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Influence of *Kluyveromyces Lactis* Arranged In Suspension and Immobilized On Obtaining Lactic Acid by Cheese Whey Fermentation

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Abstract. *Lactic acid has several applications in the pharmaceutical, food, cosmetic and chemical industries, and is currently used for its transformation into polylactic acid, which is a biopolymer used to produce environmentally friendly bioplastics. So, in the present research lactic acid was obtained from the fermentation of cheese whey, that is a waste from the cheese industry that generates high environmental pollution, hence, this research seeks to give it an added value. Thus, the Kluyveromyces lactis strain was isolated from the cheese whey, which was also used for the fermentation process and the obtaining of lactic acid, using proteinized (fresh) and deproteinized cheese whey, the strains arranged in suspension and immobilized in order to determine if those conditions have an influence on the characteristics of lactic acid. It has been confirmed that the arrangement of the strain used (in free or encapsulated form), the use of untreated and deproteinized cheese whey, and the purification conditions have an influence on the characteristics and yields of the lactic acid obtained (color, density, and the presence of other functional groups in it).*

Keywords: *Kluyveromyces lactis,* cheese whey, lactic acid, immobilized cell and suspended cell.

Introduction

Plastics are a great source of pollution since their inadequate disposal and their long time to degrade have been generating the formation of plastic islands in the oceans, being the case of Easter Island, which is considered the largest garbage island located in the Pacific Ocean [1], and considering the high use that is given to it, it is necessary to see other materials that are environmentally friendly and can be used as raw material for the production of plastics.

Among these raw materials is lactic acid, which is an organic acid that is obtained naturally by extraction of milk sugar or synthetically from cane, grape or starch sugar [2]. Lactic acid is considered one of the most important products due to its wide applications, mainly in the pharmaceutical, cosmetic, chemical and food industry [3]. Today, there is an increase in the demand for lactic acid as a raw material to produce polylactic acid (PLA), which is a biopolymer with properties that classify it as environmentally friendly [4]. Lactic acid can be produced from synthesis by fermentation or chemically. The fermentation production process has a very attractive interest due to the advantage of producing pure isomers of L (+) or D (-) lactic acid, in addition to a low cost of renewable raw materials, low energy consumption and easy conditions for its operation [4]. The fermentation can be carried out with immobilized or suspended cells. The immobilization of microorganisms is considered one of the procedures that allows increasing cell retention and cell density in bioreactors, in addition the microbial latency phase is reduced, there is a tolerance to high sugar concentrations, it improves pH control and generates a greater productivity, since purification processes would be facilitated [5]. Among the sources to obtain lactic acid is cheese whey, which is a residue from the cheese manufacturing process. Besides, without a correct treatment of cheese whey, it can cause negative consequences in the environment such as water eutrophication, soil contamination, among others [6]. In addition, 90% of the milk used in cheese production is discarded as cheese whey which contains lactose, proteins, lipids, and mineral salts [7], so it can be reused and give it an added value.

The *Kluyveromyces lactis* is a yeast that can carry out fermentations under anoxic and aerobic conditions and consume lactose [8] and it is used to produce proteins [9], organic acids and ethanol [8]. It has been determined that immobilizing strains of *Kluyveromyces lactis* generated an increase in lactose removal efficiency 3.4 times more than when they were used as a suspended cells [10].

Thus, the aim of this research is to determine if there is an influence on the physical and chemical characteristics and yield of lactic acid that obtained by cheese whey fermentation using an isolated strain from cheese whey, the *Kluyveromyces lactis* that was arranged in immobilized and suspended cells.

Experimental Procedure

Isolation and molecular identification. Homofermentative strains were isolated from cheese whey collected of cheese industry located in the city of Arequipa (Peru). The Agar MRS medium (Man, Rogosa and Sharpe medium) acquired from the Merck company was prepared, autoclaved, plated in plates and it was sowed a cheese whey sample by exhaustion on it. Growth conditions were micro anaerobiosis (in an anaerobic chamber), at 32°C for 48 h in a Witeg incubator model WIG-50. The colony of interest was replicated until isolated and GRAM staining was performed. The isolated sample was molecularly identified by PCR in the Uchumayo DNA laboratory.

