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Production of Exopolysaccharides by Lactobacillus plantarum ATCC 8014 and Concentration by Nanofiltration

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ABSTRACT

Functional food may promote specific physiological benefits besides the properties of nourishing and feeding, thanks to the presence of physiologically healthy ingredients such as probiotics and prebiotics. Amongst the probiotics is *Lactobacillus plantarum,* which belongs to the group of lactic acid bacteria (LAB). Energy obtainment by these microorganisms occurs through carbohydrate fermentation, producing mainly lactic acid but also exopolysaccharides (EPS), which presents technological application on the food industry mainly by their prebiotic properties. In order to enable the obtainment of EPS, a production process of *L. plantarum* ATCC 8014 was established in aerobic conditions in an airlift bioreactor, using supplemented tofu whey as substrate. The process was compared to the generally employed MRS medium. EPS concentration was performed by nanofiltration (NF), carried at 35 °C and 6 bars,and samples analyzed via HPLC-IR. The cultivation allowed biomass yields of up to 3.2 g/L and 2.7 g/L, and EPS yields of 350 mg/L and 210 mg/L for theTWS and MRS media, respectively. On the NF process, the final concentrated extract was obtained with VRF (volume reduction factor) of 4.0, with increases of up to 80% on EPS contents. During membrane processing, it was verified that the greatest part of the flux resistance (60%) was caused by a polarized gel layer.This study showed that the use of tofu whey is applicable for EPS productionand that nanofiltration is an efficient procedure to concentrate the prebiotic compounds obtained during the cultivation of *L. plantarum* ATCC 8014.

Key words: lactic acid bacteria, exopolysaccharide, *Lactobacillus plantarum* ATCC 8014, nanofiltration

INTRODUCTION

The interest for different products obtained through fermentative processes using microorganisms has significantly risen in the last years. Between the relevant products are functional foods, nutraceuticals, and medicines obtained from natural sources. Functional foods must present beneficial properties besides their basic nutritional features. They are consumed in conventional diets and are able to regulate bodily functions protecting against diseases such as hypertension, diabetes, cancer, osteoporosis and coronapathyes (*1*). Functional foods are all foods and beverages consumed on daily diets that can bring specific physiological benefits, due to the presence of physiologically healthy ingredients. The biologically active substances found in these foods can be classified as probiotics and prebiotics (*2*). Probiotics are non-pathogenic microorganisms that when administered in adequate amounts can exert health benefits to an individual (*3*). On the other hand, prebiotics are defined as food ingredients that may select specific intestinal microbiota species that confers benefits upon the body well-being and health (*4,5*).

Among the most notorious examples of probiotics are the lactic acid bacteria, generally present in some dairy foods (*6*). These bacteria comprise a broad group of microorganisms that exhibit specific morphologic, metabolic and physiologic features. Among these characteristics are the ability to convert sugars, organic acids, proteins or fats into flavor and aroma (*7*). In addition, these microorganisms produce interesting metabolites such as lactic acid (*8*) and exopolysaccharides (EPS), which are soluble fibers included in the group of prebiotics (*5,9*).

Lactobacillus plantarum is one species that represents a viable alternative to be used both as probiotic and prebiotic, as this microorganism secrete high quantities of EPS in the cultivation media (*10*).This microbial species can be cultivated under aeration using airlift bioreactors, which are simple to build and easy to operate (*11*), allowing for relatively inexpensive maintenance costs. The lack of mobile mechanical parts for stirring decreases the chances for contamination, as it eases cleaning and sterilization. The gas injection used for stirring and aeration suppresses the energy expenses and promotes an increase on the mass and heat transfer capacity (*12*). The tiny air bubbles inside the bioreactor increase the superficial area for oxygen transference, and the ascending air current balances the shearing forces throughout the bioreactor, resulting in higher biomass productions (*13*). Additionally, *L. plantarum* and related lactobacilli growing in the presence of air, present much higher biomass yields than under anaerobic conditions. This increase is partly due to a change on the fermentation pattern, where NADH isre-oxidized together with oxygen, yielding and extra production of ATP from acetyl phosphate via an acetate kinase reaction (*14,15*). Therefore, allied to these conditions, the characteristics of the bioreactor turn it into an attractive alternative for the use also for aerotolerant and facultative anaerobic microorganisms.

The most suitable medium for the growth of lactic acid bacteria is MRS, proposed by Man *et al*. (*16*). However, for industrial purposes and economic reasons, complex substrates of variable composition, such as other industries by-products, are also used. These substrates must be adapted for cultivation, allowing for supplementation and balancing, in order to increase the biomass yield of the selected microorganism (*17*).

Tofu whey, a by-product originated from soy cheese manufacturing has low industrial application, especially because of its high water contents. Nevertheless, it presents a considerable amount of soluble solids (*18*). This by-product may be used as substrate for the fermentation of *L. plantarum*. Furthermore, it is possible to produce metabolites, such as EPS, when the bacterium is grown in such media. Therefore, the potential of these products is being exploited by the food industry, using separation and concentration procedures to increase yield and refinement of the products.

The separation of polysaccharides, based on their molecular weight, can be used for the purification of crude extracts (*19,20*).The concentration through membranes can be used to maintain the high quality of these polysaccharides. Thus, it is based on the molecule´s selective permeation principle when forced through membranes (*21*). Nanofiltration (NF) uses membranes with very small pore diameters, more efficient on the concentration of bioactive polysaccharides (*20*). Even though such membranes have been used to concentrate compounds of small molar masses (*22-24*), they can also be used to concentrate EPS, as proposed by Camelini *et al*. (*25*). NF presents advantages over other processes such as the use of low temperatures, low energy consumption, reduction of environmental impact, due to the elimination of solvents, as well as the preservation of the properties credited to the compounds under filtration (*21,26*).

