

Effect of Temperature, pH and Stirring Speed on Growth and Cell Viability of Probiotic bacterium *Lactobacillus Plantarum*

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ABSTRACT

Lactobacillus spp. are lactic acid bacteria (LAB) commonly found in the mammalian gut microbiota and *Lactobacillus plantarum* is a Gram-positive bacterium commonly found in fermented food and is classified as probiotic [1]. This probiotic-bacterium is wide applications in the medical field because present antioxidant, anticancer, anti-inflammatory, antiproliferative, anti-obesity and antidiabetic properties [2]. Probiotics are live microorganisms that when are administered in adequate amounts, confer a health benefit on the host [3]. Probiotics are wide used in applications of the pharma industry because contributing significantly to human medicine without contributing to any side effects.

However, there are not studies were the culture conditions in bioreactors have been evaluated and particularly, as the temperature, the pH and the stirring speed affected the growth of this probiotic-bacterium. This is an important field that needs to be investigated and by this reason the objective of this study was evaluated the effect of the temperature, the pH and the stirring speed on the viable biomass production in cultures of *L. plantarum*. The effect of temperature on biomass production of *L. plantarum*, was evaluated in 250 mL Erlenmeyer flasks cultures with 100 mL of MRS medium at 34, 37 and 40°C. The maximum biomass concentration was of $3.72 \pm 0.03 \text{ g L}^{-1}$ in the temperature condition of 37°C. In bioreactor cultures, the maximum biomass concentration obtained was of $4.0 \pm 0.1 \text{ g L}^{-1}$ in the conditions evaluated of 100 and 300 rpm when the pH was kept constant at 5.0, meanwhile the cell viability was higher ($2.73 \pm 0.37 \times 10^{10} \text{ CFU mL}^{-1}$) compared with the cell viability obtained in conditions in which the pH was increased at 6.0 or 7.0 ($8.0 \pm 1.0 \times 10^9 \text{ CFU mL}^{-1}$). On the contrary, when the pH and stirring speed conditions were 7.0 and 300 rpm, the specific growth rate (μ) obtained was maximum ($0.99 \pm 0.01 \text{ h}^{-1}$). In summary, the low pH conditions favored the biomass production and the cell viability in the cultures of probiotic-bacterium *L. plantarum*. However, the μ was favored in conditions of high of pH and stirring speed (7.0 and 300 rpm). With these findings, is possible establishing adequate conditions for the production of *L. plantarum* viable biomass in bioreactor cultures for use as probiotic.

Keywords

Probiotic, *Lactobacillus plantarum*, Cell viability, Temperature.

Introduction

Lactobacillus spp. are lactic acid bacteria (LAB) commonly found in the mammalian gut microbiota, and some strains are classified as probiotic [1]. Probiotics are defined as live microorganism which, when administered in adequate amounts, confer a health benefit on the host [3]. Lactobacilli, bifidobacteria, lactococci and yeasts are classified in the category of organisms Generally Regarded As Safe (GRAS) [4]. Diverse diseases and disorders caused by

alterations in the gut microbiome can be treatment using probiotic bacteria [1,5].

In particular, *Lactobacillus plantarum* is a Gram-positive lactic acid bacterium commonly found in fermented food and used in the food industry as a potential probiotic. This probiotic-bacterium is wide applications in the medical field because present antioxidant, anticancer, anti-inflammatory, antiproliferative, anti-obesity and antidiabetic properties as is as able to tolerate acid conditions and high ethanol concentrations [2].

Another characteristic of *L. plantarum* is that present hypocholesterolaemic activity and is able to control lipid levels in the body [6]. Besides applications in the food industry, *L. plantarum* has wide applications in the pharma industry by contributing significantly to human medicine without contributing to any side effects [2]. An important field in the probiotic bacteria cultivation is establishing the adequate operation conditions in bioreactor for the growth of these microorganisms [7]. There are reports were having been observed that the pH, temperature and medium composition affect the behaviour of the microorganisms and the viable biomass is dependent of the growth phase and the culture time in the which the biomass is harvested [8].

However, until now there are not studies where the culture conditions have been evaluated in cultures of *L. plantarum*, with the objective the promoted the viable biomass production. By this reason the aim of this study was evaluated the effect of temperature, pH and stirring speed on the biomass production, the specific growth rate and the cell viability for used as probiotic.

