Chapter 29

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM MATURE COFFEE CHERIES: POTENTIAL APPLICATION IN COFFEE HUSK ENSILING

M.G.B PAGNONCELLI[1], D.BRAND [1,2], S. ROUSSOS[2], I. GAIME-PERRAUD[2], C. AUGUR[2] , C.R. SOCCOL[1]

[1] *Laboratório de Processos Biotecnológicos, Departamento de Engenharia Química, Universidade Federal do Paraná (UFPR), 81531-970 Curitiba-PR, Brasil ;* [2] *Equipe de Mycologie et de Fermentation en Milieu Solide IRD UR-119; IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925; 163 Avenue de Luminy; F-13288 Marseille Cedex 9, France*

Abstract

Coffee is one of the most profitable crops in Latin America. Brazil is the biggest producer, consumer and exporter in the world. After coffee processing only 5.8% of the total matter is used for coffee production generating a large amount of waste, were no commercial value, that can hardly pollute the environment. The coffee husk conservation can be made by silage. Specific microorganisms can produce a standard ensilage when added in the process. The main goal of this work is to study the natural microflora present in the coffee husk and coffee pulp able to ensilage these by-products. The lactic acid bacteria responsible for this process were isolated and characterised and added in an ensilage process to accelerate the acid lactic production. First, in this work, fresh fruity coffee pulp were put fermented naturally and it was observed that the pH was maintained around 4 during two months. Then many bacteria were isolated from these natural fermented materials. Morphology, fermentative and biochemistry studies were used to identify and characterise the bacteria. These bacteria were able to grown in a coffee extract and change the pH 6 to around 4.4. Then, the second study was added these isolated bacteria in processed coffee husk to ensilage. It shows that is necessary to add the lactic acid bacteria to initiate the ensilage process. In the coffee husk fermentation, 10^7 CFU were added per g of matter and the pH was maintained around 4.8; microorganisms were viable for 2 months.

Introduction

In the last ten years, the average world's coffee production was of 97 millions sacks of 60 Kg. Only two species of the *Coffea* genera are cultivated in great scale in the world, representing 100 % of commercialized coffee, *Coffea arabica* (arabica variety) and *Coffea canephora* (robusta variety). The arabica variety represents 75% of the world's production (**1-3**).

New Horizons in Biotechnology, 321-333, S. Roussos et al. (eds)

The coffee fruit also denominated coffee cherry presents different tissue layers, which covers the grain. The exocarp is the most external part of the fruit. The internal part, rich in sugars and pectin, is the mesocarp (pulp). Finally, covering the grains is the endocarp appearing as a membrane. During processing, the coffee fruit is subjected to different operations with the aim of removing the coffee grains from its envelopes (**4**). There are two technologies to obtain the de-hulled grain: through wet and dry processing. During wet processing, two residues are generated, the pulp and mucilage waters, both rich in nutrients. In dry processing, coffee husk is the sole residue originated. In both processes, pulp and husk elimination occur with the aid of microorganisms, which ferment the fruit and seeds and aid in the elimination of the envelopes (**1,5,6**).

When coffee is ready for consumption as beverage, only 5,8% of the fresh cherry weight is utilized, generating by-products of low commercial value. That may cause important environmental contamination (**7**). The utilization of agro-industrial by-products in biotechnological processes constitutes an alternative with regards to the use of the substrate. It may also help in solving the environmental pollution problem (**8**).

Various studies, utilizing coffee husk and pulp as substrate for fermentation processes, have already been undertaken. Besides the elimination of anti-nutritional factors aiming at its utilization in the nutrition of ruminants, new studies aiming at the utilization of these by-products as fermentation substrates for the production of edible mushrooms and biomolecules of commercial interest such as organic acids, vegetable hormones and aroma compounds, have been done (**9-12**).

