Vol. 17(30), pp. 915-921, 25 July, 2018
DOI: 10.5897/AJB2018.16558
Article Number: 0E5347057983
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Full Length Research Paper

Biotransformation of the residual liquid from the wet coffee benefit by Kluyveromyces marxianus

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Received 15 June, 2018; Accepted 20 July, 2018

The search for biotechnological alternatives for the use of residuals generated in the agro-industrial processing of coffee is a current problem. This study evaluated the biotransformation of the liquid residual of the humid coffee benefit using the yeast *Kluyveromyces marxianus* CCEBI 2011. It was demonstrated that this strain is able to effectively use the reducing and neutral sugars in 24 h, for a yield of 40% in the production of alcohol. Pectin oligomers were obtained with an average size between 5.4 and 5.8 units of anhydrogalacturonic acid, with potential biological activity. In the fermented residuals, a decrease of more than 30% in the concentration of caffeine was observed. The pectinolytic yeast *K. marxianus* CCEBI 2011 significantly reduces the content of soluble proteins in the liquid residual. The results indicate that the residuals of the coffee benefit are viable substrate to be used by means of alcoholic fermentation in the presence of the pectinolitic yeast *K. marxianus* CCEBI 2011, reducing its environmental impact and propitiating alternatives for their better use in the agricultural sector.

Key words: *Kluyveromyces marxianus*, pectinolitic yeast, coffee residuals.

INTRODUCTION

Coffee is one of the most traded agricultural products in the world and annually large amounts of coffee by-products are generated during coffee processing (Bhoite and Murthy, 2015). The coffee pulp is one of the main by-products derived from the wet processing of coffee; it is rich in carbohydrates, proteins, amino acids, mineral salts, tannins, polyphenols and caffeine (Dorsey and Jones, 2017). It has potentialities that are attractive to be used as raw material in different processes such as: production of bioethanol, bio-fertilizer, production of biogas, animal feed (Gurram et al., 2016; Durán et al., 2017), and as a pure or mixed substrate in the production of edible mushrooms (Oliveira and Franca, 2015). These technologies allow the use of an available and inexpensive substrate, eliminate pollution and in turn generate economic, social and environmental benefits (Chang, 2007). The yeast Kluyveromyces marxianus CCEBI 2011 has been used in the fermentation process of sugars, where endopolygalacturonases enzymes are obtained (Rivera et al., 2017). This yeast has a great biotechnological potential, especially for ethanol production (Castro and Roberto, 2014). These investigations have demonstrated the technical feasibility of co-producing ethanol and the endopolygalacturonase

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enzyme on an industrial scale, which could be valued as an alternative for the diversification of the alcohol industry (Camacho et al., 2014). Serrat et al. (2016), demonstrated the feasibility of the use of aerobic or *anoxic fermentations, with pectinolytic yeast K.marxianus* CCEBI 2011, as an alternative for the valorization of agro-industrial residues rich in pectin (coffee pulp) and of great environmental impact. In this study, the biotransformation of the liquid residual of the wet coffee by anaerobic fermentation with the pectinolytic yeast *K. marxianus* CCEBI 2011 and the changes operated on the proportions in the solid/liquid fractions of the pulp mixture of liquid coffee-residual during its fermentation as well as the changes in the sugar composition of the liquid residual and its relation with the amount of ethanol produced were evaluated and the extraction of pectic substances associated with the production of the polygalacturonase enzyme by the yeast and the changes in the content of total soluble proteins, caffeine and tannins of the liquid residual during the fermentative process were determined.

MATERIALS AND METHODS

Microorganism

K. marxianus CCEBI 2011, wild type strain isolated from coffee wet residuals, was provided by the culture collection of CEBI-Universidad de Oriente, Cuba. It was stored at 4°C in YPDA medium. This medium had the following composition (g/L): yeast extract (10), peptone (20), glucose (20) and bacteriological agar (15).

Residual

The residuals of the wet coffee benefit (pulp mixture and mucilage suspension) of the species *Coffea canephora* P. c.v. Robust were collected in the ecological pulping plant (Penagos technology, Colombia) "Cuatro Caminos" in Santiago de Cuba province and they were packed in a plastic container of approximately 20 kg and stored at -20°C.

