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Full Length Research Paper

Stability of concentrated extracts of *Hibiscus sabdarifa L*. calyx during storage at different temperatures

Moussa Ndong[1*], Ndeye Seni Faye[1], Joseph Bassama[1] and Mady Cissé[2]

[1]Section Technologies Agro-Alimentaires, UFR des Sciences Agronomiques, de l'Aquaculture et des Technologies Alimentaires, Université Gaston Berger, B.P. 234, Saint-Louis, Senegal.
[2]Ecole Supérieure Polytechnique, Université Cheikh Anta Diop, B.P. 5085, Dakar Fann, Senegal.

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***Hibiscus sabdariffa* L. (Malvaceae) calyx is a valuable food resource in Africa. This herb is rich in phenolic compounds and a good source of natural antioxidants. Concentrated extract prepared with *Hibiscus sabdariffa* L. calyx is commonly used in traditional African and Asian diets and medicines. However, the organoleptic properties of these extracts are degraded during storage. Therefore, the stability of these products is one of the problems faced by roselle calyx extracts users. Stabilization of the *H. sabdariffa* extract to prevent or reduce quality changes and to extend shelf-life of these products is a challenge to food processors. In this study, strategies for minimizing or eliminating these alterations were evaluated. Concentrated extract of hibiscus calyx (60 °Brix) was divided into three parts: control, pasteurized and addition of potassium sorbate. The samples were stored at 4, 30 or 45°C for three months. Reducing sugars, pH, titratable acidity and total anthocyanins were determined during 0, 15, 30, 45, 60, 75 and 90 days of storage. The results showed that hibiscus calyx extracts can be stored at 4°C for three months without any changes in organoleptic properties. Pasteurization and addition of potassium sorbate did not improve significantly, the stability of the concentrated extracts during storage periods at high temperature. However, these treatments extended the shelf-life at cooling temperature (4°C) without serious changes.**

Key words: *Hibiscus sabdariffa* L., calyx extracts, stability, storage, temperature.

INTRODUCTION

Nowadays, food quality is no longer limited to organoleptic, nutritional and functional quality, but also entails that foods can be transported over long distances, stored and distributed without changes. Products must therefore have sufficient shelf-life to reach consumers in good quality. Thus, novel techniques have to be developed to increase the time to keep food products intact (Singh, 2018).

Roselle, *Hibiscus sabdariffa* L, is an annual herbaceous plant that belongs to the Malvaceae family with important health properties (Ali and El-Anany, 2017). *Bissap* (*H. sabdariffa* L.) is cultivated throughout the Senegalese territory and more particularly in the central and northern regions (Cisse et al., 2009a). Red *H. sabdariffa* calyx is

*Corresponding author. E-mail: moussa.ndong@ugb.edu.sn.

used to produce a variety of food products like: jam, jelly and red soft drinks with tangy taste depending on the variety (Cisse et al., 2009b). The red color is due to anthocyanins present in the calyx. Anthocyanins are known to have nutritional and health properties (Chiu et al., 2015; Worawattananutai et al., 2014; Abdul Hamid et al., 2014; Rodriguez-Perez et al., 2017) and can be used as natural colorants in food, pharmaceutical and cosmetic products (Pinsuwan et al., 2010). The hibiscus based juices are known for their color instability during storage in correlation with changes in anthocyanin contents (Vankar and Shukla, 2011). The instability of these anthocyanins could be influenced by the quantity of solids in suspension and dissolved oxygen (Cisse et al., 2009c; Kane et al., 2015). Anthocyanins degradation during storage depends also on extraction methods and storage temperatures (Cisse et al., 2012).

Concentration process by water evaporation makes hibiscus extracts more stable. Pasteurization and food additives are methods for preservation to stabilize food products during storage. Sorbic acid and its salts kill yeasts, molds and bacteria by accumulating in microorganisms' cytoplasm, causing acidification of the cytosol (Van Beilen et al., 2014). Therefore, they preserve the organoleptic, nutritional and microbiological properties of food during storage. These changes cause a lot of losses to the value chain hibiscus.

Therefore, the objective of this study is to evaluate the impacts of pasteurization and potassium sorbate (E202) on the stability of concentrated extracts of *H. sabdariffa* during storage at 4, 30 and 45°C for 3 months.

MATERIALS AND METHODS

Production of extracts of *H. sabdariffa* L

The concentrated extracts were made with calyx of a local variety of *H. sabdariffa* L called *Bissap Vimto*. Twenty kilograms of calyx purchased from local market in Dakar, Senegal were mixed with 100 L of water and soaked for 4 h. The resulting mixture was filtered with a 0.2 mm diameter steel filter and a cotton filter to obtain a clear extract. The extract with 8.8 °Brix was then concentrated with an evaporator (AURIOL, France) to 60 °Brix. The concentrated extracts were divided into three: concentrated extract without treatment (NTC), concentrated extract with addition of 0.16% potassium sorbate (CSP) and the pasteurization of the concentrated extract at 70 C for 30 min (PC). Samples were stored in glass bottles wrapped in aluminum foil and stored at 4, 30 and 45°C. From zero time to day-90, every 15 days, up to 90 days, the concentrated calyx extracts were determined for: reducing sugars, pH, titratable acidity and total anthocyanins.

pH was determined with a pH-meter (Hanna HI 223, USA) dipped into the bottles (AOAC, 2005). The amount of titratable acid was determined by potentiometric titration using sodium hydroxide solution with a potentiometer titrator (Titroline Schott T23230 N° M 011287, Germany) (AOAC, 2005). Reducing sugars were measured with the Luff-Schoorl method (Kowalski et al., 2013). The sugars were extracted in aqueous ethanol. After eliminating the ethanol, the solutions were clarified and the sugars were determined before and after inversion. All analyses were performed in triplicate.

The anthocyanins concentrations of the hibiscus extracts were also determined during storage (Lee et al., 2005). The principle of determination was based on the properties of anthocyanins to change color depending on the pH (pH differential method). After dilution of the extract in two buffer solutions at pH 1.0 and 4.5 purchased from Fisher Scientific, USA, the absorbance was measured at 510 and 700 nm in triplicate. The anthocyanin's concentration was calculated using the following formula:

$$Ca = \frac{MW \times A \times dF \times 1000}{\varepsilon}$$

Ca: Anthocyanin concentration (mg/L); MW: molecular weight of Delphinidin 3-sambubioside, the major anthocyanin in *H. sabdariffa* extract, (597 g/mol); ε: molar extinction coefficient (26 000 L mol-1. cm-1); dF: dilution factor; A: absorbance, calculated using the formula:

A = (A1 - A2) - (A3 - A4)

Where, A1 = Absorbance measured at pH = 1 and 510 nm; A2 = absorbance measured at pH = 1 and 700 nm; A3 = absorbance measured at pH = 4.5 and 510 nm; A4 = Absorbance measured at pH = 4.5 and 700 nm.

Statistical analysis

Results are expressed as mean ± SD. The Fisher's protected least significant difference (PLSD) for multiple comparisons after one-way ANOVA was used to analyze the data (SPSS Version 14.0J, SPSS, Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS

Characterization of the *H. sabdariffa* extracts

The effect of concentration process on some physiochemical properties of the hibiscus extracts are shown in Table 1. The results showed that the evaporation process increased the Brix from 8.8 to 60 °Bx. Non-concentrated extract (NC) had a significantly higher pH (2.1±0.01) than the concentrated extract (1.4±0.04). The concentration process decreased the pH and significantly increased reducing sugars and anthocyanins in the samples.

pH variation during storage

The pH of the samples decreased during storage (Table 2). This decrease was significant ($P < 0.05$) for CSP samples stored at different temperatures at day-90 as compared to zero time. There were no differences between the pH of NTC, CSP and PC at day-90 of storage at 4, 30 and 45°C.

Titratable acidity variation during storage

Table 3 shows the changes of the titratable acidity

Table 1. Effect of the concentration process on some physico-chemical properties of *Hibiscus sabdariffa* L. extracts.

Parameters	NC	NTC
pH	2.1±0.01[a]	1.4±0.04[b]
Titratable acidity (mEq/l)	457.66±0.01[a]	3885.595±604.476[b]
Ruducing sugars (g/l)	74.66±42.15[a]	113.140±46.465[b]
Total anthocyanins (g/l)	1.58±0.30[a]	14.602±0.402[b]

Values are means ± SD, n= 3. Values in the same row with different superscripts are significantly different, P<0.05.

Table 2. pH variation during storage of *Hibiscus sabdariffa* L. extracts at 4, 30 and 45°C.

Treatment	Storage temperatures (°C)			
	Zero time	4	30	45
		After 90 days		
NTC	1.430±0.040[a]	1.415±0.007	1.400±0.014	1.455±0.007
CSP	1.600±0.010[b]	1.355±0.007	1.41±0.042	1.410±0.014
PC	1.460±0.010[a]	1.355±0.049	1.405±0.007	1.425±0.035

Values are means ±SD, n=3, Values in the same column with different superscripts are significantly different, P<0.05.

Table 3. Variation of titratable acidity (mEq/l) during storage of *Hibiscus sabdariffa* L. extracts at 4, 30 and 45°C

Treatment	Storage temperatures			
	Zero time	4	30	45
		After 90 days		
NTC	3885.595±604.476	4228.950±6.152	4276.760±36.883	4880.895±18.434[a]
CSP	3959.480±306.749	4198.540±36.854	4328.915±24.586	4563.615±61.469[b]
PC	3924.710±944.386	4233.295±61.469	4237.640±30.731	4650.54±61.462[b]

Values are means ±SD (n=3). Values in the same column with different superscripts are significantly different, P<0.05.

Table 4. Variation of reducing sugars (g/l) during storage of *Hibiscus sabdariffa* L. extracts at 4, 30 and 45°C.

Treatment	Storage temperatures (°C)			
	Zero time	4	30	45
		After 90 days		
NTC	113.140±46.465	123.36±0.01	112.56±0.01	117.955±0.007[a]
CSP	128.220±0.583	121.20±0.01	118.00±0.01	113.635±0.007[b]
PC	114.920±44.213	128.75±0.01	123.36±0.01	117.955±0.006[a]

Values are means ±SD (n=3). Values in the same column with different superscripts are significantly different, P<0.05.

between samples during storage at different temperatures. At day-90, the titratable acidity in samples stored at 4°C was non-significantly different from that on zero time. During storage at 45°C, titratable acidity significantly (P <0.05) increased in CSP and PC samples at day 90.

Evaluation of reducing sugars during storage

The concentration of reducing sugars in the hibiscus concentrates during storage is shown in Table 4. At day 90, CSP samples stored at 45°C showed the lowest concentration of reducing sugars (113.635±0.007 g/L) as

Table 5. Changes of anthocyanins levels in NTC, CSP and PC samples during storage at 4°C.

Treatment	Zero time	Day-15	Day-30	Day-45	Day-60	Day-75	Day-90
NTC	14.602±0.402^{a}	12.766±1.645	13.394±0.956	11.180±0.644	12.941±0.707	12.288±0.827	13.708±0.981
CSP	13.828±2.654^{b}	12.181±1.642	14.427±1.187	12.302±1.750	12.992±1.485	12.655±1.022	13.111±1.517
PC	12.340±0.360^{b}	11.818±1.166	14.059±0.369	12.408±1.500	12.985±1.021	12.800±1.077	13.800±1.225

Values are means ±SD (n=3). Values in the same column with different superscripts are significantly different, P<0.05.

Table 6. Changes of anthocyanins levels in NTC, CSP and PC samples during storage at 30°C.

Treatment	Zero time	Day-15	Day-30	Day-45	Day-60	Day-75	Day-90
NTC	14.602±0.402^{a}	11.597±1.823	10.922±0.955	7.473±0.443^{a}	9.871±2.064	5.748±0.323	7.222±4.836
CSP	13.828±2.654^{b}	10.030±0.889	11.613±1.886	8.724±1.101^{b}	8.337±2.695	5.504±0.648	3.852±1.619
PC	12.340±0.360^{b}	10.881±0.553	11.851±1.012	9.362±1.133^{ab}	6.765±1.562	4.082±1.656	4.487±0.815

Values are means ±SD (n=3). Values in the same column with different superscripts are significantly different, P<0.05.

Table 7. Changes of anthocyanins levels in NTC, CSP and PC samples during storage at 45°C.

Treatment	Zero time	Day-15	Day-30	Day-45	Day-60	Day-75	Day-90
NTC	14.602±0.402^{a}	3.327±0.974^{a}	2.110±0.251	2.110±0.251^{a}	1.536±0.558	0.795±0.413	-
CSP	13.828±2.654^{b}	4.486±0.378^{ab}	2.816±1.252	2.816±1.252^{b}	2.135±1.857	-	-
PC	12.340±0.360^{b}	3.894±0.637^{b}	3.288±1.006	3.288±1.006^{b}	1.454±0.367	-	-

Values are means ±SD (n=3), Values in the same column with different superscripts are significantly different, P<0.05.

compared to NTC and PC stored at the same temperature (117.955±0.007 and 117.955±0.006 g/L, respectively).

Variation of anthocyanin during storage

As shown in Table 5, at 4°C, all the treatments kept most of the anthocyanin levels [13.708 g/L (NTC), 13.111 g/L (CSP) and 13.800 g/L (PC)]. Table 6 shows the effect of temperature storage (30°C) on the anthocyanin levels of hibiscus extract samples. From the results, all the treatments affected the level of anthocyanin. The residual level of anthocyanin was 49, 31 and 26% for NTC, PC and CPS, respectively. Table 7 shows that the highest destruction of anthocyanin among the storage temperatures was at 45°C in all the samples.

DISCUSSION

The concentration process decreased pH. This process also significantly increased titratable acididy, reducing sugars and anthocyanins in the samples. These results are in accordance with Youssef and Shatta (2006). Samples of hibiscus concentrated extracts had a very low pH (1.4 to 1.6). This acidity was due to organic acids such as the succinic and oxalic acids contained in the calyces of *H. sabdariffa* (Cisse et al., 2009b). Hibiscus extracts also contain other organic acids such citric, hydroxycitric, hibiscus, malic, tartaric and ascorbic acids (Da-Costa-Rocha et al., 2014). The increase in titratable acidity during storage at 45°C did not influence significantly, the final pH of the samples. The low pH of these concentrates makes it possible to avoid growth of certain microorganisms because the minimal pH of growth for most bacteria is 4.5 with some exceptions like lactic and acetic acid bacteria which can grow at pH lower than 4. The yeasts and molds have a minimum growth pH of 1.5 to 3.5 (Oyarzabal and Backert, 2011). Pasteurization and addition of food preservatives like potassium sorbate are means to inhibit the multiplication of these microorganisms which are the main agents influencing the conservation of food products (Yang et al., 2017).

The calices also contain sugars with glucose as the major sugar present (Peng-Kong et al., 2002). Reducing sugars were shown to decrease during storage. This reduction tended to increase during storage at 30 and 45°C than at 4°C (Table 4). Microorganisms can grow rapidly at favorable temperature and use these sugars in their metabolism, leading to the reduction of the sugar content (Pokusaeva et al., 2011). Changes in reducing sugars can influence sensory acceptability of hibiscus drinks by the consumers (Bechoff et al., 2014).