Batch fermentation with strains in suspension. Batch fermentation was carried out using the inactivated strains, and untreated and treated cheese whey whose treatment had the purpose of proteins removal. The cheese whey deproteinization was carried out in an autoclave at 121 °C and 15 PSI pressure for 15 minutes, later it was taken to a 60 sieve (0.050 nm) to eliminate the greatest amount of proteins, then it was centrifuged at 4500 RPM for 10 minutes to finally go through vacuum filtration. With this procedure, it was possible to eliminate not only the proteins but also the fats (we will named it as deproteinized cheese whey).

The fermentation culture medium was composed of 0.5% p/v yeast extract, 0.9676% p/v ammonium phosphate, neutralizer (5 g/l calcium carbonate) and a 200 ml of cheese whey, which it was mixed and placed in a water bath at 65 °C for 10 minutes. It was cooled and inoculated with the native strain (3 spades). The fermentation parameters were at 32 °C and 120 RPM for 7 days.

It is important to mention that the cheese whey collected from a cheese company in the city of Arequipa (Peru) without undergoing a deproteinization process has the following physicochemical characteristics: 0.45% acidity, 0.50% ash, 3.41% lactose, 0.81% proteins, 0.85% fats and pH of 5. While deproteinized cheese whey has 0.38% acidity, 0.52% ashes, 3.57% lactose, 0.04% proteins, 0.38% fats and pH of 4.84.

Batch fermentation with strains immobilized. Batch fermentation using strains immobilized in alginate beads was carried out in deproteinized and proteinized cheese whey without adding other additives, since in preliminary tests it was observed that the alginate beads lost their stability.

Manufacture of alginate beads. Manufacture of alginate beads. The strains were cultivated in MRS broth at 32°C and 120 RPM for 24 hours. From the MRS broth a volume was taken to centrifuge in order to concentrate the bacteria at 4500 RPM for 20 minutes. The obtained pellet was washed 3 times with saline solution and then 1 ml of saline solution was added to the biomass pellet. This blend was mixed in 2.5% sodium alginate solution (1 ml of biomass in 30 ml of sodium alginate).

A calcium chloride solution at 4% was prepared and placed in a beaker on a magnetic stirrer at 120 RPM. The previously prepared mixture was added to a burette and dropped into the beaker with the calcium chloride solution. When all the mixture passed, it was left in contact for 1 h for its stabilization. Finally, the beads were washed with sterile distilled water to remove excess calcium ions before use, for which the calcium chloride was recovered.

Fermentation monitoring. Lactic acid was monitored through acidity analysis, pH variation in an OAKTON ion 2700 pH meter and lactose concentration.

Acidity analysis. A known volume from the fermentation broth was titrated with 0.1 N NaOH solution and 2 drops of phenolphthalein (indicator solution).

Lactose consumption. It was measured through Brix degrees, which is equivalent to 1 g of solute (lactose) in 100 g of solution. So, 1 ml of the fermentation medium was taken and centrifuged at 4500 RPM for 10 minutes in order to

eliminate the solids and make the strains precipitate. The suspended solution was recovered and took a few drops of the solution and placed in the refractometer to be measured.

Lactic acid purification. Lactic acid purification. Once the fermentation was finished, centrifugation was carried out in 15 ml falcon tubes for 20 min at 4500 RPM. Later, the supernatant was vacuum filtered using a Whatman 42 filter paper and then by vacuum microfiltration using a 47 mm membrane filter. The filtered sample was taken to the rotary evaporator under the conditions indicated in Table 1 at 95 RPM to concentrate the lactic acid and then proceeded to extract the lactic acid using a 500 ml separating funnel where the volume of sample and solvent (diethyl ether) were added in 1:1 ratio, shaken vigorously and left to rest until the two phases differentiated. The two phases obtained are separated, the clearest and most transparent one was taken to a rotary evaporator at atmospheric pressure and a temperature of 40°C to evaporate the diethyl ether and thus concentrate and purify the lactic acid.

Characterization of lactic acid. The samples were analyzed in the Chemistry Laboratory of the National University of San Agustin (Arequipa, Peru) by the infrared spectroscopy (FTIR) analysis according to the ASTM E1252-98 (2021) "Standard Practice for General Techniques for Obtaining Infrared Spectra for Qualitative Analysis", with a wave number range from 650 cm^{-1} to 4000 cm^{-1}. Table 1 shows the coding of the samples according to the fermentation and purification conditions in obtaining lactic acid. It should be noted that the purification was made for the systems with the highest production of lactic acid.

