International Journal of Life Sciences Vol.2 No.3. 2013. Pp. 150-155

Full Length Research Paper

Castor Seed Fermentation Using Free and Attached cells of *Bacillus subtilis* for Production of a Soup Flavouring

Chika C. Ogueke*, Clifford I. Owuamanam, Ann I. Peter-Ikechukwu , Jude O. Iwouno and Ndidiamaka Ahaotu

Department of Food Science and Technology, Federal University of Technology, Owerri. P.M.B. 1526 Owerri, Nigeria.

**Corresponding Author: Chika C. Ogueke*

Abstract

A study on the use of free and attached cells of *Bacillus subtilis* for the solid state fermentation of castor seeds for "ogiri igbo" production was conducted with the view to improving the fermentation process and product quality. Cowpea granules in association with cells of *B. subtilis* were developed as starters for the fermentation. Fermentation with free cells (broth cultures) of *B. subtilis* increased amino nitrogen from 2.67mg N/100g to 5.37mg N/100g at 96h while the attached cells increased amino nitrogen from 2.20mg N/100g to 3.88mg N/100g. The naturally fermented sample had values ranging from 2.40mg N/100g to 5.20mg N/100g at 96h. pH of the fermentations involving the free and attached cells of *B. subtilis* dropped at 24h and increased progressively until the end of fermentation with values of 7.96 and 7.27 respectively while the naturally fermented mash increased steadily from start of fermentation to 96h. Peroxide values of all the samples increased throughout the fermentation with the naturally fermented mash having the highest value (6.48 meq/kg) at 96h. Total titrable acidity of all the samples increased initially (0 – 24h) and dropped afterwards. Fermentation with *B. subtilis* alone did not optimize the fermentation process. There may therefore be need to use mixed starter containing B. subtilis and other microorganisms involved in the natural fermentation.

Key words: Amino-nitrogen; *Bacillus subtilis*; ogiri igbo; fermentation; starter cultures.

Introduction

The castor is a small annual plant. It ranges from one to seven meters in height. It has well developed roots, with green and reddish stems which become hollow with age. The fruit is a spherical capsule with small grey seeds with brown spots. Castor bean is cultivated for the seeds which yield viscous, pale yellow, non-volatile and non-drying oil. It has been used only for industrial and medicinal purposes (Ogunniyi, 2006; Ramos *et al*., 1984). It is widely used as a human laxative-cathartic agent, particularly in cases of certain radiological examinations which require prompt and thorough evacuation of the small intestine (Stubiger *et al*., 2003; Duke 1983). Its botanical name is *Ricinus communis* L of the family Eurphorbiaceae, a plant indigenous to many parts of the world. Castor oil is one of the few naturally occurring glycerides with high purity, since the fatty acid portion is nearly 90% of ricinoleic acid (Akpan et al. 2006).

Proximate analyses of un-decorticated castor seds and cake revealed that both the seed and cake contain valuable nutrients namely soluble carbohydrate: seed-7.96%, cake-24.69%, protein: seed-20.78%, cake-31.06%, fat: seed-51.20%, cake-19.40%, mineral matter: seed-7.75%, cake-11.10% (Annongu and Joseph, 2008). The decorticated seeds gave higher values of these nutrients than the un-decorticated, for instance carbohydrate: 8.86%, nitrogen: 21.87%, ether extract: 55.50%, total ash: 9.40% (Annongu and Joseph, 2008).

Castor seed fermentation for ogiri igbo is still carried out using the traditional method. This usually results in variation of quality of products as has been observed in similar fermentations (Ogueke and Nwagwu, 2007). There is also need to optimize the fermentation process with the view to industrializing the production. The use of chance fermentation as practiced traditionally makes the fermentation difficult to control and results in the contamination of products with pathogens or other microorganisms capable of producing toxins and those that cause off flavours (Ogueke and Nwagwu, 2007). These can also introduce undesirable and spoilage organisms which can cause a reduction in the product's shelf-life and increase susceptibility to spoilage. Due to these setbacks, variation in the quality and stability of the products are often observed.

Attempts have been made by a group of workers (Ogueke and Nwagwu, 2007) to produce ogiri egusi using pure cultures of the bacteria isolated from previous ogiri egusi, thus trying to introduce the use of starter cultures. The development of starter cultures for such traditional fermentations will enhance standardization of the product. The work therefore aims at fermenting castor seeds using *Bacillus subtilis* cells in association with cowpea granules, as starter cultures in 'ogiri igbo' production.