Inoculum preparation

Erlenmeyer flasks of 100 mL, containing 10 mL MGY medium following the composition (g/L): yeast extract (2.5), glucose (20), $(NH_4)_2SO_4$ (5), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2$ (0.1), and KH_2PO_4 (1) were employed and sterilized at 115°C. They were inoculated with the *K. marxianus* CCEBI 2011 strain, incubated for reactivation at 32°C, 12 h and at 150 rpm. For production experiments, 500 mL-Erlenmeyer flasks containing 200 mL of required MGY medium were inoculated for 12 h.

Substrate fermentation

The residual formed by an heterogeneous mix pulp and mucilage suspension was homogenized; the initial pH of the residual was adjusted (4.18) and was reduced to 3.5 using sulfuric acid (1 N). Three liters of the residual were transferred to bioreactors (plastic bottles of 5 L, made of poly (ethylene terephthalate), PET) and inoculated with 210 mL of the yeast inoculum, for an initial cell concentration of $4.6 \cdot 10^7$ cells/mL. The controls were performed in parallel without inoculation: 210 mL of sterile distilled water was added. The fermentation was realized in static to guarantee the microaerophilic conditions (self-induced anoxia), for 24 h at room temperature.

Processing of fermented residual

The content of the bioreactors was vigorously homogenized. The heavy solid fraction (fermented coffee pulp) was separated from the liquid fraction (suspension of fermented mucilage) by decanting using a Büchner porcelain funnel (1 mm). The respective volumes were measured and the solid fraction was weighed on a semi-analytical balance. From the liquid fraction, 500 mL were collected for the determination of the pH, chemical analysis and the determination of the polygalacturonase enzymatic activity. As a general control of “time zero", we proceeded in the same way as described for the cultures, with a representative sample of the residual (without pH adjustment or any type of additions).

Analytical methods

The liquid samples, named P0 (Residual without treatment), PC1 (Control sample, without yeast inoculum), PC2 (Control sample, without yeast inoculum, replica), PI1 (Sample inoculated with yeast) and PI2 (Sample inoculated with yeast, replica), were homogenized and centrifuged at 7500 rpm for 10 min. The supernatant was collected and the sediment was discarded. All the samples were determined in triplicate: carbohydrates (Hodge and Hofreiter, 1962), reducing sugars (Somogyi, 1952), reducing sugars in uronic acids (Anthon and Barrett, 2008), pectic substances (Bitter and Muir, 1962), tannins (APHA, 2004), caffeine (Brunetto et al., 2007), and alcohol (Conway, 1950).

An aliquot of the sample (previously cooled on ice) was precipitated at a rate of one sample volume and 3 volumes of ice-cold absolute ethanol, and centrifuged at 7500 rpm for 10 min. The precipitate was resuspended in a volume of distilled water equal to the starting volume of the sample, for the determination of soluble proteins (Lowry et al., 1951), polygalacturonase enzymatic activity (Serrat et al., 2016), and Folin-Denis test to determine the interference of proteins in the quantification of tannins (APHA, 2004).

Statistical analysis

The statistical analysis was carried out with the statistical package Statgraphics Centurion XV (Stat Point, Inc., USA). Simple linear regression analysis, comparison of regression lines and analysis of variance (ANOVA I) were performed. When necessary, the Duncan multiple range test was used to compare means.

RESULTS AND DISCUSSION

Changes in composition and the solid/liquid ratio of the fermented coffee residuals

The liquid and solid fraction volume decreased after 24 h of fermentation. However, there was an increase in the wet mass of the solid fraction (pulp) in the inoculated samples (Table 1). Table 2 shows that after 24 h of

Table 1. Volume, solid mass and pH of the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) in the microaerophilic conditions (self-induced anoxia), for 24 h at room temperature.

