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ORIGINAL PAPER

Mannitol production by lactic acid bacteria grown in supplemented carob syrup

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Abstract Detailed kinetic and physiological characterisation of eight mannitol-producing lactic acid bacteria, *Leuconostoc citreum* ATCC 49370, *L. mesenteroides* subsp. *cremoris* ATCC19254, *L. mesenteroides* subsp. *dextranicum* ATCC 19255, *L. ficulneum* NRRL B-23447, *L. fructosum* NRRL B-2041, *L. lactis* ATCC 19256, *Lactobacillus intermedius* NRRL 3692 and *Lb. reuteri* DSM 20016, was performed using a carob-based culture medium, to evaluate their different metabolic capabilities. Cultures were thoroughly followed for 30 h to evaluate consumption of sugars, as well as production of biomass and metabolites. All strains produced mannitol at high yields (>0.70 g mannitol/g fructose) and volumetric productivities (>1.31 g/l h), and consumed fructose and glucose simultaneously, but fructose assimilation rate was always higher. The results obtained enable the studied strains to be divided mainly into two groups: one for which glucose assimilation rates were below 0.78 g/l h (strains ATCC 49370, ATCC 19256 and ATCC 19254) and the other for which they ranged between 1.41 and 1.89 g/l h (strains NRRL B-3692, NRRL B-2041, NRRL B-23447 and DSM 20016). These groups also exhibited different mannitol production rates and yields, being higher for the strains with faster glucose assimilation. Besides mannitol, all strains also produced lactic acid and acetic acid. The best performance was obtained for *L. fructosum* NRRL B-2041, with maximum volumetric productivity of 2.36 g/l h and the highest yield, stoichiometric conversion of fructose to mannitol.

Keywords Carob · Lactic acid bacteria · *Lactobacillus* · *Leuconostoc* · Mannitol production

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Introduction

Mannitol, a naturally occurring polyol, is widely used in medicine, and in pharmaceutical, food and chemical industries. It can be produced by chemical, microbiological or enzymatic processes [1, 2]. Industrial chemical production is based on hydrogenation of fructose/glucose mixtures at high temperature and pressure using Raney-nickel catalyst. This process yields a mixture of mannitol and sorbitol, an isomer, which has less interesting properties and hence significantly lower market price. Moreover, separation of mannitol and sorbitol is rather difficult. The enzymatic process requires the use of redox co-factors [1, 3], which, in general, renders it unattractive. Because of these problems, mannitol production by microbial route has become attractive, even at the industrial scale.

Heterofermentative lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Leuconostoc* and *Oenococcus* produce mannitol from fructose [4, 5] and are among the most efficient microorganisms for mannitol production. In general, they can specifically produce mannitol from glucose/fructose mixtures, without making sorbitol as by-product [2], making it unnecessary to use purified substrates or complex processes for product purification. Metabolically, under adequate oxygen availability conditions, glucose can be used as an energy and carbon source, and fructose as an electron acceptor, as it can be reduced to

mannitol by means of a specific mannitol dehydrogenase (EC 1.1.1.67). Depending on the microorganism, up to 2 mol mannitol can be produced from 1 mol glucose (and 2 mol fructose), if sugar assimilation is simultaneous. This will also lead to formation of lactic acid and acetic acid and/or ethanol and carbon dioxide as other metabolic products.

Carob (*Ceratonia siliqua* L.) is a Mediterranean perennial tree producing pods that contain seeds (10%) and sugar-rich pulp (90%), which exhibits higher sugar content than sugar cane [6]. The pulp is currently an under-utilised by-product of carob locust bean gum production, and its main application is as animal feed, and to a lesser extent in traditional bakery products and beverages. Therefore higher-added-value applications are needed. Carob pulp mainly contains sucrose (the major sugar), glucose and fructose that can be water extracted and easily used to obtain sugar-rich syrups [7, 8]. These syrups are a cheap carbon source that has already been used for formulation of culture media, e.g. for xanthan, ethanol and citric acid production [9–11]. Given its sugar content, a carob-based medium may be advantageous for mannitol production.

