Appl Microbiol Biotechnol
DOI 10.1007/s00253-017-8395-1

Enhanced mannitol biosynthesis by the fruit origin strain *Fructobacillus tropaeoli* CRL 2034

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Received: 19 April 2017 / Revised: 12 June 2017 / Accepted: 13 June 2017
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Abstract Mannitol is a natural low-calorie sugar alcohol produced by certain (micro)organisms applicable in foods for diabetics due to its zero glycemic index. In this work, we evaluated mannitol production and yield by the fruit origin strain *Fructobacillus tropaeoli* CRL 2034 using response surface methodology with central composite design (CCD) as optimization strategy. The effect of the total saccharide (glucose + fructose, 1:2) content (TSC) in the medium (75, 100, 150, 200, and 225 g/l) and stirring (S; 50, 100, 200, 300 and 350 rpm) on mannitol production and yield by this strain was evaluated by using a 2^2 full-factorial CCD with 4 axial points (α = 1.5) and four replications of the center point, leading to 12 random experimental runs. Fermentations were carried out at 30 °C and pH 5.0 for 24 h. Minitab-15 software was used for experimental design and data analyses. The multiple response prediction analysis established 165 g/l of TSC and 200 rpm of S as optimal culture conditions to reach 85.03 g/l [95% CI (78.68, 91.39)] of mannitol and a yield of 82.02% [95% CI (71.98, 92.06)]. Finally, a validation experiment was conducted at the predicted optimum levels. The results obtained were 81.91 g/l of mannitol with a yield of 77.47% in outstanding agreement with the expected values. The mannitol 2-dehydrogenase enzyme activity was determined with 4.6–4.9 U/mg as the highest value found. To conclude, *F. tropaeoli* CRL 2034 produced high amounts of high-quality mannitol from fructose, being an excellent candidate for this polyol production.

Keywords Mannitol · *Fructobacillus* · Lactic acid bacteria · Central composite design · Response surface methodology

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Introduction

Lactic acid bacteria (LAB) are a diverse group of phylogenetically related microorganisms capable of converting a wide variety of carbohydrates and other carbon sources into organic acids, mainly lactic acid (Felis et al. 2015). These bacteria are intensively used in the food industry due to their important role in the formation of the organoleptic properties of fermented dairy, vegetable, cereal, and meat products as well as in averting food spoilage (Price et al. 2012). In addition, LAB can be used as cell factories for the biotechnological production of diverse functional biochemical compounds (Gaspar et al. 2013) or for the in situ food production of beneficial metabolites (LeBlanc et al. 2011; Li and Cao 2010; Ortiz et al. 2013).

Fructobacillus is a relatively new classified genus of LAB (Endo and Okada 2008) that comprises the species *Fructobacillus fructosus, Fructobacillus ficulneus, Fructobacillus durionis, Fructobacillus pseudoficulneus,* and *Fructobacillus tropaeoli*; with exception for the last one, the other species were earlier described as belonging to the genera *Lactobacillus* or *Leuconostoc* (Antunes et al. 2002; Chambel et al. 2006; Endo et al. 2011; Kodama 1956; Leisner et al. 2005). In nature, *Fructobacillus* spp. can be found in fructose-rich niches such as (i) tropical fruits, flowers, and

Published online: 03 July 2017

fermented foods made from fruits (Endo 2012), (ii) tropical ants and fruit flies that feed on tropical fruits (He et al. 2011; Thaochan et al. 2010), (iii) fermented cocoa beans (Snauwaert et al. 2013), and (iv) beehives, including honeybees, honeybee larvae, bee pollen, and fresh honey (Endo and Salminen 2013; Koch and Schmid-Hempel 2011; Rokop et al. 2015). In contrast to other LAB, most fructobacilli can utilize only a limited number of carbon sources such as fructose, glucose, and mannitol (Chambel et al. 2006; Endo et al. 2009, 2011). Moreover, growth on glucose is possible only in the presence of an external electron acceptor, e.g., oxygen (Endo 2012). The absence of alcohol dehydrogenase gene *adhE* in *Fructobacillus* spp. renders this genus incapable of NAD^+ regeneration through the conversion of acetaldehyde to ethanol (Endo et al. 2014). Thus, NAD^+ is regenerated through the conversion of fructose into mannitol, this reaction being catalyzed by the enzyme mannitol 2-dehydrogenase (MDH) (Endo 2012; Ortiz et al. 2013, 2017).

Mannitol has multiple industrial applications, being mainly used as a low-calorie sweetener in food technology and as a potent osmotic diuretic in medicine (Makhija et al. 2011). Food manufacturers mostly prefer mannitol, due to its good water solubility scope, low mold ability, lesser sensitivity towards humidity and, good wetting characteristics. Due to its low calorie content, mannitol is increasingly gaining prominence among people suffering from diabetics. While the global mannitol market size was valued at USD 209.4 million in 2015, the mannitol market revenue in the USA is expected to be duplicated in the next 10 years (http://www.grandviewresearch.com/industry-analysis/mannitol-market).

The current industrial process for mannitol production includes the application of high temperature (120–160 °C), nickel catalyst, and hydrogen gas to a mixture of fructose and glucose, resulting in a 25:75 mixture of mannitol and sorbitol, respectively, wherefrom mannitol separation becomes difficult and expensive (Makhija et al. 2011). Recent technological advancements to produce mannitol from raw materials have been applied (Ortiz et al. 2012). Whereas innovations are under process for production of this polyol using LAB, the increase in yield coupled with high-purity quality is expected in the near future.

The ability of fructobacilli and other LAB species to produce mannitol provides an opportunity for the in situ mannitol biosynthesis in fermented functional food products or to synthesize it as food or pharmaceutical compound. Thus, the biotechnological production of mannitol by microbial fermentation may be an excellent alternative to the current chemical process. In addition to *Fructobacillus* spp., other LAB species such as *Lactobacillus fermentum*, *Lactobacillus intermedius*, and *Lactobacillus reuteri* have been found to be efficient mannitol producers (Ortiz et al. 2012, 2015, 2017; Rodríguez et al. 2012; Saha and Nakamura 2003).

In this work, the mannitol production ability of the strain *F. tropaeoli* CRL 2034 isolated from fig was monitored in bioreactors using a rich medium with a fructose/glucose mixture as carbon source. As microbial mannitol biosynthesis may be induced by adverse environmental conditions, such as osmotic and oxidative stress (Chaturvedi et al. 1997; Kets et al. 1996; Sand et al. 2015), the total (high) saccharide content of the growth medium and the stirring speed were the factors studied to evaluate their effect on mannitol biosynthesis and to find the optimal conditions for its production by the assayed *Fructobacillus* strain. Response surface method with central composite designing together with the desirability function approach was used as experimental optimization strategy (Brzozowski and Lewandowska 2014; de Olmos et al. 2015). In addition to analyzing the carbohydrate consumption and mannitol biosynthesis, the activity of MDH was assessed during the exponential and stationary phase of growth during fermentation under the obtained optimized culture conditions. Finally, pure mannitol was easily isolated when growing the strain under these conditions.

