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BIOSURFACTANT PRODUCTION FROM *lactobacillus fermentum* NBRC 15885 STRAINS ISOLATED FROM WHEY

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ABSTRACT

This study is focused on the production of biosurfactants from fermentation of agro-industrial wastes by *Lactobacillus fermentum* NBRC 15885 strains which were isolated from whey produced in a cattle region of Colombia (Bajo Cauca Antioqueño). Biosurfactant production was studied by means of a full factorial experimental design (3x2x4) with three replicates. Selected factors were microbial strain, agro-industrial waste used as a carbon source (whey or molasses) and culture broth. Surface tension of supernatant was selected as response variable of the experimental design. The lowest value of surface tension was obtained when molasses was used as the main source of carbon (34,3 mNm^{-1} on average). In this case, the biosurfactant concentration in the supernatant quantified by high-performance liquid chromatography was 890 mgL^{-1}. Surface tension values below the positive control (40 mNm^{-1}) were also obtained when whey was used in the process. The results obtained showed that cheese whey and molasses can be used as a relatively inexpensive renewable substrates to produce biosurfactants from *Lactobacillus fermentum* strains.

Keywords: biosurfactant, agro-industrial waste, surface tension, fermentation.

INTRODUCTION

Surfactants are widely used in several industries, such as food, cosmetic, oil, health and pharmaceutical (Nitschke & Sousa e Silva, 2016) (Rodriguez, Teixeira, & Oliveira, 2006). Most commercial surfactants are chemically derived from petroleum. It has been estimated that the production of surfactants consumes about 7,4 billion kg of petrochemical products and emits about 31,6 billion kg of CO_2 (Patel, 2003) (Reznik, *et al.,* 2010). Additionally, these synthetic surface-active compounds are generally toxic and non-biodegradable. In recent years, such drawbacks have motivated the scientific community to seek surfactants that are more environmentally friendly, such as those produced from microbial production, known as biosurfactants (Santos, *et al*., 2008). These compounds have shown several advantages such as low toxicity, greater biodegradability, greater selectivity, biocompatibility, structural diversity, and ability to function in wide ranges of temperature, pH and salinity (Desai & Banat, 1997).

Biosurfactant production is considered one of the key technologies for development in the 21st century (Santos, Rufino, Luna, Santos, & Sarubbo, 2016). Its implementation can lead to the development of sustainable industrial processes focused on renewable resources and "green" products (De Almeida, *et al.,* 2016). The feasibility of biosurfactant production at commercial scale can be improved by using cheaper renewable substrates such as distillery wastes, animal or vegetable fat, molasses, vegetable oils, soapstock, starchy effluents, dairy industry waste (whey) and raw glycerol (Cornea, *et al.*, 2016) (Mukherjee, Das, & Sen, 2006) (Sen & Swaminathan, 2004).

In recent decades, there has been an increase in scientific interest regarding the isolation of microorganisms (yeasts, bacteria, and some filamentous fungi) that produce tensoactive compounds with different molecular structures and surface activities (Banat, *et al.,* 2010). Satpute et al. presented a review of around 46 reports related to biosurfactants production from *Lactobacillus spp.* (Satpute, *et al.,* 2016). According to these authors, very few scientific reports are available on biosurfactant production from this kind of microorganisms by using cheap and renewable substrates. Waste products with high potential to be employed in biosurfactant production are molasses and whey.

Molasses is a by-product of sugarcane and beet processing, commonly used for animal feeding and ethanol production. Their high sugar content (48%–56%) makes molasses adequate for biosurfactant production by different microorganisms. Al-Bahry *et al.* used molasses as the sole carbon and energy source for biosurfactant production from *Bacillus subtilis* B20, obtaining a product yield of 2,29 gL^{-1} (Al-Bahry, *et al*., 2013). They showed that molasses derived biosurfactants could reduce the surface tension of supernatants (from 60 mNm^{-1} to 25 mNm^{-1}) while maintaining significant stability under a wide range of temperatures, pH and salinity. Mouafo et al. investigated the potential of three indigenous bacterial strains (*Lactobacillus delbrueckii* N2, *Lactobacillus cellobiosus* TM1, and *Lactobacillus plantarum* G88) for the production of biosurfactants using sugar cane molasses and glycerol as substrates (Mouafo, Mbawala, & Ndjouenkeu, 2018). The different biosurfactants produced with molasses exhibited an acceptable reduction of the surface tension of water (from 72 mNm^{-1} to values ranged from 47,50 to 41,90 mNm^{-1}). Preliminary characterization of these crude biosurfactants revealed that they were mainly glycoproteins.

