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Original

Evaluation of lacto-serum mesophylls with lactic acid activity in medium with *Canna indica* L

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ABSTRACT

Objective. Evaluate lacto-serum as a source of mesophils with lactic acid activity in complex culture medium with sago starch (*Canna indica* L.). **Materials and methods.** Three culture media were analyzed for mesophilic lacto-serum bacteria differentiated by the inclusion of sago starch (*Canna indica* L.) (0, 0.5 and 1%). Control treatments were refrigerated (-4°C) and non-refrigerated (18°C) lacto-serum. Mesophilic growth, pH, acidity level, and total soluble solids were evaluated at 24 h intervals for 5 days; and lactic acid concentration at the end of the test. **Results.** The pH and total soluble solids (°Brix) decreased in all treatments, the % acidity increased over time and at the end of the trial the highest content of lactic acid was 32.5 and 37.2 g / L for the formulations with 0.5 and 1% sago starch respectively. Mesophilic growth was low in the 1% starch formulation and high in the non-refrigerated serum. **Conclusions.** Lacto-serum is a good source of mesophiles with lactic acid activity, reaching up to 37.2 g/L lactic acid in complex culture medium including 1% sago starch.

Keywords: Starch; Lactic acid bacteria; bacterial counting; microbial growth; culture media; whey (Sources: MeSH, NLM, CAB).

RESUMEN

Objetivo. Evaluar lactosuero como fuente de mesófilos con actividad ácido láctica en medio de cultivo complejo con almidón de sagú (*Canna indica* L.). **Materiales y métodos.** Se analizaron tres medios de cultivo para bacterias mesófilas de lactosuero diferenciados por la inclusión de almidón de sagú (*Canna indica* L.) (0, 0.5 y 1%). Los tratamientos control fueron suero refrigerado (-4°C) y sin refrigerar (18°C). Se evaluó el crecimiento de mesófilos, pH, nivel de acidez, y sólidos solubles totales con intervalos de 24 h durante 5 días; y concentración de ácido láctico al finalizar el ensayo. **Resultados.** El pH y los sólidos solubles totales (°Brix) disminuyeron en todos los tratamientos, el porcentaje de acidez aumentó con el tiempo y al finalizar el ensayo el mayor contenido de ácido láctico fue de 32.5 y 37.2 g/L para las formulaciones con 0.5 y 1% de almidón de sagú respectivamente.

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El crecimiento de mesófilos fue bajo en la formulación con 1% de almidón y alto en el suero sin refrigerar. **Conclusiones.** El lactosuero es una buena fuente de mesófilos con actividad ácido láctica lográndose hasta 37.2 g/L de ácido láctico en medio de cultivo complejo con inclusión de 1% de almidón de sagú.

Palabras clave: Almidón; bacterias ácido lácticas; conteo bacteriano; crecimiento microbiano; medio de cultivo; suero de leche (*Fuentes: MeSH, NLM, CAB*).

INTRODUCTION

Lactic acid bacteria (LAB) have been found in the lacto-serum obtained after the manufacture of cheese, this has prompted the investigation of these native microorganisms because lactic acid is commonly used in the food industry (additive) and chemical (solubilizer)(1). The use of LAB from their massification in culture media that include in their formulation sources or nutritional components where they develop naturally, could reduce the production costs of inoculants, increase bacterial growth, the production of lactic acid during fermentation and favor the development of microorganisms that are not cultivable in laboratory conditions, by the metabolic adaptation of the bacteria to the physical and chemical characteristics of the medium. For this reason, the latest findings suggest that the massification of native microorganisms should be done by simulating the natural conditions where the bacterial populations remain (2).

In spite of being scarce the researches where complex culture media formulated with lacto-serum are evaluated for the production of lactic acid from the massification of native LAB, there are reported media that include carbon sources with not defined composition, such as chicory inulin (*Cichorium intybus* L.), agave inulin (*Agave* spp.) and albedo of orange peels (*Citrus sinensis*) (1). Orange juice (*Citrus sinensis*), guava pulp (*Psidium guajava* L.), noni pulp (*Morinda citrifolia* L.) and milk powder have also been used in the formulation of complex media for LAB (3). Other carbon sources that have been used in the massification of microorganisms with industrial potential are molasses (4), orange peel and juice (5) and sugar beet (6), which usually contain sugars such as sucrose, glucose and fructose.

