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Application of response surface methodology and central composite rotatable design (CCDR) for modelling the influence of agro-industrial waste in lactic acid biosynthesis

Aplicação da metodologia de superfície de resposta e do delineamento composto central rotacional (DCCR) para modelagem da influência de resíduos agroindustriais na biossíntese de ácido láctico

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ABSTRACT
Lactic acid (LA) is one of the most important organic acids, with a wide range of industrial and biotechnological applications and can be produced by chemical synthesis and microbial culture. However, the biotech pathway is generally preferred because it provides an optically pure product. In this context, the purpose of this work was to evaluate LA biosynthesis by *Lactobacillus amylovorus* using molasses as carbon source (CS) and corn steep liquor as nitrogen source (NS) in a central composite rotatable design (CCRD) varying the concentration CS and NS, as well as to validate the model. The method for microbial culture followed an experimental design of the CCRD type, conducted without agitation, at 37ºC in Erlenmeyer flask, with pH in spontaneous evolution. The results showed that, using molasses and corn steep liquor as alternative sources, LA production ranged from 2.8 to 4.6 g/L, respectively, with the most favourable condition being 40.0 g of molasses and 250 g of corn steep liquor. It was possible, from the experimental design, to ascertain the selection of the best conditions for the microbial culture, demonstrating the feasibility of replacing CS and NS by agro-industrial waste, thus reducing the cost of producing LA.
Keywords: corn steep liquor; experimental design; *Lactobacillus amylovorus*; molasses.

RESUMO
O ácido láctico (AL), um dos ácidos orgânicos mais importantes, com uma ampla gama de aplicações industriais e biotecnológicas, pode ser produzido por síntese química e cultura microbiana. No entanto a via biotecnológica é geralmente preferida, porque fornece um produto oticamente puro. Nesse contexto, o objetivo deste trabalho foi avaliar a biossíntese de AL por *Lactobacillus amylovorus*, utilizando melaço como fonte de carbono (FC) e milhocina como fonte de nitrogênio (FN), por meio de um delineamento composto central rotacional (DCCR), variando a concentração de FC e FN, bem como a validação do modelo. O método para a cultura microbiana seguiu um delineamento experimental do tipo DCCR, conduzido sem agitação, a 37ºC, em frasco de Erlenmeyer, com pH em evolução espontânea. Os resultados mostraram que, usando melaço e milhocina como fontes alternativas, a produção de AL variou de 2,8 a 4,6 g/L, respectivamente, sendo a condição mais favorável 40,0 g de melaço e 250 g de milhocina. Foi possível obter, com base no delineamento experimental, a seleção das melhores condições para a cultura microbiana, demonstrando a viabilidade de substituição de FC e FN por resíduos agroindustriais, reduzindo assim o custo de produção de AL.
Palavras-chave: delineamento experimental; *Lactobacillus amylovorus*; melaço; milhocina.

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INTRODUCTION

Lactic acid (LA) is one of the most important organic acid which is being extensively used around the world in a wide range of industrial and biotechnological applications (GHAFFAR *et al.*, 2014). Approximately 70% of LA produced is used in the food industry because of its role in the production of yogurt and cheese (MARTINEZ *et al.*, 2013). LA is also used as an acidulant because its mild acidic taste when compared with other acids used in food, and as a preservative in olives and pickled vegetables. It is also used as flavouring agent, pH regulator, and inhibitor of residual bacteria in food processing, such as for sweets, breads, soft drinks, beer, and other products. LA has applications in the leather tanning industry, in the descaling processes, in the textile industry as a mordant (fixative) for dyeing, and can replace ethylene glycol in antifreeze. In the chemical industry, LA can be converted to ethanol, propylene glycol, and acrylic polymers (KOMESU *et al.*, 2017). In the pharmaceutical industry, LA is used in implants, pills, dialysis, surgical sutures, and controlled drug release systems. In the cosmetic industry, LA is used in the manufacture of hygiene and aesthetic products because of moisturizing, antimicrobial, and rejuvenating effects on the skin. In recent years, the demand for LA has increased considerably because of its use as a monomer in the preparation of polylactic acid (PLA), which is a biodegradable and biocompatible polymer that is used in a wide variety of applications. PLA has an increasingly important role in reducing the net emission of carbon dioxide and the petroleum demand (ZHENG *et al.*, 2017).