Whey is a by-product of the dairy industry whose disposal can represent a major pollution problem, especially in countries or regions with a strong cattle sector. A high amount of lactose (approximately 75%) is found in lactic whey. Other whey components, such as proteins, vitamins, and organic acids, are good sources for microbial growth and biosurfactant production (Santos, Rufino, Luna, Santos,

& Sarubbo, 2016). Dubey et al. investigated the production of biosurfactants from industrial distillery and whey wastes by using an oily sludge isolate *Pseudomonas aeruginosa* strain BS2 (Dubey, Juwarkar, & Singh, 2005). The yields of biosurfactant obtained were 0,91 and 0,92 gL^{-1} using the distillery and whey wastes, respectively. The isolated biosurfactant reduced the surface tension of water from 72 to 27 mNm^{-1}. Rane et al. investigated the potential of *Bacillus subtilis* ANR 88 to grow on different cheap carbon sources (molasses, whey, and extracts of potato peels, orange peels, banana peels, and bagasse) (Rane, Baikar, Kumar, & Deopurkar, 2017). They found that biosurfactant production in a medium containing glucose as sole source of carbon was 0,207 gL^{-1} and the same with molasses as carbon source was 0,241 gL^{-1}. When whey was used as sole source of carbon, biosurfactant production failed. Gudiña et al. studied biosurfactant production by *Lactobacillus agilis* CCUG31450 using the conventional Man-Rogose-Sharpe (MRS) medium for lactic acid bacteria and cheese whey as an alternative culture medium (Gudiña, Fernandes, Teixeira, & Rodrigues, 2015). The yields of biosurfactant obtained were 0,84 and 0,96 gL^{-1} using the MRS medium and whey, respectively. A preliminary chemical characterization by Fourier transform infrared spectroscopy indicated that the biosurfactant produced was a glycoprotein. *Lactobacillus agilis* CCUG31450 produced a biosurfactant that reduced the surface tension of water to 42,5 mNm^{-1}.

To the best of our knowledge, there are not reported investigations related to biosurfactant production from *Lactobacillus fermentum* strains using cheaper renewable substrates. This study is focused on the production of biosurfactants from fermentation of sugar cane molasses and whey by *Lactobacillus fermentum* NBRC 15885 strains which were isolated from whey produced in a cattle region of Colombia (Bajo Cauca Antioqueño). Biosurfactant production was studied by means of a full factorial experimental design. Selected factors were microbial strain, agro-industrial waste (source of carbon) and culture broth.

MATERIALS AND METHODS

Microorganisms and Culture Broth

Three strains of *Lactobacillus fermentum* NBRC 1885 isolated from whey produced in a cattle region of Colombia (Bajo Cauca Antioqueño) were previously selected from eighteen strains by a preliminary screening based on classical methods (bacterial adhesion to hydrocarbons, surface tension and drop collapse). Bacterial strains were stored at -70°C in Man-Rogose-Sharpe (MRS) broth, supplemented with 20% (v/v) glycerol as a cryoprotectant. MRS broth composition was: peptone (18 gL^{-1}), yeast (4 gL^{-1}), glucose (20 gL^{-1}), Tween 80 (1 mlL^{-1}), K_2HPO_4 (2 gL^{-1}), $MgSO_4$ $7H_2O$ (0,2 gL^{-1}), triammonium citrate (2 gL^{-1}) and sodium acetate (3 gL^{-1}). Bacterial strains identification was carried out at the Institute of Lactic Products of Asturias (Spain). Bacterial strains DNAs were analyzed by PCR (Polymerase chain reaction). This molecular technique allows amplifying a single or some few copies of a piece of DNA (the 16S ribosomal region). Bacterial strains belong to the collection of the research group Bioali (Universidad de Antioquia-Colombia) and were stored in the laboratory of biotechnology of the Tecnoparque SENA network.