Table 1. Coding of the proposed fermentation and purification systems to obtain lactic acid

Code	Strain Disposition	Sodium alginate [%]	$CaCl_2$ [%]	Culture medium				LA concentration conditions	
				DCW + additives	CW + additives	DCW	CW	Temperature [°C]	Time [h]
SF1	Suspended	-	-	X	-	-	-	-	-
SF2	Suspended	-	-	-	X	-	-	40	5
SF3	Suspended	-	-	-	X	-	-	45	5
PF1	Immobilized	2.5	4	-	-	X	-	40	5
PF2	Immobilized	2.5	4	-	-	X	-	45	5
PF3	Immobilized	2.5	4	-	-	-	X	-	-

Note: LA: Lactic acid, DCW: Deproteinized Cheese Whey and CW: Cheese Whey

Results and Discussion

Isolation and molecular identification. The isolated strain belongs to a yeast that presents 100% similarity with the ITS gene of *Kluyveromyces lactis* and its phylogenetic analysis indicates that it is *Kluyveromyces lactis*. Their characteristics are depicted in Table 2 and Fig. 1 shows the colonies of the *Kluyveromyces lactis* and their observation using GRAM staining where it is observed that they are GRAM positive.

Table 2. Characteristics of the strain isolated from cheese whey (Kluyveromyces lactis)

Characteristics	Strain AII	Characteristics	Strain AII
Pigmentation	White	Margin	Entire
Size	0.5 – 4mm	Texture	Dry
Form	Circular	GRAM	+
Elevation	Convex	Catalase	+

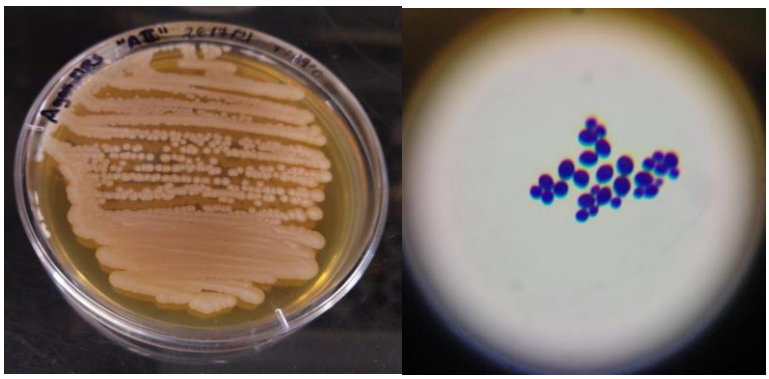

Fig. 1. Isolated yeast Kluyveromyces lactis (left) and its observation in an optical microscope at 100X (right)

Batch fermentation with strains in suspension. Fig. 2 shows the production of lactic acid versus the pH variation that occurs in the batch fermentation for 169 h. The SF2 system is the one that presented the highest production of lactic acid, reaching up to 12.45 ± 0.91 g/L, which corresponds to the use of proteinized or untreated cheese whey, with the highest production at 47 h. While when using deproteinized cheese whey, the obtaining of lactic acid was limited due to the pH value, which in this case the pH tends to neutrality, that has been seen to affect the production of lactic acid. Unlike the case when it is used proteinized cheese whey where it is observed that the pH value tends to decrease reaching a pH value of 5 where the greatest production of lactic acid is generated.

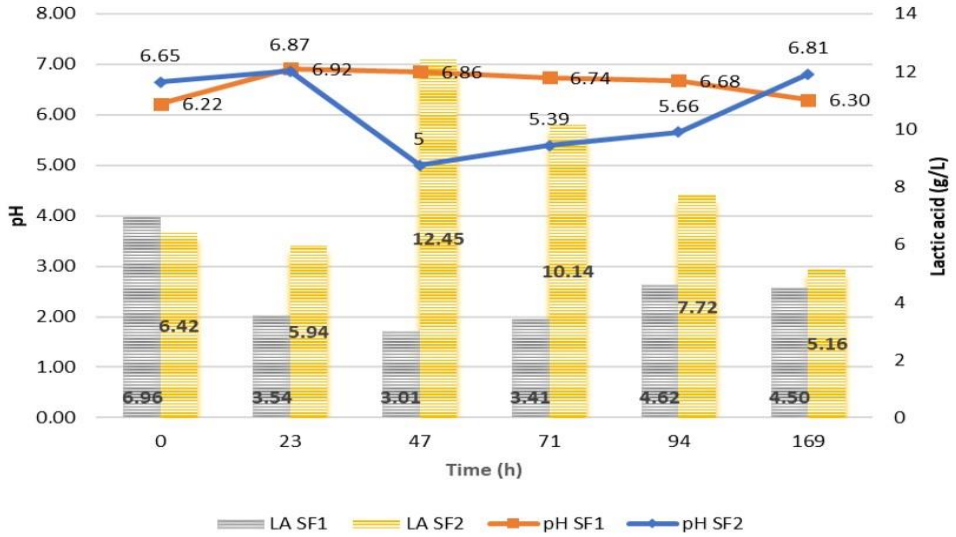

Fig. 2. Lactic acid yield (g/L) of Kluyveromyces lactis and its pH variation during the cheese whey fermentation