Sample	Total volume (mL)	Liquid volume (mL)	% v/v	Solid volume (mL)	% v/v	Solid mass (g)	% m/v	pH
PO	550	350	63.6	420	76.4	886,88	27.5	3.5
PC1	3227	1900	58.9	2300	71.3	1083,25	33.6	3.97
PC2	3227	2000	62.0	2500	77.5	1287,6	39.9	4.02
PI1	3227	1860	57.6	2220	68.8	1232	39.4	3.94
PI2	3127	1500	48.0	2200	70.4	171,108	31.1	3.97

Table 2. Volume ratio of the liquid fraction/mass of solids (L/kg) of the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) in the microaerophilic conditions, for 24 h at room temperature.

Sample	Pulp (L/kg)	Cherry coffee (L/kg)
PO	2.05	0.82
PC1	2.14	0.86
PC2	1.85	0.74
PI1	1.44	0.58
PI2	1.22	0.49

fermentation, there was a significant decrease in the volume of liquid and increase of the wet mass of the solid fraction in the pulp inoculated with pectinolytic yeast. Initially, we had the solid fraction (pulp) in equilibrium with the liquid medium (suspension of mucilage). The liquid medium presents fermentable sugars consumed by the yeast, which at the same time excretes the endopolygalacturonase enzyme to the medium (Serrat et al., 2002). This enzyme hydrolyzes the pectins in the liquid medium and in the pulp promoting that more soluble solids are present in the pulp, including oligopectinates, which migrate to the liquid medium where they will again be fermented. This should cause a flow of water, probably mediated by osmosis, from the aqueous medium to the pulp, thus decreasing the volume of free liquid in the residual and increasing the wet mass of the solids (pulp) responsible for absorbing the liquid from the residual.

The pH of the residual was adjusted from 4.18 to 3.5 and after 24 h of fermentation, pH increased, probably due to the action of microorganisms (bacteria) or other biochemical changes that occurred in the fermentation. Bacterial death and its lysis, as a consequence of the prevailing adverse conditions (acid pH, ethanol), is anevent that manifests itself with an increase in pH due to the release of biogenic amines to the medium.

Transformation of total reducing sugars, neutral sugars and alcohol production

Glucose and fructose are among the sugars that contribute to the increase in reducing power (Shao and Lin, 2018); the pectic substances also contribute to the increase in reduction. It can be observed (Figure 1) that in the inoculated samples there was an almost total consumption of the reducing sugars initially present. These sugars are used by the microorganism as a source of carbon and energy. This result is important, since it indicates the efficiency of the fermentation process by *K. marxianus* CCEBI 2011, which in just 24 h almost depletes the carbon source present in the culture medium, these results coincide with those observed by Rodríguez et al. (2008), where a consumption of almost 84% of the sugars present in the medium was observed. It is reported that this yeast is thermotolerant (Hack and Marchant, 1998), so temperatures higher than 35°C favor the growth. This significant decrease in sugars in the medium corresponds with the production of alcohol, which is only observed in the pulps inoculated with the yeast. For an alcohol yield of 40%, usual in this yeast the production of alcohol coincides with the decrease observed in neutral and reducing sugars in the inoculated samples. These results coincide with those observed by Hadiyanto et al. (2014) where the highest concentrations of biomass and ethanol were reached at 30°C, with 0.186/h, Yp/s of 0.21 (g/g), and Yx/s of 0.32 (g/g).

Changes in the composition of total uronic acids and endopolygalacturonase activity

One of the most significant biotechnological products that can be obtained with the yeasts *K. marxianus* is the endopolygalacturonase enzyme (Fonseca et al., 2008). For the quantification of enzymatic activity in the different samples, semipurification of the enzyme is necessary by precipitation with ethanol or acetone in order to reduce the background of reducing sugars present in the culture medium, which interfere in the test. Pectin hydrolysates have a growth-stimulating effect on plants; so the pectins present in the coffee pulp and mucilage were hydrolyzed in the liquid fraction as oligonogalacturonides, then this liquid residual could be used as a stimulator in the growth of the plants. Another application, reported by numerous authors, is related to the value as prebiotics of these oligomers in animal feed. The concentration of total