Hibiscus calyces are rich in anthocyanin used as

natural colorant and functional food ingredient (Idham et al., 2012). These anthocyanins are responsible for the red color of *H. sabdariffa* calyx products. The concentration of anthocyanin is influenced by the storage conditions and has an impact on the shelf-life (Sinela et al., 2016). Samples stored at 45°C for three months lost more than 99% of their anthocyanin content, while those kept at 4°C retained almost all their anthocyanins. Temperature is an essential factor in the degradation of anthocyanins. Indeed, temperature was also the determining factor in the deterioration of the phenolic pigments (Fernandez-Lopez et al., 2013; Al-Sanabani et al., 2016). In the case of anthocyanin, high temperatures favor the opening of the heterocyclic anthocyanins with the formation of chalcones which are colorless products (Dziezak, 1986). The degradation of these pigments causes disappearance of the chromatic characteristics since they determine the color of the products (Camelo-Mendez et al., 2016). Pasteurization, addition of potassium sorbate and the pH of hibiscus extracts affected anthocyanin stability. Food preservatives such as potassium sorbate were shown earlier to have a slight influence on the anthocyanins stability (Moldovan and David, 2014). High pressure processing and thermal pasteurization were reported to influence polyphenols and anthocyanins stability during storage (Marszalek et al., 2017). West and Mauer (2013) showed that the stability of anthocyanins in solution was inversely correlated to increasing pH and temperature and was related to the common destruction of anthocyanins and ascorbic acid in solution. Finally, the use of low temperature (4°C) besides pasteurization and/or addition of potassium sorbate could extend the shelf-life of these concentrates without changes.

In conclusion, concentrated hibiscus extracts can be stored at an optimal temperature of 4°C for 3 months without serious changes in the extracts. Pasteurization and addition of potassium sorbate to the concentrated extracts of *H. sabdarifa* did not improve significantly, the stability of these products during storage at higher temperature.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of pretreatments on the microbial and sensory quality of weaning food produced from blends of sorghum and soybean

Msheliza E. A.[1*], Ilesanmi J. O. Y. [2], Hussein J. B.[2] and Nkama I.[3]

[1]Department of Home Economics Education, Federal College of Education (Technical), Gombe, Gombe State, Nigeria.
[2]Department of Food Science and Technology, Modibbo Adama University of Technology, Yola, P. M. B. 2076 Yola, Adamawa State, Nigeria.
[3]Department of Food Science and Technology, University of Nigeria, Nsukka, Enugu State, Nigeria.

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This study investigated the effect of pre-treatment on the microbial and sensory quality of weaning foods produced from blends of sorghum and soybean. Sorghum and soybean were fermented, roasted and fermented/roasted, then milled into flours. Untreated sorghum and soybean flours were also produced separately and serve as the control. A portion of sorghum was malted and milled to obtain malted sorghum flour of which 5% was added to each sample except the control samples. Weaning foods were prepared in the following ratios: (75:20:5), (65:30:5) and (55:40:5) of sorghum: soybean: malt for the treated samples and the control samples contained (80:20), (70:30) and (60:40) of untreated sorghum : soybean. The microbial and sensory qualities of the samples were determined. The result of the microbial analysis of the formulated weaning food blends showed that all samples indicated a safety for consumption. The appearance of both treated and untreated were not significantly different ($p<0.05$) while the means scores of the blends in texture, taste, aroma and overall acceptability were significantly different ($p<0.05$). Fermentation and roasting used in this study showed that the processing methods had marked effect on the microbial and sensory quality of the weaning foods produced, thus, its recommendation for domestic processing of weaning foods.

Key words: Pretreatments, weaning food, sorghum, soybean, microbial and sensory qualities.

INTRODUCTION

Weaning is the gradual introduction of foods other than breast milk into a baby's diet from the age of six months. Weaning foods are foods widely used during the transition from consuming solely human milk or infant formulas to the introduction of a mixed diet. The foods are solely introduced to complement breast milk, progressively replace it and eventually adopt the child to adult diets. Usually, the breast milk which is the baby's first food is inadequate to maintain the rapid growth and development of the baby after six months of age of the

*Corresponding author. E-mail: esthermusadurkwa@gmail.com. Tel: +2348163917067.

child. Hence, there is the need to introduce appropriate weaning foods to the child which will supply the additional safe sources of energy and protein to complement breast milk and fully aid the growth and development of the child. Failure to feed the baby with appropriate food could lead to malnutrition, a problem that is common with most children in the developing countries of which Nigeria is one (Asma et al., 2006; Eshun et al., 2011).

Local foods and fortifying ingredients such as maize, millet, sorghum, soybean and cowpea have been utilized creatively with acceptability in mind (Mosha and Vicent, 2004; Mugula and Lyimo, 2000). In Nigeria, traditional weaning food consist of monocereal grains prepared from either millet, sorghum and maize is referred to as “Ogi” or “Akamu” which is of poor nutritional value (Abdulkadir and Danjuma, 2015; Rombouts and Nouts, 1995). Cereals are the most important staple food being the major sources of carbohydrates. Compositionally cereals consist of 12 to 14% water, 65 to 75% carbohydrates; 2 to 6%, lipids and 7 to 12% protein on dry weight basis. In their natural form, whole grain cereals are also significant contributor of vitamins, minerals like manganese, zinc, copper and magnesium and considerable iron but its bio-availability is low which results in incidence of iron deficiency anemia. However, processes like fermentation has improved the chemical bio-availability of iron (Oyarekua, 2011).

Abdulkadir and Danjuma (2015) reported that the major problem associated with infant during transitional phase of weaning is protein energy malnutrition (PEM) which results into condition such as marasmus or kwashiorkor. The formulation and development of nutritious weaning food from the combination of commonly used cereals with plant protein source like legumes can be used to prevent malnutrition during weaning, since cereals are deficient in lysine which are limited in legumes, whereas legumes are highly rich in lysine. The effects of the fortification are highly beneficial since nutritive value of products is also improved (Wang and Daun, 2006). The use of legumes seeds may be the beginning of a series of formulation which will lead to a substantial drop in dependency of animal source on nutritious foods. Unfortunately, legumes seeds contain anti-nutritional factors like enzymes inhibitors phytates, oxalate, saponin and polyphenolic compound, all of which limit their utilization (Abdulkadir and Danjuma, 2015). Although, remarkable improvement in the nutritive value and quality of legumes seeds have been achieved through de-hulling, heat treatment, germination, fermentation, soaking and partial hydrolysis of proteolytic enzymes (Akinrele and Bassir, 1967).

Soybeans and sorghum are typically processed prior to human consumptions. It is important that the anti-nutritional components are denatured prior to their consumptions. Numerous methods of eliminating anti-nutritional factors have been developed and tested (Newkirk, 2010). Most of the toxic and anti-nutrient effects could be removed by several processing methods such as soaking, germination, boiling, autoclaving, fermentation, genetic manipulation and other processing methods (Soetan, 2008). Fermented cereals are particularly important as weaning foods for infants and as dietary staples for adults. Ikemefuna et al. (1991) reported that a combination of cooking and fermentation improved the nutrient quality and drastically reduced the anti-nutritional factors to safe levels much greater than any of the processing methods tested. Ikemefuna et al. (1991) also reported that soaking and fermentation decreased the tannins content because these processes produce enzymes that break down complexes to release free tannins, thus, the free tannins were leached out. Roasting which is the application of heat in measured amounts, denatures the trypsin inhibitors, hemagglutinins (lecithins) and possibly allergenic proteins without damaging the quality and digestibility of the protein in the meal (Newkirk, 2010). Roasting also create a convenient method of increasing fat content in the diet without the need to physically handle a liquid oil product.

The aim of this study is to examine the effects of pretreatments (fermentation and roasting) on the microbial and sensory quality of weaning food produced from blends of sorghum and soybean.

MATERIALS AND METHODS

Sorghum and soybean grains were purchased from Gombe Main Market, Gombe State, Nigeria. All samples were kept in a moisture free environment until when needed. The chemicals and reagents used were of analytical grade and they were obtained from the laboratories of Food Science and Technology, Modibbo Adama University of Technology, Yola, Adamawa State and Home Economics Education Department, Federal College of Education (Technical), Gombe, Gombe State, Nigeria.

Preparation of samples

The sorghum and soybean grains were manually cleaned (separately) to remove sand, foreign seeds, broken and infested seeds, dirt and other contaminants.

Fermented sorghum

One kilogram (1 kg) of sorghum was dehulled after tempering using the commercial grains huller at Gombe Main Market. After dehulling, the grains were winnowed and washed to remove the hulls and germs. The grains were steeped at room temperature (32 ± 2°C) for 72 h and the steep water decanted. The fermented grains were then dried in hot air oven (Model: TO008GA-34, AKAI-TOKOYO, JAPAN) set at 60°C for 10 h to halt the fermentation process. It was milled to produce the fermented sorghum flour which was packaged in a clean polyethylene bag (Adebayo-Oyetoro et al., 2012).

Fermented soybean

One kilogram (1 kg) of soybean was soaked for 12 h in 3 L of clean water until the seed coat became soaked and wet to facilitate dehulling. Mortar and pestle were used for dehulling. The dehulled

soybean was washed to remove the seed coat. The soybean was allowed to ferment naturally in a clean covered plastic bucket for 72 h and the steep water decanted. The fermented grain was then dried in hot air oven (Model: TO008GA-34, AKAI-TOKOYO, JAPAN) at 60°C for 10 h to halt the fermentation process. It was then milled to produce the fermented soybean flour which was packaged in clean polyethylene bag until needed.

Roasted sorghum

Whole grains of sorghum weighing 1 kg were dehulled using the commercial grains huller at Gombe Main Market. After dehulling the grains, they were winnowed, washed, drained and then partially sun dried. The sorghum was then traditionally roasted using an open thick aluminum pot. Commercial grinding machine was used to mill the grains into flour. A local sieve of about 1 mm in diameter was used to sieve the flour in order to obtain a fine particle size. The flour was packaged in a plastic container and sealed until needed.

Roasted soybean

Whole grains of soybean weighing 1 kg were soaked for 12 h in 3 L of clean water until the seed coat became soaked and wet to facilitate dehulling. Mortar and pestle were used for dehulling. The dehulled soybean was washed to remove the seed coat, drained and then sun dried. The soybean was then traditionally roasted using an open thick aluminum pot. An attrition mill was used to mill the dehulled grain into fine flour and to pass through a sieve of about 1 mm mesh screen. The roasted soybean flour was packaged in a plastic container until when required.

Fermented/roasted sorghum and soybean flour

One kilogram (1 kg) of sorghum grain was dehulled and dry cleaned. This was then fermented in a plastic bucket for 72 h. Thereafter, it was sun dried and roasted traditionally using an open thick aluminum pot. It was then milled into flour using attrition mill and stored in a plastic container until needed. These procedures were used for the preparation of fermented/roasted soybean.

Malting of sorghum

The sorghum was malted as described by Badau et al. (2006). The grains were steeped at room temperature (32 ± 2°C) for 12 h. The steeped liquor was changed after 6 h. One air rest period of 1 h was applied after 6 h of steeping. After steeping, the grains were immersed in 0.1% (v/v) solution of commercial bleach (hypo), that is, 5 ml of 3.5% sodium hypochlorite. After sterilization, the grains were wrapped in a wet piece of cotton cloth and placed on a wet jute bag. Another wet jute bag was used to cover the grain wrapped in the wet cloth. The sorghum grains were allowed to germinate at room temperature (32 ± 2°C) for 72 h. During germination, small and the grains were turned by moving a clean wooden rod inside the germinating grains. For the first 48 h of malting, the sample was quantity of water (15 ml) was sprayed on the germinating grains moistened twice a day at 08:00 and 16:00 hour by spraying a mist of water on it for about 5 s and then it was turned over. On the seventy-two hour of germination, the sample was spread in the morning (8 h) and turned over in the afternoon (16 h).

At the end of the germination, the germinated grains were dried to moisture content of 5.45 ± 0.48% in an oven set at 50°C for 24 h. The dried germinated grains were polished by removing shoots and rootlets. Rootlets and shoots were separated from the kernels by rubbing between the palms in a local sieve of about 1 mm mesh size. This allows the rootlets and shoots to escape but retain the kernels. The polished malt was then milled into fine flour in a commercial milling machine and sieved using a local sieve of about 1 mm mesh screen. The malted sorghum flour was packaged in an air tight plastic container until when it was needed.

Formulation of weaning foods

The weaning foods were formulated in ratios shown in Table 1.

Microbial analysis

Materials that were used were sterilized by autoclaving at pressure of 1 kg/cm^2 (121°C) for 15 min as described by Jideani and Jideani (2006). Glass wares were sterilized by dry-heat at temperature of 160°C for 60 min. The work-place was aseptically cleaned (with antiseptic and careful control) to ensure microbial reduction inhibiting the place. Media after preparation were sterilized at 1 kg/cm^2 (121°C) for 15 min and placed in water bath at 100°C to avoid gelling before use. Besides, Bunsen burner was used to "heat fix" smears, sterilize wire loop and other equipment. Inoculation methods such as streaking and poor plating were used to culture and subculture. Preliminary observations and tests were carried out followed by secondary observation.

Serial dilution

A prepared 1 g of sample was transferred into a bottle containing 9 ml of distilled water to form the stock solution as described by Jideani and Jideani (2006). From the stock solution, 1 ml was aseptically transferred into subsequent bottles containing sterile distilled water using sterile pipette, that is, from first to second, second to third and third to fourth until after required serial dilution was made.

Standard plate count method (SPCM)

From the prepared sample, 1 ml of the diluent from a test tube was transferred aseptically into cleaned and sterilized labeled plate (pour plate count) using sterile pipette. The lid of each was aseptically opened to introduce about 15 ml molten medium (Nutrient agar or MacConkey agar) into the plate (Petri dish) to ensure total bottom covering of the plate. It was swirled gently, cooled and solidified before incubating it at 35°C for 24 h as described by Jideani and Jideani (2006). The number of microorganism per gram of the original sample (colony forming unit per gram [Cfu/gram]) was obtained using:

$$\text{Colony forming unit per gram (Cfu/g)} = \frac{number\ \ of\ colonies}{volume\ \ transferred\ \ to\ plate} \times dilution\ blank\ factor \quad (1)$$

Table 1. Formulation of the weaning foods.

Samples	Sorghum (%)	Malted sorghum (%)	Soybean (%)
F1, R1 & FR1	55	5	40
F2, R2 & FR2	65	5	30
F3, R2 & FR3	75	5	20
U1	60	-	40
U2	70	-	30
U3	80	-	20

F1, F2 and F3 = Fermented samples (55:40:5, 65:30:5 and 75:20:5 respectively); R1, R2 and R3 = roasted samples (55:40:5, 65:30:5 and 75:20:5 respectively); FR1, FR2 and FR3 = fermented and roasted samples (55:40:5, 65:30:5 and 75:20:5 respectively); U1, U2 and U3 = untreated samples (60:40, 70:30 and 80:20 respectively).

Sensory evaluation of weaning food gruel

The gruel of the developed weaning food was served to 20 weaning mothers who were asked to rank the gruel on the basis of some quality attributes (taste, texture, appearance, aroma and overall acceptability) using 9 points Hedonic scale, where 1 = 'dislike extremely' and 9 = 'like extremely'. Weaning food gruels were served to panelist in transparent plastic cups and they were asked to rinse their mouth with fresh room temperature water provided, before next serving. The containers with the samples were coded and kept far apart to avoid crowding and for independent judgment. The panelists were selected based on the basic requirements of a panelist, such as availability for the entire period of evaluation, interest, willingness to serve, good health (not suffering from colds), not allergic or sensitive to the product emulated (Badau et al., 2005).

