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Kinetic parameters of *Lactobacillus plantarum* and *Saccharomyces boulardii* growing in a beet molasses culture media

Parámetros cinéticos del cultivo de *Lactobacillus plantarum* y *Saccharomyces boulardii* en un medio de cultivo a base de melaza de betabel

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Abstract

Regular consumption of probiotic microorganisms as part of the diet can improve health and mitigate the effects of metabolic syndrome diseases. It is then essential to design production processes for this type of microorganisms, which are optimal from the point of view of production time and cost. This paper presents a study of the effect of using a prebiotic-enriched culture medium, formulated with beet molasses, which manages to enhance the growth of two types of probiotic microorganisms, *Saccharomyces boulardii*, and *Lactobacillus plantarum*. In addition to its effect on cellular biomass growth, a kinetic model for both strains growth is also presented. This model includes factors such as the *S. boulardii* capability to hydrolyze saccharose into their respective monosaccharides and the inhibition effect of lactic acid production on *L. plantarum* growth. Finally, the simulation of a mixed culture production scheme is proposed for both microorganisms to take advantage of the yeast's capability to hydrolyze saccharose to not require the addition of extra glucose to the culture medium.

Keywords: Bioreactor, fed-batch, mixed cultures, prebiotics, probiotics.

Resumen

El consumo regular de microorganismos probióticos como parte de la dieta, puede contribuir a mejorar la salud y a mitigar los efectos de las enfermedades del síndrome metabólico. Es por esto que resulta importante diseñar procesos de producción de este tipo de microorganismos, que sean óptimos desde el punto de vista de tiempo y costo de producción. En este trabajo se presenta un estudio del efecto del uso de un medio de cultivo enriquecido en prebióticos, formulado con base en melazas de betabel, que logra potenciar el crecimiento de dos tipos de microorganismos probióticos, *Saccharomyces boulardii* y *Lactobacillus plantarum*. Además de su efecto en el crecimiento de biomasa celular, se presenta también un modelo cinético para el crecimiento de ambos microorganismos, en el que se incluyen factores como la capacidad por parte de *S. boulardii* para hidrolizar sacarosa en sus respectivos monosacáridos, y la inhibición en el crecimiento de *L. plantarum*, debido a la producción de ácido láctico. Finalmente, se propone la simulación de un esquema de producción para ambos microorganismos, en una modalidad de cultivo mixto, con la finalidad de aprovechar la capacidad de la levadura de hidrolizar la sacarosa, para no requerir de la adición de glucosa suplementaria al medio de cultivo.

Palabras clave: Biorreactor, cultivo mixto, lote alimentado, prebióticos, probióticos.

1 Introduction

The human intestinal tract is a complex matrix where the host cells, nutrients, and microorganisms simultaneously interact (Walter and Ley, 2011). The intestinal mucosa is naturally colonized by more than 500 species of microorganisms, which corresponds to more than half of the wet weight of the colorectal material (Yatsunenko *et al*., 2012). The colonization of gut microbiota starts from birth and continues for the rest of the host life. Gut microbiota is responsible for many of the intestinal functions, and a dysbiosis leads to sickness and abnormal metabolic functions of the host (Carding *et al*., 2015).

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The human metagenome, which comprehends a mixture of human and microbial genes, is responsible for the degradation of some diet components, such as polysaccharides and fatty acids. The interactions that occur between probiotics and their host can be classified as communication axes between the species of microorganisms and specific organs. The response of enteroendocrine cells of the gastrointestinal tract to food consumption is connected through signaling with specific peptides and short-chain fatty acids of microorganisms in membrane receptors such as GPR41 expressed by enteroendocrine cells. These interactions generate protection of the epithelial cells, regulation of the fatty acids transport system, and stimulation of intestinal angiogenesis, among others (Nicholson *et al*., 2012). This metagenome is composed of two large families of bacteria, *Bacteroides*, and Firmicutes (Nicholson *et al*., 2012; Walter and Ley, 2011; Yatsunenko *et al*., 2012). Firmicutes are facultative aerobic microorganisms, also conceived as lactic acid bacteria, which include bacteria of the genera *Prevotella, Lactobacillus, Enterococcus*, and *Clostridium. Bacteroides* are anaerobic microorganisms, which comprise the genera *Bacteroides* and *Parabacteroides*, both families in constant change in the host (Mariat *et al*., 2009).

Since the discovery of probiotics, it has been proposed that the consumption of these living microorganisms could result in health benefits (Sánchez *et al*., 2017). Probiotics must survive the passage through the digestive system of the host and proliferate within all digestive tract and mucosa (Klarin *et al*., 2005). To achieve survival and cell adhesion, probiotics intake in the host diet has to be in concentrations not less than 10^8 UFC/ml (Kailasapathy and Chin, 2000; Martinello *et al*., 2017).

