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Effect of processing methods on the nutritional values and anti-nutritive factors of *Adenanthera pavonina* L. (Fabaceae) seeds

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This research aimed at determining the effect of processing on the nutritional and anti-nutritional values of "food tree" [*Adenanthera pavonina* L. (Fabaceae)] seeds, a highly nutritional and underutilized legume. The seeds were separated into three groups namely, boiled, roasted and raw. Quantitative analysis was carried out to measure their proximate, minerals, vitamins and anti-nutrients compositions. Analysis of variance was used to analyze the treatment groups and Duncan's multiple range tests to determine significant difference at P ≤ 0.05. The results show that processing significantly affected the nutritional and anti-nutritional constituents. The values for proximate composition in the raw, roasted and boiled seeds were: proteins (15.79±0.04, 18.86±0.02 and 23.25±0.02, respectively), carbohydrates (56.60±0.02, 54.89±0.02 and 52.05±0.02 respectively), fat (9.78±0.02, 11.70±0.02 and 11.40±0.02, respectively), crude fibre (9.80±0.04, 9.70±0.04 and 5.85±0.02, respectively), moisture (3.88±0.03, 0.10±0.02 and 3.20±0.02, respectively) and ash (4.03±0.01, 4.75±0.02 and 4.25±0.01, respectively). The values for mineral compositions in the raw, roasted and boiled seeds were: calcium (25.61±0.34, 30.34±0.02 and 80.88±0.02, respectively), magnesium (18.97±0.01, 22.76±0.01 and 60.68±0.02, respectively), phosphorus (7.00±0.06, 6.40±0.15 and 5.80±0.10, respectively), potassium (3.31±0.00, 2.43±0.02 and 4.23±0.02, respectively) and iron (0.41±0.02, 0.41±0.01 and 1.23±0.01, respectively). The values for vitamin compositions in the raw, roasted and boiled seeds were: β carotene (1458.33±0.01, 416.67±1.20 and 416.67±0.08, respectively) and vitamin E (22.50±0.02, 9.24±0.02 and 12.69±0.01, respectively). The mean values for anti-nutrient compositions in the raw, roasted and boiled seeds were: tannin (1.21±0.00, 0.049±0.00 and 0.15±0.00, respectively), phytate (5.16±0.02, 3.50±0.01 and 1.50±0.02, respectively), oxalate (0.34±0.00, 0.13±0.00 and 0.11±0.00, respectively), cyanide (1.17±0.00, 0.95±0.00 and 0.32±0.00, respectively) and trypsin inhibitor (0.92±0.01, 0.36±0.01 and 0.90±0.01, respectively). The results show that processing changed the nutritional constituents and reduced the anti-nutrients in the seeds of *A. pavonina* and boiling proven to be the best processing method.

Key words: Processing, techniques, nutritional, anti-nutritive, *Adenanthera pavonina*, seeds.

INTRODUCTION

Food legumes constitute a major source of nutrients such as proteins, lipids, carbohydrates, and other important

substances such as fibre, minerals and vitamins (Deshpande, 1992) which are necessary for human and animal health. Similarly, they contain anti-nutritional components such as saponins, tannins, phytates, lectin/haemagglutanin, oxalates, polyphenol, among others, which hinder the body from digesting the nutrients in pulses. These toxins cause food poisoning to human beings and animals (Osifo, 1974). According to Olusanya (2008) and Geil and Anderson (1994), legumes contain some toxic components such as anti-trypsin factors which impair the digestion of proteins and hence prevent its efficient utilization. Phytates, oxalates and cyanides cause various physiological disorders like increase in relative weight of pancreas and liver, and also diarrhea (Arija et al., 2006). Fortunately, many of these toxic components are destroyed by heat provided by different food processing methods (Olusanya, 2008).

Boiling and roasting are important household food processing methods. Boiling is a method of cooking food in water such that it bubbles vigorously, while roasting is achieved in an uncovered pan without water to produce a well-browned exterior and a moister cooked interior. These processing treatments increase the nutritional quality of food plants and are also effective in eliminating the anti-nutritional factors in them and thus the need for their proper processing to levels where they are safe for human and animal consumption (Hotz and Gibson, 2007; Nzewi and Egbuonu, 2011).

Adenanthera Pavonina L. (Fabaceae) is a woody Southeast Asian species of legume mostly known for its edible seeds (Arzumand et al., 2010). It is endemic to India and Southeast China, where it is considered as an alternative nutrients source for animals and humans, but has been introduced into tropical and sub-tropical areas of the world including Malaysia, Polynesia and eastern and West Africa. The plant is known as “food tree” because its seeds and leaves are valued for food, and the seeds, which when roasted are said to taste like soy bean, possess high percentage of proteins, fatty acids, minerals and other nourishing properties (Olajide et al., 2004; Senga et al., 2013).

It has been used in traditional medicine practices to treat many diseases such as asthma, boil, diarrhoea, gout, inflammations, rheumatism, tumor and ulcers, and as a tonic (Ghani, 2003; Arzumand et al., 2010). Several parts of the plant have been verified for its medicinal importance hence, the bark and leaves are used in the treatment of gonorrhea, ulcers and rheumatism. The powdered seeds are applied as a poultice to abscess and to promote suppuration (Hussain et al., 2010; Sujit et al., 2010). Physicochemical characterization of the seed oil showed appreciable amounts of neutral lipids and unsaturated fatty acids including linoleic, oleic and lignocerotic acids (Robert et al., 2004). However, report on its antinutritive constituents is totally lacking.

In Nigeria and other parts of West African where it has been introduced, the tree has been economically utilized as source of timber and wood fuel. However, there is no report on utilizing any part of it, including the seeds as food. Therefore, the present research was to analyze the nutritional potentials of the seeds in combating malnutrition and food insecurity.

MATERIALS AND METHODS

Source of plant material

Dried pods of *A. pavonina* L. were obtained from the Botanic Garden, Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State in May, 2016. The seeds were separated from the dried pods, cleaned and freed from foreign matters and air dried. The dried seeds were stored in air-tight bottles for further studies.

Preparation of samples

The preparation of the various sample groups was done following the methods of Ajeigbe et al. (2012).

Boiling

Whole seeds (100 g) were weighed using digital weighing balance and soaked in distilled water for overnight. The soaked seeds were boiled for 1 h and then rinsed with distilled water. Further boiling was done for another 2 h before the seeds were dried using Gallenkamp hot air oven at 40°C for 15 min.

Roasting

With the aid of a digital weighing balance, 100 g whole seeds were weighed and roasted in Gallenkamp hot air oven at 120°C for 1 h. The roasted seeds were allowed to cool.

Raw sample

Raw processing was done by drying 100 g whole seeds in hot air oven at 40°C. The prepared samples were separately ground with Thomas hammer mill blender to obtain powdered particle size of 1 mm. The powdered samples were stored in air-tight bottles at room temperature for further analysis.

Nutritive analysis

Determination of proximate composition of seed samples

The proximate composition of the samples was done following the standard methods as recommended by Pearson (1976) and the Association of Official Analytical Chemists (AOAC, 1990).

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Crude protein

The crude protein content of foods or plant sample was determined by using the Micro Kjedahl Nitrogen Method (Pearson, 1976). The method involves digestion of samples, distillation of digests and titration of distillate.

Crude fat (using Soxhlet apparatus)

Two grammes aliquot of the processed sample was weighed into a 250 ml clean flask and put into the thimble. The boiling flask was filled with 250 ml n-hexane. The Soxhlet apparatus was set and refluxed for about 3 h. The thimble was removed with care and the hexane was collected in the top container of the set up and drained into a container for re-use. When the flask was almost free of hexane, it was removed and dried at 105°C to a constant weight. It was transferred from the oven into a desiccator and allowed to cool, and then weighed.

Fibre

Two grammes aliquot of the sample was weighed and 150 ml of heated H_2SO_4 was added and heated to boiling for 30 min and filtered. The residue was washed three times with hot water. Pre-heated KOH (150 ml) was added and the residue was heated to boiling. Few drops of anti-foaming agent were added and boiled slowly for 30 min. The residue was filtered and washed three times with hot water, then washed with acetone, dried at 130°C for 1 h and weighed.

Moisture (using oven method)

A crucible was thoroughly washed and dried in the oven, then cooled in a dessicator and weighed. Two grammes of the sample was weighed into the crucible. The crucible and the content was transferred into a hot air oven and dried at 105°C to a constant weight. The sample was then cooled in dessicator and the weight of the crucible and the content was taken, recorded and calculated.

Ash (using muffle furnace)

Two grammes aliquot of the sample was put into a weighed crucible and pre-ashed to drive off most of the smoke. The pre-ashed sample was transferred into a furnace at 550°C and allowed to ash until white ash was obtained. The desiccator was cooled and reweighed. Ash content was calculated.

Carbohydrate

Carbohydrate content was determined by the difference in the percentage composition of protein, crude fat, ash, moisture and crude fibre (AOAC, 1990).

Determination of mineral composition

Calcium

Calcium was determined using Pearson (1976) method. 25 ml of the sample was pipetted into a conical flask, a pinch of EBT was added, 2 ml of the NaOH solution was also added and the mixture was titrated with standard EDTA solution.

$$\text{Ca (mg/100 g)} = \frac{\text{T x N x E x 1000}}{\text{Volume of sample used}}$$

Where, T = Titre value; M= molarity of EDTA; E = equivalent weight of calcium.

Magnesium

Magnesium was determined using Pearson (1976) method. Aqueous extract of the sample (25 ml) was pipetted into a conical flask and a pinch of EBT was added and then shaken. This was followed by the addition of 2 ml buffer. The mixture was then titrated using 0.01 M EDTA.

$$\text{Mg (mg/100 g)} = \frac{\text{T x N x E x 1000}}{\text{Volume of sample used}}$$

Where T = Titre value; M= molarity of the standardized EDTA; E = equivalent weight of magnesium.

Phosphorus

Phosphorus was determined using Pearson (1976) method. Aqueous extract of the sample (5 ml) was pipetted into a test tube and 5 ml of the molybdate solution was added and the absorbance read at 420 nm. The concentration was calculated using the standard curve.

Potassium (using flame photometer)

Potassium was determined using Pearson (1976) method. The instrument was switched on and allowed for about 20 min to stabilize. The gas was then turned on, distilled water was aspirated through the siphon in order to zero the instrument and the samples were aspirated and the emission recorded. The concentrations were calculated using sodium and potassium calibration curve for sodium and potassium readings, respectively.

Iron

Iron was determined using Pearson (1976) method. The sample (10 ml) was added into a 100 ml flask and made up to 50 ml with de-ionized water. Concentrated HCl (20 ml) was added followed by the addition of 1 ml of hydroxylamine solution. About 0.5 g glass beads was added and heated to boiling point till the volume reduced to 2 ml. Ammonium acetate buffer solution (10 ml) and 2 ml of phenanthroline were added and the content made up to 100 ml mark with de ionized water.

Determination of vitamin composition

Vitamin A (β-carotene)

One gram of the sample was weighed. Then, the proteins were first precipitated with 3 ml of absolute ethanol before the extraction of vitamin A with 5 ml of heptane. The test tube containing this was shaken vigorously for 5 min. On standing, 3 ml from the heptane layer was taken up in a curvette and read at 450 nm against a blank of heptane. The standard was prepared and read at 450 nm wavelength using UV/Vis spectrophotometer (Model: CE 2041), and vitamin A calculated from the standard (Pearson, 1976).

Table 1. Mean proximate constituents observed in raw and processed (roasted and boiled) seeds of *A. pavonina*.

Sample	Protein (%)	Carbohydrate (%)	Crude fat (%)	Crude fibre (%)	Moisture (%)	Ash (%)
Raw	15.79 ± 0.004^{a}	56.60 ± 0.02^{c}	9.78 ± 0.02^{a}	9.80 ± 0.04^{b}	3.88 ± 0.03^{c}	4.03 ± 0.019^{a}
Roasted	18.86 ± 0.02^{b}	54.89 ± 0.02^{b}	11.70 ± 0.02^{c}	9.70 ± 0.04^{b}	0.10 ± 0.02^{a}	4.75 ± 0.02^{c}
Boiled	23.25 ± 0.02^{c}	52.05 ± 0.02^{a}	11.40 ± 0.02^{b}	5.85 ± 0.02^{a}	3.20 ± 0.02^{b}	4.25 ± 0.01^{b}

Mean value with different superscript alphabets in each column are significantly different from each other by DMRT ($P < 0.05$).

Vitamin E

One gram of each sample was macerated with 20 ml of petroleum ether for 10 min. The macerated samples were allowed to stand for 1 h with intermittent shaking at every 10 min and thereafter, centrifuged for 5 min. Three millilitres of supernatant was transferred into triplicate test tubes, evaporated to dryness and then re-dissolved with 2 ml of ethanol and shaken. One millilitres of 0.2% ferric chloride in ethanol, 1 ml of 0.5% and dipyridyl in ethanol and 1 ml of ethanol were added and the resultant solution was made up to 5 ml. The solution was mixed thoroughly by shaking and absorbance was taken at wavelength of 520 nm using UV/Vis spectrophotometer (Model: CE 2041), against the corresponding blank.

Anti-nutritive analysis

Tannins

This was determined as described by Pearson (1976). Distilled water (10 ml) was added to 1 g of the test sample and shaken at 5 min interval for 30 min. The solution was centrifuged to get the extract. Two and half millilitre of the supernatant was transferred into a test tube and 2.5 ml of standard tannic acid solution was also transferred into a 50 ml flask. One millilitre Folin-Denis reagent was added into the flask, followed by 2.5 ml of saturated Na_2CO_3 solution and the solution was made up to the mark. Absorbance was read after 90 min incubation at room temperature using UV/Vis spectrophotometer (Model: CE 2041).

Phytate

The sample (0.5 g) was extracted with 100 ml of 2.4% HCl for 1 h at room temperature. The extract (5 ml) was pipetted into a test tube and diluted with 25 ml of distilled water. 0.7 M sodium chloride (15 ml) was added and the absorbance was read at 520 nm using UV/Vis spectrophotometer (Model: CE 2041). The value was calculated from a prepared standard curve and blank (Pearson, 1976).