Materials and methods

Isolation of LAB from figs

Wild ripe fig fruits were aseptically collected in Tucumán (Northwestern Province of Argentina), put into sterile bags, and immediately transported to the laboratory. LAB and fructophilic lactic acid bacteria (FLAB) were isolated by direct plating and by culture enrichment according to the methods described by Endo et al. (2009). Ninety milliliters of peptone water [0.1% (*w*/*v*) bacteriological peptone] were added to 10 g of cut ripe figs and homogenized for 1 min using a stomacher (Stomacher® 400, London, UK). For direct bacterial isolation, serial dilutions of the obtained suspension were plated onto deMan, Rogosa and Sharpe (MRS) (De Man et al. 1960) and MRSf [MRS containing 2% (*w*/*v*) of fructose instead of glucose] agar (1.5 *w*/*v*), both supplemented with 0.1 g/l cycloheximide and 0.1 g/l sodium azide to inhibit fungal and yeasts, and coliforms and Gram-negative microorganisms, respectively. On the other hand, bacterial isolation by culture enrichment was done by adding 5 ml of fructose–yeast extract–peptone (FYP) broth (Endo et al. 2009) to small pieces of fig sample and incubating at 30 °C for 24 h. Afterwards, 100 μl of the enriched culture was inoculated into fresh FYP broth for further incubation at 30 °C until visible bacterial growth. Subsequently, serial dilutions were plated onto FYP agar containing 5 g/l of $CaCO_3$. All plates were incubated at 30 °C; MRS and MRSf plates were incubated anaerobically (Anaerobic System AnaeroGen™, Oxoid Ltd., UK) for 24 to 72 h, while FYP plates were incubated

aerobically for 24 to 72 h. Different colonies were randomly picked and purified by streaking on suitable media.

Molecular typing of LAB isolates

Isolates were initially analyzed by Gram staining, microscopic morphology, and catalase activity. Gram-positive and catalase-negative isolates were selected as presumptive LAB, and further subjected to molecular typing and subsequent identification. Genomic DNA was extracted by using the commercial DNA extraction kit NucleoSpin® 96 Tissue (Macherey-Nagel GmbH & Co. KG, Germany), and used as template for molecular analysis. Isolates were classified as different strains according to the profiles obtained by rep-PCR fingerprinting (amplification of repetitive bacterial DNA elements through the polymerase chain reaction) of their genomic DNA with the primer $(GTG)_5$ (5′-GTGG TGGTGGTGGTG-3′) (Versalovic et al. 1994). The PCR reaction mixture (25 μl) consisted of 5 μl buffer [5X Green GoTaq® reaction buffer (Promega, WI, USA)], 1 μl of primer (63 mM), 7.35 μl nuclease free water, 2.6 μl $MgCl_2$ (50 mM), 4 μl BSA (1 mg/ml), 2.5 μl DMS [100% (*v*/*v*)], 1.25 μl mixture of deoxyribonucleoside triphosphates (dATP, dTTP, dCTP, and dGTP, 25 mM), 1 μl template DNA (50 ng/μl), and 0.3 μl Taq DNA polymerase (Promega), and the thermocycler program was as follows: 95 °C for 5 min; 30 cycles of 94 °C for 1 min, 40 °C for 1 min, and 65 °C for 8 min; and a final extension step at 65 °C for 16 min. The PCR products were electrophoresed in a 1.5% (*w*/*v*) agarose gel (15 × 20 cm) for 16 h at a constant voltage of 55 V in 1× TAE buffer [40 mM Tris–Acetate, 1 mM EDTA (pH 8.0)]. The rep-PCR profiles were visualized after staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) under UV trans-illuminator (Syngene, Cambridge, UK), and digital image documentation was done using a CCD camera (Canon, Tokyo, Japan). The fingerprints were analyzed by the BioNumerics V4.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived from the profiles.

Screening for mannitol-producing strains

One representative LAB isolate of each $(GTG)_5$-PCR fingerprint cluster was grown in two different culture media for determining mannitol production: (i) a formulated fruit simulation medium (FSM), with the following composition (g/l): glucose, 10.0; fructose 10.0; sorbitol, 5.0; malic acid, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.05; K_2HPO_4, 1.0; vegetable peptone, 10.0; and Tween 80, 1 ml; pH 6.10; and (ii) the slightly modified FYP (Endo et al. 2009) broth containing (g/l): vegetable peptone, 5.0; yeast extract, 10.0; glucose, 20.0; sodium acetate, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; sodium chloride, 0.01; and Tween 80, 0.5 ml/l). Cell-free supernatants (CFSs) of 24-h cultures grown in FSM and FYP were analyzed for mannitol content by high-pressure anion exchange chromatography (HPAEC) with pulsed amperometric detection as described by Camu et al. (2007). The strain that exhibited the highest mannitol synthesis was further identified and selected for optimization experiments (Table 1).

Molecular identification of the selected mannitol-producing LAB strain

Molecular identification of the selected LAB strain was performed by sequencing the 16S ribosomal RNA gene using the primer pair pA (5-AGAGTTTGATCCTGGCTCAG-3) and pH (5-AAGGAGGTGATCCAGCCGCA-3) (Edwards et al. 1989). PCR products were electrophoresed in a 1.0% (*w*/*v*) agarose gel at 100 V for 45 min in 1× TAE buffer, stained and visualized as described above. The size of DNA fragments of approximately 1500 bp was estimated using a standard 1-kb DNA ladder (1 Kb Plus DNA Ladder, Invitrogen™, Carlsbad, CA, USA). The nucleotide sequence was afterwards determined (Macrogen, Amsterdam, The Netherlands). The obtained rRNA gene sequence was compared with those available at the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/) by using the BLAST (Basic Local Alignment Search Tool) software (http://www.ncbi.nlm.nih.gov/BLAST), and identified as *F. tropaeoli*. The sequence obtained for this strain is available under the GenBank accession no. KY859794.

Culture conditions, experimental design, and statistical analyses

The strain *F. tropaeoli* CRL 2034 was deposited in the Culture Collection of Centro de Referencia para Lactobacilos (CERELA-CONICET), San Miguel de Tucumán, Argentina. Stock cultures of this strain were stored at −20 °C in a medium containing (g/l): skim milk, 100.0; yeast extract, 5.0; glucose, 10.0; and 10.0% (*v*/*v*) glycerol. Before experimental use, cultures were propagated (2%, *v*/*v*) twice in FYP and incubated at 30 °C for 15–17 h.

For each experiment, FYP medium was supplemented with a variable total saccharide content (75, 100, 150, 200, or 225 g/l) and inoculated with cells to reach an initial count of approximately 10^7 CFU/ml. All experimental fermentations were carried out at 30 °C and pH 5.0 with variable stirring settings (50, 100, 200, 300, or 350 rpm).