There is also a strong correlation effect that can be observed between the auto-aggregation phenomenon and the pH and transit time through the digestive system. This dependence gives probiotics such as *L. plantarum* the capacity to maintain their survival under starvation conditions and a wide pH range through the intestinal tract (Melgar-Lalanne *et al*., 2018). Meanwhile, *Saccharomyces boulardii* is a non-pathogenic yeast, with a probiotic effect, which has anti-microbial, enzymatic and metabolic activity, that may help alleviate even symptoms related to multiple sclerosis. This is achieved by increasing the effect of the enzymes present in the intestinal mucosa, improving the secretory activity of IgA, and the release of enzymes that contribute to the metabolism of carbohydrate absorption (Aghamohammadi *et al*., 2019). The main characteristic of *L. plantarum* and other *Lactobacillus* microorganisms, is that they produce lactic acid as the final electron acceptor in carbohydrate metabolism. Additionally, lactic acid bacteria (LAB) have the capacity to consume different carbohydrate sources, hexoses and pentoses (González-Leos *et al*., 2020), and also have limited abilities to synthesize amino acids from limited nitrogen sources, which makes them dependent on their concentration in the culture medium. LAB growth in minimal mineral media is usually slow since they prefer enriched culture media (De Man, J. C. Rogosa, M. and Sharpe, 1960). Many of these LAB also can synthesize proteolytic enzymes (membrane-anchored extracellular serine proteinases) as well as a wide variety of intracellular peptidases (Christensen *et al*., 1999). In addition, the simultaneous culture of LAB with different types of yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii*, has the potential to improve the growth performance of the LAB, due to the yeast capacity to secrete specific enzymes to promote pentose and hexoses fermentation (A. Nawaz *et al*., 2020).

Beetroot (*Beta vulgaris* L.) is a biannual vegetable that stores energy reserves into their root. This vegetable belongs to the family of *Chenopodiaceae* and branches a pair of cotyledons from which later develop a genuine oval to corniform leaves of dark green or reddish-brown color (Chhikara *et al*., 2019). The juice has significant amounts of vitamin B, iron, magnesium, and potassium, essential for human health (Domínguez *et al*., 2017). Beetroot stores mainly sugars and starches and therefore is attractive as a carbon and nutrient source for bioethanol production (Šantek *et al*., 2010) and microorganisms of industrial interest (Gamage *et al*., 2016; Kailasapathy and Chin, 2000). Beetroot is a highly industrialized crop; by processing its roots, large sugar productions can be achieved, even comparable to production from sugar cane (Renouf *et al*., 2008; Valli *et al*., 2012). Also, beetroot has large amounts of betalains, betacyanins, and betaxanthins, which are a class of water-soluble nitrogenous pigments (Kujala *et al*., 2002). Beetroot pigments are particularly attractive because of its antioxidant properties (Domínguez *et al*., 2017). All of these factors are of particular interest since Mexico has a large production of beetroot, about 70 thousand tons in 2016, being Puebla and Jalisco, the primary producing states. The waste generated in the industrialization of beets is commonly used as biofertilizers and for the extraction of antioxidant

pigments (Battistella Lasta *et al*., 2019; Lasta *et al*., 2019). Then the use of beets as the basis for culture medium formulation for probiotics growth and maintenance, particularly for strains of the genus *Lactobacillus* and *Saccharomyces*, result especially attractive to the food biotechnology industry. There is strong evidence that phenolic compounds modulate the composition of gut microbiota improving a variety of biochemical signaling between strains resulting in cell growth (de Llano *et al*., 2017; García-Hernández *et al*., 2018).

Given the current context, it is essential to generate solutions based on natural products with minimal environmental impact. In this work, we propose the use of a culture medium formulated with residues from beet industrialization to maximize the production of probiotic strains of *Lactobacillus plantarum* and *Saccharomyces boulardii*. In addition, we propose a growth kinetic model for *L. plantarum* and *S. boulardii*, that can be used to design optimal production schemes for these probiotics.

2 Materials and methods

2.1 Microorganisms and growth conditions

2.1.1 Microorganisms and growth conditions

Lactobacillus plantarum BG112 (LP) is a food and pharmaceutical strain from SACCO®, Tlajomulco, México. *Saccharomyces boulardii* CNCM I-745 (SB) was isolated from Floratil®. LP and SB cells were grown in 250-ml Erlenmeyer flasks with 50 ml of MRS broth (De Man, J. C. Rogosa, M. and Sharpe, 1960) and 50 ml of Yeast-Peptone-Dextrose broth (YPD) (Gamage *et al*., 2016), respectively, to reactivate lyophilized powder and conduct all experiments. Growth conditions were 37 °C, 150 rpm, pHC = 6.5 with NaOH (10M). For both strains, the inoculum propagation used consecutive cultures of 24-12-6 h growth to obtain cells in the middle of the exponential growth phase.