Materials and Methods.

Material collection and preparation

Approximately 15kg of castor seeds (*Ricinus communis*) were purchased from Ihiagwa market in Owerri, Imo State, Nigeria in August, 2012. Seeds obtained were dehulled and sorted to remove bad seeds, hulls and extraneous materials. The seeds were stored at 4^{O}C until used for the study. Approximately 5.0kg of cowpea (*Vigna unguiculata*) was purchased from Owerri main market, Imo State, Nigeria and the seeds were properly sorted before use.

Bacillus subtilis used for the study

Bacteria were isolated from various ogiri egusi samples from three states of the consuming region of South Eastern Nigeria. Microbiological analyses were conducted immediately by suspending 10g of sample in 90 mL sterile maximum recovery diluent(MRD Oxoid CM0733) and homogenized using a Stomacher (A.J.Seward, BA6021) for 1 to 2 min at normal speed. Ten-fold dilutions were then prepared and 0.1ml of suitable dilution was spread onto Nutrient agar (NA, Oxoid CM0003). Plates were incubated aerobically at 37^{0}C for 24 - 48 hours. Representative dominant colonies were picked and purified by streaking on same Nutrient agar. Stock cultures were kept in Nutrient broth containing 20% glycerol and stored at -20^{0}C.

Prior to the genotyping, the Bacillus isolates were phenotyped according to Ouoba *et al.* (2008). The bacteria were chosen based on their phenotypic characteristics including colony and cell morphology, cell motility, presence of endospore, Gram and catalase reaction after growth on Nutrient agar (NA; Oxoid CM0003) for 24h. Fermentation profiles of carbohydrates (API 50CHB galleries; BioMerieux, Basingstoke, UK) were also determined to assist typing. This was done according to the manufacturer's instruction and results were analyzed using the apiweb software. Those identified as *Bacillus subtilis* were used for the fermentation.

Before use the identified *Bacillus subtilis* were streaked on Tryptone Soy agar (BIOTEC) and incubated at 35^{O}C for 18h. The cultures were then washed by suspending the cells in 10 mL of 0.1M potassium phosphate buffer (pH 7.0). This was followed by centrifugation at 14000xg for 5 min using an MSE centrifuge (Minor, UK) (Njoku *et al.*, 1990).

Preparation of carrier granules

The method of Isu and Abu (2000) was used with minor modification as shown below. Cowpea (*Vigna unguiculata*) granules were prepared by immersing 500 g of well sorted raw seeds of black-eyed cowpea in 5 liters of portable water, autoclaved at 121°C for 15 min, cooled, drained and air-dried. The boiled seeds were spread evenly on an aluminium foil and placed in a hot-air oven (Weiss-Gallenkamp, UK) at 80°C for 24-48 h, then cooled. The cooled seeds were dry-milled using a warring blender, sieved into different particle sizes using a laboratory sieve (Endocotts Ltd, UK) and particles sizes of 700µm was collected. One gram (1g) each was dispensed into MacCartney bottles and sterilized in a hot-air oven (Weiss-Gallenkamp, UK) at 100°C for 18-24 h (with the lid loosely closed).

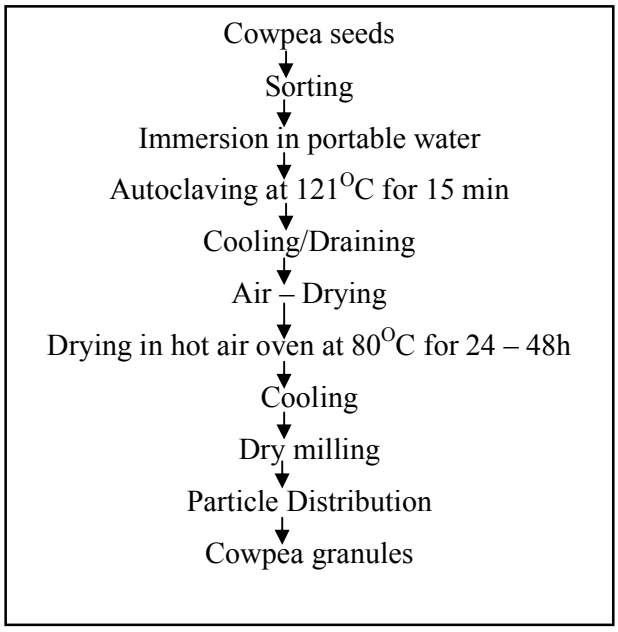

Fig 1: Flow diagram for production of Cowpea granules (carrier)

