polymers and cosmetics. The consequences of inactivation were examined at pH 7.0 and 5.0.

The sugar consumption patterns of the double mutant (deficient in both lactate dehydrogenases), at pH 7.0, clearly deviated from patterns of the *Lb. fermentum* parent strain (Figure 33). Whereas the parent strain consumed glucose and fructose in a typical 0.5:1 ratio and both fructose and glucose ran out at approximately the same time, the glucose consumption of the double mutant was severely decelerated compared to the fructose consumption. When the initial fructose (20 g/L) was consumed by the mutant cells (about t = 7 h), only about 55% of the initial glucose (10 g/L) was consumed. The glucose-to-fructose consumption ratio was thus decreased to about 0.3:1. Moreover, after t = 6 h the glucose consumption rate became very low.

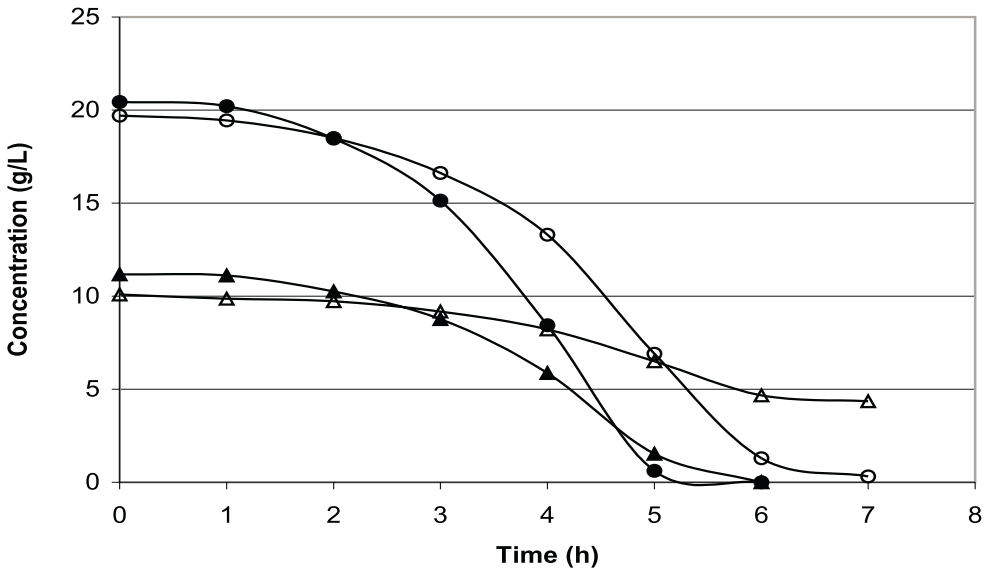


Figure 33. Sugar consumption by *Lb. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase negative mutant (GRL-1032). The temperature, pH and agitation were set at 37°C, 7.0 and 200 rpm, respectively. Legends: *circles*, fructose (g/L); *triangles*, glucose (g/L); *closed symbols*, parent strain; *open symbols*, mutant.

Importantly, similar mannitol production levels were obtained with both strains (Figure 34). In contrast to expectations, the D/L-lactate dehydrogenase negative mutant also produced some D-lactate (0.5 g/L compared to 6.4 g/L of total lactate produced by the parent strain). Furthermore, pyruvate (2.0 g/L) and 2,3-butanediol (0.4 g/L) were also produced. The formation of lactate in the mutant cultivations was puzzling. The inactivation of the lactate dehydrogenase genes had been checked thoroughly: First, no lactate dehydrogenase activities were detected in cell lysates from mutant cell cultures. Second, using PCR and three different sets of primers, it

was ensured that inactivation had occurred at the DNA level. Third, applying northern blotting, the m-RNAs encoding for both lactate dehydrogenases clearly detected in the parent cells, were not detected in the mutant cells. Moreover, the accuracy of the carbon- and redox balances (Table 14) supported the fact that the lactate formed was actually produced from pyruvate. Thus, the formation of D-lactate could not be explained, but it was speculated that another enzyme, with D-lactate dehydrogenase side-activity, was responsible for reducing pyruvate to lactate in these cells.

