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Table of Content: Volume 17 Number 30 25 July, 2018

ARTICLES

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Full Length Research Paper

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

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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

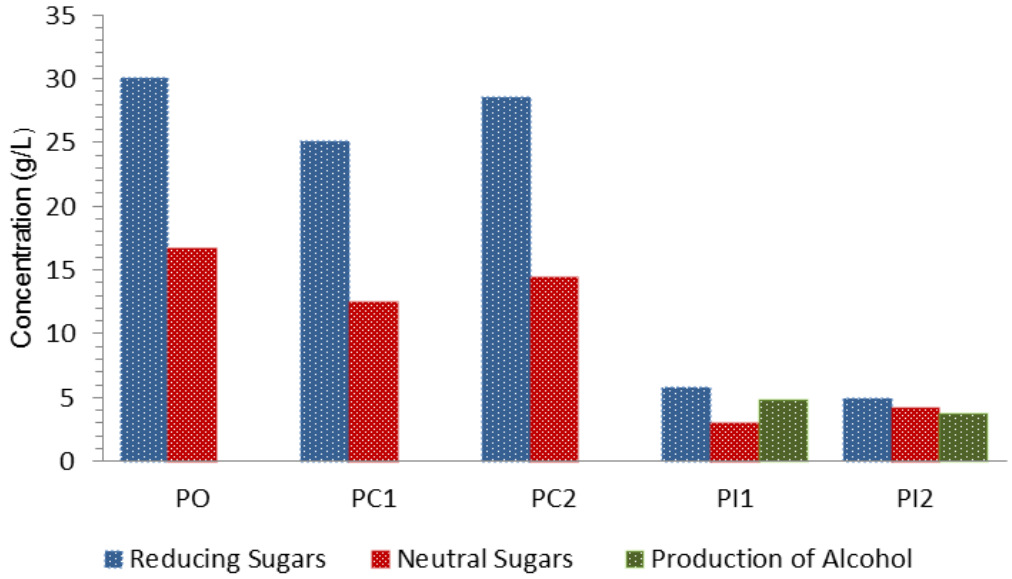

Figure 1. Concentration of reducing sugars, neutral sugars and alcohol in the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

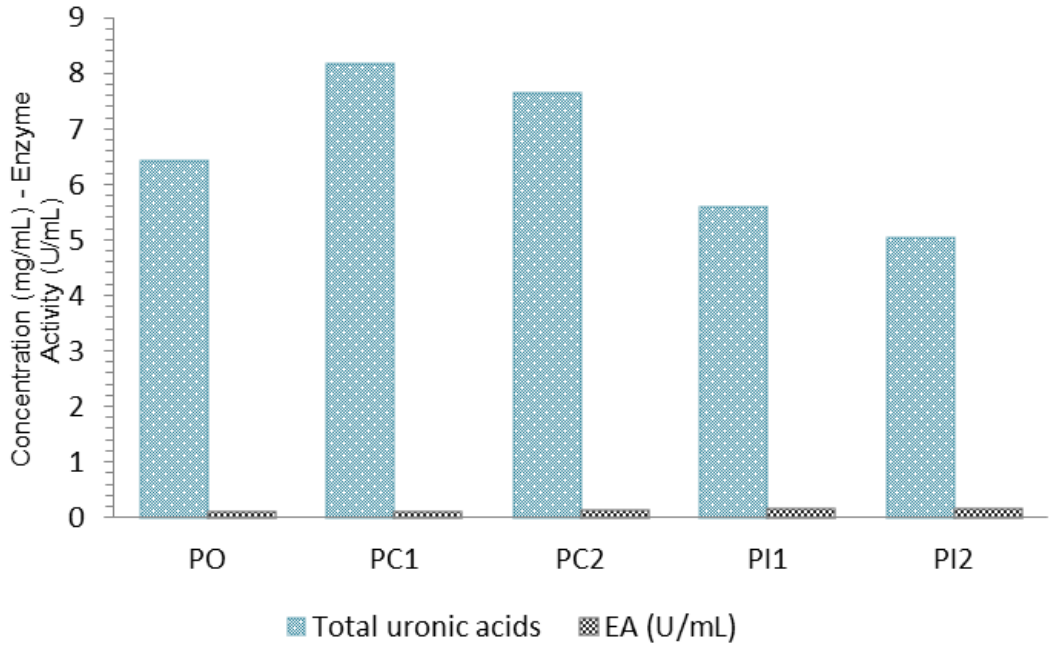

Figure 2. Concentration of pectic substances (such as total uronic acids) and enzymatic activity in samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

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

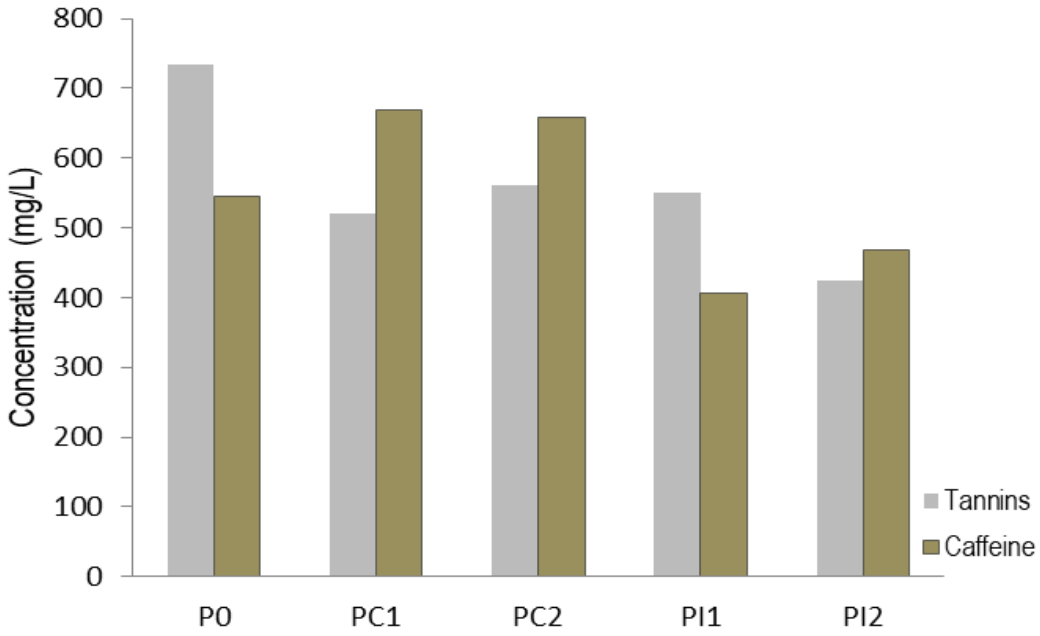

Figure 3. Concentration of total tannins and caffeine in the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

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.

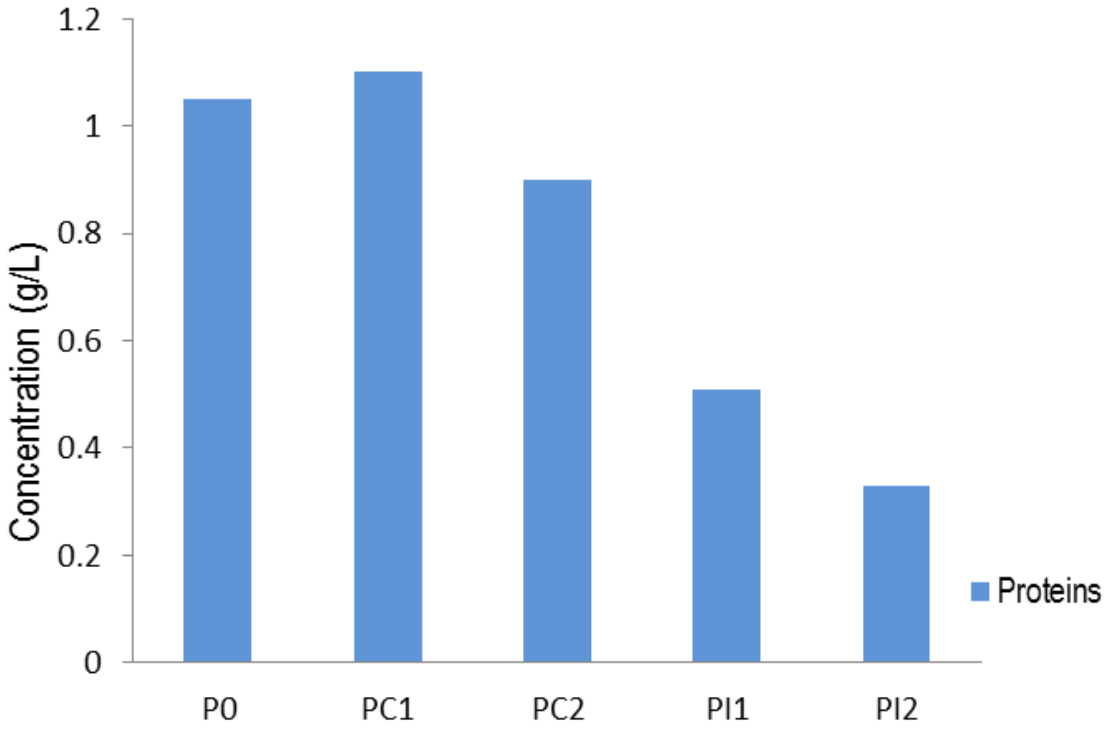

Figure 4. Concentration of total proteins in the samples: pulp time zero (PO), pulp control 1 (PC1), pulp control 2 (PC2), pulp inoculated 1 (PI1) and pulp inoculated 2 (PI2) after 24 h of fermentation.