The fermentation process, besides conserving and protecting these foodstuffs from pathogenic microorganisms, can improve product digestibility and in certain cases eliminate its toxicity (**13**). Silage is a process by which fermentation of the available sugars with the production of organic acids, mainly lactic acid by lactic bacteria in anaerobic conditions, is achieved (**14,15**). The objective of a silage process is to minimize loss of dry matter and nutritional value and to avoid the development of undesirable microorganisms (**16**).

The lactic bacteria responsible for the fermentation process are normally present on the plant tissue to be ensiled. Nevertheless, the utilization of starters improves the standardization of the fermentation, controlling the plant microbial flora, mainly the aerobic flora and accelerating pH fall (**15,17-19**).

The lactic bacteria can ferment carbohydrates by two means. The homo-fermentative where the final product of the process is exclusively lactic acid and hetero-fermentative, where, besides lactic acid, the presence of other metabolites such as acetic, propionic and butiric acid and ethanol can occur (**20-22**).

The present work had as objectives, the isolation and identification of lactic bacteria present in mature coffee cherries, the evaluation of the potential of these bacteria in the fermentation of sugars available in coffee husk, the determination of the acidification capacity of the media due to the production of organic acids and finally the utilization of strains as starters for the silage of coffee husk.

Coffee husk characterization

The chemical composition of coffee husk may vary depending on the cultivar. In Table 1 the components present in coffee husk of two different cultivars are presented. Sample 1 corresponds to the husk given by Café Damasco and sample 2 was supplied by Cocamar. Analyses were carried out according to the methodologies described by Nelson (**23**), Somogyi (**24**), Goering and Van Soest (**25**) and AOAC (**26**).

For the utilization of a raw material as substrate in a fermentative process, its chemical composition should be considered. The substrate should give the necessary conditions to the development of the microorganisms employed, aiming at a fermented product of high quality. After fermentation, the process should result in a food of high nutritional value.

The values obtained for proteins demonstrate that this substrate possesses an important concentration of organic nitrogen that may be utilized as animal feed. The values obtained for ashes reveal appreciable quantities of minerals in coffee husk, which are favorable to the development of microorganisms. These mineral values are related to the presence of calcium and phosphorus, which meet the 1:3 proportion, which is the ideal condition for the utilization the substrate as animal feed. In order for silage to take place, it is necessary for the material to be used to have approximately 12% of available sugars (**14**). The reducing and total sugars present in sample 2 demonstrated values relatively low, probably to the fact that the cherries, when harvested for processing, were not mature enough. Coffee husk sample 1 was characterized as an ideal substrate for lactic fermentation, because the quantity of reducing sugars showed superior values to the minimal demanded for good ensiling.

Table 1. Chemical composition of coffee husk samples.

Components	Sample 1	Sample 2
Protein (g%)	7.65	9.48
Lipids (g%)	1.57	1.59
Ashes (g%)	6.70	9.62
Calcium (g%)	0.25	0.51
Phosphorus (g%)	0.08	0.12
Reducing sugars (g%)	23.85	3.80
Total sugars (g%)	27.45	8.69
Neutral detergent fibers (g%)	29.14	60.15
Acid detergent fibers (g%)	21.78	49.92
Lignin (g%)	5.83	9.30
Cellulose (g%)	15.27	32.40
Hemicellulose (g%)	7.37	10.23

The physico-chemical analyses of coffee husk samples utilized demonstrate that this residue could be considered as a good substrate for the development of bioprocesses utilizing microorganisms (**8-12**).

Isolation of bacteria from coffee cherries

Mature coffee cherries, manually depulped, were submersed in sterile water and then were placed to ferment naturally for 48 hours at 35°C, with agitation of 150 rpm. Part of the fermented material was transferred to MRS broth and incubated at 35°C for 48 hours (**27**). The isolation occurred in MRS agar after successive dilutions on inoculation plates.

