

Microbiological, Physicochemical and Enzymatic Changes in Fermented African Locust Bean (*Parkia biglobosa*) Seeds Using *Bacillus subtilis* and Additives

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Abstract. Traditional fermentation of dried seeds of *Parkia biglobosa* is used to produce a local product called ‘iru’. It is a condiment consumed mostly in Western Nigeria. In this study, the effect of *Bacillus subtilis* BC4333 starter cultures and different additives, such as local softening agents called ‘kuuru’, *Hibiscus sabdariffa* seed cotyledon and ash from cashew (*Anacardium occidentale*) plants on the microbiological, physicochemical, and enzymatic properties of fermented *Parkia biglobosa* seeds were investigated. The dried seeds of *Parkia biglobosa* were processed by boiling the cotyledons with *Hibiscus sabdariffa*, ‘kuuru’ and *Anacardium occidentale* (cashew plant) ash using natural fermentation and *Bacillus subtilis* strain BC4333 as a starter culture. The fermentations were carried out at 35⁰C for 36 hrs. Commercially fermented *Parkia biglobosa* seed (called ‘iru-pete’) was used as control. All the samples were analyzed for microbial, physicochemical and enzymatic properties using standard biochemical techniques. ‘Iru’ produced using ‘kuuru’ (IFK) had the highest microbial count of 7.88 log CFU/g, while the microbial count of ‘iru’ produced with ash (IFA) and ‘iru’ produced with dried seeds of *Hibiscus sabdariffa* (IFH) were 7.69 log CFU/g and 7.75 log CFU/g respectively. The pH of the fermented products ranged between 7.06 and 7.91, while the unfermented sample (UFS) had pH 6.68. The unfermented sample had the highest total titratable acidity (TTA) of 2.6×10⁻²N while the ‘iru’ samples produced using ‘kuuru’ (IFK) had the least TTA of 1.0×10⁻²N. Fermentation significantly (P<0.05) increased the moisture content of the products. Starter culture fermented ‘iru’ (F14) had the highest protease, amylase and phytase activity of 232.3UPR, 0.41UAM and 71.85UPH respectively. Findings from this study suggest that ‘iru’ produced by addition of *Bacillus subtilis* strain BC4333 had the best desirable biochemical qualities when compared to the other tested additives and thus recommended for commercial scale.

1. Introduction

‘Iru’ is a fermented product of *Parkia biglobosa* (also known as African locust beans) seeds which is consumed as a condiment mostly in Western part of Nigeria. The unfermented *Parkia biglobosa* seeds are rich in protein, vitamins and minerals [1]. At some stages in the preparation of the ‘iru’, fermentation is required to bring out the desired nutritional value and other organoleptic properties such as taste, flavour and texture [2] as well as to enhance the enzymatic activities of the product [3]. There are two varieties of ‘iru’, the soft type known as ‘iru-pete’ and the hard type known as ‘iru-woro’.

The processing of iru-pete at local level is achieved by addition of agents, such as “kuuru” and ash from dried plants during the second boiling of the seeds [4]. “kuuru” is a fermented product of dried seeds of *Hibiscus* sp. Previous studies have shown that ‘kuuru’ negatively impact on the nutritional components/quality of the fermented products. There is need to apply modern biotechnological techniques like the use of starter cultures to improve the traditional food processing techniques [5]. The use of starter cultures has been found to reduce fermentation time as well as guarantee product rich nutritional value in the traditional manufacturing process. *Bacillus subtilis* is one of the predominant organisms in fermented African locust beans [6].

Considering the possible impacts of additives on the qualities of the final fermented product of African locust bean seeds (“iru”), it is pertinent to scientifically elucidate the effects of ‘kuuru’, ash, dried seeds of *Hibiscus sabdariffa* and starter culture *Bacillus subtilis* on the microbial, physico-chemical and enzymatic activities of the fermented products.

2. Materials and Methods

2.1. Source of materials

African locust bean seeds (ALB) and ‘kuuru’ a local softening agent were purchased from retailers at King’s Market, Ado-Ekiti, Ekiti State. The dried seeds of *Hibiscus sabdariffa* commonly known as ‘isapa’ amongst the Yorubas were collected from a farmer in Ado – Ekiti. The ash used for this study was obtained by burning a branch of cashew (*Anacardium occidentale*) tree. The pure culture of a *Bacillus subtilis* strain was obtained from Food Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTECH), Pathumthani, Thailand [8].