There are two optical isomers of LA: L(+)-lactic acid and D(–)-lactic acid. LA is classified as GRAS (generally recognized as safe) for use as a food additive by the US FDA (Food and Drug Administration), but D(–)-lactic acid is at times harmful to human metabolism and can result in acidosis and decalcification (WEE *et al.*, 2006). LA can be produced by chemical synthesis or by microbial culture, as shown in figure 1. Although racemic DL-lactic acid is always produced by chemical synthesis from petrochemical resources, an optically pure L(+)- or D(–)-lactic acid can be obtained by microbial synthesis of renewable resources when the appropriate microorganism that can produce only one of the isomers is selected (HOFVENDAHL & HAHN-HÄGERDAL, 2000).

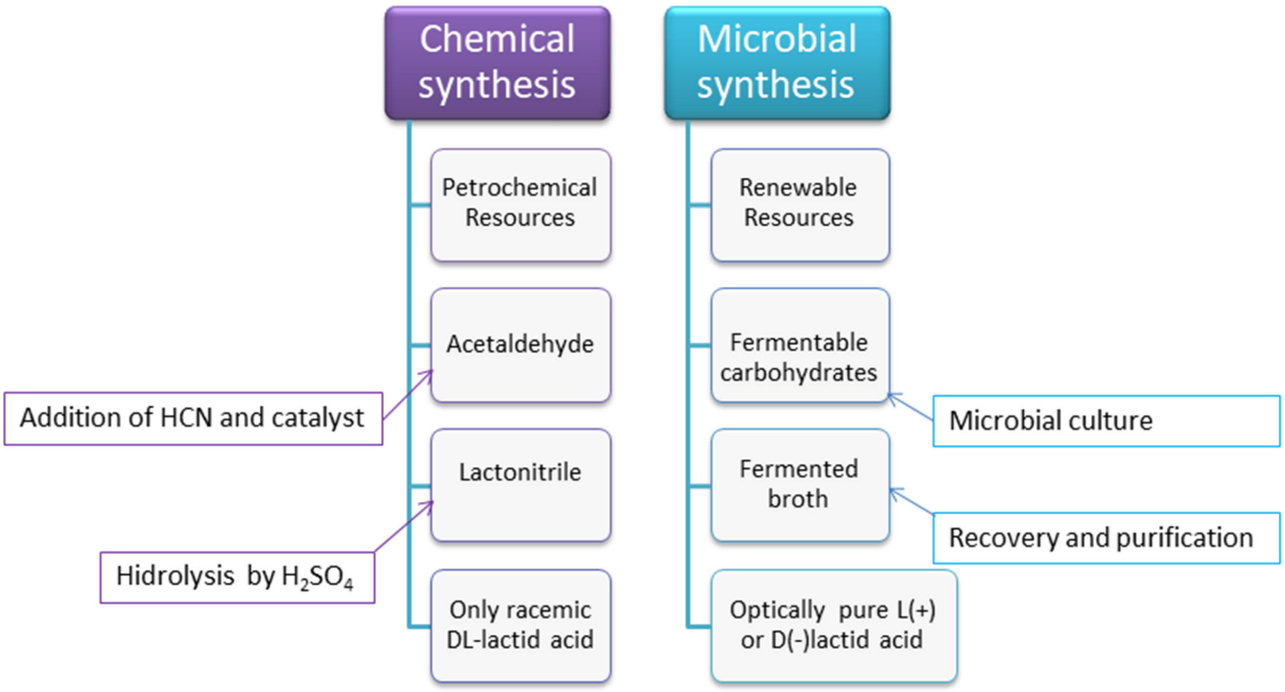

Figure 1 – Overview of the two manufacturing methods of lactic acid: a) chemical synthesis and b) microbial synthesis (WEE *et al.*, 2006).