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as means ± standard error (SE). Analysis of variance (ANOVA) was carried out to determine any significant differences in measurements using the SPSS statistical software (SPSS 20.0 for Windows; SPSS Inc., Chicago, IL, USA) and considering the confidence level of 95%. The significance of the difference between the means was determined using the Duncan's multiple range test, and the differences were considered to be significant at $p< 0.05$ (Hussein et al., 2016).

RESULTS AND DISCUSSION

Effect of pretreatments on the safety of the formulated weaning foods from blends of sorghum and soybean

The result of microbial analysis as presented in Table 2 showed that pretreatments (fermentation, roasting and the combination of fermentation and roasting) have positive effect on the microbial load of the weaning foods samples as compared to the untreated samples. The total plate count (TPC) ranged from 1.30×10^3 to 2.83×10^3 (cfu/g) with sample F55:40:5 having the highest value while sample R55:40:5 had the lowest value. In the fermented samples, there was a significant decrease in the TPC in all the blends as the percentage inclusion of soybean increased. The percentage reduction in TPC of fermented sample was 4.59%. There was also a significant decrease in the TPC of all the roasted blends as the percentage inclusion of soybean increased. The same trends of decrease were observed for fermented and roasted samples. The percentage reduction in TPC of fermented sample was 3.70% and fermented/roasted was 4.75%. While the untreated samples shows a significant increase in the TPC of all the blends as the percentage inclusion of soybean increased. The higher TPC of fermented samples to fermented/roasted, roasted and untreated samples were observed to be due to the multiplication of microorganism during fermentation process. The TPC of all the roasted and fermented/ roasted samples were very low. This was observed to be due to high temperature of roasting as most microorganisms cannot survive higher temperature.

There was no detectable yeast and moulds growth in fermented, roasted and fermented/roasted samples. The untreated samples showed very low growth of 5.00×10^1 cfu/g, 4.80×10^1 cfu/g and 4.60×10^1 cfu/g for samples U60:40, U70:30 and U80:20, respectively. These results are much lower than the FAO/WHO limits of 10^4 to 10^6 cfu/g for bacteria and 10^2 to 10^4 cfu/g for moulds in weaning foods (Abdulkadir and Danjuma, 2015). The yeast and moulds growth in the untreated samples were observed to be present as contaminants and do not appear after the pretreatments. The expected decrease or elimination was also reported by Mbata et al. (2009) for fermentation of maize flour fortified with Bambara groundnut. This shows clearly the importance of these pretreatments used in the aspect of weaning food processing. The microbial analysis of the formulated weaning food blends reveals that all the formulation indicated a safety of the products for consumption and this was due to higher standard of personal hygiene and quality maintenance of manufacturing practice observed during the preparation. Nwokoro and Chukwu (2012) highlighted the importance of adequate hygiene during the preparation of food and also link between infection

Table 2. Microbial load of the weaning foods prepared from the blends of sorghum and soybean (cfu/g).

Sample blends		Total plate count	Yeast and moulds count
Fermented	F55:40:5	2.70×10^3	No growth
	F65:30:5	2.74×10^3	No growth
	F75:20:5	2.83×10^3	No growth
Roasted	R55:40:5	1.30×10^3	No growth
	R65:30:5	1.33×10^3	No growth
	R75:20:5	1.35×10^3	No growth
Fermented and roasted	FR55:40:5	2.00×10^3	No growth
	FR65:30:5	2.00×10^3	No growth
	FR75:20:5	2.10×10^3	No growth
Untreated	U60:40	2.30×10^3	5.00×10^1
	U70:30	2.20×10^3	4.80×10^1
	U80:20	2.10×10^3	4.60×10^1

F55:40:5, F65:30:5 and F75:20:5 = fermented samples; R55:40:5, R65:30:5 and R75:20:5 = roasted samples; FR55:40:5, FR65:30:5 and FR75:20:5 = fermented and roasted samples; U60:40, U70:30 and U80:20 = untreated samples.

Table 3. Sensory qualities of the weaning foods prepared from the blends of sorghum and soybean.

Sample blends		Appearance (na)	Texture	Taste	Aroma	Overall acceptability
Fermented	F55:40:5	7.45 ± 0.42	7.40 ± 0.32^{a}	4.35 ± 0.57^{b}	4.05 ± 0.55^{d}	5.45 ± 0.46^{b}
	F65:30:5	7.15 ± 0.47	6.95 ± 0.41^{a}	4.65 ± 0.55^{b}	4.70 ± 0.45^{cd}	5.30 ± 0.58^{b}
	F75:20:5	7.75 ± 0.27	7.35 ± 0.29^{a}	4.85 ± 0.57^{b}	6.00 ± 0.50^{bc}	5.25 ± 0.63^{b}
Roasted	R55:40:5	7.80 ± 0.30	6.95 ± 0.37^{a}	8.15 ± 0.30^{a}	7.70 ± 0.40^{a}	7.85 ± 0.43^{a}
	R65:30:5	6.85 ± 0.52	6.75 ± 0.45^{a}	7.80 ± 0.28^{a}	7.35 ± 0.49^{ab}	7.80 ± 0.32^{a}
	R75:20:5	7.35 ± 0.48	7.20 ± 0.45^{a}	7.60 ± 0.39^{a}	7.35 ± 0.47^{ab}	7.65 ± 0.42^{a}
Fermented and roasted	FR55:40:5	6.60 ± 0.50	5.35 ± 0.43^{b}	7.50 ± 0.41^{a}	7.15 ± 0.27^{ab}	7.70 ± 0.31^{a}
	FR65:30:5	6.60 ± 0.40	5.05 ± 0.46^{b}	7.30 ± 0.50^{a}	7.05 ± 0.46^{ab}	7.65 ± 0.51^{a}
	FR75:20:5	6.75 ± 0.49	4.85 ± 0.55^{b}	7.25 ± 0.46^{a}	6.90 ± 0.58^{ab}	7.55 ± 0.54^{a}
Untreated	U60:40	6.95 ± 0.56	6.90 ± 0.45^{a}	7.20 ± 0.48^{a}	6.70 ± 0.60^{ab}	7.00 ± 0.60^{a}
	U70:30	7.15 ± 0.43	7.60 ± 0.35^{a}	7.20 ± 0.37^{a}	6.35 ± 0.46^{ab}	7.65 ± 0.51^{a}
	U80:20	6.75 ± 0.54	6.65 ± 0.55^{a}	7.25 ± 0.48^{a}	6.35 ± 0.44^{ab}	7.50 ± 0.43^{a}

Means in the same column bearing different superscripts are significantly different ($p<0.05$); F55:40:5, F65:30:5 and F75:20:5 = fermented samples; R55:40:5, R65:30:5 and R75:20:5 = roasted samples; FR55:40:5, FR65:30:5 and FR75:20:5 = fermented/roasted samples; U60:40, U70:30 and U80:20 = Untreated samples.

and nutrition.

Also, the indication of very low microbial contents of the products can be attributed to the low moisture content of the products, which is an indication of low water activity preventing microbial growth. Similar results were reported by Amankwah et al. (2009) for formulation of weaning food from fermented maize, rice, soybeans and fish meal.

Effect of pretreatments on sensory evaluation of the formulated weaning foods from blends of sorghum and soybean

Table 3 shows the results obtained for sensory qualities of the weaning foods prepared from the blends of sorghum and soybean. All the weaning mothers were conversant with the factors governing the qualities of the weaning foods. The sensory attributes of the weaning blends show that there were significant differences ($p<0.05$) among the means scores of the blends in texture, taste, aroma and overall acceptability, while there were no significant difference ($p>0.05$) among the means scores in appearance. In terms of appearance, the scores ranged from 6.60 to 7.80 with the sample FR55:40:5, fermented/roasted having the lowest score, while sample R55:40:5 roasted had the highest score, thereby the most preferred by the panelists. The

appearance of both treated and untreated were significantly preferred ($p<0.05$) despite no addition of 5% malt to untreated samples. Similar result was reported by Badau et al. (2005) for weaning food gruel produced from pearl millet and legumes.

The scores for texture ranged from 4.85 to 7.60 with the sample FR75:20:5, fermented/roasted having the lowest score while the most preferred by the panelists was sample U70:30, untreated. This is due the viscous nature of the untreated blend upon cooling as observed by the panelist. In terms of taste, the scores ranged from 4.35 to 8.15 with sample F55:40:5 fermented having the lowest score while sample R55:40:5, roasted, had the highest score, thereby the most preferred by the panelists. With regards to the aroma, the scores ranged from 4.05 to 7.70 with the sample F55:40:5, fermented, being the least preferred by the panelists and sample R55:40:5, roasted, was the most preferred by the panelists. The taste and aroma of the roasted samples preferred by the panelists is due to the flavoring agent and the least preferred taste and aroma of fermented blends were observed to be due to higher acidity resulting from the prolonged fermentation time. The weaning food blends from roasted samples had significantly ($p<0.05$) higher mean score in appearance, taste, aroma and overall acceptability followed by fermented and roasted samples. These relatively higher mean scores of the roasted samples followed by fermented and roasted samples could be probably due to the roasted flavour and aroma imparted on soybean during roasting coupled with astringency and aromatic compounds conferred on the sorghum by the fermentation process. Similar results were reported by Adedeji et al. (2015) that fermentation imparts desirable flavors, esters, ketones, aldehydes and aromatic compounds as well as characteristic astringency on products. But in this study, relatively lower mean sensory scores were obtained for fermented samples in terms of taste. These lower mean scores were due to beanny flavour imparted by the soybean supplementation. Both treated and untreated samples when reconstituted were smooth and homogeneous, without noticeable lumps. This was the reason why untreated samples were accepted coupled with the fact that the weaning mothers are used to these untreated ones. The overall acceptability scores ranged from 5.25 to 7.85 with the sample F75:20:5 fermented having the lowest score while sample R55:40:5, roasted, had the highest score thereby the most preferred by the panelists.

Conclusion

Weaning foods from blends of fermented (sorghum and soybean) and roasted (sorghum and soybean) grains were produced. The microbial analysis of the formulated weaning food blends revealed that all the formulations are safe for consumption. From the results of the sensory evaluation, it was concluded that fermentation and roasting processes improved the degree of acceptability of the various weaning foods blends. Therefore, every raw material to be used for the production of weaning foods should be pretreated for safety and enhancement of organoleptic quality.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Production and functional property of maize-millet based complementary food blended with soybean

Akinsola A. O.[1*], Idowu M. A.[2], Babajide J. M.[2], Oguntona C. R. B.[3] and Shittu T. A.[2]

[1]Department of Home Economics, Federal College of Education (Special), Oyo, Oyo State, Nigeria.
[2]Department of Food Science and Technology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.
[3]Department of Nutrition and Dietetics, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

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Traditional complementary foods are mainly based on cereal grains which when cooked get gelatinized and swollen thereby making the diet viscous and bulky for infants and young children. This study was carried out to investigate the effect of fermentation, germination, and roasting methods on the functional properties of maize-millet-soybean mix with a view to producing less bulky and nutrient dense complementary food. Fermented, germinated, roasted, and untreated (control) grains were dried in an air oven at 55°C for 48 h to 10% moisture content, milled and sieved separately into fine flours (450 microns). Four complementary food samples were formulated and analyzed for wettability, dispersibility, water absorption capacity, swelling power, solubility index and pasting properties. Results showed that fermentation, germination, or roasting methods significantly ($p<0.05$) affect functional property of the complementary food samples. The swelling power of fermented sample was higher, while solubility of germinated sample was higher than other samples. The water absorption capacity of the complementary food samples ranged from 1.27 in germinated maize-millet-soybean to 1.61 in control sample. Fermented sample had the highest peak, trough and final viscosities, while germinated sample had the least. The study showed that germination significantly reduced water holding capacity and swelling power of the complementary food, and is recommended for producing nutrient dense complementary from maize-millet-soybean mix.

Key words: Complementary food, gelatinize, functional property, processing method.

INTRODUCTION

Complementary foods play a vital role on child growth and development since it complements for both nutritional and developmental needs of the infant when breast milk alone is no longer sufficient (Temesgen, 2013). According to WHO (2003), good quality weaning food must have high nutrient density, low viscosity, bulk density and appropriate texture along with high energy, protein and micronutrient contents and should have a consistency that allows easy consumption (Balasubramanian et al., 2014). Several studies have

*Corresponding author. E-mail: akinsola4gold@gmail.com, Tel: 2348034800734.

reported that most of the complementary foods consumed by the infants in many parts of world are deficient in essential macronutrients and micronutrients leading to malnutrition, which is one of the serious problems in developing countries. Protein energy malnutrition (PEM) generally occurs during the crucial transitional phase when children are weaned from liquid (that is, breast milk) to semi-solid or fully adult (family) foods. Milk and cereals are nutrient-rich sources that are commonly used in complementary foods (Aderonke et al., 2014), but are associated with different medical conditions like allergies and lactose and gluten intolerance.

Various blends of cereal grains with legumes have been developed through fermentation, germination, and or roasting. While functional properties of such food products during product formulation have been frequently neglected, more emphasis is placed on nutritional quality and quantity. Functional properties of complementary foods vary with type of grains, processing and cultural practices of the people. Ikegwu (2010) reported that type of starch granules present in the complementary foods significantly affect functional properties when its absorb water, swell and thickens to form a paste, with an accompanying change in appearance of the heated suspension. The transition from a suspension of starch granules to a paste when heated is accompanied with increase in viscosity and bulkiness. Many factors may also influence the degree and kind of association that may occur. The amount of water-bound associated with starch granules which increase as the heat starts to disrupt the inter-granules influences the swelling and functional properties of the flour.

The increase in paste viscosity when a hot paste is cooled is governed by the retrogradation tendency of the starch granules, and largely determined by the affinity of hydroxyl groups in one molecule for another which occurs mainly between the amylose molecules (June et al., 1991). Moreover, the high cost of commercial complementary foods coupled with household food insecurities and global economic meltdown now demands for effective strategies for improving the nutritional status of infants and young children by promoting the use of high quality complementary foods which could be of better functional properties and high nutrient dense, low dietary bulk and viscous at cottage level production. This study is therefore designed to develop nutrient-dense, safe, low-cost complementary food from the combination of fermentation, germination, and roasting methods on the functional properties of maize-millet-soybean mix for possible use as a complementary food for infants and young children.

MATERIALS AND METHODS

The yellow maize (*Zea mays*), finger millet (*Eleusine coracana*), and soya beans (*Glycine max*) used in this study were purchased at Lafenwa Market, Abeokuta, Ogun State, Nigeria.

Preparation of control samples

The three raw materials of four kilogram each were divided into four portions and each portion of the raw material was subjected to processing method of fermentation, germination, and roasting, while the fourth portion served as control. A portion of each of the raw material were thoroughly cleaned to remove extraneous material, winnowed, washed, drained and dried at 55°C for 48 h to bring the moisture content to about 10%. The dried samples were first re-winnowed before milling using locally fabricated machine and sieved to approximately mesh size of 450 microns. The maize, millet, and soybean flours obtained were mixed at a ratio of 50:30:20, respectively, and served as control samples.

Preparation of fermented flours

Each portion of the raw materials was soaked with distilled water in volume of water three times its weight (ratio of 1:3 weight/volume) and allowed to ferment in closed plastic bucket at 28°C for 48 h as described by Adeyemi and Beckley (1986). After fermentation, each of the raw materials was drained in a cleaned plastic sieve for 10 min, re-washed, and dried at 55°C for 48 h to bring the moisture content to about 10%. The sample was then packaged separately in airtight plastic container till further use.