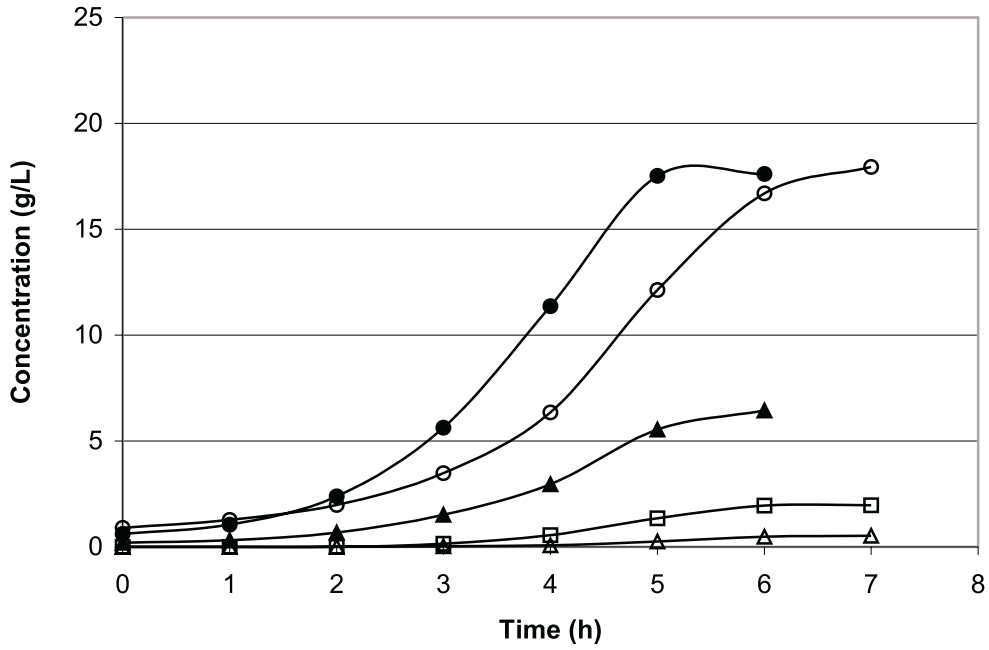


Figure 34. Concentration of mannitol, pyruvic and lactic acid in cultivations with *Lb. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase negative mutant (GRL-1032); Run 1 of 2. The temperature, pH and agitation were set at 37°C, 7.0 and 200 rpm, respectively. Legends: *circles*, mannitol (g/L); *triangles*, lactic acid (g/L); *squares*, pyruvic acid; *closed symbols*, parent strain; *open symbols*, mutant.

The production of 2,3-butanediol is common among bacteria. A low affinity aceto-lactate synthase first converts pyruvate into α-acetolactate, which is then converted to acetoin by α-acetolactate decarboxylase. Two moles of pyruvate are needed for the production of one mole α-acetolactate. Finally, acetoin is reduced to 2,3-butanediol by acetoin reductase, whilst NADH is simultaneously oxidized to NAD^+. Diacetyl can also be produced from α-acetolactate by non-enzymatic conversion. This important aroma compound is usually formed in trace amounts and was not detected in the samples using HPLC analysis.

Table 14. *Lb. fermentum* NRRL-B-1932 compared to a D/L-lactate dehydrogenase negative mutant (GRL-1032) in a growth medium containing both fructose and glucose at 37°C and pH 7.0.