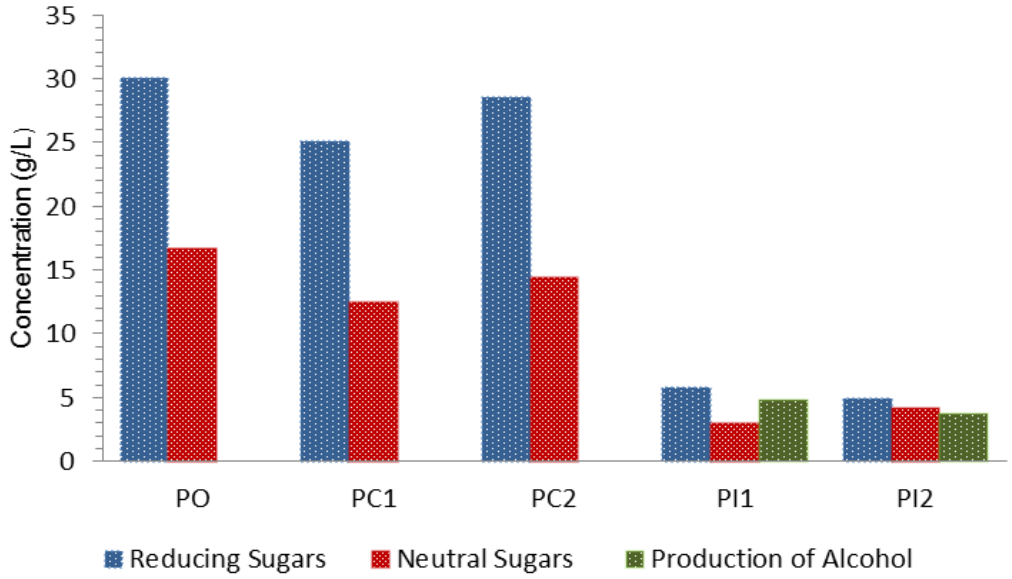

Figure 1. Concentration of reducing sugars, neutral sugars and alcohol in the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

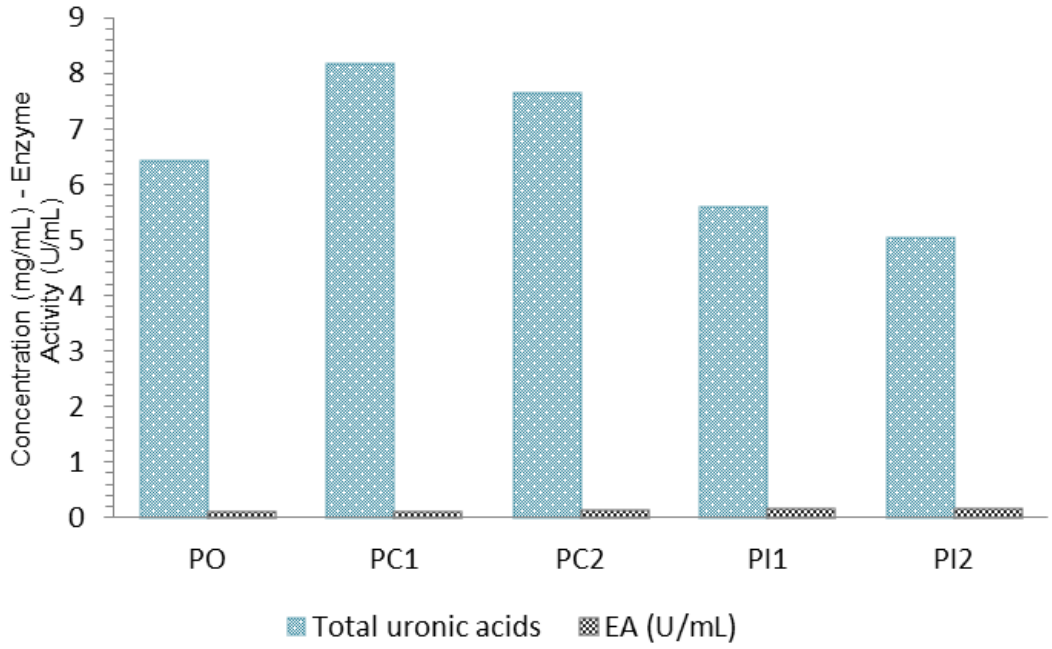

Figure 2. Concentration of pectic substances (such as total uronic acids) and enzymatic activity in samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