Biosurfactant Production

Lactobacillus strains (LAB) were incubated in a rotary shaker at 37°C and 200 rpm for 24 h. The optical density (OD) in the spectrophotometer was adjusted to 0,1 at 600 nm to assure the same cell concentration in all fermentations. After fermentation, culture broth was centrifuged at 12 000 xg for 15 min to obtain a cell-free supernatant which was acidified with HCl (6M) to pH=2, overnight at 4°C. To obtain a pellet, the supernatant was centrifuged and washed with water at pH=7.

Biosurfactant production was studied by means of a full factorial experimental design (3x2x4) with three replicates. A factorial design is employed to evaluate the effect of two or more variables with multiple levels, allowing the analysis of the main effects and the interaction of factors. Selected factors were: microbial strain (three strains: LAB05 (5), LAB10 (10) and LAB12 (12)), agro-industrial waste (two substrates: whey (1) and molasses (2)) and culture broth (four culture media: M1 (1), M2 (2), M3 (3) and M4 (4)). Table 1 shows the composition of the culture broths. Since three replicates were carried out, a total of 72 experiments were performed in random runs to minimize errors due to possible systematic trends in the factors. Surface tension of supernatant was selected as response variable of the experimental design. This property was determined by the "Du-Nöuy ring method" using a tensiometer (K100 series Kruss). Experimental data was evaluated by an analysis of variance conducted at a 95% confidence level. Statistical analysis was performed by using the Minitab® 15 Statistical Software.

Although all microbial strains were isolated from whey, this factor was considered in the experimental design because this agro-industrial waste was provided from different suppliers (cattle farms). In this work, whey was studied as a double purpose material: source of microbial strains and substrate.

Surface tension reduction is the most important parameter in screening for microorganisms with potential for industrial production of biosurfactants. The criterion commonly used for selecting biosurfactant producers is the capacity to generate metabolites that reduce the surface tension of water below 40 mNm^{-1} (Batista, Mounteer, Amorin, & Tótola, 2006).

Biosurfactant Quantification and Characterization

The concentration of biosurfactant in the supernatant was directly quantified by high-performance liquid chromatography (HPLC) using a Thermo Scientific chromatograph with a quaternary pump, a Zorbax SB-C18 column and an ultraviolet detector PDA. Phosphate Buffer 20% (pH 2, 20 mM KH_2PO_4), with a flow of 1 ml/min, was used as mobile phase. Sample injection was 20 µL, and tests were carried out for 30 minutes at a wavelength of 205 nm (Cheng, et al., 2013). The calibration curve was obtained by dissolving samples of surfactin standard (98% Sigma

Aldrich) in an elution buffer at four concentrations (100, 200, 300 and 400 ppm). Surfactin is recommended as a standard since it is one of the best studied biosurfactants (Varjani & Upasani, 2017). To obtain a preliminary molecular characterization of the biosurfactant, FTIR tests were performed using a Thermo scientific Smart iTR Nicolet iS10 spectrophotometer. All measurements consisted of 5 scans using a range of wave number from 600 to 4000 cm^{-1} (Thavasi, Jayalakshmi, & Banat, 2011).

Table-1. Culture broth compositions.