In a previous work [12], 30 bacterial strains from genera *Lactobacillus*, *Leuconostoc* and *Weissella* were screened for mannitol production in carob-based syrups. From these, a group of eight strains were identified as potential mannitol producers that must be studied deeply to unveil their potential advantages and limitations concerning use as industrial mannitol producers using carob syrup medium.

In this work, a detailed kinetic and physiological characterisation of these eight strains using a carob-based culture medium was carried out, to evaluate their performance and metabolic capabilities, and to select the most promising strain.

Materials and methods

Raw material

Carob pulp was obtained from a local de-seeding factory (Chorondo e Filhos L.da, Loulé, Portugal). Carob pods were purposely collected to contain the two main varieties grown in Portugal, Mulata and Galhosa (80% and 20%, respectively) to produce a defined and representative lot. The pods were industrially de-seeded into kibbles and then stored at room temperature in burlap sacks. On average, the kibbles present the following composition (g/100 g dry weight): sucrose, 42.68; glucose, 8.54; fructose, 5.97; pinitol, 5.22; lignin, 18.86; cellulose, 7.64; hemicellulose, 0.26; protein, 4.70; fat, 0.61; ash, 3.11; and others (by difference), 2.41 [12].

Medium preparation

Carob syrup was obtained by aqueous extraction using an orbital incubator (Infors Unitron HT, Switzerland), at 50°C for 5 h as described elsewhere [12] and stored at −20°C. Before use, precipitates were removed by centrifugation at 7,500 *g* for 25 min in a Beckman Coulter centrifuge (Fullerton, USA) followed by filtration using Whatman no. 41 filter paper.

Sucrose hydrolysis and sterilisation of carob syrup were performed by correcting syrup pH to 3 using 5 M HCl, followed by 15 min at 121°C in an autoclave. Carob syrups were chemically characterised as described below.

The pH of hydrolysed carob syrups was corrected to 6.5 by addition of sterile NaOH. These hydrolysates were supplemented with MRS nutrients, except ammonium citrate, which was replaced by 50 mM sodium citrate buffer (pH 6.2) to keep pH values in a range acceptable for bacterial growth. Besides sodium citrate, the final medium contained (per litre): 10 g peptone, 8 g beef extract, 4 g yeast extract, 1 ml Tween 80, 5 g $NaCH_3COO$, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$ and 2 g K_2HPO_4. The supplements and citrate buffer were sterilised separately (121°C, 15 min) and added aseptically to the pH-corrected carob hydrolysates, yielding a dilution of the original carob syrup hydrolysate of 1:2.

Microorganisms and culture conditions

Eight heterofermentative lactic acid bacteria (LAB) were used in this study. The strains and the corresponding growth temperatures are summarised in Table 1. Stock cultures were maintained in liquid nitrogen, and inocula were prepared using MRS growth medium. Pre-inoculum was obtained by re-suspending the cells in MRS growth medium and incubation at the required temperature for 48 h. For inocula preparation, 100-ml Erlenmeyer flasks containing 50 ml MRS medium were inoculated with 1 ml pre-inoculum. Both pre-inoculum and inoculum were incubated without agitation.

One millilitre of 16 h inoculum culture was used to seed 100-ml Erlenmeyer flasks containing 50 ml culture medium. All cultures were incubated in an orbital shaker (100 rpm) for 30 h at the same temperature as the inoculum. Samples were withdrawn at a specific time to determine cell growth, consumption of sugars and formation of products.

All experiments were performed, at least, in duplicate. Replicates always differed by less than 10%, and typically by less than 5%.