For experimental design and data analysis, the Minitab-15 statistical package was used. As *Fructobacillus* strains strictly require an external electron acceptor such as fructose or O_2 for

Table 1 Mannitol production by representative isolates from genotypically different clusters grown in FYP medium at 30 °C for 24 h

Cluster	Representative strain	Mannitol production (g/l ± SD)
I	F-H2-402	ND
II	F-H2-401	7.76 ± 0.36
III	H1-167	7.98 ± 0.24
IV	H1F-106	8.31 ± 0.13
V	H3-213	ND
VI	H3F-206	ND
VII	F-H2-438-2	8.89 ± 0.11
VIII	F-H3-466	8.10 ± 0.15
IX	F-H3-450	9.46 ± 0.27
X	H2-200	8.78 ± 0.11
XI	H3F-182	ND
XII	H3-241	ND
XIII	F-H3-442	8.21 ± 0.43

SD standard deviation, *ND* not detected

glucose dissimilation (Endo et al. 2014), the effects of total saccharide content (TSC) and stirring (S) on the mannitol production and yield responses were evaluated by response surface methodology (RSM) with central composite design (CCD) to find the optimal cultivation conditions. RSM methods are often used to find the optimal factor settings that produce the best response and to examine the relationship between the controllable factors and the responses. The levels of the two studied fixed factors (TSC and S) were defined according to a 2^2 full-factorial CCD with 4 axial points ($\alpha = 1.5$) and 4 replications of the center point, leading to 12 random experimental runs. This design allowed estimating the main effects and interaction and quadratic effects for each factor (TSC and S). The experiments were conducted using FYP medium using a variable TSC [glucose + fructose (1:2)] and variable S as indicated above (Tables 2 and 3). All fermentations were carried out in a 1.5-l bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ, USA) at 30 °C and pH 5.0. Samples were aseptically withdrawn at 24 h, and both linear and quadratic effects of the proposed factors (TSC and S) on both responses (mannitol production and yield) were analyzed.

After the collection of experimental data, a regression model was established by using RSM; the adequacy of the mathematical model and the incidence of the values of fixed factors and their interactions and significant relationships were statistically analyzed with an established significance level of 5%, as values of $p < 0.05$ considered significant by applying analysis of variance (ANOVA). Contour plots and plots of surface response were a complementary resource to figure out the factor values affecting each response. Subsequently, and as to optimize many responses a balance among them should be achieved, a multiple response prediction analysis using

Table 2 Independent variables and levels evaluated applying central composite design (CCD) for mannitol production by *Fructobacillus tropaeoli* CRL 2034

Variables	Levels				
	$-\alpha$	−1	0	+1	$+\alpha$
TSC (g/l)	75	100	150	200	225
S (rpm)	50	100	200	300	350

TSC total saccharide content, *S* stirring

the combined desirability function (D) was performed to optimize both studied responses simultaneously. The desirability function approach enables to transform a multiple response issue into a single response issue by using mathematical transformations. Desirability functions are different according to the objective. In this work, the objective was the maximization (the larger the best) of responses. Firstly, each response was transformed into a number between 0 and 1, named as individual desirability (d); a d value closer to 1 means a more desirable response. Secondly, individual desirabilities for each studied response had been combined [$D = (d_1 d_2 \ldots d_m)\ 1/m$], obtaining a combined desirability (D), and finally the optimization of D led the optimal factor values to optimize target responses.

Finally, to confirm the results shown by the applied optimization process, a validation experiment was conducted at the predicted optimum levels of TSC and S.

Table 3 Experimental conditions and results of CCD for mannitol production and yield by *F. tropaeoli* CRL 2034 as function of total saccharide content and stirring

Run	Variables		Responses	
	TSC	S	Mannitol production (g/l)	Yield (%)
1	100	300	54.13	76.60
2	150	200	87.93	98.55
3	150	50	80.93	86.46
4	150	200	72.93	72.84
5	75	200	47.06	90.26
6	200	100	84.97	69.31
7	150	200	80.67	86.17
8	225	200	94.32	78.54
9	150	200	80.41	80.85
10	200	300	98.39	76.43
11	150	350	74.46	75.35
12	100	100	57.76	83.24

TSC total saccharide content (g/l), *S* stirring (rpm), % g mannitol/g fructose consumed × 100

Microbiological and analytical analyses

For each single fermentation run, samples were aseptically withdrawn to monitor the fermentation process by measuring bacterial growth kinetics and metabolite production.

Bacterial growth

Bacterial growth of *F. tropaeoli* CRL 2034 was monitored in 0-, 2-, 4-, 6-, 8-, 10-, 12-, and 24-h cultures by measuring the optical density at 600 nm (OD_{600}) and cell dry mass determination (g/l) by using a thermogravimetric infrared humidity analyzer MA100C-000230 V1 (Sartorius AG, Göttingen, Germany). Cell viability was determined by plating serial dilution samples on FYP agar (FYP plus 1.5%, *w*/*v*, agar) and expressed as colony-forming units per milliliter (CFU/ml) after 48–72 h of incubation at 30 °C. Cell morphology was controlled by using a light microscope (CX, Olympus, Tokyo, Japan) using oil immersion (×100).

Metabolite analyses

CFSs of 24-h fermentation experiments were obtained by centrifugation (9200×*g*, 10 min) and further analyzed for residual carbohydrate, organic acid (lactic acid and acetic acid), and mannitol concentrations. The consumption of glucose and fructose and the production of mannitol and organic acids were determined by high-performance liquid chromatography (HPLC) as described before (Ortiz et al. 2015b). Briefly, all determinations were done using a Smartline pump 100, a WellChrom K-2301 refractive index (RI) detector, a Smartline autosampler 3800 Plus (Knauer GmbH & Co., Berlin, Germany) and a ZC-90 oven (Zeltec, Buenos Aires, Argentina). A Rezex RPM-Monosaccharide Pb^{+2} (300 × 7.8 mm) column (Phenomenex Laboratories Inc., Torrance, CA, USA) at 85 °C with Milli-Q water as mobile phase (flow rate of 0.6 ml/min) was used for determining carbohydrates and mannitol, whereas a Rezex ROA-Organic Acid H^+ (8%) (Phenomenex Laboratories Inc.) column at 41 °C with 5 mM H_2SO_4 as mobile phase (flow rate of 0.6 ml/min) was used to measure organic acids. Data were analyzed by the *Eurochrom Basic Edition for Windows* software. Mannitol yield was calculated as the ratio between the amount of mannitol produced and the amount of fructose consumed in a defined time interval × 100 [(g/g) × 100], representing the conversion efficiencies of fructose into mannitol (Ortiz et al. 2012).

Preparation of cell-free extracts

For cell-free extract (CFE) preparation, cells from 5 ml of fermented culture at each sampling point were harvested by centrifugation (9,200×*g*, 10 min), washed three times with cold 50 mM sodium phosphate buffer (pH 5.5), and finally mixed with glass beads (150–212 μm, Sigma-Aldrich Chemical Co., MO, USA) in a 1:2:1 (mg cells/μl buffer/mg beads) ratio. Subsequently, cells were disrupted using a Mini Bed Beater-8 (Biospec Products Inc., OK, USA) for 10 min (with 2 min disruptions on ice every 2 min) at maximum speed. Cell debris and glass beads were removed by centrifugation (14,700×*g*, 5 min, 4 °C), and supernatants were immediately used as CFE for enzymatic activity determination.

Measurement of mannitol 2-dehydrogenase activity

Mannitol 2-dehydrogenase (MDH) activity was measured in 0-, 2-, 4-, 6-, 8-, 10-, 12-, and 24-h cultures as described before (Ortiz et al. 2015). Briefly, the enzymatic activity was determined in a 200-μl volume; the assay mixture contained 50 μl of 200 mM sodium phosphate buffer (pH 5.5), 50 μl of 2 mM NADPH (Sigma-Aldrich Chemical Co.), 50 μl of Milli-Q water, and 10 μl of 0.5 mg protein/ml CFE. The reaction mixture was acclimatized at 30 °C for 2 min, and the reaction was initiated by adding 40 μl of 1 M fructose (Sigma-Aldrich Chemical Co.). MDH activity was determined spectrophotometrically on a Cary 50 MPR microplate reader (Varian Inc., CA, USA); the consumption of NADPH was monitored by measuring the absorbance at 340 nm (ε340, 6220/M cm) for 5 min. One unit (U) of MDH activity was defined as the amount of enzyme needed to catalyze the consumption of 1 μmol of NADPH per min (reducing the fructose) under the experimental conditions set. Specific MDH activity was expressed in units per milligram of protein (U/mg).