	Parent	Mutant
Time to fructose depletion (h)	5.5	7
Final cell dry weight (g/L)	3.8 ± 0.0	2.7 ± 0.1
Maximum specific growth rate (1/h)	0.81 ± 0.01	0.60 ± 0.02
Volumetric fructose consumption rate (g/L/h) [a]	2.5 ± 0.1	1.5 ± 0.1
Specific fructose consumption rate (g/g cdw/h) [a]	4.2 ± 0.0	4.1 ± 0.1
Yield of mannitol from fructose (mol-%)	89.0 ± 1.3	88.1 ± 0.5
Volumetric mannitol productivity (g/L/h)	3.5 ± 0.0	2.6 ± 0.0
Fraction of D-lactate (%)	53.2 ± 1.0	100
Fraction of L-lactate (%)	46.8 ± 1.0	nd
Specific D-lactate dehydrogenase activity (U/mg)	4.1 ± 0.1	nd
Specific L-lactate dehydrogenase activity (U/mg)	0.1 ± 0.0	nd
Mannitol (C-mol/C-mol sugar)	0.601 ± 0.010	0.683 ± 0.002
Lactic acid (C-mol/C-mol sugar)	0.187 ± 0.000	0.021 ± 0.001
Acetic acid (C-mol/C-mol sugar)	0.099 ± 0.001	0.080 ± 0.001
Pyruvic acid (C-mol/C-mol sugar)	nd [b]	0.080 ± 0.002
Ethanol (C-mol/C-mol sugar)	0.023 ± 0.000	0.006 ± 0.000
2,3-Butanediol (C-mol/C-mol sugar)	nd [b]	0.021 ± 0.001
Carbon dioxide (C-mol/C-mol sugar)	0.061 ± 0.000	0.053 ± 0.001
Biomass (C-mol/C-mol sugar)	0.032 ± 0.012	0.058 ± 0.007
Carbon balance	**1.004 ± 0.001**	**1.003 ± 0.000**
NAD/NADH balance	**0.99 ± 0.00**	**0.98 ± 0.02**

[a] Between t = 1 and t = 3 hours. The specific consumption rates were calculated using a logarithmic mean of the biomass (see Chapter 7.9).
[b] *nd* = not detected.