In this respect, the present study compared the biomass and EPS production of *L. plantarum* ATCC 8014 grown on two different media: MRS and crude tofu whey supplemented with nutrients. The aim of the study was to establish an efficient process for the production and concentration of EPS using nanofiltration, allowing for its application in nutritional food preparation.

MATERIAL AND METHODS

Microorganism

Lactobacillus plantarum strain ATCC 8014, obtained from the Tropical Collection of Cultures André Tosello (Campinas, SP, Brazil) was selected for the study. Lyophilized bacteria cells were rehydrated, cultivated in MRS medium, and stored in Eppendorf tubes with 20% glycerol at -20 °C until use.

Inoculum preparation and cultivation media

The starter culture was obtained by adding 2 mL of the recovered *L. plantarum* ATCC 8014 cells into 18 mL of nutrient broth. Forty-eight hours later this pre-inoculum was transferred to 180 mLof each of the tested media: MRS and STW (supplemented tofu whey), and incubated at 30 °C for 18 h. This volume was chosen because it represents the proper amount to be inoculated into a 5 L airlift bioreactor. The whole procedure was performed withan initial pH of 6.0, and with controlled air supply.

The tofu whey was obtained at Tofutura Indústria de Alimentos Ltda. Its composition was determined and is presented in Table 1. The tofu whey was supplemented by adding (g/L)11.5 glucose, 1.0 yeast extract, 0.2 $MgSO_4$, 0.05 $MnSO_4$, 1.4 K_2HPO, 3.0 sodium citrate, and 1.0 mL/LTween 80.

Table 1. Tofu whey (TW) chemical composition.

Cultivation in the airlift bioreactor

To enable and standardize the production of EPS, the cultivation of *L. plantarum* ATCC 8014 was performed in an airlift bioreactor with controlled pH and aeration, using the MRS and STW media supplemented with 0.3 mL/L polypropylene glycol antifoam. The pH was maintained at 6.0 by automatically adding H_2SO_4/NaOH 0.1 mol/L. Dissolved O_2levels were automatically controlled, maintaining the concentration above critical during the entire cultivations by varying airflow rates.The respiration rate of a cell is progressively limited when the dissolved oxygen concentration is set below the critical level. For this study, the critical level was determined by interrupting aeration in a particular moment during cultivation and recording the probe´s oxygen readings reduction. During this short period during which the assay was developed, the biomass is considered constant. Correspondingly, as long as oxygen is available, the respiration rate is also constant. In the exact moment the oxygen concentration line drops, the respiration starts to reduce, indicating the critical value. During cultivation, samples of 30 mL were collected at regular time intervals and frozen at-4 °C for the determination of biomass, EPS production, and glucose consumption.

Biomass and glucose determination

Biomass concentration was determined by gravimetric procedures. Samples of known volume were filtered through pre-weighted filter paperand dried at 55 °C to constant weight. The concentration of glucose on the samples was determined via the GOD enzymatic method, based on Trinder's classical reaction. The determination of the absorbance was performed at 480 nm and a glucose standard curve made with concentrations varying from 0 to 3 g/L.

Kinetic and stoichiometric parameters

Biomass specific growthrate (μ_X) profiles, defined by Eq. (1), were obtained with the geometrical calculations from the derivatives (*27*). Similarly, the specific production rate of EPS (μ_P) was obtained, defined by Eq. (2). The biomass yield ($Y_{X/S}$) and EPS yield ($Y_{P/S}$) were calculated using Eq. (3) and Eq. (4), respectively.

$$\mu_X = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

$$\mu_P = \frac{1}{X}\frac{dP}{dt} \quad (2)$$

$$Y_{X/S} = \frac{X - X_0}{S_0 - S} \quad (3)$$

$$Y_{P/S} = \frac{P - P_0}{S_0 - S} \quad (4)$$

Where X = biomass concentration (g/L), P = EPS concentration (g/L), S = glucose concentration (g/L) and t = time (hours). Subscript "*0*" indicates initial concentrations. Mean values (μ_{xmed} and μ_{pmed}) were obtained by the arithmetic means of μ_X and μ_P determined by Eq. 1 and 2, respectively.

EPS quantification

The high molar mass polysaccharide on the samples were determined by HPLC-IR using a TSK gel 5000 PW column (7.8 × 300 mm, Tosoh Corporation) coupled with a pre-column. Analyses were done in a Perkin Elmer Series 200 chromatograph with a refractive index Perkin Elmer series 200a detector coupled to a UV detector at 280 nm, with a mobile phase of 0.2 mol/L NaCl, and flux of 1 mL/min. Two volumes of ethanol (PA) were added to each 500-µL sample volume for the precipitation of polysaccharides, and subjected to centrifugation. A standard curve was performed with standard molecular weight dextrans (12-2000 g/mol, Sigma, USA) with concentration of 3 mg/mL and injection of 20 µL. Samples were analyzed at a concentration of 3 mg/mL, dissolved in the mobile phase.