Therefore, it can be deferred that for this strain of *Kluyveromyces lactis*, the acidic pH favors the production of lactic acid. This behavior, where a higher yield of lactic acid is generated at pH less than 5, has also been observed by Hun et al. [11] where *K. lactis* produced 4.70 g/L of lactic acid at 36 hours and at 30°C, but if the generation of this strain is compared with ours, 12.45 ± 0.91 g/L was obtained at 47 hours and then it was decreasing while the pH was ascending. It is worth mentioning that Hun et al. [11] used a known lactose concentration (60 g/L) and determined that this concentration influences the obtaining of lactic acid, while in this research the cheese whey was used as a source of lactose, whose initial concentration was 3.41 % for proteinized cheese whey and 3.57% for deproteinized cheese whey, allowing its use and giving it an added value to one of the most polluting dairy residues.

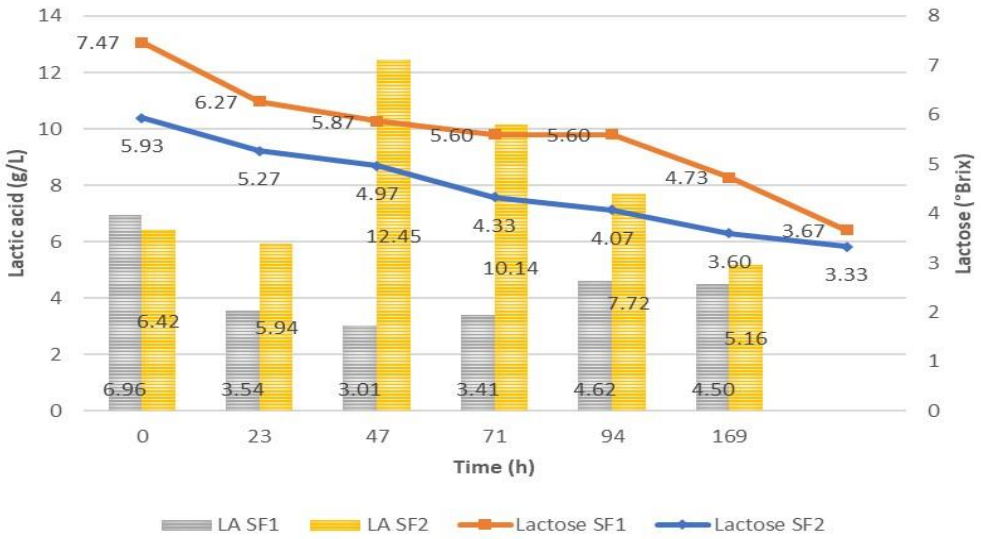

Fig. 3. Lactic acid yield (g/L) of Kluyveromyces lactis and its lactose consume during the cheese whey fermentation

Fig. 3 the consumption of lactose is depicted when lactic acid is generated. It is observed that despite the fact that the medium with deproteinized cheese whey who had the greatest amount of lactose decreases, but a high amount of lactic acid is not generated, this due to the effect of pH. While the medium with proteinized cheese whey, it has a lower amount of lactose but generates a greater amount of lactic acid. Therefore, it can be inferred that the use of proteinized and deproteinized cheese whey influences the lactose metabolism of the yeast Kluyveromyces lactis and therefore the production of lactic acid. The lactose most consumed (56.16%) was of the medium with the proteinized cheese whey, while the medium with deproteinized cheese whey consumed 43.84% lactose. This greater consumption of lactose goes hand in hand with the amount of lactic acid obtained, being the culture medium with proteinized or fresh cheese whey the one that allowed obtaining a greater amount of lactic acid.