Protein content (mg) of CFE was determined by using the Bio-Rad dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions; bovine serum albumin was used as standard.

Mannitol isolation and purification

CFS from 24 h fermentation culture under optimized conditions was obtained by centrifugation (9200×*g*, 20 min). As mannitol precipitates at a minimum concentration of 180 g/l at 4 °C, the isolation was done following two procedures: Firstly, 200 ml of CFS was concentrated 2.5-fold by evaporation (50 °C, 50 rpm for 50 min) using a rotary evaporator Hei-VAP Advantage (Heidolph, Schwabach, Germany). Then, the CFS was cooled down to 4 °C for 72 h to induce crystallization. Crystals were recovered by vacuum filtration using a vacuum flask and a Büchner funnel with filter paper (Double Rings 102 Medium Qualitative Flow, Hangzhou Xinhua Paper Industry Co., LTD, Zhejiang, China) washing the crystals with cold water. Filtration was performed immediately after the cooling step to prevent crystal dissolution. In the second procedure, 100 ml of CFS was subjected to a clarification step by adding 5% (*w*/*v*) of activated coal, mixed for

5 min, filtered, and further concentrated at 50 °C, 50 rpm for 25 min. Then, mannitol was precipitated and recovered as described above. Mannitol crystals were observed by means of a binocular loupe (Wild Heerbrugg, Heerbrugg, Switzerland) and an optical microscope (Zeiss, Göttingen, Germany).

The purity of the isolated mannitol was assessed by determining the melting temperatures of the crystals with a heating microscope with temperature readings in the eyepiece (Mikroskop Heiztisch 350, Ernst Leitz GMBH, Wetzlar, Germany).

Results

Isolation, identification, and selection of the *F. tropaeoli* strain

Three hundred and seventeen colonies were picked from MRS, MRSf, and FYP plates from which 126 Gram-positive and catalase-negative isolates were classified as putative LAB. The dendogram containing the $(GTG)_5$-PCR fingerprint profiles of the LAB isolates from fig samples appeared distributed into 13 clusters displaying genotypically different LAB strains (Fig. 1). One representative isolate of each profile group representing distinctive LAB strains was selected to screen for mannitol production as mannitol is synthesized from fructose, one of the main carbohydrate component in fruits.

From the 13 genotypically different LAB isolates, only representative strains belonging to 8 clusters were able to produce mannitol in the range of 7.76–9.46 g/l; the amount of mannitol produced was similar in both FSM and FYP media and strain dependent (Table 1). The best mannitol producer strain was the isolate FYP-H3-450 (Fig. 1, cluster IX), which was able to synthesize 9.46 ± 0.27 g/l of mannitol from 10 g/l of fructose present in the culture medium. This strain was molecularly identified as *F. tropaeoli* and selected for the subsequent mannitol production studies using the FLAB-specific FYP medium. The strain (CRL 2034) was deposited in the Culture Collection of CERELA-CONICET and the 16S rDNA sequence (1242 bp) in the GenBank database (accession number KY859794).

Optimization of mannitol production and yield by CCD and multivariate analysis

The CCD matrix and the obtained experimental data to determine the effects of the factors studied, TSC and S, on the responses of mannitol production and yield by *F. tropaeoli* CRL 2034 are shown in Table 3. The ANOVA, regression analysis, linear and quadratic effects of the factors TSC and S, and significance of the model coefficients were analyzed for each response (Tables 4 and 5). Significant p values for linear effects of TSC on mannitol production were found while no effect was noticed for the S factor. Moreover, the interaction between both factors on this response was not significant ($p > 0.05$) (Table 4). Table 5 shows that the main effect of TSC was 50.25 (g/l) on mannitol production. This means that, on average, mannitol production increased by 50 g/l when TSC increased from 100 to 200 g/l, which was also reflected in the surface graphics (Fig. 2). This value was very significant regarding the variation of the experimental data observed. When analyzing the model coefficients for mannitol yield, the p values were >0.05, indicating that no effect of the two factors studied on mannitol yield was detected. The response surface plot and contour plot displaying complementary information about mannitol production and yield are shown in Figs. 2 and 3, respectively. In summary, the mannitol production adjusted univariate model showed that only TSC provoked great differences on this response; in contrast, neither TSC nor S showed significant effects on the mannitol yield (Tables 4 and 5).

The fit of the model was checked by the adjusted coefficient of determination (adj. R^2), which explained 88.85 and 0.00% of the variability of mannitol production and yield, respectively. These results indicated that when analyzing both responses individually, the model equations were suitable for predicting mannitol production but not mannitol yield.

The trends of the responses studied were inversely related to the increase in the factors, meaning that the increase of TSC and/or S caused an increase in mannitol production and a decrease in mannitol yield (Figs. 2 and 3); thus, a multivariate analysis with desirability function (D) approach to optimize both responses studied was carried out. Individual responses are transformed into desirability (d) values and the D (0–1) is afterwards computed as the geometric mean of both d. The response surrounds the target when D gets closer to 1. D was calculated by setting TSC and S within the experimental range to maximize the two responses evaluated (Fig. 4). Implementation of multiple response optimization led to obtaining theoretical values of TSC and S at 165 g/l and 200 rpm, respectively, with predicted values of mannitol production and mannitol yield at 85.03 g/l [95% CI (78.68, 91.39)] and 82.97% [95% CI (72.34, 93.59)] (Table 6). The D calculated value was 0.588.

Validation of the model

To validate the adequacy of the model, a verification experiment was carried out for the predicted optimum parameters (Fig. 5). The optimized culture conditions enabled obtaining a mannitol production value of 81.9 g/l and a yield of 77.5%, in outstanding agreement with the theoretical expected values. The percentage errors for mannitol production and yield were 3.68 and 5.51%, respectively, suggesting a suitable model. Experimental values of both responses showed the high