Nanofiltration of EPS

For the concentration of total polysaccharides, a new NF membrane was used in a tangential flow operating equipment. The organic membrane is composed of polyvinylidene fluoride with molar mass cut-off of 150-300 g/mol, and filtration area of 0.9 m² (Model HL2521TF, GE Osmonics®, Philadelphia, U.S.). Sixteen litters of polysaccharide extract were submitted to NF during approximately 90 min. This time length was necessary since the system operated with no permeate recycling. Concentrate and permeate samples were collected in different times, referring to VRF values of 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0. Operation parameters controlled during NF were temperature (35±1 ºC) and transmembrane pressure (6 bars). This control was necessary to preserve the extract properties. Aliquots of 40 mL of the different VRF retentate were added to 80 mL of ethanol 96 °GL to precipitate total polysaccharides, which were determined through gravimetry. The VRF calculation, the permeate flux J(L/m^2/h), the process efficiency related to the polysaccharide retention percentual, membrane resistance calculation, and the influence of pressure and temperature on the process were determined following Camelini *et al.* (*25*).

RESULTS AND DISCUSSION

Bioreactor cultivation and EPS production

Data from Fig. 1 refer to biomass yield and EPS production for *L.plantarum* ATCC 8014 cultivated in a 5 L airlift bioreactor in both MRS and STW media.Glucose, the only source of carbon monitored, was totally consumed in about 30 hours of cultivation.The pH during cultivation was maintained constant at 6.0±0.1 and temperature close to 30±1 °C.The bioreactor maintained the O_2 concentration above critical for both batches (10% of saturation, experimentally determined).

Fig. 1. Growing parameters for *Lactobacillus plantarum* ATCC 8014under aeration on MRS (a) and STW (b) media in airlift bioreactor.

After 30 h of cultivation (glucose total consumption), total biomass production was 2.7 g/L and 3.2 g/L, with biomass yields ($Y_{X/S}$) of approximately 0.15 and 0.34 for the MRS and the STW media, respectively (Table 2). Feltrin *et al.* (*28*) obtained biomass production of 2.2 g/L for *L.plantarum* ATCC 8014 cultivatedin MRS medium for 24 h at 35 °C with agitation. Aside from these main differences, *L. plantarum* ATCC 8014 grown on STW had an average specific growth rate (μ_{Xmed}) of 0.038/h, which is about 2 times shorter than that obtained for the MRS .Nevertheless, the average specific production of EPS (μ_{Pmed}) was similar for both media. Furthermore, glucose conversion into biomass and EPS was much higher in the STW then in MRS (Table 2). Even though there was larger biomass production for the MRS (Fig. 1), the production of EPS was similar for both media. As biomass increases, so does the EPS production for both cultivations. This result is similar to that obtained by Zhang et al. (*29*). However, in MRS (Fig. 1a), the relation was not proportional after glucose was gone, indicating higher biomass amounts produced from the protein sources of the medium, in relation to EPS. These results show that under poor nutritional conditions, such as is the case for STW, the microorganism converts more energy into EPS since this compound can be opportunely metabolized as source of carbon and energy. Preliminary results (data not shown) comparing anaerobic versus aerobic cultivation of *Lactobacillus plantarum* ATCC 8014 in MRS have already indicated higher biomass (15%) and EPS (25%) yields when the cultivation is done aerobically.

Table 2. Parameters evaluated during the cultivation of *Lactobacillus plantarum* ATCC 8014 in airlift bioreactor in MRS and STW media.

The relation between EPS concentration and biomass (*X*) production was presented in Fig. 2. For MRS, the relation reached the peak at 17 h of cultivation, significantly dropping afterwards. In the STW, this peak was reached at 15 h of cultivation, with a less significant drop then that observed for the MRS medium.

Fig. 2. Relation between the concentration of EPS and biomass of *Lactobacillus plantarum* ATCC 8014 grown under aeration in airlift bioreactor.

One plausible explanation for the reduction in substrate conversion into EPS (relative to biomass) observed in the MRS may be related to the low levels of dissolved oxygen (DO) observed after 20 h of cultivation (10% of saturation). The system that controls air flow in the airlift bioreactor reached maximum capacity [1.20 vvm (volume of air/volume of the bioreactor, per minute), equals to a k_La of 70/h determined by the dynamic method], 2 h after the DO concentration reached the set point (17% saturation), and it was unable to maintain the concentration above critical afterward (Fig. 3). As for the STW, since the microbial cells presented lower growth rate, the DO concentration was kept above critical during the entire cultivation. Therefore, the drop observed in the relation EPS/*X* (Fig. 2) for the STW maybe related to a potential nutritional limitation of the medium. Even supplemented, the final nutritional level for the STW was much lower than the MRS.This hypothesis reinforces the results of Ounis *et al.* (*30*), who showed that tofu whey, even when supplemented with 5 g/L yeast extract and 20 g/L sucrose, promoted growth that was 4 times smaller than in MRS. Those authors obtained maximal specific growth rate 2.5 times higher in MRS in anaerobiosis, than in the aerobic conditions of the present study. Our study and the one of Ounis *et al.* (*30*) have measured different cultivation parameters. We have not determined the production of derived acids, while Ounis and collaborators (*30*) did not determine EPS production. Therefore, it is still to be determined if the lower growth rates observed here for the MRS, aside from the fact we used a different bacterial isolate, is due to aeration or not. An additional production of ATP under aerobiosis may stimulate the production of EPS, and consequently lower growth rates.