The isolated strains were classified initially by the technique of Gram. The group of strains chosen for further studies were those that showed positive reaction to Gram dye, with rod or coccus-rod morphology. The selected strains were preserved in liquid nitrogen.

The natural fermentation of coffee cherries after 48 hours showed turbidity, indicating the presence of a great number of microorganisms. From the isolated strains, 20 were selected, showing coccus rod morphology and Gram positive reaction, and classified in later fermentative studies (fig 1).

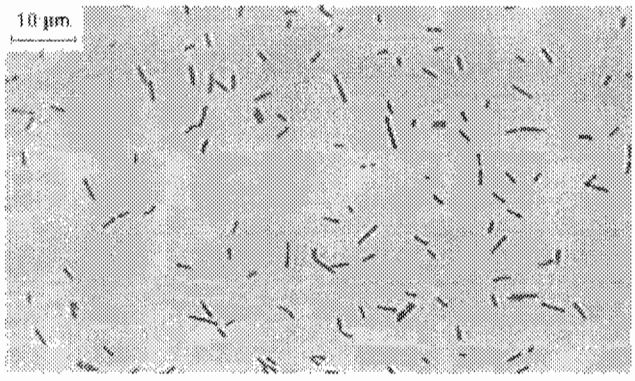

Figure 1. Morphology of Gram-positive bacteria (x1000) isolated from coffee cherries

Selection of bacteria isolated in liquid media containing coffee husk extract

In order to evaluate the potential of each isolated strain in utilizing the sugars present in coffee husk, a natural media was prepared from coffee husk, by cooking 100g of husk (sample 1) in 1L of distilled water at 100°C for 1 hour. The extract obtained was filtered and the pH adjusted to 7.00. The final volume was completed to 1L with distilled water.

Fermentative potential of bacteria in coffee husk extract

The strains were inoculated 10% (v/v) in a liquid medium of coffee husk extract, with initial pH of 6.0 or 7.0 and incubated at 35°C for 48 hours.

This study was carried out with the objective of evaluating the potential of bacteria isolated in relation to its capacity to ferment sugars present in coffee husk. After 48 hours acidification of the culture medium was observed. This is a typical process of acidic bacteria action, where the microorganism is able to transform the reducing sugars of the culture medium into organic acids (**14**). Figure 2 shows the acidification capacity of coffee husk extract by different isolated strains.