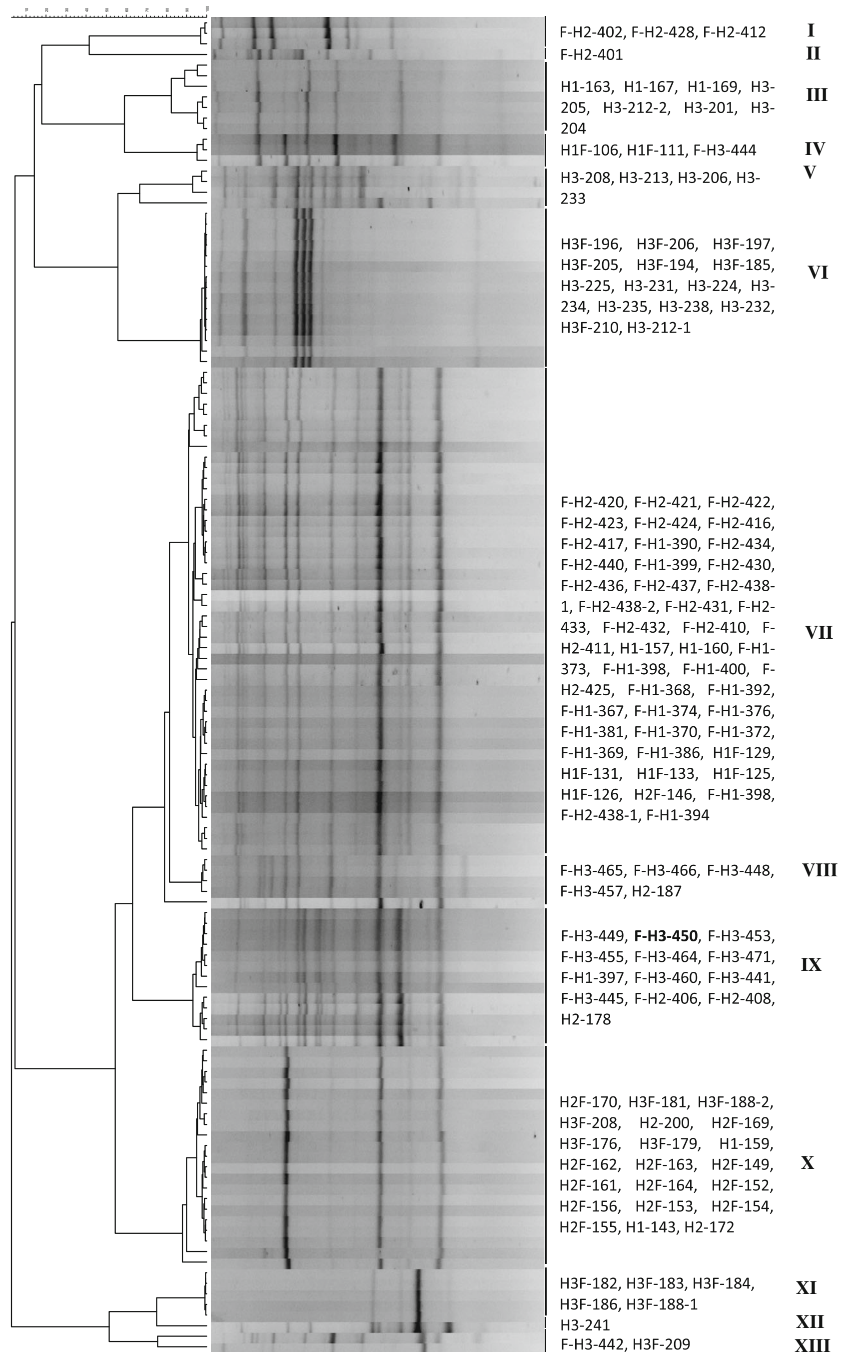

Fig. 1 Cluster analysis of the generated $(GTG)_5$-PCR fingerprints of 126 LAB isolated from fig fruit (clusters *I-XIII*). Species identification of the representative strain with highest mannitol production was carried out by partial 16S rRNA gene sequence analysis. F-H3-450 (in *bold*, cluster *IX*) was the selected strain for mannitol studies

predictive ability of the dual optimization, confirming the adequacy of the selected approach.

Bacterial growth and fermentation kinetics of *F. tropaeoli* CRL 2034 grown under the predicted optimized culture conditions are shown in Fig. 5. The exponential growth phase (as determined through cell counts, cell dry mass and optical density) ranged from 4 to 8 h of fermentation, with a μ_{max} of 0.99/h, and was coincident with the highest decrease of the carbon sources fructose and glucose, which were completely depleted after 24 h of incubation. Production of organic acids, namely lactic acid and acetic acid, started after 6 h of incubation, showing a 2:1 lactic acid/acetic acid concentration ratio from 8 h to the end of the experiment. The maximum concentrations (26.8 and 13.3 g/l, respectively) were obtained after 24 h of fermentation. Fructose was mainly used by *F. tropaeoli* CRL 2034 as an alternative electron acceptor for mannitol production, which began after 4 h of incubation and continued beyond bacterial growth; the maximum value (81.9 g/l) being reached at 24 h (Fig. 5b). In contrast, the specific MDH activity values were highest (4.9 U/mg protein) during the log phase, whereas a slight decrease was detected from that period on (Fig. 5b).

Table 4 Analysis of variance (ANOVA) and regression analysis for mannitol production and yield by *F. tropaeoli* CRL 2034

Source			Mannitol production					Mannitol yield				
			DF	Adj SS	Adj MS	*F* value	*p* value	DF	Adj SS	Adj MS	*F* value	*p* value
Model	Linear		5	2615.89	523.18	18.54	0.001	5	262.66	52.53	0.67	0.664
			2	2384.39	1192.20	42.24	0.000	2	148.95	74.47	0.94	0.440
		TSC	1	2384.39	2384.39	84.48	0.000	1	118.16	118.16	1.50	0.267
		S	1	0.00	0.00	0.00	0.996	1	30.79	30.79	0.39	0.555
	Square		2	158.88	79.44	2.81	0.137	2	66.32	33.16	0.42	0.675
		TSC*TSC	1	158.10	158.10	5.60	0.056	1	13.49	13.49	0.17	0.694
		S*S	1	15.93	15.93	0.56	0.481	1	63.28	63.28	0.80	0.405
		2-way interactions	1	72.61	72.61	2.57	0.160	1	47.39	47.39	0.60	0.468
		TSC*S	1	72.61	72.61	2.57	0.160	1	47.39	47.39	0.60	0.468
Error			6	169.35	28.22			6	473.61	78.93		
	Lack-of-fit		3	56.87	18.96	0.51	0.705	3	124.29	41.43	0.36	0.791
	Pure error		3	112.48	37.49			3	349.32	116.44		
Total			11	2785.23				11	736.27			

Mannitol production: R^2 = 93.92%, R^2_{adj} = 88.85%, $R^2_{(pred)}$ = 78.14%; mannitol yield: R^2 = 35.67%, R^2_{adj} = 0.00%, $R^2_{(pred)}$ = 0.00%

TSC total saccharide content, *S* stirring, *DF* degrees of freedom, *SS* sum of square, *MS* mean square

Mannitol isolation and purification

Mannitol was relatively easily isolated after cool precipitation. Two isolation procedures were followed with and without a clarification step using activated coal before concentration. Although non-clarified crystals were needle-shaped (Fig. 6a-d) and clarified crystals were prism-shaped (Fig. 6e-h), the melting temperature (Tm) of both types of crystal samples showed high purity according to their Tm of 165 ± 1 °C (water) and 168 ± 1 °C (water), respectively.

Discussion

Mannitol is a polyol or sugar alcohol derived from D-fructose, naturally produced by certain organisms including LAB (Ortiz et al. 2013). From industrial and commercial standpoints, mannitol is a highly interesting compound, as it has several applications in foods, pharmaceutical, and medical fields. The prospective of a more economical biotechnological production compared with the current chemical synthesis is driving to flourishing new research on mannitol biosynthesis by fermentation using LAB strains. Among LAB, heterofermentative species stand out by being excellent mannitol producers with high yields from glucose/fructose (1:2) mixtures (Makhija et al. 2011).