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Full Length Research Paper

Evaluation of *Curcuma zerumbet* (Zingibaraceae) rhizome extracts sub-acute toxicity on Wistar rats

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Plant extracts have been lately used by the population to treat various types of diseases, and this has been notably encouraged by the World Health Organization (WHO). *Curcuma zerumbet* (Zingiberaceae) belonging to the family of the Zingiberaceae, is herbaceous, perennial and, utilized by the population to treat gastric disorders. However, data on the subacute toxicity of this species are scarce in the literature. Therefore, the present work aimed to ascertain the subacute toxicity of different doses of the aqueous and hydroalcoholic extracts from *C. zerumbet.* These extracts were orally administered through gavage in Wistar rats for 28 consecutive days. This study followed the instructions put forth by Guideline 407 (subacute toxicity) of Organization for Economic Cooperation and Development (OECD). In specific pharmacological tests for acute and subacute toxicity in rats, it can be stated that *C. zerumbet* extracts doses greater than 5 g/kg neither caused mortality nor presented oral toxicity. Therefore, the extracts toxicity parameters analyzed in different doses on the groups of animals have shown no significant difference from those found in the control group. This allows one to conclude *C. zerumbet* (Zingiberaceae) rhizome aqueous and alcoholic extracts in high doses to harbor very low toxicity within a short time.

Key words: Curcuma, subacute toxicity, extracts.

INTRODUCTION

The knowledge regarding the medicinal properties of plants has been passed and enhanced from generation to generation in rural and urban communities. The diversity of medicinal plant species has fostered the use of the ones harboring phytotherapy properties due to the belief that all that is "natural" causes no ill effects (Oliveira et al., 2011). However, the use of plant extracts is not totally free of risks, since in addition to harbouring bioactive compounds, the same plant may contain toxic substances in different amounts and concentrations.

The species Curcuma zerumbet (Zingiberaceae) is a perennial herb belonging to the family Zingiberaceae,

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spontaneously occurring in Southeast Asia and Northeast India (DAS et al. 2013). In the state of Amazonas, this plant is found in some municipalities, being utilized in ornamentation or in the form of teas for treating gastric disorders (Castro et al., 2017).

In Brazil, most genera are used as ornamentation, food and pharmaceuticals. They stand out in the treatment of cervical cancer (Epstein et al., 2010), flatulencies (Alsarhan et al., 2012), gastritis (Neamsuvan, 2012), hepatic disorders (Singh et al., 2012), cough (Abe and Ohtani, 2013), rashes skin infections (Rahmatullah et al., 2011) hepatitis and Inflammation (Schaffer et al., 2011). C. zerumbet subacute toxicity phamacological studies have shown very few scientific evidences. Thus, the toxicological study, regardless of pharmacological results, is essential on account of the existing ethnopharmacological and toxicological data. This is why the present work was undertaken with the objective to assess the subacute toxicity of C. zerumbet extracts, being orally administered to rats through gavage.

MATERIALS AND METHODS

Collection and herbization of *C. zerumbet* Roscoe (Zingiberaceae)

The *C. zerumbet* species samples were collected in the community of Tarumã-mirim, Ramal do Pau Rosa, Latitude 2°43'17"S, Longitude 60°08'19"W, located in the state of Amazonas/Brazil. The exsiccate was sent to the Botany unit of the National Research Institute of Amazonia (INPA) to be identified and deposited in its Herbarium under No. 265800. The rhizomes were hygienized, washed in running water and dried in an air circulating oven at 45°C for 24 h.

How extracts were obtained

The aqueous extract was obtained by the infusion method using 50 g of Curcuma diluted in 1000 mL of distilled water, while the hydroalcoholic extract was obtained by the maceration method using 200 g of Curcuma in 2000 ml ethanol: water (1: 1) for one period of 72 h. After extractions, the extracts were dehydrated through lyophilization using the LS 3000 (TERRONI®) lyophilized apparatus for 48 h (Castro et al., 2017).

Extracts sub-acute toxicity analysis

The determination of the pharmacological activity was done by a test model established by the World Health Organization (WHO) and in accordance with the technical Standards established and approved by the Commission for Ethics in Research on the Use of Animals (CEUA / INPA) under protocol number 003/2013; the animals used in the experiments were albino rats weighing 200 - 400 g, from the Central Bioterium of INPA.

The aqueous and hydroalcoholic extracts subacute toxicity was analyzed using male albino Wistar rats, from the Inpa's Central Bioterium. During the experimental period (28 days), the animals were kept in polypropylene boxes, under controlled photoperiod (12 h light / 12 h dark) at 23 ± 2°C. These animals were fed ration and water with extracts (aqueous or hydroalcoholic) *ad libitum* orally, through gavage (OECD, 2008b). During this period, we analyzed the ration and water consumption variation as well as the body mass of the animals (OECD, 2008a). The animals were randomly divided into seven groups, with each group containing six rats, according to the following protocol: (i) Group 1: control; (ii) Group 2: fed with 100 mg/Kg of aqueous extract; (iii) Group 3: fed with 1000 mg/Kg of aqueous extract; (iv) Group 4: fed with 5000 mg/Kg of aqueous extract; (v) Group 5: fed with 100 mg/Kg of hydroalcoholic extract; (vi) Group 6: fed with 1000 mg/Kg of hydroalcoholic extract; (vii) Group 7: fed with 5000 mg/Kg of hydroalcoholic extract;
After 28 days, the animals were submitted to 12 h of fasting, and blood collection by intracardiac puncture was performed after anesthesia with intraperitoneal administration of ketamine / xylazine (10/10 mg/kg). The blood was stored in collection tubes with 10% sodium EDTA and the following parameters were analyzed:

(i) Hematological: total leukocyte, neutrophils, lymphocytes, eosinophilia, monocytes, red blood cells, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cells distribution slope according to the erythrocyte volume (RDW-SD), mean red blood cell volume coefficient of variance (RDW-CV) and mean platelet volume (MPV) platelet count.
(ii) Biochemical: aspartate aminotransferase (AST or TGO), alanine minotransferase (ALT or TGP), albumin, glucose, urea, creatinine, total cholesterol, triglycerides, uric acid.
At the end of the experimental period, all animals were sacrificed by decapitation and the following organs were removed, weighed, measured and observed macroscopically: liver, small intestine, kidneys, spleen, heart, and lung.

Statistical analysis

Data were represented as means ± standard deviation of the means of five animals per group of experiment *in vivo*. The differences between the means were determined by one way analysis of variance (ANOVA), followed by the Tukey test. Analyses were performed using the Windows program, Graph Pad Prism version 6.0 (Graph Pad Software). The value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Extracts sub-acute toxicity analysis

Subacute toxicity assessment is a widely used methodology to ascertain and classify substances as to their ability to cause acute damage to living organisms in high doses, especially pathological and pathological injuries, and may contribute to establish safety parameters - together with other toxicity data – pertaining to human health (Zatta et al., 2009). As regards plants, this method is useful for identifying the toxicity it may present and thereby prevent people from believing natural products to be devoid of toxic or adverse effects (Silveira et al., 2008; Cunha et al., 2009; Farsi et al., 2013). The present work, through experimental trials with rats being orally administered with aqueous and alcoholic extracts at doses of 100 to 5 g/kg, has demonstrated the lack of pharmacological parameters or toxicological effects prone to lead them to mortality. This indicates that extracts from *C. zerumbet* harbor very low toxicity. In Table 1, the results of the biochemical parameters

Table 1. Biochemical parameters of Wistar rats after oral application of Curcuma zerumbet rhizome aqueous and hydroalcoholic extract during 28 days of treatment.

