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Produção de *Lactobacillus rhamnosus* BRM 029693 em fermentação em batelada

Production of *Lactobacillus rhamnosus* BRM 029693 in feed-batch fermentation

Producción de Lactobacillus rhamnosus BRM 029693 en fermentación por lote de alimentación

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Rafaella Martins de Freitas
ORCID: https://orcid.org/0000-0003-2508-3108
Instituto Federal de Educação Ciência e Tecnologia do Ceará, Brasil
E-mail: rafaella_mf@yahoo.com.br
Juliane Döering Gasparin Carvalho
ORCID: https://orcid.org/0000-0002-0199-7864
Universidade Federal do Ceará, Brasil
E-mail: julianedgc@gmail.com
Laura Maria Bruno
ORCID: https://orcid.org/0000-0002-0875-7716
Embrapa Agroindústria Tropical, Brasil
E-mail: laura.bruno@embrapa.br
Gustavo Adolfo Saavedra Pinto
ORCID: https://orcid.org/0000-0002-3672-8795
Embrapa Agroindústria Tropical, Brasil
E-mail: gustavo.saavedra@embrapa.br

Resumo

O objetivo deste trabalho foi produzir biomassa de *Lactobacillus, rhamnosus* BRM 029693, em fermentador, com o intuito de ampliar sua escala de produção. *Lactobacillus rhamnosus* BRM 029693, foi cultivado em fermentador em diferentes condições de aeração (em superfície e profundidade), empregado um substrato comercial (caldo MRS) e substrato simplificado formulado no próprio laboratório; e em diferentes condições de pH (4,7, 5,2,

5,7). A produção de biomassa em escala de fermentador utilizando o MRS sintético, aeração de 1 v.v.m. na superfície do meio e sem controle de pH foi a maior (3,2 g/L) entre todos os ensaios. Quando o meio MRS foi substituído por MRS simplificado, a produção de biomassa foi de apenas 0,7 g/L. O pH também influenciou na produção de biomassa, que foi de 0,9 g/L quando o pH da fermentação foi controlado em pH 4,7.

Palavras-chave: Biomassa; Cultivo de *Lactobacillus*; Bactéria ácido lática.

Abstract

The objective of this work was to produce *Lactobacillus rhamnosus* BRM 029693 biomass in a fermenter with the aim to expand its production scale. *Lactobacillus rhamnosus* BRM 029693 was grown in a fermenter under different aeration conditions (in surface and depth) using a commercial substrate (MRS broth) and simplified substrate formulated in the laboratory itself, and under different pH conditions (4.7, 5.2, 5.7). The highest biomass production on a fermenter scale occurred using synthetic MRS, 1 v.v.m. aeration on the medium surface and without pH control (3.2 g/L) among all the tests. The biomass production was only 0.7 g/L when the MRS medium was replaced by simplified MRS. The pH also influenced the biomass production, reaching 0.9 g/L when the fermentation pH was controlled at pH 4.7.

Keywords: Biomass; *Lactobacillus* growth; Lactic acid bacteria.

Resumen

O objetivo deste trabalho foi produzir biomassa de Lactobacillus, rhamnosus BRM 029693, em fermentador, con el intuito de ampliar su escala de producción. Lactobacillus rhamnosus BRM 029693, foi cultivado em fermentador em diferentes condições de aeração (em superfície e profundidade), empregado um substrato comercial (caldo MRS) y substrato simplificado formulado no próprio laboratório; e em diferentes condiciones de pH (4,7, 5,2, 5,7). Una producción de biomasa en escala de fermentador utilizando MRS sintético, emisión de 1 v.v.m. na superfície do meio and sem control of pH foi a maior (3,2 g / L) entre todos los ensaios. Quando o meio MRS foi sustituído por MRS simplificado, una producción de biomasa de apenas 0,7 g / L. El pH también puede influir en la producción de biomasa, que es de 0,9 g / L, o el pH de la fermentación controlada de pH 4,7.