Component	M1	M2	M3	M4
Peptone	18 gL^{-1}	10 gL^{-1}	5 gL^{-1}	10 gL^{-1}
Yeast	4 gL^{-1}	2,5 gL^{-1}	3 gL^{-1}	3 gL^{-1}
Tween 80	1 mlL^{-1}	-	1 mlL^{-1}	1 mlL^{-1}
Dipotassium hydrogen phosphate	2 gL^{-1}	-	2 gL^{-1}	2 gL^{-1}
Triammonium citrate	2 gL^{-1}	-	2 gL^{-1}	2 gL^{-1}
Anhydrous sodium acetate	3 gL^{-1}	-	3 gL^{-1}	3 gL^{-1}
Magnesium sulphate $7H_20$	0,2 gL^{-1}	0,25 gL^{-1}	0,2 gL^{-1}	0,2 gL^{-1}
Anhydrous magnesium sulphate	0,0034 gL^{-1}	-	0,0034 gL^{-1}	0,0034 gL^{-1}
Iron (II) sulphate	-	-	0,0034 gL^{-1}	0,0034 gL^{-1}
Saccharose	20 gL^{-1}	20 gL^{-1}	20 gL^{-1}	20 gL^{-1}
Meat extract	-	5 gL^{-1}	-	-
Lactose	-	5 gL^{-1}	-	-
Ascorbic acid	-	0,5 gL^{-1}	-	-
Sodium Glycerophosphate	-	19 gL^{-1}	-	-

RESULTS AND DISCUSSIONS

This section focuses on the analysis of the best conditions to produce biosurfactants from *Lactobacillus fermentum* NBRC 15885 according to the proposed experimental design. In addition, a basic characterization of the biosurfactant produced is carried out.

Best Conditions for Biosurfactant Production

Biosurfactants produced by *Lactobacillus strains* have been less studied than those produced by *Bacillus* and *Pseudomonas* species. This situation is mainly because biosurfactants derived from LAB are less effective in reducing surface tension to low or ultralow values. According to Gudiña *et al.*, this kind of biosurfactants are suitable for reducing surface tension to values around 36-45 mNm^{-1} (Gudiña, Fernandes, Teixeira, & Rodrigues, 2015). The measured values of surface tension for the different combinations of factors and their replicas (matrix of 72 experiments) are shown in Table-2. As can be seen in this table, for several cases, surface tension values below the positive control (40 mNm^{-1}) were obtained confirming the presence of surface-active compounds in the supernatant. In addition, most values of this property are within the expected range 36-45 mNm^{-1}.

Since the factorial design was executed randomly with replicas, results were analyzed using analysis of variance (ANOVA) as statistical tool. This technique consists of calculations that provide information about levels of variability within a regression model and form a basis for tests of significance. ANOVA calculations are displayed in an analysis of variance table, which has the format shown in Table-3.

Since the p-value for the F test statistic of the model is less than 0,05, there is a statistical relationship between the response variable and the selected factors with a 95% confidence level. In addition, it can be inferred from the data in Table-3 that both the main effects and the double interactions were significant since the p-value for all of them was less than 0,05. Those results indicate that any change in the level of any factor will affect the value of the response variable (see Figure-1) and that such change will also depend on the level of the factors due to the interactions effect (see Figure-2). The value of the determination coefficient (R squared) indicates that 93,05% of the variability in the response variable is explained by the three factors analyzed.

To guarantee the validity of the results, it must be verified that the assumptions of the model such as normality, independence and constant variance are fulfilled. From Figure-3, it can be inferred that the assumptions were correct and therefore the obtained model is valid.

The combination of levels that allows achieving the lowest value of surface tension was determined by

analyzing the interaction among factors (Figure-3). This combination corresponds to strain LAB 12 (12), culture broth M2 (2) and molasses as agro-industrial waste (1). To verify that this combination of factors allows obtaining repeatable values of the response variable within the range predicted by the model, five additional runs were performed at the same conditions of temperature (37°F), time (24 hours) and agitation speed (200 rpm). The five values of surface tension obtained were 34,2, 33,8, 34,5, 35,2 and 33,6 mNm-1 (34,3 mNm^{-1} in average).

Figure-2 (interaction effects plot) allows analyzing the interaction between pairs of factors, for example, culture broth and agro-industrial waste. Culture media are aqueous solutions where microorganisms develop. Microorganism growth only occurs in the presence of the required nutrients. Each combination of culture broth and agro-industrial waste represents a media where the agro-industrial-waste is the main source of carbon and the culture broth is a group of nutrients required to guarantee an adequate growth of the microorganisms. Looking for decreasing the costs of production of biosurfactants at industrial scale, the objective is to replace expensive nutrients and sources of carbon and nitrogen by cheaper alternative substrates, in this case, molasses and whey.