2.2. Preparation of starter cultures

The inoculum was prepared by growing the strains of *Bacillus subtilis* BC4333 in 50ml Nutrient Broth (NB) in 250ml conical flasks for 24 hours under agitation (200 rpm) at 35°C. The turbid cultures were centrifuged at (10,000rpm), 4°C for 10mins. The supernatant was decanted and the cells pellet was re-suspended in 5ml of sterile distilled water. The optical densities (OD) of broth cultures were measured at 540 nm with Pye Unicam SP6-250 visible spectrophotometer. The volume of the inoculum required to inoculate 300g of substrate to give a final inoculation ratio of 10^4 cells per gram of substrate, was calculated [7].

2.3. Laboratory production of ‘iru’

The method described by [8] was adopted. The African locust bean seeds were soaked in water for 15 min, boiled using pressure pot for 3 h and dehulled to remove the testa. Three hundred grams (300 g) each of the dehulled seeds were weighed into six different 1L-beakers. The 300 g cotyledons in first beaker was poured into pressure pot and boiled for 1 h, drained, oven-dried at 75°C for 24 h and was labeled as (UFS) unfermented substrate. The 300 g cotyledons in beaker 2 was also poured into pressure pot and boiled for 1 h, drained and aseptically poured into a sterile of 10 cm × 20 cm × 10 cm rectangular-shaped aluminum fermenting can and was labeled as naturally fermented ‘iru’ (NFK). Five grams (5 g) each of finely ground *H. sabdariffa* seeds, ash, and ‘kuuru’ were added to seeds in beakers 3, 4 and 5, respectively, poured into separate pressure pots, and was boiled in water for 1 h. After boiling, it was poured aseptically into sterile fermenting cans of the same dimension used above and they were labeled as; IFH (‘iru’ fermented with *Hibiscus sabdariffa* seeds), IFA (‘iru’ fermented with ash) and IFK (‘iru’ fermented with ‘kuuru’), respectively. The sixth batch of 300 g cotyledons was also boiled under pressure for 1 h, drained and poured aseptically into sterile aluminum fermenting can. It was inoculated with 1.0 ml of the starter culture *B. subtilis* BC4333 and labeled as F14. The six samples were fermented at 35°C for 36 h. Commercial sample of ‘iru-pete’ (CIP) served as control. All the seven samples were analysed for changes in microbial counts, some physicochemical and enzymatic properties using standard techniques.

2.4. Microbial counts

Nutrient agar was used for the growth of microorganisms. The microbial analysis carried out included isolation of microorganisms from the samples and determination of total microbial counts (microbial load) using direct microscopic observation of the isolates [9].

2.5. pH determination

The pH of each homogenate was determined with a Pye Unicam pH meter (Model PW9409). Five grams (5g) of each sample was homogenized and mixed with 100ml of distilled water. Determination was done in triplicates.

2.6. Total titratable acidity determination

The suspension from the pH determination was filtered and 20 ml of the filtrate was titrated against 0.1M NaOH using 1 drop of phenolphthalein as indicator [10]. The determination was done in triplicate

2.7. Moisture content determination

Five grams of each sample was weighed separately into pre-weighed aluminum foil. The foil paper and its content was put in oven at 160°C for 1hour and weighed intermittently until a constant weight was achieved. The new weight was subtracted from the weight of the wet sample. The percentage moisture content was then calculated [11].

2.8. Extraction of crude enzymes

The crude enzymes were prepared by adding 40ml of distilled water to ten grams (10 g) of 'iru' samples and ground into slurry, using mortar and pestle. The slurry was centrifuged at 2000 rpm, for 10min at 4°C. The supernatant was decanted and used for enzyme assays; excess was stored in a freezer at -2°C. Extracellular enzymes assayed include, protease, amylase lipase, pectinase, phytase and galactanase.