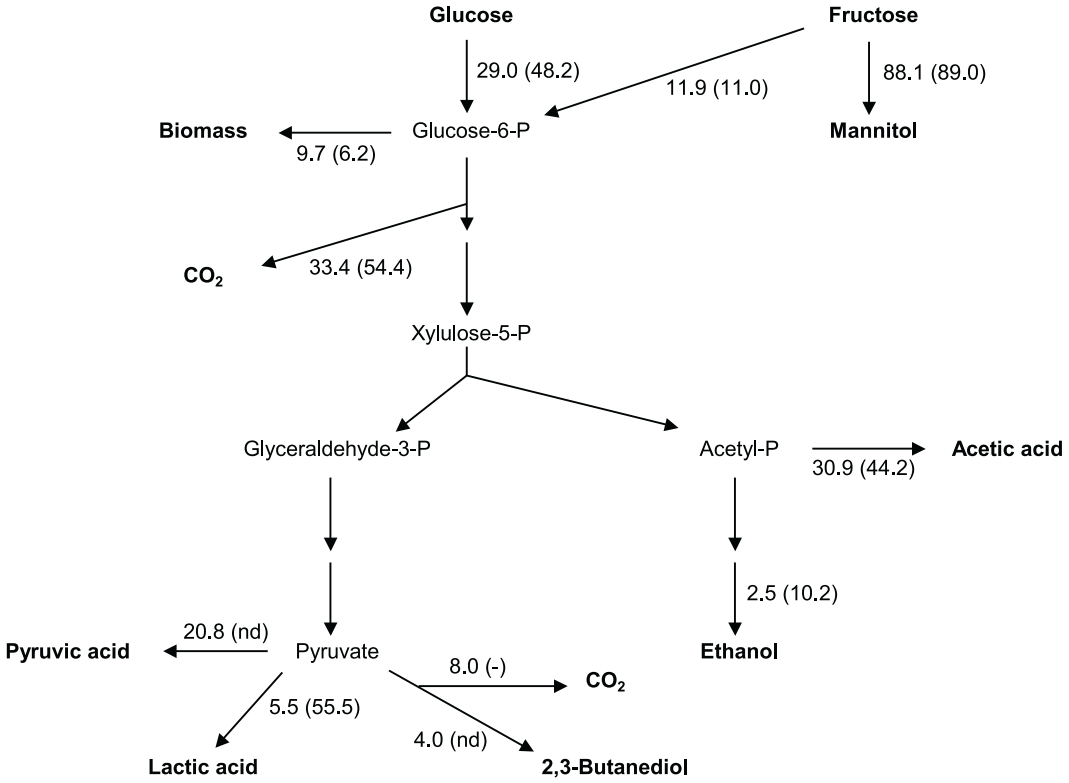


Figure 35. The primary metabolism of *Lb. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase negative mutant (GRL-1032) at 37°C and pH 7.0. The values represent yields on fructose (mol/mol×100). Fructose, glucose, mannitol, lactic acid, acetic acid and ethanol were measured with HPLC, whilst the biomass and carbon dioxide were calculated as described in Appendix 1. The respective values of the parent strain are shown in brackets. *nd* = not detected.

Furthermore, the double mutant grew significantly slower than the parent strain. Although the specific fructose consumption rate was not affected, the lack of the lactate dehydrogenases drastically affected the glucose catabolism and the redox balance of the cells. In the parent cells, all NADH needed for reduction of fructose to mannitol is formed when glucose-6-P is metabolized to ribulose-5-P (upper branch). All excess NADH formed in this branch of the primary metabolism is re-oxidized to NAD^+ in the production of ethanol from acetyl-CoA. As shown in Figure 35, 88.1 mol mannitol per mol fructose consumed was produced by the mutant cells. An equal amount of NADH is required for the reaction to proceed, but only 66.8 mol NADH per mol fructose was produced in the upper branch. Therefore, to complete the fructose-to-mannitol reaction, NADH (21.3 mol/mol) typically used in lactate production (i.e. formed when glyceraldehyde-3-P is metabolized to pyruvate) was now re-oxidized by mannitol dehydrogenase. Due to the lack of the lactate dehydrogenases, pyruvate was thus accumulated in the growth medium. Also, 2,3-butanediol was produced from pyruvate. To fulfill the remaining redox imbalance a small amount of ethanol was produced, whilst acetyl-P was mainly de-phosphorylated to acetate.

Table 15. *Lb. fermentum* NRRL-B-1932 compared to a D/L-lactate dehydrogenase negative mutant (GRL-1032) in a growth medium containing both fructose and glucose at 37°C and pH 5.0.

	Parent	Mutant
Time to fructose depletion (h)	4.75	6
Final cell dry weight (g/L)	4.7 ± 0.2	3.7 ± 0.1
Maximum specific growth rate (1/h)	0.91 ± 0.02	0.72 ± 0.02
Volumetric fructose consumption rate (g/L/h) [a]	3.6 ± 0.0	2.0 ± 0.1
Specific fructose consumption rate (g/g cdw/h) [a]	3.9 ± 0.1	4.8 ± 0.5
Yield of mannitol from fructose (mol-%)	90.6 ± 1.2	91.4 ± 2.2
Volumetric mannitol productivity (g/L/h)	3.7 ± 0.1	2.9 ± 0.1
Fraction of D-lactate (%)	56.7 ± 0.5	100
Fraction of L-lactate (%)	43.3 ± 0.5	nd
Specific D-lactate dehydrogenase activity (U/mg)	4.9 ± 0.4	nd
Specific L-lactate dehydrogenase activity (U/mg)	1.0 ± 0.0	nd
Mannitol (C-mol/C-mol sugar)	0.565 ± 0.000	0.650 ± 0.000
Lactic acid (C-mol/C-mol sugar)	0.185 ± 0.003	0.096 ± 0.001
Acetic acid (C-mol/C-mol sugar)	0.100 ± 0.001	0.096 ± 0.001
Pyruvic acid (C-mol/C-mol sugar)	nd [b]	nd [b]
Ethanol (C-mol/C-mol sugar)	0.027 ± 0.001	0.006 ± 0.001
2,3-Butanediol (C-mol/C-mol sugar)	nd [b]	0.040 ± 0.002
Carbon dioxide (C-mol/C-mol sugar)	0.063 ± 0.001	0.071 ± 0.001
Biomass (C-mol/C-mol sugar)	0.026 ± 0.005	0.045 ± 0.000
Carbon balance	**0.996 ± 0.005**	**1.003 ± 0.004**
NAD/NADH balance	**1.01 ± 0.01**	**0.98 ± 0.01**

[a] Between t = 1 and t = 3 hours. The specific consumption rates were calculated using a logarithmic mean of the biomass (see Chapter 7.9).
[b] *nd* = not detected.