Lacto-serum, besides being a natural source of LAB, corresponds to a liquid medium that provides amino acids and carbon in the form of lactose mainly (7), therefore, it is an industrial by-product that can be used for the formulation of bacterial culture media at low cost due to its high production, approximately 9 kg of whey are obtained for 1 kg of manufactured cheese, that is, between 80-90% of the volume of milk used (8,9).

In Colombia, the oversupply of this by-product and the low demand due to the lack of transformation processes and technology for the elaboration of specialized products, force the producer to discard it, which has repercussions in the contamination of soils and water sources due to the excessive contribution of bases, acid pH and high degradability rate (7). Among the main advances in the use of lacto-serum are reported the obtaining of protein concentrate (9,10), bioactive peptides with antimicrobial, antioxidant and antihypertensive function (7), ethanol production (11), vitamins, surfactants, enzymes, antibiotics and polysaccharides (112,13).

But, in spite of the technological advances, the major limitation is the high cost and low adaptation of the technologies in traditional farm systems. Therefore, it is necessary to expand the research related to the use of lacto-serum with applications that can be replicated in the field as, for example, the development of inoculants in complex culture media formulated with inputs that are found in the farms, such as sago (*Canna indica* L.), a rustic plant with a wide world distribution from which starch can be extracted that tolerates acidic media (14,15). The evaluation of culture media with non-conventional materials that are natural sources of microbial populations with industrial application allows functionalizing products considered waste and taking advantage of promising bacteria. For that reason, the objective of this work was to evaluate lacto-serum as a source of mesophiles with lactic acid activity in complex culture medium with sago starch.

MATERIALS AND METHODS

Obtaining the whey and formulation of the culture medium. The lacto-serum used was supplied by producers of peasant cheese from Colombia, Andean region, Boyacá department, from farms specialized in dairy cattle raising.

Fifteen subsamples of 100 mL of lacto-serum (with less than 1 h of extraction) were taken in amber bottles and transported under refrigerated conditions (-4°C) and protected from direct light to the laboratory of Animal Nutrition of the Pedagogical and Technological University of Colombia, Tunja, coordinates 5°55'56'' S and 73°35'49'' W, located at an altitude of 3000 msnm. The samples were filtered to eliminate the residues of the whey. The samples were filtered to remove cheese residues, and mixed into a single sample that was distributed in 500 mL beakers that had the following components: $CaHPO_4$, $CaCO_3$, $CaHPO_4$, molasses, and starch from sago tuber (*Canna indica* L.) extracted by streaking (15) that was incorporated at three high (1%), medium (0.5%), and low (0%) levels. Controls were refrigerated (C1) (4°C) and unrefrigerated (C2) (18°C) serum (Table 1). The culture media were incubated at room temperature (18°C) without shaking in an incubation chamber for 5 days to simulate field conditions; no external isolates were inoculated.

Table 1. Formulations of bacterial culture media with different carbon source on wet basis.

Component (%)	C1	C2	0	0.5	1
Lacto-serum	100	100	99.5	94	93.5
Sago	0	0	0	0.5	1
$CaCO_3$	0	0	0.3	0.3	0.3
$CaHPO_4$	0	0	0.2	0.2	0.2
Molasses	0	0	5	5	5

C1: refrigerated whey (4°C), C2: non-refrigerated lacto-serum (18°C), 0: low level with 0% sago starch, 0.5: medium level with 0.5% sago starch, 1: high level with 1% sago starch

Chemical composition of carbon sources and culture media. The analyses were made in dry base, for this, liquid samples were dried by forced convection at 40°C before making the compositional analysis. Protein digestibility was determined by in vitro method (16), dry matter (DM) content with drying oven at 60°C and moisture content (H) by difference (17); ash (Cen) by ashing method (118), ethereal extract by Soxhlet method, protein by Kjeldahl, reducing sugars and non- reducing sugars by Benedict's quantitative test (19), total soluble solids (20), and presence of sugars by Molish Felling and Selivanoff method in test tubes (21,22).

For the Molisch test, 2 mL of the unknown solution (1 g sample in 50 mL distilled water) was mixed with 2 drops of Molish's reagent and allowed to react for 30 min. For Fehling's test, 6 drops of Fehling's reagent and 1 mL of the unknown solution were mixed and then the test tubes were placed in boiling water for 30 min. For the Sellivanoff test, 1 mL of unknown sample was added with 5 mL of Sellivanoff's reagent and it was left in rest during the same time, and in the Benedict's test, 1 mL of reagent was used with 5 mL of unknown solution, then the evaluation of the colorations was made as described in table 2 to interpret the results.