Palabras clave: Biomasa; Cultivo de Lactobacillus; Bactéria del ácido láctico.

1. Introduction

Lactic acid bacteria (LAB) are used in the production of various fermented foods (mainly dairy products) and can be divided into two major biotechnological groups: starter cultures, which are those that lead to fermentation; and non-initiator or adjunct cultures, which act in developing desirable characteristics of the product, such as improving the taste and texture (Vandera *et al*. 2019).

It is widely known that raw milk cheeses develop a more intense flavor than pasteurized milk cheeses (Pasquale *et al*., 2019) due to a reduction in the diversity of lactic acid bacteria present in raw milk during pasteurization. Therefore, bacterial biodiversity can be considered a fundamental factor in maintaining typical characteristics of traditional cheeses, which generates a demand for developing autochthonous cultures specifically developed for typical cheeses worldwide (Carafa *et al.*, 2019; Guarcello *et al.*, 2016 and Bruno *et al.*, 2017).

Coalho cheese is a traditional product from the Northeast region of Brazil and has economic, social and cultural importance, since emigrants who seek a closeness with their territory of origin demand it in their consumption. Artisanal coalho cheese is made with raw milk, but according to Brazilian legislation, the milk used in manufacturing unripened cheese must be pasteurized or heat treated (Brasil, 1996).

Bruno et al. (2017) selected *Lactobacillus* strains to be used as an adjunct culture in producing coalho cheese made with pasteurized milk. However, the development of a microbial culture for application in the industry also involves expanding the bacterium's cultivation scale to obtain biomass in quantity, separation and recovery of cells from the culture medium, and its conservation by processes which not only enable preserving the culture for a long period of time, but which also facilitates its commercialization.

In view of the above, the objective of this work was to evaluate the biomass production parameters of *Lactobacillus rhamnosus* BRM 029693 in a 2 L fermenter, aiming to expand the cultivation scale to obtain biomass.

2. Material and Methods

Lactobacillus rhamnosus BRM 029693 bacterium isolated from artisanal coalho cheese and belonging to the Collection of Microorganisms of Interest to the Tropical Agroindustry was used in all experiments. The stock culture of the microorganism was

maintained in a 10% maltodextrin and 10% lactose solution at -80 °C, then thawed, and a 0.1% aliquot was activated in MRS broth at 37 °C, under agitation (150 rpm) for 24 hours.

An aliquot (100µL) of the stock culture was inoculated in 100mL of MRS broth and incubated at 37 °C under agitation (150rmp) in a rotary shaker (Tecnal TE-240) for 24h. This culture was used as an inoculum for fermentations.

The fermentations were carried out in a simple batch mode in a 3L bioreactor (BioFlo/CelliGen 115, New Brunswick Scientific) using a 2L working volume and 1% inoculum.

The influence of aeration on biomass production was assessed by varying the aeration on the surface of the medium or by injecting air at depth. The fermentations were carried out in duplicate using MRS (Kasvi) as a culture medium and aeration of 1 v.v.m. at 37 °C/48 hours, without pH control. Aliquots of 30 mL were removed at times 0, 18, 24, 42 and 48 hours to determine the biomass concentration (g/L).

The simplified MRS medium (10 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, 2 g/L ammonium citrate, 2 g/L magnesium sulfate) was evaluated as a substrate alternative for the MRS broth. The same fermentation conditions used above were maintained. The biomass concentration was determined at times 0, 18, 24, 42 and 48 hours.

Fermentation was carried out at three different pH values (4.7; 5.2 and 5.7) in a simplified MRS medium at 37 °C/24 h, with aeration of 1 v.v.m. and agitation of 150 rpm to determine the pH influence on biomass production. The pH was controlled by adding 1N NaOH solution. The biomass concentration determination was performed at times 0.16, 20 and 24 hours.