Batch fermentation with immobilized strains. Batch fermentation with immobilized strains. Lactic acid production using immobilized Kluyveromyces lactis from cheese whey fermentation for 168 hours is shown in Fig. 4. As in the previous case, the immobilized yeasts had the highest generation of lactic acid (35.70 g/L) when the pH decreased, while when the pH increased, the lactic acid generated was quite limited. This is because the influence of pH on the generation of lactic acid is very important since it affects two aspects of microbial metabolism, which is the metabolism of enzymes and the transport of nutrients within cells [12]. Therefore, it is verified that the acidic pH favors the metabolism of lactose and the production of lactic acid using Kluyveromyces lactis. This is an advantage over other strains since in the case of Lactobacillus casei, Enterococcus lactis, Enterococcus faecalis, Enterococcus camelliae and Weissella paramesenteroi at pH 4 the least generation of lactic acid occurs while at pH 5 increases and rises to its maximum at pH 6 and 7, however, at pH 8 decreases again using deproteinized cheese whey, being Lactobacillus casei the one that generated the most lactic acid with 44.25 g/L after 10 hours of fermentation [13]. Therefore, these fermentation conditions and substrate source will depend on the selected strain. This is observed in the case of some strains of the genus Lactobacillus where it has been determined that the use of cheese whey limits the production of lactic acid, so it is important the nutrient supplementation to cheese whey [2,14]; contrary to what is observed in this research.

It is important to mention that in this research no supplementary additives were used in the culture medium because when placing the alginate beads containing the yeast their stability was affected and disintegrated, therefore, it was decided to use the deproteinized and proteinized cheese whey without any additives. It has seen that the pH of the medium rose while in this case (cheese whey without additives) its initial pH was lower (5.40). It is also important to mention that it was determined that at 4% calcium chloride concentration generated the highest lactic acid yield whose results are shown in this article. When using the immobilized strains, it is observed how the generation of lactic acid increases significantly compared to using the strains in suspension and, unlike the strains in suspension, in this case the highest generation is obtained from the deproteinized cheese whey.

If Fig. 4 is observed, the pH even reaches a value of 3.64 where the highest value of lactic acid is obtained (35.70 ± 0.15 g/L), so the stability of immobilized Kluyveromyces lactis when generating lactic acid in acid conditions is favored since one of the advantages of immobilization is tolerance to extreme conditions, high sugar concentrations, improves pH control, generates greater productivity, and facilitates purification processes [5]. Therefore, the immobilization of Kluyveromyces lactis has greatly favored the production of lactic acid and this encourages a use that can be given to Kluyveromyces lactis since it is currently more used and exploited to produce recombinant proteins on a large-scale industry [9].

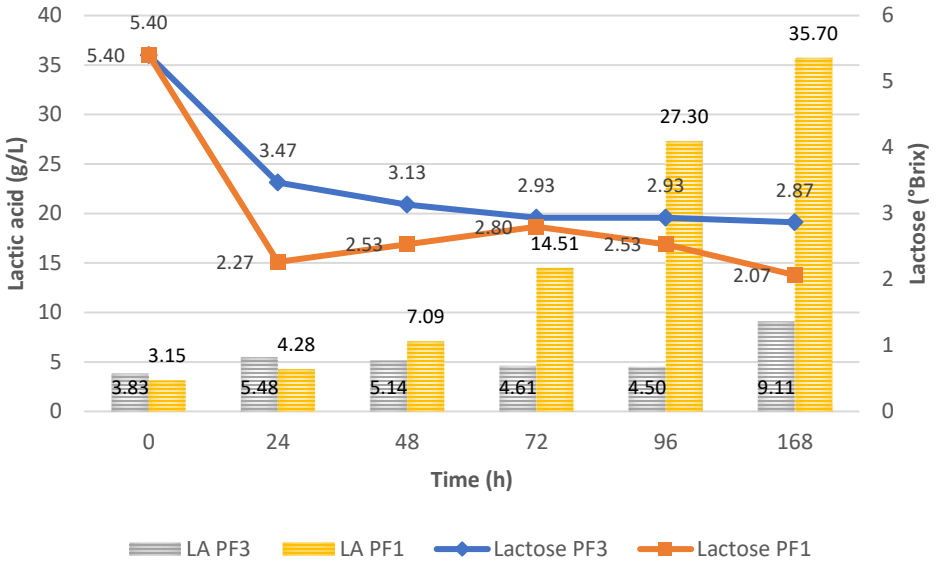

Fig. 4. Lactic acid yield (g/L) of inmovilized Kluyveromyces lactis and pH variation during the cheese whey fermentation