Parameter	Groups						
	1	2	3	4	5	6	7
Glucose (mg/dL)	118±2.5	117±1.5	120±1.5	121±1.4	115±2.8	119±2.4	122±1.6
Cholesterol (mg/dL)	88 ±2.7	86±1.8	88±2.4	90±2.5	83±1.9	92±3.6	93±2.5
Triglycerides (mg/dL)	79±2.3	76±1.4	79±1.3	82.9±2.7	75±1.5	78±1.2	86±1.3*
Uremia (mg/dL)	37±4.4	45±2.4	40±2.0	46±1.5*	39±2.3	43±1.7	46±1.8*
Albumina (g/dL)	3.8±1.0	3.1±1.7	3.2±2.8	3.3±1.9	3.3±1.7	3.2±1.8	3.2±1.5
Uric Acid Úrico (mg/dL)	1.2±1.8	1.5±2.7	0.9±1.5	1.3±1.6	2.3±1.3	1.2±1.8	1.1±2.8
Creatinine (mg/dL)	0.78±1.8	0.67±1.5	0.75±1.2	0.69±1.8	0.55±1.0	0.76±1.8	0.79±1.9
TGP/ALT (U/L)	49±4.3	44±3.6	51±2.0	56±1.5	48±2.6	51±2.6	57±2.9
TGO/AST (U/L)	134±5.0	133±2.5	138±1.3	140±3.6	126±1.8	139±2.7	142±1.4

1, control; 2, dose of 100 mg/kg of the aqueous extract of the rhizome of *C. zerumbet*); 3, dose of 1000 mg / kg of the aqueous extract of the rhizome of *C. zerumbet*; 4, 5000 mg/ kg dose of *C. zerumbet* rhizome aqueous extract; 5, dose of 100 mg/kg of the hydroalcoholic extract of the rhizome of *C. zerumbet*;; 6, dose of 1000 mg/Kg of the hydroalcoholic extract of the rhizome of *C. zerumbet*;; 7, 5000 mg/kg dose of the hydroalcoholic extract of the rhizome of *C. zerumbet*; values expressed as mean ± S.D.1.

after the application of the extracts for a period of 28 days of experiment. Table 1 shows that the study groups total cholesterol did not differ significantly but triacylglycerol differ significantly when hydroalcoholic extract was administered orally at the dose of mg/kg whereas no change was observed when the rats were fed with the aqueous extract.

This finding may be both related to hydroalcoholic extract concentration and the animals feeding, since the latter was not controlled.

Urea and/or creatinine are eliminated by the kidneys and; in chronic kidney disease there is reduced functioning of the kidneys, with both substances being accumulated in the blood (Dusse and Freitas, 2015). Many factors can significantly change the plasma urea values without being related to the renal function especially protein-rich diet, such as the one found in rodents ration. Elevated urea values in conjunction with creatinine indicate processes that lead to decreased renal blood flow or to gastrointestinal bleeding with obstructive post-renal processes such as tumors and stenosis of the urinary tract (Sodré et al., 2007). Since there were no significant increases on urea levels, uremia is possibly related to total protein content and not to renal disease. Proteins are degraded to urea and as still observed in Table 1. The latter had their levels significantly increased for groups (4) and (7) as compared to control (1).

Renal (pre-renal, renal and post-renal) and extra-renal (protein intake) factors may interfere with increased serum urea concentration. Therefore, this increase in urea levels in the animals that consumed higher doses of the extracts, both aqueous and hydroalcoholic, may be suggested by extra renal factors such as the ingestion of dietary protein by animals, since urea is the main end product of protein catabolism or toxicity index for the high dose groups of aqueous and hydroalcoholic extracts.

Plasma albumin is a good indicator of nutritional status and, in this case, the values obtained in this research are within the normality standards for the species under study. According to the literature, healthy rats bear albumin content in the range of 3.4 to 4.3 g dL^{-1} (Gautier et al., 2014). There was no effect on AST and ALT levels, which are considered sensitive indicators of hepatocellular damage and when within limits may provide a quantitative assessment of the degree of liver damage (Al-habori et al., 2002). Therefore, it is possible to deduce, that the aqueous and hydroalcoholic extract caused no damage to the kidneys or liver, at doses below 5000 mg / kg. The hematological parameters assessment can be used to determine the extent of deleterious effects brought about by strange compounds, including plant extracts, on an animal's blood constituents (Ashafa et al., 2012).

Low hemoglobin, erythrocytes and hematocrit concentrations may indicate anemia, recent bleeding or fluid retention, causing hemodilution. Yet, a decreased platelet count (thrombocytopenia) can result from a series of pathological situations, such as the increased destruction of these cells, due to the use of certain drugs, immune disorders, disseminated vascular coagulation and even mechanical lesions (Dougan et al., 2008). The leukocyte differential is used to assess the distribution and morphology of white blood cells, providing more specific information about the immune system than the leukocyte count alone does (Prinyakupt and Pluempitiwiriyawej, 2015).

Hematological parameters were measured in this study after 28 days of administration of the extracts. The treated animals showed no significant decreases in red blood cell, hemoglobin, hematocrit and platelets levels(Table 2) and the differential count of white cells and lymphocytes, as compared to control's.

Table 2. Wistar rats hematological parameters after oral application of the aqueous and hydroalcoholic extract during 28 days of experiment.

Parameter	Groups						
	1	2	3	4	5	6	7
Leu. (10^3/µL)	8.8±2.5	8.2±1.9	7.0±2.7	5.3±2.9	9.4±1.4	9.4±1.5	9.5±1.5
Hem. (10^6/µL)	6.3±1.6	5.8±2.6	5.9±1.3	5.6±1.5	6.4±1.4	6.1±1.6	5.9±1.3
Hemo. (g/dL)	13.9±1.5	13.3±2.2	13.9±2.0	12.7±1.3	14.6±2.0	14.3±2.0	13.6±1.6
Hema. (%)	37.7±1.5	37.1±1.9	38±2.0	35.9±2.1	40.6±1.7	39.5±2.4	38.4±1.4
M.C.V. (*f*)	59±2.5	63.8±1.7	63±1.3	63.2±1.6	63.4±2.3	62.5±1.9	63.7±1.6
M.H.C. (pg)	21.7±1.9	22.8±2.3	23.3±2.4	22.3±1.7	22.8±1.4	23.3±1.6	22.9±2.0
M.C.H.C. (g/dL)	36.8±1.6	35.8±1.9	36.5±1.4	35.3±1.6	35.9±1.7	36.2±1.8	35.4±1.4
R.D.W. (%)	12.1±2.0	13±1.3	12.9±1.6	13.6±2.0	12.7±1.4	12.6±1.5	13.9±1.6
Plat. (10^3/µL)	899±1.5	711±1.5	722±1.5	773±1.4	723±1.5	669±1.5	906±1.5
Neu. (%)	11.4±2.3	12±2.6	12.1±1.7	14±1.4	13±0.3	12±0.7	11.9±1.4
Lymph. (%)	81.2±1.7	77.0±2.2	80.7±1.9	77±1.4	77±0.8	76±1.5	82.2±2.7
Mon.(%)	6.5±1.3	3±1.1	7.0±1.4	5±2.3	5±1.6	5±1.7	3.3±1.5
Eus. (%)	0.6±0.6	1±0.5	0.0±0.2	0.1±1.5	0.0±1.0	0.1±2.0	0.4±2.4
Bas.	0.3±04	0.0±1.0	0.2±0.1	0.0±1.2	0.0±0.2	0.0±1.7	0.2±1.7

1, control; 2, Dose of 100 mg/kg of the aqueous extract of the rhizome of *C. zerumbet*); 3, dose of 1000 mg / kg of the aqueous extract of the rhizome of *C. zerumbet*; 4, 5000 mg/ kg dose of *C. zerumbet* rhizome aqueous extract; 5, dose of 100 mg/kg of the hydroalcoholic extract of the rhizome of *C. zerumbet*;; 6, dose of 1000 mg/Kg of the hydroalcoholic extract of the rhizome of *C. zerumbet*;; 7, 5000 mg/kg dose of the hydroalcoholic extract of the rhizome of *C. zerumbet*; values expressed as mean ± S.D.1.