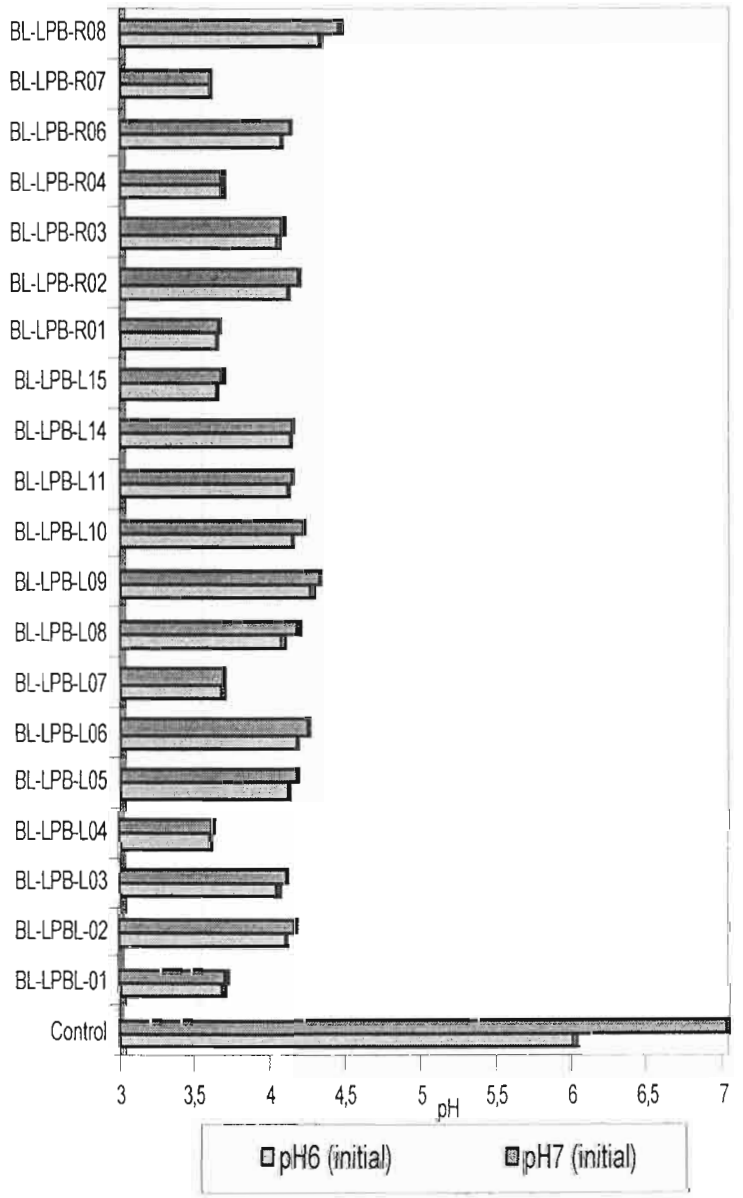

Figure 2. pH in coffee husk extract cultivated with isolated lactic acid bacteria.

It was observed that the majority of the strains studied (BL-LPB-L02, L03, L05, L06, L08, L09, L10, L11, L14, R02, R03, R06 e R08) possess a metabolism that allow the production of acid, reducing the pH of the medium to values close to 4.00. However, a second group represented by the strains BL-LPB-L01, L04, L07, L15, R01, R04 and R07, showed greater acidification power, reducing the pH to values close to 3.50. The strains that represent the second group, by presenting a greater capacity of acidification probably have a bigger potential in being utilized as starters in the silage process of coffee husk.

Identification and quantification of the metabolites produced in coffee husk extract

After the fermentation process, coffee husk extracts, with initial pH 7.00, were centrifuged at 11400 rpm for 15 minutes. The supernatant was diluted in the proportion of 1:10 and filtered through a 0.22 μm membrane and analyzed by high performance liquid chromatography, by utilizing a HPX-87H column (300 x 7.8 nM) – BioRad Aminex, with a temperature of 60°C. The mobile phase utilized was H_2SO_4, 5 nM.

Metabolic products were identified and quantified by comparison to standards such as lactic acid, acetic acid, propionic acid, butyric acid and ethanol. Table 2 shows the products of the metabolism of different isolated bacterial strains through the analysis by HPLC of fermented coffee husk extract.

Some strains produced high concentrations of lactic acid in coffee husk extract, reaching concentrations of 17 g/L, as was the case of the strain BL-LPB- R01. These strains, considered good producers of lactic acid, produced minimal quantities of other metabolites, therefore they can be classified as homo-lactic. On the other hand, another group of strains studied, produced low amounts of lactic acid, and excessive amounts of other metabolites. This was the case of strain BL-LPB-L14 producing 8.14 g/L of lactic acid, 3.08 g/L of acetic acid, 3.77 g/L of propionic acid and 0.97 g/L of ethanol. The strains belonging to this group could be classified as hetero-lactic. Of the 20 isolated strains, 7 presented a homo-fermentative metabolism and the remaining were hetero-fermentative.

Table 2. Metabolites produced by lactic bacteria.