2.1.2 Culture media formulation

Two different culture media were used for each strain; both were based on beet molasses. The base culture media was formulated with beet molasses to improve cellular biomass production, with a lower cost of carbohydrate source. Beetroot residues are reduced in size with knife mill until it reaches an average size of 2.5 cm. Afterward, juice and solids obtained in the previous step were cooked in T-304 stainless steel kettles with a capacity of 16 L (INTERTECNICA MMV-4-AS / CR, Mexico). After cooking, they were processed by means of a JERSA pulper (Mexico), model L, and subsequently filtered by means of a filter mat. Finally, the product obtained from the filtration was boiled until obtaining molasses of 50 ºBx. The final composition of beet molasses culture media (MBT medium) consisted of saccharose (40 g) (Merck), beet molasses (100 g), green tea extract (100 g) (Zoma Tea Collection®), yeast extract (5.71 g), and tap water to a final volume of one liter, adjusted to a final pH of 6.5. *Saccharomyces boulardii* used base culture media (MBT), and *Lactobacillus plantarum* used enriched beet molasses culture media (EMBT) with glucose (20 g) (Merck). Culture media were sterilized at 121 °C and 15 PSI in a YAMATO (SN300, JP.) sterilizer.

2.2 Bioreactor experiments

A series of experiments were carried out at a lab-bioreactor scale to evaluate MBT and EMBT culture medium and propose joint fermentation schemes. *Saccharomyces boulardii* was cultivated in batch and *Lactobacillus plantarum* in fed-batch cultures. Afterward, mixed culture schemes were evaluated through simulation with the parameters obtained.

2.2.1 Batch cultures

Saccharomyces boulardii cultures were grown in a fully instrumented stirred tank bioreactor (Applikon, bio My-control) with a working volume of 200 ml. Growth conditions were 37 °C, 150 rpm, pHC = 6.5 with NaOH (10M). Batch operation time was 24 h, and samples were taken every hour for the first 12 hours, then a final reading at 24 hours; all samples were kept at 4 °C until further analysis for cell viability determination.

2.2.2 Fed-Batch cultures

Lactobacillus plantarum cultures were carried out in a Fed-Batch fully instrumented stirred tank bioreactor (Applikon, bio EZ-control) with a maximum working volume of 7 L. A peristaltic pump (Masterflex, USA) was used to feed the inflow of beetroot molasses into the bioreactor. The initial working volume was 2 L of sterile culture media. It was inoculated with 0.5% (v/v) of new cells in the middle of the exponential growth phase to obtain an initial cellular biomass

concentration of 1.17 g/L in the bioreactor. The inflow rate was 2.4 mL/min, and growth conditions were 37 °C, 150 rpm, pH_C = 6.5 with NaOH (10M). Samples were taken every hour for the first 12 hours, then a final reading at 24 hours; all samples were kept at 4 °C until further analysis for cell viability determination.

2.3 Growth and analyte quantification

2.3.1 Cell biomass determination

Optical density correlated to a dry-cell weight curve that quantified cell growth in a microplate reader at 660 nm (Thermo Scientific Multiskan GO, Finland). Centrifugation of samples at 5,000 rpm allowed the recovery of cell biomass pellets for optical density determination; the pellets were resuspended in a volume equal to the supernatant with a solution of peptone water 1% (w/v) and NaCl 0.85% (w/v). Supernatants volume was used for analytes concentration determination. Viable cell count was conducted in Petri dishes with MRS and YPD for LB and SB, respectively. Incubation took place at 37°C for 24h. Decimal dilution method was used as counting method and the readings were validated with a permittivity probe for viable biomass (Futura ABER Instruments, UK).

2.3.2 Glucose, saccharose and D-Lactate determination

Supernatants obtained from the separation of biomass through centrifugation were used for glucose (YSI 2365), saccharose (YSI 2703), and lactate (YSI 2329) determination. Cell samples were preserved by freezing at -40 °C until its respective analysis. The determination of soluble analytes (glucose, saccharose, and lactic acid) was performed using the YSI SELECT 2950 biochemistry analyzer (Yellow Springs, OH).

2.4 Kinetic analysis of data

The simultaneous growth of *Saccharomyces boulardii* and *Lactobacillus plantarum* offers some advantages from the operation point of view, but one of the most exciting characteristics is the ability to mutually promote their growth (Cheirsilp *et al*., 2007). For the mixed culture fermentation simulation, we considered that the microorganisms could consume both glucose and saccharose. However, in the case of *S. boulardii*, it has been observed that it is capable of hydrolyzing saccharose to form additional glucose (El Enshasy and Elsayed, 2017). This issue is particularly important because these two carbohydrates are present in the medium formulated with beetroot molasses.