Oxalate

One gram of the powdered sample was weighed and put into a test tube and 47.5 ml of water and 2.5 ml of 6 N hydrogen chloride were added to the powdered sample. It was boiled for 1 h and made up to 62.5 ml with water. The solution was cooled at room temperature and filtered. Some filtrate (12.5 ml) was taken and the pH was adjusted to the range of 4.0 to 4.5 with dilute ammonia (NH_3). The solution was heated up to 90°C, filtered and heated up again to 90°C. Then, 5 ml of calcium chloride was added to the solution with constant stirring. The solution was allowed to stand overnight. The solution was centrifuged for 5 min and the supernatants were decanted off. The precipitate was dissolved with 5 ml of 20% sulphuric acid. It was heated until about to boil. The solution was then titrated with 0.5 N standard $KMNO_4$ until a pale pink colour that persisted for 30 s was attained and the percentage oxalate was calculated (Pearson, 1976).

Cyanide

Five grams of the sample was prepared into a paste and the paste was dissolved in 50 ml of distilled water and allowed for the cyanide extraction to stay overnight, then filtered and the filtrate was used for the cyanide determination. To 1 ml of the sample filtrate in a test tube, 4 ml alkaline picrated was added and allowed to stand for 5 min. The absorbance was read at 490 nm after colour development (redish brown colour). The absorbances of the blank and standard were also read and the cyanide content of the test sample was extrapolated from cyanide standard (Pearson, 1976).

Protease inhibitor

Two grammes of the finely ground sample was extracted with 10 ml of 0.01 N NaOH for 1 h. 5 ml of benzoyl-DL arginine-p-nitro anilide hydrochloride (BAPNA) solution was hydrolyzed with 2 ml of 0.2 mg/ml trypsin (Sigma Type 11) in 0.0001 M HCl. P-nitro anilide was released as a coloured product and absorbance was read at 410 nm (Pearson, 1976).

Data analysis

The data obtained for the nutritive (proximate, mineral and vitamins) and anti-nutritive composition were statistically analyzed using one way analysis of variance (ANOVA) and reported as mean ± standard error of triplicate data. Duncan's multiple range test was used for mean separation.

RESULTS

Proximate compositions of the raw and processed (roasted and boiled) seeds of *A. pavonina* are presented in Table 1. The processed seeds had the highest values of protein, crude fat and ash while the raw seeds had more carbohydrate, crude fibre and moisture. The proximate composition also varied significantly ($P < 0.05$) between the boiled and roasted seeds.

Analysis of variance (ANOVA) showed that there is significant difference ($P < 0.05$) in the mineral composition of the processed (roasted and boiled) seeds of *A. pavonina* when compared with the raw seeds. Processing methods affected the composition of mineral

Table 2. Mean constituents of the mineral nutrients observed in raw and processed (boiled and roasted) seeds of *A. pavonina*.

Sample	Ca (mg/100 g)	Mg (mg/100 g)	P (mg/100 g)	K (ppm)	Fe (mg/100 g)
Raw	25.61±0.34^{a}	18.97±0.01^{a}	7.00±0.06^{c}	3.31±0.00^{b}	0.41±0.02^{a}
Roasted	30.34±0.02^{b}	22.76±0.01^{b}	6.40±0.15^{b}	2.43±0.02^{a}	0.41±0.01^{a}
Boiled	80.88±0.02^{c}	60.68±0.02^{c}	5.80±0.10^{a}	4.23±0.02^{c}	1.23±0.01^{b}

Mean value with different superscript alphabets in each column are significantly different from each other by DMRT (P<0.05).

Table 3. Mean constituents of the vitamin nutrients observed in raw and processed (boiled and roasted) seeds of *A. pavonina*.

Sample	β-Carotene (iu)	Vitamin E (iu)
Raw	1458.33±0.01^{b}	22.50±0.02^{c}
Roasted	416.67±1.20^{a}	9.24±0.02^{a}
Boiled	416.67±0.88^{a}	12.69±0.01^{b}

Mean value with different superscript alphabets in each column are significantly different from each other by DMRT (P<0.05).

Table 4. Mean constituents of the anti-nutrients observed in raw and processed (roasted and boiled) seeds of *A. pavonina.*

Sample	Tannin (%)	Phytate (%)	Oxalate (%)	Cyanide (%)	Trypsin inhibitor (%)
Raw	1.21±0.00^{c}	5.16±0.02^{c}	0.34±0.00^{c}	1.17±0.00^{c}	0.92±0.01^{c}
Roasted	0.49±0.00^{b}	3.50±0.01^{b}	0.13±0.00^{b}	0.95±0.00^{b}	0.36±0.01^{b}
Boiled	0.15±0.00^{a}	1.50±0.02^{a}	0.11±0.00^{a}	0.32±0.00^{a}	0.90±0.01^{a}

Mean value with different superscript alphabets in each column are significantly different from each other by DMRT (P<0.05).

nutrients in the seeds (Table 2). Processing significantly increased the percentage compositions of calcium, magnesium, iron and potassium.

Analysis of variance (ANOVA) showed significant difference (P<0.05) in the vitamin composition of the processed (roasted and boiled) seeds of *A. pavonina* when compared with the raw (control) seeds. Processing methods affected the composition of vitamin nutrients in the seeds. Processing significantly reduced the vitamins A and E constituents (Table 3).

There was significant difference (P<0.05) in the anti-nutrients composition of the processed (roastesd and boiled) seeds when compared with the raw seeds. Processing methods affected the composition of anti-nutrients in the seeds. The anti-nutrients were generally reduced in the processed seeds and the boiling gave the most significant effect (Table 4).

DISCUSSION

The results from the nutritional analysis showed that the values for the major nutrients tested are within the reported values for other legumes (Aremu et al., 2006). Protein composition of processed *A. pavonina* seeds is comparable to that found in the seeds of soybean, *Canavalia ensiformis* and cowpea (El-Adaway and Taha, 2001), and much higher than that of bambara groundnut (Akaninwor and Ogechukwu, 2004). Carbohydrate level is favorably compared with the acceptable range mean values for legumes (20 to 60%) (Aykroyed and Dought, 1964), and higher as compared to that of *C. ensiformis,* soybean and *Mucuna utilis* (Balogun and Olatidoye, 2012). The carbohydrate content gave an indication that the seeds of *A. pavonina* studied here can be considered as a rich source of energy and is able to supply the daily energy requirements of the body in children and adults (Aranda et al., 2001; Balogun and Olatidoye, 2012). The seeds of *A. pavonina* contained higher crude fats than most other legumes. Legumes generally have low fat content in the range of 1 to 2% with the exception of *Cicer arietinum, Glycine max* and pea nut (Costa et al., 2006). The same appreciable result was recorded for crude fibre, moisture content and ash.

Furthermore, processing methods were observed to significantly (P<0.05) affect the nutrient composition when compared with the raw seeds. Boiled seeds had higher amount of protein than roasted seeds. In addition,

regarding the recommended daily allowance for proteins for children, which ranges from 23.0 to 36.0 g, and for adult (44 to 56 g), it can be considered that the boiled seeds of *A. pavonina* can supplement the recommended daily intake of this nutrient, particularly for children. Therefore, this appreciable proteins content in the processed seeds suggests their usefulness as alternative source of protein nutrients. The fat composition obtained from the processed seeds of *A. pavonina* studied showed that processing significantly ($P < 0.05$) affected the fat composition of the seeds. Roasted seeds had the highest amount of fat than the boiled seeds.

The crude fibre content of the roasted seeds was significantly ($P < 0.05$) higher than the boiled seeds and the overall fibre content of *A. pavonina* was higher when compared with those of other legumes, for example, *Dolichos tribalus*, *Vigna radiata* and *Vigna unguiculata* (Aremu et al., 2006). Processing methods therefore, affected the crude fibre composition, with the roasted seeds having more amount than the boiled seeds, and this implied that more of the crude fibres were probably leached into water during boiling (Aremu et al., 2006).

The moisture composition of the roasted seeds of *A. pavonina* was significantly ($P<0.05$) lower in comparison with boiled seeds. This was expected as seeds are subjected to higher temperatures during roasting. The result indicate that roasting may favor keeping quality and acceptability of *A. pavonina* seeds as texture, taste, appearance and stability of foods depends on the amount of water they contain (Isengard, 2001).

The ash composition of the processed seeds of *A. pavonina* was reflective of the high level of some mineral elements presented in Table 2. The roasted seeds had significantly ($P < 0.05$) higher amount of ash than the boiled seeds. The low value of ash in the raw seeds may be as a result of the effects of anti-nutrients on the mineral contents of the food sample. The ash content reported here is higher as compared to the recommended values and suggested that these seeds are rich source of ash (Kala and Mohan, 2008).

Table 2 shows that processing significantly ($P<0.05$) increased some mineral contents of *A. pavonina* seeds with boiling having the most significant increased effect. This is probably because minerals are not destroyed by heat. The reduction in some cases may be as a result of leaching of minerals into the boiling water and through roasting process (Amarowicz et al., 2009). This study revealed that seeds of *A. pavonina* are rich in mineral elements including calcium, phosphorus, potassium, magnesium and iron. These minerals are necessary for cell formation, transmission of nerve impulse, fluid balance and bone formation (Ezeagu and Ologhobo, 1995).

In this study, vitamins A (β carotene) and E compositions of the seeds of *A. pavonina* were significantly ($P<0.05$) reduced by processing methods when compared with the raw seeds. This agrees with an earlier report that processing of legumes by heating lead to reduction of vitamin content (Asogwa and Onweluzo, 2010). This could be explained by the fact that vitamins are lost during processing because of their high sensitivity to oxidation, and leaching into water soluble media during storage (Davy et al., 2010).

The result of the anti-nutrient values of the processed seeds of *A. pavonina* showed that processing methods significantly ($P<0.05$) reduced the anti-nutrient composition of the seeds when compared with the raw seeds (Table 4). It has been reported that some anti-nutrients are heat labile and therefore will be reduced to a great extent by the application of heat to the food (Apata and Olegbobe, 1994), and this statement has been justified in this result as boiling most significantly reduced all the anti-nutrients to very low levels in seeds of *A. pavonina*. Roasted seeds showed higher increase in phytate content because of the increase in phosphorus concentration since phytate is the major store of phosphorus in mature seeds, while the boiled seeds showed reduced amount of phytic acid which is attributed to leaching in water.

Conclusion

Processing methods adopted in this study have been proven to have significant effects on the nutritional and anti-nutritional compositions of the seeds of *A. pavonina,* a highly nutritious and neglected legume, in agreement with earlier reports that thermal processes of legumes enhance tenderization of the cotyledons, thereby increasing palatability and nutritional value by inactivating endogenous toxic factors. In addition, it was observed that boiling gave higher significant effect than roasting and proved as more efficient method of processing the seeds. Furthermore, this study revealed that the seeds, when properly processed, have high nutritional values that can be exploited and considered as an alternative source of nutrients to reduce malnutrition among economically weaker categories of people in the developing countries.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Composting of sugar cane bagasse by *Bacillus* strains

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Composting of sugar cane bagasse with *Bacillus sp.* CMAGI2 and *Bacillus subtilis* JCM 1465^T strains was carried out during five months at horticultural center. Chemical, biochemical and microbial parameters were followed during this process. There was a difference between inoculated composts and non-inoculated compost. These bacterial additives allowed greater biodegradation compared to control compost. The inoculated composts were more degraded than the control compost with compost3 which presented the highest OM loss with 91.37%, compost1 with 90.15% and compost2 had 89.47% of OM loss. Control compost showed the lowest C/N ratio, however compost3 had the highest C/N ratio compared to compost1 and compost2. Microbiologically, *Bacillus* strains in compost1 and compost2 had probably inhibitory effect on microflora statistically if they were inoculated alone when the mixture of two strains (compost3) had no inhibitory effect on microflora during the composting process. The inoculated composts presented higher enzymatic activities than control compost, probably due to the presence of *Bacillus* strains.

Key words: Sugar cane bagasse, composting, *Bacillus sp.*CMAGI2, *Bacillus subtilis* JCM 1465^T.

INTRODUCTION

In these last years, a decrease in humus soil has been observed in most agricultural fields. This issue is a result of number of human activities and environmental phenomena such as: large use of chemical fertilizer in intensive agriculture, bad management of agricultural wastes, soil erosion, and various environmental pollutions. This problem has significantly impacted on agricultural productivity, and finding solutions for this issue has become a vital and dynamic research focus.

The use of compost has been thought to be an option, for enhancing soil quality. Composting is a biological process which converts heterogeneous organic wastes into humus like substances by mixed microbial population under controlled optimum conditions of moisture, temperature and aeration (Ryckeboer et al., 2003b; Ahmad et al., 2007; Insam and de Bertoldi, 2007;

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Jurado et al., 2015). The resulting product is called compost and can be used as soil conditioner and or organic fertilizer. It is well known that soil microbiome plays important role in compost biodegradation to specific and precise stages, however it is very difficult to count the number of microorganisms involved in this bioprocess (Ryckeboer et al., 2003b). Among the composting microorganisms, bacteria, actinomycetes and fungi constitute the major active groups. Bacteria are also the most diverse group of compost organisms, using a broad range of enzymes to chemically degrade a variety of organic matters (Ryckeboer et al., 2003b). The high surface/volume ratio of bacteria allows a rapid transfer of soluble substrates into the cell. They are usually far more dominant than larger microorganisms such as fungi (Tuomela et al., 2000; Ryckeboer et al., 2003b; Insam and de Bertoldi, 2007; Mehta et al., 2014). The ubiquitous genus *Bacillus* is often found in environment mostly in composts at any stage (Ryckeboer et al., 2003, b; Insam and de Bertoldi, 2007; Franke-Whittle et al., 2014). Their capacity to produce spores allowing to survive in unfavorable environmental condition is an advantage over other bacteria. They can produce extracellular polysaccharide hydrolysing enzymes (Priest, 1977).

The inoculation with specific microorganisms can be a useful method for enhancing the properties of compost and decreasing the composting time (Adebayo et al., 2011; de Figueirêdo et al., 2013; Jurado et al., 2015) but the use of inoculants remains controversial due to contradictory results presented by many authors (Adebayo et al., 2011; de Figueirêdo et al., 2013). Indeed, environmental and nutritional conditions are not the only parameters that can affect microbial growth; the presence of other microorganisms can influence the activity of the tested microorganism, either positively or negatively (Franke-Whittle et al., 2014). The aim of this study was to investigate the evolution of physico-chemical parameters and changes in microbial population during the sugar cane bagasse composting.