At pH 5.0, the external concentration of pyruvate increased until the mutant cells reached the late exponential growth phase, where after the pyruvate (0.9 g/L) was rapidly consumed by the cells (Table 15). At the lowered pH the mutant cells produced even more D-lactate (2.7 g/L) (Figure 36). No lactate dehydrogenase activities were detected. Furthermore, the glucose-to-fructose consumption ratio was now 0.4:1.

In conclusion, at pH 7.0 about 38.5 g fructose (213.7 mmol) and 11 g glucose (61.9 mmol) were metabolized into about 34 g D-mannitol (188.2 mmol) and 4 g pyruvic acid (44.3 mmol) in 7 hours. Hence, in this process about 76.3% (C-mol/C-mol sugar) of the total carbon present in sugars was recovered in valuable end products (D-mannitol and pyruvic acid). Similar levels were obtained earlier with the mannitol and L-lactic acid two-product process (see Chapter 8.7.2). However, the concentration of pyruvate obtained was not as high as expected and this will complicate the purification steps. The low level of pyruvate formed was primarily

due to the severe slow-down of glucose catabolism and the formation of "waste-products" such as 2,3-butanediol and lactate.

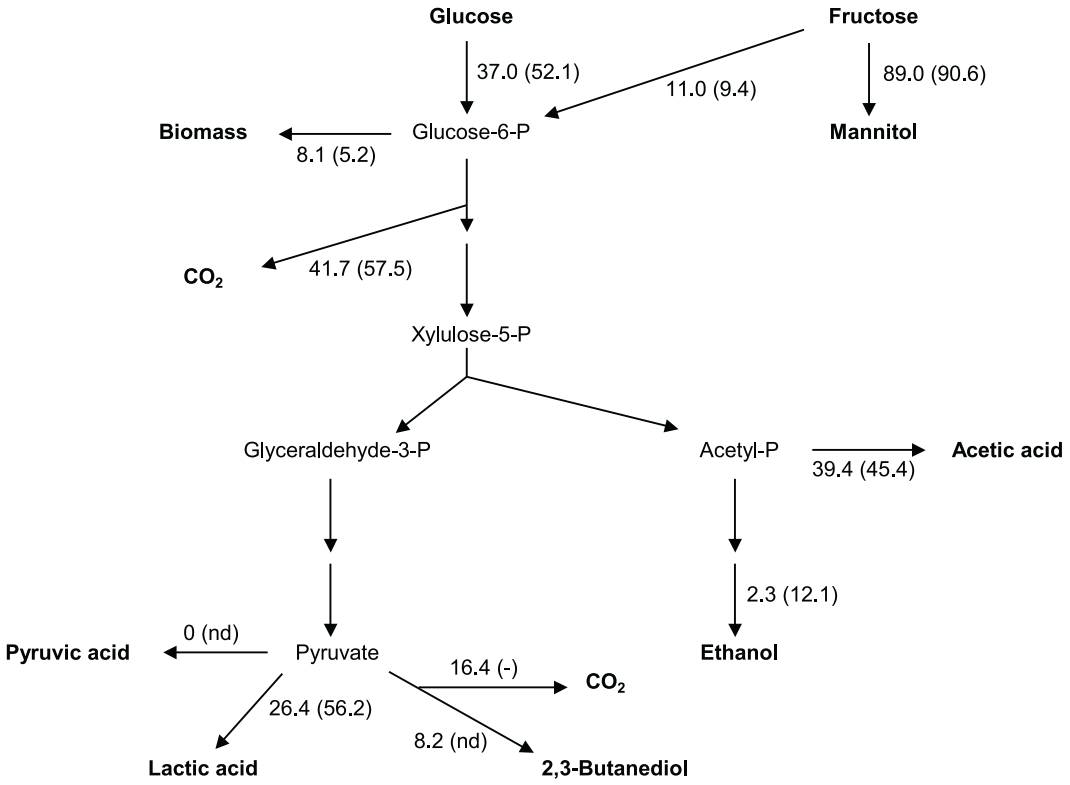


Figure 35. The primary metabolism of *Lb. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase negative mutant (GRL-1032) at 37°C and pH 5.0. The values represent yields on fructose (mol/mol×100). Fructose, glucose, mannitol, lactic acid, acetic acid and ethanol were measured with HPLC, whilst the biomass and carbon dioxide were calculated as described in Appendix 1. The respective values of the parent strain are shown in brackets. *nd* = not detected.

In earlier studies, the inactivation of both lactate dehydrogenases in *Lb. plantarum* resulted in major rerouting of glucose catabolism, which led to the production of end products such as acetoin, ethanol, acetate, mannitol and succinate (Ferain *et al.*, 1998). Moreover, Neves *et al.* (2000) studied the disruption of the lactate dehydrogenase in *Lc. lactis*. In a resting state, the mutant cells produced the same end products as above. Mannitol was transiently produced and metabolized once glucose was depleted. In these cells, mannitol was formed from fructose-6-P via mannitol-1-P. Furthermore, a lactate dehydrogenase deficiency can also be lethal to certain LAB at high glucose concentrations (*Streptococcus mutans*; Hillman *et al.*, 2000).

8.7.4 Inactivation of acetate kinase in Lb. fermentum