According to this framework, we isolated 126 presumptive LAB present in figs from Northwestern Argentina, from which 13 different strains were screened for mannitol production. The best mannitol producer was the fructophilic strain FYP-H3-450, which was genotypically identified as *F. tropaeoli*. Other *Fructobacillus* species such as *F. pseudoficulneus* has been previously isolated from figs by Endo et al. (2009). These authors have shown that FLAB

Table 5 Coefficient values for mannitol production and yield by *F. tropaeoli* CRL 2034

Term	Mannitol production						Mannitol yield					
	Effect	Coef	SE Coef	*T* value	*p* value	VIF	Effect	Coef	SE Coef	*T* value	*p* value	VIF
Constant		80.42	2.65	30.38	0.000			84.20	4.43	19.02	0.00	
TSC	50.25	25.12	2.73	9.19	0.000	1.00	−11.19	−5.59	4.57	−1.22	0.27	1.00
S	0.03	0.02	2.73	0.01	0.996	1.00	−5.71	−2.85	4.57	−0.62	0.56	1.00
TSC*TSC	−20.52	−10.26	4.34	−2.37	0.056	1.07	−6.00	−3.00	7.25	−0.41	0.69	1.07
S*S	−6.51	−3.26	4.34	−0.75	0.481	1.07	−12.98	−6.49	7.25	−0.90	0.41	1.07
TSC*S	19.17	9.59	5.98	1.60	0.160	1.00	15.50	7.70	10.00	0.77	0.47	1.00

TSC total saccharide content, *S* stirring

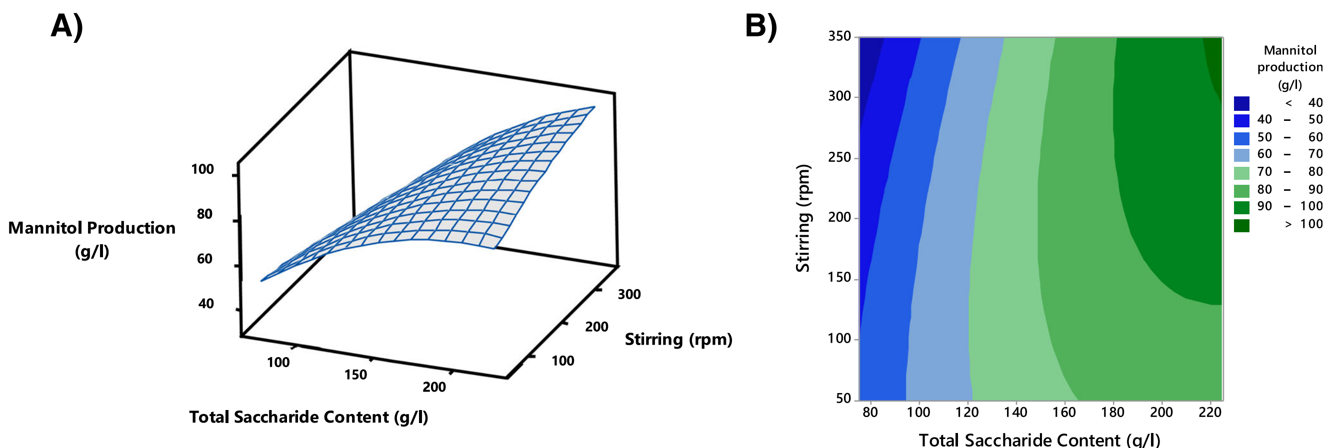

Fig. 2 Response surface plot (**a**) and contour plot (**b**) of interaction between the factors total saccharide content and stirring and their effect on mannitol production by *F. tropaeoli* CRL 2034

have unique biochemical properties comparing to LAB, such as the synthesis of mannitol from fructose that is used as an electron acceptor in the presence of glucose.

To exploit the potential of a microorganism for the production of a specific metabolite, it is crucial to optimize its culture parameters. Since several authors have reported that mannitol is accumulated in bacteria as an osmoprotective or antioxidant compound in response to environmental stress (Wisselink et al. 2002), this study aimed to evaluate the carbohydrate content of the medium (as osmotic stress) and stirring (as oxidative stress) as an attempt to promote and optimize mannitol biosynthesis by the fruit isolate *F. tropaeoli* CRL 2034.

Commonly, the influence of different parameters on fermentations is studied with conventional *one variable at a time* (OVAT) methods, but as these procedures are time-consuming and do not include mutual interaction among variables (Lim et al. 2007), the tendency of applying statistical optimization methods is growing, due to the suitability of this approach for a vast range of processes. Here, we used RSM with CCD to determine the main and interactive effects of factorial combination of TSC and S on mannitol production and mannitol yield by the *Fructobacillus* strain used and to model and optimize the conditions studied of the production process. The studied fructophilic strain was able to synthesize high amounts of mannitol with values close to 100 g/l after 24 h incubation, one of the highest values reported by a LAB strain to date. By applying RSM with CCD, the experimental results obtained for mannitol production (81.9 g/l) and mannitol yield (77.5%) were strongly consistent with the predicted results (mannitol production of 85.03 g/l; mannitol yield of 82.97%). These methodologies have been successfully used in the optimization of various processes involving LAB. For instance, exopolysaccharide production by *L. confusus* TISTR 1498 from sugarcane juice has been optimized (Manochai et al. 2014). Also, the optimal nitrogen concentration and culture medium pH value to enhance the biosynthesis of the intracellular prolyl endopeptidase enzyme by *L. acidophilus* 5e2 have been studied (Brzozowski and Lewandowska 2014).

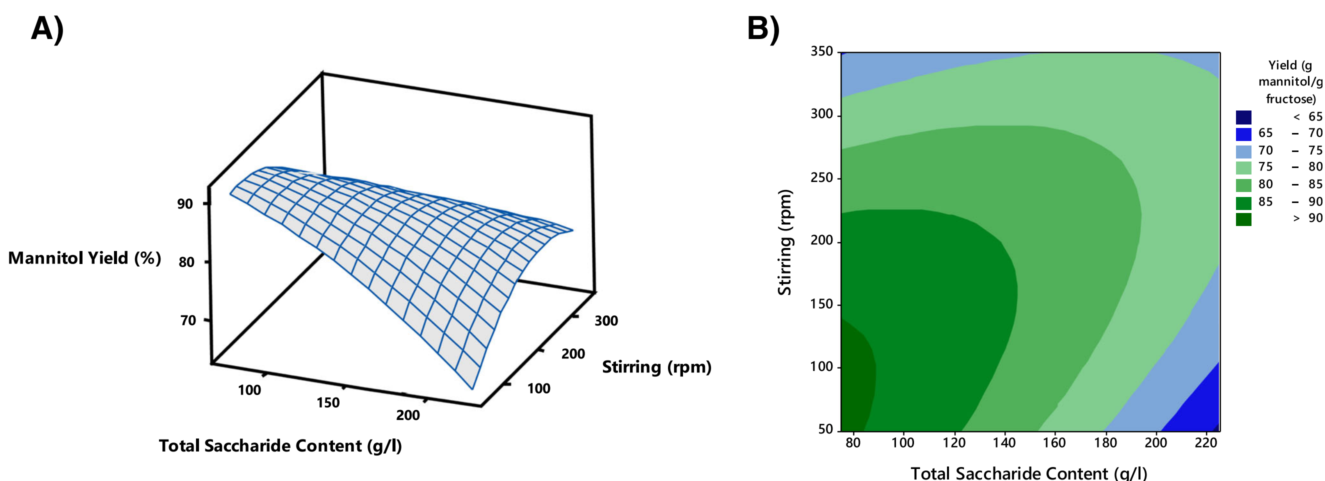

Fig. 3 Response surface plot (**a**) and contour plot (**b**) of interaction between the factors total saccharide content and stirring and their effect on mannitol yield obtained by *F. tropaeoli* CRL 2034