For the SB growth kinetic model, the simple Monod equation was considered, in function of glucose concentration, being glucose the principal substrate for the yeast (Eq. 1). In the case of LP, the kinetic model is more complicated than the one proposed for SB growth, since the bacteria can consume both glucose and saccharose, but at the same time, its growth is inhibited by the presence of lactic acid in the medium. Therefore, the kinetic model (See Table 1. Model nomenclature) requires to include these phenomena, so the use of a Han-Levenspiel model (Han and Levenspiel, 1988) is proposed (Eq. 2).

$$\mu_{Sb} = \mu_{max_{Sb}} \cdot \frac{C_{glu}}{C_{glu} + K_{glu_{Sb}}} \tag{1}$$

Table 1. Model nomenclature.

Symbol	Variable	Units	Symbol	Variable	Units
μ_i	Specific growth rate	1/h	K_H	Hydrolysis specific reaction rate	L/g-h
μ_{max}	Maximum specific growth rate	1/h	C_i	Concentration	g/L
K_{glu_i}	Glucose saturation concentration	g/L	K_{suc_i}	Saccharose saturation concentration	g/L
X_i	Biomass concentration	g/L	P	Product concentration	g/L
m_i	Total mass	g	$Y_{X_i/glu}$	Biomass glucose yield	g/g
P_{lim}	Lactic acid limit concentration	g/L	$Y_{X_i/suc}$	Biomass saccharose yield	g/g
V_r	Reactor volume	L	$Y_{X_i/P}$	Biomass product yield	g/g
$\dot{v}_{in}$	Media inlet volumetric flow	L/h	i	Sb = *S. boulardii* Lp = *L. plantarum* glu = glucose suc = saccharose	-

$$\mu_{Lp} = \mu_{max_{Lp}} \cdot \frac{C_{glu}}{C_{glu} + K_{glu_{Lp}}} \cdot \frac{C_{suc}}{C_{suc} + K_{suc_{Lp}}} \cdot \left(1 - \left(\frac{P}{P_{lim}}\right)\right) \quad (2)$$

Considering that the yeast is capable of hydrolyzing saccharose to form glucose and consumes glucose as a substrate for its growth, these effects must be incorporated into the mass balances of all present species: cell biomass, glucose, and saccharose. The equations related to both substrates consider the effects of the microorganism growth and the substrate feed in the case of fed-batch operation (Eq. 3 - 5).

$$\frac{dm_{X_{Sb}}}{dt} = X_{Sb} \cdot \mu_{Sb} \cdot V_R \quad (3)$$

$$\frac{dm_{suc}}{dt} = -K_H \cdot C_{suc} \cdot X_{Sb} \cdot V_R + \dot{v}_{in} \cdot C_{suc_in} \quad (4)$$

$$\frac{dm_{glu}}{dt} = \left(K_H \cdot C_{suc} \cdot X_{Sb} - \frac{1}{Y_{X_{Sb/glu}}} \cdot \mu_{Sb} \cdot X_{Sb}\right) \cdot V_R + \dot{v}_{in} \cdot C_{glu_in} \quad (5)$$

Similarly, in the case of the bacteria, the mass balance considers the same system states present in the yeast kinetic model, in addition to the lactic acid concentration. These mass balances were formulated on a mass basis and not concentration, as is usually done; this approach gives versatility to the model so that it can describe several modes of operation, such as batch or fed-batch (Eq. 6 - 7). The model also includes mass balances for cellular biomass and produced lactic acid (Eq. 8 - 9).

$$\frac{dm_{glu}}{dt} = -\frac{1}{Y_{X_{LP/glu}}} \cdot \mu_{Lp} \cdot X_{Lp} \cdot V_R + \dot{v}_{in} \cdot C_{glu_in} \quad (6)$$

$$\frac{dm_{suc}}{dt} = -\frac{1}{Y_{X_{LP/suc}}} \cdot \mu_{Lp} \cdot X_{Lp} \cdot V_R + \dot{v}_{in} \cdot C_{suc_in} \quad (7)$$

$$\frac{dm_{X_{Lp}}}{dt} = X_{LP} \cdot \mu_{Lp} \cdot V_R \quad (8)$$

$$\frac{dm_P}{dt} = \frac{1}{Y_{X_{LP}}} \cdot \mu_{Lp} \cdot X_{Lp} \cdot V_R \quad (9)$$

Finally, the total mass balance for the reactor is included to consider the reactor volume change due to the inlet feed (Eq. 10). This equation is used for both microorganisms.

$$\frac{dV_R}{dt} = \dot{v}_{in} \quad (10)$$

The model kinetic parameters were calculated using least squares; the numerical integration of the mass balances was done using the Matlab® *ode23s* function, and for solving the least-squares problem, the *fmincon* optimization function, using the Nelder-Mead method.