Strains	Lactic acid (g/L)	Acetic acid (g/L)	Propionic acid (g/l)	Butyric acid (g/l)	Ethanol (g/l)
BL-LPB-L01	15,10	0,58	0,26	0	0
BL-LPB-L02	6,88	3,27	0,38	0	0,93
BL-LPB-L03	5,83	4,36	0,32	0	0,75
BL-LPB-L04	13,73	1,20	0,26	0	0

BL-LPB-L05	6,57	3,12	0,34	0	0,87
BL-LPB-L06	7,78	3,12	0,34	0	0,74
BL-LPB-L07	14,15	1,32	0,29	0	0
BL-LPB-L08	7,71	3,10	0,29	0	0,86
BL-LPB-L09	6,99	2,68	0,36	0	0,77
BL-LPB-L10	6,19	3,20	0,34	0	0,77
BL-LPB-L11	6,98	3,19	0,39	0	0,94
BL-LPB-L14	8,28	3,08	3,77	0	0,97
BL-LPB-L15	15,98	1,14	0,30	0	0,68
BL-LPB-R01	17,05	0,50	0,41	0	0
BL-LPB-R02	6,02	2,84	0,49	0	0,46
BL-LPB-R03	6,45	2,62	0,37	0	0,61
BL-LPB-R04	14,26	0,42	0,38	0	0
BL-LPB-R06	6,20	2,69	0,33	0	0,44
BL-LPB-R07	15,16	0,75	0,47	0	0
BL-LPB-R08	4,60	2,15	0,51	0	0

When comparing acidification of the culture medium by the different bacterial strains, it was observed that the strains belonging to the first group had greater capacity to acidify the culture medium, maintaining the final pH around 3.50. As for the hetero-lactic strains, the pH of the medium was close to 4.20. By analyzing these results it was deduced that homo-fermentative bacteria had a greater capacity to acidify the fermentative process. In any silage process, the fast fall of pH is a primordial factor. Lactic bacteria that have greater potentialities for utilization in this process are those that have the best ability for quick acidification.

Biochemical identification of the isolated lactic bacteria

Biochemical identification of lactic bacteria was based on the capacity of these microorganisms to metabolize certain sugars as sole carbon source in their fermentative metabolism to produce organic acids. The biochemical profile of the isolated strains was traced using 49 different carbon sources (API 50CHL – BioMerieux).

Culture media contained the indicator bromocresol purple, which enabled the visualization of the media acidification process. Interpretation of results was based on statistical comparison of the fermented sugars by the bacteria.

Table 3 shows the results of the identification of lactic bacteria which presented a reliability superior to 90% in the use of API 50 CHL.

Table 3. Identification of Lactic Acid Bacteria.

Strains	Species	Probability
BL-LPB-L01	*Lactobacillus plantarum* 1	99,9
BL-LPB-L02	*Lactobacillus brevis* 3	94,3
BL-LPB-L03	*Lactobacillus brevis* 3	94,8
BL-LPB-L04	*Lactobacillus plantarum* 1	99,9
BL-LPB-L05	*Lactobacillus brevis* 3	96,3
BL-LPB-L06	*Lactobacillus brevis* 3	94,8
BL-LPB-L07	*Lactobacillus paracasei ssp paracasei* 3	99,7
BL-LPB-R01	*Lactobacillus plantarum* 1	99,2
BL-LPB-R01	*Lactobacillus brevis* 3	94,8

All the isolated bacteria showing rod morphology and by producing lactic acid as major product of the metabolism belong to the *Lactobacillus* genera. These were then further divided into three different species.

Coffee husk silage

The strains that produced the highest acidification of the fermentation media with coffee husk extract, were selected to be tested as starters of coffee husk silage. The strains utilized in the process were *Lactobacillus plantarum* (BL-LPB-R01) and *Lactobacillus paracasei spp paracasei* (BL-LPB-L07). The inoculation rate utilized was 10^6 CFU/g of coffee husk on a dry weight basis.

Coffee husk utilized in the experiment was sample 1. The husk was re-hydrated in water, to a moisture content between 60 and 70%, inoculated with the selected lactic bacteria and incubated at room temperature.