An acetate kinase negative mutant was constructed as described in Chapter 7.7.2. The aim of such a mutation was to facilitate purification in a two-product system producing mannitol and either lactate or pyruvate. Crossover integration of an in-activation plasmid at the acetate kinase site was, however, not achieved. Preliminary test tube experiments (50 mL) were performed using the OSCP medium. The mutants required selection pressure, and thus the media used in mutant cell cultivations contained 5 μg/mL erythromycin. The initial pH of the growth medium was 6.2 and the temperature was controlled at 42°C. Samples for measurement of optical density were taken once an hour. After 6 hours of cultivation, the experiment was ended and the culture broths analyzed for primary metabolites. The cells were disrupted by sonication and the cell lysates were analyzed for acetate kinase activity and total protein concentration.

No acetate kinase activity was detected in the mutant cells, whereas the specific activity of the parent strain was 5.5 U/mg. The acetate kinase negative cells grew significantly slower than the parent cells. At t = 6 hours, the optical densities of the mutant and parent cells were 0.5 and 2.0, respectively. This was partly a consequence of erythromycin present in the mutant cell culture medium. Moreover, the acetate kinase negative cells consumed only about 22.9 mol glucose per 100 mol fructose (parent strain = 41.7 mol/100 mol), which indicated that the lack of the acetate kinase also negatively affected the rate of glucose catabolism (a similar effect was observed with cells lacking both lactate dehydrogenases; see Chapter 8.7.3).

No acetate was detected in culture broth of acetate kinase negative cells. The lack of acetate kinase activity also resulted in decreased mannitol yield (89% (mol/mol), parent strain; 78%, mutant). The redox balance of the mutant cells was satisfactorily fulfilled, but on the other hand, when the carbon balance was examined it was found that a significant amount of acetyl-P was rerouted to unknown metabolites. It was believed that the missing acetyl-P was first converted into acetyl-CoA, without disrupting the redox balance, and then incorporated to some kind of storage compound. Acetyl-CoA is the starting point for many such compounds (e.g. lipids).

Although acetate production was successfully blocked, the mutation resulted in decreased mannitol yield and decreased glucose-to-fructose consumption ratio. If such a mutation would be transferred into e.g. a mannitol and L-lactate two-products system, such properties would be unwanted. It is also believed that these changes would not disappear, if resting cells were to be used in the bioconversion instead of growing cells.