2.5 *Mixed culture simulation*

Considering SB capacity to hydrolyze saccharose to obtain glucose, it is possible to cultivate both SB and LP in a mixed culture and take advantage of the generated glucose for both biomasses production. This production scheme represents a considerable advantage since the beet molasses formulated culture medium, may now be used to produce *L. plantarum* without the need to add extra glucose, thus reducing the cost associated with the process. In order to evaluate the production potential of a mixed culture scheme, production simulations were developed for batch and fed-batch modes, using the obtained kinetic parameters. Equations 1 - 10 were integrated using the Matlab® *ode23s* function, with a 1 g/L initial concentration for both biomasses, 70 g/L for saccharose, 1.6 g/L for glucose, and no initial lactic acid present. The initial reactor volume was 2 L. Two different scenarios were evaluated, first a batch fermentation with constant volume, and then a fed-batch scheme, with a culture media inlet flow of 2.4 mL/min; the substrate concentration in the inlet flow was the same as the initial conditions.

3 Results and discussion

The evaluation of the formulated culture medium included a series of experimental essays to compare growth, substrate consumption for both strains (SB and LP) and lactic acid production for LP. In addition to the beet molasses medium, commercial MRS and YPD were used for the growth of LP and SB, respectively, for comparison purposes. Figure 1A shows the comparison of the growth behavior between cultures of LP in the beet molasses and the commercial MRS culture media.

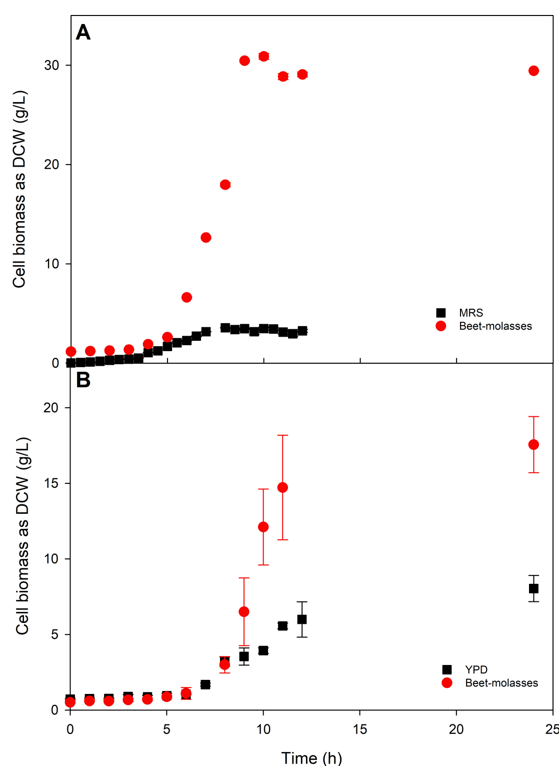

Fig. 1. Biomass formation of probiotic strains: A) *Lactobacillus plantarum* and B) *Saccharomyces boulardii*.

The obtained results showed that both culture media allowed LP a similar adaptation lag phase at least the first 5 h of culture. After the adaptation phase, beet molasses culture media significantly increased the cell biomass formation reaching values of 29.43 ± 0.24 g/L. Since MRS cultures reached 3.21 ± 0.05 g/L, the improvement obtained with the formulated beet molasses culture media is at least 9-fold compared to commercial MRS. It is inferred that prebiotics contained in beet molasses culture media such as polyphenols, prebiotic carbohydrates, and antioxidants elicited the formation of cell biomass. These observations are consistent with the report of Vodnar *et al.*, who proved catechin-rich tea extracts to improve the growth and viability of *Lactobacillus casei* during lactic fermentation and concluded that phenolic compounds acted as metabolic enhancers (Vodnar *et al.*, 2012). It is essential to mention that the used beet molasses formulated culture media contained 30 g/L of saccharose and 20 g/L of glucose compared to the commercial MRS media, which only contains 20 g/L of glucose.

Although the difference in the initial amount of carbohydrates is a factor that can stimulate biomass growth or derive lactic acid metabolism, the substrate biomass yields of the formulated medium with beet molasses were higher than those obtained in the MRS medium. The biomass to substrate yield obtained with beet molasses was ca. 0.60 g/g and the yield obtained with MRS was ca. 0.18 g/g, which represents at least an increase of 3-fold in biomass formation.

The difference in carbohydrate concentration between both culture media as well as the carbon sources (glucose and saccharose) did not modify the dynamics of carbon assimilation since they presented similar behaviors, as shown in Figure 2A. The formation of lactic acid was similar between the two-culture media tested, as shown in Figure 3. Based on this observation we infer that the carbon flux could be redirected to biosynthetic pathways and biomass formation instead of lactic acid production. Also, the remaining aerobic energetic system of the bacteria could help the cell division by providing energy through carbon metabolism and preserve a net oxidation state equal to zero in the cell.