Considering a consumption of 11.5 g/L of added glucose to the tofu whey, and a utilization of at least 60% of the sugars naturally present in this substrate (stachyose, raffinose and sucrose) and metabolized by the bacteria (*30*), we end up with a total consumption of about 16 g/L of the carbon source. Still taking into account the biomass and EPS obtained at the end of the cultivation, one can determine the global conversion rate, which, in this case, was about 35%, which is very likely if considered that an extra ATP maybe produced under such process.The differences between glucose added amounts in the composition of the two media in relation to those detected by the enzymatic method, i.e., lower values, may be related to glucose degradation reactions that occur during media sterilization, as well as reactions of glucose with proteins (Maillard).

This study showed that *L. plantarum* ATCC 8014 produced two different kinds of EPS: one with molar mass of 2.9 x 10^5 Da, and a second with molar mass of 1.7 x 10^6 Da. These EPS fractions were found in bothmedia. After the purification of the higher molar mass EPS fraction via dialysis, it was then used as the standard fraction for the HPLC-IR quantification. In a 30 h cultivation batch, the maximum yield of this polysaccharide was 210 mg/L and 350 mg/L for the MRS and STW media, respectively.

Zhang *et al.* (*29*), during experiments with *L. plantarum* in MRS, reached EPS production of 69 mg/L. The EPS fraction presented molar mass of 1.15 x 10^6 Da. On the other hand, Tallon*et al.* (*31*), using the same medium, observed that the production of polysaccharides at 25 °C (126 mg/L) was 8% smaller then at 18°c (135.8 mg/L). More importantly, the EPS fractions recovered in each temperature were also different. At 25 °C the polysaccharide presented molar mass of 8.5 x 10^5 Da, while at 18 °C it was 4 x 10^4 Da. Our results show that EPS production for both cultivation media were higher than any result reported previously in the literature, especially when using the STW medium.

Differences found for the biomass amounts, EPS fractions and molar masses in each experiment confirms the hypothesis raised by Ruas-Madiedo *et al.* (*32*) and De Vuyst and Degeest (*33*) that exogenous factors, such as carbon source, dissolved oxygen, and cultivation time, affect the EPS production and overall growth pattern of lactic acid bacteria.

Fig. 3 presents the variation of specific airflow rate and the dissolved oxygen concentration (DO) during cultivation in MRS (Fig. 3a) and STW (Fig. 3b) media.The system was kept operating automatically with a minimal airflow rate of 0.1 vvm, to maintain the DO above critical (10% saturation).

Fig. 3. Variation in the dissolved oxygen concentration and specific airflow rate during the cultivation of *Lactobacillus plantarum* ATCC 8014 in airlift bioreactor on MRS (a) and STW (b) media.

If the concentration of dissolved oxygen were to reach values close to critical, the control system would act to compensate any shortage, since the bioreactor can operate with airflow rates up to 1.20 vvm. Therefore, the fall on the DO observed in Fig.3 (a and b) is considered normal due to the increase in biomass concentration in the culture media. Even after the normal fall, the DO concentration remained above critical along the entire cultivation cycle, corroborating the fact that *L. plantarum* is a facultative anaerobic bacterium growing well under aerobic conditions. After 32 h of cultivation and when glucose was totally consumed, there was a new demand for O_2, depicted by the increase in the specific airflow rate, keeping the DO above critical for the cultivation in the STW, indicating the utilization of other sources of carbon present in the growing medium. For the MRS, the DO reached critical values after 36 h of cultivation, indicating that the demand for oxygen was higher than the bioreactor could provide. However, other factors may be involved, such as alterations in the culture medium that affect the hydrodynamics of the medium and reduce the speed of mass transfer, particularly in the final stages of cultivation.

EPS concentration by nanofiltration

Membrane separation processes have been widely used for the cultivation of lactic acid bacteria to separate lactate, lactic acid and the microorganism itself. Timer *et al.* (*22*), Dey *et al.* (*23*) and Sikder *et al.* (*24*) have described the mechanical details behind the NF process, depicting the method´s efficiency taking into account variables such as temperature, pressure, cultivation media, and concentration yields, among others.Those authors have shown that nanofiltration, coupled or not to other methods for downstream purification, is an attractive alternative for the recovery and utilization of the final product (EPS, for example). Although no work has described the utilization of this procedure for the separation of exopolysaccharides, Camelini *et al.* (*25*) used NF for the separation of EPS from the fungus *Agaricus subrufescens*, employing the same membranes and analytical equipment as those employed in the present study. Therefore, it is possible vto somewhat compare the outcomes of the extraction method and behavior of the NF membrane used in Camelini´s experiment with the one described here.

The permeate flux (*J*) during the NF of *L. plantarum* ATCC 8014extractgrown on STW medium is presented in Fig.4.

Fig. 4. Permeate flux during the nanofiltration of *Lactobacillus plantarum* ATCC 8014 extract on STW medium.

Lactobacillus plantarum ATCC 8014 extracts grown on STW medium were used as the feed for the NF. It was verified aflux decrease throughout the filtration as a function of time. Average flux was 8.8 L/(m^2/h). The process was carried for 90 min at fixed temperature of 35 ± 1 ºC and pressure of 6 bars. As expected, a decrease in *J* was observed through time (*t*) due to concentration polarization and membrane fouling, which are generally present in such membrane separation processes. Using the same membrane for the concentration of EPS from *A. subrufescens* grown on a liquid, synthetic medium, Camelini *et al.* (*25*) found an average permeate flux of 26.8 L/(m^2/h). Studies reported by Prudêncio *et al.* (*34*) and Benedetti *et al.* (*35*), who subjected bark aqueous extract and aqueous extracts of fat-free soy flour to nanofiltration (using the same membrane), showed permeated flux values of 22.8 and 12.4 L/(m^2/h), respectively. This shows that the composition of the media and the EPS structure are directly determining the permeate flux.