Preparation of sprouted flours

Another portion of each raw material was germinated using the method described by Kulkarni et al. (1991). Each cleaned and washed grain were soaked in a volume of water three times the weight of grains (3:1) for 12 h in a container at ambient temperature. The steeping water was drained off using cleaned plastic sieve. Each of them was then spread on a jute sack placed on a wooden platform and covered with another jute sack for germination at room temperature (28°C) for 48 h and watered every 12 h to enhance the sprouting processing. After 48 h, the germinated samples were collected and washed, drained and dried at 55°C for 48 h to bring the moisture content to about 10%. The sample was then packaged separately in airtight plastic container till further use.

Preparation of roasted flours

The last portion of each raw material was clean, washed and allowed to drain for 10 min, transferred to aluminum trays, and roasting at 120 ± 5°C (maize and millet), and 130 ± 5°C (soybeans) for 10 and 15 min, respectively. All the processed (fermented, sprouted and roasted) grains were allowed to cool, winnowed and milled using a milling (locally fabricated) machine and sieved to approximately mesh size of 450 microns. The maize, millet, and soybean flours obtained were mixed at a ratio of 50:30:20, respectively for each treatment. The sample was then packaged separately in airtight plastic container till further use.

Functional properties determination

Bulk density was determined by the method of Wondimu and Malleshi (1996), while wettability index was done according to the

Table 1. Functional properties of maize-millet-soybean complementary foods.

Functional property	SC	SF	SG	SR
Bulk Density	0.58±0.09[a]	0.66±0.07[a]	0.60±0.01[a]	0.59±0.00[a]
Wettability (s)	10.63±0.13[a]	11.78±1.26[a]	25.54±2.97[c]	14.91±0.98[b]
Dispersibility (%)	65.75±1.40[a]	68.75±1.37[b]	63.50±0.21[a]	70.25±2.76[b]
Foaming Capacity (cm^3)	1.84±0.23[a]	12.50±0.14[c]	14.00±0.71[d]	4.76±0.31[b]
Water Absorption Capacity (g/cm^3)	1.61±0.24[a]	1.46±0.55[a]	1.27±0.70[a]	1.45±0.23[a]

Values are mean ± standard deviation of triplicate scores. SC: Control sample; SF: Fermented maize-millet-soybean; SG: Germinated maize-millet-soybean; SR: Roasted maize-millet-soybean. Mean values in the same row with different superscript are significantly different ($p< 0.05$).

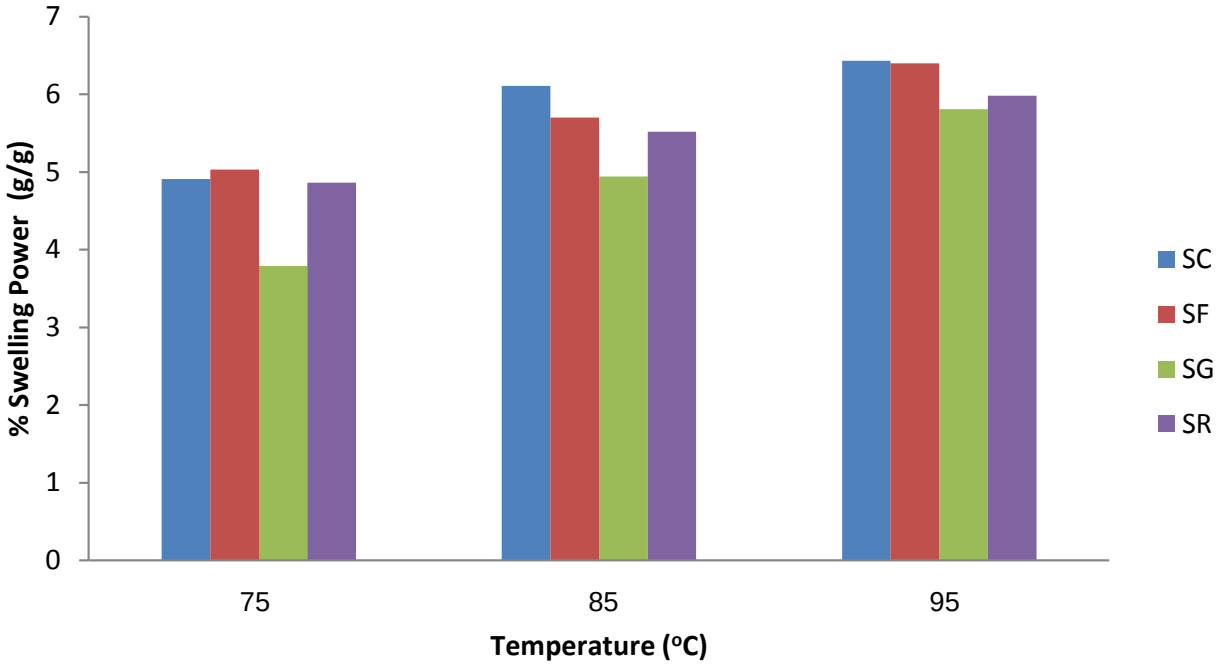

Figure 1. Swelling power of maize-millet-soybean complementary flours. SC: Control sample; SF: Fermented maize-millet-soybean; SG: Germinated maize-millet-soybean; SR: Roasted maize-millet-soybean.

procedure described by Okezi and Bello (1988). Dispersibility was carried out according to the method described by Kulkarni et al. (1991). The method of Padmashree et al. (1987) was used for the determination of foaming capacity, while water absorption capacity was determined using the method described by Sosulski (1962). The swelling power and solubility index of each sample was determined using the method of Leach et al. (1959), while pasting characteristics was determined by the method described by Ikegwu (2010).

Statistical analysis

A one-way analysis of variance and Duncan's test were used to establish the significance of differences among the mean values at the 0.05 significance level. Results were expressed as mean of triplicate analyses. The statistical analyses were performed using SPSS software (Systat statistical program version 21, SPSS Inc., USA).

RESULTS

The functional properties of the complementary food samples are presented in Table 1. Bulk density of the complementary food samples ranged from 0.58 in SC (control) sample to 0.66 in SF (fermented) sample. There is no significant difference ($p<0.05$) in the bulk density values. The wettability ranged from 10 in SC (control) sample to 18 s in SFR (fermented-roasted) sample, while dispersibility ranged from 63.5% in SG (germinated) sample to 70.25% in SR (roasted) sample. The swelling power (Figure 1) at 75°C for the complementary flours ranged from 3.79 to 5.18 for SG (germinated) sample and SF (fermented) sample, respectively, while solubility index (Figure 2) at 75°C ranged from 1.53 in SR (roasted) sample and 14.27 in SG (germinated) sample. At 85°C,

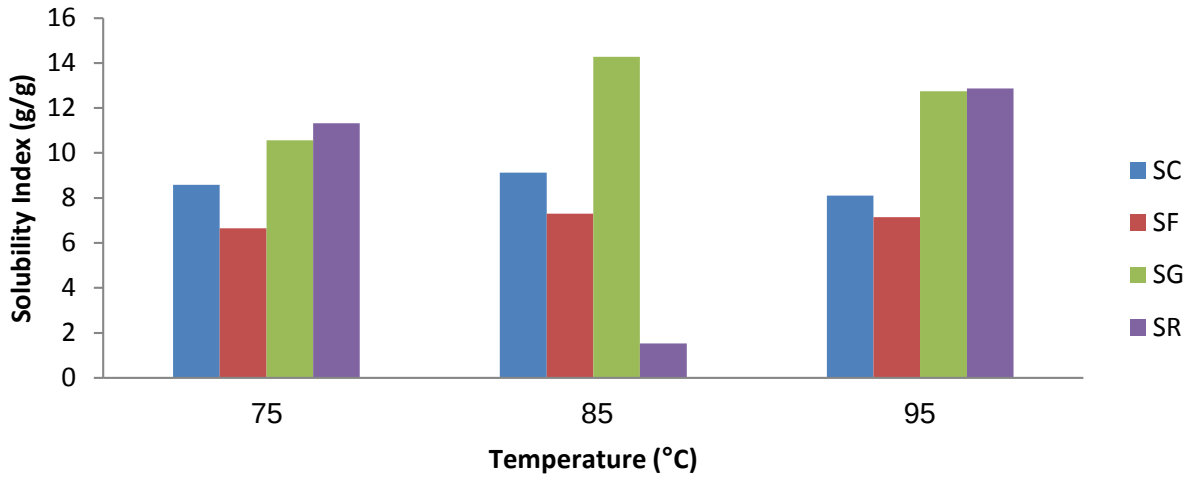

Figure 2. SC: Control sample; SF: Fermented maize-millet-soybean; SG: Germinated maize-millet-soybean; SR: Roasted maize-millet-soybean.

Table 2. Paste property of the complementary samples.

Parameter	SC	SF	SG	SR
Peak viscosity (RVU)	26.71±0.53^{d}	19.66±0.41^{c}	3.34±0.23^{a}	5.92±0.47^{b}
Trough (RVU)	21.96±0.18^{d}	18.00±0.59^{c}	2.96±0.30^{a}	4.71±0.53^{b}
Breakdown (RVU)	1.75±0.35^{ab}	1.46±0.18^{a}	6.29±0.06^{c}	1.21±0.06^{a}
Final viscosity (RVU)	42.21±0.53^{d}	32.25±0.35^{c}	0.50±0.24^{a}	18.92±0.70^{b}
Setback (RVU)	27.25±0.35^{d}	24.25±0.35^{c}	3.46±0.06^{a}	14.21±0.18^{b}
Peak Time (min)	6.54±0.53^{b}	6.94±0.04^{c}	4.17±0.00^{a}	6.86±0.03^{bc}
Pasting Temp (°C)	50.25±0.07^{a}	50.28±0.11^{a}	50.30±0.00^{a}	50.30±0.07^{a}

Values are mean ± standard deviation of triplicate scores. SC: Control sample; SF: Fermented maize-millet-soybean; SG: Germinated maize-millet-soybean; SR: Roasted maize-millet-soybean. Mean values in the same row with different superscript are significantly different ($p< 0.05$).

the swelling power of the complementary food samples ranged from 4.94 to 5.71 for SG (germinated) sample and SC (control) sample respectively, while solubility index at 85°C ranged from 1.63 in SR (roasted) sample to 13.89 in SG (germinated sample). Swelling power at 95°C ranged from 5.82 in SG to 6.17 in SC (control) sample, while solubility index at 95°C ranged from 7.23 in SF (fermented) sample to 13.22 for SR (roasted) sample, respectively.

The pasting characteristics of the complementary food samples are as presented in Table 2. Peak viscosity and trough of all the samples were significantly affected ($p>0.05$) when compared with the control sample. The result shows that germinated sample reach their peak viscosity and trough earlier than fermented and roasted samples. Breakdown viscosity ranged from 1.21 RVU to 6.29 RVU in SR and SG samples respectively, while final viscosity during heating ranged from 0.50 RVU in SG (germinated) sample to 42.21 RVU in SC (control) sample. The setback value ranged from 3.46 RVU to 27.25 RVU for SG (germinated) sample and SC (control) sample, respectively, while peak time ranged from 4.17 min in SG sample to 6.94 min in SF sample. There were significant differences ($p<0.05$) in the setback of the complementary foods. There is no significant difference ($p>0.05$) in pasting temperature of all the complementary food samples.

DISCUSSION

Fermentation, germination and roasting methods employed in this study significantly reduced ($p<0.05$) the water absorption capacity and viscosity of the complementary food samples and could enhance the volume consumed per meal. Germination reduces

swelling power better than other processing methods employed in this study. This result agreed with that of Wadud et al. (2004), who work on vegetable protein-based complementary foods, complementary foods based on cereals and legumes, and Ezeocha and Onwuka (2010), who worked on the physicochemical and nutritional quality of maize and soybean complementary foods, respectively. There were no significant differences ($p>0.05$) in bulk densities of the complementary food with values between 0.58 and 0.66. Low bulk density of food products had been reported to provide nutrient dense meal for infants and young children, as more of the products can be eaten resulting in high nutrient intake per meal for the baby (Nnam, 2000). Bulk density could also be affected by moisture content and reflects particle size distribution of the complementary flours (Wadud et al., 2004). Complementary foods were also significantly different ($p<0.05$) in their wettability and dispersibility. Germinated sample had the highest wettability among the other formulated samples, while roasted sample had the highest dispersibility. The control sample however, had the lowest wettability and dispersibility. This showed that germination and roasting could increase wettability and dispersibility of food products. These results are in agreement with Ezeocha and Onwuka (2010) work on complementary foods based on cereals and legumes that germination and/or roasting significantly affect wettability and dispersibility of soybean based complementary foods. The zero foaming capacity observed in the heat treated (roasting) complementary food sample of this study was similar to those observed with heat treated flours by other authors (Padmashree et al., 1987).

Obatolu and Cole (2000) earlier reported that mild heat treatment caused surface denaturation of protein and resulted in better foaming properties of complementary blends of soybean and cowpea with un-malted and malted maize. Prinyawiwatkul et al. (1997) also reported that denaturation decreases protein solubility, which in turn decreased foaming capacity of cowpea based formulated complementary foods. These authors also reported that foam volume and specific gravity are indices of texture lightness of food products. This result agreed with the work of Solomon (2005) and Anigo et al. (2009), who reported that heat treatment prevented foaming in dry flours or powder of sorghum, millet, and acha, while germination enhance foaming capacity in dry flour or powder of sorghum, millet, and acha. Wadud et al. (2004) and Solomon (2005) reported that processing methods, time and temperature amongst other factors affect the functional properties of a food product. The complementary foods were significantly different ($p<0.05$) in their water absorption capacity (WAC) values. The SC (control) and SF (fermented maize-millet-soybean) had the highest WAC as they retained water more than other complementary food samples. Increase in protein content after sprouting might be due to enzymatic changes, hormonal changes or a compositional change following the degradation of other constituents (D'souza, 2013). The enzymes produced during sprouting lead to the hydrolysis of starch and proteins with release of sugar and amino acids. Proteolytic enzymes improves amino acid availability mainly lysine, methionine and tryptophan (Bolanle et al., 2012). Water absorption capacity (WAC) observed in this study is probably related to the low viscosity patterns and weak internal organization resulting from starch granules as reported by Singh et al. (2003), who worked on cookie-making properties of corn and potato flours, respectively.

This study showed that germination, fermentation, and roasting methods significantly ($p<0.05$) decreased WAC of the complementary food samples. Germinated complementary food samples had the lowest WAC as compared with samples processed using other methods. This may be as a result of the malting process which hydrolyzed starch and thereby reduce the water holding capacity. The result agreed with the earlier reports of Ezeocha and Onwuka (2010) that germinated food flours generally had low WAC as a result of hydrolyzation of starch granules, thereby reducing their water holding capacity. Flour from SG samples had the least swelling power, while SC (control) sample had the highest. All the complementary food samples were significantly different ($p>0.05$) in their swelling capacity. Generally, the low level of swelling power obtained for germinated samples may have been caused by the presence of protein, lipid, and amylase activity which increased during germination of the seed. Hence, when cooked, the hydrolyzed starch swells less, retains less water, has lower viscosity, and increases the nutrient and energy densities per unit volume of the blended flour. Significant differences ($p<0.05$) were observed in the solubility index of all the complementary foods.

Flour from germinated sample had the highest value, while flour from SR (roasted) sample had the least solubility index. It is possible that the heat treatment (roasting) weakened and destroyed protein structure of SR samples thereby inhibiting its solubility. Weaning food of high viscosity and high bulk density is usually unacceptable to infants as it makes feeding taskful and causes choking. Infants can easily consume sufficient quantity of food if it is low in viscosity/bulk density because it allows incorporation of more solids in mixture leading to an increase in nutrient density of the gruel. Low viscosity and low bulk density weaning food with a high nutrient content is a desirable characteristic in complementary foods (Onweluzo and Nwabugwu, 2009). There were significant differences in peak viscosities of all the complementary flours. Peak viscosity of the SF (fermented maize-millet-soybean) was generally higher than peak viscosities of samples processed using other methods (germination and roasting).