MATERIALS AND METHODS

In this study, we used bagasse as substrate and *Bacillus sp.* CMAGI2 and *Bacillus subtilis* JCM 1465^T strains as inocula. The sugar cane bagasse was taken from the Senegalese Sugar Company (Compagnie Sucrière Sénégalaise C.S.S.) which is located to the north of Senegal (Richard-Toll, St-Louis). This substrate was chosen for its low microbial activity, which may allow an assessment of the behavior of inoculated *Bacillus* strains during the process. The composting of sugar cane bagasse was carried out at horticultural center UPPF of Senegalese Institute of Agricultural Research "Institut Sénégalais de Recherches Agricoles" (ISRA), located at Sangalkam, Dakar, Senegal.

Preparation of bacterial inoculants

B. subtilis JCM 1465^T strain spores were kindly provided by Belgian partners from Gembloux University. It was reactivated on nutrient agar (*Bio-Rad*) then preserved at 4°C and stored too in 20% glycerol at -80°C. *B. sp.*CMAGI2 was isolated from mature sugarcane bagasse compost in laboratory of Applied Microbiology and Industrial Engineering (Microbiologie Appliquée et Génie Industriel, MAGI) (Diallo et al., 2015). The bacterial biomass of inoculum (*B. sp.* CMAGI2 and *B. subtilis* JCM 1465^T) was prepared with their specific culture media growth.

B. sp CMAGI2 was cultured in medium containing: 5.0 g of glucose, 5.0 g casamino-acids, 3.0 g beef extract, 5.0 g peptone in 1 L distilled water at pH 8. *B. subtilis* was cultured in a medium containing: peptone 5.0 g, beef extracts 3.0 g in 1 L distilled water at pH 7.

The inocula were prepared by growing young colonies of the two strains in fresh sterilized culture media in 5 L flasks and incubated for two days at 40°C. The resulting bacterial cultures were diluted in 20 L of sterile water before inoculation in the process.

Composting process

Windrows composting were carried out in this project. We built four cemented composters with 5 m length, 1 m width and 60 cm depth, and filled an amount of 2 m^3 bagasse with 60% humidity. They were inoculated with 10% (20 L) bacterial culture with a final bacterial concentration of 10^9 CFU/ml and a bacterial population between 10^7 and 10^8 CFU/g of fresh compost was obtained at the beginning of composting (de Figueirêdo et al., 2013). One composter was the control compost and was non inoculated, that is, was also designed by replacing the bacterial inocula by an equal volume of sterilized distilled water, Compost1 was inoculated with *B. subtilis* JCM 1465^T, Compost2 was inoculated with *Bacillus* sp. CMAGI2 and Compost3 with the mixed culture of the two strains (*B.* sp.CMAGI2 and *B. subtilis* JCM 1465^T. At the beginning of composting, the bacterial suspensions were sprayed on the moistened bagasse and the material was turned upside down after inoculation to spread the bacteria. The organic matter was humidified every two days, and the inoculation was repeated every two months.

During the first month, the return was done manually and weekly, then from the second month bimonthly and monthly until the end. At the third month, heated cow manure taken from local family farmhouse at Sangalkam was added in order to increase the pH and decrease the C/N ratio. 2.5 volumes of bagasse and 1 volume of cow manure were mixed. The composting was carried out for 5 months.

Microbial parameters

The changes of composting microflora at different stages were determined by enumeration. Microbial samples were collected at different times (days) and were taken from different points of the windrow, and then mixed before analysis: 0, 7th, 14th, 21th, 28th, 35th, 42th, 49th, 63th, 84th, 105th, 112th, 126th and 147th day. The targeted microbial groups were: mesophilic fungi and yeasts, total mesophilic bacteria, spore forming bacteria, faecal coliforms, faecal *Streptococci*, *Clostridia*, *Salmonella* and mesophilic actinomycetes. The analysis for *Staphylococci*, *Escherichia coli*, *Vibrio*, *Listeria* and *Bacillus cereus* were carried out only in the final compost (147th day) samples.

About 25 g of fresh compost were suspended in 225 ml sterile buffered peptone water (*Scharlau, Spain)* and shaken at 150 rpm for 30 min at room temperature (22°C) to allow the microorganisms to migrate into the solution. Then the resulting suspension was diluted (ten-fold serial dilutions) using sterile buffered peptone water in test tube (9 ml) and was used for microbial counts. One milliliter

from each dilution was spread on sterile Petri dishes containing the required culture media. After incubation, the number of colony forming units (CFU) was counted and the microbial number of each sample expressed in terms of CFU/g of fresh weight.

Fungi and yeasts were counted on chloramphenicol glucose agar (Scharlau, Spain) and plates were incubated at 25 to 30°C for 48 to 72 h according to ISO 7954 and FIL-IDF 94B standards. Total bacteria were determined by the plate pouring technique using plate count agar (Liofilchem, Italy) containing sterile cycloheximide at a concentration of 250 µg/ml. They were incubated at 30°C for 3 days. The number of spore-forming bacteria was determined as above, only; diluted suspensions were first incubated at 80°C for 20 min, then plated in nutrient agar (Liofilchem, Italy) supplemented with sterile cycloheximide (250 µg/ml) for 24 h at 30°C. *Clostridia* were detected on trypticase sulfite neomycin agar (*Bio-Rad, USA*) and incubated at 44°C for 24 h. Violet red bile lactose agar (Liofilchem, Italy) was used as selective differential medium to enumerate faecal coliforms after incubation at 44°C for 24 h. Bile esculin agar (Scharlau, Spain) was used for detecting faecal *Streptococci* after incubation at 44°C for 24 h. Actinomycetes were cultured in specific medium (Wink, 2004) composed of 1 g/l L-Asparagine, 10 g/l Glycerol, 1g/l K_2HPO_4, 20 g/l agar and 1 ml salt solution (1 g $FeSO_4$ $7H_2O$; 1 g MnCl2 $4H_2O$; 1 g $ZnSO_4$ $7H_2O$ in 100 ml distilled water) adjusted at pH 7.2 and supplemented with sterile cycloheximide (250 µg/ml). The incubation was done at 30°C for 7 to 15 days.

We monitored *Salmonella* during the process as recommended by standard methods of ISO 6579. The compost suspensions (25 g in 225 ml of buffered peptone water) were used for this analysis. On the samples of final compost (day 147), we used Baird Parker medium (Bio-Rad, USA) for *Staphylococci* and plates were incubated at 37°C for 24 h; the Mossel medium (Bio-Rad, USA) was used for *B. cereus* incubated at 30°C for 24 h. *E. coli* was counted on Rose Gal 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) agar (Biokar, France) after incubation at 44°C for 24 h. *Vibrio* were cultured in thiosulfate-citrate-bile-saccharose medium (Biokar, France) at 37°C for 24 h. *Listeria* was detected as recommended by standard methods of ISO 11290.

Analytical methods

The temperature was determined with electronic temperature probe *Checktemp1 (Hanna instruments*) and it was measured at 20 cm depth (the middle of pile). The measurements were taken at three points along the windrows. The pH was determined with an electrode pH-meter Cyberscan *(*laboratoires Humeau*)* on a water extract from compost using a ratio of 10:100 (w/v) compost/distilled water. Before reading the pH values, the mixture was shaken at room temperature for 30 min. The humidity content was determined by oven-drying at 105°C until constant weight (16 h). For these parameters, the samplings were done weekly for a better monitoring of the process. Ash content (A) was determined by loss on ignition of samples at 900°C with furnace (*carbolitte*) until a constant weight (24 h). Total organic carbon (TOC) content was determined by oxidation with potassium dichromate according to Walkley and Black (1934) modified method. The total nitrogen (TN) was analyzed by the method of Kjeldahl.

Loss of organic matter (OM) during composting was determined and we considered OM decreased when ash content increased. According to Paredes et al. (1996), we calculated the following formula:

$$\text{OM loss } \% = 100 - 100\left(\frac{\text{Ai}(100 - \text{Af})}{\text{Af}(100 - \text{Ai})}\right)$$

Where, Ai is the initial ash level and Af the final ash level.

The cellulase and xylanase activities were quantified by the methods of Miller (1959) and Wood and Bhat (1988) which are colorimetric methods using carboxymethylcellulose and xylan respectively as substrates. The alkaline and acid phosphatases activities were estimated by using ρ-nitrophenylphosphate prepared in acid and alkaline buffer respectively as substrate according to Tabatabai and Bremner (1969). These analyses for enzymatic activities were performed in triplicate. They were done on days 0, 28, 56, 84, 112 and 147 and values were read with an UV/Vis spectrophotometer *Analytik Jena* (*Specord 200 Plus*) equipped with basic *WinASPECT PLUS*® software. Results were expressed in unit enzyme corresponding to 1 µmole of product liberated per gram dry weight enzyme per minute.

Statistical analysis

Data obtained in the study were the mean values of three replicates. Data were subjected to statistical evaluation using one-way ANOVA ($p < 0.05$) with XLSTAT (v2008.1.01) software and Tukey (HSD) test for multiple comparisons.

RESULTS AND DISCUSSION

Humidity, temperature and pH during composting

Humidity is a very important parameter for composting and it may become a limiting factor if not well monitored (Ahmad et al., 2007). That is the reason why, the moisture content was determined every week for a better monitoring of the composting process. The best range of moisture is 40 to 60% (FAO, 2005; Ahmad et al., 2007). An initial humidity of 60% is acceptable in the beginning of the composting process; however that should decrease up to 30% to prevent further biological activity in the final product. In this process, the four designs of composting were in the ideal range of humidity (Figure 1). With 60% humidity at the beginning of process, the four composts reached around 30% of moisture at the final stage with highest values in control compost.

Temperature is an important factor for tracking the composting process evolution. It was weekly monitored during all the process. The four composts presented the same temperature profile (Figure 2). After 2 months, the highest temperature (45.07°C) was recorded in compost3, and lowest temperature was noted after the seventh week with weak value of temperature 37.53°C in control compost confirmed statistically (p=0,013) with significant difference between control compost and compost3. These low temperature values can be attributed to the decrease in pH, which can affect microbial growth and activity.

According to Sundberg et al. (2004), low pH is an inhibiting factor in the transition from mesophilic to thermophilic phase, which explained low values of composting temperatures. But in control compost, there is no low pH value, so the recorded heat in other

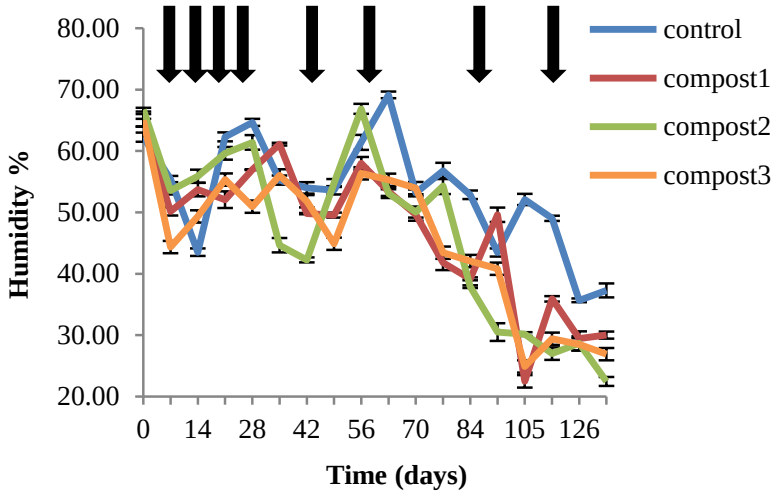

Figure 1. Humidity evolution in the four windrows composting. Arrows indicate turnings. The vertical bars represent the standard deviation of the mean calculated for triplicates.

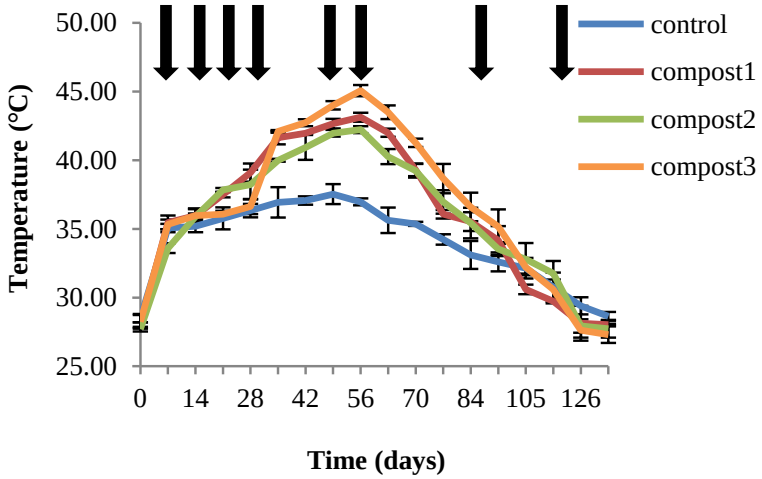

Figure 2. Temperature profile of the four composts. Arrows indicate turnings.

windrows may be caused by the activity of inoculated bacteria. At initial stage of composting, low pH was due to the low pH of the bagasse which was 3.56 (Table 1) and increased during the first month.

One month after, we observed a decrease in pH and the condition was found to be acidic in inoculated windrows which may be attributed to the presence of short chain organic acids, mainly lactic and acetic acids. These products resulted from the activity of acid-forming bacteria that break down complex carbonaceous material to organic acids as intermediate products (Tuomela et al., 2000; Beck-Friis et al., 2001).

In control compost, any decrease in pH was not observed (Figure 3) and confirmed statistically with significant difference between control compost and inoculated composts ($p < 0.05$). At the third month, after incorporation of the cow manure, the pH increased up to 7.0 in all composts corresponding to the ideal neutral final pH with a high natural buffering capacity (FAO, 2005). Evolution of composts was monitored during the experimental period of 5 months and characteristics of final composts are presented in Table 2.

According to the formula of Paredes et al. (1996); post-hoc comparisons using HSD test indicated in terms of

Table 1. Physico-chemical characterization of bagasse and cow manure before composting.

Parameter	Bagasse	Cow manure
Humidity (%)	9.58(0.83)	8.35(0.34)
pH	3.56(0.03)	8.62(0.01)
Organic carbon (%)	66.38(0.49)	33.54(1.50)
Nitrogen (%)	0.34(0.00)	2.66(0.00)
C/N ratio	195.23(1.43)	12.61(0.56)
Ash (%)	20.50(1.13)	51.55(1.32)
Organic matter (%)	79.50(1.13)	48.45(1.32)

Values in parenthesis are deviation standard of the triplicates.