The material was ensiled over a period of 30 days, with the evaluation of specific fermentative parameters at 0, 3, 10, 20 and 30 days of fermentation. Parameters surveyed included pH, production and quantification of metabolites, consumption of total and reducing sugars and viable cell count.

Inoculation of lactic bacteria facilitated the acceleration of the silage process, in the beginning of the fermentation, as can be observed in figure 3. The silage pH presented a fast fall in the first days of the process, reaching values close to 3.90 after 10 days of fermentation, staying stable until the end of the process (30 days). It can be considered that coffee husk silage can be accelerated with the use of starters. This way, the process could be concluded in only 10 or 15 days. A reduction in the time required to complete the silage process in an important factor for cost reduction resulting in an increase in productivity.

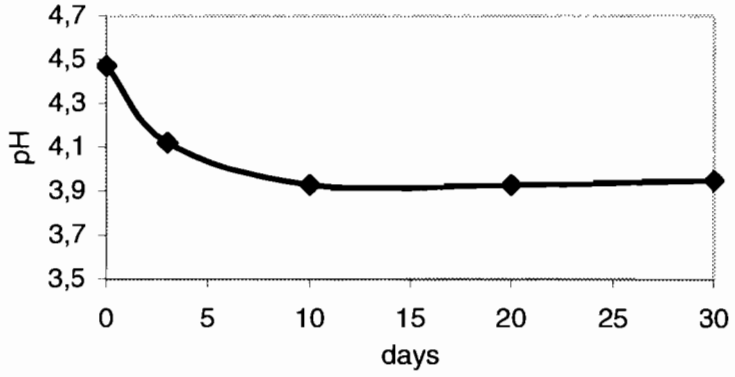

Figure 3. pH evolution of coffee husk silage.

During the silage process, lactic acid was the major metabolite. The production of other organic acids was quite reduced, due to the homo-fermentative metabolism of the starters utilized (fig 4). Ethanol was however produced, probably due to the presence of others microorganisms naturally present in this substrate, such as yeasts.

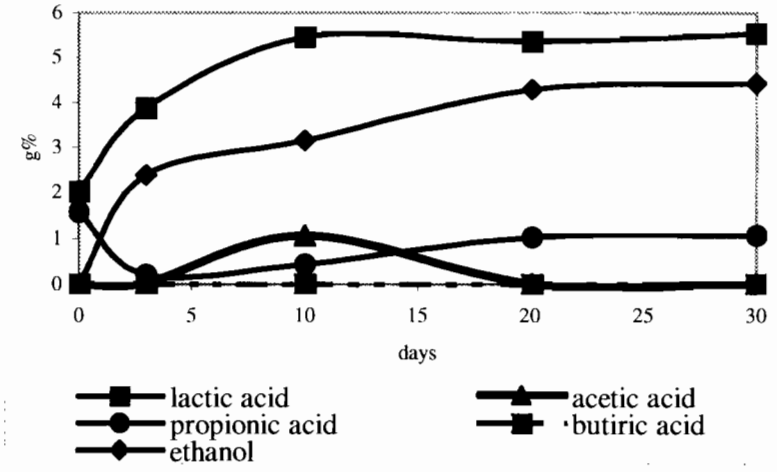

Figure 4. Evolution of the produced metabolites during coffee husk silage.

The microflora was able to metabolize most of the sugars present, within the first ten days. As can be observed in figure 5, a rapid reduction in the reducing sugars was obtained.