Table-2. Measured values of surface tension for the random combinations of factors and their replicas (matrix of 72 experiments).

Strains	Culture Broth	Agro-industrial waste	Surface tension (mNm^{-1})	Strains	Culture Broth	Agro-industrial waste	Surface tension (mNm^{-1})
5	1	Molasses	36,8	12	1	Molasses	38,2
12	3	Molasses	40,2	10	3	Whey	40,4
10	3	Molasses	41,2	5	3	Whey	42,1
12	1	Molasses	40,8	10	4	Whey	34,1
5	3	Molasses	41,9	5	1	Whey	41,7
10	1	Whey	39,1	12	1	Whey	39,8
5	4	Molasses	42,2	10	4	Molasses	39,1
12	3	Molasses	37,1	12	2	Whey	35,3
5	4	Whey	40,3	5	2	Molasses	39,1
12	4	Whey	37,9	12	3	Whey	41,8
10	2	Molasses	53,4	5	1	Molasses	37,9
5	1	Whey	43,4	5	2	Whey	39,3
10	3	Molasses	42,9	12	4	Molasses	40,2
10	3	Whey	41,2	10	2	Whey	37,7
5	2	Whey	43,1	5	2	Molasses	37,5
10	4	Whey	34,4	10	4	Molasses	38,5
5	3	Whey	45,3	5	3	Molasses	44,2
10	1	Whey	41,1	12	2	Molasses	35,3
12	2	Molasses	35,0	5	4	Whey	41,3
10	3	Molasses	38,6	12	4	Molasses	38,8
12	3	Molasses	43,9	10	1	Molasses	34,2
10	4	Molasses	41,7	12	1	Whey	38,4
12	4	Molasses	41,6	5	4	Whey	39,9
12	4	Whey	37,7	10	2	Whey	41,8
5	1	Molasses	37,4	12	2	Whey	42,2
12	1	Molasses	38,3	10	3	Whey	39,6
5	2	Molasses	36,1	12	3	Whey	42,1
5	2	Whey	42,4	10	4	Whey	34,3
12	2	Molasses	35,4	5	4	Molasses	46,3
5	3	Whey	42,7	12	1	Whey	41,2
10	2	Molasses	37,4	10	2	Molasses	45,4
5	4	Whey	39,6	12	2	Whey	35,0
10	1	Molasses	33,2	10	1	Molasses	34,6
12	4	Whey	38,0	12	3	Whey	37,3
5	3	Molasses	43,1	5	4	Molasses	43,2
10	2	Whey	39,8	12	3	Whey	42,4

Table-3. Analysis of variance.

Source	DF (Degree of freedom)	Adj. SS (Adjusted sum of squares)	Adj. MS (Adjusted mean squares	F	P
Strains	2	54,454	27,227	16,1	0,000
Culture broth	3	121,036	40,345	23,9	0,000
Agro-industrial waste	1	15,125	15,125	8,97	0,004
Strains*Culture broth	6	460,272	76,712	45,5	0,000
Strains*Agro-industrial waste	2	72,390	36,195	21,4	0,000
Culture broth*Agro-industrial waste	3	155,005	51,668	30,6	0,000
Error	48	80,913	1,686		
Total	71				
Model summary: S=1,29834, R-squared=93,05%, R-squared (adjusted)=89,72%					

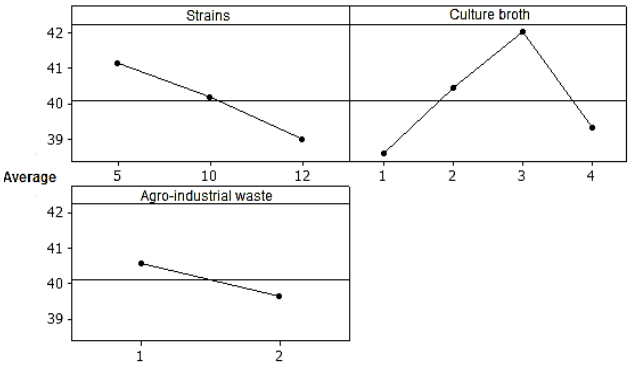

Figure-1. Main effects plot.