Peak viscosity is indicative of the strength of pastes,

which are formed from gelatinization during processing in food applications. It may also be as a result of amylase activity in the complementary flour which resulted in the viscosity changes (Niba et al., 2001). There were significant differences ($p<0.05$) in trough of the complementary flours with the fermented sample having the highest value. Trough is the minimum viscosity value in the constant temperature phase of the RVA profile and measures the ability of paste to withstand breakdown during cooling (Ezeocha and Onwuka, 2010). This study showed that fermentation increased both peak and trough viscosity more than germination, roasting method. These results agreed with the reports of Wadud et al. (2004), Ezeocha and Onwuka (2010), and Ikegwu (2010), that germinated food products were less bulky, highly nutritional, and good functional property than fermented, or fermented combined with roasted food sample.

There were significant differences ($p<0.05$) in breakdown viscosity among the complementary food samples. Higher breakdown viscosity values were obtained in germinated sample compared with samples processed using other methods as a result of alpha-amylase that was developed during malting which might have degraded the starch granules in the raw food material. Adebowale et al. (2005) reported that the lower the breakdown viscosity, the lower the ability of the sample to withstand heating and shear during cooking. The results of this study showed that all the complementary samples had low breakdown viscosity. Hence, the blended flours would be able to withstand heating and shear stress during cooking because of their low peak and breakdown viscosity values. Less stability of starch paste or gel after cooling is often accompanied with low value of breakdown viscosity as earlier reported by Shimelis et al. (2006). This implies that gels of flour sample from SF (42.3 RVU) may be more stable after cooling compared to gels of flours obtained from using other processing methods (10.5 – 26.1 RVU).

There were significant differences ($P>0.05$) in final viscosities of the complementary flours. Final viscosity indicates the ability of starch-based food to form a viscous paste or gel after cooking and cooling (Ikegwu, 2010). The fermented sample had highest final viscosity, while germinated (SG) sample had the least value. The marked increase in final viscosity observed in the fermented sample might be due to alignment of chains of amylose in the starch as reported by Ikegwu (2010), and Niba et al. (2001) who reported that final viscosity are important in determining ability of a sample of material to form a gel during processing could be used to improve and optimize food texture; hence measurement of final and peak viscosities is relevant in food formulation. This study showed that fermentation and germination could influence setback viscosity of the complementary flours. High value (27.3 RVU) of setback viscosity was obtained in control sample, while least value (3.5 RVU) was obtained in germinated sample. Setback viscosity is an indication of gel stability and potential for retrogradation and syneresis, while Sanni et al. (2004) reported that the lower the setback during cooling of paste is a reflection of the retrogradation tendency of the paste. This means that control (SC) sample will retrograde faster after reconstitution for feeding while germinated (SG) sample will retrograde slowly. Also, fermented sample will retrograde faster than germinated and roasted samples.

There were significant differences ($p<0.05$) in the peak time of all the complementary foods. The same trend as observed for peak, trough, and final viscosities was also observed for peak time in all the complementary foods. The fermented sample had higher peak time than germinated and roasted samples. This is in agreement with the reports of Aguilera and Rojas (1996) that thermal time significantly has effect on rheological properties of whey protein cassava starch gels. Complementary food is usually prepared into gruel before been given to the child. The pasting temperature obtained in this study, shows that there were no significant differences ($p>0.05$) in all the complementary flours. Pasting temperature is the temperature at onset of rise in viscosity of a starch-based food product or material. When the temperature is above the gelatinization temperature, starch granules began to swell and viscosity on shearing (Adebowale et al., 2005; Ikegwu, 2010). These results are in agreement with the findings of Gernah et al. (2012) and Victor (2014), who reported that processing methods like sprouting and fermentation are valuable in reducing the viscosity of infant gruels, increase total solids and nutrient density of weaning food. This decrease in viscosity due to sprouting might be due to the enzymatic breakdown of macromolecules such as polysaccharides and polypeptides to smaller units, such as dextrins and peptides, respectively (Gernah et al., 2012).

Pasting characteristics is important in predicting the behaviour of food paste during and after cooking. Pasting temperature is one of the pasting properties which provide an indication of the minimum temperature required to cook a given sample, energy cost involved and other components stability (Ikegwu et al., 2009). Attainment of the pasting temperature is essential in ensuring swelling, gelatinization, and subsequent gel formation during processing. This result showed that the formulated complementary sample form a paste in hot water below boiling point, and functional property of the germinated sample was better than other processing method samples in terms of wettability, dispersibility, water absorption capacity, swelling and pasting property.

Conclusion

The study showed that production of maize-millet-soybean complementary food for infants and young

children using germination method is better than fermentation and roasting methods.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Freeze and spray drying of *Scaptotrigona bipunctata* (Lepeletier, 1836) pollen - development and physicochemical characterization

Maria Josiane Sereia[1*], Ana Luíza Sereia[2], Rejane Stubs Parpinelli[2], Érica Gomes de Lima[2], Adriele Rodrigues dos Santos[2], Fernando Antônio Anjo[2], Cláudio Gomes da Silva Júnior[2], Vagner de Alencar Arnaut de Toledo[2]

[1]Federal University of Technology – Paraná (UTFPR), Via Rosalina Maria dos Santos, 1233, ZIP 87301-899, Campo Mourão, Paraná, Brazil.
[2]State University of Maringá (UEM), Av. Colombo, 5790, ZIP 87020-900, Maringá, Paraná, Brazil.

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This study was carried out to evaluate human consumption of the pollen of *Scaptotrigona bipunctata* colonies stored in pots; it was lyophilized and spray-dried in arrays of arabic gum and hydrolyzed collagen which have been widely applied to get products with best technological features and highest biological activity. After the samples were prepared they were evaluated by physicochemical analysis, and the data were compared. *S. bipunctata* pollen lyophilized had product with higher antiradical activity than the pollen dried using spray dryer which only quantified the antioxidant activity of the antioxidant compound that was encapsulated and stabilized in the solid matrix material.

Key words: Biological activity, encapsulated pollen, lyophilized pollen, phenolic profile, stingless bees.

INTRODUCTION

Native bees occur in many tropical regions of the world, including all of Latin America and Africa, Southeast Asia and the North of Australia. However, much of the diversity of the species occurs in the Americas, with approximately 400 types described (Villas-Bôas, 2012). Bee breeding is practiced in almost all regions of Brazil, in the North and Northeast of the country; it is a sustainable alternative or additional form of income for small and medium farmers; it is greatly important in the use and sustainable management of the forest environment and preservation of biodiversity of the ecosystems (Venturieri, 2008).

The stingless bee, *Scaptotrigona bipunctata* (Lepeletier, 1836) belongs to the Meliponinae group popularly known as tubuna bee or straw cane due to the entrance of the colony that has a funnel shape, made of dark cerumen (Costa and Imperatriz-Fonseca, 2000). In the nest, the pots where they store honey and pollen can reach 2.5 up to 3.0 cm in height and the rearing combs can be built helically, with the presence of real cells on the border. They form very populous colonies comprising 2,000 to 50,000 bees, with highly defensive behavior; this often makes humans to destroy their nests, easily found in cavities pre-existing in tree trunks, mainly in the States

*Corresponding author. E-mail: vagner_abelha@yahoo.co.uk.

of Ceará, Minas Gerais, Rio Grande do Sul, Paraná, and São Paulo (Costa and Imperatriz-Fonseca, 2000). There is little knowledge about its management in rational hives, biology and pollen use as a floral resource in the different environments in which they inhabit.

In addition to honey, native stingless bees allow the potential exploitation of products and by-products with a high value, such as propolis, pollen and geopropolis (Silva et al*.,* 2014). The pollen of native stingless bees is deposited in the colony in specific pots, facilitating its exploitation. Natural pollen collected from flowers is processed in these pots by bees, which deposit enzymes in the pollen that initiates its digestion and helps its natural preservation, and then it is called 'saburá' in Brazil (Brasil, 2001; DerMardersian et al., 2005; Lima Neto et al., 2017).

Pollen has long been used, especially among natural food supporters, as a supplement to human diet for its richness in relation to proteins, lipids, vitamins and minerals (Komosinska-Vassev et al., 2015; Bogdanov, 2016). Although the chemical composition of a food such as pollen, which is rich in protein and possesses phytotherapeutic properties, is of great interest, it is rarely commercially available. When processed is sold in dehydrated portions, or blended with honey (Villas-Bôas, 2012). Another problem regarding the use by the food industry of natural products such as pollen is the fact that many of the constituents responsible for its biological properties, such as phenolic compounds and pigments with antioxidant activity, are highly susceptible to oxidation and volatilization and unstable in the presence of heat, light, and oxygen (Bobbio and Bobbio, 2001).

The rising of powdered foods has driven the food industry to carry out research activities in technology and innovation aimed at leveraging sales. Thus, it would be possible to convert poorly soluble granular structures into an easily dissolved, attractive fine powder, which preserves the nutritional part of the food, at a low cost and with a short operating time. This can be crucial in the decision to purchase this product for a large number of consumers and, in the short or medium term, can contribute to encouraging the management of stingless bees, the improvement of pollen production techniques and also contribute to the greater appreciation of products of Brazilian native stingless bees. Therefore, this study aimed to develop and characterize a new product from the encapsulation of the pollen of *S. bipunctata*, in arabic gum and hydrolyzed collagen matrices using spray-dryer and lyophilization drying methods.

MATERIALS AND METHODS

The experiment was carried out at Universidade Tecnológica Federal do Paraná (Paraná Federal Technology University), Campo Mourão Campus, Brazil from July 2015 to June 2016. Approximately 10 kg of pollen was collected from different beehives of *S. bipunctata* in the Mandirituba region of the state of Paraná. After collection, the pollen was manually cleaned and wrapped in polyvinyl chloride (PVC) bags, which were hermetically sealed and stored in a freezer at -20ºC until use. Synth brand arabic gum wall material (treatment I) and Sanavita brand hydrolyzed collagen (treatment II) were used for the production of microcapsules. In treatment III the pollen was lyophilized.

Spray drying method

The production of the microcapsules (Treatments I and II) was carried out according to Rocha et al. (2012). The encapsulating material and the pollen were dispersed in water at a 2:1:3 (m/m/m) ratios, and then filtered a standard fine mesh sieve (150 µm). For the production of microcapsules, the mixture was heated to a temperature of 70°C and mechanically shaken at 4000g for 5 min with a FISATOM model 713D shaker (Brazil). This movement continued during a cooling process in an ice bath until the mixture reached a temperature of 10ºC.

The spray drying process of the samples was performed using a LM-LABMAQ model MSD 1.0 (Brazil) spray dryer with a 1.00 mm diameter atomizer, incoming gas temperature of 130°C, drying air flow of 3.60 and a sample of 0.50 L/h. The samples were packaged in an amber flask and kept at room temperature.

Drying in a lyophilizer method

The pollen was dispersed in water at a 1:1 (m/m) ratio to obtain an ultra-rapid separation, with the minimum degradation of the product to be dried, filtered with a standard fine mesh sieve, placed in Petri plates and frozen in a PANASONIC (Brazil) ultra-freezer to -83ºC. Drying by lyophilization (treatment III) was performed in a LIOTOP lyophilizer; model L101 (Brazil), for 72 h at a temperature ≤ -40°C - compressor temperature and a pressure of approximately 50.00 µHg. The samples were kept in a desiccator and then crushed in a mortar, sieved and packaged in an amber flask and kept at room temperature.

Physical-chemical composition of the resultant products

Each parameter was analyzed in three repetitions: Moisture (%), total sugars, reducing and non-reducing (%), lipids (%), fixed mineral residue (%), proteins (%), total fiber (%), total energy value (TEV), ash (%), pH, water activity, hygroscopicity (g/100g). Apparent density (g/cm^3) was measured according to the Instituto Adolfo Lutz (2005) and AOAC (2012). The calories content (Kcal) and energy value (Kj) were calculated according to the National Sanitary Surveillance Agency (Anvisa, 2005).

Total phenols, total flavonoids and antioxidant activity

Obtaining pollen extracts

Using 50.00 mL falcon tubes, 0.40 g of the encapsulated samples was diluted in 10 mL of 80.00% methanol acidulated with 0.20% concentrated hydrochloric acid - 1:25 (w/v). For the lyophilized pollen, a 0.40 g sample was diluted in 20.00 mL of the same diluent, to create the lyophilized pollen extract, with an initial concentration of 1:50 (w/v).

The tubes were sealed, stirred in a QL901 BIOMIXER (Brazil) vortex mixer for 2 min and then placed in a CRISTÓFOLI Ultron2 model (China) ultrasonic bath, for 20 min to rupture the microcapsules. The encapsulated pollen extracts were placed in a NOVATECNICA model NT825 (Brazil) refrigerated centrifuge at 3000 *g* for 20 min and the supernatant was used for analysis.

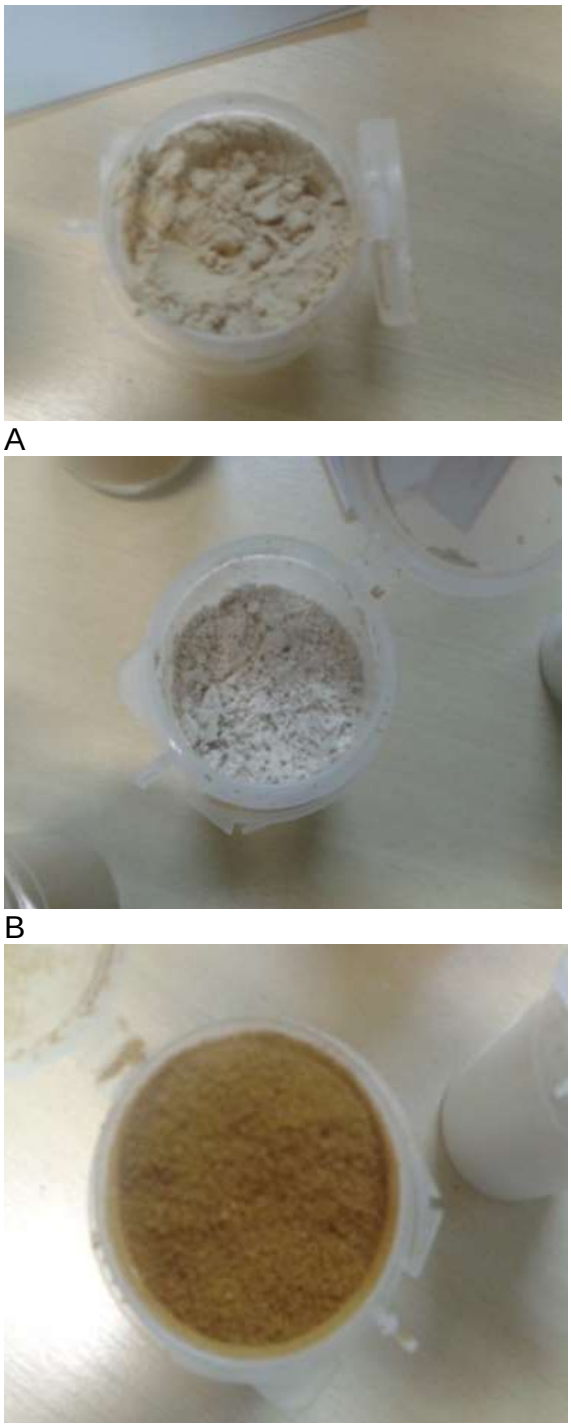

Figure 1. Microcapsules of pollen in arabic gum A, microcapsules of pollen in hydrolyzed collagen B and lyophilized pollen C.