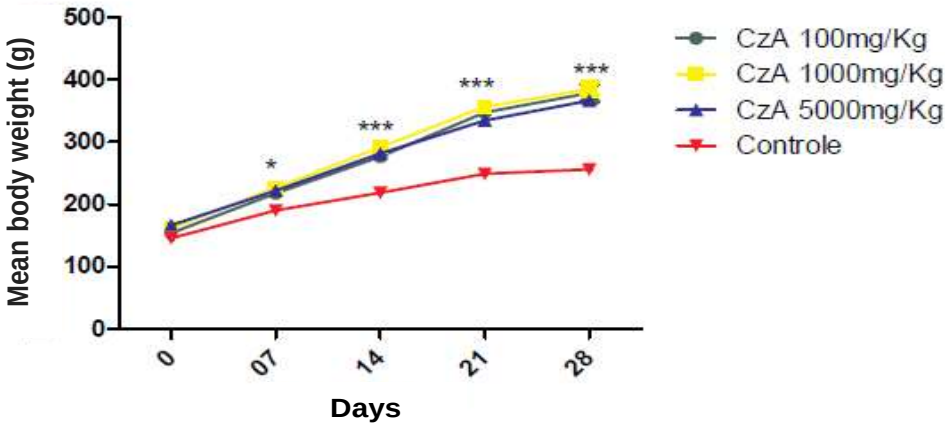

Figure 1. Animals body mass assessment for 28 days of oral treatment with aqueous extract from *Curcuma zerumbet.*

In the study by Hossen et al. (2017), the MCV and HCM concentration levels increased when animals were fed for a period of 28 days with species Curcuma Longa. While the other hematological data remained unchanged. In this study, this was not observed. In the study by Salama et al. (2013) this species biochemical and hematological presented no significant difference between the control groups. Thus, corroborating the findings put forth by the present work.

For daily habits (feed consumption and fecal production), there was no significant variation between the groups treated with the aqueous and hydroalcoholic extract and the control group. However, there was change in the body mass parameter, when compared to that shown by the control. The mean body mass presented by the animals treated with the aqueous extract at doses of 100, 1000 and 5000 mg/Kg increased from 155 ± 5.8 to 363 ± 7.8; 166 ± 8.4 to 370 ± 7.5 and 166 ± 6.9 to 350 ± 8.7 respectively; while that presented by the control increased from 145 ± 5.7 to 241 ± 6.5 showing a statistical difference from the seventh day of application onwards (Figure 1) while the mean body mass of the animals observed in the hydroalcoholic extract experiment increased from 147 ± 8.9 to 366 ± 7.8

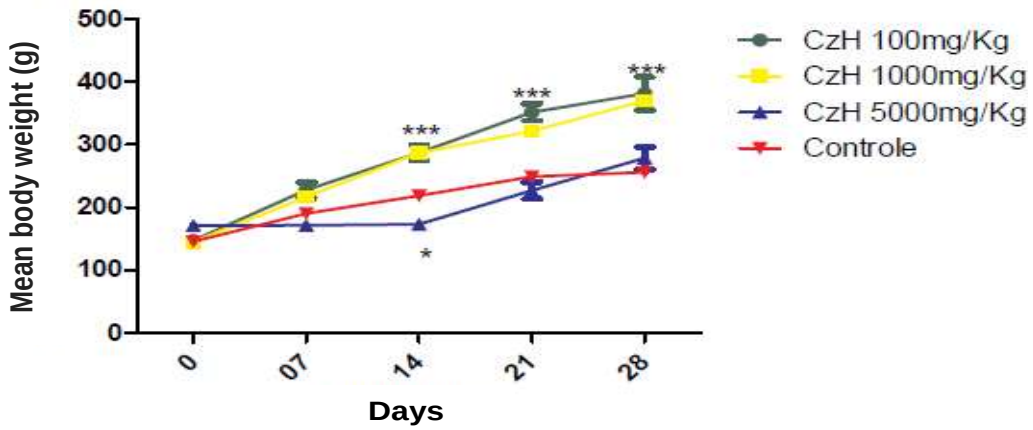

Figure 2. Animals body mass assessment for 28 days of oral treatment with the hydroalcoholic extract from *C. zerumbet.*

at the dose of 100 mg / kg; 144 ± 8.8 to 345 ± 9.8 at the dose of 1000 mg / kg and 171 ± 7.0 to 252 ± 7.8 at the dose of 5000 mg / kg. These findings may be related to the extracts-borne protein and carbohydrate levels. Figure 2, shows the animals to present a significant difference in the body mass from the 14th day of the experiment onwards, as compared to that of the control at doses of 100 and 1000 mg/kg. The animals evaluated at the dose of 5000 mg/kg exhibited a constant body mass in the first weeks of the experiment. They exhibited the same gain of body mass as that of control animals, from the 14th day onwards. It is known that systemic toxicity can be identified by the decrease in the body mass of the animals and by changes in water and feed intake, which have shown to be paramount when undertaking a substance or extract's toxicity assessment, since it provides data pertaining to the animals' health status as a whole (Valadares, 2006). Nevertheless, these animals showed no changes on their food and water intake. The macroscopically analyzed and weighed organs exhibited no statistical differences between the control groups and the experimental doses. Thus, the tested *C. zerumbet* extracts showed no evidence of harboring any toxicity.

Conclusion

It can be concluded that the aqueous and hydroalcoholic extract from the rhizome of *C. zerumbet* (Zingiberaceae) harbors low, short term toxicity in high doses. In cases of prolonged use, further studies are required to ascertain the outcome of such histopathologic changes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biosurfactant production by *Bacillus subtilis* UFPEDA 86 using papaya (*Carica papaya* L.) waste as substrate: Viability studies and pH influence of the culture medium

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Biosurfactants are surface-active compounds derived from microorganisms and offer several advantages over chemical surfactants, such as low toxicity, good biodegradability and ecological acceptability. Even though interest in biosurfactants is increasing, these bioproducts do not compete economically with synthetic surfactants due to the overall costs of the bioprocess. The use of inexpensive raw materials is an important approach to reduce these costs since the substrate price account for 10 to 30% of the final product expenses. In this study, papaya (*Carica papaya* L.) waste was used as a substrate to produce biosurfactant by *Bacillus subtilis* UFPEDA 86 strain. In addition, culture medium pH was corrected from 5.25 to 6.8 in order to analyze the influence of this variable on the biosurfactant production. The submerged fermentation was carried out on a shaker incubator at 37°C, 200 rpm for 96 h. Biomass and substrate concentration, surface tension, emulsification index and critical micelle concentration were analyzed. The strain was well adapted to both substrates studied, without and with pH correction. Using the broth without pH correction (pH=5.25), a maximum cell concentration of 1.07 g L^{-1} at 36 h a 25.5% surface tension reduction, emulsification index of around 61% and critical micelle concentration of about 35 mg L^{-1} was obtained. Using the broth with pH correction to 6.8, better results; maximum cell concentration of 1.14 g L^{-1} at 24 h, a 32.5% surface tension reduction, emulsification index of around 66% and critical micelle concentration at about 35 mg L^{-1} was obtained. The papaya waste proved to be an effective substrate in the biosurfactant production by *B. subtilis* UFPEDA 86 and the pH variable proved to be of great importance in the yield of this process.

Key words: Biosurfactant, submerged fermentation, papaya waste, *Bacillus subtilis*.

INTRODUCTION

Surfactants are amphipathic molecules, consisting of hydrophobic and hydrophilic portions, which act preferentially at the interface between fluid phases with different polarities, promoting the reduction of surface and interfacial tensions between immiscible compounds. Its amphiphilic capacity increases solubility and mobility of hydrophobic and organic compounds insoluble in aqueous solutions (Ghojavand et al., 2008; Singh et al.,

2007; Ying, 2006). Thus, surfactants are applied in a wide variety of industrial processes involving emulsification, foam, detergency, wetting, dispersion or solubilization (Nitschke et al., 2004).

Most of the surfactants produced are chemically derived from petroleum. However, increasing environmental concerns have led to the search of natural surfactants as an alternative to the traditional synthetic products (Barros et al., 2007). Natural surfactants synthesized by microorganisms are called biosurfactants and offer several advantages over chemical surfactants such as low toxicity, biodegradability, ecological acceptability, stability to extreme temperature, pH or salinity conditions and the possibility of production from renewable sources (Lobato, 2013; Rocha, 2017). In addition to these characteristics, biosurfactants have a wide variety of potential applications, which make them even more interesting. The main applications of these biological compounds are in the advanced oil recovery and in the bioremediation fields, however, they can still be used as emulsifiers, functional ingredient, microbiological, pharmaceutical and therapeutic agent, and as additives in health and beauty products (Ghojavand et al., 2008).

Biosurfactants have a wide variety of chemical structures, including glycolipids, lipopeptides, phospholipids, fatty acids or neutral lipids, among others (Geys et al., 2014; Gudiña et al., 2013). *Bacillus* species produce a broad spectrum of lipopeptide biosurfactants, which are cyclic molecules consisting of a variable-length fatty acid (hydrophobic fraction) bound to a short peptide chain (hydrophilic fraction) of seven or ten amino acids. Among them is the surfactin, a lipoheptapeptide produced by *Bacillus subtilis* strains and one of the most effective biosurfactants known so far (Singh et al., 2009).