The effects of temperature (Fig.5) and pressure (Fig.6) on the permeate flux were characterized by the difference between the permeate fluxes for water and that for the STW extract, altering the temperature from 25 to 45 °C, and the pressure from 1 to 7 bars. Fig.5 shows the flux as a function of temperature at constant pressure of 6 bars. Pure water permeability is a function of membrane structure.

Fig. 5. Permeate flux as a function of temperature on water and *Lactobacillus plantarum* ATCC 8014 extract on STW medium.

Higher permeability suggests that the membrane have larger pore size and/or pore density that promote water molecules to go through it (*36*). However, once the feed solution was replaced by the *L.plantarum* ATCC 8014 extract, the permeate flux was also affected by the interactions between the microbial compounds (causing fouling phenomena), membrane polymer materials, pressure, and temperature of the system. The difference between the permeate fluxes for water and the extract clearly indicates the formation of a polarized gel layer and the effect of fouling during the process. These phenomena together reduced the permeate flux around 2.5-folds, while operating at temperatures of 45 °C. It is also possible to notice a slight increase on the permeate flux, around 1.8-folds, when temperature increases. The effects of temperature on the permeate flux has already been reported, since higher temperatures reduce the viscosity of the extract and therefore its facility to permeate through the membrane (*37*). Furthermore, temperature affects the flexibility of the membrane polymeric chains, making the polymeric material more flexible (*38*). However, the variation in temperature of the feed stream must be applied following certain principles with the view to avoid the degradation or losses of the product nutrients. A temperature limit also needs to be recognized as a function of the thermo resistance of the membrane, and economic aspects related to energy consumption (*39*).

Fig.6 shows the effects of pressure on the flux under a constant temperature of 35 °C, demonstrating that the flux increases with pressure, at rates of about 30% in 7 bars of pressure.The flux of water through the membrane increased linearly with pressure, with R^2 = 0.99, ensuring that membranes were properly compacted, and the *L. plantarum* ATCC 8014 extract permeate flux wasnotably smaller than the water permeate flux under the same operating conditions. This indicates that the effect of concentration polarization is noteworthy for *L. plantarum* ATCC 8014 extract. Concentration polarization occurs when pressure is large enough to transport the solutes with high molecular mass to the membrane active layer, creating barrier to the

permeation rate by solute back diffusion from the membrane to the feed (*40,41*). The concentration of retained solutes at the membrane surface leads to a reduction in effective pressure,increasing the filtration resistance and osmotic pressure effects. This behavior was confirmed with the flux resistance studies, where polarization concentration represent around 60% of permeate flux decreases.

Fig. 6. Permeate flux as a function of pressure on water and *Lactobacillus plantarum* ATCC 8014 extract on STW medium.

Fig. 7 presentsdata on EPS concentrated by NF. The results for EPS content in the concentrate collected in different VRFs were different from the results obtained for the feed extract. The data show that the EPS contents on the concentrate in different VRF values over time is equivalent to a linear equation, that is, the higher the VRF, the higher the amount of precipitated EPS after NF. The highest retention index (*R*), close to 0.90 (90%), was achieved when VRF was 4.0. After NF, and taking the average for the duplicates, it was observed an increase of about 5 times on the EPS content in the concentrate, with a yield of 12.4 g/L at VRF 4.0. Thus, it was verified that the tofu whey increases the EPS production and the NF was efficient to concentrate the prebiotic compounds from the *L. plantarum* ATCC 8014 cultivation medium. Camelini *et al.* (*25*) observed a similar behavior on the membrane concentration of different aqueous fungi extracts, with retention percentages up to 92% at VRF's 5, 6, and 7. Studies performed by Ledur Alles *et al.* (*41*) showed retention indexes close to 70% after nanofiltration of yacon fructooligosaccharides. The same authors reported that diafiltration did not influence largely the fructooligosaccharide retention index (it increased from 68.8% without, to 70.5% with diafiltration).

Fig. 7. EPS concentration of *Lactobacillus plantarum* ATCC 8014 extract processed by nanofiltration (35 °C / 6 bars).

Compounds fractionation by nanofiltration depends on numerous factors like membrane pore size distribution and filtration conditions (pressure, temperature and tangential velocity). Goulas *et al.* (*42*) reported that an increase in saccharide retention was observed when the work pressure was increased, due to increased solvent flux and membrane compaction. Another factor influencing nanofiltration may be the membrane material and pore size distribution. Membranes composed of polyvinylidene fluoride (hydrophobic), as the one used in the present study, appear to exert better retention of saccharides than cellulose (more hydrophilic) membranes, as the used by Ledur Alles et al. (*41*).

In relation to the membrane resistances, calculated according to Camelini *et al.* (*25*), most of the flux resistance was caused by the presence of a polarized layer. The difference between water and extract permeated flux, observed in the Fig. 5, indicates the formation of a polarized gel layer, phenomena usually present in membrane separation processes. This phenomenon is responsible for around 60% of the flux decline. A second major component was the membrane resistance itself (40%). Resistance due to fouling was responsible for only 1% of the total resistance. Working with the filtration of extracts from *A. subrufescens*, Camelini et al. (*25*) showed that approximately 90% of the permeate flux resistance was due to the formation of a polarized gel layer, followed by the membrane resistance (10%). In the same study, resistance caused by fouling was null. The small influence of fouling in both experiments indicates that the membrane can be fully recovered at the end of the experiments, allowing for reuse.