Fig. 5 shows how lactose consumption occurs while lactic acid is generated, the lactose consumption is higher for PF1 system, which corresponds to the use of deproteinized cheese whey, achieving a removal of 61.67%, while in proteinized cheese whey fell to 46.85%. Therefore, when using proteinized cheese whey without any supplements, lactose metabolism and its uptake are affected, despite the fact that the pH values were low. Which allows us to differ that if proteinized cheese whey is going to be used for Kluyveromyces lactis, nutritional additives and suspended cells are required but for deproteinized cheese whey no other additives are required and the strain must be immobilized, the latter being more favorable since it would reduce costs. Therefore, the suspended and immobilized cell, the use of treated and untreated cheese whey, and the addition of nutritional supplements affect lactose metabolism and therefore lactic acid production.

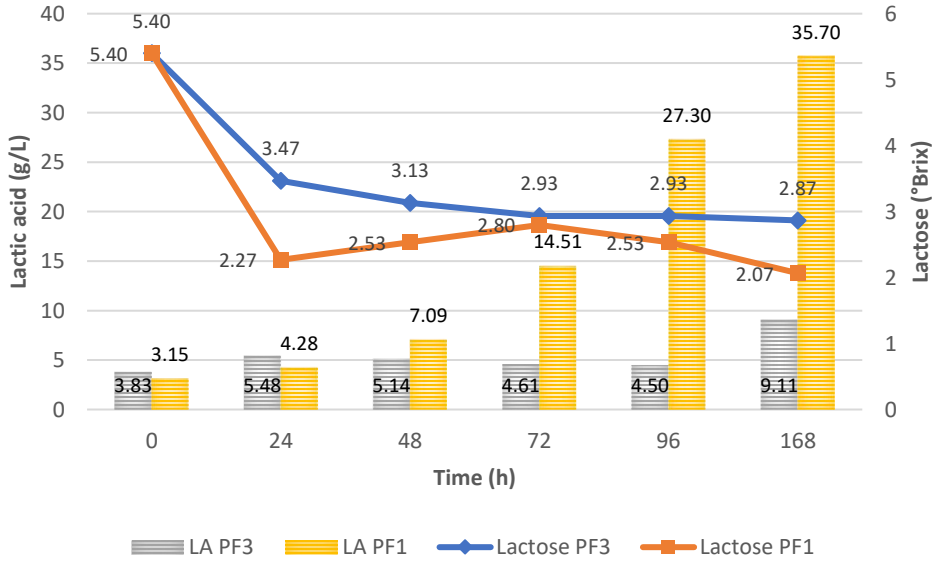

Fig. 5. Lactic acid yield (g/L) of inmovilized Kluyveromyces lactis and its lactose consume during the cheese whey fermentation

Purification and characterization of lactic acid. Lactic acid purification was carried out for the systems that presented the highest yield (SF2 and PF1) and the temperature and concentration time of lactic acid was varied, the characteristics and performance of them are shown in Table 3.

Table 3. Yield and physical characteristics of Lactic Acid

Code	Concentration conditions	Strain Disposition	Initial Volumen [ml]	LA [ml]	Yield [%]	Density [g/ml]	Colour
SF2	40°C – 5h	Suspended	90	3.5	3.89	0.97	Whitish
SF3	45°C – 5h	Suspended	120	3.0	2.50	1.21	Whitish
PF1	40°C – 5h	Immobilized	32	2.7	8.44	0.71	Yellowish
PF2	45°C – 5h	Immobilized	43	1.4	3.26	0.98	Whitish

Table 3 shows that a whitish color is obtained in three samples and yellowish in PF1 sample. These colors were also obtained with homofermentative strains and purified by a microfiltration process [15]. However, it is mentioned that using nanofiltration a lighter color is obtained and by reverse osmosis a transparent color is obtained, in addition this generates a higher concentration of lactic acid [15]. Regarding the density values, it is observed that this also varies according to the concentration conditions used, the density being closest to the theoretical value (1.2 g/cm3) in SF3 sample whose conditions are at 45 °C for 5 h with a value of 1.21 g/cm3. Regarding the PF2 sample (immobilized strains) at 45 °C for 5 h, it had a higher density value than the PF1, this probably indicates that at 45°C for 5 h the lactic acid purification is favored. However, the highest purification yields for both, suspension, and encapsulated strains, are achieved at 40 °C for 5 hours, with the highest yield for the PF1 system (deproteinized whey and immobilized strains). It should be noted that this work has observed what was indicated by Orozco Olivarez [15] who specified that the lactic acid purification step in fresh or proteinized whey samples is more difficult due to the lack of deproteinization and prior filtration, which has been also observed in the purification stage, in addition to the fact that not using encapsulated strains the filtration stage has been slower than when using the encapsulated strains. Therefore, the use of deproteinized or non-deproteinized cheese whey, as well as strains without or encapsulated favors the time in which lactic acid purification delays.