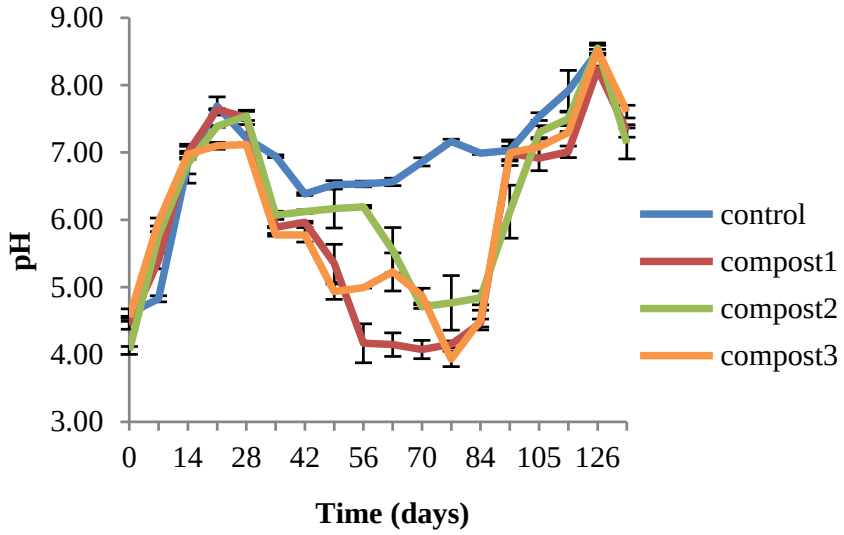

Figure 3. Changes in pH for the four types of composting.

OM loss that compost3 presented the highest value (91.37% OM loss), followed by compost1 and compost 2 with 90.15 and 89.47% OM loss, respectively. The control compost which showed the lowest OM loss (85.55%) presented a significant difference ($p < 0.05$) compared to the inoculated ones (Figure 4). Thereby, control compost was less degraded compared to others. This result is in agreement with that of C/N ratio, which indicated that control compost was less degraded (Figure 5). These two parameters (MO and the C/N ratio) are considered as maturity parameters.

Microbial evolution

This process involves complex microbial community which plays key role during composting (Insam and de Bertoldi, 2007). Many factors determine microbial evolution during composting like temperature, pH, humidity and aeration.

Fungi and yeasts

Mesophilic fungal populations were low at the beginning of composting process in all windrows. A rapid growth of this microbial group was observed in the first four weeks of the experiment, and the highest fungal population was obtained at the 28th day. The control presented the highest fungal population (1.26×10^{13}CFU/g), followed by compost3 (1.91×10^{12} CFU/g) and compost2 (3.25×10^{10} CFU/g) (Figure 6). Compost1 presented the lowest fungal population with 1.0×10^{9} CFU/g. After the 28th day, a drastic decrease in fungal population was observed, which may be due to the increasing temperature, since fungi are very sensitive to the high temperature (Tuomela et al., 2000; Mehta et al., 2014). Surprisingly, compost3 in which the highest temperature was recorded presented

Table 2. Physical and chemical characterization of the four different final composts.

Parameter	Control	Compost1	Compost2	Compost3
Humidity%	37.29(1.14)	30.01(0.57)	22.44(0.72)	26.89(0.20)
pH	7.48(0.07)	7.32(0.09)	7.13(0.23)	7.61(0.09)
Nitrogen%	0.82(0.03)	1.12(0.00)	1.24(0.14)	1.09(0.09)
C/N ratio	37.56(3.03)	19.12(0.92)	16.41(1.49)	21.05(1.74)
Organic matter	36.17(0.80)	22.38(0.58)	26.72(0.12)	24.51(0.59)
Na%	0.22(0.01)	0.14(0.01)	0.24(0.01)	0.20(0.00)
Mg%	0.35(0.00)	0.27(0.00)	0.30(0.01)	0.40(0.00)
K%	0.68(0.00)	0.62(0.01)	0.70(0.02)	0.80(0.01)
Ca%	1.19(0.02)	0.74(0.02)	0.93(0.02)	1.16(0.00)
P(ppm)	551(0.00)	240.34(0.00)	251.27(0.00)	419.86(0.00)
Color	Brown	Brown	Brown	Brown
Odor	Damp earth	Damp earth	Damp earth	Damp earth

values in parenthesis are deviation standard of the triplicates.

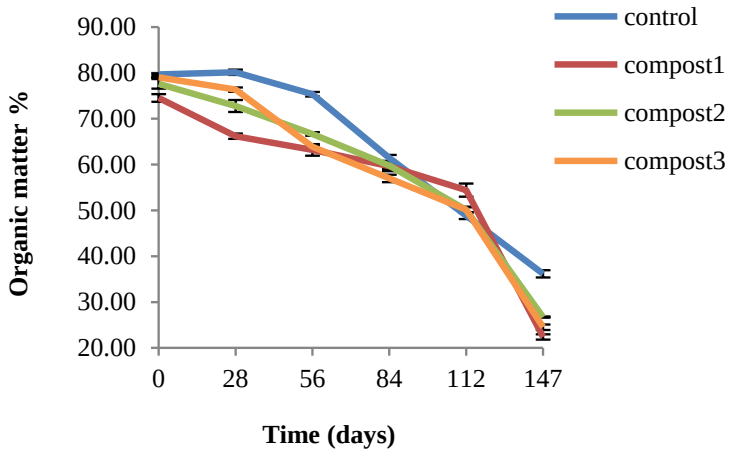

Figure 4. Evolution of organic matter during composting.

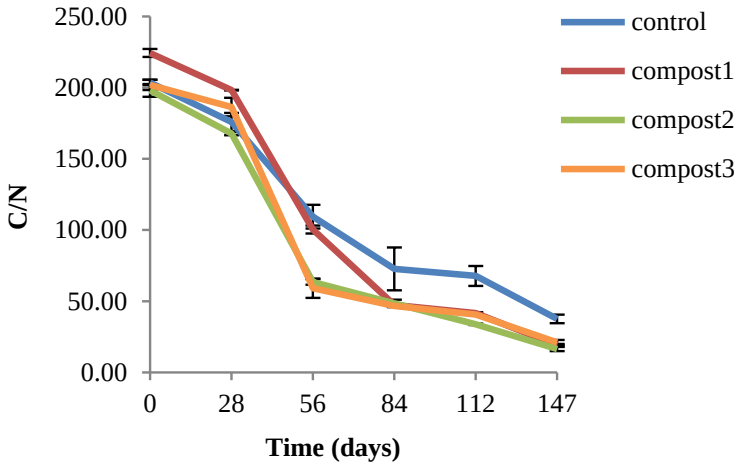

Figure 5. C/N ratio during composting.

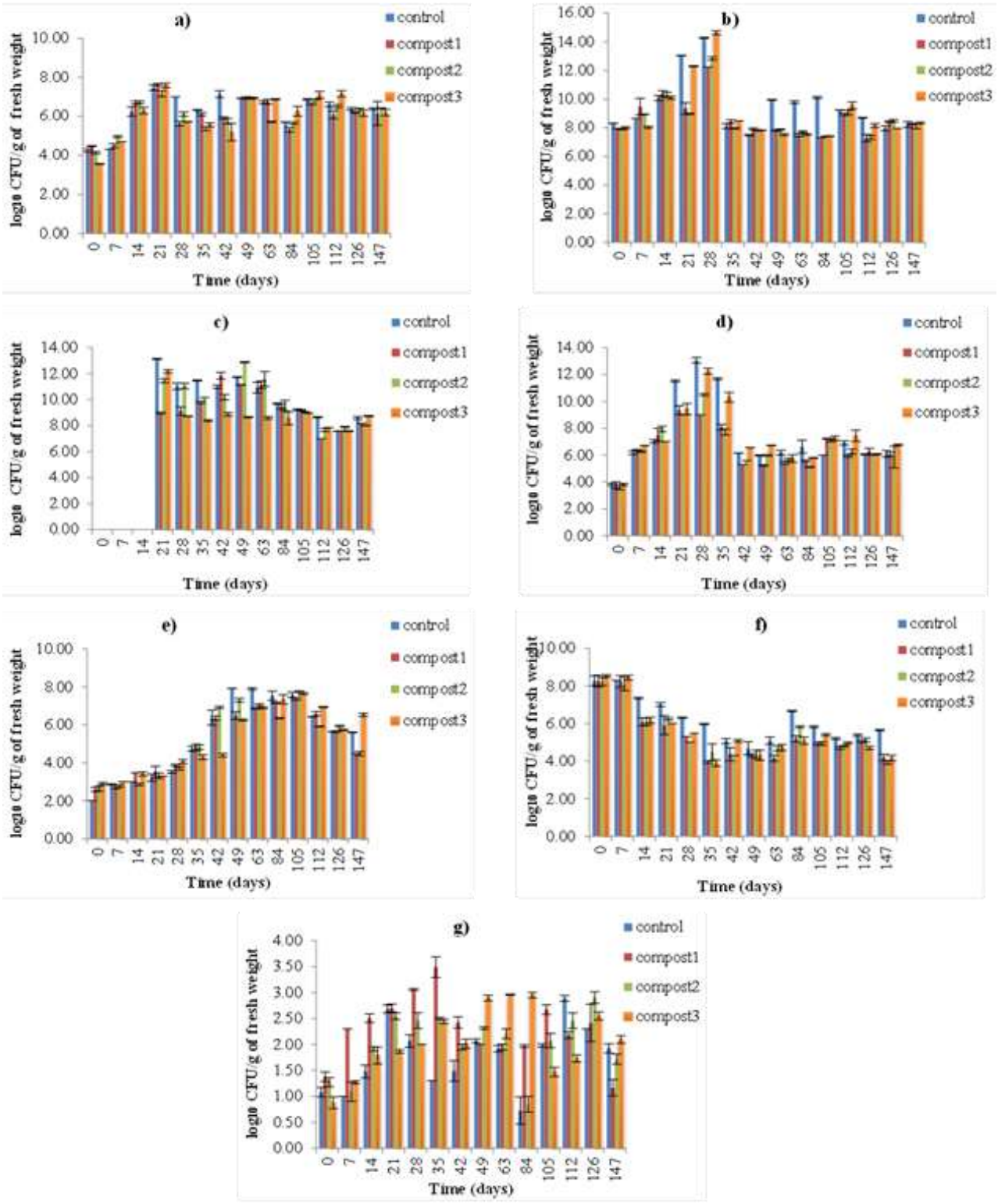

Figure 6. Evolution of different microbial groups during different composting. (a) spore-forming bacteria. (b) Bacteria. (c) Actinomycetes. (d) fungi and yeasts. (e) Faecal coliforms. (f) Faecal Streptococci. (g) Clostridia. The vertical bars represent the standard deviation of the mean calculated for triplicates.

the highest fungal population compared to other inoculated composts. When the pH increased and the temperature decreased, mesophilic fungal population increased and recolonized again the substrate. The number of fungal population presented significant difference ($p < 0.05$) across different composts (Table 4).

Mesophilic bacteria

During composting, bacteria are mainly responsible for substrate decomposition and heat generation (Tuomela et al., 2000; Insam and de Bertoldi, 2007; Mehta et al., 2014). Initial bacterial population was near 10^7 to 10^8 CFU/g in the different windrows. Bacterial growth occurred in the first four weeks with significant difference between control compost, compost3 and inoculated composts with single strain ($p < 0.05$). After 28^{th} day a decrease in bacterial population was observed. The highest bacterial population was found in compost3 (4.52×10^{14} CFU/g) followed by control compost (1.93×10^{14} CFU/g), and then compost2 and compost1 had no difference statistically. From the fourth week, the mesophilic bacterial population began to decrease in all windrows probably due to the decreasing pH and elevation of temperature. Among the four designed composting, control compost had the most important number of bacteria, this may be explained by the fact that, its pH had never fallen to acidity level throughout the composting process (Table 4).

Spore-forming bacteria

During the first month, the density of spore-forming bacteria increased from 10^4 to 10^7 CFU/g in all windrows. A progressive increase of spore-forming bacteria was observed because of the increasing temperature and the decreasing of pH in all windrows. However, the population of sporulated bacteria of control compost was higher than the others ($p < 0.05$) (Table 4). Probably, this was due to nutrient competition phenomenon available for bacteria or these bacteria could not use the readily degradable cellulosic substrate. When the temperature decreased and the pH increased meaning favorable environment, there was a germination of spores and a decrease of spore-forming bacteria.

Actinomycetes

Actinomycetes play key role in degradation of organic compounds such as cellulose, lignin, chitin and proteins (Epstein, 1997). In this process, they appeared from the third week and reached 1.37×10^{13} CFU/g in control compost and the lowest population was noted in compost1 with 9.59×10^8 CFU/g. Their non-appearance during the initial stage might be due to their slow growth rate compared to bacteria or fungi. This result is consistent with many reports in which actinomycetes appeared during the thermophilic phase as well as the cooling and maturation phases of composting (Tuomela et al., 2000). During this composting in all windrows, actinomycetes population presented significant difference in all composts ($p < 0.05$).

Faecal coliforms

The count of faecal coliforms is a good indicator of the sanitary quality of soil, food and environment. A low faecal coliform population was noted at initial stages, then an increase up to 10^8 CFU/g was noted probably due to recontamination, redistribution by equipment used for windrows turnings or water used for humidification. When the temperature cooled down in last month, coliforms decreased to 4.05×10^5 in control compost, 3.0×10^4 in compost1, 3.14×10^4 in compost2 and 3.43×10^6 in compost3 ($p < 0.05$) (Table 3).

Faecal Streptococci

Faecal *Streptococci* are ubiquitous and are the best indicators of faecal pollution. The number of *Streptococci* decreased considerably from 2.09×10^8 in control compost, 2.16×10^8 in compost1, 1.83×10^8 in compost2 and 3.42×10^8 in compost3 ($p < 0.05$) at initial stages to 4.58×10^5 in control compost, 1.65×10^4 in compost3, 1.52×10^4 in compost1 and 8.52×10^3 in compost2 ($p < 0.05$) at the end. Similar results were reported by Hassen et al. (2001).

Clostridia

Clostridia are telluric bacteria and involved in biodegradation of soil. *Clostridium* converts organic compounds to sugar, acids and alcohol and play important role in compost maturation (Ryckeboer et al., 2003b; Franke-Whittle, 2014), that is, anaerobic microorganisms decompose organic matter after aerobic bacteria had consumed oxygen in composts or when gas exchange is very slow. During the composting process, the count in compost1 was higher than the others, compost2 and compost3 had the same number and control compost presented the lowest number ($p < 0.05$) (Table 4).