Determination of phenolic compounds

The phenolic compounds were determined using the Singleton et al. (1999). The total phenol concentration was determined by interpolating the absorbance of the samples based on a calibration curve constructed with standard gallic acid - GA (Brazil). A calibration curve (0.00 to 1500.00mg GAE/L, r^2 = 0.9976) was constructed and the results expressed in mg GAE/g of pollen. Absorbance was measured in quartz cuvettes with a length of 765 nm in an Ocean Optics USB-650 (USA) UV-VIS spectrophotometer.

Total flavonoids

The flavonoid content present in the encapsulated and lyophilized pollen extracts was determined by Alothman et al. (2009). Total flavonoid concentration was determined by interpolating the absorbance of the samples based on a calibration curve constructed with standard quercetin, Sigma-Aldrich, 95.0% purity (USA). A standard calibration curve (50.00 to 500.00 mg QE/L, r^2 = 0.9941) was constructed and the results expressed in mg QE/g of pollen. Absorbance was measured in quartz cuvettes at 510 nm in an Ocean Optics USB-650-UV-VIS spectrophotometer.

Antioxidant and antiradical activities

Antioxidant activity was determined according to Roginsky and Lissi (2005). Antiradical effectiveness was evaluated with the DPPH (2,2-diphenyl-1-picryl-hydrazyl) method (Mensor et al., 2001). The scavenging activity of the DPPH free radical was expressed in terms of $EC_{50,}$ which represents the minimum concentration necessary for the antioxidant to reduce the initial concentration of DPPH by 50.00%.

Statistical analysis

The design was completely randomized, with three treatments, and the data were submitted to variance analysis at a 5% significance level. The means were compared by the Tukey test using SAS ver. 9.3 (2012).

RESULTS AND DISCUSSION

Characteristics of the resultant products

The *S. bipunctata* encapsulated pollen samples in hydrolyzed collagen and arabic gum matrices and lyophilized pollen, while not sensorial evaluated, had a fine texture; it was homogenous, had slightly yellow color, variable aroma and citrus flavor and floral, and had characteristic of natural pollen (Figure 1 a, b, c).

Physicochemical composition of the resultant products

The results of the physicochemical parameters of the *S. bipunctata* encapsulated pollen samples in hydrolyzed collagen and arabic gum matrices are presented in Table 1.

The means of the physicochemical composition of the dried pollen samples using the spray dryer, hydrolyzed collagen and arabic gum wall material and lyophilized pollen differed statistically ($p<0.05$) in all tested characteristics (Tables 1 and 2).

Table 1. The physicochemical parameters of pollen of *Scaptotrigona bipunctata* encapsulated in hydrolyzed collagen and arabic gum matrices and lyophilized

Treatment	M (%)	RS (%)	TRS (%)	NRS (%)	L (%)	GF (%)	P (%)	Cal (Kcal)	EV (Kj)	A (%)
Pollen with hydrolyzed collagen	4.64^{a} ±0.52	7.51^{a} ±0.26	8.86^{a} ±0.19	1.30^{a} ±0.22	2.08^{a}±0.15	2.12^{a}±0.02	82.25^{a}±0.76	383^{a}±0.00	1609^{a}±0.00	1.35^{a}±0.09
Pollen with gum arabic	4.11^{a}±0.42	6.05^{b}±0.13	18.52^{b}±0.44	11.97^{b}±0.36	1.74^{a}±0.16	62.90^{b}±0.71	10.28^{b}±0.22	131^{b}±0.00	550^{b}±0.00	2.45^{b}±0.11
Lyophilized pollen	2.61^{b}±0.11	16.08^{c}±0.16	17.67^{c}±0.20	1.52^{a}±0.17	5.56^{b}±0.34	41.43^{c}±0.45	30.04^{c}±0.51	241^{b}±0.00	1012^{b}±0.00	2.69^{b}±0.15
F Value	22.23	2403.30	946.84	1610.71	249.32	899.20	14165.10	ns*	ns*	115.28
P Value	0.0017	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

M %, moisture; RS %, reducing sugar; TRA %, total reducing sugar; NRS %, non-reducing sugar; L %, lipids; GF %, gross fiber; P %, proteins; Cal Kcal, calories in 100g; EV kJ, energy value in 100g; A %, ashes. ns, not significant. Means followed by different letters in the same column differ significantly from one another based on the Tukey test P< 0.05.

Table 2. The physicochemical parameters of pollen of *Scaptotrigona bipunctata* encapsulated in hydrolyzed collagen and arabic gum matrices and lyophilized.

Treatment	pH	wa	H (%)	AD (g/mL)	Tpe (mgGAE/g)	TFl (mgQE/g)	AA (mg/g)
Pollen with hydrolyzed collagen	4.77^{a} ±0.02	0.25a±0.002	15.14^{a}±0.12	0.49^{a} ±0.01	93.33^{a} ±8.86	9.52^{a} ±7.64	1.94^{a} ±0.15
Pollen with gum arabic	3.97b ±0.03	0.30b ±0.004	12.33b±1.47	0.45b±0.00	56.57b±4.49	7.76a±7.87	2.55b±0.09
Lyophilized pollen	3.74c±0.03	0.25a±0.002	11.13b±0.27	0.39c±0.01	241.57c±1.70	50.60b±5.22	0.69c±0.02
F Value	1240.78	1347.52	16.99	97.13	849.20	185.91	267.58
P Value	<0.0001	<0.0001	0.0034	<0.0001	<0.0001	<0.0001	<0.0001

wa, water activity; H %, hygroscopicity; AD g/mL, apparent density; TPe mgGAE/g, total phenols; FlT mgQE/g, total flavonoids; AA mg/g, antioxidant activity; ns, not significant. Means followed by different letters in the same column differ significantly from one another based on the Tukey test P< 0.05.

The chemical composition of stingless bee pollen, that quantified its components in a natural form, dried in the oven (Bogdanov, 2016) using a spray dryer or lyophilization is rare, making it difficult to compare with the data obtained in this study. However, despite this lack of information, it was observed that *S. bipunctata* spray dried using hydrolyzed collagen and arabic gum as capsule wall material had a greater protein and fiber content, respectively, while pollen dried in a lyophilizer presented a more balanced chemical composition in terms of carbohydrates, protein, fiber and lipids, low moisture content and hygroscopicity (Figure 1). These differences are attributed to the chemical drying process employed, and also to the chemical composition of the materials used as walls.

The mixing of hydrolyzed collagen in water resulted in less viscous solutions that were more suitable for the encapsulating process by promoting the efficiency of the atomization in the drying stage; it resulted in a greater opacity of the encapsulating matrix. The use of hydrolyzed collagen considerably increased the protein content of the final product, resulting in nutritional value. This makes it an excellent option as a supplement. arabic gum, meanwhile, is a polysaccharide consisting essentially of D-galactose, L-arabinose, L-ramnose, water and glycoproteins (Andrade et al., 2013) which possess important technological functions such as acting as a soluble dietary fiber as well as a body or texture agent in industrial preparations.

Rebelo et al. (2016) reported values for moisture from 37.12 to 53.39%; protein from 24.00 to 37.63%; lipids from 6.47 to 10.81%; ashes from 2.74 to 4.03%; crude fiber from 9.30 to 13.65%; carbohydrates from 25.66 to 44.27% energy from 331.33 to 350.47 kcal/%; pH from 3.34 to 3.70; total solids from 46.60 to 62.87% and water activity from 0.85 to 0.91, respectively. Barajas et al. (2012) found a mean protein content of 28.00%; fat from 4.00 to 5.00%; ash, 2.10 and 3.30% in pollen from mountainous and pasture regions of Colombia, but both with a mean daily

temperature of 14ºC.

In pollen samples *in natura* of different species of native stingless bees collected in the Amazon region, average values of 36.90% moisture, 19.50% proteins, 4.00% lipids, 2.10% ash and 37.50% carbohydrate were found (Oliveira et al., 2007). However, Bogdanov (2016) reported that dried bee pollen at 40°C has average 32.8% proteins, including 11.50% essential amino acids, 40.7% reducing sugars, 3.70% sucrose, 12.80% lipids, 0.19% vitamin C, 0.07% β-carotene and a maximum of 6.00% moisture; regardless of its floral origin, it is rich in essential nutrients (Bogdanov, 2016). Melo and Almeida-Muradian (2011) determine the moisture content of bee pollen dried samples by six methods, and reported moisture values of 3.96% with oven drying at 100ºC and 10.02% with lyophilization.

Some countries have official standards for the identification and evaluation of the bee pollen quality, such as: Brazil (Brazil, 2001), Bulgaria (Bulgarian Standard 2567111-91), Poland (PN- R- 78893) “Obnóza pylkowe.” - Polish bee pollen legislation (Campos et al., 2008). These countries have established minimum requirements for the moisture content of dried pollen: Brazil (2001), maximum 4.00%, Bulgaria, maximum 10.00% and Poland, maximum 6.00%. Campos et al. (2008) proposed a classification of the pollen from the *A. mellifera* for commercial purposes based on its moisture content. The original product had an initial content of 20.00 to 30.00%, which dropped to no greater than 6.00% following drying at temperatures not above 42°C.

Sugars in pollen can vary from 13.0 to 55.0% including glucose, fructose and sucrose (Bogdanov, 2016), 25.70% (Kędzia and Hołderna-Kędzia, 2012), or up to 40.70% (Komosinska-Vassev et al., 2015). In the present study, the reducing sugar, total reducing sugar and sucrose contents were greater in the pollen samples dried with a lyophilizer than those obtained from a spray dryer (Table 2).

Comparing the lipid, fiber, protein, caloric and energetic values (Tables 1 and 2), the final products obtained displayed distinct technological and nutritional characteristics. The fiber and protein grade was significantly greater in pollen samples encapsulated with arabic gum and in collagen, respectively, while lipid content was significantly greater in the lyophilized pollen samples. Szczesna (2006) obtained a mean content of 5.10%, of which 0.40% was represented by long-chain essential fatty acids such as linoleic, linolenic and arachidonic, while Komosinska-Vassev et al. (2015) reported a mean total lipid content of 12.80%.

Mineral content is almost 1.60%, including the macro-nutrients calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K) and micronutrients iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), silicon (Si) and selenium (Se), the latter in a quantity of 0.02% (Kędzia and Hołderna-Kędzia, 2012). In dried pollen samples taken from different regions of Bulgaria, Dinkov and Stratev (2016) reported the minimum values of 0.91% and maximum values of 2.30% from the Karlovo and Sliven and Lovech regions, respectively. Komosinska-Vassev et al. (2015) described that mean mineral content of 4.00% in pollen. Both the minerals and the type of wall material used can provoke changes in the chemical composition of the final product, reducing its initial pH which is, on average, from 4.00 to 6.00 (Brazil, 2001) (Table 2).

For water activity (Table 2), all values were within the expected range for atomized and lyophilized products, and also within the recommended range to ensure the microbiological, enzymatic, non-enzymatic and oxidative stability of the resultant products (Bobbio and Bobbio, 2001). The pollen extract encapsulated in arabic gum displayed a greater water activity than pollen encapsulated with collagen, and lyophilized pollen. Silva et al. (2013) concluded that the water activity depended on the chemical nature of the hydrocolloids used as wall material. In this case, the chemical structure of the hydrolyzed collagen, due to having a high number of hydrophilic groups along the protein polymer chain, resulted in a higher water binding capacity than the arabic gum encapsulated pollen, significantly reducing the water activity of the pollen encapsulated with collagen. The intensity of this interaction is essential for biocontrol during the storage and marketing of products subjected to drying processes, as moisture content affects water activity, enzymatically and through microbiological stability, and hence the shelf life of the product, as it is the component that causes rapid fermentation and food spoilage (Isengard et al., 2006).

Hygroscopicity and appearance varied similarly in all three treatments (Table 2). The dried particles encapsulated with arabic gum exhibited a lower hygroscopicity tendency than the particles encapsulated in collagen or the lyophilized pollen which, in a saturated environment, can result in the modification of the chemical composition and appearance, favoring microbial multiplication. In studies on the atomization of other types of food, the mean variation in the density values ranged from 0.74 to 0.92g/mL (Botrel et al., 2012).

Total phenols, flavonoids and antioxidant activity

The maximum values of total phenols, flavonoids and antioxidant activity of the lyophilized pollen were 241.57 mg GAE/g , 50.60 mg QE/g and 0.69 mg/g, respectively, which were significantly higher ($p < 0.05$) than the values of the pollen samples encapsulated with hydrolyzed collagen or arabic gum (Table 2). Drying with a lyophilizer in a vacuum was more efficiently concentrated the functional components than drying with a spray drier, in which the particles were formed by the aggregation of substantial quantities of wall material. Bogdanov (2016) reported that pollen should be dried at the lowest

temperatures possible, with a maximum of 30°C, with the best alternative being freeze drying.

The results presented a correlation between water content and wall material and the total polyphenol content and antioxidant activity of the pollen preparations. Prelipcean (2012) reported that the storage conditions and the concentration of methanol used as a solvent observed that the total polyphenol content (mg GAE/g) varied from 24.73 to 28.8, and from 21.67 to 26.50, using 96 and 70% methanol, respectively. Stoia et al. (2015) studied that the total phenolic compound content in pollen and propolis in five locations in Romania observed a maximum value of 4.93 ± 0.88 mg GAE/g from dried pollen mass, significantly less than 9.71 ± 0.80 mg GAE/g found for propolis. All these values were significantly lower than those found in the present study for *S. bipunctata* pollen. Although, it was difficult to compare the results found in this study with the previous studies, as the authors used various solvents and extraction conditions and work with samples from different regions with different concentrations of polyphenols.

Menezes et al. (2010) analyzed that the antioxidant potential of pollen produced by Africanized honeybees from different species of plants had values from 0.72 to 1.99 mg QE/g of pollen from *Mimosa pudica* and from 1.55 and 2.50 mg QE/g from *Eucalyptus* pollen. Moreira et al. (2008) described the values of antioxidant activity for propolis extracts of 33.00 mg/g for a concentration of 0.001 g/L and 94.00 mg/g for a concentration of 0.02 g/L.

Silva et al. (2013) reported the antioxidant activity of propolis extract dried by spray dryer with different proportions of arabic gum and modified starch, with concentrations from 2.5 to 5.00 mg/mL. Nori et al. (2011) applied the same technique to encapsulate propolis extracts using protein isolated from soya and pectin as encapsulating agents and obtained propolis in powder form. This was stable, and had its own antioxidant and antimicrobial activity against *Staphylococcus aureus,* and the property of controlled release in foods.

When analyzing the mean antioxidant activity values of the samples dried in a *spray dryer* with hydrolyzed collagen and arabic gum, it was possible to identify significant differences ($p<0.05$). Such reactions are the bases of the method used in this study for the evaluation of antioxidant activity. Moreira et al. (2008) attributed the differences observed to external factors such as handling, storage, and temperature.

The use of hydrolyzed collagen in the encapsulation of pollen allowed an increase in phenol concentration, which was accompanied by an increase in antioxidant activity. An antioxidant to be applied in industrial formulations should be used at low concentrations and present a low risk to the consumer (Bobbio and Bobbio, 2001). These attributes motivated this investigation, which attempts to make available to the market a concentrated option of possible compounds to be consumed as a supplement, industrial ingredient or gastronomic delicacy.