Fig. 6 shows the spectrum of the PF1 and PF2 samples that corresponds to the lactic acid produced using the deproteinized cheese whey and the yeast encapsulated in alginate beads, the presence of the characteristic peak of the OH group at 3361.7 cm^{-1} is observed (PF2 sample) and at 3373.3 cm^{-1} (PF1 sample) and at 1702.1 cm^{-1} (PF2 sample) and 1703.2 cm^{-1} (PF1 sample) which corresponds to C=O. However, for both cases a peak is observed around 1638 cm^{-1} that corresponds to C=O, which indicates that there are impurities in the lactic acid. In both samples, the C-O-C group was detected at 1275.0 cm^{-1} (PF2 sample) and at 1271.5 cm^{-1} (PF1 sample) and the C-H group at 1389.3 cm^{-1} (PF2 sample) and at 1388.3 cm^{-1} (PF1 sample). According to the C=O peaks and these last peaks probably correspond to DL-lactic acid monomer since the peaks are like those indicated by Nikolic et al. [16]. Among both, the PF1 sample could be considered as the one with the highest purity since it tends to have a sharper peak at 1702 cm^{-1} and has a higher yield (Fig. 5).

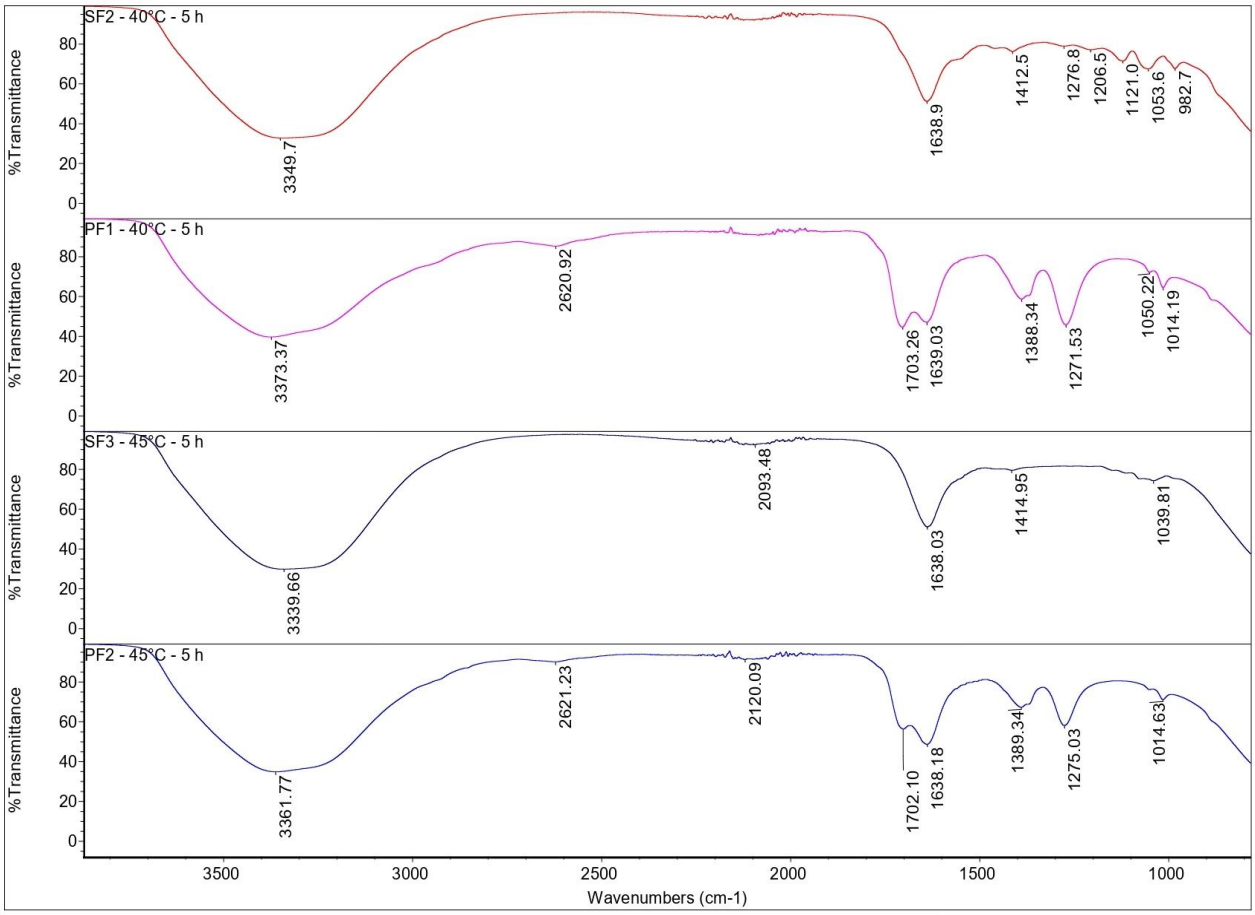

Fig. 6. FTIR spectras of SF2 (40°C), PF1 (40°C), SF3 (45°C) and PF2 (45°C)

Fig. 6 also shows the spectra of SF2 and SF3 samples that correspond to lactic acid obtained from proteinized cheese whey broth and inoculated with non-activated yeast. According to the spectra, a clear difference is observed when using suspended and immobilized strains. In the case of non-encapsulated yeasts, the disappearance of the peak is observed at 1275.1 cm^{-1} (PF1 sample) and at 1271.6 cm^{-1} (PF1 sample) which corresponds to the C-O-C group. For both samples, the presence of OH, C=O and C-H group is observed at 1414.9 cm^{-1} (SF3 sample) and at 1412.5 cm^{-1} (SF2 sample), and there is a difference between them in the range of 1500 to 650 cm^{-1}, where are peaks for the SF2 sample (40°C for 5 h) that belongs to C-O at 1206.5, 1121, 1053.6 and 982.7 cm^{-1}, while for the SF3 sample only there are two peaks at 1638.0 cm^{-1} (C=O group) and at 1414.9 cm^{-1} (C-H group). As in the previous cases, lactic acid is not totally pure and if both samples are compared, the SF2 sample could be selected, which according to the peak at 1120 cm^{-1} corresponds to DL-lactic acid at 85% purity [17] with a higher yield.

Conclusion