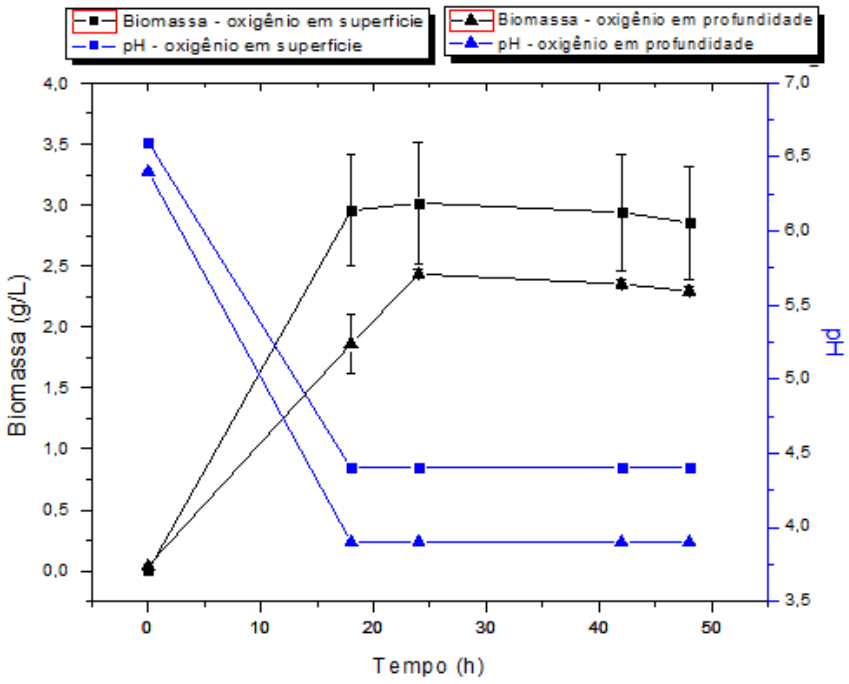
Biomass production was estimated by determining the absorbance at 600 nm of samples taken at different fermentation times, which were then transformed into dry weight (g/L) according to a previously constructed standard curve, and which correlates absorbance and biomass in dry weight.

3. Results and Discussion

The influence of aeration on *L. rhamnousus* BRM 029693 biomass production was evaluated by varying the aeration mode carried out on the medium surface or by injecting air at depth. The biomass production by the BRM 029693 strain after 24 hours of fermentation was 3.2 g/L when oxygen was inserted into the medium surface, and 2.4 g/L when the gas was injected at depth (Figure 1). It was also observed that there was a greater decline in pH in

the first 24 hours of fermentation when oxygen was injected into the bottom of the vat, which justifies the lower amount of biomass produced in this condition.

Figure 1 - *Lactobacillus rhamnosus* BRM 029693 biomass production in MRS medium in the bioreactor with baffles at 37 °C/48 h, under agitation (150 rpm) and with 1 v.v.m aeration on the medium surface and at depth.