Table 3. Microbial parameters of the final composts.

Parameter	Control compost	Compost 1	Compost 2	Compost 3
Spore-forming bacteria (CFU/g)	9.0×10^5	6.5×10^5	8.0×10^5	5.0×10^4
Fungi and Yeasts (CFU/g)	1.40×10^6	1.22×10^6	2.17×10^6	5.74×10^6
Bacteria(CFU/g)	1.8×10^8	1.85×10^8	1.35×10^8	2.15×10^8
Actinomycetes (CFU/g)	4.03×10^8	1.50×10^8	1.04×10^8	5.37×10^8
Faecal coliforms (CFU/g)	4.05×10^5	3.0×10^4	3.14×10^4	3.43×10^6
Faecal *Streptococci* (CFU/g)	4.58×10^5	1.65×10^4	8.52×10^3	1.52×10^4
Clostridium (CFU/g)	85	15	53	126
Salmonella (25g)	absence	absence	absence	absence
Staphylococci (CFU/g)	0	0	0	0
E.coli (CFU/g)	0	0	0	0
B. cereus (CFU/g)	0	0	0	0
*Listeria (*25 g)	Absence	Absence	Absence	Absence
Vibrio (CFU/g)	0	0	0	0

Table 4. Microbial number of different composts during the composting process.

Log_{10} CFU/g of fresh weight	Control compost	Compost 1	Compost 2	Compost 3
Fungi and yeasts	103.380^a	91.137^b	92.663^b	101.880^a
Bacteria	133.957^a	121.237^c	120.763^c	125.803^b
Spore-forming bacteria	88.343^a	84.653^b	84.707^b	85.293^b
Actinomycetes	113.027^a	103.477^c	109.533^b	97.063^d
faecal coliforms	74.350^a	71.953^b	71.850^b	73.633^a
faecal streptococci	86.797^a	74.643^c	76.103^{bc}	76.967^b
Clostridium	24.920^c	32.217^a	28.250^b	28.900^b

^{a,b,c,d}represent significant differences ($p<0.05$) in the same line.

***Salmonella* were not detected at any stage during the whole composting process**

Except *Clostridium*, microbial population was found to be higher in control compost probably due to low temperature which was recorded in control compost. It was followed by compost3. The compost1 and compost2 had the lowest count and often equal statistically for most microbial groups with ($p < 0.05$). Probably, *Bacillus sp.* CMAGI2 and *Bacillus subtilis* JCM 1465^T had an inhibitory effect on microbial flora if inoculated alone. For instance in compost3, where a mixed culture of the two strains was inoculated, microbial population was higher than that of compost1 and compost2 inoculated with a single strain. When these two *Bacillus* strains were inoculated together, they probably had no inhibitory effect on microflora (Table 4).

Enzymes are biomolecules involved in specific catalyzing biological reactions (Alef and Nanniperi, 1995). During the composting, secretions and changes in enzymatic activities were caused by the action of many microorganisms. These enzymes participate in return in complex microbial successions. Enzymes are the main mediators of various biodegradation processes (Goyal et al., 2005). In this study, three important enzymes involved in organic matter biodegradation were targeted: cellulases, xylanases, acid and alkaline phosphatases.

The initial cellulases activities were very low in all designed composts. On day 56, these activities were equal in compost1 and compost3 and started to increase ($p < 0.05$) compared to cellulase activity of control compost and compost2. Highest values were significantly different in windrows and noted on 112^{th} day. Indeed, compost3 scored a cellulase activity of 3.1369 $U.g^{-1}.mn^{-1}$ while compost2 and compost1 scored 2.5691 and 2.1532 $U.g^{-1}.mn^{-1}$ respectively (Table 5). The lowest value was recorded in control compost with 1.7734 $U.g^{-1}.mn^{-1}$. That may be explained by low level of nitrogen reported to be a limiting factor in cellulose degradation elsewhere (Tuomela et al., 2000). Increase in cellulase activity

Table 5. Evolution of cellulase, xylanase, acid phosphatase, alkaline phosphatase activities during composting.

Time (days)	Cellulase (U.g^{-1}dry matter)						Xylanase (U.g^{-1}dry matter)						Acid phosphatase (mU.g^{-1}dry matter)						Alkaline phosphatase (mU.g^{-1}dry matter)					
	0	28	56	84	112	147	0	28	56	84	112	147	0	28	56	84	112	147	0	28	56	84	112	147
Control compost	0.08	0.07	0.15	0.12	1.77	0.47	0.21	0.26	0.12	0.2	1.29	0.46	20.96	33.11	49.23	45.88	42.14	47.31	0.02	3.94	0.44	0.59	1.89	1.01
Compost1	0.11	0.15	0.25	0.51	2.15	0.37	0.10	0.15	0.30	0.61	12.18	1.01	29.18	65.86	72.83	47.27	21.15	15.94	0.07	1.95	0.38	1.60	1.26	1.02
Compost2	0.13	0.09	0.16	0.43	2.57	0.60	0.30	0.14	0.24	0.52	11.46	0.81	26.72	69.47	45.93	24.47	13.35	27.07	0.03	3.22	2.41	3.07	1.63	1.34
Compost3	0.09	0.15	0.28	0.58	3.14	0.68	0.17	0.22	0.43	0.39	7.24	1.33	26.53	53.10	72.62	35.31	33.72	23.48	0.07	2.48	2.47	1.92	1.93	1.13

observed on the 112th day is attributable to the incorporation of cow manure which increased the compost microflora. Since sugar cane bagasse contained about 50% cellulose, 25% hemicellulose and 25% lignin (Tuomela et al., 2000; Insam and de Bertoldi, 2007), we can understand why these low values were obtained.

Xylanases activities presented the same trend as cellulases activities, that is, the highest activity was remarked on day 112. This similar trend between cellulases activities and xylanases activities were also observed by Zeng et al. (2010).

Compost1 and compost2 had no significant difference and scored the highest values with 12.181 and 11.455U.g^{-1}.mn^{-1}, but they presented difference compared to control compost and compost3 with xylanases values 1.291 and 7.237 U.g^{-1}.mn^{-1} respectively.

The slow biodegradation kinetics of cellulose and hemicellulose can be explained by the environmental condition under which the experiment was carried out. In fact, the optimun degradation activity of these enzymes are obtained in low pH and high temperature (around 50°C) (Schinner and von Mersi, 1990). Also, like any enzymatic reaction, concentration, location and mobility of the enzymes in the compost impact in enzymatic activity (Hayano, 1986). Presence of more easily-decomposable substrates lead to the suppression of components rich with xylan and cellulose decomposition. Hence, we can assume that the strong activity on day 112 was caused by high microbial diversity noted during the cooling and maturation phases of composting (Ryckeboer et al., 2003b; Insam and de Bertoldi, 2007).

Phosphatases catalyse the hydrolysis of phosphate esters and are enzymes with relatively broad specificity, capable of acting on a number of different structurally related substrates, but at widely different rates (Alef and Nanniperi, 1995).

In the phosphatase assay, phosphomonoesterases were searched and were classified as acid, neutral and alkaline phosphatases; the first two enzymes have been detected in animal, microbial and plant cells and the other has been found only in microorganisms and animals (Alef and Nanniperi, 1995)..

In this process, we noted high activities of acid phosphatase during the first month. Statistically, all windrows presented significant values with 69.47 mU.g^{-1} for compost2, 66.59 mU.g^{-1} for compost1, 53.10 mU.g^{-1} for compost3 and 33.11 mU.g^{-1} for the control compost for the second month for the others and then declined. This phenomenon is similar to the studies of Albrecht et al. (2010). The highest value was observed in compost1 with 72.83 mU.g^{-1}.mn^{-1} to the second month.

Initial alkaline phosphatases were very low and its activity increased in the first month with significant difference in all windrows when the pH increased. The highest alkaline phosphatase activity was recorded in control compost with 3.94 mU.g^{-1}.mn^{-1} and the lowest value of enzyme was 1.95 mU.g^{-1}.mn^{-1} in compost1. The activity of this enzyme decreased in the second month, increased again in the third month and decreased again at the end in all composts.

Compost1, compost2 and compost3 showed higher enzymatic activities than control compost. These results are consistent with the evolution of organic matter and C/N ratio and confirmed by the study of Albrecht et al. (2010). These three inoculated composts were found to be better degraded than the non inoculated control compost, implying that the the use of the two *Bacillus* strains would enhance organic matter biodegradation rate, and shorten the composting process.

Conclusion

The aim of this study was to use two *Bacillus* strains (*Bacillus* sp. and *B. subtilis*) as inocula during a composting with bagasse as substrate. These strains were tested as to whether they could enhance the biodegradation rate of sugar cane bagasse. Physicochemical and microbiological parameters were monitored to follow maturation of composts. Interestingly, *Bacillus* strains presented a high metabolic activity, and could reduce the composting time of sugarcane bagasse. Besides, these strains decreased the microbial microflora count when they were inoculated alone with significant difference compared to control and the other with mixed bacterial culture windrows. The obtained products would contribute and increase the fertilization of soils.

Conflict of Interests

The authors have not declared any conflict of interests.

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Kinetic models and parameters estimation study of biomass and ethanol production from inulin by *Pichia caribbica* (KC977491)

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The growth kinetics and modeling of ethanol production from inulin by *Pichia caribbica* (KC977491) were studied in a batch system. Unstructured models were proposed using the *logistic equation* for growth, the *Luedeking-Piret* equation for ethanol production and modified *Leudeking-Piret* model for substrate consumption. Kinetic parameters (X_0, μ_m, m, n, p and q) were determined by nonlinear regression, using Levenberg-Marquart method implemented in a Mathcad program. Since the production of ethanol was associated with *P. caribbica* cell growth, a good agreement between model predictions and experimental data was obtained. Indeed, significant R^2 values of 0.91, 0.96, and 0.95 were observed for biomass, ethanol production and substrate consumption, respectively. Furthermore, analysis of variance (ANOVA) was also used to validate the proposed models. According to the obtained results, the predicted kinetic values and experimental data agreed well. Finally, it is possible to predict the development of *P. caribbica* using these models.

Key words: *Pichia caribbica*, inulin, bioethanol, numerical simulation.

INTRODUCTION

Bio-ethanol being a clean, safe and renewable resource has been considered as a potential alternative to the ever-decreasing fossil fuels (Martin et al., 2002; Wyman, 1994). Various substrates are available for the ethanol production but their choice depends on the cost and the production process profitability (Quintero et al., 2015). Most of the industrial processes are currently based on hexose carbohydrates from starch or sucrose-containing biomass (Kumari and Pramanik, 2012; Duhan et al., 2013). Among these substrates, inulin has received a major interest since it is present as a carbohydrate reserve in a large variety of plant roots and tubers such

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as Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium intibus*), dahlia (*Dahlia pintana*) and dandelion (*Taraxacum officinal*) (Cabezas et al., 2002; Singh and Bhermi, 2008).

The bioconversion of biomass to ethanol is executed following two steps: hydrolysis of solid substrate to reducing sugars and the fermentation by yeast or bacteria to convert fermentable sugars to ethanol (Torget et al., 1991; Kara Ali et al., 2013). The bioprocess which involves microbial cells is complex in nature and is a critical step for better yield achievement (Mahajan et al., 2010). Behavior of the microbial system can be evaluated by the development of kinetic models and experimental designs (Voll et al., 2011; Xu et al., 2011). The use of kinetic models is interesting to reduce the number of experiments needed to assess the extreme operation conditions and for optimization and control (Lin and Tanaka, 2006). Two different categories of mathematical model; the structured and unstructured models, can be considered for modeling a microbial process (Nielsen et al., 1991; Gadjil and Venkatesh, 1997; Murat and Ferda, 1999; Lei et al., 2001). Structured models take into account some basic aspects of cell structure, function and composition. By contrast, in unstructured models, only a global parameter such as cell mass is employed to describe the biological system, cell growth or product formation. Usually, theoretical models have been proposed and used for the elucidation of metabolic steps and for the calculation of kinetic parameters (Ghosh et al., 2012). To our knowledge, this is the first report to study *Pichia caribbica* (KC977491) growth kinetics and the modeling of ethanol production from inulin by this yeast strain. The main objectives were to: (I) Produce biomass and ethanol by *P. caribbica* (KC977491) in a batch system; (II) Propose unstructured models for growth and ethanol production to predict a process of fermentation by *P. caribbica* (KC977491); (III) Validate the obtained results between the theoretical unstructured models and experimental data.

MATERIALS AND METHODS

Yeast and culture media

The yeast *P. caribbica* (KC977491) used in this work was isolated from arid soil area and identified previously (Kara Ali et al., 2013). This strain was grown in a medium containing 100 ml of YPD (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L), incubated at 30°C for 24 h under agitation of 150 rpm. Cells (11 ml/ DO_{600} = 9) were further transferred into flasks containing 100 ml of the fermentation medium composed of (g/L): inulin 30, yeast extract 4, peptone 4 and initial pH 5. The culture was incubated at 37°C under agitation of 150 rpm for 5 days.

Assay techniques

Fructose and ethanol analysis

After the fermentation period, the biomass was separated from medium using centrifugation technique at 5,000 rpm and 4°C for 5 min. The supernatant were cleaned by cellulose acetate membrane (0.2 µm, Minisart Sartorius), then, the fructose consumption and ethanol production were investigated by HPLC under subsequent conditions following the CWBI protocol: Agilent 1110 series (HP Chemstation software) with a Supelcogel C-610H column preceded by a Supelguard H pre-column (oven temperature 40°C). 0.1% H_3PO_4 solution (in milliQ water) was used as the isocratic mobile phase at a flow rate of 0.5 ml $min^{-}1$ and a differential refractive index detector (RID) was heated at 35°C. The process lasted for 35 min at a maximum pressure of 60 bars. The standard curves were prepared using the different concentrations of fructose and ethanol (from 0.125 to 4 g/l) for both of them.

Cell mass analysis

The biomass concentration of *P. caribbica* was determined by the dry weight method (Buono and Erickson, 1985). The cells obtained as mentioned previously were washed twice with water and dried by incubation at 105°C until constant weight.

Mathematical approach

Kinetic models

A mathematical model is a collection of mathematical relationships which describe a process. Practically in each model, a simplification of the real process is made. Mathematical models have proven to be very useful in gaining insight in processes (Philippidis et al.,1992; Santos et al., 2012) for instance by comparing different models and their ability to describe experimental data (Auer and Thyrion, 2002; Amribt et al., 2013). Furthermore, models have been successfully used for optimization or control of processes (Yip and Marlin, 2004). Different types of models can be distinguished for the different goals and depending on the available information. Some characteristics which are of interest for modeling bioprocesses are illustrated in Table 1.