Table 2. Evaluation of the Fehling, Molisch and Sellivanoff reactions.

Test	Observation	Interpretation
Molisch	A violet ring forms between the phase separating liquids.	Positive for the presence of carbohydrates
Fehling	A red precipitate forms in thesolution	Reduction of sugar present
Sellivanoff	Solution with reddish coloration	Presence of ketohexoses and aldopentoses
Benedict	Precipitate forms with red or yellowcoloration.	Positive for the presence ofreducing carbohydrates

Adapted from Pawar (22)

Mesophilic growth. The growth of native bacteria of the lacto-serum was evaluated, for this, 1 mL of each sample was diluted up to 10^{-9} in 0.8% saline solution, and they were sowed in *Compact Dry* for mesophilic aerobes®, three replicates per treatment. Monitoring was performed starting at 0 h and at 24 h intervals for 5 days. The plates were incubated at 37°C for 48 h and later the colony forming units (CFU) were counted (22,23).

Lactic acid concentration. To determine the total lactic acid concentration at the end of the trial, the Reflectoquant® lactic acid test(Supelco, USA) was used. For this, the liquid samples were diluted in distilled water (1:100) and a test strip was immersed and subsequently placed in the reading area of the equipment.

Variation of pH and acidity. The acidity of the treatments was obtained by titration with NaOH, and phenolphthalein was used as indicator, then, with the following formula was estimated the acidity percentage of lactic acid (24).

% Acidez=(V NaOH x NaOH)x(PMácido/(valencia x 1000))/A x 100

PMacid= molecular weight of the acid
A= aliquot of sample in mL
NaOH= NaOH concentration

While the pH was evaluated with an OAKTON® potentiometer (OAKTON Instruments, Vernon Hills, IL, USA) that was calibrated before each measurement (118). These analyses were performed at 24 h intervals for 5 days.

Total soluble solids content (°Brix). A MA887® refractometer (Milwaukee Instruments, USA) was used and 25 mL samples were analyzed in an erlenmeyer flask, three replicates per treatment. Measurements were taken at 24 h intervals for 5 days.

Statistical analysis and experimental design. Five treatments were evaluated, corresponding to the formulations with lacto-serum and two controls, each one with 3 replicates in a completely randomized design; each repetition was treated as an experimental unit. The data were organized in an excel table and from the obtained averages the graphs were made, the statistical model of greater adjustment was determined for the pH and microbial growth variables. Analysis of variance (ANDEVA) was also done and Tukey's test was applied where there were significant differences ($p<0.05$) with the statistical software SPSS Statistics.

RESULTS

Chemical composition of carbon sources and culture media. The results of the chemical composition of sago starch, lacto-serum, molasses and culture media are shown below (Table 3).

Mesophilic growth. The initial content of mesophilic CFU in the lacto-serum ranged from 123 ± 10 to 302.6 ± 8.19. The bacterial growth differed during the time between formulations with significant differences($p<0.05$). At the end of the trial, the highest CFU value was observed in the non-refrigerated lacto-serum (C2) and the lowest growth was obtained in the formulation with 1% sago starch. In all the culture media, the bacterial growth adjusted to a sigmoid curve ($p<0.001$) (Table 4).

Table 3. Compositional quality of mixed ingredients and culture media on a dry basis.