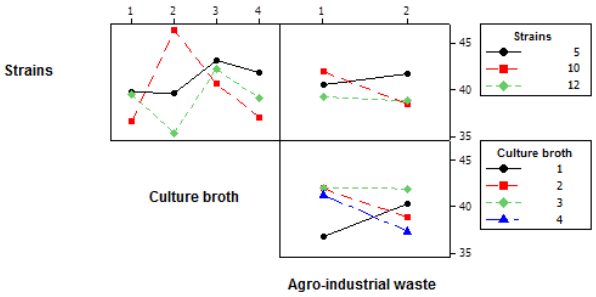

Figure-2. Interaction effects plot.

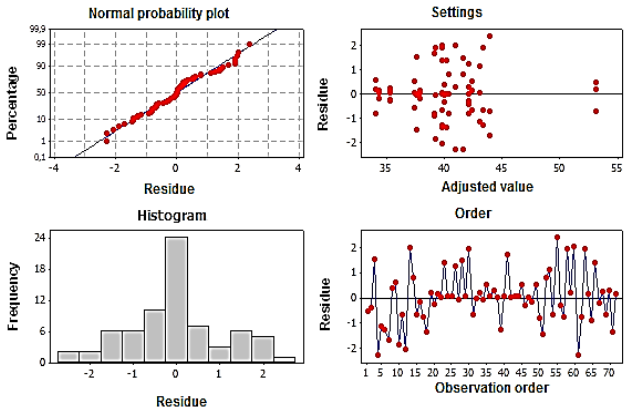

Figure-3. Residue analysis.

As shown in Figure-2, when molasses was used, better results (lower values of surface tension) were obtained by using the culture broths M2 and M4 than when the standard culture broth M1 was used. The combination molasses-M2 allowed reducing the content of peptone and yeast in the culture media. In this case, the media was supplemented with other sources of nitrogen and phosphorous such as meat extract and sodium glycerophosphate. On the other hand, when the combination molasses-M4 was used, the reduction in the peptone and yeast contents was offset by supplementing the culture media with iron sulphate, which has been reported as an efficient additive for increasing biosurfactant production (Rodriguez, Teixeira, & Oliveira, 2006). Surface tension values below 40 mNm^{-1} were also obtained when whey was used. It indicates that this agro-industrial waste can be also used as an adequate source of carbon in the formulation of culture media for biosurfactant production by Lactobacillus fermentum strains. As shown in Figure-2, when whey was used, better results were achieved when the strain LAB 12 (12) and the standard broth M1 (1) were used. Those results suggest that the best performance of whey can be achieved when it is mainly supplemented with peptone and yeast.

Biosurfactant Quantification and Characterization

The results presented in this section correspond to the sample for which the lowest surface tension value was obtained (strain 12, M2 broth and molasses). The concentration of biosurfactant in the supernatant was determined by means of the calibration curve shown in Figure-4. This curve was obtained from chromatograms of samples of surfactin standard diluted at four concentrations (100, 200, 300 and 400 ppm). Figure-5 shows the chromatogram of surfactin standard at 100 ppm. It is observed five peaks between 9 min and 17 min. Figure-6 shows the chromatogram of the biosurfactant synthesized in this work.

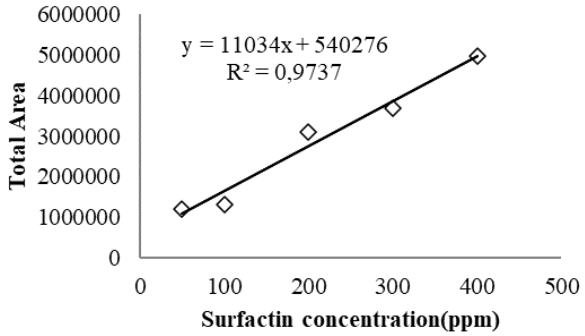

Figure-4. Calibration curve for biosurfactant quantification.