The higher influence of the polarized gel layer can occur due to the presence of polysaccharides, but also due to salts and residual carbon from the cultivation medium (*25*). It is important to point out that the resistance caused by this phenomenon can be substantially reduced by altering the permeate flux characteristics, usually by providing low pressures and high shear rates at the membrane surface, in tangential filtration mode. Thus, it is possible to obtain higher permeate fluxes with the same feed, modifying only the operational conditions. On the other hand, fouling is considered an irreversible phenomenon, which increases the operational costs, decreasing membrane permeability (*43*).

It is noteworthy to mention that the tangential filtration has a number of advantages. Among them, low energy consumption, simplicity during operation, high selectivity, and separation efficiency. Furthermore, it is possible to obtain high quality products due to the use of low process temperatures, which maintains the structural and nutritional characteristics of the final products (*37*).

CONCLUSIONS

The work revealed that the production of biomass from *L. plantarum* ATCC 8014 can be efficiently obtained using airlift bioreactors. The cultivation of the bacterium in this equipment presented a specific growth rate of 0.04/h, with an index for substrate conversion into biomass of $0.26 g_X/g_S$. This conversion was mainly directed to EPS production.The study also showed that it is possible to replace the regular growing media normally used for the cultivation of *L. plantarum* (MRS, etc.) with supplemented tofu whey. This substrate has shown to have the proper conditions that stimulate the growth of the tested bacterium. The production of two EPS fractions, one with 2.9×10^5 Da and another with 1.7×10^6 Da molar masses was observed. After purification in HPLC-IR, the EPS production reached a yield of 350 mg/L in the STW medium. For the production of total polysaccharides, especially those with high molar masses, a procedure based on nanofiltration allowed the concentration of extracts of up to five times the initial values. This procedure reduces the ethanol amounts needed for the precipitation of these molecules, increasing, consequently, the EPS yield in more than 80%.

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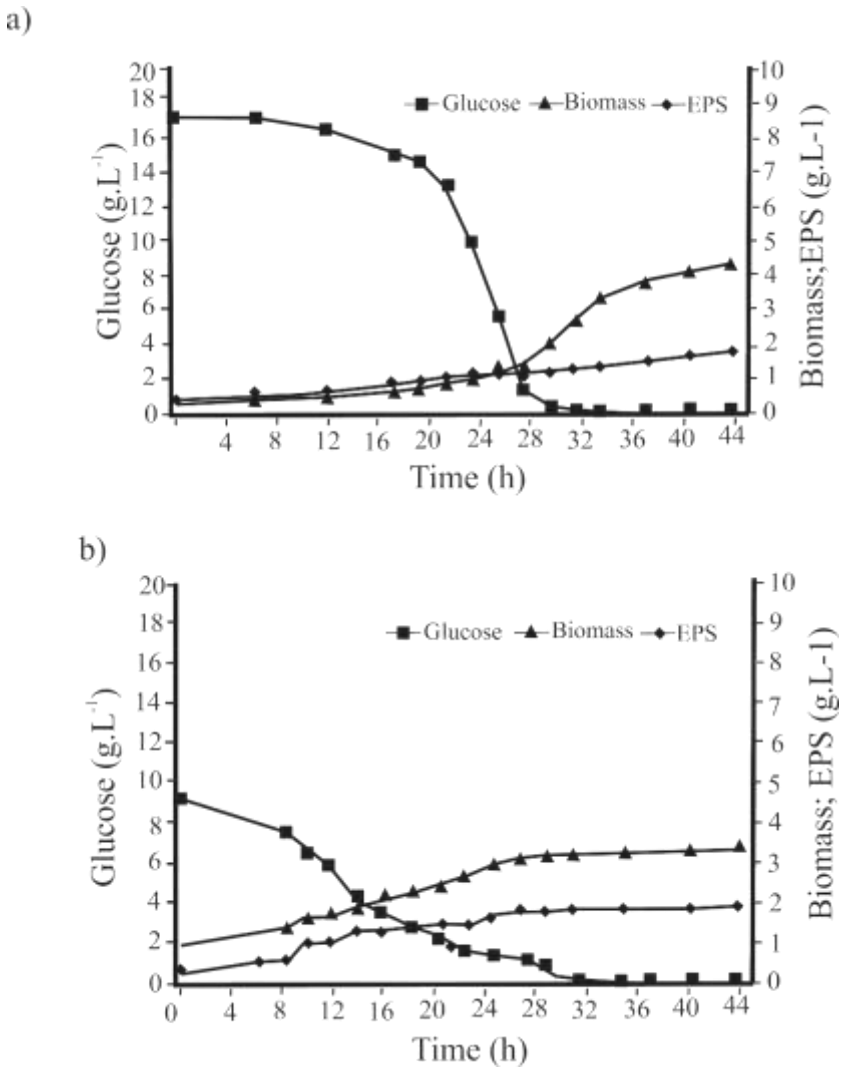

Fig. 1. Growing parameters for *Lactobacillus plantarum* ATCC 8014under aeration on MRS (a) and STW (b) media in airlift bioreactor.