Parameter	Carbon source			Culture medium		
	lacto-serum	Sago	Molass a	0	0.5	1
Humidity (%)	97.8 ±0.95	6.1 ±0.08	25.2 ±0.31	78.5 ±0.01	76.2 ±0.02	73.9 ±0.02
Dry matter (%)	2.2 ±0.95	93.9 ±0.08	74.8 ±0.31	21.5 ±0.02	23.8 ±0.02	26.1 ±0.04
Crude protein (%)	44.1 ±0.92	1.9 ±0.05	1.1 ±0.13	15.2 ±0.15	14.1 ±0.13	13.3 ±0.12
Ash (%)	13.3 ±0.01	0.6 ±0.35	18.4 ±0.21	15.3 ±0.11	14.3 ±0.05	13.4 ±0.08
Ether extract (%)	6.6 ±0.95	0.5 ±0.02	2.7 ±0.14	3.7 ±0.01	3.6 ±0.05	3.3 ±0.06
Reducing sugars	0.1 ±0.07	0.4 ±0.04	0.2 ±0.01	0.1 ±0.01	0.2 ±0.01	0.2 ±0.01
Non-reducing sugars	0.1 ±0.02	0.1 ±0.01	0.1 ±0.01	0.1 ±0.01	0.1 ±0.01	0.1 ±0.01
Total soluble solids (°brix)	7.4 ±0.26	14. 2 ±0.03	36.4 ±0.02	24.07 ±1.03	23.41 ±0.09	22.84 ±0.09
Protein digestibility (%)	90 ± 0.02	63 ± 0.91	55 ± 0.57	87.4 ± 0.08	85 ± 0.37	80 ± 0.16
Molish Test	+	+	+	+	+	+
Felling Test	+	+	+	+	+	+
Selivanoff Teste	+	+	+	+	+	+
Benedict Test	+	+	+	+	+	+

±: standard error of the mean

Table 4. Mesophilic growth (CFU/mL) in culture media with different carbon source.

T	Day					Model	R^2 (%)
	1	2	3	4	5		
C1	582 ±8.1 a	584 ±12.9a	1054 ±11.4b	1600 ±5.4b	1591 ±11.6b	$Y=1720.17/(1+e^{(x-1.9993)/0.8258)})$	92.87
C2	569 ±17.6b	583 ±17.1b	2042 ±75.4a	2402 ±60.3a	2950 ± 47.1a	$Y=3062.55/(1+e^{(x-2.7140)/0.7958)})$	98.54
0	518 ± 12.6c	528 ±9.4c	1054 ±14.3b	1048 ±11.7d	1224 ±15.8c	$Y=1371.15/(1+e^{(x-2.0169)/1.4163)})$	96.93
0.5	536 ±19.4c	571 ±6.6b	1027 ±89.5c	1016 ±12.4d	1128 ± 58.3d	$Y=1228.51/(1+e^{(x-1.6064)/1.3525)})$	97.88
1	390 ± 15.2d	405 ±10.5d	1029 ±19.3c	1080 ±15.6c	1119 ±17.2d	$Y=1196.41/(1+e^{(x-2.0510)/0.8625)})$	95.07

C1: refrigerated lacto-serum, C2: non-refrigerated lacto-serum, 0: low level with 0% sago starch,lacto-serum, $CaCO_3$, $CaHPO_4$; 0.5: medium level with 0.5% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$, 1: high level with 1% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$. Means with different letters indicate significant difference in each sampling point, according to Tukey's test ($p<0.05$). ±: represents the standard error of the difference between means. R^2: coefficient of determination. X= time elapsed during bacterial growth.

Variation of pH, acidity and lactic acid concentration. The pH differed significantly among the formulations ($p<0.05$). The non-refrigerated lacto-serum (C2) and the formulations with 0.5 and 1 % sago starch had a more acidic initial pH compared to the refrigerated lacto-serum (C1) and formulation 0. From the second day there were significant differences ($p<0.05$) between the pH of the refrigerated lacto-serum (C1) and the other formulations. It was observed that the pH of the culture media decreased adjusting to an exponential decay curve ($p<0.0001$) and at the end of the trial the lowest value was for the non-refrigerated serum (4.3 ± 0.01) (Table 5).

Table 5. pH variation in culture media with different carbon sourcefor mesophilic bacteria.

T	Day					Model	R^2 (%)
	1	2	3	4	5		
C1	7 ±0.12c	6.9 ±0.01a	6.7 ±0.25a	6.7 ±0.26a	6.6 ±0.27a	$Y=7.5463*e^{(-0.0519*x)}$	96.98
C2	6.9 ±0.04ab	6.6 ±0.04b	5.1 ±0.31b	4.8 ± 0.29b	4.3 ± 0.01c	$Y=8.2460*e^{(-0.1337*x)}$	97.24
0	7.1 ±0.02bc	6.8 ±0.01ab	4.8 ±0.06b	4.6 ±0.04b	4.6 ±0.04bc	$Y=8.1482*e^{(-0.1298*x)}$	96.02
0.5	6.7 ±0.03d	6.4 ±0.01c	5.1 ± 0.02b	4.5 ±0.05b	4.4 ±0.02bc	$Y=7.6166*e^{(-0.1176*x)}$	97.67
1	6.6 ±0.01d	6.2 ±0.01d	5.3 ±0.05b	4.7 ±0.01b	4.6 ±0.04bc	$Y=7.3921*e^{(-0.1027*x)}$	97.73