Inoculum preparation and standardization of cultures

Cultures from 18h Tryptone Soy agar plates (BIOTECH) were examined for purity by microscopic examination of slides prepared from randomly selected colonies. A loopful of the 18h old culture was inoculated in a flask containing 50mL of Tryptone Soy broth (Oxoid) and was incubated aerobically for 48 h at 35°C. Cells from the broth culture were harvested and washed three times

with 0.1 M potassium phosphate buffer by centrifugation at 14000xg for 5min. The cells were then aseptically re-suspended in 20mL sterile 0.1M potassium phosphate buffer (pH 7.0). The cell suspension was then standardized using a spectrophometer (Model S2100UV, UNICO, New Jersey, USA) at 550nm wavelength such that 1.0mL of the suspension contained about 3.0 x 10^8 cells.

Transfer of standardized cultures to cowpea granules (carrier)
With a sterile pipette 0.2mL of the standardized suspension of *B. subtilis* was aseptically transferred into different parts of 1.0g of the sterile cowpea granules in the Mac Carthney bottles in an inoculation chamber. The contents of the bottles were thoroughly mixed by inversion of the bottles at the rate of 20 times per min for 5 min (Monela *et al.*, 1985). They were then transferred to a rotary shaker to shake them for 5 min (Husmark and Ronner, 1990). The granules with attached Bacillus cells were dried using sterile air from a monopoint dryer (Type 4547, Braun Ag, UK) until a final moisture level of 10% was attained. The attached cells were stored at 4^OC until used for the study. These served as starter cultures. Standardized broth cultures of *B. subtilis* were also prepared (1.0mL of broth culture contained about 3.0 x 10^8 cells) and used to ferment castor seeds for ogiri igbo production.

Fermentation of castor seeds using starter cultures and standardized broth cultures of *B. subtilis*
Two kilograms of dehulled castor seeds were properly sorted and cleaned, and extraneous materials removed. The seeds were boiled for 6 h until the endosperm softened, then drained and cooled to about 30°C, placed into a sterilized container and inoculated with 10g of the starter cultures of *B. subtilis*. This was aseptically mixed and allowed to ferment for four days at ambient temperature (30-32°C). For the fermentation using standardized broth cultures, 2.0kg of the castor seeds were treated as above except that the boiled and cooled seeds were inoculated with 2.0 mL of the standardized broth cultures.

Natural fermentation of castor seeds for ogiri igbo
This was also prepared as above. However, after boiling and cooling the seeds were allowed to ferment naturally without the addition of the starter cultures or the standardized broth cultures. The fermentations were monitored for the amino nitrogen content, pH, titratable acidity, and peroxide value. All analyses were carried out in triplicate.

Determination of pH
The pH of each fermentation process was measured as follows. Five grams of fermenting seeds was taken from the bulk and mashed in 45mL of sterile deionized water (Njoku *et al*., 1990). The pH was measured using a digital pH meter (Jenway pH/MV/Temperature Meter, Model 3510, Essex, UK) at ambient temperature (30-32°C). Before use the pH meter was standardized using buffers (4.0 – 9.0).

Determination of titratable acidity of fermenting seeds
Chilled distilled water (2 mL) was added to 5 g of fermenting mash in a chilled mortar. The sample was crushed with a pestle and then transferred to 100 mL flask. An additional 10 ml of chilled extractant was added and thereafter the flask was stoppered and shaken on a Staurt flask shaker at medium speed for 15 min. The extracts were then centrifuged at 2300 × g in a bench-top MSE centrifuge at 5°C. The residue was re-suspended in 8 mL of chilled extractant and subjected to the same procedure. The supernatants were combined and made up to 20 mL with chilled extractant (Njoku and Okemadu, 1989). Ten milliliters of the extract was then titrated with 0.1 M NaOH using phenolphthalein as indicator. A blank was prepared using 10 ml distilled water. The titrable acidity was calculated as percent lactic acid/100g of sample.