The authors recommended further research with this product such as: submitting the products to sensory testing to understand if pollen encapsulation can attenuate characteristic flavor and odor. Other encapsulation matrices such as starch, maltodextrins and other polysaccharides generate more economically accessible. Kinetic release assays and the testing of the involved mechanisms should be done; the spray dryer drying parameters need to be improved and the antimicrobial activity of the products obtained needs to be evaluated.

Conclusion

From a nutritional perspective, regardless of the type of drying used, the preparations of *S. bipunctata* pollen studied can be considered an excellent option as a nutritional supplement. This substance significant has phenolic content and capacity to eliminate free radicals, with promising nutritional and physiological implications with a positive effect on health promotion. However, lyophilized *S. bipunctata* pollen resulted in a product with considerably greater anti-radical activity than pollen dried in a spray dryer.

The use of hydrolyzed collagen considerably increased the final protein content, enhancing its nutritional value and resulting in an excellent option for supplement. Meanwhile, the arabic gum helped to obtain a product rich in fiber and with a low energy value, encouraging its use as an ingredient or additive.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Enhancement of eritadenine production using three carbon sources, immobilization and surfactants in submerged culture with shiitake mushroom (*Lentinula edodes*) (Berk.) Singer)

Byron Durán-Rivera[1*], José Rodrigo Moreno-Suárez[2], Felipe Rojas Rodas[1], Kelly Marcela Valencia Jiménez[3] and Dagoberto Castro–Restrepo[1]

[1]Facultad de Ingeniería. Unidad de Biotecnología vegetal. Universidad Católica de Oriente. Colombia.
[2]Facultad de Agronomía. Grupo de investigación en sanidad vegetal. Universidad Católica de Oriente. Colombia.
[3]Centro de la ciencia y la investigación farmacéutica. CECIF. Colombia.

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In this study, the effect of three carbon sources (mannitol, minced potato and sucrose), two immobilization substrates (alginate and wood cylinders), and three surfactants (Tween 20, Tween 40 and Tween 80), were evaluated on eritadenine production using shiitake (*Lentinula edodes*) mycelium under submerged cultivation, in shake flasks within 20 days. Eritadenine and biomass were measured by HPLC and gravimetrically, respectively. Alginate immobilization of mycelium promoted significant enhancement of eritadenine yields of 88 mg/L, compared to the control (8.7 mg/L) and wood immobilization (14.8 mg/L). Likewise, eritadenine yields (72.4 mg/L) were enhanced by adding surfactant tween 20 to the broths in 0.5%, than control (8.7 mg/L) without surfactant. Tween 40 and 80 did not improve eritadenine yields, but both produced better biomass values (superior to 5 g/L) than the control (3.9 g/L). All carbon sources (sucrose, mannitol, mince potato, and glucose as control) produced similar low eritadenine yields, with best results (10.2 mg/L) by sucrose, although glucose produced the best biomass yields of 3.9 g/L. Also, carbon sources and the best biomass values did not show significant effect on eritadenine production. pH values in the best eritadenine yielding fermentations went down from 6 to 3-4, but pH had a low correlation with eritadenine yields. Finally, all data obtained in the present study are useful for optimizing culture conditions, towards industrialization of this important health improver metabolite (eritadenine).

Key words: Eritadenine, shiitake mushroom, submerged culture, immobilization, surfactants.

INTRODUCTION

Edible shiitake mushroom has been massively consumed for thousands years in East Asian countries especially in China, Korea and Japan. This mushroom is the second most consumed in the world after the bottom mushroom

*Corresponding author. E-mail: byronsk8@gmail.com.

(*Agaricus* spp.), with annual production of two millions of tons (Hwang et al., 2012), mainly because the mushroom contains various bioactive substances very beneficial to health. Among other benefits, shiitake is well known as lipid-lowering agent, antibacterial, antiviral, anticancer, and by its excellent nutritional properties (Bisen et al., 2010; Rasmy et al., 2010; Yang et al., 2013; Gil-Ramirez et al., 2018).

Eritadenine from shiitake is a secondary metabolite consisting of a purine alkaloid with an oxidized sugar fragment, which has been shown to be successful in decreasing lipids like cholesterol and triglycerides levels in blood (Yang et al., 2013). Thus, in mice, eritadenine prevents hyperhomocysteinemia, because of its action on triglycerides metabolism (Yang et al., 2013). Similarly, a reduction in serum cholesterol up to 20% in a week is obtained in rats, daily supplemented with 0.005% eritadenine in their food (Shimada et al., 2003; Shu-Lei et al., 2012). Also eritadenine has other interesting biological effects, like inhibition of parasite *Cryptosporidium* sp. (Čtrnáctá et al., 2010), and inhibition of the enzyme, S-adenosyl-l-homocysteine hydrolase (SAHH) (Yamada et al., 2007) and angiotensine (Afrin et al., 2016). In that sense, eritadenine has been proposed as pharmaceutical ingredient (Enman et al., 2011).

Eritadenine have been obtained successfully by using submerged culture of shiitake mycelium; however yields are still unsatisfactory for an industrial scale production. For instance, Enman et al. (2008) reported yields of 10.2 mg/L after 20 days of incubation in a simple broth composed of malt extract and yeast extract in shake flasks. Later, better yields of 25 mg/L were obtained in similar broth, but supplemented with a dried distillers grain and soluble water extract (DDGS), incubated at similar conditions in shake flasks and bioreactors (Enman et al., 2012).

Nutritional conditions, fungal immobilization and surfactants could increase eritadenine production in shiitake mycelium under submerged cultivation, as well as some reports have demonstrated improved yields of metabolites in many filamentous fungi (Kirby et al., 2014; Noreen et al., 2016; Hameed et al., 2017). For example, nitrogen and carbon sources influence positively the production of the antioxidant ergothioneine in shiitake (Tepwong et al., 2012a; b), as well as the production of the anticancer alkaloid chaetominine with *Aspergillus fumigatus* (Zhang et al., 2016). Additionally, fungal immobilization have improved activity and production of laccase enzyme by *Trametes versicolor* and *Coriolopsis polyzona* cultures (Alaoui et al., 2008; Ünal and Kolankaya, 2013; Noreen et al., 2016); have enhanced penicillin production with *Penicillium* (Weber et al., 2012), and gluco-amylases used in food industry by *Aspergillus* cultures (Papagianni et al., 2002). Furthermore, the addition of surfactants has promoted higher yields of terpenes in *Saccharomyces* sp. (Kirby et al., 2014), has enhanced significant laccases activity in *Armillaria* sp. (Hadibarata and Kristanti, 2013), and have improved yields of monakolin K in *Monascus* (Zhang et al., 2014). Nevertheless, little is known about the effect of surfactants, cell immobilization and various carbon sources in shiitake submerged culture for eritadenine production. This study aimed to evaluate eritadenine yields obtained with shiitake mycelium in submerged cultures, employing three carbon sources, three surfactants, and two fungal immobilization supports.

MATERIALS AND METHODS

Fungal strain and propagation

Shiitake mushroom strain LEUCO4, was obtained by culturing the mycelium cloned from carpophores bought in a local supermarket in Colombia. The mycelium was identified taxonomically by 18SrRNA method, sequencing the ITS1 and ITS4 regions. The sequences obtained corresponded to a *Lentinula edodes* (Berk.) Singer strain (data not published yet), after alignment of sequence in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A portion of mycelium was transferred to petri dishes with malt extract agar (MEA) (Oxoid Limited, Hampshire, UK), and incubated at 24°C within 12 days to grow (Stamets, 2000), and were then cut out to make 0.6 cm diameter agar-mycelium disks, used in all the inoculations.

Liquid basal media

As liquid basal media, a broth composed of 20 g/L of malt extract, 2 g/L of yeast extract (Oxoid Limited, Hampshire, UK) and 20 g/L of D-glucose were used, at a pH of 6±0.1. This was prepared according to the method described by Enman et al. (2008), and used as control.

Carbon sources assay

Flasks of 250 mL were filled with liquid basal media and used to evaluate different carbon sources. Instead of D-glucose, flasks were supplemented with three different carbon sources; D-mannitol, sucrose (both from Sigma-Aldrich Corp., USA) and mince potato. Minced potato was obtained by blending potatoes (from local markets, Colombia) for 5 min, according to method described by Sambamurthy and Nageswarra (1971). All the flasks were inoculated with six agar-mycelium disks and incubated within 20 days in an orbital shaker at 120 rpm and 24°C.

Surfactants assays

The effect of surfactants on eritadenine production was evaluated using tween 20, 40 and 80 (Merck Corp., Germany). Surfactants were added in a concentration of 5% to flasks with 100 mL of liquid basal media. Flasks were inoculated with six 0.6 mm agar-mycelium disks, and incubated within 20 days in an orbital shaker at 120 rpm and 24°C.

Immobilization assays

Two different fungal immobilization supports were used to produce eritadenine: (1) wood and (2) calcium alginate. Wood immobilization method was carried out using wood [*Jacaranda copaia* (Aubl.) D.

Don] disks, with 0.6 cm diameter and 0.5 cm of thickness. Then disks were soaked in distilled water until they reached 50% of moisture, using gravimetric method. Next, the autoclaved cold disks were placed over solid sterile MEA in a petri dish, and inoculated with a fragment of mycelium from a MEA culture; then the disks were incubated for 16 days at 24°C, to obtain wood-mycelium disks. Six wood disks were used to inoculate 250 mL Erlenmeyer's containing 100 mL basal broth and placed in orbital shaker for 20 days, at 24°C and 120 rpm. Alginate immobilization method was prepared by using a solution of calcium alginate (Phytotechnology Laboratories, Shawnee Mission, KS, USA) at 3% and circular agar-mycelium disks cut from a MEA culture (0.6 cm diameter and 0.5 cm of thickness). The disks were submerged in the alginate solution and mixed with 0.1 M $CaCl_2$ for 40 min, until the polymerization reaction occurred, according to the method described by Shide et al. (2004). The 1 cm in diameter beads obtained were put inside 250 ml flasks containing 100 ml of basal broth, and incubated together with the wood immobilization flasks in identical conditions.

Biomass measurement

Biomass was separated from broths using vacuum filtration using Wattman #4 filter. The biomass obtained was dried at 60°C in an oven for 24 h, and weighted in a Sartorius Practtum 313-1s scale (Germany). Biomass was expressed as dry weight (DW).

HPLC analysis

Filtrated broths from the last step were employed for eritadenine quantification. HPLC analysis were achieved adapting the method described by Enman et al. (2008), using a HPLC Agillent Technologies 1200 Series apparatus (USA). Samples were injected through a C18 column (5 µm, 150 mm×4.6 mm) using a mobile phase consisting of acetronitrile with a gradient from 2 to 60%, during the first 10 min; and 0.1% trifluoroacetic acid (TFA) from 60 to 2%, from 10 to 11 min (both solvents from Merck Corp., Kenilworth, NJ, Germany). Temperature was kept at 23°C and detection wavelength was 260 nm. An eritadenine standard (Santa Cruz Biotechnology Inc., Dallas, TX) was used for the calibration curve.

Statistical methods

All data were tested using variance analysis ANOVA and Tukey multiple range test ($p<0.05$), using R project 3.1.3 software (https://www.r-project.org/).

RESULTS AND DISCUSSION

Eritadenine yields by carbon sources

Eritadenine from three carbon sources (mannitol, sucrose and minced potato) were compared with the eritadenine standard, as shown in Figure 1. All carbon sources produced eritadenine after 20 days of incubation, as shown in Table 1. Maximal yield was obtained with sucrose, although, there were non-significant differences between the carbon sources and the control composed by D-glucose ($P<0.05$). These yields were similar than those reported by Enman et al. (2008) who obtained 10.23 mg/L of eritadenine after 20 days of incubation, with a shiitake strain cultured in the same basal broth of the present experiments. These results contrast with the improved ergopeptides production, promoted by using mannitol and potato mince in broths, as carbon sources in submerged cultivation of *Claviceps* fungi (Sambamurthy and Nageswara, 1971). The results indicated that carbon source is not a very determinant factor for eritadenine production with shiitake, but because eritadenine contains five nitrogen atoms, then probably nitrogen and other complex nutritional sources play a more important role in biosynthesis of such molecule than carbon sources (Enman et al., 2012). For instance, Enman et al. (2012) obtained slightly better yields of eritadenine (25 mg/L) than the present study, adding 10% of a cereals water extract (DDGS) to the broths. Since the extract contains proteins, fats and ashes, the authors hypothesized that some of those substances have a stimulatory effect on eritadenine biosynthesis. Similarly, higher ergothioneine (a potent antioxidant) yields, are produced by shiitake mycelium, by the addition of some amino acids (Tepwong et al., 2012b), or ammonium sulphate (Jang et al., 2016) as nitrogen sources in broths. Also, Mantle (2009) enhanced significantly the production of indolediterpenoid metabolites with *Claviceps* fungi under submerged cultivation, by adding tryptophan as precursor in the biosynthesis pathway and Zhang et al. (2016) demonstrated the importance of amino acids addition as precursors for the anticancer alkaloid chaetominine production with *Aspergillus* sp., in a dosage dependent manner, wherein high dosages probably produce inhibition of enzymes of the pathway, and low yields of the metabolite.