2.8.1. Protease activity

The assay of protease activity was carried out using the method of [9]. The substrate, a 1% casein solution was prepared in 0.05M citrate phosphate buffer (pH 7.0). The solution was denatured by heating at 100°C in a water bath for 15min. One mililitre (1ml) of 1% casein solution was pipetted into different test tubes and allowed to equilibrate at 35°C in a water bath for 15 mins. Then 0.2 ml of the crude enzyme solution was added to each tube and incubated for 1h. The reaction was terminated by adding 3 ml of cold 10% trichloro acetic acid (TCA). The control contained only casein solution during incubation at 35°C, the TCA was added, before the addition of enzyme solution. The undigested proteins were allowed to precipitate by standing the tubes at 4°C for 1h. The tubes were centrifuged at 3000rpm at 4°C for 15min. The supernatant fluid was analyzed for un-precipitated protein by direct reading of the absorbance at 280nm with a spectrophotometer (Unicam 1700). Tyrosine was used for the standard curve. One unit of proteolytic activity was arbitrarily defined as the amount of enzyme causing a release of 1 mg of Tyrosine in an hour at 35°C.

2.8.2 Amylase activity

Amylase activity was assayed by the method of [12]. A 1% starch substrate solution was made in 0.02M sodium phosphate buffer, pH 6.9. A 0.2 ml of the enzyme solution was added to 1ml of the substrate solution in a test tube and the mixture was incubated at 40°C for 1h. The amount of reducing sugars released in the reaction mixture was estimated by adding 2ml of 3,5-Dinitrosalicylic acid (DNSA) reagent, heating the mixture in water bath at 100°C for 15min. The amount of reducing sugars produced was read from a standard curve, prepared with known concentrations of maltose. One unit of enzyme activity (UAM) was arbitrarily defined as the amount of enzyme that will release 10µg of reducing sugars (maltose) per hour Bernfield, 1955 [12].

2.8.3. Lipase activity

Lipase activity was assayed by modifying the method of [13]. Olive oil (Sigma, Bangladesh) was used as substrate. The substrate was prepared by reflux using vortex mixer to mix 0.2 ml of olive oil with 0.2M sodium acetate buffer pH 5.0. A 0.2 ml of enzyme solution was added to 0.2 ml of the substrate in a test tube; and incubated at 37°C for 1h. The reaction was terminated by adding 8ml of absolute ethanol. The mixture obtained was titrated with 0.02M NaOH, using 2 drops of phenolphthalein (0.1g in 50ml absolute ethanol and 5ml water) as indicator. Blanks were prepared by adding 8ml of absolute alcohol before adding the enzyme solution. The difference in titre value of blank and reaction mixture gave the amount of alkali required to neutralize the liberated fatty

acids. One unit of lipase activity (ULP) was defined as the amount of enzyme that will release 1 μg of fatty acids per minute.

2.8.4. Pectinase activity

The pectinase activity was assayed by modifying the method of [14]. A substrate solution of 0.5% pectin was prepared in 0.1M NaOH (5g of pectin was added slowly to approximately 600 ml of boiling 0.1M NaOH to dissolve). It was cooled rapidly and diluted to 1 litre and then stored in the refrigerator. To 2.5 ml of the substrate (kept at 30°C and pH adjusted to 7.0 using 0.01M of NaOH), was added 0.2ml of crude enzyme. The reaction was allowed to proceed for 1h, after which the pH was measured and the solution titrated with 0.01M NaOH to pH 7.0. One unit of enzyme activity (UPE) was the amount of enzyme that will release 1 μg glucose per minute.

2.8.5. Phytase activity

The phytase enzyme was assayed quantitatively following the method of [15]. The reaction mixture contained 0.5 ml of enzyme solution and 1 ml of phytic acid in 0.2 M acetate buffer pH 7.2. The reaction was incubated at 35°C for 1h and stopped by adding 1 ml freshly prepared color reagent (mixture of 25 ml of 10% w/v ammonium molybdate solution $\text{H}_24\text{Mo}_7\text{N}_6\text{O}_{24}\cdot 4\text{H}_2\text{O}$, 1% (v/v) of 25% ammonium solution, with 25 ml 0.235% (w/v) ammonium vanadate solution (NH_4VO_3), 2% (v/v) nitric acid solution (70 ml 65% nitric acid and 130 ml distilled water), stirred slowly while adding 16.5 ml 65% nitric acid, cooled to room temperature and adjusted to 100 ml with distilled water). The color developed was determined by spectrophotometer (Unicam 1700) at 415 nm. One unit of the phytase activity (UPH) was the amount of enzyme that released 1 μg inorganic phosphate released per minute.