Depending on the application, one form of the optically pure LA is preferable over the other. Additionally, microbial LA fermentation offers advantages in terms of the utilization of renewable carbohydrate biomass, low production temperature, low energy consumption, and the production of optically high pure LA by selecting an appropriate strain (ABDEL-RAHMAN *et al.*, 2011). The significant advantage of biotechnological production by culture rather than by chemical synthesis is that low cost raw materials can be used such as molasses, starchy wastes, cellulose and other materials rich in carbohydrates (ANURADHA *et al.*, 1999; VISHNU *et al.*, 2000). In an effort to reduce production costs, researchers have developed different processes, of which direct culture stands out as one of the most cost effective processes for lactic acid production. In a recent revision, there is an indication of amylolytic lactic acid bacteria for a single phase in lactic acid conversion, in which the direct conversion of biomass into lactic acid was studied, giving special interest to the simultaneous degradation of biomass complexes (saccharification) and the use of sugar for the production of lactic acid (culture) (REDDY *et al*., 2008).

Lactic acid is easily obtained through the biotechnological process, mainly using strains from low cost raw materials (NARAYANAN *et al.*, 2004). Generally, the process occurs in two phases: the saccharification, followed by culture by *Lactobacillus*. The direct conversion of starch into lactic acid by bacteria with both amylolytic acid and lactic production can eliminate the two phase process, making it more economically feasible (ALTAF *et al.*, 2007).

Approximately 3.5 billion tons of agricultural wastes are produced per year worldwide. These biomasses are potential alternative sources of carbohydrates for culture, with normally low acquisition costs when compared to refined sugars, and are readily available, making them especially interesting (ZHANG *et al*., 2007). Some agricultural wastes considered as potential substrates for production are: cotton husks, maize cobs and stalks, beetroot, molasses, cassava bagasse, molasses spent in washing, hydrolysed corn fiber and wheat bran (KOTZAMANIDIS *et al*., 2002; SAHA & NAKAMURA, 2003; NAVEENA *et al*., 2005; ROJAN *et al*., 2005).

The yeast extract commonly used as a source of nitrogen is a suitable nutrient for ensuring the growth of these bacteria but its high cost poses a limitation on its application in industrial processes, thus, it is desirable to find nutrients that are cost effective for use in industrial processes. Some low cost nutrients, such as fish wastes (MARTONE *et al*., 2005), corn steep liquor which is the water resulting from soaking the corn (RIVAS *et al*., 2004), sub products from the beer industry (PAULI & FITZPATRICK, 2002) and hydrolysed proteins from cheese whey (FITZPATRICK *et al*., 2003) have been used for producing LA.

In a recent study, Jaramillo *et al*. (2018), using a Central Composite Rotatable Design (CCRD), obtained a quadratic model to describe the relationship between DLA production and the components of the medium and showed the significant effect of sodium acetate, meat extract, yeast extract, glucose and dipotassium phosphate in the production of DLA.

The objective of this study was to evaluate the biosynthesis of LA by *Lactobacillus amylovorus* using different carbon sources (CS) and nitrogen sources (NS) coming from agro-industrial wastes, through a central composite rotatable design (CCRD) varying the concentration of molasses (CS) and corn steep liquor (NS) and validating the model.

MATERIAL AND METHODS

MICROORGANISM AND SUBSTRATES

The microorganism used in this study was *Lactobacillus amylovorus* CCT 2948, an amylolytic strain of the Andre Tosello Foundation – Tropical Cultures Collection (FAT, Campinas, SP, Brazil). For strain maintenance, we used tube cultivation in MRS (Mann, Rogosa and Sharpe) complete medium, containing peptone (10.0 g/L), yeast extract (4.0 g/L), meat extract (8.0 g/L), glucose (20.0 g/L), sorbitan monooleate (1.0 mL/L), dipotassium phosphate (2.0 g/L), sodium acetate (2.0 g/L), triammonium citrate (2.0 g/L), magnesium sulfate (0.20 g/L) and manganese sulfate (0.05 g/L). The agro-industrial wastes tested were molasses and corn steep liquor from Ingredion (Westchester, Illinois, USA).

CENTRAL COMPOSITE ROTATABLE DESIGN (CCRD)

A CCRD was carried out in this study, with three central points, coming to a total of 11 experiments. The distances from the axial points were ± 1.41.

The *Lactobacilus amylovorus* cultivation for the production of lactic acid was conducted in a medium similar to the MRS medium, with peptone, meat extract, yeast extract by corn steep liquor, with the glucose being substituted by molasses, as shown in tables 1 and 2.

Table 1 – Concentrations (g/L) of carbon and nitrogen sources used in the CCRD.