uronic acids in the samples maintained before and afterthe fermentationis is as shown in Figure 2. Fragments of pectin oligomers with an average size between 5.4 and 5.8 units of anhydrogalacturonic acid can be observed in the inoculated samples. These oligomers are pectins with biological activity (Li et al., 2016) and have a concentration in order of 5 g/L, which indicates that this preparation can be used directly for agriculture purpose and animal feed.

Enzyme activity is low as compared to what was reported for this yeast. González (2005) obtained an accumulation of PG of 15.4 ± 1.6 U/mL. It may be due to

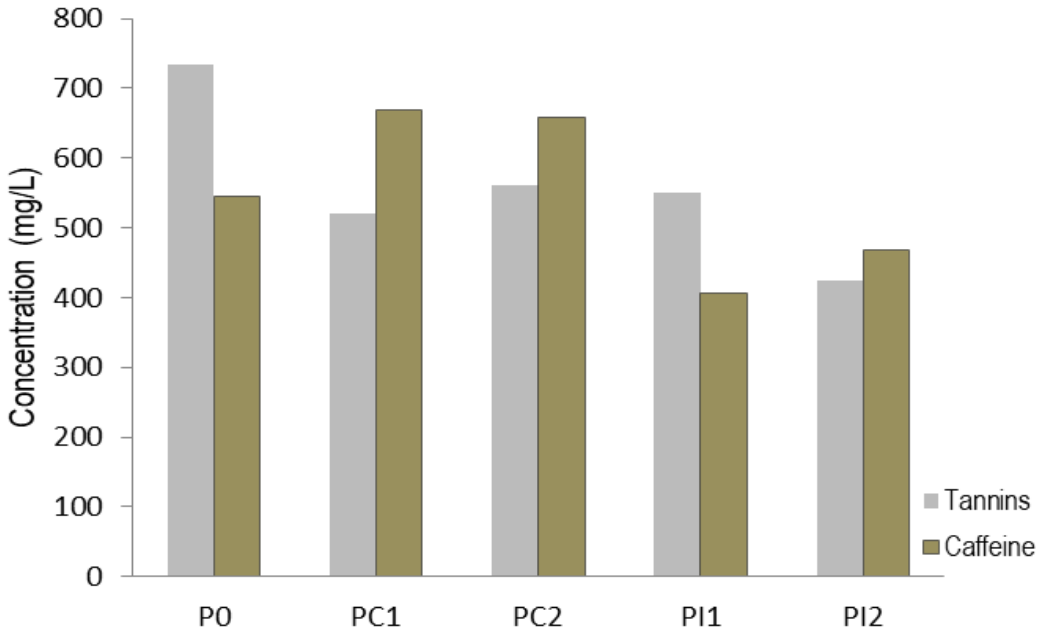

Figure 3. Concentration of total tannins and caffeine in the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

the conservation and manipulation of the samples, with several freeze-thaw cycles, which influenced the activity.

Changes in the concentration of caffeine, tannins and total proteins

The increase in the concentration of caffeine in the control samples in relation with the initial sample can be observed as shown in Figure 3. This may be due to the action of microorganisms of the natural microflora of the residual. At the same time, a significant decrease of more than 30% can be observed in the concentration of caffeine in the inoculated samples, which is favorable for animal feed and for its use as prebiotics, because the caffeine present in the pulp is anti-physiological, so the use in animal feed is limited. This result suggests that K. marxianus CCEBI 2011 probably degrades or uses caffeine as a source of nitrogen, which would be a novel fact in the physiology of yeasts.

In the tannins, no significant variation was observed between the inoculated and non-inoculated samples. These tannins present in fermented samples in important quantities, in order of half a gram per liter, can be considered attractive and they could be a source for the preparation of antioxidants. The yeast influences significantly the reduction of proteins in samples (Figure 4). This may be due to the fact that they may have been used for their consumption as a source of carbon, nitrogen or aminoacids. These results coincide with that observed by González (2005).