Biomass yields by carbon sources

All carbon sources tested produced different biomass yields compared to the control, as shown in Table 1. In this study, glucose (control) generated the highest yields, followed by potato mince; while sucrose and mannitol produced similar lower values. Biomass production under submerged culture was similar and slightly lower than that reported in previous studies (Tepwong et al., 2012b; Enman et al., 2008). The production of biomass with mince potato was higher than sucrose and mannitol. This is probably due to the protein contents of the potato tuber, which is a suitable nutritional source. Further, mince potato, is commonly used to produce shiitake biomass under submerged culture (Aminuddin et al., 2007) and in petri dishes (Mahamud and Ohmasa, 2008), in the form of potato dextrose agar (PDA), the most used media for edible mushrooms (Zagrean et al., 2017). For shiitake, potato acts mainly as a carbon source, due to its high starch content, but also it contains 2.4% of protein (Loyola et al., 2010). In submerged cultures, it is well established that nitrogen is a limiting factor to shiitake

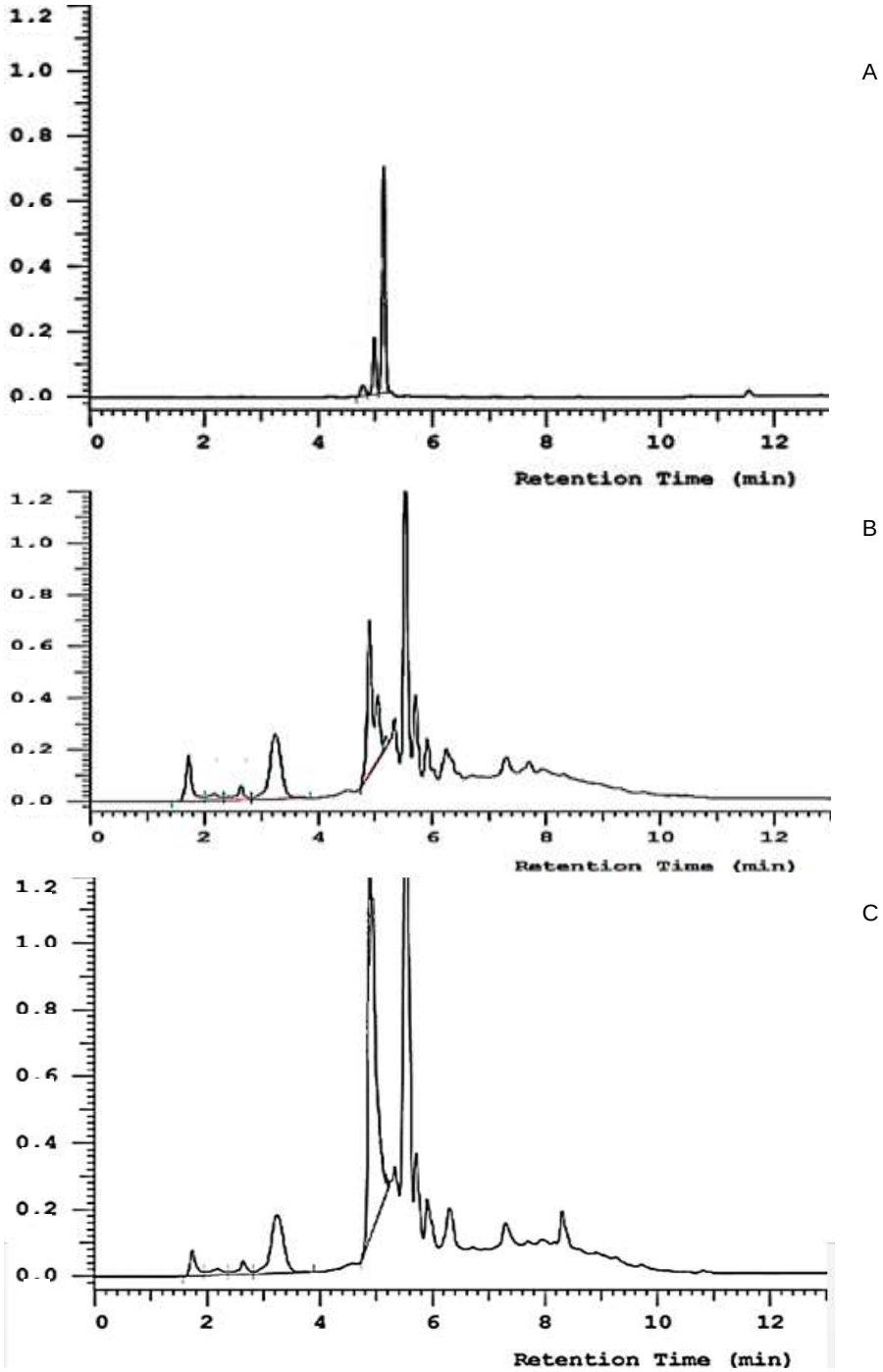

Figure 1. HPLC Chromatograms. (A) Standard reactive (Santa Cruz Biotecnology Inc), eritadenine corresponds to the peak at 4.5-4.6 min. (B) Chromatogram for mycelium cultured with surfactant tween 40 in basal broth. (C) Chromatogram for immobilized mycelium assay in alginate.

growth, even more than carbon sources (Tepwong et al., 2012b), moreover various hydrolytic and oxidative enzymes are more active, when nitrogen sources are provided to shiitake, which enhance metabolism and consequent growth (Pedri et al., 2015). Furthermore, in the case of D-mannitol these results are contradictory to others studies. D-Mannitol is reported as one of the best carbon sources to produce biomass in submerged cultures with important medicinal fungi like shiitake (Tepwong et al., 2012b), *Hydnum repandum L.* (Peksen

Table 1. Eritadenine and biomass yield by shiitake mycelium with three carbon sources, three surfactants and two immobilization supports in submerged cultivation.

Treatment	Eritadenine yield (mg/L)	Biomass (g/L)	Final pH
Alginate immobilization	88.06 ± 3.61[a]	-*	3.21 ± 0.07
Wood immobilization	14.83 ± 2.01[cd]	-*	3.36 ± 0.36
Tween20	72.40 ± 3.11[b]	2.90 ± 1.08[bc]	4.00 ± 0.14
Tween 40	13.06 ± 1,58[cd]	5.68 ± 0.23[a]	3.59 ± 0.34
Tween 80	18.66 ± 0.90[c]	5.00 ± 0.16[a]	3.25 ± 0.21
Sucrose	10.20 ± 1.91[d]	1.39 ± 0.04[d]	3.44 ± 0.01
Mannitol	8.43 ± 0.72[d]	1.09 ± 0.07[d]	3.37 ± 0.02
Potato, minced	8.00 ± 1,40[d]	2.35 ± 0.11[c]	3.57 ± 0.00
Control	8.73 ± 6.16[d]	3.93 ± 0.25[b]	3.55 ± 0.02

Values show averages of three data with their standard deviation. Similar groups have the same letter according to Tukey test (P≤0.05). *Not evaluated

et al., 2013) and *Amanita caesarea* (Scop: Fr.) (Daza et al., 2006). In this study D-mannitol generated lower biomass values, probably due to the complexity of the structure (an alditol), which is more difficult to metabolize than simple saccharides. Mannitol must be broken down to monosaccharides before entering the respiratory pathway. Accordingly, Tepwong et al. (2012b) obtained slightly better shiitake biomass yields with fructose or glucose than mannitol in submerged culture.

Finally, shiitake biomass in submerged culture has been obtained in higher yields using alternative nutritional sources. Lopez-Peña et al. (2013) obtained 9.5 g/L of biomass using a vid wood polar extract rich in polyphenols and some protein content. Harris-Valle et al. (2007) obtained 7 g/L using a polar vid wood extract. Nevertheless, those authors do no mention clearly the substance in the extracts responsible for the enhancement of growth, but possibly in polar vegetal extracts, polar molecules like polyphenols promote growth in shiitake (Beltrán-García et al., 2001). Supplementary, Hassegawa et al. (2005) reaffirmed the enhancement of shiitake biomass production by adding molasses to the broths, although the same result is not obtained when sucrose (main component of molasses) is used.

Eritadenine yields with surfactants

Similarly to others, surfactants have specific effects on fungal metabolism (Jakovljevi et al., 2014; Lazim et al., 2016). In this study, surfactant Tween 20 enhanced significantly eritadenine yield, as shown in Table 1. Eritadenine yield obtained with surfactant Tween 20 was two times that reported previously (Enman et al., 2008, 2012). Tween 20 did not enhance biomass production. Similarly, Kirby et al. (2014) found higher production of terpene in surviving yeast cells (*Saccharomyces* sp.), after being submitted to an inhibitory concentration of Tween 20 (up to 10%). As in *Saccharomyces* sp., maybe in shiitake, Tween 20 acts as selective pressure factor, stimulating growth of resistant cells that are producer of higher quantity of metabolites like eritadenine.

On the other hand, the positive enhancing effect on eritadenine yields promoted by Tween 20, lies also in the consequent permeation of fungal cell membranes. Surfactants in liquid media change the permeability of cellular membranes, which promote oxygen and nutrients to enter at a faster rate to cells, releasing metabolites continuously in the media (O´sulivan et al., 2004). Also, Tween 20 can acts as stress factor, stimulating shiitake to produce eritadenine. Accordingly, in *P. chrysogenum* metabolic changes are observed as stress result of adding detergents to liquid broths, with consequent production of different organic acids (Jakovljevi et al., 2014)

Finally, Tween 40 and 80 did not enhance eritadenine yields, but biomass production did significantly. Similar growth stimulation by Tween 80 in submerged cultures have been observed in *Beauveria* (Mwamburi et al., 2015), and *Armillaria* sp. (Hadibarata and Kristanti, 2013). Probably, surfactants enable mycelial cells to absorb nutrients at a faster rate promoting better cell multiplication (Lazim et al., 2016), which can be happening here for shiitake. The present results with tweens found an interesting tendency: Tween 20 promotes more metabolites, but not the growth, whilst tween 80 promotes good growth, but few metabolite production. Accordingly, in *Saccharomyces* sp. occurs the same: Tween 80 has no growth inhibitory effect, but tween 20 inhibits it, although the tolerant cells are at the same time good terpene producers (Kirby et al., 2014).

Effect of immobilized conditions on eritadenine yields

Calcium alginate promoted significantly eritadenine

Table 2. Correlation matrix for eritadenine content, biomass and pH, after 20 days of submerged shiitake mycelium cultivation.

	Eritadenine (mg/L)	Biomas	pH
Eritadenine (mg/L)	1	-0.019	**0.516**
Biomass		1	-0.068
pH			1

production to 88 mg/L as shown in Table 2. This concentration is more than eight times the eritadenine concentration reported by Enman et al. (2008), and almost three times that reported by the same authors in 2012. Nonetheless, fungal immobilization on wood disks was not successful and produced similar yields than carbon sources (Table 1). This is probably the first time calcium alginate is used for immobilization of shiitake mycelium under submerged conditions for eritadenine production. Maybe in shiitake cells, the alginate gel matrix produce some type of enzyme protection when conditions become unfavorable, resulting in a promoted eritadenine biosynthesis pathway. A superior stability of Aspergillus glucosydases exposed to pH 3,5 have been obtained by enzyme immobilization (Gonzales-pombo et al., 2014); similarly immobilized L-asparaginases from *Penicillium* show higher activity in pH 9 and 60°C in comparison to the same enzyme not immobilized (El-Refai et al., 2016). Besides, alginate is known as better support than others for immobilization of microorganisms and enzymes, for example Ünal and Kolankaya (2013) found that the superior activity and stability in a longer time period in *Trametes* sp. laccase, immobilized in alginate compared to kappa-carrageenan. On the contrary, wood seems to be a non-favorable support for eritadenine production, as various authors describe usage of different common immobilization supports to produce metabolites in submerged cultures with filamentous fungi, but with very different results, as higher enzymes production by immobilizing in polyurethane (PUF) rather than pine wood (PW) with *Dichomitus squalens* (P. Karst.) D.A. Reid, (Susla et al., 2007); also fivefold manganese peroxidase production was observed when *Irpex lacteus* (Fr.) Fr cultures were immobilized in PUF than in PW (Kasinath et al., 2003). Polyurethane like other polymers (e.g. Alginate) acts only as attachment place to the fungi, while wood acts not only as attachment place, but also as nutrient source to the fungi (Susla et al., 2007), nonetheless wood acts only as a carbon source mainly made of cellulose and other difficult to break polymers; then possibly its very low nitrogen content limits the shiitake mycelium to grow and its production of some metabolites, besides shiitake breaks wood slower in cultures with no nitrogen, than in nitrogen supplemented cultures, because some nitrogen sources stimulate more expression of enzymes like oxidases and hydrolases (Pedri et al., 2015). Besides, significant growth and enhanced production of the antioxidant erothioneine by shiitake in submerged culture have been evidenced, when monosaccharides based broths were supplemented with various amino acids as nitrogen sources (Tepwong et al., 2012b). The present results suggested that nutritional and culture conditions for high biomass yields, are not necessarily the same conditions for high eritadenine yields.

Finally, fungal immobilization has been a very well established technique for culturing different fungi in submerged culture, which enhances fungal metabolites yields; like the enzymes laccases with *Coriolopsis polyzona* (Pers.) with Ryvarden (Alaoui et al., 2008); the antibiotic peniciline with *Penicillium* (Weber et al 2012), and gluco-amylases with *Aspergillus* sp. (Papagianni et al., 2002). Additionally, immobilization offers various advantages like greater resistance of entrapped biomass to sudden physical-chemical changes, with consequent recyclability of biomass for various batches before losing metabolite productivity (Rodriguez, 2009; Gonzales-pombo et al., 2014, El-Refai et al., 2016). in this sense, Noreen et al. (2016) reported optimal activity at pH 3 and 60°C for alginate immobilized *Trametes* sp. laccase, compared to non-immobilized laccase with optimal activity in pH 4.5 and 45°C; and similarly better stability of alginate beads have been observed during *Aspergillus* fermentations to produce glucoamylases (Papagianni et al., 2002). In the present study, this resistance and stability of biomass and beads, seems to be present in shiitake submerged cultures, according to the improved yields of eritadenine obtained in alginate immobilization experiment.

Effect of final pH on eritadenine and bimass yields

pH was reduced from the initial value after 20 days of fermentation, as reported previously in fermentations with shiitake (Enman et al., 2012; Pedri et al., 2015), as a direct consequence of active aliphatic acids production, typical of wood decomposer fungi like shiitake (Hakala et al., 2005). The samples with tween 20 that produced the best eritadenine yield (72.40 mg/L) had final pH of 4.0, and samples with alginate immobilization (88 mg/L) of 3.2, indicating a reduction in pH, as shown in Table 1. Similarly, the best eritadenine yields of 25 mg/L obtained by Enman et al. (2012), reduced pH to 3.5 to 3.6. These

results indicate that the optimal pH for eritadenine production is in the range of 3 to 4.

On the other hand, in the case of biomass production there was a pH reduction as well. The best treatments for biomass production were tween 40 and tween 80 (5.68 and 5 g/L, respectively)and reduced pH to the range of 3.25 to 3.59. Similar results were reported previously, establishing superior growth rate at a pH of 3.5 to 4, as optimum values for shiitake biomass production in submerged culture (Hassegawa et al., 2005; Quaicoe et al., 2014).

In that order of ideas, pH values to obtain eritadenine and biomass are different. As shown in Table 2, the correlation analysis for pH and biomass did not give a significant value, while pH and eritadenine yield presented a medium correlation of 0.5. These results corroborate the conclusion of Enman et al. (2008 and 2012) that is not possible to obtain high eritadenine and biomass yields at the same time. Thus, the implementation of different incubation and nutritional factors, are necessary to obtain high eritadenine or biomass yields. This situation seems to happen with the surfactants evaluated. For instance, tween 20 produced the highest eritadenine yields, but not the higher biomass productions, while tween 80 was vice versa.

Additionally, in this study, possibly the more favorable pH value to produce eritadenine may stimulate certain growth morphology, that influences the pathway in a positive manner. pH is known as a very important factor affecting morphology and growth (Gibbs et al., 2000), at the same time morphology affects metabolites production by shiitake in submerged cultivation, such as higher quantities of ergothioneine are produced, when bigger pellets are formed, in dependent manner of stirring speed (Tepwong et al., 2012a). Similarly, when shiitake is cultured in bioreactors and shake flasks, pellets and free dispersed mycelium are formed respectively, with certain differences in eritadenine yields not statistically analyzed (Enman et al., 2008), with higher yields in free dispersed mycelium cultures. On the other side, as the purine molecules (like in eritadenine) are biosynthesized from aminoacides, the different yields of eritadenine in different final pH may be a result of the different profile of amino acids that are present in different pH values (Aminuddin et al., 2013).

Conclusion

Addition of surfactants to broths enhanced significantly eritadenine and biomass yield after 20 days of fermentation. Tween 20 increased eritadenine yields with about eight times, compared to broths without the surfactant. Tween 40 and Tween 80 incremented biomass yields (two-folds), but these surfactants did not increase eritadenine yields. Similarly, immobilization of the mycelium in alginate enhanced significantly eritadenine production in submerged culture by six times, compared to non-immobilized fermentations, whilst wood immobilization did not promote these same results. Finally, to enhance significantly eritadenine yields, it is necessary to bear in mind a correct pH value (in the range 3-4), combined with optimized broths, and incubation parameters like immobilization and surfactants towards obtaining eritadenine in producing good yields. Future studies regarding eritadenine production by submerged cultures should explore the relationship between yields and some nitrogen sources, lipids, salts, and growth morphology of shiitake mycelium. In that sense, the knowledge of the role of these parameters could offer new approaches towards improvement of this important biotechnological process. This information will be very useful in future commercial eritadenine production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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