Source	-1.41	-1	0	+1	+1.41
Molasses	10.0	14.4	25.0	36.5	40.0
Corn Steep Liquor	10.0	14.4	25.0	36.5	40.0

Table 2 – CCRD aimed at optimizing lactic acid production by molasses and corn steep liquor.

Experiment	Carbon Source	Nitrogen Source	[C]	[N]
M 1	Molasses	Corn steep liquor	-1	-1
M 2	Molasses	Corn steep liquor	1	-1
M 3	Molasses	Corn steep liquor	-1	1
M 4	Molasses	Corn steep liquor	1	1
M 5	Molasses	Corn steep liquor	0	0
M 6	Molasses	Corn steep liquor	0	0
M 7	Molasses	Corn steep liquor	0	0
M 8	Molasses	Corn steep liquor	-1.41	0
M 9	Molasses	Corn steep liquor	1.41	0
M 10	Molasses	Corn steep liquor	0	-1.41
M 11	Molasses	Corn steep liquor	0	1.41

The assays were conducted in Erlenmeyer flasks (250 mL), containing 100 mL of medium and incubated at 37°C, without static culture.

DETERMINATION OF LACTIC ACID AND SUBSTRATE CONSUMPTION

These parameters were obtained through high performance liquid chromatography (HPLC) in Merck-Hitachi equipment model P-7000 If.

STATISTICAL ANALYSIS

The result, analysed in terms of lactic acid production, was later submitted to a statistical analysis using the Statistica 7 program.

RESULTS AND DISCUSSION

The influence of molasses (CS) and corn steep liquor (NS) on the production of LA in 84 h cultivation were carried out using a CCRD, where the codified and real values of starch and corn steep liquor concentrations used in experiments as well as the final concentration of lactic acid, are presented in table 3.

Table 3 – CCRD with lactic acid production values, in 84 h of microbial culture, using molasses as a carbon source and corn steep liquor as a nitrogen source.

	Assay	Molasses (g/L)	Corn steep liquor (g/L)	Lactic acid (g/L)
	M1	14.4	14.4	2.8
Complete	M2	35.6	14.4	4.2
	M3	14.4	35.6	3.2
	M4	35.6	35.6	4.5
	M5	25.0	25.0	3.8
Central	M6	25.0	25.0	3.7
	M7	25.0	25.0	3.7
	M8	10.0	25.0	3.1
Axial	M9	40.0	25.0	4.6
	M10	25.0	10.0	3.1
	M11	25.0	40.0	3.7

According to the results, it can be verified that two test conditions for the production of LA stood out: the experiment M4, in which a combination of 35.6 g/L of molasses and 35.6 g/L of starch was used obtaining an LA production of 4.5 g/L, and the M9, which varied the molasses concentration to 40.0 g/L and the corn steep liquor to 25.0 g/L resulting in a total of 4.6 g/L LA in 84 h.

The M2 assay, which used 35.6 g/L of molasses and 14.4 g/L of corn steep liquor also gave a good result, with a value above 4 g/L for LA production. This result can be considered favourable as there was the least visual occurrence of waste accumulation in terms of culture, leading to lower costs in the extraction and purification of LA.

The M1 experiment showed a lower value in LA production, in which the concentrations of molasses and of corn steep liquor used were 14.4 g/L for both sources, suggesting that there were nutrient restrictions, since it was the condition with the lowest CS and NS concentrations.

Through the p-value estimation supplied by ANOVA (table 4), it was verified that for the tested values, when individually analysed, only the molasses in quadratic form did not exercise significant influence. The corn steep liquor, both in linear as well as quadratic form, obtained a p-value lower than the significance level established for this test, noting that the corn steep liquor concentration exercises significant influence on the production of lactic acid. However, the same was not observed for the interaction of the two factors, which leads to the conclusion that these do not exercise significant influence on the production of lactic acid.

Table 4 – Variance analysis (ANOVA) showing the significant variation in the parameters: molasses concentration and corn steep liquor concentration.

Factors	Sum of squares	Degrees of freedom	Mean squares	Fcalc	p-value
Molasses (L)	2.808	1	2.808	265.955	0.000016*
Molasses (Q)	0.025	1	0.025	2.414	0.180952
Corn steep liquor (L)	0.291	1	0.291	27.630	0.003308*
Corn steep liquor (Q)	0.134	1	0.134	12.721	0.016098*
ME x MI	0.001	1	0.001	0.144	0.719896
Error	0.052	5	0.010		
Total SS	3.366	10			

* = Significant; L = linear; Q = quadratic.