Determination of peroxide value
Fifteen grams of fermenting seeds was mashed and 5 g each of the mashed seeds was placed into a 250mL glass – stoppered Erlenmeyer flask, and 30mL of solvent mixture (3 parts by volume of Glacial acetic acid + 2 parts by volume of Chloroform) was added. The flask was swirled to dissolve the mashed seeds in the solution. Five hundred microliters of saturated potassium iodide solution was added and the solution was allowed to stand (with occasional shaking) for 1 min, then 30mL of distilled water was added. The mixture was titrated by gradually adding 0.1N sodium thiosulphate and constantly shaking vigorously. The titration continued until the yellow color had almost disappeared. Five hundred microliters of starch indicator solution was added. The titration was continued, shaking the flask vigorously at near end point until the iodine from the chloroform layer was liberated. The sodium thiosulphate was then added until the blue color had just disappeared. The tubes were placed in boiling water to boil for about 30 sec. A blank was also prepared at the same time. The peroxide value was calculated and presented as milliequivalent of peroxide per 1000g of sample.

Amino nitrogen determination
The formol titration method (Pham and Del – Rosario, 1983) was used. Three grams of fermenting seeds was taken and mashed. Two grams of the mashed seeds was placed in a conical flask, and then 0.5 mL of phenolphthalein (0.5%) and 0.4mL of neutral saturated potassium oxalate were added. The mixture was kept to stand for few minutes and this was neutralized with 0.1M NaOH to a standard pink color. Two milliliters of 40% formaldehyde solution was added and allowed to stand for few minutes (until mixture was colorless). This was then titrated with 0.1M NaOH to pink color. The titer value obtained was designated 'V_1'. A blank was run by titrating a mixture of 2mL of formaldehyde solution and 10mL distilled water with 0.1M NaOH to obtain 'V_2'. The amino nitrogen was calculated and presented as mg N/100g of sample.

Analysis of data

The data obtained from this study were statistically analyzed using Analysis of Variance (ANOVA). The means were separated using Fischer's least significant difference (LSD) at $p \leq 0.05$ confidence level. Microsoft Excel 2007 was used for the analysis.

Results and discussion

Effect of fermentation on pH

The pH values obtained during the fermentation are shown in table 1. The pH values of the sample fermented by the free and attached cells of *Bacillus subtilis* initially decreased within 24h and increased steadily thereafter while the pH of naturally fermented sample increased from the start of fermentation till the end of fermentation. This initial drop in pH could be attributed to the production of organic acids by the bacteria from the utilization of the small amount of carbohydrate present in the seeds. Castor seeds contain 7.96% carbohydrate (Annongu and Joseph, 2008). Such trend has also been observed by Ogueke *et al.* (2013) during ogiri egusi production using *B. subtilis*. In a mixture of substrates microorganisms are known to utilize substrates in a hierarchical manner with the carbohydrates being the first to be utilized. It could be that after the exhaustion of the carbohydrate the bacteria then started breaking down the proteins to amino acids using their proteases, and increased deaminase activity following utilization of amino acids as carbon and energy sources by the fermenting bacteria (Njoku and Okemadu, 1989; Ogueke and Nwagwu, 2007; Ogueke *et al*., 2013). These activities resulted in increase in pH.

Table 1: Mean values of pH change during fermentation of castor seeds

Fermentation Treatment	Fermentation Period (Hour)					
	0	24	48	72	96	LSD
Starter cultures	6.0	5.36	5.93	7.1	7.27	0.75
Broth cultures	6.3	5.58	7.11	7.58	7.96	0.42
Natural	6.15	6.28	6.7	7.03	7.26	0.50
LSD	0.18	0.48	0.60	0.30	0.36	

At the end of fermentation their pH values were in the alkaline range, typical of other indigenous fermentations of proteinaceous foods (Steinkraus, 1995; Ogunsanwo *et al*., 1989; Barber and Achinewhu, 1992; Adewusi *et al*., 2004; David and Aderibigbe, 2010). At 48h only the broth culture fermented sample had its pH above 7.0. This shows that use of the attached cells may not be adequate for fermenting castor seeds for ogiri igbo production. It could be that the nature of the substrate did not allow optimum activity of the bacteria. The broth cultures because they may have been at their optimum activity at inoculation made them to be able to attack the seemingly difficult substrate.

Effect of fermentation on amino nitrogen

The effect of fermentation on the amino nitrogen content is shown in table 2. The amino nitrogen content of the samples increased with fermentation time. However, these results were lower than those obtained during the fermentation of melon seeds for ogiri egusi production using free and attached cells of *B. subtilis* (Ogueke *et al., 2013*). The lower values obtained in this study could be that the nature of the substrate affected the activities of the bacteria. It could also be that the optimum fermentation may be achieved with not just the *B. subtilis* alone but in combination with one or two microorganisms involved in the natural fermentation. The highest amino nitrogen content (5.37mg N/100g) was obtained from samples fermented with broth cultures of *B. subtilis* while the least value of 3.88mg N/100g was obtained from samples fermented with attached cells of *B. subtilis*.