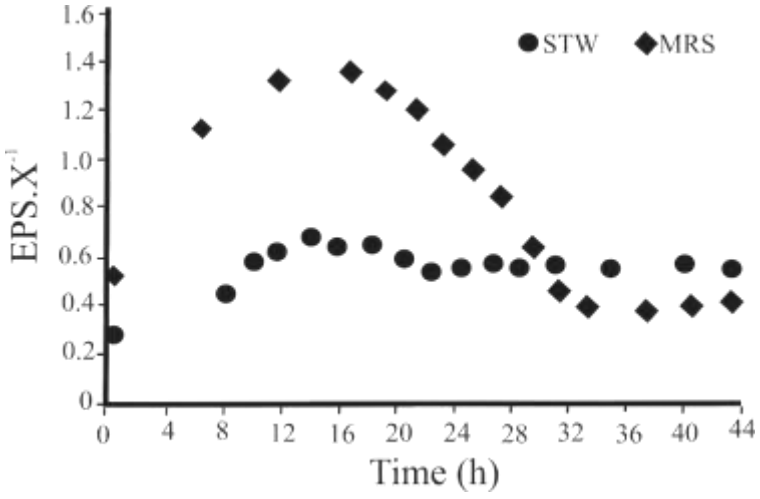

Fig. 2. Relation between the concentration of EPS and biomass of *Lactobacillus plantarum* ATCC 8014 grown under aeration in airlift bioreactor.

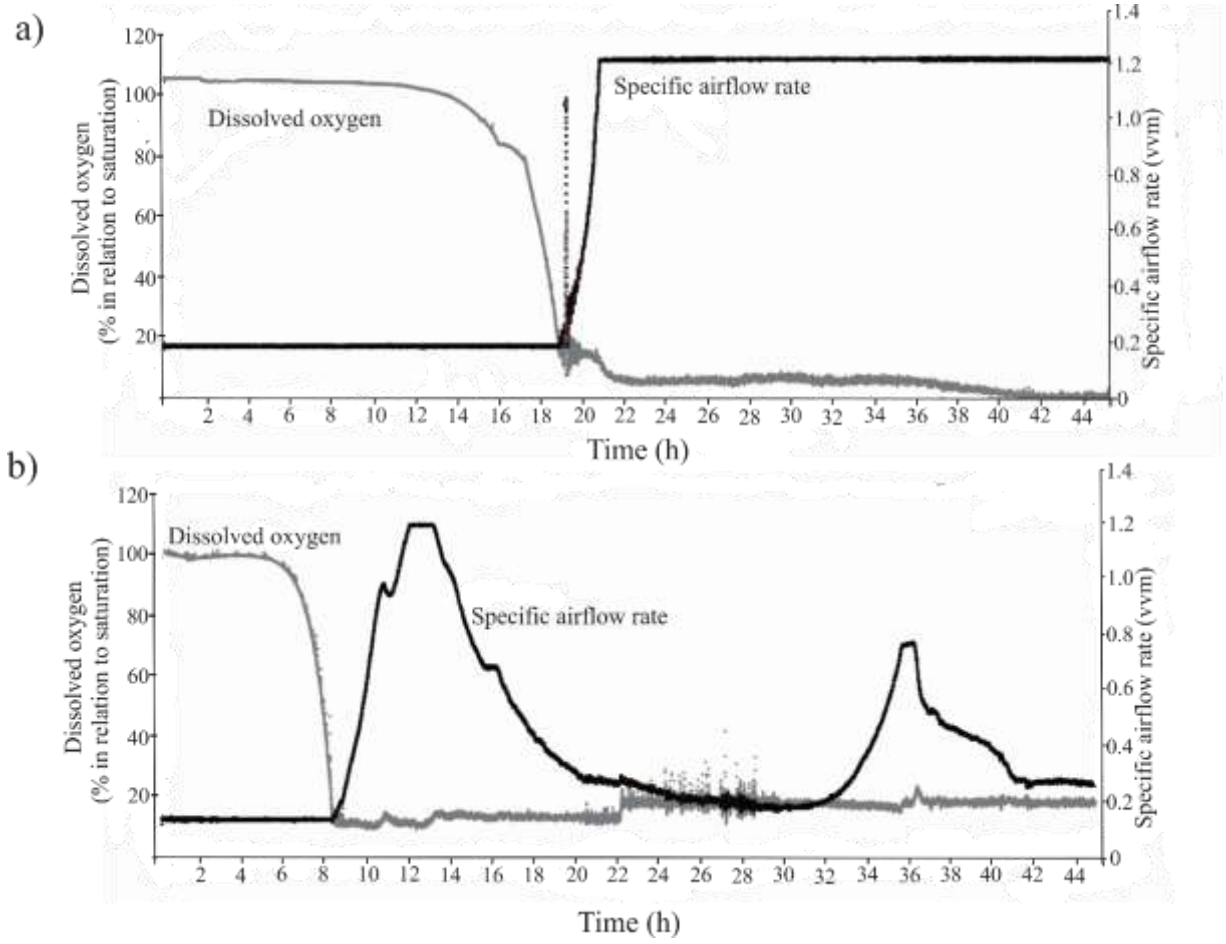

Fig. 3. Variation in the dissolved oxygen concentration and specific airflow rate during the cultivation of *Lactobacillus plantarum* ATCC 8014 in airlift bioreactor on MRS (a) and STW (b) media.