Materials and Methods

Microorganism and culture medium

Lactobacillus plantarum was isolated from breast milk and was cryopreserved at -70°C in a 30% glycerol solution. The cultures of *L. plantarum* grown in MRS medium (Man, Rogosa and Sharpe) (Difco) and prepared as following: 55 g of MRS Broth was weighted and dissolved in 1.0 liter (L) of distilled water and after the solution was sterilized at 121°C during 15 minutes. When was necessary grown the bacterium in solid cultures, Petri dishes were filling with MRS-Agar (Difco Laboratories) at concentration of 70 g per liter of product.

Culture condition

Flasks cultures

L. plantarum was cultivated under anaerobic conditions in 250 mL Erlenmeyer flasks, with a filling volume of 100 mL MRS medium at 100 rpm in a shaking incubator (LabTech). In these cultures, the effect of temperature was evaluated in the range from 34 to 40°C . The bacterial growth was measurement by optical density at a wavelength of 620 nm and was correlated with a patron curve for determinate biomass concentration in g L^{-1} (cell dry weight).

Bioreactor cultures

L. plantarum cultures were grown in an Applikon bioreactor containing 2 L of MRS medium at $37 \pm 0.5^{\circ}\text{C}$, with variable stirring speed from 100 to 300 rpm. The bioreactor was equipped with two impellers, a Rushton turbine and a propel of $\text{Di}/\text{T} = 1/3$ (where Di is the impeller diameter, and T the bioreactor diameter). The cultures were done under fully anaerobic conditions and to guarantee these conditions in the bioreactor, a constant flow of nitrogen was injected by the head of the bioreactor during all culture time. The pH was measured with an Ingold probe (Applikon, ADI 1010) and controlled at 5.0, 6.0, and 7.0 ± 0.1 by an on/off system using a peristaltic pump and adding 4N NaOH or 20 % (v/v) H_3PO_4 solutions. According to the central composite design 2^2 used in this study, each experimental condition was evaluated by duplicate and

the results presented are the average of the independent runs.

+ pH -	100 7.0		300 7.0
		200 6.0	
	100 5.0		300 5.0

- Stirring speed (rpm) +

Table 1: Central composite design 2^2 that was used in this study in the which pH and stirring speed were the evaluated factors.

Analytical determinations: biomass concentration, optical density measurement and cell viability

The biomass concentration was estimated using a correlation between the biomass concentration and optical density. The biomass concentration was quantified as cell dry weight (in grams per liter) and the optical density was measurement at a wavelength of 620 nm (Victor X3 multimode plate reader, Perkin Elmer). A patron curve was made in the interval of optical density from 0.1 to 0.9 absorbance units and biomass concentration from 0.2 to 3.5 g L^{-1} . The cell viability was measurement by plate counting using MRS-Agar Petri dishes. A serial dilution in the order since 106 to 108 was done and only the dishes where the colonies forming units were between 25 and 250 were considerate in this study. Later of plate, the dishes were incubated at 37°C during 48 hrs at least and later the colonies form units (CFU) were count.

Results

Effect of temperature on biomass production in shaken flaks cultures

The effect of temperature on biomass production of *L. plantarum*, was evaluated in 250 mL Erlenmeyer flasks cultures with 100 mL of MRS medium at 34, 37 and 40°C . The maximum biomass concentration was of $3.72 \pm 0.03 \text{ g L}^{-1}$ in the temperature condition of 37°C . Both at 34 and 40°C , the biomass production was of 3.40 ± 0.13 and $3.27 \pm 0.11 \text{ g L}^{-1}$, respectively (Figure 1). These findings indicate that in the condition of 37°C , the biomass production was favored compared with the others temperature conditions evaluated in shake flask cultures. Due that *L. plantarum* is a probiotic-bacterium, the better culture conditions are those that favor the biomass production and by this reason is important grown the bacterium at 37°C . In summary, at 37°C was possible to obtain a higher maximum biomass concentration compared with the obtained at 34 and 40°C .