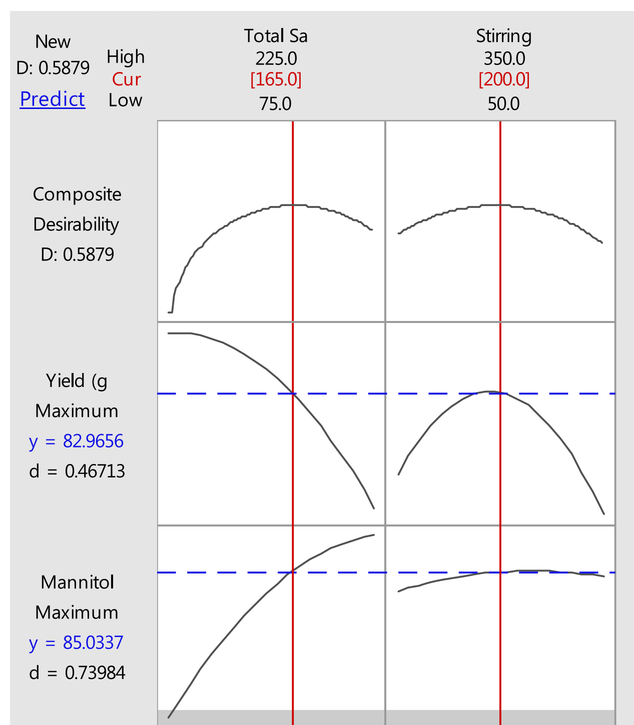

Fig. 4 Optimization plot of multiple response prediction analysis for simultaneous optimization of mannitol production and mannitol yield by *F. tropaeoli* CRL 2034

Moreover, CCD has also facilitated the maximization of lactic acid production by a strain of *Lactococcus lactis* by setting the optimal values of inoculum size, temperature, and skim milk dry matter added for the fermentation process (Hassaïne et al. 2014). More recently, the moisture content, temperature, and microbial inoculum for soy-paste fermentation by *L. paracasei* subsp. *paracasei* and *Bifidobacterium longum* strains have been optimized by applying an analogous approach (de Olmos et al. 2015). Similar methodologies have been reported in optimization studies of mannitol production by LAB. von Weymarn et al. (2002b) applied RSM to evaluate the effect of temperature and pH on specific mannitol productivity and yield by the strain *Leuconostoc mesenteroides* ATCC 9135, achieving high volumetric mannitol productivity (26.2 g/l h) and mannitol yield (97 mol%) using high-cell density membrane cell-recycle cultures. Also, the effects of four salt nutrients (ammonium citrate, sodium phosphate, magnesium sulfate, and manganese sulfate) on the production of mannitol by *L. intermedius* NRRL B-3693 have been tested by using a fractional factorial design, reaching a production of 200.6 ± 0.2 g mannitol using 300 g of fructose per liter in 67 h under optimized conditions (Saha 2006).

The ANOVA of the univariated regression equations models of this design indicates that TSC was the most significant positive factor for both responses investigated, whereas S was not significant in any of the two cases. The goodness of fit of the model for mannitol production, as assessed by determining R^2, showed that 93.92% of the sample variation for this response was attributable to independent factors, whereas for mannitol yield, the model could describe only 35.67% of the variation. Literature data suggest that a good fit of a model involves a $R^2 \geq 80\%$ (Betiku et al. 2016); thus, the model applied for mannitol production was significant although not for mannitol yield.

The results obtained in this work for mannitol production by *F. tropaeoli* CRL 2034 showed that this strain is a promising mannitol producer to scaling up the production process. Some *Lactobacillus* and *Leuconostoc* strains have been used in various attempts to improve the microbiological production of mannitol during batch fermentation processes (Makhija et al. 2011; Ortiz et al. 2013). *L. intermedius* NRRL B-3693 showed a mannitol production of 198 g/l from a high fructose initial concentration (300 g fructose/l, pH 5.0, 37 °C, 130 rpm) as this strain, as well as *F. tropaeoli* CRL 2034, was able to grow under high osmotic pressure (Saha and Nakamura 2003). Mannitol production by the strains *Lactobacillus* sp. Y-107 (100 g fructose/l, pH 8.0, 35 °C, 120 h) and *Leuconostoc* sp. Y-002 (50 g fructose/l, pH 6.0, 35 °C, 25 h), isolated from kimchi, showed values of 73 and 26 g of mannitol from 100 g of fructose/l with yields of 86 and 65%, respectively (Yun and Kim 1998). Contrary to the *Fructobacillus* strain of this study, those isolates were not able to use carbohydrate concentrations at higher levels than 100 g/l, displaying low osmotolerance. *L. fermentum* NRRL B-1932 was capable of producing mannitol with yields of 86, 89, and 94 mol% when growing at 25, 30, and 35 °C, respectively, in a

Table 6 Optimized values of total saccharide content and stirring factors, and predicted responses values for mannitol production and yield by *F. tropaeoli* CRL 2034

Variable	Setting			
Total saccharide content	165			
Stirring	200			
Response	Fit	SE Fit	95% CI	95% PI
Yield (g mannitol/g fructose)	82.97	4.34	(72.34, 93.59)	(58.77, 107.16)
Mannitol production (g/l)	85.03	2.6	(78.68, 91.39)	(70.56, 99.50)

Fit fitted values, *SE Fit* standard error of the fit, *CI* confidence interval, *PI* prediction interval

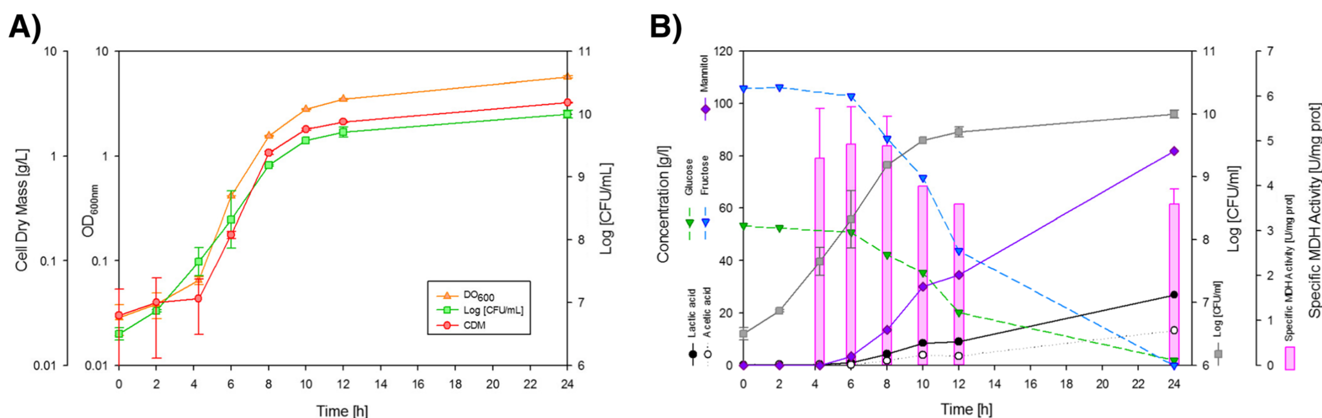

Fig. 5 Cell growth and fermentation profile of *F. tropaeoli* CRL 2034 grown under optimized conditions (total saccharide content 165 g/l, stirring 200 rpm) for mannitol production and yield during incubation at 30 °C and pH 5.0 for 24 h. **a** Growth kinetics. **b** Sugar consumption, organic acid and mannitol production, and specific MDH activity