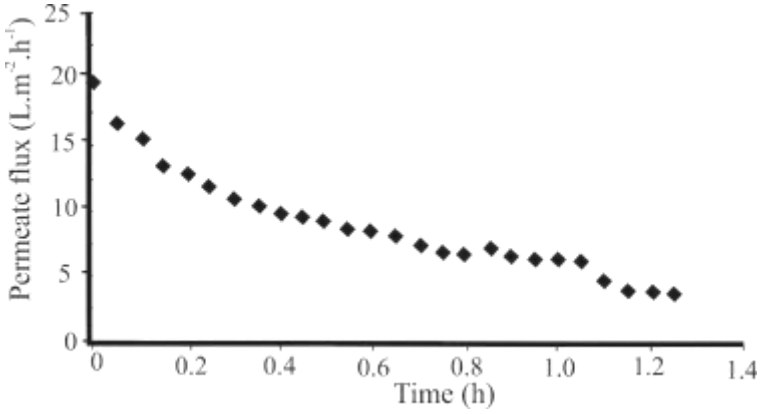

Fig. 4. Permeate flux during the nanofiltration of *Lactobacillus plantarum* ATCC 8014 extracts on STW medium.

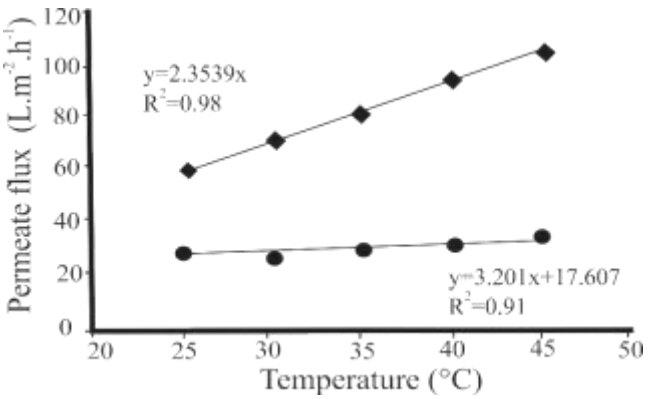

Fig. 5. Permeate flux as a function of temperature on water (♦) and *Lactobacillus plantarum* ATCC 8014 extracts on STW medium (●).

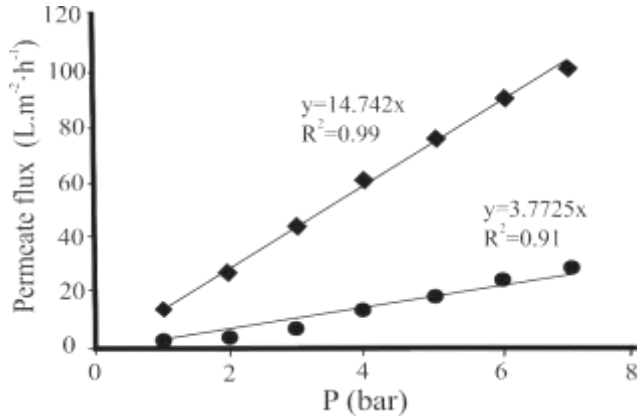

Fig. 6. Permeate flux as a function of pressure on water (♦) and *Lactobacillus plantarum* ATCC 8014 extracts on STW medium (●).

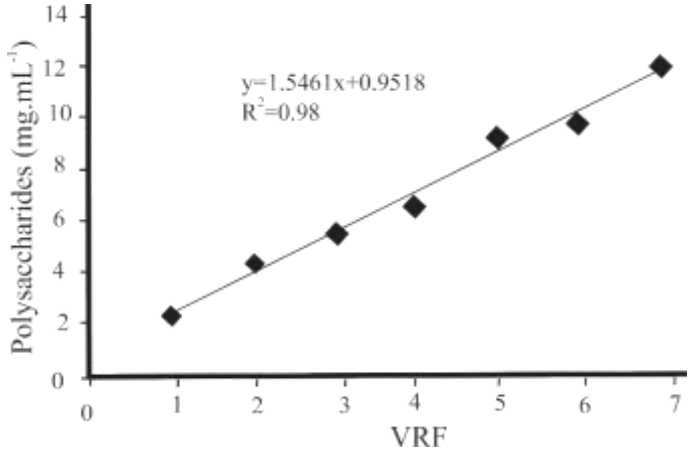

Fig. 7. EPS concentration of *Lactobacillus plantarum* ATCC 8014 extract processed by nanofiltration (35 °C / 6 bars).

Table 1. Tofu whey (TW) composition.

Analytical determination (AOAC methods)	TW
Proteins (% m/v)	0.35 ± 0.02
Oligosaccharides*(% m/v)	0.85 ± 0.02
Lipids (% m/v)	<0.10
Humidity	98.44 ± 0.01
Ashes (% m/v)	0.36 ± 0.01
Total acidity (mL N 100/mL)	1.78 ± 0.04
pH	5.2 ± 0.01
Soluble solids (°Brix)	2.1 ± 0.01
Conductivity (μS/cm)	3.75 ± 0.07

* Sucrose, raffinose and stachyose determined by HPLC.

Table 2. Parameters evaluated during the cultivation of *Lactobacillus plantarum* ATCC 8014 in airlift bioreactor in MRS and STW media.

Culture media	Kinetic and stoichiometric parameters				
	μ_{Xmed} (1/h)	μ_{Pmed} (1/h)	$Y_{X/S}$ (g_{biom}/g_{gluc})	$Y_{P/S}$ (g_{EPS}/g_{gluc})	$(EPS/X)_{med}$
MRS	0.074	0.040	0.15	0.04	0.82
STW	0.038	0.039	0.34	0.15	0.59

$(EPS/X)_{med}$ obtained by the arithmetic mean of the data from Fig. 2.