9. CONCLUSIONS

Mannitol has a steady market, but the methods for producing mannitol are still evaluated and developed. Commercial production of mannitol relies on catalytic hydrogenation of fructose or fructose-glucose mixtures. However, the hydrogenation process has several drawbacks. In the hydrogenation process, fructose is reacting with a metal catalyst under high pressure and temperature. The hydrogen needed is supplied in the form of hydrogen gas. With the best metal catalysts available, only about 50% of fructose is reduced to mannitol, while the rest is reduced to sorbitol. Metal catalysts are non-specific in regards to the substrates, i.e. they catalyze a variety of reactions depending on the substrates present in the reactor. Besides that the catalyst must be of high quality, also the fructose and hydrogen gas must be of high purity to avoid further yield losses and unwanted side-reactions.

A complicated purification process adds additional costs to the catalytic hydrogenation process. First, ion exchange must be used to remove the metal catalyst. Second, high temperature reactions result in color formation and extra purification steps are needed to remove the color impurities. Third, although a big solubility difference between mannitol and sorbitol favors efficient separation of these two compounds, production of high purity mannitol (or sorbitol) requires additional purification steps. Moreover, pure fructose is seldom used as a starting material in commercial production. Instead cheaper fructose-glucose mixtures are used, in which case mannitol becomes the side product of the process. It is thus obvious that the current chemical mannitol production methods are both laborious, ineffective and relatively expensive.

Alternative production methods based on both enzymatic and microbial techniques have been studied. Enzymatic processes for mannitol production often applies so-called cofactor regeneration systems, but the use of these systems on a commercial level, is restricted by factors such as strong end product inhibition, high K_m value of key enzyme for fructose and low volumetric productivities. A noteworthy microbial production process was developed by Ojamo *et al*. (2000). High yields and volumetric productivities were achieved with this bioprocess alternative. However, some drawbacks remained, like cell leakage from the reactor column and slow fructose consumption at low concentrations.

In this work, a well-known enzymatic reaction, in which heterofermentative LAB reduce fructose into mannitol, was applied to develop a commercially competitive mannitol bioprocess. Several LAB species were compared in their ability to produce mannitol and based on the resulting data, an efficient strain belonging to the *L. mesenteroides* species was identified. When this strain was grown in a simple batch process good yields but only moderate productivities were achieved. To increase the productivity, more sophisticated bioprocess alternatives were studied. Using membrane cell-recycle bioreactor techniques and optimizing the critical process parameters, productivities over 20 g/L/h were achieved. In Table 16, the new bioprocess is compared to the traditional catalytic hydrogenation process.

Table 16. Comparison of a catalytic hydrogenation process and the bioprocess described in this thesis.

Catalytic hydrogenation	Microbial process
- non-specific catalyst	- specific catalyst (enzyme)
⇒ high purity raw materials required	⇒ low purity raw materials can be used
- ultra pure hydrogen gas required	- no gases added
- mannitol yield from fructose ~50%	- mannitol yield from fructose ~95%
- mannitol is the side product	- mannitol is the main product
- impurities in process solution: sorbitol and metal catalyst	- impurities in process solution: organic acids, ethanol and sugars
⇒ difficult to separate	⇒ easy to separate
- chromatographic separation required	- no chromatographic steps required
- yield of crystalline mannitol from initial sugar ~39%	- yield of crystalline mannitol from initial sugar ~52%
- side products formed per kg mannitol crystals produced ~1.6 kg	- side products formed per kg mannitol crystals produced ~0.7 kg

Regardless of whether the yield or productivity carry more weight from an economical point of view, both parameters ought to be met by the new bioprocess. It is naturally important to remember that a supply of glucose is also needed in the bioprocess and hence, the maximum theoretical yield for the bioreaction phase is only about 67% of mannitol from the sugar consumed (the real yield for the new bioprocess was about 61-62%).

The functionality of the new bioprocess was tested on two levels. First, on a laboratory-scale, the process was run in a semi-continuous mode a total of 14 batches. Hence, it was shown that stable yields and productivities were possible to achieve in successive batches using the same initial biomass. Second, moving from a 2-L laboratory-scale to a small pilot-scale (100 L), no changes in essential process parameters were observed. It is thus assumed that the new bioprocess will pass further scale-up phases and is applicable for commercial production-scale. Moreover, the scalability of this process concept is expected to be better than e.g. the process using immobilized cells.