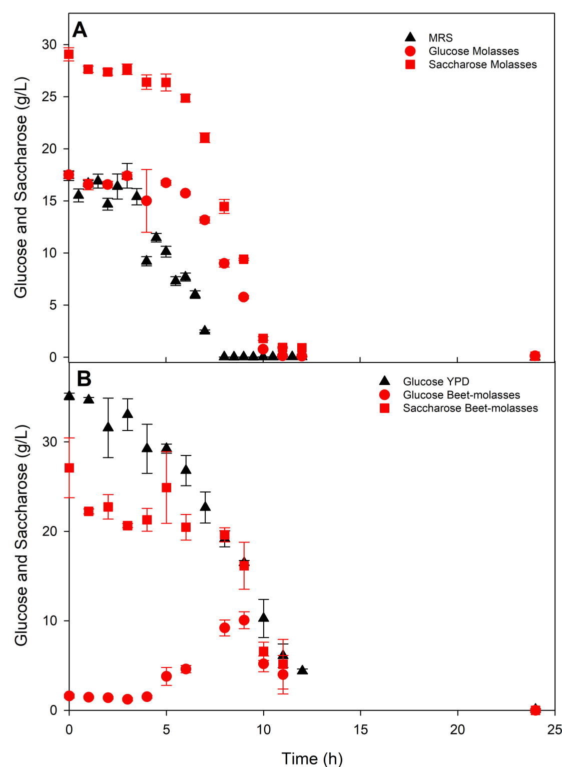

Fig. 2. Substrate consumption of probiotic strains: A) *Lactobacillus plantarum* and B) *Saccharomyces boulardii*.

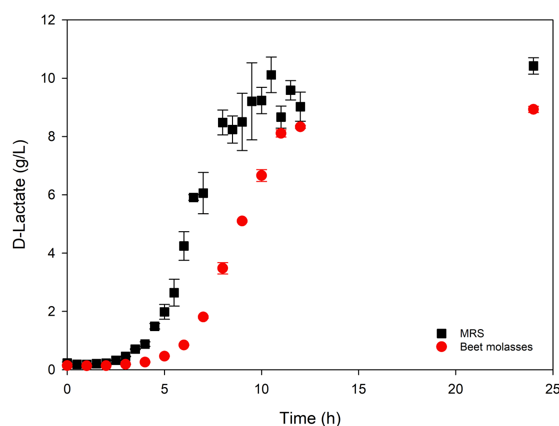

Fig. 3. D-Lactate formation in *Lactobacillus plantarum* cultures.

A series of batch cultures were performed to compare the growth of SB between the beet molasses culture media and a yeast-specific prepared medium (YPD). Figure 1B, shows SB cell biomass formation, and it is visible that beet molasses culture media enhanced as well the growth of SB compared to the one observed in YPD cultures. SB strain exhibited an adaptation lag phase of 8 h and from this time, beet molasses culture media favored SB growth, achieving up to 17.55 ± 1.85 g/L, which represents double biomass formation compared to 8.04 ± 0.86 g/L in YPD cultures. It is worth the mention that YPD culture media had 40 g/L of initial glucose compared to the 30 g/L of saccharose used in the base beet molasses media. On the one hand, as shown in Figure 2B, SB used glucose available in YPD during the first 12 h of culture.

On the other hand, SB started the culture with a simultaneous saccharification and fermentation process, hydrolyzing saccharose into monosaccharides (glucose and fructose) for growth. It was observed a slight variation in the rate of saccharose consumption compared to the one observed in YPD cultures, as well as an increase in the biomass to substrate yields obtained; 0.35 and 0.20 g/g for the culture media with beet molasses and YPD, respectively.

3.1 *Effect of beet molasses culture media formulation on biomass yield*

Batch cultures of SB exhibited a Monod-shape kinetic with a simultaneous process of saccharification and growth (Figure 4A). SB cell biomass had a lag phase of at least 6 h, and immediately cells entered the exponential growth phase until 11 h of culture. Biomass cells reached 17.55 ± 1.85 g/L at the end of the culture (25 h). SB cultures started with 69.48 ± 8.56 g/L of saccharose. Hydrolysis of saccharose occurred from the beginning of the culture; invertase produced by yeast generated glucose. From simultaneous saccharification, the glucose obtained reached 10.06 ± 0.95 g/L. As a result, cells consumed saccharose rapidly in the first 10 h of culture. As shown in Figure 4A, the process became dynamic in the first 12 h.

Lactobacillus cultures showed a different behavior from that presented by SB-yeasts (Figure 4B). Since experiments were conducted as fed-batch cultures, its growth was much faster with an adaptation phase of 4 h, and an exponential growth phase observed until 9 hours of fermentation. LP biomass cell achieved 30.89 ± 0.30 g/L at 10 h of culture. After this time, biomass cells remained at the same concentration levels, compensating the dilution factor related to culture media feed; the feeding strategy was 2.4 mL/min of a mixture of saccharose and glucose from beet molasses culture media. The production of lactic acid was obtained from the beginning of the cell culture since it is a product of the primary metabolism, obtaining a behavior similar to the formation of biomass.