Effect of temperature on specific growth rate (μ) in shaken flaks cultures

Other parameter important that determinates the process time in the cultures of probioticbacterium is the specific growth rate (μ) and by this reason the effect of temperature on μ of *L. plantarum* was evaluated in shaken flaks cultures. When the bacterium was cultivated at 34°C , the μ was of $0.20 \pm 0.04 \text{ h}^{-1}$, meanwhile for the temperature conditions of 37 and 40°C , there are not differences in this parameter and was of $0.47 \pm 0.01 \text{ h}^{-1}$ in both conditions. Interestingly, the specific growth rate increased when the culture

temperature was changed from 34 to 37 and 40°C. This behavior indicate that the growth of *L. plantarum* is dependent of culture temperature and the growth of this bacteria in better at 37 and 40°C compared with the growth obtained in the condition of 34°C. Taking into consideration that the maximum biomass production was obtained at 37°C and the specific growth rate was higher at 37 and 40°C that at 34°C, the temperature condition choose for the bioreactor cultures was 37°C.

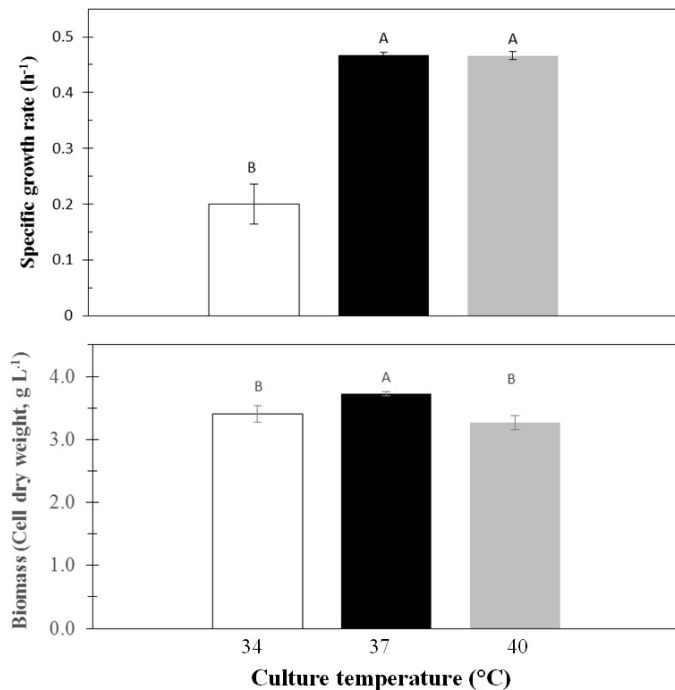


Figure 1: Specific growth rate (μ) and maximum biomass concentration obtained in cultures in 250 mL Erlenmeyer flask with 100 mL of MRS medium under anaerobic conditions at 34, 37 and 40 °C.

Effect of the pH and the stirring speed on biomass production in bioreactor cultures

The effect of the pH and the stirring speed on the growth of the probiotic-bacterium *L. plantarum* was evaluated in bioreactor cultures at 37°C and with 2.0L of MRS medium. Culture samples were taken each two hours, the experiments were made by duplicate and the cultures were stopped until that stationary phase was reached. Interestingly, there is not effect of the stirring speed on biomass production both at low and high pH condition evaluated. The maximum biomass concentration obtained was of $4.0 \pm 0.1 \text{ g L}^{-1}$ in the conditions evaluated of 100 and 300 rpm when the pH was kept constant at 5.0 in the bioreactor (Figure 2). Also, the biomass concentration was practically constant ($3.7 \pm 0.21 \text{ g L}^{-1}$) in the interval of 100 to 300 rpm and under pH conditions of 6.0 and 7.0. With respect to pH of the culture, was observed that this factor had a negative effect on the maximum biomass concentration. In this work, we findings that the biomass concentration was higher ($4.0 \pm 0.1 \text{ g L}^{-1}$) in the culture conditions where the pH was minimum (pH=5.0) and independent of stirring speed condition. Meanwhile that when the pH of the culture was kept constant at 6.0 and 7.0 values, the biomass concentration decreased and was of $3.7 \pm 0.21 \text{ g L}^{-1}$ (Figure 2).

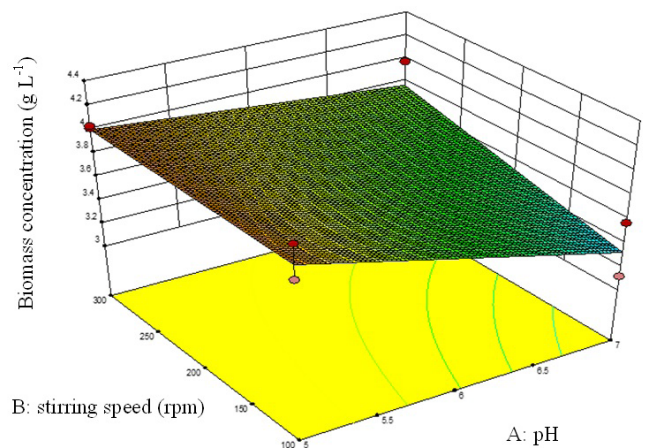


Figure 2: Effect of pH and stirring speed on the biomass maximum concentration (Xmax) of *L. plantarum* obtained in bioreactor cultures with 2.0 L of MRS medium under anaerobic conditions.