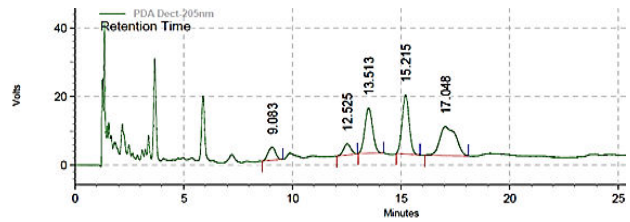

Figure-5. HPLC chromatogram of surfactin standard.

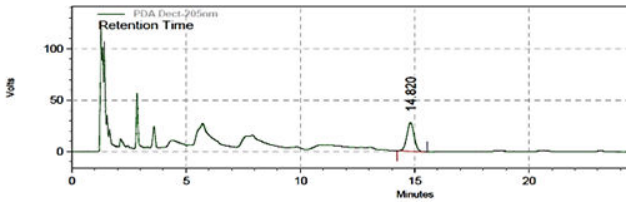

Figure-6. HPLC chromatogram of biosurfactant synthesized by *Lactobacillus fermentum* NBRC 15885 at 37°C, 200 rpm for 24 h, using molasses as carbon source.

Considering the total area of the chromatogram shown in Figure-6, a concentration of 89 ppm is obtained by interpolation in the calibration curve. Since a dilution ratio of 1:10 was taken, the actual biosurfactant concentration is 890 ppm. This value is within the same order of magnitude of those reported by Rodrigues et al. for biosurfactants produced from *Lactococcus lactis* with whey and molasses, 693 ppm and 1735 ppm, respectively. (Rodriguez, Teixeira, & Oliveira, 2006).

FTIR has been a widely used technique to obtain a preliminary characterization of complex biosurfactant mixtures produced by different *lactobacilli* strains (Gudiña, Fernandes, Teixeira, & Rodrigues, 2015). Figure 7 presents the infrared spectra of the biosurfactant produced. Wide absorbance with wave numbers ranging approximately from 3000 cm^{-1} to 3700 cm^{-1} is observed. The broad peak at 3271 cm^{-1} indicates the presence of -OH, –CH and –NH groups, characteristic of carbon-containing compounds with amino groups as occurs with peptides (Das, Mujherjee, & Sen, 2008) (Yilmaz, Ergene, Yalcin, & Tan, 2009). The peak at 2925 cm^{-1}, corresponding to C-H stretching, indicates the presence of bonds occurring in aliphatic chains. At 1629 cm^{-1}, a well-defined peak caused by bond vibration of the ester group (C=O) is observed (Yilmaz, Ergene, Yalcin, & Tan, 2009). Adjacent to this peak, there is a smaller but also well-defined peak at 1531 cm^{-1}, which indicates the presence of the nitro group (N=O) common in proteins. Other significant peak observed at 1307 cm^{-1} corresponds to C-O stretching in sugars such as polysaccharides. According to the functional groups identified, the biosurfactant can be classified as a nonionic. Its hydrophilic groups consist of mono- or polysaccharides, carboxylic acids, amino acids or peptides and its hydrophobic groups are saturated, unsaturated, or hydroxylated fatty acids (Desai & Banat, 1997).

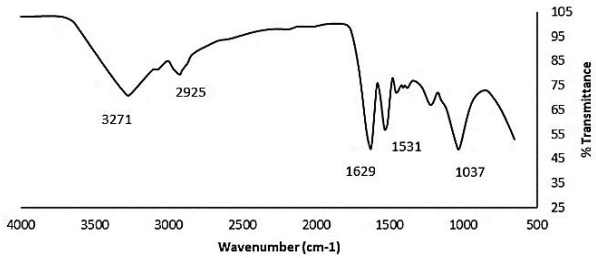

Figure-7. FTIR spectrum of biosurfactant synthesized by *Lactobacillus fermentum* NBRC 15885 at 37°C, 200 rpm for 24 h, using molasses as carbon source.

CONCLUSIONS

Results obtained showed that cheese whey and molasses can be used as relatively inexpensive renewable sources of carbon to formulate culture media to produce biosurfactants from *Lactobacillus fermentum* strains. It was also demonstrated that whey can be used as a double purpose material: source of microbial strains and substrate.

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