In spite of the increasing interest in biosurfactants, these compounds do not compete economically with synthetic surfactants due to the high process costs associated with inefficient methods of bioproducts recovery and the use of costly substrates, which account for about 10 to 30% of the overall production cost (Lobato, 2013; Tuleva et al., 2009). A method to reduce such costs is the use of alternative substrates with a good balance of carbohydrates and lipids to provide efficient microorganism growth conditions and biosurfactant synthesis. In this sense, by-products of agroindustry activity are great substrate sources for biosurfactant production and their use can diminish the environmental problems caused by their inadequate disposal, as well as the costs associated with effluent treatments (Gallert and Winter, 2002; Makkar and Cameotra, 2002; Mukherjee and Das, 2005). Thus, the use of agroindustrial wastes brings value to these materials and contributes to the sustainability of their economical chain (Rocha, 2017).

Papaya (*Carica papaya* L.) peels, for example, provide a good alternative substrate for fermentation since they have a good nutritional balance and are usually discarded in the fruit processing industries (Gondim et al., 2005; Lima et al., 2008). According to Gondim et al. (2005), the main macronutrient present in the papaya waste is carbohydrate, which makes this residue an excellent energy source and a good substrate for fermentation. In addition, the peels of this fruit have pH around 5.5, which makes them less acidic when compared to the other fruits peels and therefore less aggressive to the microorganism. According to data provided by the quality control of Brasfrut® (Feira de Santana, BA, Brazil), about 65 tons of papaya wastes were eliminated by this company in 2016. Despite the good characteristics of the papaya waste and the sustainability involved in its use, no study is reported in the literature using these residues as a substrate for the biosurfactant production.

In this context, the present work aimed to evaluate the biosurfactant production by *Bacillus subtilis* strain UFPEDA 86 using papaya (*C. papaya* L.) waste from the industrial fruit processing as substrate, and to analyze if the pH modification of the culture medium is effective in the biosurfactant production. Cell growth, substrate consumption, surface tension reduction, emulsification index and critical micelle concentration were evaluated during 96 h of fermentation.

MATERIALS AND METHODS

Microorganism maintenance

B. subtilis UFPEDA 86 used in this study was kindly provided by the Department of Antibiotics of the Federal University of Pernambuco, Brazil. The strain was maintained in Luria-Bertani agar medium, as proposed by Cold Spring Harbor protocols (2010), with modifications. The modified medium was composed of 10.0 g L^{-1} tryptone; 5.0 g L^{-1} of yeast extract; 5.0 g L^{-1} NaCl and 20.0 g L^{-1} agar. The pH of the medium was adjusted to 6.8 using 1 M NaOH or 1 M HCl. The inoculation was performed in a laminar flow chamber and the tubes were incubated at 37°C for 24 h and then stored at 4°C. This procedure was repeated monthly for strain maintenance.

Pre-inoculum and inoculum

The broths used as pre-inoculum and inoculum, proposed by Bugay (2009, modified), had the same composition: 20.0 g L^{-1} glucose; 3.0 g L^{-1} KH_2PO_4; 7.0 g L^{-1} K_2HPO_4; 0.2 g L^{-1} $MgSO_4.7H_2O$; 1.0 g L^{-1} $(NH_4)_2SO_4$ and 1.0 g L^{-1} of yeast extract, pH corrected to 6.8 using 1 M NaOH or 1 M HCl. The pre-inoculum was prepared by

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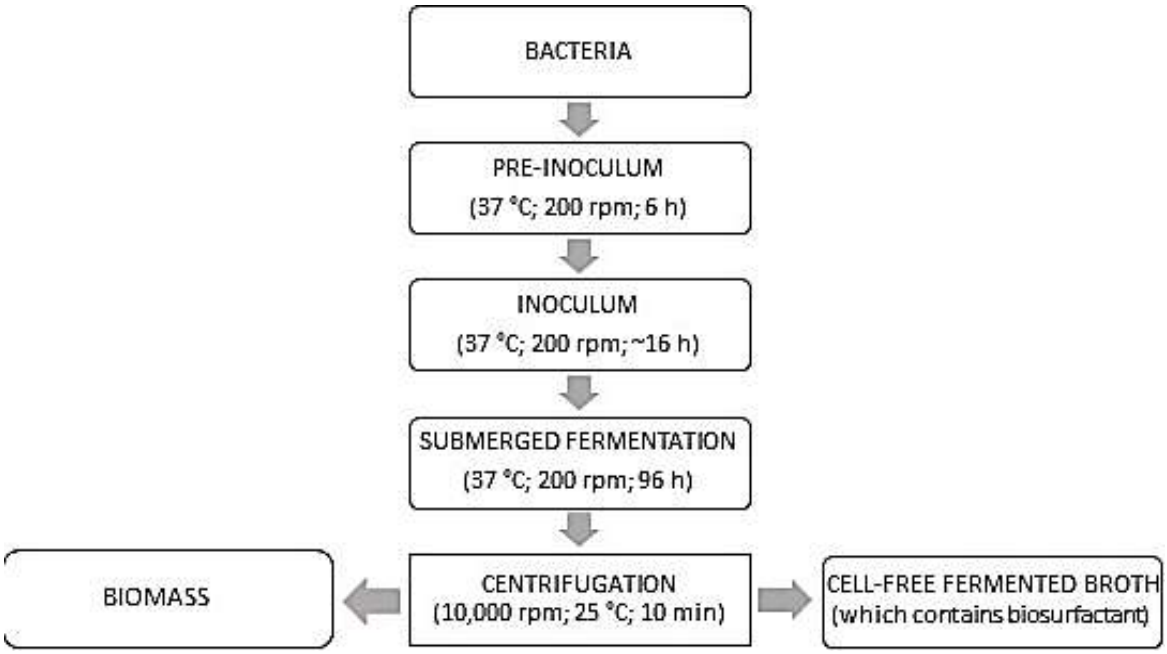

Figure 1. Biosurfactant production steps.

transferring three culture loops to a 125 mL Erlenmeyer flask containing 30 mL of the medium and then brought to an incubator under orbital shaking (Tecnal® TE-424) at 37°C and 200 rpm for 6 h. For the inoculum, 250 mL Erlenmeyer flask containing 50 mL of the medium was used. At this stage, an aliquot corresponding to 10% (v/v) of the inoculum (5 mL) was withdrawn from the pre-inoculum and transferred to the Erlenmeyer flask which was also incubated under orbital shaking at 37°C and 200 rpm for about 16 h.

Substrate preparation

The papaya (*Carica papaya* L.) waste used in this work was obtained from Brasfrut®, a fruit processing industry located in the city of Feira de Santana, Bahia, Brazil. The residues were homogenized with distilled water in a 2 L domestic blender (Philips® 500 W) at the concentration of 250 g L^{-1} (Souza et al., 2012). Then, they were filtered and centrifuged (Hitachi Ltd. CR22G III) at 10,000 rpm at 25°C for 10 min until the removal of all the solid particles and obtaining of the aqueous extract (broth) used as substrate in the fermentation. In order to check the influence of pH on the biosurfactant production, a similar medium with pH corrected to 6.8 was prepared.

Biosurfactant production

The production was carried out by submerged fermentation in an orbital shaker incubator at 37°C and 200 rpm during 96 h. Samples were collected at regular intervals to monitor the biomass concentration, substrate concentration, surface tension variation, emulsification index and critical micelle concentration. The sampling intervals were: 4 in 4 h in the first 12 h and then 12 in 12 h until complete 96 h of culture. The samples were collected and centrifuged at 10,000 rpm at 25°C for 10 min to separate the biomass from the supernatant (cell-free fermented broth), which contained the biosurfactant. Figure 1 shows, in a simplified way, the biosurfactant production steps.

Determination of biomass concentration and cell productivity

The biomass concentration was determined by the dry mass method (Tríboli, 1989). In this method, 50 mL of the samples were transferred to pre-weighed tubes and then centrifuged at 10,000 rpm at 25°C for 10 min. The pellet formed was used to determine the cell concentration and the supernatant was separated for further analysis. The pellet was washed with distilled water and centrifuged 3 times to remove residues from the supernatant. After washing, the samples were placed in a drying oven (Tecnal® TE-392/2) at 65°C for 24 h until constant weight. After this time, the tubes were placed in a desiccator for 5 min and weighed. The biomass concentration (g L^{-1}), [X], was expressed according to Equation 1.

$$[X] = \frac{m_{dry} - m_{empty}}{50} \times 1000 \quad (1)$$

Where, m_{dry} = mass of the tube with dry biomass (g); m_{empty} = mass of the empty tube (g).

The cell productivity (Px; g L^{-1} h^{-1}) was determined by Equation 2 described by Schmidell et al. (2001).

$$P_X = \frac{X_m - X_o}{t_f} \quad (2)$$

Where, X_m = maximum cell concentration (g L^{-1}); X_o = initial cell concentration (g L^{-1}) and t_f = total time of the fermentation (h).