The capital costs for the new bioprocess are low compared to the capital costs of typical bioprocesses. Due to a low contamination risk not much effort must be put into maintaining aseptic conditions and only moderately equipped reactors are needed. Also, no gassing systems are required and only the basic parameters (temperature and pH) need to be controlled. Moreover, the purification comprises basic well-established unit operations. The production costs of the new mannitol bioprocess, on the other hand, are strongly dependent on the price of fructose. One key benefit of microbial processes, in general, is that low-purity raw materials can be used without affecting the yields or productivities. In the case of mannitol production, this will drastically reduce the production costs and is a highly relevant factor, when the new bioprocess alternative is evaluated against the chemical hydrogenation processes. Furthermore, the use of *L. mesenteroides*, especially in the food industry, is commonly accepted.

The use of LAB as hosts for production of recombinant proteins will most likely grow in importance in the future as new cloning tools are developed. Significant progress has been made with homofermentative LAB, where e.g. *Lc*. *lactis* has become a very popular cloning host. Cloning of heterofermentative LAB is also a topic of current research efforts (e.g. Bourel *et al*., 2001). In order to find applications for the glucose catabolism of the new mannitol bioprocess, metabolic engineering techniques were applied. For instance, mutants producing mannitol and either optically pure L-lactate or pyruvate were constructed. Hence, the total yield of valuable products from sugar consumed was significantly improved in both cases.

In the future, efforts should be placed in finding sources of readily available low-purity fructose syrups. Ideally this syrup would contain both fructose and glucose (e.g. side streams from a glucose to fructose isomerization process). The effects of using a low purity raw material source must then be carefully validated. After the sugars were depleted from the medium, the cells were observed to slowly consume mannitol. Hence, correct timing of the product recovery by filtration is vital. However, without an on-line sugar analysis system, the correct timing was difficult to estimate. Although some indications were obtained from the base consumption rate, in the future, other on-line analysis methods must be considered and studied. Finally, the reaction rates by which fructose was reduced to mannitol were surprisingly high. Hence, it would be sensible to study the use of this reduction power for reduction of other substrates to their respective high-value reduced forms.

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Calculation of biomass and carbon dioxide

Reaction:

1	-Fru + Mtol - NADH = 0
2	-Fru + G6P = 0
3	-Glu + G6P = 0
4	-G6P + 6/4.63 × X = 0
5	-G6P + X5P + 2 × NADH + CO_2 = 0
6	-X5P + GAP + AP = 0
7	-AP + HAc = 0
8	-AP + EtOH - 2 × NADH = 0
9	-GAP + Pyr + NADH = 0
10	-Pyr + HLac - NADH = 0
11	-Pyr + HPyr = 0
12	2 × Pyr + BD + 2 × CO_2 - NADH = 0

Substrates:

S1	Glu	fructose
S2	Fru	glucose

Metabolites:

M1	NADH	nicotinamide adenine dinucleotide (reduced form)
M2	G6P	glucose-6-P
M3	X5P	xylulose-5-P
M4	AP	acetyl-P
M5	GAP	glyceraldehyde-3-P
M6	Pyr	pyruvate

Products:

P1	Mtol	mannitol
P2	X	biomass
P3	CO_2	carbon dioxide
P4	HAc	acetic acid
P5	EtOH	ethanol
P6	HLac	lactic acid
P7	HPyr	pyruvic acid
P8	BD	2,3-butanediol

The stoichiometric coefficients were placed in matrices and the matrices transposed. The transposed metabolite matrix was set to be zero. Following equations were obtained:

$n_X = 6/4.63 \times (n_{fru} - n_{mtol} + n_{glu} - n_{HAc} - n_{EtOH})$,

$n_{CO2} = n_{HAc} + n_{EtOH} + 2 \times n_{BD}$,

$NAD/NADH = (3 \times n_{HAc} + 3 \times n_{EtOH}) / (n_{mtol} + 2 \times n_{EtOH} + n_{HLac} + n_{BD})$.

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