Conclusions

During the fermentation of the residuals of the wet coffee benefit generated with low water consumption technology by the pectinolitic yeast *K. marxianus* CCEBI 2011, a significant reduction in the volume of liquid in the residual is produced while increasing the mass of the fermented pulp; both changes are related to a reduction in polluting potential of these residues and their better management. *K. marxianus* CCEBI 2011 effectively ferments in 24 h the reducing and neutral sugars present in the residuals of the wet coffee benefit under microaerophilic conditions with an alcohol yield close to 40%. During the fermentation of the coffee residuals, this yeast accumulates in the liquid fraction, pectin oligomers with an average size between 5.4 and 5.8 units of anhydrogalacturonic acid biologically active at concentrations in the order of 5 mg/mL and the fermentation of the residuals of the wet benefit of coffee with *K. marxianus* CCEBI 2011 leads to a decrease of more than 30% in the concentration of caffeine, which is very favorable from the perspective of the use of residuals in animal feed and in its use as prebiotics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

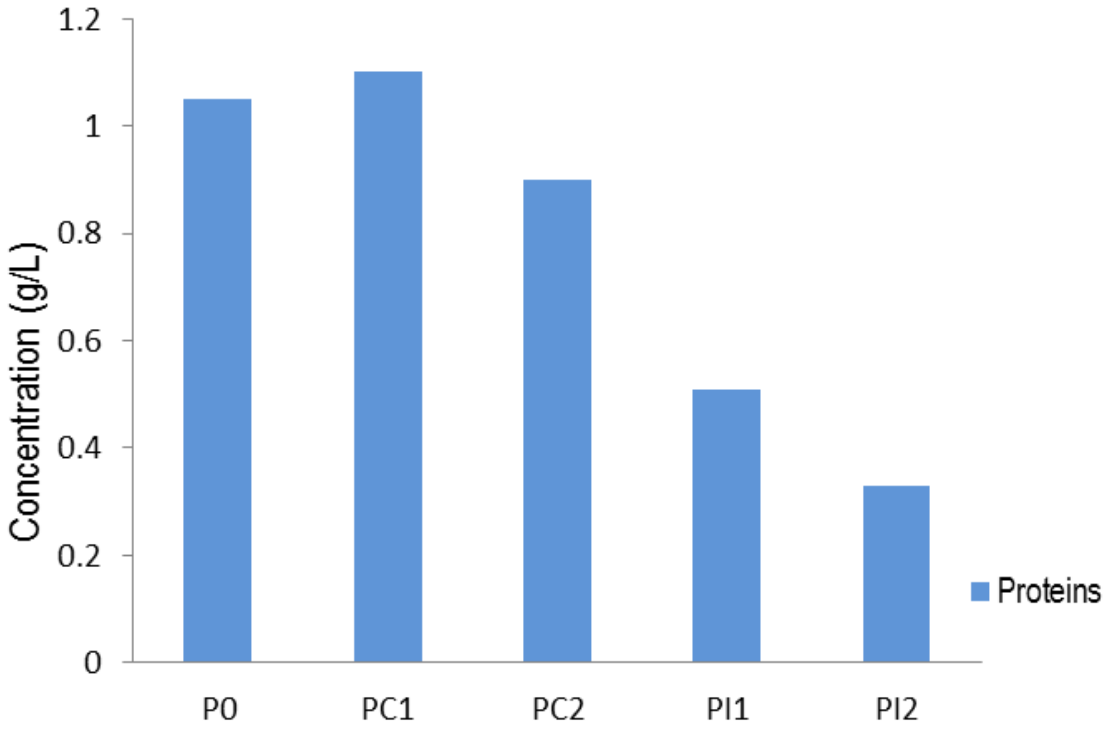

Figure 4. Concentration of total proteins in the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

ACKNOWLEDGEMENTS

Financial support from Ministry of Science and Technology, High Education and Technical Vocational Training (MCTESTP-Maputo, Mozambique) through HEST Project-World Bank is gratefully acknowledged.

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