2.8.6. Galactanase activity

Galactanase activity was assayed by the method of [16]. A 1% solution of arabinogalactan was prepared in 0.02M sodium phosphate buffer pH 7.0. A 0.2 ml of the substrate was added to 0.2 ml of the enzyme solution. The reaction mixture was incubated at 35°C for 1h. The enzyme reaction was terminated with 2ml of DNSA (dinitro-salicylic acid) reagent and then heated in water bath at 100°C for 5 minutes. The mixture was cooled and then read in a colorimeter at 540nm against reagent blank containing buffer.

3. Results

Fig. 1 shows the microbial load of the fermented products and unfermented substrate Addition of 'kuuru', dried seeds of *Hibiscus sabdariffa* and ash enhanced microbial population of the products when compared with the naturally produced 'iru' (7.50 log CFU/g). Hence, 'iru' produced using 'kuuru' (IFK) had the highest microbial count of 7.88 log CFU/g while the microbial count of IFA and IFH were 7.69 log CFU/g and 7.75 log CFU/g respectively, the unfermented sample had the least microbial count of 3.50 log CFU/g.

The pH of the fermented products using different additives and unfermented substrate are shown in Fig. 2. The unfermented sample had the least pH of 6.68. There was a significant increase in the pH of the product when 'kuuru' was added when compared with the naturally fermented product (7.52). However, addition of 'ash' (7.35) and dried seeds of *Hibiscus sabdariffa* (7.06) led to a significant reduction in pH of the product while the commercially produced 'iru-pete' had a pH of 7.18.

The total titratable acidity (TTA) of both the fermented products and unfermented sample are presented in Fig. 3. The unfermented sample had the highest acidity of $2.55 \times 10^{-2}\text{N}$. The addition of 'ash' and dried seeds of *Hibiscus sabdariffa* led to a significant increase in the TTA, while addition of 'kuuru' led to a significant reduction ($P < 0.05$) in TTA of the fermented products. The sample produced with strain BC4333 had an acidity value of $1.18 \times 10^{-2}\text{N}$.

The moisture content (%) of the samples is presented in Fig. 4. All the fermented products had higher moisture content than the unfermented sample. The CIP had the highest moisture content of 58%, while the unfermented sample had the least moisture content of 41.67 %.

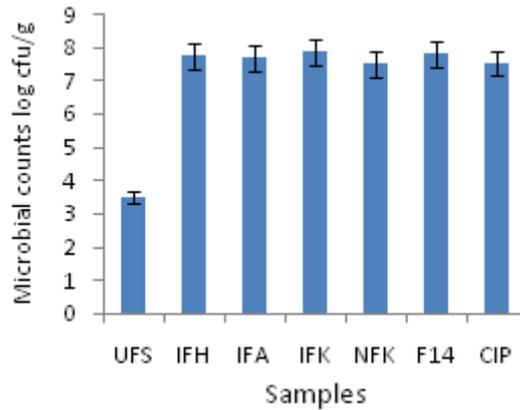


Figure 1. The microbial load (log CFU/g) of unfermented and fermented *Parkia biglobosa* seeds. **KEY:** UFS=Unfermented sample; IFH='iru' fermented using *Hibiscus sabdariffa* seeds; IFA='iru' fermented using ash; IFK= 'iru' fermented using 'kuuru'; NFK= naturally fermented product; F14='iru' fermented using *Bacillus subtilis* strain BC4333; CIP= commercial 'iru-pete'

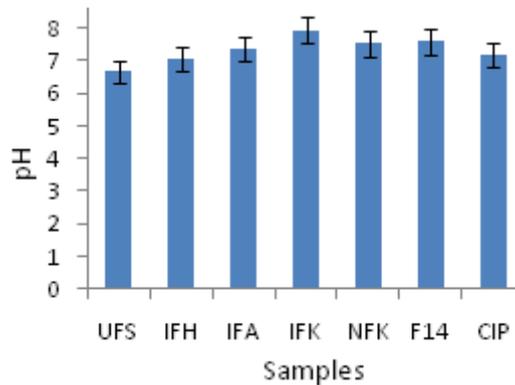


Figure 2. The pH of unfermented and fermented *Parkia biglobosa* seeds with different additives. **KEY:** UFS=Unfermented sample; IFH='iru' fermented using *Hibiscus sabdariffa* seeds; IFA='iru' fermented using ash; IFK= 'iru' fermented using 'kuuru'; NFK= naturally fermented product; F14='iru' fermented using *Bacillus subtilis* strain BC4333; CIP= commercial 'iru-pete'