Microbial growth kinetics

The logistic equation is a very common unstructured model in macroscopic description of cell growth processes (Parente and Hill, 1992). It accounts for the inhibition of growth which occurs in many batch processes (Benkortbi et al., 2007). In this study, the logistic equation was adapted to investigate *P. caribbica* (KC977491) growth. It can be described as follows:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where X is the biomass concentration (g/L), X_m is the maximum biomass concentration (g/L), μ_m is the maximum growth rate (h^{-1}) and t is the time (h). The integration of the biomass production rate with the use of the initial condition (at t = 0, X = X_0) gives a sigmoidal variation of X as a function of t which may represent both an exponential and a stationary phase (Equation 2):

$$X = \frac{X_0 e^{\mu m t}}{\{1-(X_0/X_m)(1-e^{\mu m t})\}} \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Ethanol production kinetics

The kinetic of product formation was based on the *Luedeking-Piret* model, initially developed for the fermentation of gluconic acid by different types of microorganisms (Luedeking and Piret, 1959). It is

Table 1. Some growth models reported in the literature.

Kinetic models	Symbols used	Authors
$\mu = \mu_{max}\frac{S}{K_S + S + S^2/K_i}$	**μ**: is the specific growth rate (h^{-1}) **μ_{max}:** is the maximum specific growth rate (h^{-1}) **S**: is the substrate concentration (g/L) **K_S**: is the Substrate saturation constant (g/L) **K_i**: is the substrate inhibition constant (g/L)	Jackson and Edwards (1975)
$\mu = \mu_{max}\frac{S^n}{S^n + K_S}$	**n**=Constant of the process	Moser (1983)
$\mu = \mu_{max}\frac{S}{S + K_S}\{1 - \frac{S}{S_m}\}^n$	**S_m** : is the maximum substrate concentration above which growth is completely inhibited (g/L) **n:** is an empirical constant	Luong (1987)
$\mu = \mu_{max}\frac{S}{K_m + \left(1 + \frac{P}{K_p}\right)S}$	**K_m**: is the Michaelis constant **K_p**: is the lactate inhibition constant for cell growth (g/L) **P** : is the product concentration (g/L)	Ishizaki and Ohta (1989)
$\mu = \mu_{max}\left(\frac{S}{S + K_S}\right).\left(\frac{K_i}{S + K_i}\right).(1 + \frac{P - K_i}{P_m - P_i})$	**K_i** : is the substrate inhibition constant (g/L) **P_m**: is the maximum inhibitory lactate concentration (g/L) **P_i** : is the threshold level of lactate before an inhibitory effect (g/L)	Boonmee et al. (2003)
$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)^f.\left(1 - \frac{P}{P_{max}}\right)^h$	**f**: is a parameter related to the toxic power for biomass **h**: is a parameter related to the inhibitory product	Altiok et al. (2006)

an unstructured model, which combines growth and non-growth associated contribution towards product formation. Thus, the product formation depends upon the growth rate (dX/dt) and instantaneous biomass concentration (X) (Equation 3).

$$\frac{dP}{dt} = m\frac{dX}{dt} + nX \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Where "P" is the product concentration (g/L), "m" (g/g) and "n" (1/h) are the *Luedeking-Piret* equation parameters for growth and non-growth associated product formation respectively. A carbon substrate is used to form cellular material and metabolic products as well as for the cellular maintenance.

The product formation rate equation (Equation 4) can be expressed by integrating Equation 3 using Equation 2 with the initial conditions P = 0 at t = 0:

$$p = mX_0\left\{\frac{e^{\mu m t}}{\left\{1-\left(\frac{X_0}{X_m}\right)(1-e^{\mu m t})\right\}}\right\} + n\frac{X_m}{\mu_m}\ln\{1 - \frac{X_0}{X_m}(1 - e^{\mu_m t}) \quad \ldots\ldots \quad (4)$$

Substrate consumption kinetics

Kinetics substrate consumption can be described as follows:

$$-\frac{dS}{dt} = p\frac{dX}{dt} + qX \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

Where, p= $1/Y_{X/S}$ (g/g) and q is maintenance coefficient (1/h). Equation (5) is rearranged as follows:

$$-dS = p\,dX + q\int X(t)\,dt \quad \ldots\ldots\ldots\ldots\ldots \quad (6)$$

Substituting Equation 2 in Equation 6 and integrating with initial conditions ($S = S_0$; t = 0) give the following equation:

$$S = S_0 - pX_0\left\{\frac{e^{\mu m t}}{\left\{1-\left(\frac{X_0}{X_m}\right)(1-e^{\mu m t})\right\}} - 1\right\} - q\frac{X_m}{\mu_m}\ln\{1 - \frac{X_0}{X_m}(1 - e^{\mu_m t}) \quad (7)$$

Model of parameters estimation

Kinetic models which describe the microbial process on a particular substrate are nonlinear which in turn makes parameter estimation relatively difficult. Though few models can be linear, their utilization is limited because of the error associated with the transformation of dependent variable and therefore resulted in inaccurate parameter estimations. Hence, the nonlinear least-squares regression is often used to estimate kinetic parameters from nonlinear expressions. The parameter estimation obtained from the linear kinetics expressions can be used as initial estimation in the iterative nonlinear least-squares regression using the least square curve fit in order to fit the models developed and to estimate the parameters (substrate consumption, biomass and product formation).

Fitting procedures and parametric estimations calculated from the results were carried out by minimization of the sum of quadratic differences between experimental and model-predicted values, using the nonlinear least-squares Levenberg-Marquardt method (Marquardt, 1961) with a developed Mathcad program. The coefficient of determination R^2 was estimated to assess the accuracy of the estimated parameters achieved by fitting the experimental values to the proposed mathematical models. If R^2 approximate to 1, this coefficient justifies an excellent consistency of these equations (Annuar et al., 2008). Furthermore, the ANOVA

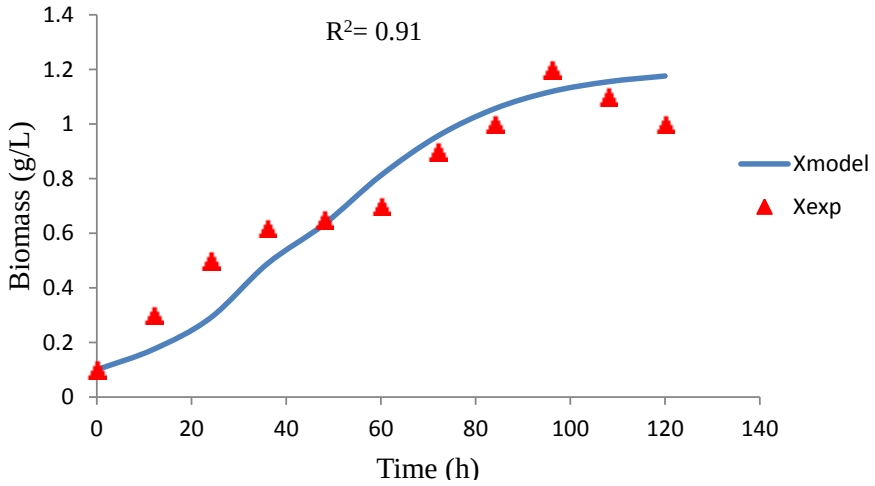

Figure 1. Comparison between predicted and experimental growth kinetics.

Table 2. Analysis of variance for the growth model.

Source of variation	Sum of squares (SS)	Degree of freedom (DF)	Mean square (MS)	F-value	Critical F value (Fcrit)
Regression	1.68333955	1	1.68333955	**101.694608**	**5.11735501**
Error	0.148976	9	0.01655289		
Total	1.83231555	10	0.18323155		

test was also carried out to evaluate the accuracy of the models. The two basic data measures of variation sources are: Variation due to the regression and variation due to residuals. The statistical F-value is a ratio of the relative regression variation/relative residual variation. Thus, if F value is significantly greater than critical F value, this indicates that the regression model is accepted.

RESULTS AND DISCUSSION

Many researchers have attempted to model yeast fermentation and different approaches have been considered (Aiba et al., 1968; Ghose and Tyagi, 1979; Hoppe and Hansford, 1982). However, it is not easy to choose a single best fitting. In order to choose the best model it is important to consider how well it describes the transition from exponential to stationary phase of the process model (Kostov et al., 2012).

Microbial growth

The logistic equation of biomass growth (Equation 2) is used to fit the batch fermentation growth data. Figure 1 compares the predictive model related to cell growth with the experimental data recorded during batch fermentation of *P. caribbica* (KC977491). The maximum biomass concentration (1.2 g/L) was obtained after 96 h of fermentation and a complete depletion of fructose in the medium. In addition, a Levenberg-Maquardt method is used in Mathcad to obtain μ_{max} by minimizing the difference between experimental growth and calculated one using Equation 2. The program gives the value of μ_{max} = 0.052 h^{-1}. This value is relatively low compared to those reported in several studies. Indeed, the μ_{max} value from *Saccharomyces diastaticus* (strain LORRE316) was in the interval of 0.1 and 2 h^{-1} with optimum of 0.9 h^{-1} (Wang and Sheu, 2000). Otherwise, the production of ethanol using *Saccharomyces cerevisiae* (ATCC4126) has showed a μ_{max} of 0.28 h^{-1} (Bazua and Wilke, 1977). Moreover, the μ_{max} related to *S. cerevisiae* ITD00196 reached 0.58 h^{-1} in a batch system (Jiménez-Islas et al., 2014). The variation of this parameter may be explained by the type of microorganisms, the substrate consumption and the environmental conditions.

The analysis of Figure 1 shows that there is an adequacy between the experimental data and those predicted (R^2 = 0.91). Also, the analysis of variance (ANOVA) results for the growth model are presented in the Table 2. F-value (101.694608) is greater than critical F value (5.11735501), which proved the acceptance of this test. On the basis of the obtained results, a good correlation coefficient (R^2 = 0.91) and a significant ANOVA test shows that the proposed logistic model is adequate to explain the sigmoidal profile of the yeast growth. According to the literature, the study proposed by Dodic et al. (2012), was carried out used logistic

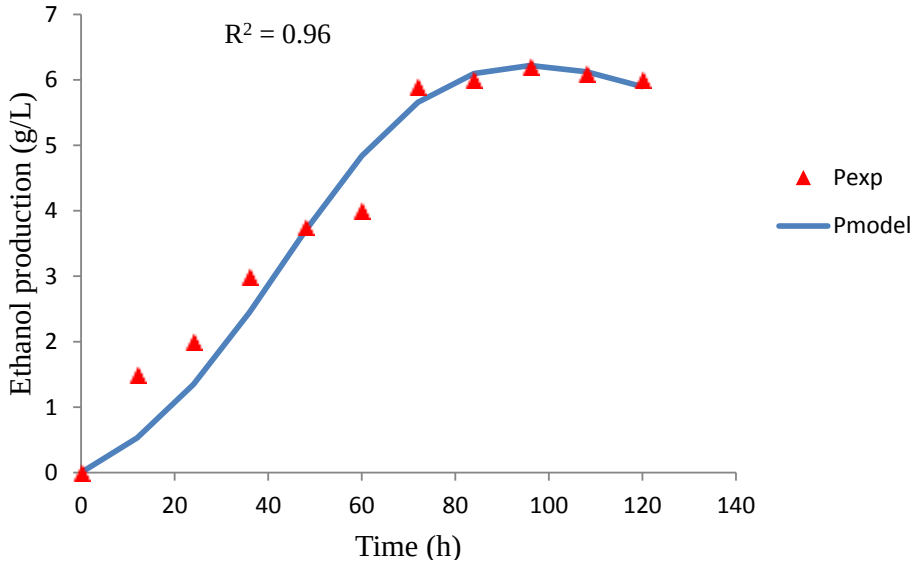

Figure 2. Comparison between predicted and experimental ethanol formation kinetics.

empirical kinetic model to describe batch fermentation of raw juice. The results show a good agreement with experimental data (R^2 = 0.99), thus, the logistic equation was found to be an appropriate kinetic model for successfully describing yeast cell growth in batch fermentation of raw juice system.

Ethanol production

The Equation 4 is applied to simulate the product formation, thus, Figure 2 shows the comparison of predicted model and experimental data for ethanol production by *P. caribbica*. The ethanol concentration reached its highest values in 96 h (6.2 g/L) from experimental data. Using the same procedure as above, the programs returns the values of 7.725 g/g for the growth associated rate constant 'm' and - 0.088 1/h for the non-growth associated rate constant 'n'.

These results show that the degree of growth associated constant rate 'm' is much greater than the non-growth associated rate constant 'n'. Similar results were achieved by Jiménez-islas et al. (2014). The simultaneous cell growth and ethanol production suggest that it is a growth-associated product. This result is in accordance with that of Thatipamala et al. (1992) who found that when using glucose as substrate, ethanol and biomass were produced simultaneously. In contrast, Ahmad et al. (2011) performed a series of experiments to show that ethanol batch fermentation is a non-growth-associated process that uses glucose. However, these authors used a forced aeration of 0.075 vvm in the culture medium and an agitation speed of 75 rpm, whereas, in our experiments, air was only transferred naturally from air phase to liquid phase. This discrepancy can be explained by the fact that when oxygen is absent, *S. cerevisiae* produces ethanol in order to reoxidize $NADH^+$ to NAD^+; however, in presence of oxygen, it acts as a final electron acceptor.

Moreover, the analysis of Figure 2 shows that there is a good agreement between model predictions and experimental data, effectively a correlation coefficient (R^2) value for ethanol production was 0.96. The analysis of variance (ANOVA) results for the ethanol production model are presented in the Table 3.

ANOVA of the regression model (Table 3) demonstrates the fitness of this model due to the F-value of 95.485816 greater than critical F value (4.4589701).

A good R^2 (0.96) for ethanol production and a significant ANOVA test confirmed that the model provides the relevant prediction The same results were obtained in several researches using the same model (Annuar et al., 2008). In addition, Jiménez-Islas et al. (2014) found the effects of pH and temperature on ethanol production from red beet juice by two strains: *S. cerevisiae* ITD00196 and *S. cerevisiae* ATCC 9763. This study was predicted by using the *Luedeking-Piret* model for ethanol production and validated only by a correlation coefficient (R^2). The authors concluded that this model was found to describe quantitatively this study due to a high level of correlation (R^2 = 0.97).