Analytical methods

Glucose, fructose, mannitol and pinitol were analysed by high-performance liquid chromatography (HPLC; Waters,

Table 1 Lactic acid bacteria strains used in this study

Microorganism	Strain	Growth temperature (°C)
Lactobacillus		
Lactobacillus intermedius	NRRL B-3692	37
Lactobacillus reuteri	DSM 20016	37
Leuconostoc		
Leuconostoc fructosum	NRRL B-2041	30
Leuconostoc ficulneum	NRRL B-23447	30
Leuconostoc citreum	ATCC 49370	30
Leuconostoc lactis	ATCC 19256	30
Leuconostoc mesenteroides subsp. *cremoris*	ATCC 19254	30
Leuconostoc mesenteroides subsp. *dextranicum*	ATCC 19255	30

Milford, USA) using a Sugar-SP0810 column (Shodex, Japan). The column was maintained at 80°C, and the sugars were eluted with ultra-pure water at flow rate of 1 ml/min. The HPLC system was a Waters LC1 module 1 plus (Millfort, MA, USA) equipped with both a refractive index (RI) and an ultraviolet (UV) detector set at 280 nm. For lactic acid, acetic acid, iso-butyric acid, citric acid, ethanol and hydroxymethylfurfural (HMF) analyses, an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) with a de-ashing Micro-guard pre-column (Bio-Rad) was used. The column was maintained at 50°C, and the mobile phase was 5 mM H_2SO_4 at flow rate of 0.6 ml/min. All compounds were analysed with a RI detector, and HMF was also analysed by the UV detector. All samples were filtered through 0.22-μm Gelman membrane filters prior to analysis.

Total phenolic compounds were determined by using the Folin–Ciocalteu colorimetric method according to Singleton and Rossi [13]. Briefly, 100 μl sample was mixed with 5 ml 1/10 (v/v) diluted Folin–Ciocalteu reagent and 4 ml 7.5% Na_2CO_3. Absorbance was measured at 765 nm after 15 min incubation at 45°C. Total phenolic compounds are expressed as g gallic acid equivalents (GAE)/l. Assays were carried out in triplicate. Cell growth was evaluated by measuring absorbance at 600 nm. At the beginning and at the end of fermentations, biomass dry weight was determined gravimetrically. Two millilitres of each sample was centrifuged in dried Eppendorf tubes, washed twice with 2 ml filtered water and dried overnight at 100°C.

Calculations

Volumetric consumption rate of sugars (Q_S, g/l h) was calculated as total monosaccharides (glucose and fructose) consumed in a defined time interval. The specific growth rate (μ, h^{-1}) was calculated by linear regression of the curve of ln(OD/ODi) versus time for the exponential growth phase. The biomass (Q_X) and mannitol (Q_{ManOH}) volumetric production rates (g/l h), hereinafter referred to as the biomass and mannitol productivities, were calculated as the increase in cell mass and mannitol concentrations, respectively, for a designated time interval. The mannitol yield ($Y_{ManOH,}$ g/g) was calculated as the ratio between mannitol increase and fructose consumption, for a designated time interval.

Results and discussion

Composition of hydrolysed carob pulp syrups and carob pulp base medium

Carob syrups used in this work were obtained by water extraction of carob pods under operational conditions previously optimised, and typically contain sucrose (accounting for more than 50% of total sugars) together with free fructose, glucose and pinitol in a total close to 200 g/l [12]. As a high content of free fructose and glucose are required for efficient mannitol production, carob syrups have to be hydrolysed. Table 2 summarises the composition of the hydrolysed carob pulp syrups used in this work for culture medium preparation after the sterilising hydrolysis. More than 90% of the original sucrose was converted to fructose and glucose, leading to an increase of concentration of both sugars, at least twofold. Under these mild conditions, some degradation of hexoses also occurred, and HMF was found as the main degradation product, although at low concentration. Phenolic compounds were also found, but in relative low amounts.