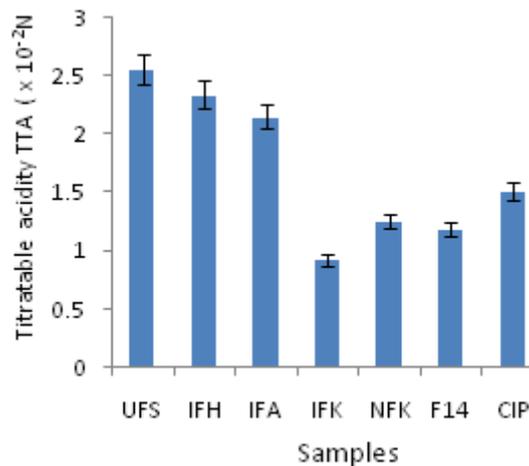


Figure 3. Total titratable acidity (TTA) of the unfermented and fermented *Parkia biglobosa* seeds with different additives.

KEY: UFS=Unfermented sample; IFH='iru' fermented using *Hibiscus sabdariffa* seeds; IFA='iru' fermented using ash; IFK= 'iru' fermented using 'kuuru'; NFK= naturally fermented product; F14='iru' fermented using *Bacillus subtilis* strain BC4333; CIP= commercial 'iru-pete'

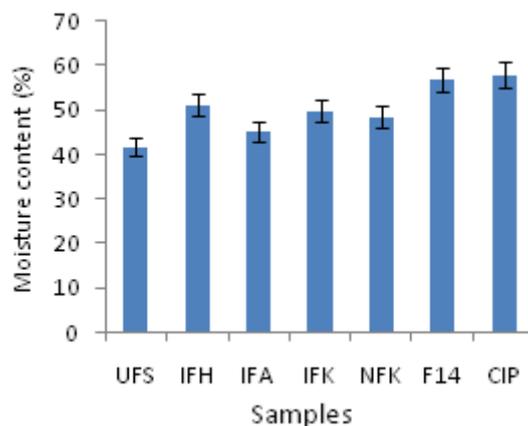


Figure 4. Moisture content (%) of the unfermented and fermented *Parkia biglobosa* seeds with different additives.

KEY: UFS=Unfermented sample; IFH='iru' fermented using *Hibiscus sabdariffa* seeds; IFA='iru' fermented using ash; IFK= 'iru' fermented using 'kuuru'; NFK= naturally fermented product; F14='iru' fermented using *Bacillus subtilis* strain BC4333; CIP= commercial 'iru-pete'.

The F14 sample had the highest protease activity of 232.39 mg/ml followed by CIP with activity of 61.49 mg/ml. IFK had protease activity of 57.02 mg/ml while UFS had the least activity of 5.24 m/ml. Also, sample F14 had the highest amylase activity of 0.41 ± 0.02 , followed by CIW with amylase activity of 0.38. Sample IFK had amylase activity of 0.20 ± 0.03 , while the UFS had the least amylase activity of 0.02mg/ml.

The UFS had the highest lipase activity of 0.80 mg/ml followed by IFA sample lipase activity of 0.67 mg/ml and NFK with activity of 0.50 mg/ml. Samples F14 and IFH had the lowest lipase activities of 0.17mg/ml. IFK and CIP also had the same lipase activities of 0.25 mg/ml. The lipase activity of the NFK sample is significantly higher than the lipase activity of F14 and IFH sample.

The IFA sample had the highest pectinase activity (1.76 mg/ml), followed by F14 with pectinase activity of 1.17mg/ml; while UFS had the least activity of 0.50mg/ml.