C1: refrigerated lacto-serum, C2: non-refrigerated lacto-serum, 0: low level with 0% sago starch,lacto-serum, $CaCO_3$, $CaHPO_4$; 0.5: medium level with 0.5% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$, 1: high level with 1% sago starch, whey, $CaCO_3$, $CaHPO_4$. Different letters per column indicate significant differences according to Tukey $p<0.05$. ±: represents the standard error of the difference between means. R2: coefficient of determination. X= time elapsed during bacterial growth.

There were significant differences in the acidity level of the formulations at 24 h intervals ($p<0.05$). On the first day the most acidic culture media were those including 0.5 (0.2582 ± 0.01) and 1 % (0.3063 ± 0.01) of sago starch, the other treatments were between the range 0.1021 - 0.8707 ($p<0.05$) (Figure 1). At the end of the trial the least acidic treatment was the refrigerated lacto-serum (0.1262 ± 0.01) and the highest acidity was observed in the media with 0.5 (0.8708 ± 0.01) and 1% (0.8407 ± 0.01) of sago starch.

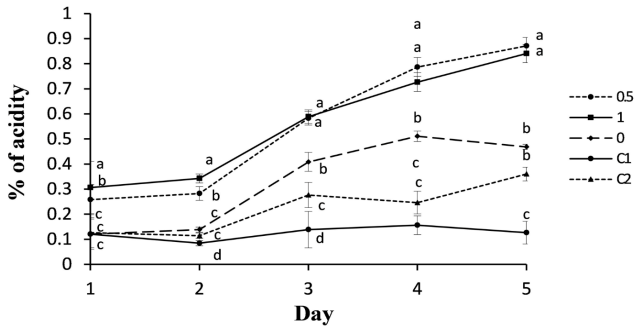

Figure 1. Percentage of acidity of the culture media for mesophilic bacteria during fermentation. Means with different letters indicate significant difference at each sampling point, according to Tukey's test($p<0.05$). Vertical bars represent the standard error.

The percentage of acidity was related to the total lactic acid content at the end of the trial, with the highest value being observed in the culture medium with 1% sago starch, with significant differences between treatments ($p<0.05$) (Table 6).

Table 6. Lactic acid production in culture media with different carbon source for mesophilic bacteria after 5 days of fermentation.

Treatment	Lactic acid (g/L)
C1	12.6 ± 0.02 d
C2	16.9 ± 0.01 c
0	18 ± 0.01g c
0.5	32.5 ± 0.02 ab
1	37.2 ± 0.01 a

C1: refrigerated lacto-serum, C2: non-refrigerated lacto-serum, 0: low level with 0% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$; 0.5: medium level with 0.5% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$, 1: high level with 1% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$. Different letters per column indicate significant differences according to Tukey $p<0.05$. ±: represents the standard error of the difference between means.

Variation of total soluble solids (°Brix). The total soluble solids content (°Brix) was above 6.4°Brix (Figure 2), and decreased with time. There were statistically significant differences between treatments by sampling point ($p<0.05$).

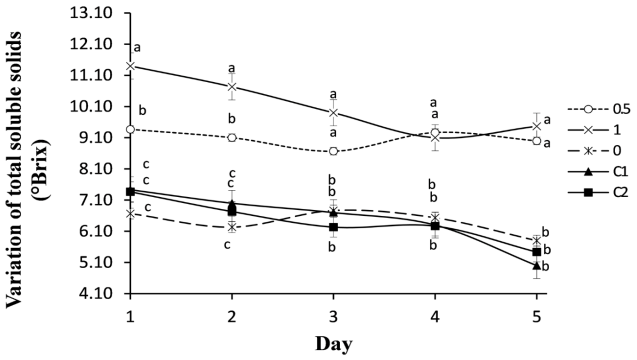

Figure 2. Total soluble solids content in the blends over time. C1: refrigerated lacto-serum, C2: unrefrigerated lacto-serum, 0: low level with 0% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$; 0.5: medium level with 0.5% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$, 1: high level with 1% sago starch, lacto-serum, $CaCO_3$, $CaHPO_4$. Means with different letters indicate significant difference in each sampling point, according to Tukey's test ($p<0.05$). Vertical bars represent the standard error.