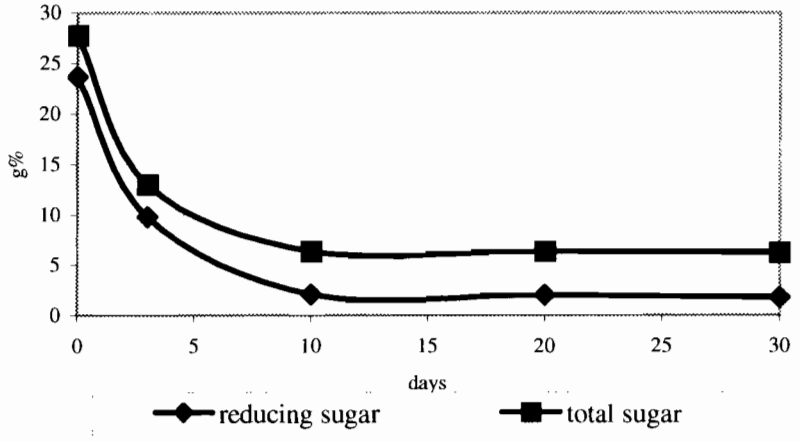

Figure 5. Evolution of total and reducing sugars during coffee husk ensilage.

Figure 6 shows the growth curve of the lactic bacteria during the silage process. In the first days an intense multiplication of cells took place, followed by a decline after 10 days of fermentation. The fall may be explained by the great acidification of the medium in the beginning of the fermentation.

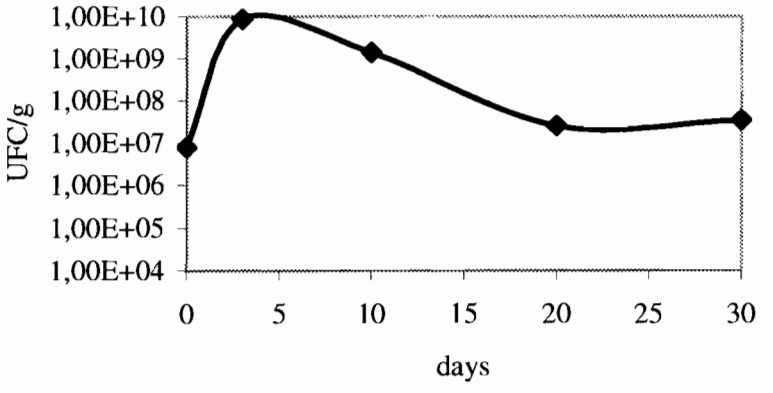

Figure 6. Lactic Acid Bacteria growth curve.

Conclusions

It was possible to isolate from mature coffee cherries a great number of bacteria. Among them, lactic acid bacteria were classified into homo-fermentative and hetero-fermentative groups. The isolated strains were able to produce organic acids from the soluble sugars present in coffee husk. From the 20 selected strains, 8 showed a homo-fermentative metabolism, being classified as homo-lactic, as lactic acid was the major metabolite. The selection of bacteria from coffee husk extract media allowed the selection of bacteria with high acidification power, classified as homo-lactic, able of reduce the final pH of the medium to values around 3.50. The hetero-lactic strains reduced the pH of the medium to values close to 4.20.

The biochemical tests undertaken with the 50 CHL kit allowed the identification of three different species of *Lactobacillus*. The strains were further characterized as *L. plantarum, L. brevis* and *L. paracasei.*

The addition of starters in the silage process of coffee husk accelerated the silage process. After the first ten days of the fermentation, variations were minimal and the pH remained constant until the end of the process.

Strains BL-LPB-R01 and BL-LPB-L07 were tested as starters, demonstrating great potential to be utilized in processes of coffee husk silage.

Acknowledgment

M.G.B Pagnoncelli and C.R. Soccol thank CAPES and CNPq, respectively, for a scholarship, the latter under the Scientific Productivity Scheme.

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Pagnoncelli M.G.B., Brand D., Roussos Sevastianos, Gaime Perraud Isabelle, Augur Christophe, Soccol C.R. (2003).

Isolation and identification of lactic acid bacteria from mature coffee cheries : potential application in coffee husk ensiling.

In : Roussos Sevastianos (ed.), Soccol C.R. (ed.), Pandey A. (ed.), Augur Christophe (ed.).

New horizons in biotechnology. Dordrecht : Kluwer, 321-333.

ISBN 1-4020-1718-9