Xiaodong *et al.* (1997) cultivated *Lactobacillus amylovorus* in different carbon sources such as corn starch, cassava starch, rice starch, starch from wheat and potato and obtained a yield of 10.1, 7.8 and 7.9 g/L of LA for maize, wheat and rice, respectively. It should be noted that, in this study, only carbon source has been replaced, using conventional nitrogen sources from the MRS culture medium.

Through the estimation of the p-value provided by ANOVA (table 4), it was verified that, for the values tested, when individually analysed, only molasses in quadratic form did not exert significant influence. Corn steep liquor, in both linear and quadratic form, showed a p-value lower than the significance level established for this test, demonstrating that the concentration of corn steep liquor exerts a significant influence on the production of lactic acid. However, the same was not observed for the interaction of the two factors, which leads to the conclusion that they do not exert significant influence on LA production.

As some factors of ANOVA presented as significant, the regression coefficients can be calculated in order to build the model. The linear and quadratic coefficients and their interactions are part of the model to compose the response surface graph. The results of the regression coefficients of the model are presented in table 5.

Table 5 – Coefficients of regression of lactic acid production response, using molasses as carbon source and corn steep liquor as nitrogen source.

Factors	Coefficients of regression	Standard error	t(5)	p – value
Mean	3.712	0.059	62.582	0.000000*
Molasses (L)	0.593	0.036	16.308	0.000016*
Molasses (Q)	0.067	0.043	1.553	0.180952
Corn steep liquor (L)	0.191	0.036	5.256	0.003308*
Corn steep liquor (Q)	-0.154	0.043	-3.566	0.016098*
ME_1/MI_2	-0.019	0.051	-0.379	0.719886

* = Significant; L = linear; Q = quadratic.

It was verified, by regression analysis, that the corn steep liquor concentration is a variable that, both in linear form as well as quadratic form, has a significant influence on LA production, while the molasses concentration in quadratic form and the molasses/ corn steep liquor interaction have no significant influence on LA production (table 5). The adjustment of the model was also expressed by the correlation coefficient R2 which was 0.984, indicating that 98.4% of the variability in the response can be explained by the model (Eq. 1).

$$[AL]=3.712+0.593*[ME]+0.667*[ME]^2+0.191*[MI]-0.154*[MI]^2\ [ME]\ *\ [MI] \quad (1)$$

Where: [*AL*] = lactic acid concentration, [*ME*] = molasses concentration, [*MI*] = corn steep liquor concentration.

By just considering the significant factors, the equation can be rewritten as presented in Eq. 2.

$$[AL]=3.712+0.593*[ME]+0.191*[MI]-0.154*[MI]^2 \quad (2)$$

As the models are predictive in the experimental region, the response surface graph shows the interaction between molasses concentrations and corn steep liquor in the production of LA by fermentation (figure 2a). It can be observed that LA production was greater using molasses concentrations (CS) in the range of 35 to 40 g/L, and corn steep liquor (NS) from 18 g/L, while the

use of low concentrations of molasses and corn steep liquor simultaneously led to low LA production. Even under favorable conditions of temperature, pH and anaerobiosis, the results showed that lower LA concentrations were produced in all experiments when compared to other authors. This may have occurred due to the complex nutritional requirements of the genus *Lactobacillus* (HOLZAPFEL & WOOD, 1995).