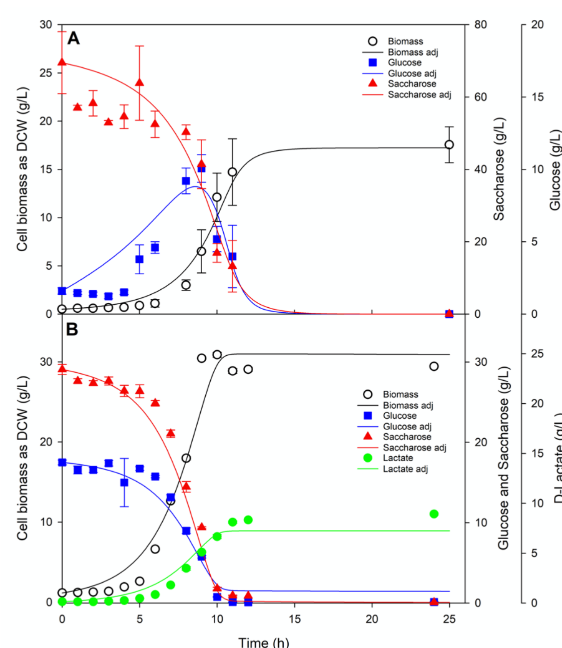

Fig. 4. Growth of probiotic strains: A) Batch fermentation of *Saccharomyces boulardii* in MBT and B) Fed-Batch fermentation of *Lactobacillus plantarum* in EMBT.

Lactic acid concentration obtained was 8.93 ± 0.10 g/L at the end of the culture. LP consumed both glucose and saccharose present in the beet molasses but at different specific uptake rates.

3.2 Kinetics of cell growth and saccharose hydrolysis in S. boulardii batch fermentation

The growth kinetics of *S. boulardii* was studied in a batch bioreactor where it was observed that the glucose concentration increased during the first 9 hours of culture due to the saccharose hydrolysis provoked by the presence of yeast-produced invertases (Figure 4A). The saccharose hydrolysis presented first-order kinetics with respect to the concentration of saccharose and cell biomass, and its reaction rate constant has a value of K_H = 0.0216 L/g-h. These results are consistent with the rate of invertase formation reported by El Enshasy (2017) in a similar study, for culture media in which the only carbohydrate source is saccharose (El Enshasy and Elsayed, 2017).

Additionally, the initial glucose concentration in the culture medium was minimal, but when the yeast growth entered its exponential phase, the glucose reached its maximum concentration. Cell biomass achieved a concentration responsible for the increased intake of glucose, and this was greater than that of its production due to the hydrolysis of saccharose, decreasing its concentration until it nearly disappears. This behavior in the growth of cell biomass is consistent with Monod type kinetics, with a specific growth rate of $\mu_{max_{Sb}}$ = 0.662 1/h and saturation constant of de $K_{glu_{Sb}}$ = 6.163 g/L (fittings had correlation index values of $R^2 > 0.98$ for biomass). Finally, after 15 h, the microorganisms stationary stage is observed, and a yield of $Y_{X_{Sb/glu}}$ = 0.458 g/g (fittings had correlation index values of $R^2 > 0.85$ for glucose). Thus, the kinetic behavior of the growth of *S. boulardii* can be adequately represented with the proposed model (Eq. 1,3,4 and 5), as observed in Figure 4A.

3.3 Kinetics of cellular biomass growth for L. plantarum in fed-batch fermentation

The growth kinetics of LP was studied in a fed-batch bioreactor (Figure 4B) where it was observed that, due to the presence of both glucose and saccharose in the culture media, the cellular biomass reached the exponential growth phase earlier, unlike the case of SB, which had to hydrolyze saccharose previously. Unlike SB, LP is capable of feeding directly on both carbohydrates, saccharose, and glucose; this behavior makes it necessary to consider both substrates in the growth kinetics model (Eq. 2). For this model, the calculated specific growth rate is $\mu_{max_{Lp}}$= 0.191 1/h, and the saturation constants for glucose and saccharose are $K_{glu_{Lp}}$= 3.07 g/L and $K_{suc_{Lp}}$ = 4.95 g/L, respectively (fittings had correlation index values of $R^2 > 0.98$ for biomass). In addition, it is necessary to include a term to represent the growth inhibition produced by high concentrations of lactic acid, obtaining a relatively high limit of lactate concentration, P_{lim} = 66.53 g/L. These values are similar to those found by Passos (1994), and reaffirm the behavior described by the author, who mentions that the specific mortality rate of the microorganism increases with the lactic acid concentration (Passos *et al*., 1994). Table 2 summarizes the kinetic parameters obtained.

In addition to the kinetic parameters, the parameters related to the mass balances represented in equations 6 - 10, confirm that LP has a higher affinity for glucose than for saccharose. In addition, high biomass/substrate yields are observed: $Y_{X_{Lp/glu}}$ = 1.90 g/g and $Y_{X_{Lp/suc}}$ = 1.05 g/g (fittings had correlation index values of $R^2 > 0.98$ for all species).