Substrate consumption

In this study, Equation 7 is applied to predict the consumption of the fructose substrate. However, *P. caribbica* is able to convert inulin to fructose, which was converted, after that, to ethanol. The hydrolysis of inulin in fructose by inulinase enzyme secreted by this yeast

Table 3. Analysis of variance (ANOVA) for the ethanol production model.

Source of variation	Sum of squares(SS)	Degree of freedom (DF)	Mean square (MS)	F-value	Critical F value (Fcrit)
Regression	58.5522127	2	29.2761064	**95.485816**	**4.45897011**
Error	2.452813	8	0.30660163		
Total	61.0050257	10	6.10050257		

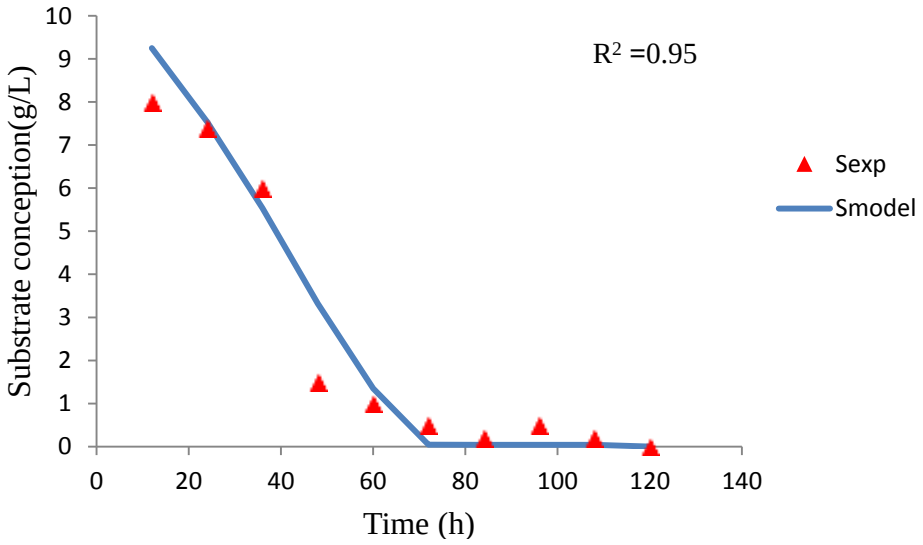

Figure 3. Comparison between predicted and experimental fructose consumption kinetics.

Table 4. Analysis of variance (ANOVA) for the substrate consumption model.

Source of variation	Sum of squares (SS)	Degree of freedom (DF)	Mean square (MS)	F-value	Critical F value (Fcrit)
Regression	159.57095	2	79.7854749	**91.2945575**	**4.45897011**
Error	6.991477	8	0.87393463		
Total	166.562427	10	16.6562427		

was previously studied using two medium containing separately pure chicory inulin and artichoke extract (Kara Ali et al., 2016).

The comparison of predicted model and experimental data for substrate consumption modeling during batch fermentation by *P. caribbica* is shown in Figure 3.

In the beginning, the initial fructose concentration was 8 g/L after 12 h (conversion inulin into fructose by *P. caribbica*). Biomass concentration and ethanol production (Figures 1 and 2) increased with a decrease in the fructose level (Figure 3). Fructose consumption had been gradually reduced from the beginning of the fermentation until t_{120h} when it ran out. In addition, the program used in this study, gives the values of p =14.735 g/g and q = -0.077 1/h, these values were calculated in another kinetic study (Pazouki et al., 2008). Thus, the bio-decolorization of distillery effluent in a batch culture was conducted using *Aspergillus fumigatus*. A simple model was proposed using the *Leudeking-Piret* kinetics for substrate utilization, the equation coefficients calculated were p = 1.41 (g/g) and q = 0.0007 (1/h). The difference between these values may be explained by the types of microorganism, fermentation period and the rate of substrate consumption to obtain the energy necessary for the maintenance of the cells in stationary phase.

It can be observed from Figure 3 that there is a good adequacy between model prediction and experimental data (R^2 = 0.95). The analysis of variance (ANOVA) results for the ethanol production model are presented in the Table 4. The F value (91.2945575) is larger than critical F value (4.45897011); this result clearly shown that, this model was applicable to this particular system (a good correlation coefficient R^2 and a significant ANOVA test). The experimental data reported by Oghome and

Kamalu (2012), using modified Leudeking-Piret model, were also studied; the correlation coefficients, R^2 and adjusted R^2 are 0.6849 and 0.9827 respectively, which indicates that this model fits the experimental data very well.

Conclusion

Microbial fermentation is complex and it is quite difficult to understand the complete details process. The model proposed in this study appears relevant to describe the biomass, ethanol production and substrate consumption versus fermentation time. The growth pattern followed the logistic model and the parameters were proved. Ethanol production was represented by *Luedeking-Piret* model; it was noticed that ethanol production by *P. caribbica* (KC977491) was growth associated. High significance of coefficient of determination (R^2) was observed with the experimental and predicted results. The statistical analyses using ANOVA were done by means of statistical F-value test which indicates the sufficiency of the regression models. Therefore, the models developed may be useful for controlling the growth, ethanol production and substrate consumption kinetics at large fermentation scale using this strain.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Protection of *Lactobacillus acidophilus* under *in vitro* gastrointestinal conditions employing binary microcapsules containing inulin

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In this research, microcapsules based on low acyl gellan (LAG) and sodium alginate (SA) containing inulin were developed in order to assess its protective effect on the viability of *Lactobacillus acidophilus* under *in vitro* gastrointestinal conditions.The results showed that microencapsulated cells display significantly (P<0.05) higher resistance to simulated gastrointestinal conditions (SGIC) than free cells. Besides, the incorporation of inulin into the wall matrix resulted in improved survival after 5 h incubation in SGIC. These results represent an alternative to vehiculate probiotics in food, especially in solid food due to the size of the microcapsules. Therefore, these microcapsules can contribute to possible industrial applications in the development of new alimentary products.

Key words: Inulin, microcapsules, probiotic, simulated gastrointestinal conditions, sodium alginate, low acyl gellan.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in appropriate concentrations provide health benefits to the host because they colonize the human gut in adequate amounts (10^6 CFU/mL) (Tripathi and Giri, 2014; WHO/FAO, 2002). These health benefits include therapeutic effects such as alleviating symptoms of lactose malabsorption, reducing the level of serum cholesterol, irritable bowel syndromes and colon cancer, besides enhancing resistance to gut infections (Kailasapathy and Chin, 2000; Sanders et al., 2013). All these effects are caused by inhibiting pathogen growth and stimulating the host's immune response (Figueroa et al., 2011). However, the incorporation and viability of these bacteria in food products still represent a technological challenge for researchers during the development of new probiotic products, because the viability of probiotics often decreases sharply during gastric transit due to the strong acidic conditions (Holkem et al., 2016).

One effective method to protect probiotic bacteria from

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the environmental factors encountered during the passage through the human gastrointestinal tract is the microencapsulation using various polysaccharides as wall material (that is, gellan gum and sodium alginate). Gellan gum is an anionic extracellular heteropolysaccharide produced by the bacterium *Sphingomonas paucimobilis* and consists of repeating units of a tetrasaccharide (1,3-β-D-glucose; 1,4-β-D-glucuronic acid; 1,4 β-Dglucose; and 1,4-α-L-rhamnose). It is available in two forms: High acyl gellan (HAG) and low acyl gellan (LAG). When HAG is exposed to strong alkali treatment at high temperature, the acyl groups are hydrolyzed and LAG is obtained. These structural differences between HAG and LAG allow great diversity of its textural properties. Therefore, HAG forms soft, elastic gels; while LAG gum forms strong gels (González et al., 2012). With regard to alginates, they are polysaccharides produced by brown algae (*Laminaria digitata, Laminaria hyperborea, Ascophyllum nodosum* and *Macrocystis pyrifera*). Alginates are widely used in the industry due to their non-toxic and gelling properties. Chemically, alginates are an anionic linear copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) joined by β 1-4 links and structured in blocks that can be homopolymeric (M or G) or heteropolymeric (MG) (Rosas et al., 2013). Within the most important applications of alginates in biotechnology is the ability to create stable gels through the ionic interaction between two adjacent chains with monovalent or divalent cations, forming junction zones that stabilize the gel structure (Fabich et al., 2012; Tavassoli et al., 2016).

Different methods for probiotic microencapsulation have been reported, including spray-drying, ionic gelantion, extrusion and complex coacervation (Champagne and Fustier, 2007; Martín et al., 2015). Internal ionic gelation (IIG) has been used for microorganisms microencapsulation due to its low cost, mild formulation conditions and high cellular retention making this technique one of the most promising ones (Cook et al., 2012). The microencapsulation using IIG does not require specialized equipment, complex techniques or the use of expensive reagents; moreover, IIG protects the microencapsulated cells from the acidic condition facilitating the gradual cell release in the target place (Chavarri et al., 2010; Cook et al., 2011; Guerin et al., 2003; Kanmani et al., 2011). Therefore, the aim of this study was to evaluate the *Lactobacillus acidophilus* survival into microcapsules containing inulin as a prebiotic compound under simulated gastrointestinal conditions.

MATERIALS AND METHODS

Microencapsulation

Microcapsules were obtained using a technique based on the formation of a water–oil emulsion. The dispersion (aqueous phase) was prepared with a mixture of 25SA/75LAG at 0.8% w/v, incorporating 1 mL of the cell suspensión (*L. acidophilus*) and 30 mM of Ca^{++}. Then, the dispersion was added into the oil phase (sunflower oil and 0.1% v/v of surfactant) under constant agitation in a stirring plate followed by the incorporation of 1 mL of δ-gluconolactone up to pH 4 in order to start the internal ionic gelation process. The microcapsules were harvested by centrifugation at 5000 rpm for 5 min, and the pellets were washed twice with saline solution to remove the oil residues.

Microcapsule morphology and size

Twenty micro liter of the microcapsules were used to determine the diameter employing a Leica DM500 microscope with a digital camera. The samples were diluted in sterile saline prior to the optical analysis and the captured images were analyzed using the software Image Pro-Plus ver 5.1. The average size of microcapsules was evaluated by measuring 100 microcapsules.

Microencapsulation efficiency

The microcapsules suspension were centrifuged at 5000 rpm in order to separate the free cell from microencapsulated cells. Then, the bacterial concentration in the supernatant was determined and encapsulation efficiency (% EE) was calculated according to Equation 1 as proposed by Gonzalez et al. (2015).

$$EE\ (\%) = (A-B)/A \times 100 \quad (1)$$

In this equation, A is the total bacterial concentration in the suspension and B is the free bacterial concentration in the supernatant.

Viability of *L. acidophilus* microencapsulated

Since the encapsulation process may affect the viability of probiotics, in the present study, the viability of *L. acidophilus* was enumerated before being subjected to simulated gastrointestinal conditions. The microencapsulated bacteria were released from the microcapsules based on the method proposed by Sheu and Marshall (1993). The microcapsules (1 g) were suspended in 9 mL of phosphate buffer (pH 7, 0.1 M) and homogenized for 5 min at 14,000 rpm using a high-speed homogenizer (Ultra-Turrax, model T50) and the breaking of the microcapsules was confirmed by optical microscopy. The enumeration of the viable cells was carried out by the drop plate method after 48 h incubation at 37°C on MRS agar under anaerobic condition. After the incubation time, the viable probiotic cells were counted and expressed in log colony forming units per gram (log CFU g^{-1}).

Viability of free and microencapsulated *L. acidophilus* subjected to simulated gastric and intestinal juices

One gram of microcapsules was subjected during 1 h to simulated gastric juice (SGJ) which is prepared by adjusting the pH of 0.2% (w/v) NaCl solution to 3 through the addition of 1.0 M HCl solution in order to mimic the stomach condition (Cheow et al., 2014). Afterwards, the same microcapsules were also added to simulated intestinal juice (SIJ) (6.8 g of KH_2PO_4 in deionized water at pH 7.0) for 4 h, resulting in a total simulated gastrointestinal transit time of 5 h (Graff et al., 2001). It is interesting to mention that all the tests were performed at 37°C in order to simulate the body temperature and the solutions employed were prepared on the same analysis day. The survival of free and microencapsulated *Lactobacillus acidophilus* was conducted according to the aforementioned

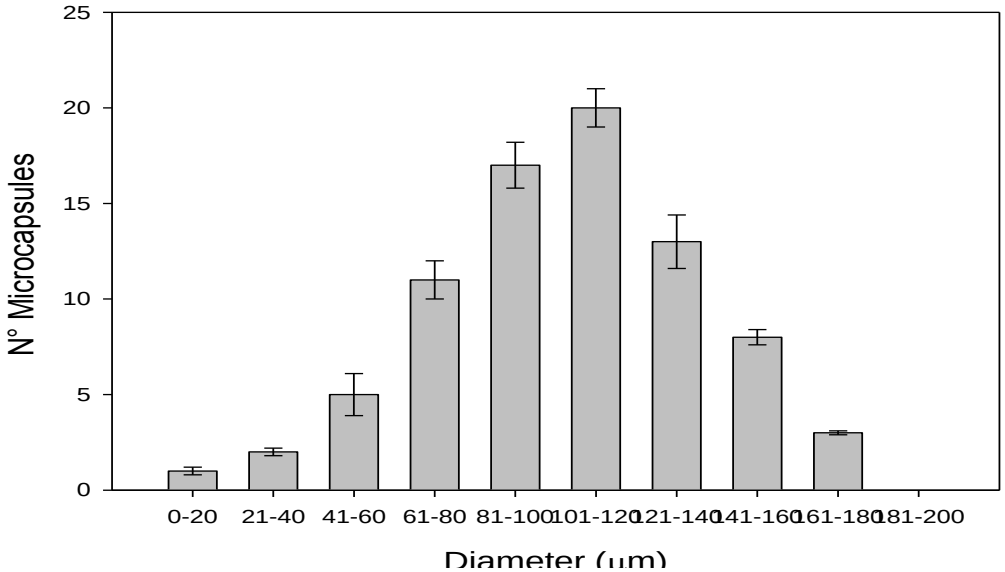

Figure 1. The size distributions of the microcapsules based on SA and LAG.

technique.

Statistical analysis

All the experimental data were subjected to analysis of variance (ANOVA- one way) using the software SPSS (ver. 17 for Windows) followed by Tukey's mean comparison test at a level of 5% significance. All the tests were carried out in triplicate and the data expressed as the mean ± standard deviation.