medium containing fructose (20 g/l) and glucose (10 g/l) (von Weymarn et al. 2002a). *L. reuteri* CRL 1101 and *L. mucosae* CRL 573 (before misidentified as *L. fermentum* CRL 573) were capable to produce mannitol; a maximal production (22.22 and 56.84 g/l) and yield (75.7 and 93.5 mol%) were achieved at constant pH 5.0 for both strains, respectively (Rodríguez et al. 2012). Furthermore, to give added value to by-products of other industries and/or to reach lower production-costs, alternative substrate sources or nutrients for mannitol production have been investigated. Cashew apple juice has been used as substrate for mannitol production by *Leuc. mesenteroides* B-512, achieving a value of 18 g/l with a yield of 67% (Fontes et al. 2009). *Leuc. fructosum* NRRL B-2041 was able to produce mannitol (43.7 g/l) in a medium supplemented with hydrolyzed carob syrup, reaching a mannitol yield of 100% (Carvalheiro et al. 2011). The use of sugarcane molasses as low-cost substrate allowed obtaining a maximal mannitol concentration of 32.8 g/l after 24 h of fermentation with *L. reuteri* CRL 1101 (Ortiz et al. 2012). Among the current available data, which allow asserting that mannitol production is strain-dependent, the results of this study show that *F. tropaeoli* CRL 2034 is a very promising mannitol producer, displaying high production values and yields under optimized culture conditions.

Analyzing strain behavior and mannitol production during the optimal fermentation process, the mannitol synthesis by *F. tropaeoli* CRL 2034 began during the log growth phase increasing continuously until 24 h of fermentation; similar results were observed for *L. intermedius* NRRL B-3693 that converted fructose into mannitol from the early growth phase (Saha and Nakamura 2003). In the presence of a glucose/fructose mixture (1:2 ratio), the *Fructobacillus* strain studied consumed glucose as carbon source, producing lactic acid and acetic acid instead of ethanol, and used fructose almost completely as electron acceptor with the accompanying production of a large amount of mannitol, showing conversion

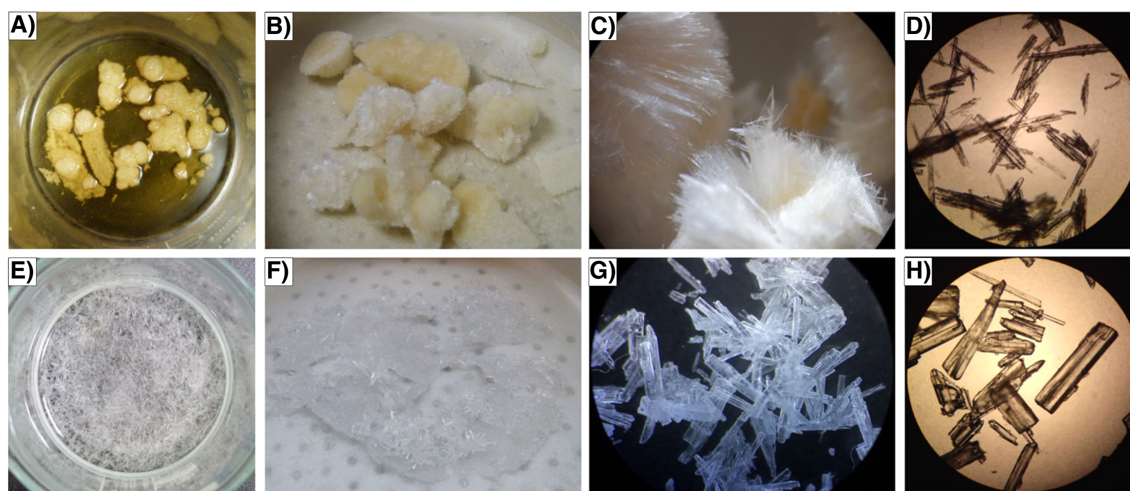

Fig. 6 Mannitol isolation from concentrated **a**, **b** non-clarified culture supernatant and **e**, **f** clarified culture supernatant of *F. tropaeoli* CRL 2034 after incubation at 4 °C. **c**, **d** Needle-shaped mannitol crystals from non-clarified culture supernatant as observed by binocular loupe (**c**) and optical microscope (**d**, magnified ×40). **g**, **h** Prism-shaped mannitol crystals obtained from clarified culture supernatant observed by binocular loupe (**g**) and optical microscope (**h**, magnified ×40)

efficiencies of 77.5%. The absence of ethanol production has been attributed to the lack of an alcohol/acetaldehyde dehydrogenase gene (*adhE)* in the *Fructobacillus* genus (Endo et al. 2014). The strain *L. intermedius* NRRL B-3693 showed a comparable behavior (Saha and Nakamura 2003). Concerning the MDH activity, the highest values (4.6–4.9 U/mg protein) were found between 4 and 8 h of fermentation that corresponded with the log and early stationary growth phases; these values being the highest registered so far from a MDH enzyme crude extract (Ortiz et al. 2013). Studying the correlation between mannitol production and MDH activity of *L. reuteri* CRL 1101 has shown that the highest MDH activities (3.646–2.064 U/mg cell protein) are obtained during the log growth stage (4 h), independently of the fermentation conditions assayed (Ortiz et al. 2012).

Mannitol isolation was easily achieved by concentrating the culture supernatant 2.5-fold, an amount twice lower than that used with *L. reuteri* CRL 1101 (Ortiz et al. 2015); in contrast, there was no need for a concentration step before cool precipitation when using *L. intermedius* NRRL B-3693 (Saha and Nakamura 2003). The shape of the crystals obtained from non-clarified supernatant was similar to the small white needle-like crystals reported before (Ortiz et al. 2015; Saha and Nakamura 2003), which differed from the crystals obtained from clarified supernatant that were larger and prism-shaped. Interestingly, both types of mannitol crystals obtained in this study presented melting points close to the melting point of the pure compound (168 °C) (Company 1967–1968), indicating the high purity of the fermentation product regardless the application of the activated coal clarification step. This extra step at industrial scale processes would represent an additional cost; so, it could be ignored depending on the destination and use of the final product.

To conclude, in this work, a RSM-CCD combined methodology was used to produce high amounts of high-purity mannitol from a fructose-rich medium using the fructophilic strain *F. tropaeoli* CRL 2034. To our knowledge, this is the first report of a statistical optimization of the culture conditions for mannitol production by a *Fructobacillus* strain, setting the background for the biotechnological production of mannitol at industrial scale.

Acknowledgements We are grateful to Prof. Dr. César Catalán from the Universidad Nacional de Tucumán for equipment supply and assistance during the melting temperature determination of mannitol crystals.

Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

Funding The study received support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 003) and Fondo para la Investigación Científica y Tecnológica (FONCyT, Préstamo BID PICT 2014-312) from Argentina; the Tallin University of Technology, Estonia, for awarding KA the internship scholarship to work in CERELA; and the Belgian Argentinean Fermented Foods (BAFF) project from the Vrije Universiteit Brussel, Belgium. LRR is recipient of a doctoral fellowship from CONICET.

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