After supplementation, the concentration of components in the hydrolysates was reduced to a half, and carob-pulp-based medium had average composition (per litre) as follows: 44.4 g fructose, 47.6 g glucose, 7.7 g pinitol, 2.4 g acetic acid, 0.85 g iso-butyric acid and 0.34 g HMF. Organic acids, such as iso-butyric acid, and furan derivatives such as HMF, may act as fermentation-inhibiting

Table 2 Composition of the carob syrup hydrolysates used for culture medium preparation

Compound	Concentration (g/l)
Sucrose	11.4 ± 2.1
Fructose	89.2 ± 1.9
Glucose	96.0 ± 1.8
Pinitol	15.6 ± 0.3
Acetic acid	0.70 ± 0.10
Iso-butyric acid	2.05 ± 0.08
Hydroxymethylfurfural	0.67 ± 0.02
Phenolic compounds	1.62 ± 0.04

The concentrations of these compounds in the culture medium are half of those reported in this table, due to the addition of the required supplements

compounds. However, at these low levels and initial pH they may not exert a strong inhibition effect, suggesting the suitability of hydrolysates for fermentation purposes; hence, no detoxification procedure was applied.

Kinetic profiles

To evaluate mannitol production by selected strains using a carob-based medium, the kinetic profiles for growth, sugars, mannitol and other co-products were studied. Figures 1 and 2 show the results obtained for *Lactobacillus* and *Leuconostoc* strains, respectively. Amongst *Lactobacillus* strains, fructose and glucose were simultaneously consumed, but fructose assimilation rate was always higher. *Lb. intermedius* displayed the fastest growth and maximum specific growth rate, two times higher than those for *Lb. reuteri*, which was also confirmed by the higher final cell density achieved. The maximum mannitol concentrations obtained were similar for both microorganisms, although mannitol accumulation by *Lb. intermedius* occurred at a higher rate, which is consistent with fructose depletion. As expected, lactic acid and acetic acid were also produced, mainly when glucose consumption was higher. Ethanol was also found, but only in minor amounts, typically a maximum concentration around 1 g/l. *Lb. intermedius* was an exception, as it presented more significant production (5 g/l) for the final fermentation stages, mainly after fructose depletion and onset of single glucose metabolism. Ethanol production from glucose has been already described for *L. intermedius* strain [4]

All *Leuconostoc* strains preferably consumed fructose over glucose, in similar trend to lactobacilli. The remaining glucose concentration varied depending on the strain. Higher fructose consumption rates were found for *L. ficulneum* and *L. fructosum*, which together with *Lactobacillus* strains were the best mannitol producers. Maximum specific growth rate were similar for all *Leuconostoc* strains ($\sim$0.2 h^{-1}), but the final biomass concentration achieved also depended on the strain.

As for *Lactobacillus*, these strains also produced lactic acid and acetic acid. For all LAB tested, at the maximum mannitol concentrations, aliphatic acids ranged between 9.9 and 18.5 g/l and 14.9 and 27.0 g/l for acetic acid and lactic acid, respectively.

Kinetic and stoichiometric parameters

Table 3 shows the results for kinetic and stoichiometric parameters obtained for *Lactobacillus* and *Leuconostoc* strains grown in carob-based medium. All strains produced mannitol at high yields (>0.70 g mannitol/g fructose) and relatively high volumetric productivities (>1.31 g/l h), and displayed high fructose consumption (>91%). Independent of the genus, strains could be divided into two groups: one for which glucose assimilation rates were always below 1.05 g/l h (glucose consumption lower than 55%), and a