The starter culture fermented sample (F14) had the highest phytase activity of 71.85 mg/ml, the phytase activity of NFI, F14 and IFA were 62.29 mg/ml, 48.09 mg/ml and 26.89 mg/ml respectively while UFS had the least phytase activity of 9.90mg/ml. The CIP, IFA and F14 samples had galactanase activity of 0.48 mg/ml, 0.74 mg/ml and 0.14 mg/ml respectively. However, UFS had the least galactanase activity of 0.08 mg/ml.

Table 1. Extracellular enzyme activities of the unfermented and fermented *Parkia biglobosa* seeds using different additives.

Samples	Protease (UPR mg/ml)	Amylase (UAM mg/ml)	Lipase (ULP mg/ml)	Pectinase (UPE mg/ml)	Phytase (UPH mg/ml)	Galactanase (UGA mg/ml)
UFS	$5.25^h \pm 0.00$	$2.00e \pm 0.00$	$800.00a \pm 0.00$	$500.00g \pm 0.00$	$9.90g \pm 0.00$	$80.00g \pm 0.00$
IFH	$8.55^g \pm 0.31$	$4.00^c \pm 0.01$	$170.00^c \pm 0.00$	$580.00^f \pm 0.00$	$19.02^f \pm 0.12$	$110.00^f \pm 0.00$
IFA	$19.35^f \pm 0.43$	$33.00^c \pm 0.02$	$670.00^a \pm 0.00$	$1760.00^d \pm 0.00$	$26.89^e \pm 0.00$	$740.00^g \pm 0.00$
IFK	$57.02^d \pm 0.00$	$20.00^d \pm 0.03$	$250.00^d \pm 0.00$	$1080.00^d \pm 0.00$	$48.09^c \pm 0.30$	$180.00^d \pm 0.00$
NFK	$47.08^c \pm 1.16$	$36.00^{bc} \pm 0.03$	$500.00^b \pm 0.00$	$670.00^e \pm 0.01$	$62.29^b \pm 0.05$	$580.00^b \pm 0.00$
F14	$232.39^a \pm 0.00$	$41.00^a \pm 0.00$	$170.00^c \pm 0.00$	$1170.00^c \pm 0.00$	$71.85^a \pm 0.00$	$140.00^e \pm 0.00$
CIP	$61.49^c \pm 0.00$	$33.00^c \pm 0.00$	$250.00^d \pm 0.00$	$530.00^g \pm 0.06$	$48.09^c \pm 0.30$	$480.00^c \pm 0.00$

KEY: UFS, Unfermented substrate; IFH, 'iru' produced using *Hibiscus sabdariffa* seeds; IFA, 'iru' produced using ash; IFK, 'iru' produce with 'kuuru'; NFK, naturally fermented product; F14, 'iru' fermented with *Bacillus subtilis* strain BC4333; CIP, commercial 'iru-pete'; Values in column having the same superscript do not differ significantly at 0.05. UPR = Unit of protease activity, UAM = Unit of amylase activity, ULP = Unit of lipase activity, UPE= Unit of pectinase activity, UPH = Unit of phytase activity and UGA = unit of Galactanase activity.

4. Discussion

Originally 'kuuru' was added to soften the cotyledon but when the effects of different additives on the bacteria count of 'iru' during fermentation were investigated, the high bacterial load of 'iru' produced with 'kuuru' (7.93 log CFU/g) shows that 'kuuru' might have enhanced the growth of the organisms involved in the fermentation. The increase in pH of 'kuuru' fermented product might be due to the alkaline nature of the 'kuuru' [17]. The increase in pH also encouraged the growth of *Bacillus* spp, which grows well at pH 7.0-8.0.

The higher amount of protease, amylase and phytase activities in 'iru' fermented with the use of the starter culture could be due to higher number of organisms during fermentation. The use of *Hibiscus sabdariffa* seeds and wood ash as additives did not enhance protease, amylase and phytase activities during fermentation. However, protease and phytase activities were higher when 'kuuru' alone was added. Addition of 'kuuru' showed a significant decrease in enzymatic activities during fermentation, which of course should be discouraged from use [18]. The highest lipase and pectinase activities in 'iru' produced using ash might be attributed to the fact that the ash promoted the activities of the enzymes [19]. The alkaline medium created by 'kuuru' might have obliterated the enzyme assay.