Table 2: Mean values of amino nitrogen of fermenting castor seeds (mg N/100g)

Fermentation Treatment	Fermentation Period (Hour)					
	0	24	48	72	96	LSD
Starter cultures	2.2	2.65	2.69	3.67	3.88	1.01
Broth cultures	2.67	2.92	3.39	5.02	5.37	0.8
Natural	2.4	2.98	3.77	4.93	5.2	0.72
LSD	0.16	0.18	0.51	0.75	0.82	

Effect of fermentation on peroxide value

Peroxide values obtained during fermentation are shown in table 3. The values increased throughout the fermentation period. The least value (6.04 meq/kg) was obtained from the samples fermented with the broth cultures of *B. subtilis*. This still indicates that the broth culture preparation could be best suited for the fermentation of castor seed for ogiri igbo production. The values obtained in all the samples were well below the threshold value of 30 milliequivalent of active oxygen per kg of oil in oil rich food materials (such as castor seeds) (Gotoh and Wada, 2006). Therefore the chance of the products going rancid is very low since the peroxide value is used as a measure of the extent to which rancidity can occur (Ihekoronye and Ngoddy, 1985). However, the mash fermented naturally exhibited higher peroxide values throughout the fermentation.

Table 3: Mean values of peroxide value of fermenting castor seeds (Meq/kg)

Fermentation Treatment	Fermentation Period (Hour)					
	0	24	48	72	96	LSD
Starter cultures	6.17	6.18	6.22	6.27	6.28	0.13
Broth cultures	6.18	6.24	6.28	6.24	6.04	0.09
Natural	6.16	6.31	6.34	6.39	6.48	0.09
LSD	0.01	0.06	0.05	0.08	0.1	

Effect of fermentation on total titrable acidity

Effect of fermentation on total titrable acidity is shown in table 4. There was an initial increase in the values obtained in all the samples with the naturally fermented mash having the highest value of 0.73% at 24h. Thereafter the values decreased. The initial increase may be due to the utilization of the carbohydrates with the production of organic acids (Ogueke and Nwagwu, 2007). Earlier works by David and Aderibigbe (2010) and Ogueke *et al*. (2013) have shown similar trends. The decrease in total titrable acidity observed afterwards could be attributed to "alkaline fermentation" (Steinkraus, 1995) as well as increase in protease and deaminase activities (Njoku and Okemadu, 1989) which would result in ammonia production.

Table 4: Mean values of total titratable acidity (% lactic acid) of fermenting castor seeds

Fermentation Treatment	Fermentation Period (Hour)					
	0	24	48	72	96	LSD
Starter cultures	0.35	0.71	0.39	0.23	0.24	0.02
Broth cultures	0.31	0.67	0.35	0.19	0.20	0.05
Natural	0.37	0.73	0.41	0.25	0.24	0.04
LSD	0.03	0.12	0.04	0.06	0.03	

Conclusion

Castor seeds fermentation is an indigenous fermentation carried out in homes using local implements. Starter cultures were developed for the fermentation by attaching *B. subtilis* cells to cowpea granules. Fermentation with free cells (broth cultures) produced higher levels of amino nitrogen and pH, thus indicating that the free cells performed better in the fermentation. However, the amino nitrogen levels were low showing that fermentation probably did not come to completion due to the nature of the substrate. It is therefore recommended that more work be carried out using mixed cultures of the microorganisms involved in the natural fermentation.

References

Adewusi, S. R. A., Omafuvbe, B. O., Falade, O. S. and Osuntogun, B. A. (2004). Chemical and biochemical changes in African locust bean (*Parkia biglobosa)* and melon seeds (*Citrullus vulgaris*) during fermentation to condiments. Pakistan Journal of Nutrition. **3 (3): 140 - 145.**

Akpan, U.G., Jimoh, A. & Mohammed, A.D. (2006). Extraction, Characterization and Modification of Castor Seed Oil. Leonardo Journal of Sciences. **(8): 43-52.**

Annongu, A. A. and Joseph, J. K. (2008). Proximate Analysis Of Castor Seeds And Cake. Journal of Applied Science and Environmental Management. **12(1): 39 – 41.**

Barber, L. I. and Achinewhu, S. C. (1992). Microbiology of ogiri production from melon seeds (*Citrullus vulgaris*). Nigerian Food Journal. **10: 129 - 135.**

David, O. M. and Aderibigbe, E. Y. (2010). Microbiology and proximate composition of 'ogiri', a pastry produced from different melon seeds. New York Science Journal. **3 (4): 18 - 27.**

Duke, J.A. (1983). *Ricinus communis* L. Handbook of Energy Crops 1-6. (online) http://www.hort.purdue.edu/newcrop/duke_energy/Ricinus_communis.html. (March 3, 2006).