Table 2. Kinetic parameters.

Kinetic parameter	*Saccharomyces boulardii*	*Lactobacillus plantarum*
μ_{max_i}	0.6623 1/h	0.191 1/h
K_{glu_i}	6.1632 g/L	3.070 g/L
P_{lim}	-	66.530 g/L
K_H	0.0216 L/g-h	-
K_{suc_i}	0.0033 g/L	4.950 g/L
$Y_{X_{i/glu}}$	0.4576 g/g	1.900g/g
$Y_{X_{i/suc}}$	0.4846 g/g	1.050 g/g
$Y_{X_{i/P}}$	-	4.240 g/g

This yield values may be due to intracellular invertases capable of hydrolyzing saccharose for its subsequent consumption. Finally, the biomass/product yield, $Y_{X_{Lp/P}}$ = 4.24 g/g produced, suggests that the microorganism is maintained in a cell replication mode, rather than lactic acid formation. The kinetic behavior of LP growth is consistent with the observed experimental behavior (Figure 4B). The continuous feeding of culture media to the bioreactor manages to keep the concentration of cellular biomass constant, thus prolonging the exponential growth phase of the microorganism.

3.4 *boulardii and L. plantarum mixed culture simulation comparison*

Despite the fact that SB is able to grow effortlessly in the beet molasses formulated culture medium, this is not the case for LP, since the low initial glucose concentrations considerably retard its growth. However, considering the ability of SB to hydrolyze saccharose to obtain glucose, for both microorganisms to benefit from, the option of producing both microorganisms in a mixed culture results promising.

From the simulations of the mixed culture production o SB and LP in a batch bioreactor, it can be seen that the saccharose concentration decreases while the glucose concentration increases, due to the hydrolysis phenomena (Figure 5A).

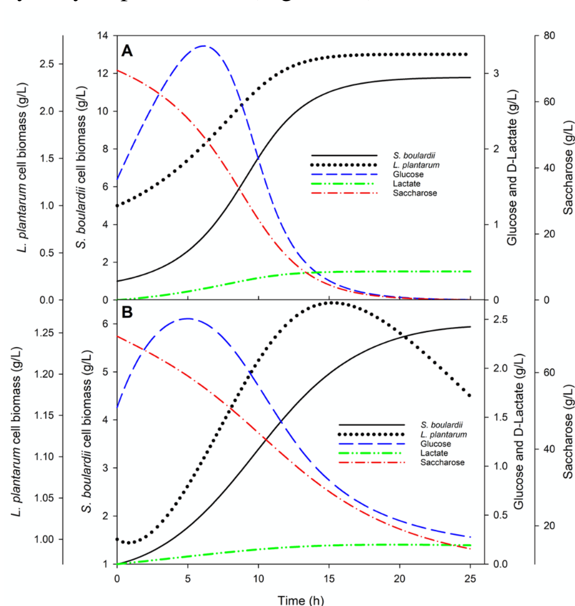

Fig. 5. Figure 5. Simulation of mixed cultures of *Saccharomyces boulardii* and *Lactobacillus plantarum*: A) Batch and B) Fed-Batch.

This increase in glucose concentration allows both microorganisms to grow at similar speeds and achieve similar cellular biomass concentrations and low lactic acid production since the metabolic status of the cells remains in biosynthetic pathways and cell division. In addition, consistent with the behavior reported by (Cheirsilp *et al*., 2007), the improvement in the LP cellular biomass growth, is not only influenced by the sachharose hydrolysis by the yeast, but there is also a buffering effect in lactate production, thus delaying the inhibition. This reduction in the lactate production is also observed in both simulations, batch and fed-batch.

On the other hand, in the case of biomass production in a fed-batch reactor simulation, similar behavior can be observed. Nonetheless, the continuous feeding of the culture medium into the bioreactor gradually dilutes all present species, reaching lower concentrations than in the case of the batch reactor (Figure 5B). These results suggest that it is possible to design production schemes in which the feeding of the culture medium is not carried out in a constant way but rather by means of an optimal variable flow path, which allows having suitable concentrations of both substrates in the medium, promoting faster cell growth.

Conclusions

The proposed kinetic model for the growth of *Saccharomyces boulardii* manages to describe its observed growth behavior in a growth-promoter culture medium formulated from beet molasses. This model contemplates the hydrolysis of saccharose to produce glucose, and thus promote cellular growth. Furthermore, the model proposed for the growth of *Lactobacillus plantarum*, adequately describes the growth of the microorganism, considering both substrates present in the culture medium, saccharose, and glucose, as well as the possible inhibition that occurs at high lactic acid concentrations. The simulations of the mathematical model describe the behavior of both microorganisms in mixed culture in this culture medium and allow the design of different production schemes that can combine the advantages of batch and fed-batch production, such as variable culture medium feeding flow.

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