Source: Authors,

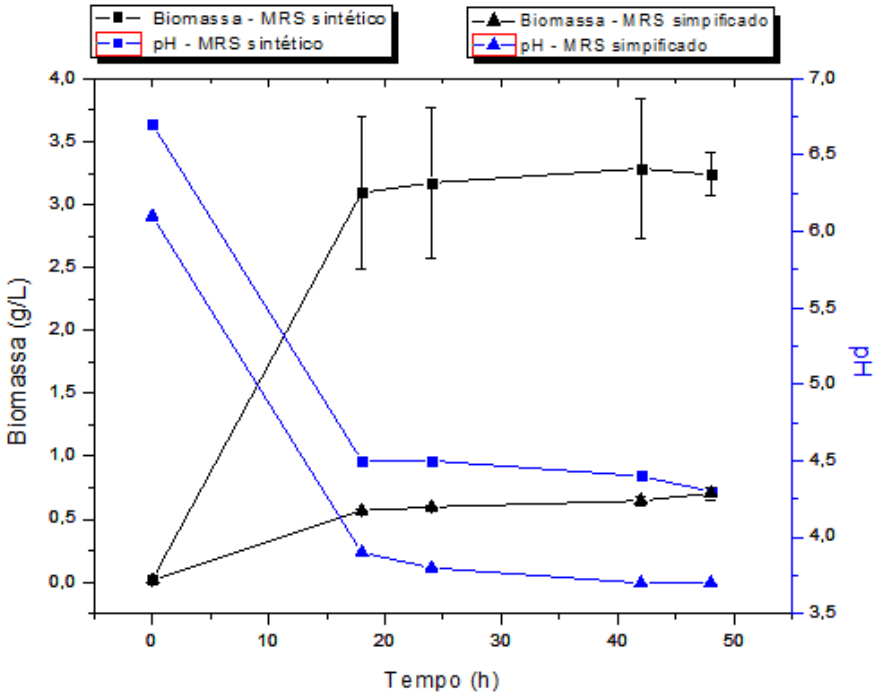
Although lactic acid bacteria are known as oxygen tolerant anaerobes, aerobic cultures and respiration metabolism have been shown to improve technological and stress response properties in microorganisms such as *Lactobacillus lactis* (Lechardeur *et al.* 2011 and Perdersen *et al.*, 2015) *Lactobacillus plantarum* (Zotta *et al*., 2014) and some members of the *Lactobacillus casei* group (Ianniello *et al.*, 2016).

Ianniello *et al.* (2016) investigated the influence of oxygen on the growth of the *Lactobacillus casei* N87 strain in a 3L bioreactor at 37 °C for 24 hours and observed that the use of oxygen injection combined with the supplementation of the culture medium contributed to a greater biomass production (5.28 g/L) when compared to the anaerobic process (4.06 g/L).

On the other hand, Zotta *et al.* (2012) evaluated the influence of aeration on the growth of *Lactobacillus plantarum* WCFS1 and its tolerance to stress, finding that there was no significant difference in biomass production in the aerobic and anaerobic processes.

The simplified MRS medium with a lower nutrient content composition was evaluated as a lower cost substrate to be used in producing *L. rhamnosus* biomass (Figure 2). Biomass production in the simplified MRS medium was 0.5 g/L in the first 24 hours of fermentation and 0.7 g/L at the end of the process. After adding the inoculum, the initial pH of the medium (6.5 ± 0.2) dropped to a pH value of 6.0 ± 0.2 (Figure 2), possibly due to the absence of a buffering agent in its formulation. The pH of fermentation in simplified MRS medium remained lower than the pH of fermentation in commercial MRS medium throughout the process.

Figure 2 - Biomass production of the *Lactobacillus rhamnosus* BRM 029693 strain in a commercial and simplified MRS medium in a bioreactor without baffles at 37 °C/48 h, under agitation (150 rpm) and with 1 v.v.m. aeration on the medium surface.