Determination of substrate concentration (sugars concentration)

The sugars quantification was performed by the 3,5-dinitrosalicylic acid (DNS) method, which determines reducing sugars, as

proposed by Miller (1959). The samples were initially hydrolyzed in test tubes by the addition of 1 mL of the supernatant and 0.5 mL of pure HCl. The tubes were taken to a water bath for 10 min at 70°C and then cooled on ice bath for approximately 5 min. After this period, 2 mL of 4 N NaOH was added and the tube content was completed to 10 mL using distilled water.

After hydrolysis, aliquots of 200 µL of the hydrolyzed samples were transferred to test tubes containing 200 µL of DNS reagent. The tubes were then brought to a water bath at 100°C for 10 min followed by ice bath for 5 min. After this procedure, it was added 5 mL of distilled water, followed by agitation in a vortex (Phoenix® AP56) and absorbance reading in a spectrophotometer (Shimadzu Corp. UV-1800) at 540 nm. A sample containing 0.5 mL of distilled water was used as blank in place of 0.5 mL of the hydrolyzed sample. A calibration curve was constructed by correlating the concentration of glucose and fructose with the absorbance reading.

Determination of the surface tension

The surface tension of the cell-free fermented broth was monitored for 0, 4, 8, 12, 24, 36, 48, 60, 72, 84 and 96 h by the Du Noüy ring method using tensiometer (Kruss K20) at temperature of ± 25°C (Kuyukina et al., 2001).

Determination of emulsification index

Cell-free fermented broths were mixed to hydrophobic compounds - soy oil, gasoline and diesel - in test tubes in the ratio of 4:6, respectively, and homogenized in a vortex at full speed for 1 min. The tubes were allowed to stand at room temperature for 24 h and at the end of the time measurements were made of the height of the emulsified layer and total height of the liquids in the tube using a ruler. The emulsification index was obtained by Equation 3 described by Cooper and Goldenberg (1987).

$$EI_{24}\ (\%) = \frac{EL}{TH} \times 100 \quad (3)$$

Where, EI_{24} = emulsification index (%); EL = height of the emulsified layer (cm); TH = total height of the liquids (cm).

Determination of critical micelle concentration (CMC)

Different concentrations of the cell-free fermented broth containing surfactin produced after 24 h of fermentation were obtained by performing several dilutions of this broth in distilled water (Santa Anna et al., 2002). Surface tension of the resulting solutions was measured at 25°C, as described previously. The CMC was determined by plotting the surface tensions (mN m^{-1}) as a function of the concentration (mg L^{-1}) and it was found at the intersection point between the two lines that best fit the pre- and post-CMC data (Gudina et al., 2010).

RESULTS AND DISCUSSION

Biomass concentration, cell productivity, substrate concentration and surface tension

The curves shown in Figure 2, plotted using Origin 8.1 (OriginLab CO., MA, USA), represent the cell growth (biomass), the substrate consumption and the surface tension behavior along 96 h of fermentation using the broth prepared from papaya (*Carica papaya* L.) waste as substrate without pH correction (Figure 2A) and with the pH adjusted to 6.8 (Figure 2B).

When analyzing the biomass curve in Figure 2A, the following stages of microbial growth are defined: Latency phase (lag phase) from 0 to 2 h; exponential phase (or log phase) from 2 to 36 h; stationary phase from 36 to 60 h and decline phase from 60 h.

The small lag phase demonstrates that the *Bacillus subtilis* UFPEDA 86, although usually cultivated in media containing glucose as the main carbon source, was easily adapted to the substrate used in this study, demonstrating the versatility of this microorganism when using more complex substrates.

Observing the biomass curve in Figure 2B, for fermentation with pH adjusted to 6.8, the following stages of microbial growth are defined: Latency phase (lag phase) nonexistent in the time interval analyzed; exponential phase (or log phase) from 0 to 24 h; stationary phase from 24 to 60 h and decline phase from 60 h.

The absence of the lag phase demonstrated that the microorganism was adapted almost instantaneously to the complex medium used in this work. This fast adaptation may be due to the change in the pH of the medium since the microorganism was cultivated at a pH close to neutrality and this one was maintained during fermentation. In this way, this variable showed important influence in terms of process optimization, once the exponential phase, which is associated with the formation of the bioproduct, was more quickly achieved.

The maximum cell concentration obtained using the broth without pH correction was 1.07 g L^{-1} at 36 h and 1.14 g L^{-1} using the broth with pH correction at 24 h, which shows that *Bacillus* was able to develop in both culture media proposed in this study. The biomass productivities, for the broths without pH correction and with 6.8 pH were 0.011 and 0.012 g L^{-1} h^{-1}, respectively. The difference in biomass productivities in the two fermentation configurations studied was very low. However, in the broth fermented at 6.8 pH, this parameter was reached 12 h earlier, compared to the broth with no pH adjustment. In this way, the industrial relevance of pH correction of the culture medium has once again been demonstrated, since there will be time savings in the formation of the bioproduct.

In terms of cell performance, Silva et al. (2015) studied the growth of *Bacillus subtilis* UFPEDA 86 for 48 h using glucose and sodium nitrate as carbon and nitrogen sources and obtained a maximum cellular concentration of 0.4 g L^{-1} and productivity of 0.008 g L^{-1} h^{-1}, values lower than what was obtained for the medium used in this study, which demonstrates that the strain had a good suitability when it used papaya waste as substrate, as well as the good nutritional source that the papaya waste is.

Analyzing the substrate curves (Figure 2), it was

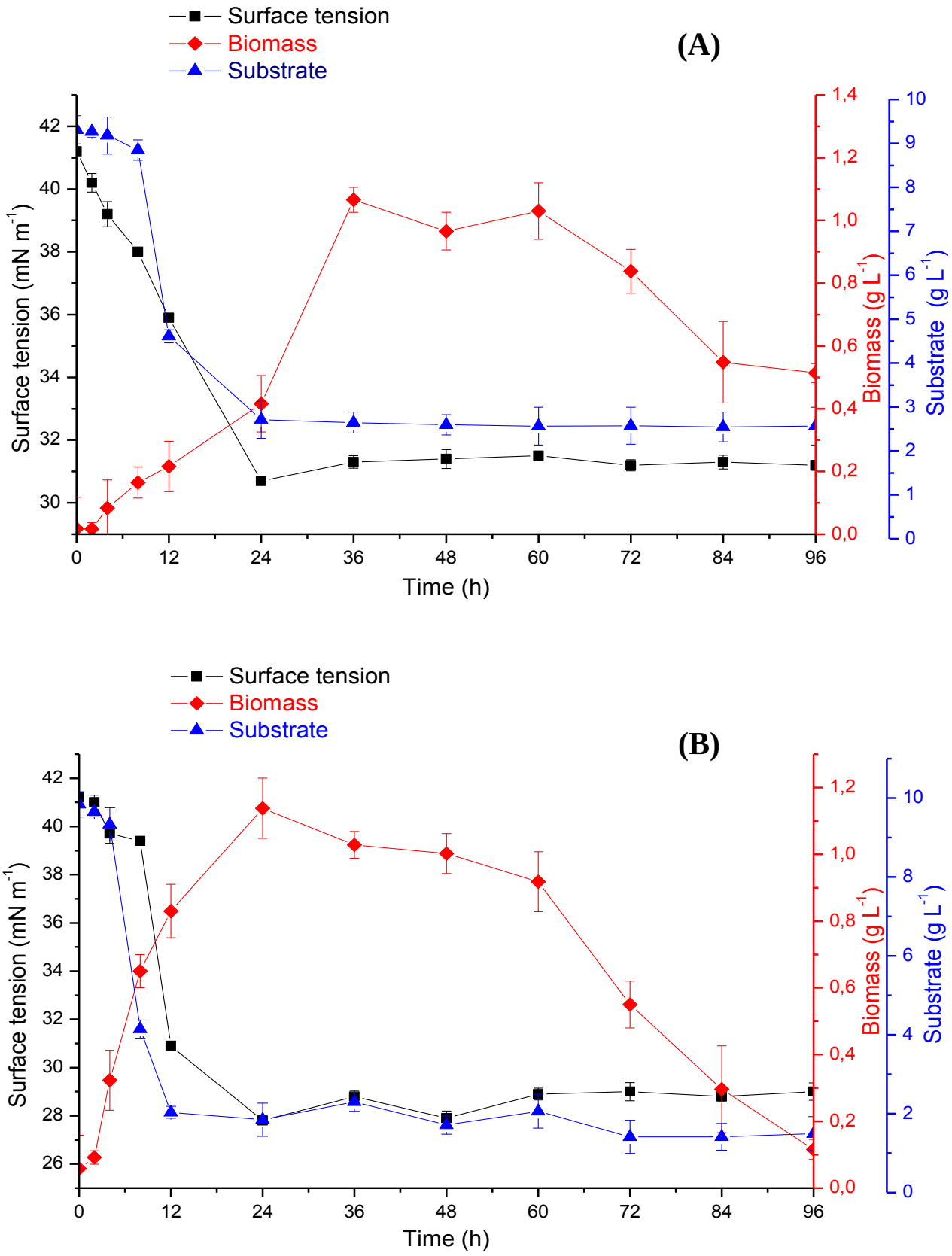

Figure 2. Biomass concentration, substrate consumption and surface tension along 96 h of culture using the aqueous extract prepared from papaya (*Carica papaya* L.) waste as substrate without pH correction (A) and with the pH adjusted to 6.8 (B).

observed that the highest consumption occurred during the exponential phase of microbial growth, as expected, because at this stage, in addition to consuming the substrate for cell maintenance, the microorganism also uses it for development. It was also noted that the consumption was high, with almost substrate exhaustion which may have caused a series of processes that affect the microorganism survival. These processes include: induction of chemotaxis and motility, degradative enzymes synthesis, development of genetic competence, production of peptidic antibiotics and sporulation (Marahiel et al., 1993).