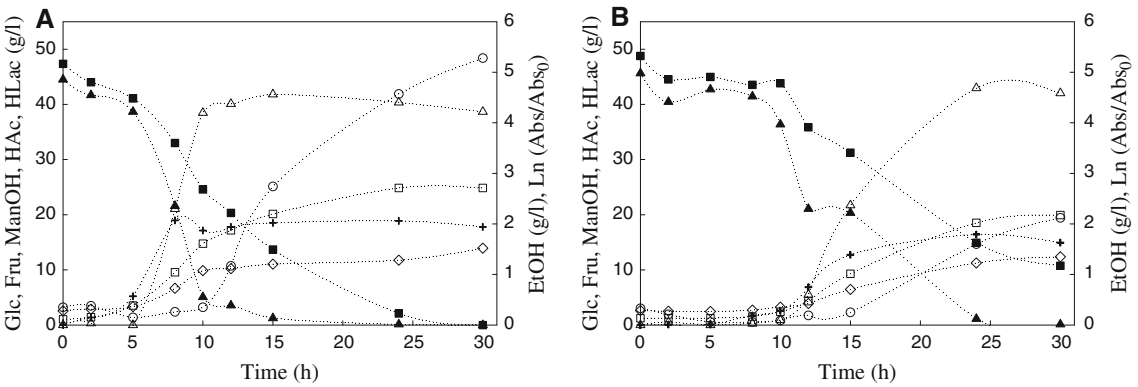

Fig. 1 Fermentation profiles for mannitol production by *Lactobacillus* strains in carob-syrup-based medium. *Lb. intermedius* (**a**); *Lb. reuteri* (**b**). Glucose (*filled squares*), fructose (*filled triangles*), mannitol (*open triangles*), lactic acid (*open squares*), acetic acid (*open diamonds*), ethanol (*open circles*), cell density (*plus symbols*). Data points are the average of at least two independent replicates. *Lines* are guides to the eye only and do not have any statistical significance