Source: Authors,

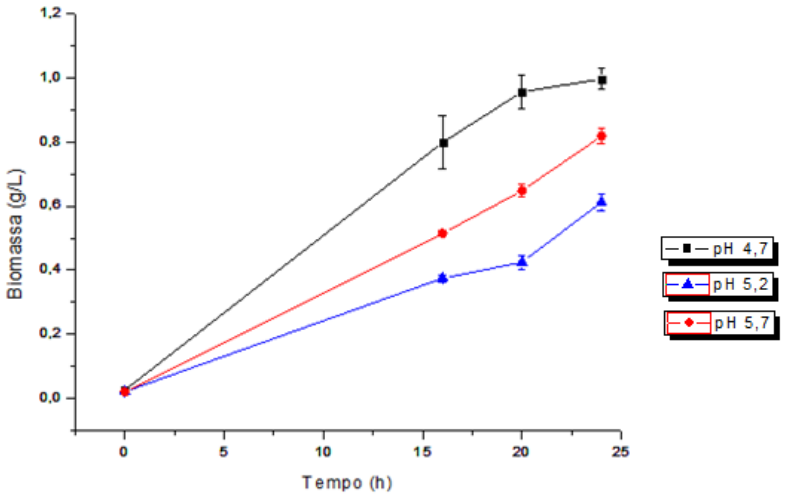
Hwang *et al.* (2015) used a similar culture medium (10 g/L glucose, 10 g/L yeast extract, 10 g/L soy peptone, 2 g/L ammonium citrate, 0.2 g/L magnesium sulfate, 5 g/L sodium acetate, 0.05 g/L manganese sulfate, and supplementation with 10 ml/L corn syrup) to

the simplified MRS in producing *Lactobacillus acidophilus* cells and obtained a maximum biomass production of 4.54 g/L in dry weight.

Berecka *et al.* (2011) studied the effect of various components of the culture medium on *Lactobacillus rhamnosus* biomass production and determined that a medium composed of 15.44 g/L glucose, 3.92 g/L sodium pyruvate, 8.0 g/L meat extract, 1.88 g/L potassium phosphate, 4.7 g/L sodium acetate and 1.88 g/L ammonium citrate resulted in 23 g/L of dry matter biomass after 18 hours of cultivation in a bioreactor, with a mixed source of carbon, glucose and sodium pyruvate being the preferred substrate for the growth of this microorganism.

As the growth of *Lactobacillus* sp. is inhibited by the lactic acid produced and one of the strategies used to keep the pH unchanged is to control it by adding a neutralizer (Aguirreezauriatza *et al*., 2010), cultivation of the BRM 029693 strain was carried out in a simplified highest biomass production of 0.9 g/L was obtained with pH control at pH 4.7 after 24 hours of fermentation. Although pH control influenced biomass production, it still fell far short of the amount obtained in cultivation with synthetic MRS, which suggests the need to reformulate the alternative medium. Coghetto *et al*. (2016) used the liquid fraction resulting from the washing steps and separation of the isolated soy protein production as a substrate and obtained *Lactobacillus plantarum* biomass production of 10.85 ± 0.03 g/L after 30 hours of fermentation, with 4.5 v.v.m. aeration, temperature of 25 °C and pH controlled at 5.5. Brinques *et al*. (2010) studied the optimization of biomass and lactic acid production by *Lactobacillus plantarum* in submerged bioreactor systems using supplemented cheese whey as a carbon source and reported a biomass production of 10.2 g/L (dry weight) after 48 hours at 34 °C, 3.5 v.v.m. aeration, 200 rpm agitation and pH controlled at 5.2.

Figure 3 - Biomass production of the *Lactobacillus rhamnosus* BRM 029693 strain in a simplified MRS medium in a bioreactor without baffles at 37 °C/48 h, under agitation (150 rpm) with 1 v.v.m. aeration on the medium surface and pH controls at 4.7, 5.2 and 5.7.



Source: Authors,

4. Final Considerations

The agitation parameters and the aeration form were studied for *Lactobacillus rhamnosus* BRM 029693 biomass production, with agitation of 150 rpm and 1 v.v.m aeration into the medium surface being determined as the optimal conditions. The substrate also influenced the cell production, which decreased to less than 50% when the fermentation was carried out in a simplified MRS medium, with less availability of nutrients. The pH control at 4.7 during fermentation positively influenced the amount of biomass produced, but there is a need to improve the formulation of the simplified MRS in order to increase biomass production.

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Percentage of contribution of each author in the manuscript

Rafaella Martins de Freitas – 30%

Juliane Döering Gasparin Carvalho – 20%

Laura Maria Bruno – 35%

Gustavo Adolfo Saavedra Pinto– 15%