Effect of the pH and the stirring speed on specific growth rate (μ) in bioreactor cultures

In this study, we evaluated the effect of pH and stirring speed on the specific growth rate of *L. plantarum* using a factorial design where the pH and the stirring speed were the factors evaluated and two levels for each factor as well as a central point condition was included. Interestingly, the specific growth rate (μ) was of $0.78 \pm 0.05 \text{ h}^{-1}$ in the conditions of low pH and stirring speed (5.0 and 100 rpm, respectively) evaluated (Figure 3). On the contrary, when the pH and stirring speed conditions were 7.0 and 300 rpm, the μ obtained was of $0.99 \pm 0.01 \text{ h}^{-1}$. However, in the cultures where the pH was increased from 5.0 to 7.0 and the stirring speed decreased from 300 to 100, the μ decreased in all the cases and this rate was of $0.90 \pm 0.02 \text{ h}^{-1}$ (Figure 3).

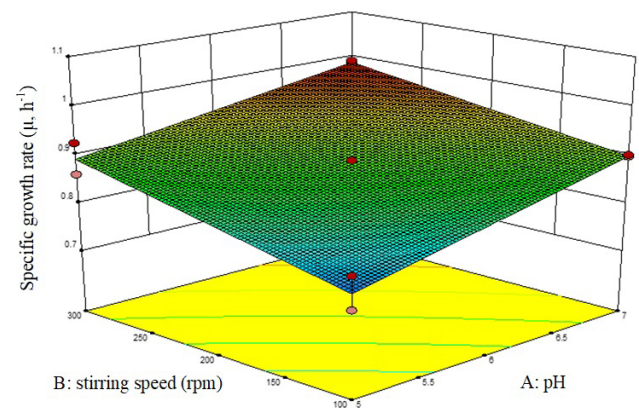


Figure 3: Effect of pH and stirring speed on the specific growth rate (μ) of *L. plantarum* in cultures in bioreactor with 2.0 L of MRS medium under anaerobic conditions.

Effect of pH and the stirring speed on cell viability of *L. plantarum* in bioreactor cultures

An important parameter in the cultures of probiotic bacteria is the cell viability due that according to the definition of probiotic are live microorganisms that administered in adequate amounts; confer a health benefit on the host. By this reason in this study, the cell viability at the end of the cultures was evaluated by count in

plate (Figure 4). In the conditions where the pH was controlled at 5.0, the cell viability was higher compared with the cell viability obtained in conditions in which the pH was increased at 6.0 or 7.0. For example, when the pH was kept at 5.0 and the stirring speed of 100 rpm, the cell viability was $1.98 \pm 0.07 \times 10^{10}$ CFU mL⁻¹. The cell viability was of the same order in the cultures where the pH was of 5.0 and the stirring speed of 300 rpm ($2.73 \pm 0.37 \times 10^{10}$ CFU mL⁻¹). In the pH conditions of 6.0 and 7.0, the cell viability decreased one order of magnitude and was of $8.0 \pm 1.0 \times 10^9$ CFU mL⁻¹, independently of the stirring speed (from 100 to 300 rpm) employed in the cultures (Figure 4).

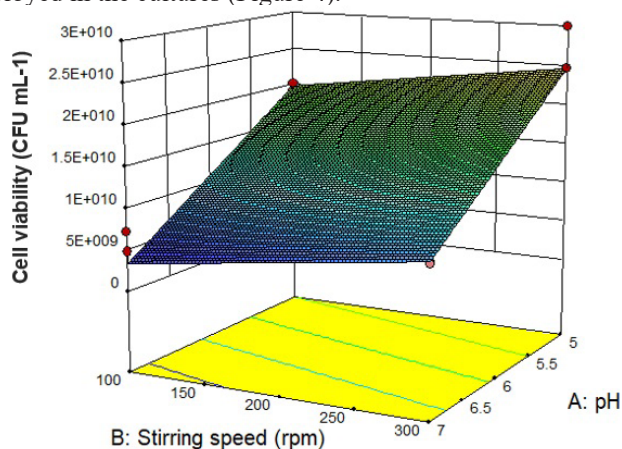


Figure 4: Effect of pH and stirring speed on the cell viability of *L. plantarum* obtained in bioreactor cultures with 2.0 L of MRS medium under anaerobic conditions.