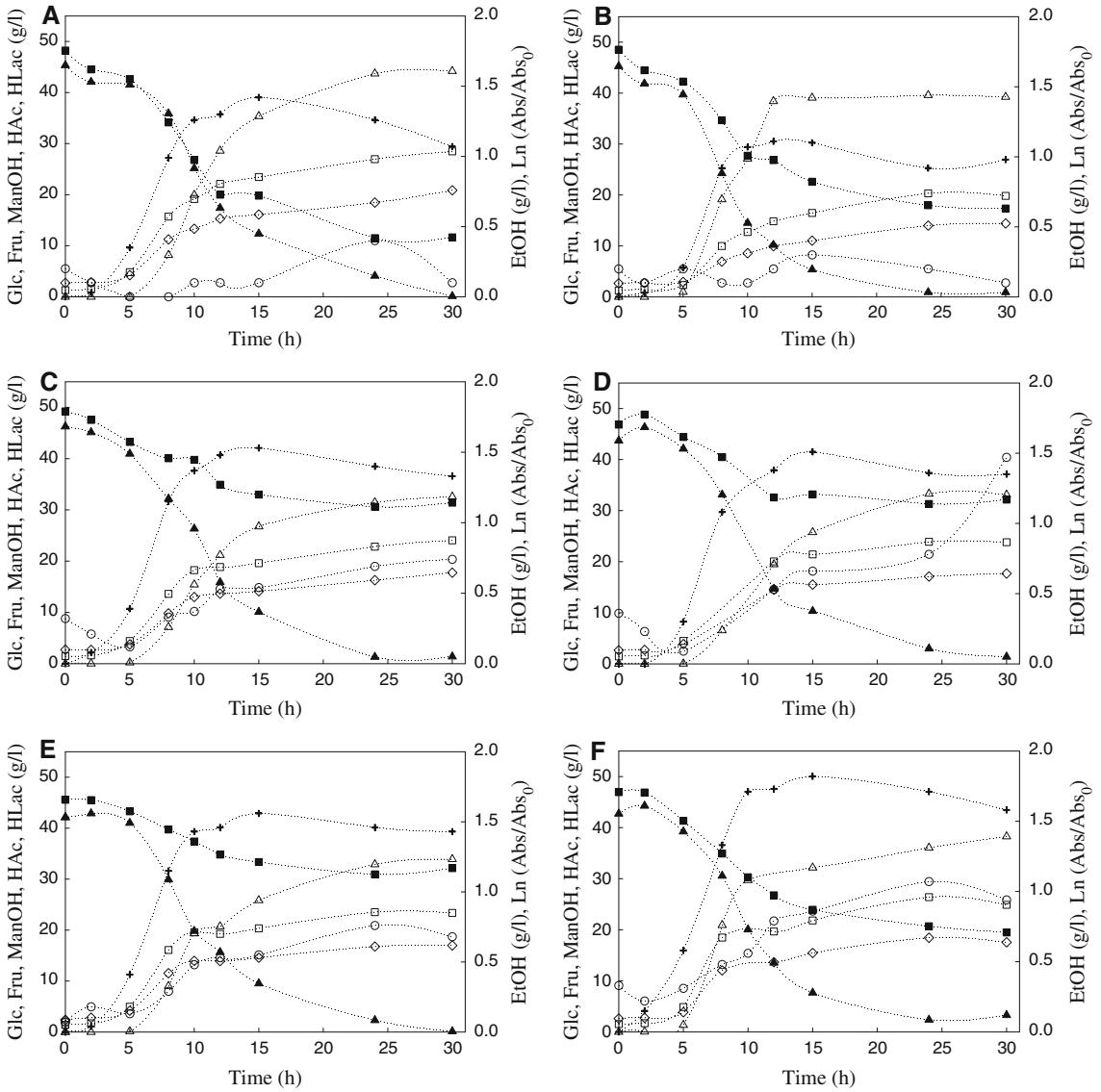

Fig. 2 Fermentation profiles for mannitol production by *Leuconostoc* strains in carob-syrup-based medium. *L. fructosum* (**a**), *L. ficulneum* (**b**), *L. citreum* (**c**), *L. lactis* (**d**), *L. mesenteroides* subsp. *cremoris* (**e**), *L. mesenteroides* subsp. *dextranicum* (**f**). Glucose (*filled squares*), fructose (*filled triangles*), mannitol (*open triangles*), lactic acid (*open squares*), acetic acid (*open diamonds*), ethanol (*open circles*), cell density (*plus symbols*). Data points are the average of at least two independent replicates. *Lines* are guides to the eye only and do not have any statistical significance

second for which they ranged between 1.41 and 1.89 g/l h (glucose consumption ranging from 63% to 96%). The group exhibiting the faster glucose assimilation (*Lb. intermedius*, *Lb. reuteri*, *L. fructosum* and *L. ficulneum*) also exhibited higher mannitol production rates and yields as well as ratio of mannitol to aliphatic acids (lactic acid and acetic acid) above 1. In fact, the rates for glucose consumption and mannitol production were positively correlated ($r = 0.86$, data not shown).

These results clearly indicate that these two groups may have different metabolic capabilities, with strains that exhibited slower glucose consumption producing, in general, higher acetic acid concentrations. The highest ratio of mannitol to other products was obtained for *Lb. reuteri* DSM 20016, but this is not due to higher mannitol production but rather to higher biomass production yield and lower lactic acid production.

The ratios of mannitol to other co-products (lactic and acetic acids) obtained in this work were, however, lower than the values (higher than 2), reported by Saha [14]. It is worth noting that those reported values were obtained for higher fructose concentrations and different ratio of glucose to fructose, which is also a key parameter for efficient mannitol production. Von Weymarn et al. [15] described

Table 3 Kinetic and stoichiometric parameters for growth and formation of products for *Lactobacillus* and *Leuconostoc* strains in carob-syrup-based medium

Microorganism	Consumption (%)		Q_S (g/l h)	Q_X (g/l h)	Q_{ManOH} (g/l h)	Y_{ManOH} (g/g)	ManOH/ HLac	ManOH/ (HLac + HAc)
	Glc	Fru						
Lb. intermedius	96	100	3.74	0.27	1.68	0.91	1.6	1.1
Lb. reuteri	69	98	3.27	0.16	1.79	0.96	2.3	1.4
L. fructosum	76	91	3.25	0.14	1.82	1.06	1.6	1.0
L. ficulneum	63	98	3.12	0.09	1.65	0.89	1.7	1.2
L. citreum	38	97	2.65	0.07	1.31	0.70	1.4	0.8
L. lactis	33	93	2.34	0.08	1.39	0.82	1.4	0.8
L. mesenteroides subsp. *cremoris*	32	95	2.27	0.003	1.37	0.83	1.4	0.8
L. mesenteroides subsp. *dextranicum*	55	94	2.73	0.003	1.50	0.89	1.4	0.8