RESULTS

Microencapsulation

The microencapsulation method employed in this work is based on the emulsion between two phases, one hydrophobic and one hydrophilic containing the anionic polysaccharide, where by agitation, a great number of drops are originated which are gelled by acidification with δ-gluconolactone, since ion calcium is released from the calcium carbonate. The obtained microcapsules showed a unimodal behavior; which may be explained by the slow release of calcium ions from calcium carbonate because of the slow disruption of the gluconolactone.

Figure 1 depicts the number versus intervals of obtained microcapsules size. A unimodal behavior with particle sizes between 20 and 180 µm was observed. The microcapsule size is an important physical parameter since it can influence the sensorial attributes as aroma, texture and appearance when microcapsules are applied into food matrices. Microcapsules minor to 100 µm are desirable in liquid food, so as to avoid negative sensorial impact (Burgain et al., 2011).

Figure 2 shows the morphology of the obtained microcapsules with SA and LAG using calcium carbonate as a Ca^{2+} donor which was with spherical in shape and the outside surface with regular surfaces without the presence of deformations.

In order to determine the microencapsulation efficiency of the microencapsulation process, two counts were carried out. The initial count corresponds to the number of microorganisms added to the biopolymer dispersion (aqueous phase) and the second one was determined after the microcapsules were harvested. It is worth to mention that no negative effect was observed as there was no significant difference ($p < 0.05$) among the obtained CFU values before and after microencapsulation process; due to that, high averages of efficiency percentages were obtained (94.32 to 95.76%). Nonetheless, the encapsulation efficiency of the microcapsules was slightly improved when the prebiotic was incorporated into the microcapsule; thus, the loss of probiotic in microencapsulation process was reduced.

Viability of *L. acidophilus* microencapsulated

It was noted that there was no significant difference ($P < 0.05$) among efficiency and viability values of *L. acidophilus* encapsulated in binary microcapsules before incorporation to simulated gastrointestinal juices. It means that all the microencapsulated bacteria were able to grow, thereby yielding the beneficial effect associated with the probiotic intake. This also indicates that the microorganisms did not suffer pronounced damage during the microencapsulation process, showing that IIG is a feasible and adequate technique to produce microcapsules containing probiotics.

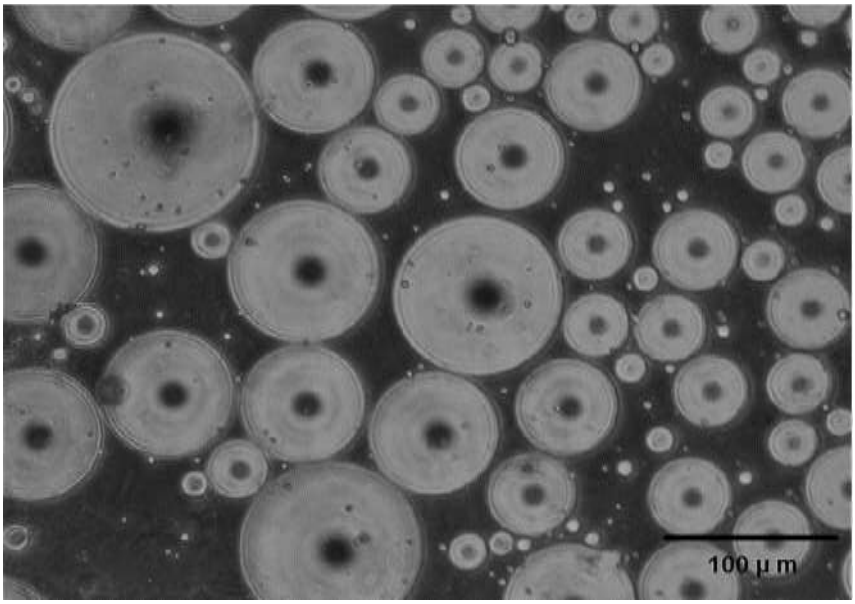

Figure 2. The optical micrographs at 10× of the binary microcapsules.

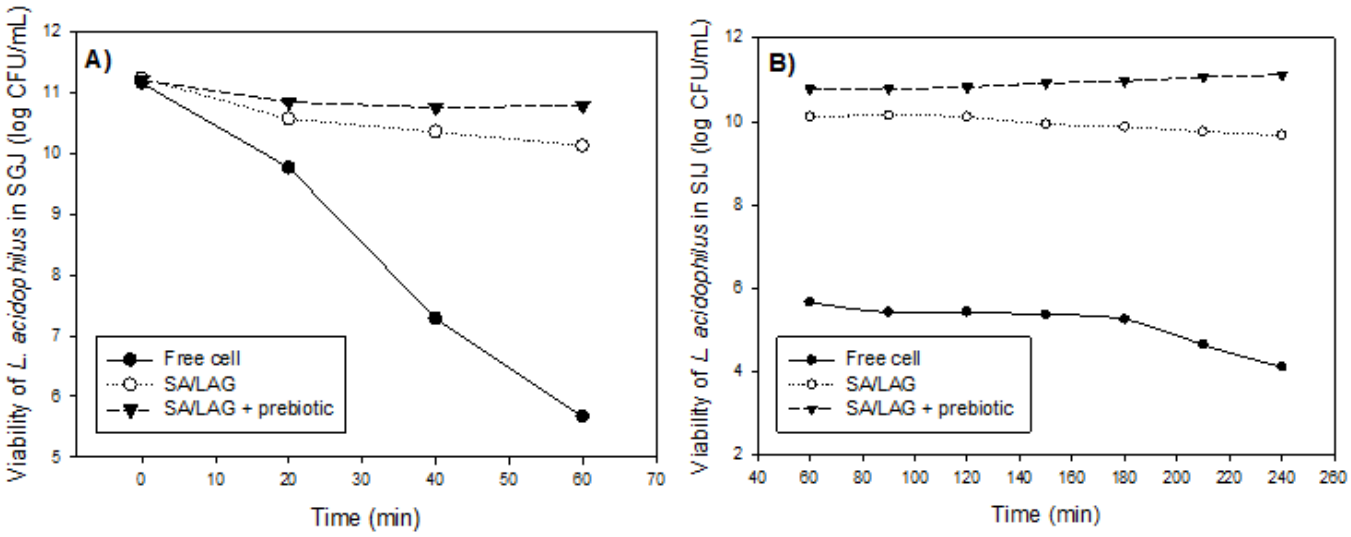

Figure 3. Viability of *L. acidophilus* free and microencapsulated in simulated gastrointestinal fluid (A: SGJ; B:SIJ).

Viability of free and microencapsulated *Lactobacillus acidophilus* subjected to gastric and intestinal conditions

Microcapsules containing *L. acidophilus* were initially exposed to SGJ for 1 h and then, the same microcapsules were transferred to SIJ for a further 4 h in order to mimic the gastrointestinal transit environment, equal procedure was realized for cells in free status. Figure 3 shows the results for the viability of *L.acidophilus* exposed to SGJ conditions for the free cells, microencapsulated and microencapsulated along with inulin. It was noted that the presence of inulin in the microcapsules provided the highest level of protection to the encapsulated cells, where 10.78 log CFU/ mL of the encapsulated cells survive to the SGJ during 1 h followed by cell microencapsulated alone with with 10.12 log CFU/mL, while free cells decreased sharply its viability until 5.67 log CFU/mL. Therefore, it is extremely important to protect *L. acidophilus* by microencapsulation These findings indicate that microcapsules based on mixture SA/LAG incorporated with inulin are stable under. acid solution likely by the interaction between biopolymer and the prebiotic. It should be clear that initial counts

before the incorporation to the SGJ were 11.23 log CFU/mL for the *L. acidophilus* microencapsulated, 11.15 log CFU/mL for free cells and 11.20 log CFU/mL for microencapsulated cell containig the prebiotic.

After immersion in SGJ, the difference between the viable number of *L. acidophilus* in free status, microencapsulated alone and microencapsulated along with inulin became highly significant ($P<0.05$) with longer incubation time.

At the end of the exposure time of *L. acidophilus* (free, microencapsulated and microencapsulated along with prebiotic) to SGJ conditions, the same probiotic bacteria was subjected to SIJ conditions at pH 7.0 as can be seen in Figure 3B. After submitting the *L. acidophilus* microencapsulated to SIJ conditions, they showed a significant decrease ($P< 0.05$) of 0.45 log CFU/mL when compared to the initial count, that is before the intestinal simulation. Most likely, some microcapsules were broken or there was a penetration of gastrointestinal juices into the microcapsules killing the probiotic. With regard *to L. acidophilus* microencapsulated along with inulin, an increase was observed ranging from 10.78 to 11.12 log CFU/mL; this is likely by a possible consumption of inulin by the probiotic or by a controlled release from microcapsules when the environmental pH rise. Conversely, the count of *L. acidophilus* in free status showed a reduction ($P< 0.05$) of 1.55 log CFU/mL when compared to the initial count before simulation.

In general terms, the number of the microencapsulated cells (both with and without inulin) that remain viable is approximately 6.27 log CFU/mL higher than free cells after being subjected to SIJ; which means that microcapsules protected from the acidic condition found in the gastrointestinal transited to *L. acidophilus* at the end of incubation period (5 h).

DISCUSSION

Characterization of microcapsules loaded with *L. acidophilus*

All the microcapsules revealed spherical shapes, as was displayed in Figure 2. The microcapsules had an average diameter of 102.82 µm being higher than those reported by Holkem et al. (2016) who reported mean diameters of 77.84 µm on microcapsules based on alginate. In the present study, smaller diameters were obtained than those obtained by Cai et al. (2014), who microencapsulated *L. acidophilus* with alginate by emulsification obtaining microcapsules with mean sizes ranging from 323 to 343 mm. Similar results were also published by Song et al. (2013), who studied microencapsulation of yeast by internal gelation and found microcapsule size with diameters between 35 and350 mm. Likewise, Wang et al. (2016) reported large size of microcapsules (1.5 mm) loaded with *Lactobacillus plantarum*, employing SA with or without inulin as inner layer and skim milk as outer layer. It should be noted that the diameters of microcapsules may affect the texture of the food products in which they are applied. For example, diameters about 100 µm are desired for most applications due to a better protection against acidic conditions as those found on the gastrointestinal transit (Arup et al., 2011; Champagne and Fustier, 2007).

The encapsulation efficiency (% EE) found in the present study had a mean value of 94.87%. These results are in agreement with those publised by Holkem et al. (2016) who found % EE values of 89.71% for Bifidobacterium BB-12 microencapsulated by IIG using alginate as a wall material. Likewise, Pitigraisorn et al. (2017) reported % EE values of 95.3% for *L. acidophilus* microencapsulated on non coated alginate beads. Nevertheless, in the current research, the efficiency was shown to be greater than that found in the studies of Zou et al. (2011) who microencapsulated *Bifidobacterium bifidum* F-35 obtaining values ranging from 43 to 50% using alginate microcapsules prepared by a similar technique of microencapsulation.

Viability of *L. acidophilus* microencapsulated

*L. acidophilus*is is a probiotic bacterium whose viability is significantly reduced at low pH values (Lee and Salminen, 2009). To overcome this problem, one objective of microencapsulation is to provide protection to probiotic cells during exposure at low pH (Çabuk and Harsa, 2015). However, microcapsules made of alginate tend to be highly porous leading to loss of core material. For this reason, blends of alginate and other polymers are employed in order to reduce wall porosity (Burgain et al., 2011).

Microcapsules loaded with *L. acidophilus* were initially exposed to SGJ at pH 3 for 1 h after which the same microcapsules were transferred to SIJ at pH 7 for a further 4 h. It should be noted that initial viable counts were in agreement with the recommended minimum values for the addition to a food probiotic product, as suggested by Aureli et al. (2011) and Salminen et al. (2011), who declared that the ingestion of probiotic cells should be around 8 to 9 log CFU/g to obtain beneficial effects on the health.

Various authors have reported that the microencapsulation with SA is effective for the survival of probiotics in acidic conditions (Ding and Shah, 2007; Doleyres and Lacroix, 2004). Maciel et al. (2014) reported an increase in the viability of *L. acidophilus* microencapsulated with sweet whey or skimmed milk by spray-drying during exposure to simulated gastrointestinal conditions at pH 2 to 7.

Etchepare et al. (2016) microencapsulated *L. acidophilus* in alginate beads with resistant starch (Hi-maize) and investigated the probiotic survival under

simulated gastrointestinal conditions. These authors reported that probiotic populations reduced to approximately 5.4 to 5.8 log CFU/g after exposure to simulated gastrointestinal fluids, which is similar to the values reported in the present study.

It was noticable that the barrier effect produced by microcapsules against acid conditions improve the probiotic viability because high count were obtained when *L. acidophilus* was microencapsulated along with inulin followed by the cells microencapsulated alone. Conversely, free cells showed a marked reduction in the population of *L. acidophilus*; these values demonstrate that the microorganism was fragile under acid conditions, which justify the microencapsulation to improve probiotic survival under gastric and intestinal conditions. Similar results were observed by Wang et al. (2016) who evaluated the viability of *L. plantarum* into microcapsules made of alginate containing inulin; these authors found that the microcapsules added with inulin resulting in reduction of the probiotic population to 0.4 log CFU/g, but when the inulin is absent, the probiotic population is reduced to 0.9 log CFU/g.

The enhancement of the *L. acidophilus* viability against gastrointestinal fluids could be due to the reduction of gastric fluid penetration into the microcapsule core, and the negative charges of the carboxylate groups that enhanced the buffer effect against infiltrated acid. Therefore, it could be a potentially effective matrix in protecting probiotics through the harsh environment it exists. It can be hypothesized that LAG formed the backbone of the microcapsules, while the SA is the factor governing the viability of encapsulated cells in SGJ due to alginate dissolution (Déat-Lainé et al., 2012). Alginate is converted into an insoluble layer of porous alginic acid, which at higher pH values dissolves and releases the active compounds (George and Abraham, 2006) in the desired location (intestine) (Park et al., 2014). Also, high obtained viability could be caused by SA particles dispersion which reduce the diffusion of oxygen into the microcapsules and thereby protect the microorganisms from oxygen exposure (Salminen et al., 2016).

In the present study, microcapsules based on LAG and SA loaded with *L. acidophilus* and inulin produced by IIG, represent an alternative to vehiculate probiotics in food, especially in solid food due to the size of the microcapsules.

Conflicts of Interests

The authors have not declared any conflict of interests.

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