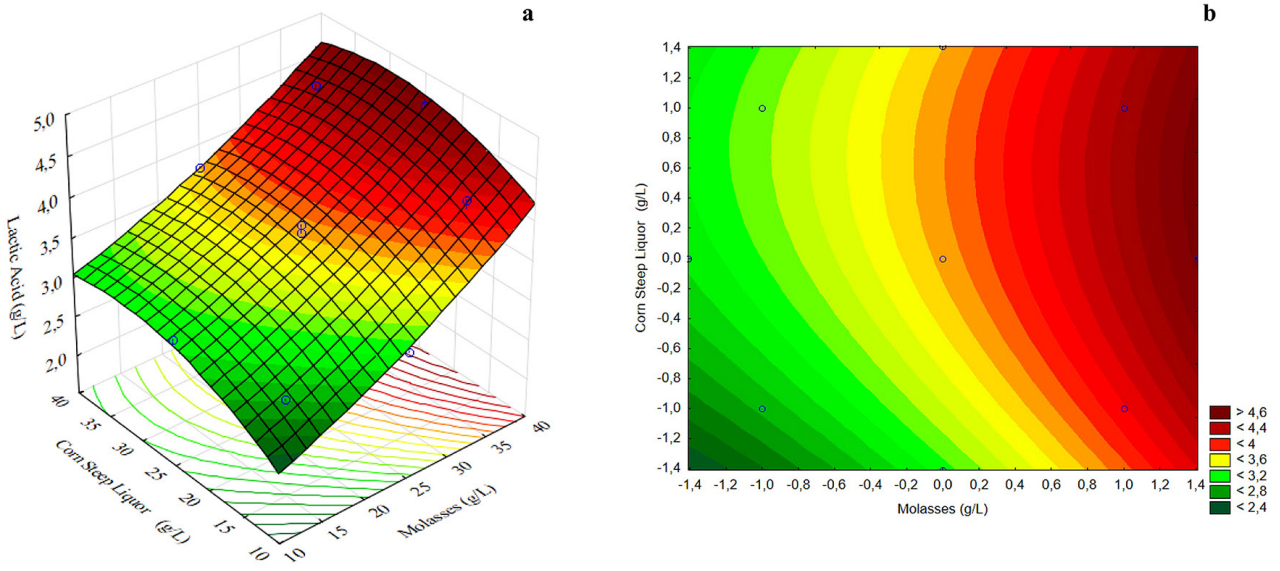

Figure 2 – Response surface graph showing the interaction between molasses concentration (CS) and corn steep liquor (NS) on lactic acid production (a) and contour curves of lactic acid production, using molasses as CS and corn steep liquor as NS (b).

In a similar study to this work, using *Lactobacillus amylovorus* at 40°C, LA concentrations equal to 4.2 and 4.8 g/L were obtained, using cassava and potato starch, respectively (XIAODONG *et al*., 1997), in the same order of magnitude of the best results achieved in this manuscript.

In the visualization of the response surface graph, the inability to confirm an optimal value for the LA production was verified, due to the fact that increased molasses concentrations can yield higher concentration values. These results suggest that a move should be made to a new experimental region, having the higher molasses concentration values at the maximum level used in this design. This can also be seen in the contour curves shown in figure 2b.

Through Eq. 1, LA concentrations can be theoretically predicted in an 84 h time period, thus enabling the applied model to be validated. Theoretic concentrations of LA were calculated varying the codified concentrations of molasses and corn steep liquor, starting from -1.41 up to +1.41, with 0.2 point intervals. Thus, for the model validation, we decided to use the codified concentrations of molasses and corn steep liquor equivalent to +1.41 and -0.8, respectively, which are in the region of the highest LA concentrations predicted by the model, which equals 40.0 g/L molasses and 18.6 g/L corn steep liquor. As corn steep liquor hampers product purification, it was decided to work with the lower concentration within the region of maximum concentrations.

The result obtained theoretically by the model for this condition was 4.3 g/L, while the mean value obtained experimentally was 4.16 ± 0.59 g/L (table 6). Since the value obtained experimentally was statistically equal to the predicted value of the model, we can consider that this model is valid.

Table 6 – Concentrations of LA predicted by the model and obtained experimentally, using molasses as source of carbon and corn steep liquor as a source of nitrogen.

	Concentration of LA (g/L)	
	Predicted	**Experimental**
Molasses/ Corn steep liquor	4.30	4.16 ± 0.59

CONCLUSION

The result obtained from the investigated CCRD enabled the best conditions to be selected for carrying out the microbial culture process. The regression coefficients showed that the nitrogen source exerts a strong influence, interfering directly in LA production. The carbon source, although important, presents a lower level of significance, molasses being the most suitable source to use as glucose replacement in the culture process. The use of CCRD not only helped in selecting the variable with the most influence in LA production, but enabled knowledge of the concentrations in which the evaluated carbon and nitrogen sources should be added to the medium to increase LA production. Aimed at proposing an economic and efficient microbial culture process for the production of LA through biotechnological route for the exchange of high cost nutrients such as glucose, peptone and yeast extract by low cost renewable resources, it was possible to replace them by agro-industrial wastes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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