Glc glucose, *Fru* fructose, Q_S consumption rate of sugars, Q_X biomass productivity, Q_{ManOH} mannitol productivity, Y_{ManOH} mannitol yield on fructose, *ManOH/HLac* ratio of mannitol to lactic acid, *ManOH/(HLAc + HAc)* ratio of mannitol to lactic acid + acetic acid. All values calculated after 24 h

for *L. mesenteroides* an optimum ratio of glucose to fructose of 0.55:1. Also, Saha [16] reported that lactic acid production by *Lb. intermedius* surpassed mannitol concentration when sugar cane molasses (ratio of glucose to fructose of 1:1) were used for culture medium preparation.

The maximum mannitol concentration (43.7 g/l) was obtained with *L. fructosum* (Fig. 2a). This strain also exhibited the highest volumetric productivity (2.36 g/l h, at 15 h) and conversion efficiency of fructose to mannitol of 100%. This productivity is higher than the obtained for *L. pseudomesenteroides* ATCC 12291 grown in media containing glucose and fructose (100:50) under similar culture conditions [17], and the yield was higher than values previously reported for *Lb. intermedius* B-3693 grown in fructose-based medium [4] and the same *L. pseudomesenteroides* [17]. Although stoichiometric conversion of fructose to mannitol has been also previously reported for *Lb. sanfranciscensis* [5, 18], that strain exhibited lower productivity. Given that the initial performances with those strains were further optimised, namely using pH-controlled bioreactors (preventing use of citrate buffer that was typically partially consumed, data not shown), or using different operation modes, such as fed-batch, cell recycling and optimised culture media, it is expected that *L. fructosum* may also present a significant performance improvement [14, 15, 19, 20].

The mannitol volumetric productivities obtained for the group exhibiting lower mannitol production (*L. citreum*, *L. lactis, L. mesenteroides* subsp. *cremoris* and *L. mesenteroides* subsp. *dextranicum*) were similar to values reported for *L. mesenteroides* ATCC 9135, *L. pseudomesenteroides* ATCC 12291, *Lb. brevis* ATCC 8287 and *Lb. fermentum* NRRL 1932 [15]. According to the present results, these syrups, although containing acetic and iso-butyric acids, HMF and some phenolics that were also solubilised during extraction process of sugars, did not have an apparent inhibitory effect on growth of these microorganisms. For this reason, increasing the syrup concentration seems to be a possibility to achieve higher performance. Furthermore, the levels attained by the produced lactic and acetic acids did not hinder fructose consumption. As these acids can be recovered from the fermentation broth (e.g. by electrodialysis [4]), their co-production may be an advantageous trait, as it will be possible to design a multi-product process.

Conclusions

The eight mannitol-producing LAB strains tested were able to grow and produce mannitol with relatively high efficiency in carob-syrup-based medium. Kinetic and physiological characterisation of these strains was performed, and the results obtained suggest that the studied strains can be divided into two groups based on their glucose consumption and mannitol production rates, as well as ratio of mannitol to aliphatic acids. The highest volumetric productivity was obtained for *L. fructosum*, which was also one of the most robust strains tested. This strain is currently being used for mannitol process optimisation in carob-based media.

Given that the levels attained by the produced acids did not hinder fructose consumption, their co-production may be an advantageous trait. The design of a multi-product process is now possible, as these acids are also marketable compounds, in particular lactic acid, which has a large and expanding market for industrial production of bioplastics.

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