A yeast was isolated from the cheese whey collected from an artisanal cheese company in the city of Arequipa (Peru), which was molecularly identified as *Kluyveromyces lactis*. This strain corresponds to Gram positive, with an optimal growth temperature of 32 °C, with tolerance to acidic pH and mainly to neutral and alkaline pH and

with a high resistance to salinity reaching a tolerance up to 12% of saline concentration which give it an advantage against extreme environments.

The *Kluyveromyces lactis* strain was used to obtain lactic acid from proteinized and deproteinized cheese whey and it was shown that there is an influence on using either of them. In addition, it was shown that using the strains arranged in suspension and immobilized has an influence on the lactic acid yields and in their physicochemical characteristics. It has been determined that the lactic acid production is favorable at acidic pH, being the SF2 system (proteinized cheese whey) the one that presented the highest production of lactic acid reaching up to 12.45 ± 0.91 g/L at 47 h. While when using immobilized strains, a greater tolerance to acidic pH and the highest production of lactic acid (35.70 ± 0.15 g/L) at 168 h is observed. Which allows us to differ that if proteinized cheese whey is going to be used for *Kluyveromyces lactis*, nutritional additives and suspended cells are required, while for deproteinized cheese whey the additives are not required but the strain must be immobilized being this last one more favorable since it would reduce costs

Regarding purification, the highest yield was achieved with the PF1 system (deproteinized cheese whey, immobilized strains and concentrated at 40°C for 5 h) with a value of 8.44% followed by the SF2 system (broth with proteinized cheese whey, strains in suspension and concentrated at 40°C for 5 h. Likewise, it is observed that from the fermentacion until purification steps have an influence on the physicochemical characteristics of lactic acid since there is variation in its density, color, yield and in the FTIR spectra. Being the SF3 system who showed a density closer to that of commercial lactic acid.

According to the FTIR analysis, all the samples present characteristic peaks of lactic acid (OH, C=O and CH groups) in all the spectra, therefore, lactic acid was obtained in all cases. There are differences with the presence of some peaks when *Kluyveromyces lactis* is used in suspension or encapsulated, as well as, in the different concentration conditions for purification.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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