The measurements of surface tension over time is an indirect method to monitor the production of biosurfactants and are therefore of great importance in the study of these bioproducts. As the microorganism grows, it synthesizes the biosurfactant and this metabolite is excreted in the metabolic broth, reducing the surface tension (Lima et al., 2016). Analyzing the behavior of the surface tension for the curve with no pH correction (Figure 2A), it was observed that there was a decrease from 41.2 to 30.7 mN m^{-1}, which means a reduction of approximately 25.5% in 24 h of culture. When the behavior of this variable was observed for the curve with pH correction (Figure 2B), a decrease of 41.2 to 27.8 mN m^{-1} was noted, which means a reduction of about 32.5% in the same 24 h of culture. These values indicate favorable results for the substrate used in the present work since the literature indicates that effective biosurfactants should reduce the surface tension of the medium by at least 20% (Ehrhardt, 2015).

A study by Rocha (2007) using a *B. subtilis* (LAMI007) strain and nutrient broth and diluted cashew juice (varying concentrations of 10 to 50%) as substrates showed no biosurfactant was produced; once biosurfactant production did not occur, there will not be cellular growth and alteration of the surface tension during the 72 h of fermentation, indicating that the proposed medium need supplementation in order to form the bioproduct. In the present study the papaya waste was homogenized at a concentration of 25% (m/v), and cellular growth and reduction of the surface tension occurred, that is, the medium offered sufficient nutrients for the formation of the bioproduct.

Rocha (2007) also carried out the supplementation of the diluted cashew juice with nitrogen sources and verified that there was a reduction in surface tension of about 11.94% after 48 h of cultivation, a lower value and in a longer time than that obtained in the present work.

The study conducted by Ehrhardt (2015) using a strain of *B. subtilis* and pineapple wastes as substrate in the biosurfactant synthesis showed a decrease in surface tension from 64.54 to 48.25 mN m^{-1} after 24 h, which means a reduction of about 25.2%, a value similar to that found in the present study using the papaya waste as substrate without pH correction. However, this value was lower than that obtained when the papaya waste broth with pH corrected to 6.8 was used, which indicates once again the importance of controlling the pH variable to obtain better yields in the fermentation process.

Emulsification index

The emulsification index is also an indirect measurement of the biosurfactant production and it is of great importance since it provides quick results on the formation of biosurfactant and also allows the evaluation of its stability, regarding the emulsion maintenance. Figure 3, plotted using Origin 8.1 (OriginLab CO., MA, USA), shows the emulsification indexes along 96 h of fermentation of papaya residues with no pH modification (Figure 3A) and with 6.8 pH (Figure 3B). The hydrophobic compounds used for emulsification were soy oil, gasoline and diesel.

Analyzing the results of Figure 3, it was observed that both fermented broths were able to emulsify the three tested hydrophobic compounds after 12 h of fermentation, and the highest emulsification was observed for soy oil, whose values were higher than 50% in all the essays. The good emulsification in soy oil suggests a potential use of the produced biosurfactant in the food industry as emulsifying agents, conferring the formation of desired food consistency and texture, as well as phase dispersion (Banat et al., 2000). The emulsification index in soy oil shown in Figure 3A was around 61% and in Figure 3B was around 66% in 12 h of fermentation. In both cases, the emulsification index was bigger than what was found by Silva et al. (2015), who used glucose and sodium nitrate as carbon and nitrogen sources and obtained emulsification index of 34.5% in the fermentation time of 24 h using sunflower oil as a hydrophobic compound. The highest emulsification indexes were obtained in the exponential phase of growth, as expected, because it was in this stage that the most pronounced reduction of the surface tension was observed, indicating a higher biosurfactant production.

The high emulsification index obtained by the soy oil suggests a higher affinity of the biosurfactant produced in the present work by the functional groups found in this oil that is basically formed by fatty acids of 16 and 18 carbons, as reported by Fonseca and Gutierrez (1974). Gasoline and diesel are made up of mixtures of hydrocarbons ranging from 4 to 12 carbons for gasoline and 8 to 16 carbons for diesel, as described by the National Agency for Petroleum, Natural Gas and Biofuels (2016). Figure 3 also shows that the emulsification index for gasoline was superior to the one found for diesel in all tests even though both were formed by a mixture of hydrocarbons. The difference in the emulsification behavior of diesel and gasoline may suggest a better emulsification of short chain hydrocarbons regarding the biosurfactant produced in this paper, as already reported by Barros et al. (2008), but other studies are required for

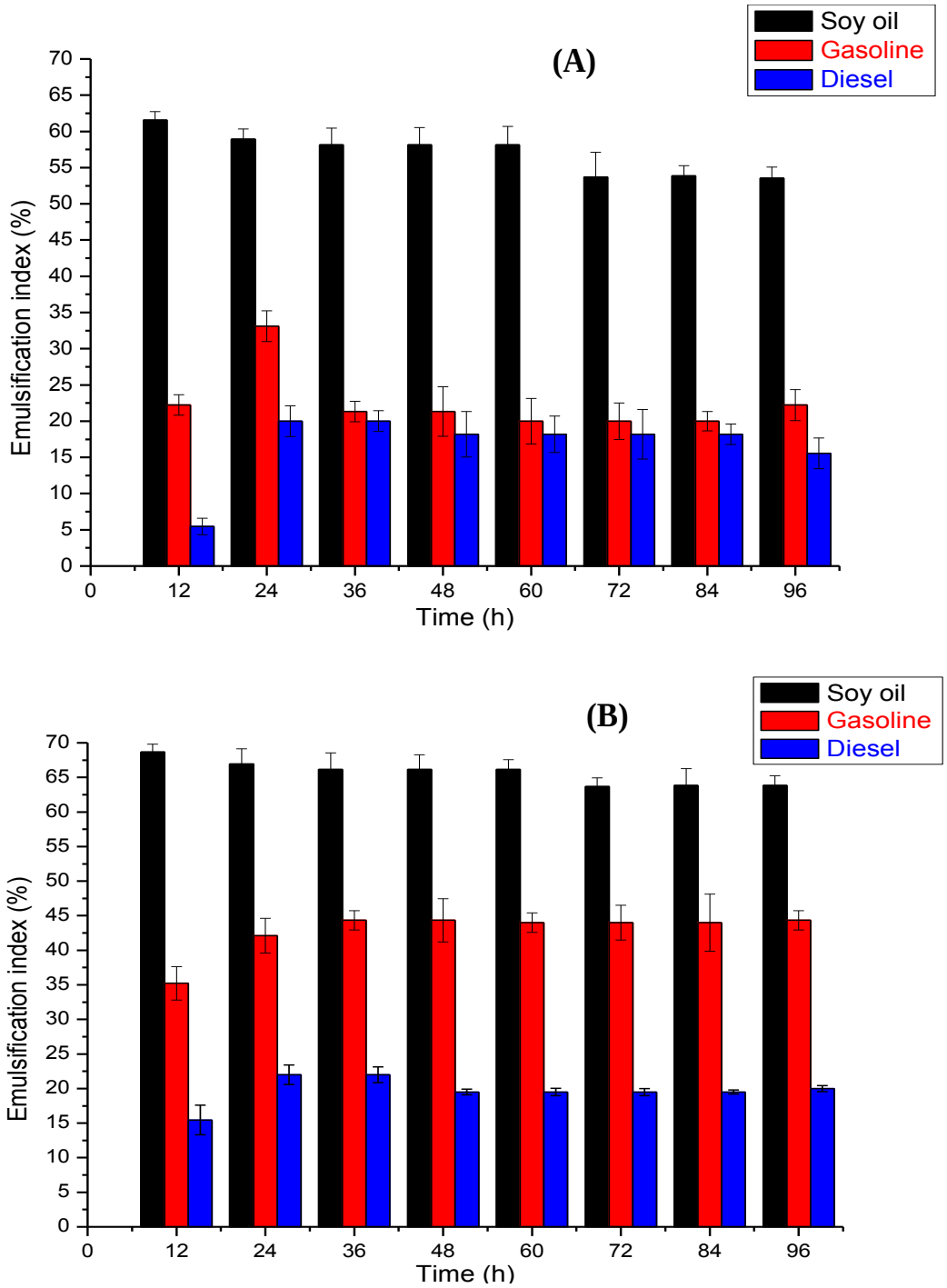

Figure 3. Emulsification indexes of the supernatants along 96 h of fermentation using soy oil, gasoline and diesel as hydrophobic compounds for the papaya wastes without pH correction (A) and with pH correction to 6.8 (B).

this analysis.