DISCUSSION

The chemical composition of the lacto-serum evaluated was superior to the observed in whey used for the formulation of biopreparations with probiotic function (2.82% of ethereal extract and 4.55% crude protein) (25) and lacto-serum generated in Germany and the European Union, due to the high content of soluble solids, which is related to the quality of milk and separation of casein in a short time.

The nutritional qualities of sago starch were similar to the reported in Colombian starch extracted by the traditional method of grating (15) and it was within the ranges for starch of good quality according to the Codex parameters and the United States Department of Agriculture, where it is considered a functional food (26).

The chemical composition of molasses was similar to the one used in processes of obtaining products of microbial origin, although its quality varies due to the effect of the varieties of cane, fertilization and the sugar refining process (4,27).

The evaluation parameters of the fermentation process differed by treatment. The initial pH in the media was higher than the reported in lacto-serum (5.54-5.76) (11), but it was reduced during the fermentation process since sugars like glucose, lactose, fructose, and sucrose are used as energy sources during the microbial growth of mesophilic bacteria that produce lactic acid (118,11), it is reported total consumption of carbon sources after 10 days (6).

The depletion of sugars was proportional to the decrease in the content of total soluble solids, but, higher values of °Brix were observed in the formulations with 0.5 and 1 % of sago starch due to the inclusion of molasses that can contribute up to 50% of total sugars in dry matter (6).

The decrease in the pH of the lacto-serum can be stopped after refrigeration as it was observed in C1, due to the fact that the low temperatures stop the microbial metabolism and consequently the proliferation of bacteria, this allows that the lactose content is stabilized for up to four weeks (5,11), and it is due to the fact that the speed of bacterial growth has a lineal relation with the temperature (28).

The decrease of pH in the other formulations was stabilized from the third day, thanks to the inclusion of $CaCO_3$, because it acts as a neutralizing agent, for example: with the inclusion of 0.5% $CaCO_3$ in fermentations in solid state the pH is maintained on 4.8 (18). But, the inclusion of $CaCO_3$ can have antimicrobial activity due to the effect of the formation of carbonic acid (4), this can have limited the growth ofmesophilic bacteria in the evaluated culture media.

It is reported that the carbon source influences the amount of lactic acid produced in bacterial massification media, for example,*Lactobacillus rhamnosus* can use glucose and sucrose, however, with extracts of carob (*Ceratonia siliqua*) that contain 35 g/L of sucrose and30 g/L of hexoses, it only consumes hexose and produces up to 22 g/Lof lactic acid, compared with media with sucrose (16 g/L lactic acid) (229). The results obtained show that the mesophiles of the lacto-serum can produce higher values than these, standing out the formulations with 0.5 % (32.5 ± 0.02 g/L) and 1 % (37.2 ± 0.01 g/L) of sago starch. However, these values are lower than those observed in a fed fermentation system (57 g/L) formulated with corn liquor (45 mL/L), manganese sulfate (0.075 g/L) and lacto-serum lactose (60 g/L) (30), which is related to the depletion of nutrients and the structure of the sugars provided by the raw materials. Due to the fact that the main componentof molasses is sucrose, which must be converted to fructose and glucose to be assimilated (4), and sago starch contributes sugars in form of amylopectin and amylose, which must be unfolded to glucose, however, the efficient use of carbon sources is directly related to the microorganism, besides, additional nutrients as sulfur and nitrogen can favor the microbial growth (6,14,20).

In conclusion, the enrichment of lacto-serum with mineral sources, molassesand *Canna indica* L. 1% favor the growth of mesophilic aerobic bacteria that can produce up to 37.2 g/L of lactic acid. In this medium was presented the lowest growth of microorganisms but the highest production of lactic acid, which indicates that lactic acid bacteria (BAC) that do not belong to the group of mesophilic aerobes as the facultative anaerobic BAC could contribute to the generation of product, therefore it is suggested that in future tests it is determined the BAC that grow in these culture media. In general, the type of substrate, the contribution of protein, sugars and minerals are factors that intervene in the growth of specialized microorganisms and in the products that are generated during the fermentation process.

Conflict of interest

The authors declare that there is no conflict of interest for the publication of this article.

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