Discussion

In a recent study was observed that a mixture of *L. plantarum*, *Pediococcus acidilactici*, and *P. pentosaceus* reduced *Salmonella* populations on Alfalfa Sprouts. Also, this mixture of probiotic-bacteria did not compromise the yield, seedling length, or pH of the sprouts and did not influence the sprout quality [9]. The temperature affected the growth and biomass production, but the better temperature condition was 37°C. Under this condition, the biomass production and the specific growth rate were favored (Figure 1). These results are consistent due that this probiotic-bacterium was isolated from breastmilk and 37 °C is the temperature of the human body. By this reason, the growth of *L. plantarum* was optimal in these conditions of temperature. There are reports where has been demonstrated that the growth of *Lactobacilli* is adequate in temperatures between 32 to 37°C [7,8]. Probiotic bacteria present diverse mechanisms of action for exert the potential beneficial effect of the probiotic on the health of the host [4]. Previously, has been reported that *L. plantarum* is a bacterium able to produce lactic acid decreasing the pH in the medium. It is possible that the lactic acid production is a mechanism to exerts against pathogenic bacteria preventing or limiting their colonization. Also, the acetoin production contributes to pH homeostasis in *L. plantarum* according has been reported previously [10]. Interestingly, in this

study we observed that the viable biomass production was favored in conditions with pH low. Both the biomass concentration and the cell viability obtained under pH low conditions were maximum for *L. plantarum*. Despite that the specific growth rate was maximum when the pH and stirring speed conditions in the cultures were high (7.0 and 300 rpm), under these cultures conditions both the cell viability and the biomass production decreased compared with the results obtained at low pH conditions (Figures 2 and 4).

Conclusion

In summary, the low pH conditions favored the biomass production and the cell viability in the cultures of probiotic-bacterium *L. plantarum*. Also, temperature conditions influenced on the biomass production and specific growth rate, but the better temperature for the culture of *L. plantarum* was 37°C. With these findings, is possible establishing adequate conditions for the production of *L. plantarum* viable biomass in bioreactor cultures to be used as probiotic supplement in functional foods or for seed protection against Gramnegative pathogenic bacteria.

References

1. Rout GK, Jayanta KP, Sushanto G, et al. Benefaction of probiotics for human health: A review. *J Food Drug Anal.* 2018; 26: 927-939.
2. Arasu MV, Al-Dhabi NA, Ilavenil S, et al. In vitro importance of probiotic *Lactobacillus plantarum* related to medical field. *Saudi J Biol Sci.* 2016; 23: 6-10.
3. Hill C, Guarner F, Reid G, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol.* 2014; 11: 506-514.
4. Butel MJ. Probiotics, gut microbiota and health. *Médecine et maladies infectieuses.* 2014; 1-8.
5. Daliri EB-M, Lee BH. New perspectives on probiotics in health and disease. *Food Science and Human Wellness.* 2015; 4: 56-65.
6. Nami Y, Vaseghi R, Manafi M, et al. Hypocholesterolaemic activity of a novel autochthonous potential probiotic *Lactobacillus plantarum* YS5 isolated from yogurt. *LWT- Food Science and Technology.* 2019; 111: 876-882.
7. Wallenius J, Uuksulainen T, Salonen K, et al. The effect of temperature and pH gradients on *Lactobacillus rhamnosus* gene expression of stress-related genes. *Bioprocess Biosyst Eng.* 2011; 34: 1169-1176.
8. Romero-Gil V, Bautista-Gallejo J, Rodríguez-Gómez F, et al. Evaluating the individual effects of temperature and salt on table olive related microorganisms. *Food Microbiol.* 2013; 33: 178-184.
9. Rossi F, Lathrop A. Effects of *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus* on the Growth of *Listeria monocytogenes* and *Salmonella* on Alfalfa Sprouts. *J Food Prot.* 2019; 82: 522-527.
10. Tsau JL, Guffanti AA, Montville TJ. Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. *Appl Environ Microbiol.* 1992; 58: 891-894.