Critical micelle concentration

An important distinction to be made is between an effective biosurfactant and an efficient biosurfactant. According to Oliveira et al. (2013), effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The

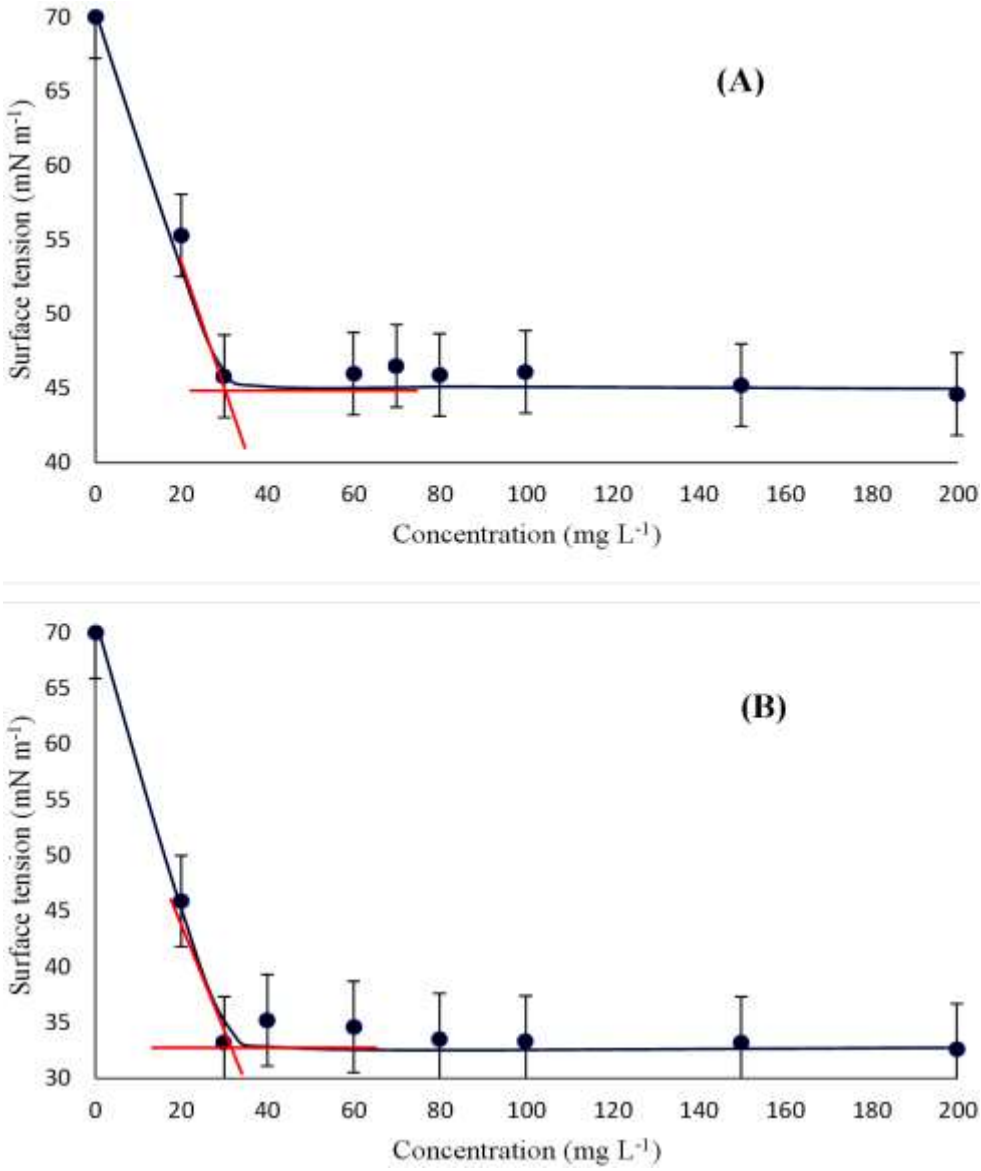

Figure 4. Critical micelle concentration for the fermented medium without pH correction (A) and with pH correction to 6.8 (B).

latter can be determined from the critical micelle concentration (CMC) of the biosurfactant.

CMC is the amphiphilic component concentration in solution at which the micelles formation is initiated. At low biosurfactant concentrations in the fluid, the tendency of the molecules is to agglomerate on the surface. However, the higher the concentration of these compounds on the surface, the greater the tendency to form micelles. At a maximum concentration, the surface will be saturated with biosurfactant to the point that the addition of more biosurfactant will characterize the formation of this molecular aggregate. The lower the CMC, the better the efficiency of the biosurfactant whose concentration values range from 1 to 200 mg L^{-1} (Costa, 2005; Ferreira, 2016). It is important for several biosurfactant applications to establish their CMC, because above this concentration no further effects are observed in the surface activity. Figure 4, plotted using Excel 2016 (Microsoft® CO., WA, USA), shows the critical micelle concentration of the broths without pH correction (Figure 4A) and with 6.8 pH (Figure 4B), using the cell-free fermented broth after 24 h of culture.

Analyzing Figure 4, it is noted that the critical micelle concentration for with and without pH correction corresponded to approximately 35 mg L^{-1}. However, it can be seen in Figure 4A that the CMC is given by the point whose surface tension corresponds to 45.1 mN m^{-1} and in Figure 4B the CMC is obtained at the point where

the surface tension corresponds to 33.3 mN m^{-1}. Although the biosurfactant concentrations obtained were the same (same efficiency), the biosurfactant produced using the substrate with pH correction to 6.8 was more effective in reducing surface tension, which enforces the importance of the substrate and pH variables in the bioprocess.

Reis et al. (2004) investigated biosurfactant production by *B. subtilis* ATCC 6633 using commercial sugar, sugarcane juice, mannitol and soy oil. The commercial sugar had a greater reduction of the surface tension and CMC of 78.6 mg L^{-1} in 48 h of culture, that is, a higher concentration and a longer fermentation time than what was achieved in the present study. Ferreira (2016) used a biosurfactant produced by *B. subtilis* using algaroba (*Prosopis juliflora*) as a substrate for advanced oil recovery and obtained CMC value of 50 mg L^{-1}, also higher than what was obtained in the present study.

Oliveira et al. (2013) obtained CMC from cell-free fermented broths using different initial concentrations of clarified cashew apple juice as substrate and found values between 10 and 65 mg L^{-1}. Such variations in CMC values for surfactin have been commonly described by other authors who explained that such changes depend on the nature of the solvent used to dissolve surfactin as well as the purity of the surfactin preparation. From the results obtained in the present study, 35 mg L^{-1} are within the range obtained by the aforementioned author and are in agreement with results more commonly described in literature ranging from 1 to 200 mg L^{-1} according to Costa (2005), which shows the potential of using papaya (*Carica papaya* L.) wastes as a carbon source for the production of surfactin by *B. subtilis* UFPEDA 86.

Conclusion

Papaya (*C. papaya* L.) waste was effective as a substrate source in biosurfactant production by *B. subtilis* UFPEDA 86, since they caused a significant reduction in the surface tension of the medium. In addition, cell growth occurred quickly, which demonstrated the adaptability of the microorganism to this alternative substrate. By correcting the pH of the culture medium to 6.8, a better adaptation of the microorganism and, consequently, a greater reduction of the surface tension was observed, which shows that the pH variable is of great importance regarding the yield of this bioprocess. The results suggest that papaya (*C. papaya* L.) waste can be a valuable substrate for biosurfactant production by *B. subtilis* and can also be an opportunity to reduce the environmental pollution caused by its disposal from the agro-industrial processing. In addition, both the waste producer and the biosurfactant producer will benefit, since the waste will have added value however they will not be as high as that of the conventional substrates.

Therefore, the use of low cost waste to produce a valuable bio-product provides a way to seek technological innovations by reducing costs and increasing profits, which is the main goal of efficient and accurate agricultural management.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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