Gotoh, N. and Wada, S. (2006). Importance of peroxide value in assessing food quality and food safety. Journal of the American Oil Chemists' Society. **83(5): 473 – 474.**

Husmark, U. and Ronner, U. (1990). Forces involved in adhesion of *Bacillus cereus* spores to solid surfaces under different environmental conditions. Journal of Applied Bacteriology. **69: 557 – 562.**

Ihekoronye, A.I. and Ngoddy, P.O (1985). Integrated food science and technology for the tropics. Macmillian Publishers, London. **pp.123 - 127.**

Isu, N. R. and Abu, G. O. (2000). An evaluation of the effect of Bacillus cells and Bacillus spores in association with cowpea granules as starter cultures for the fermentation of African oil bean (*Pentaclethra macrophylla Bentham*) to 'ugba'. Plant Foods for Human Nutrition. **55: 127 – 138.**

Monela,B., Halfpap, L. and Birdsell, D. (1985). Langmuir and scatchard parameters do not describe the binding of *Actinomyces viscosus* treated hydroxyapatite. Journal of General Microbiology. **131: 2619 – 2626.**

Njoku, H. O., Ogbulie, J. N. and Nnubia, C. (1990). African oil bean (*Pentaclethra macorphylla Bentham*) for ugba production. Journal of Food Science. **49: 13-26.**

Njoku, H. O. and Okemadu, C. P. (1989). Biochemical changes during the natural fermentation of the African oilbean (*Pentaclethra macrophylla*) for the production of ugba. Journal of Science of Food and Agriculture. **49: 457 – 465.**

Ogueke, C. C. and Nwagwu, A. (2007). Comparative study of melon seeds (*Citrullus vulgaris*) fermented with mixed cultures and pure cultures of bacteria isolates from ogiri egusi. Life Science Journal. **4 (4): 41 - 46.**

Ogueke, C. C., Owuamanam, C. I., Peter-Ikechukwu, A. I. and Ahaotu, I. (2013). Free and attached cells of *Bacillus subtilis* as starters for production of a soup flavouring ("ogiri egusi"). Malaysian Journal of Microbiology. **9(1): 103 – 110.**

Ogunniyi, D.S. (2006). Castor Oil: A vital industrial raw material. Bioresource Technology. **97: 1086-1091.**

Ogunsanwo, B. M., Fabya, O. O., Idowu, O. R., Ikotun, T. and Akano, D. A. (1989). The fate of aflatoxins during the production of ogiri, a West African fermented melon seed condiment from artificially contaminated seeds. Nahrung. **33 (10): 983 - 988.**

Ouoba, L.I.I., Parkouda, C., Diawara, B., Scotti, C. and Varnam, A.H. (2008). Identification of Bacillus spp from Bikalga, fermented seeds of *Hibiscus sabdariffa*: phenotypic and genotypic characterization. Journal of Applied Microbiology. **104: 122–131.**

Pham, C. B. and Del-Rosario, R.R. (1983). The preparation of protein hydrolysate from deffated coconut and soya-bean meals. Effect of process variable on the amino-nitrogen released and flavour development. Journal of Food Technology. **18: 21.**

Ramos, L.C.D., Tango, J.S., Savi, A. and Leal, N.R. (1984). Variability for Oil and Fatty Acid Composition in Castor bean Varieties. Journal of American Oil Chemists Society. **61: 1841-1843.**

Steinkraus, K. H. (1995). Handbook of indigenous fermented foods. Cornell University, New York. **pp. 349 - 356.**

Stubiger, G., Pittenauer, E. and Allmaier, G. (2003). Characterization of Castor Oil by On-line and Off-line Non-aqueous Reverse-phase High-performance Liquid Chromatography – Mass Spectrometry (APCI and UV/MALDI). Phytochemical Analysis. **14: 337-346.**