5. Conclusion

Thus, this study confirmed that the addition of 'kuuru' during fermentation of *Parkia biglobosa* seeds to produce 'iru-pete' is undesirable; since it has been shown to reduce the enzymatic activities of the fermented product. The research also confirmed the benefit of *Bacillus subtilis* BC4333 as starter culture in the production of 'iru-pete' since it enhances its enzymatic activities. Hence, *B. subtilis* BC4333 can be recommended for local production of 'iru-pete' by rural women.

References

- [1] S.A. Odunfa, Microorganisms associated with fermentation of African locust bean (*Parkia filicoidea*) during iru preparation, J. Plant Foods. 3(4) (1981) 245-250.
- [2] I.T. Ademola et al., An assessment into physical and proximate analysis of processed locust bean (*Parkia biglobosa*) preserved with common salt, Pak. J. Nutr. 10(5) (2011) 405-408.
- [3] E.Y. Aderibigbe, S.A. Odunfa, Growth and extracellular enzyme production by strains of *Bacillus* species isolated from fermenting African locust beans, iru, J. Appl. Microbiol. 69(5) (1990) 662-671.
- [4] G.P. Oyeyiola, Microbiology of the fermentation of 'iru pete' and 'iru woro' obtained from local producers, MIRCEN Journal of Applied Microbiology and Biotechnology. 4(4) (1988) 439-445.
- [5] T.R. Omodara, E.Y. Aderibigbe, Effect of starter culture and different components of 'kuuru' on nutritional quality of fermented *Parkia biglobosa*, Inter. J. Appl. Microbiol. Biotechnol. Res. 2 (2014) 73-78.
- [6] O.K. Achi, Traditional fermented protein condiments in Nigeria, Afr. J. Biotechnol. 4(13) (2005) 1612-1621.
- [7] E.Y. Aderibigbe, W. Visessanguan, D. Jureeporn, Sourcing starter cultures for *Parkia biglobosa* fermentation III: preparation of starter cultures for industrial scale fermentation, Brit. Microbiol. Res. J. 6(4) (2015) 215-224.
- [8] M.J. Ikemebomeh, R. Kok, Mass balance of the processing and fermentation of the African locust bean (*Parkia filicoidea* Welw.), Can. Inst. Food Sci. Tech. J. 17(1) (1984) 48-50.

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- [9] P.O. Olutiola, R.I. Nwagwugwu, Growth, sporulation and production of maltase and proteolytic enzymes in *Aspergillus aculeatus*, Transactions of the British Mycological Society. 78(1) (1982) 105-113.
- [10] M.A. Joslyn, Methods in food analysis: physical, chemical, and instrumental methods of analysis, Academic Press, London, 1970.
- [11] C.K. Odoh et al., Assessment of mould contamination and physico-chemical properties of crude palm oil sold in Jos, Nigeria, Food Sci. Nutr. 5(2) (2017) 310-316.
- [12] P. Bernfield, Amylases X and B, in: S.P. Colowicz, N.O. Kaplan (Eds.), Methods in Enzymology, Vol. 1, Academic Press, New York, 1955, p.149-158.
- [13] F.M. Young, B.J.B. Wood, Biochemical changes in experimental soy sauce Koji, International Journal of Food Science and Technology. 12(2) (1977) 163-175.
- [14] C.H. Hills, H.H. Mottern, Properties of tomato pectase, J. Biol. Chem. 168(2) (1947) 651-663.
- [15] A.J. Engelen et al., Simple and rapid determination of phytase activity, Journal of AOAC International. 77 (1994) 760-764.
- [16] E.E. Trevelyan, D.P. Procter, J.S. Harrison, Detection of sugar in paper chromatogram, Nature. 166(4219) (1950) 444-445.
- [17] S.A. Odunfa, G.F. Oyeyiola, Microbiological study of the fermentation of *ugba*, a Nigerian indigenous fermented food flavor, J. Plant Foods. 6(3) (1985) 155-163.
- [18] E.Y. Aderibigbe, B. Schink, S.A. Odunfa, , Extracellular proteinases of *Bacillus* spp. isolated from fermented African locust bean seeds, *Iru*, Food Microbiol. 7(4) (1990) 281-293.
- [19] Z.B.B. Ateslier, K. Metin, Production and partial characterization of novel thermostable esterase from a thermophilic *Bacillus* sp., Enzyme Microbiol. Technol. 38(5) (2006) 628-635.