Single and Mixed Starter Culture Fermentation of *Oso* (fermented Seeds of *Cathormion Altissimum*)

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Abstract

Oso is a fermented food made from spontaneous fermentation of Cathormion altissimum seeds. It is a local staple food of the people of Yewa in Ogun state. The lack of consistency in the product due to uncontrollable fermentation process remains a challenge, hence the objective of this study. 38 strains of micro-organisms, of which 15 Bacillus subtilis strains, 10 Bacillus licheniformis strains, ten Staphylococcus aureus strains and three Leuconostoc mesenteroides involved in the spontaneous fermentation of Oso, were tested for suitability as starter cultures. Enzyme activities using the plate assay method and APIZYM kit (Biomerieux, France) were used to determine the suitability of the strains for use as starter cultures. The quality of the starter culture fermented sample was measured based on the sensory evaluation test. The toxigenic potential of the selected strains were carried out by haemolytic test on sheep blood agar. Protease activities ranged from 9.5 to 20.9 mm. Out of the 14 combinations of starters, single and mixed strains, only five of the combinations (BS, BL, BS+BL, BS+BL+LM, BS+BL+SA+LM) were accepted based on results of sensory evaluation. The five combinations of starters (BS, BL, BS+BL, BS+BL+LM, BS+BL+SA+LM) and the spontaneously fermented sample were not significantly different (P > 0.05). They were rated the same and the best. The sample fermented with only the Bacillus subtilis and the one fermented with only Bacillus licheniformis were not significantly different (P > 0.05). Results of the present investigation indicate the potential of single and mixed strains of micro-organisms as starter culture for the fermentation of Oso.

Keywords: starter culture, APIZYM kit, toxigenic potentials, enzyme, sensory evaluation.

Fermentation De Levain Unique Et Miste d'Oso (Graines Fermentés De *Cathormionaltissimum*)

Résumé

Oso est un aliment fermenté issu de la fermentation spontanée de graines de Cathormionaltissimum. C'est un aliment de base local de la population de Yewa dans l'État d'Ogun. Le manque de cohérence du produit dû à un processus de fermentation incontrôlable reste un défi, d'où l'objectif de cette étude. 38 souches de micro-organismes, 15 souches de Bacillus subtilis, 10 souches de Bacillus licheniformis, 10 souches de Staphylococcusaureus et trois Leuconostocmesenteroides impliqués dans la fermentation spontanée d'Oso, Les activités enzymatiques utilisant la méthode de dosage sur plaque et le kit APIZYM (Biomerieux, France) ont été utilisées pour déterminer l'aptitude des souches à une utilisation en tant que levain. La

qualité de l'échantillon fermenté de levain a été mesurée sur la base du test d'évaluation sensorielle. Le potentiel toxigénique des souches sélectionnées a été réalisé par test hémolytique sur gélose au sang de mouton. Les activités de protéase allaient de 9,5 à 20,9 mm. Parmi les 14 combinaisons de souches du levain, de souches uniques et de souches mixtes, cinq seulement (BS, BL, BS + BL, BS + BL + LM, BS + BL + SA + LM) ont été acceptés sur la base des résultats de l'évaluation sensorielle. Les cinq combinaisons de levures (BS, BL, BS + BL, BS + BL + LM, BS + BL + SA + LM) et l'échantillon spontanément fermenté n'étaient pas significativement différentes (P> 0,05). Ils ont été classés les mêmes et les meilleurs. L'échantillon fermenté avec seulement le Bacillus subtilis et celui fermenté avec seulement le Bacillus licheniformis n'étaient pas significativement différents (P> 0,05). Les résultats de la présente étude indiquent le potentiel de souches uniques et mixtes de micro-organismes en tant que levain pour la fermentation d' Oso.

Mots clés: levain, kit APIZYM, potentiels toxicogènes, enzyme, évaluation sensorielle.

Introduction

Seeds of legumes are alkaline fermented foods that are widely consumed in Africa. The seeds of *Cathormion altissimum* are legumes fermented to produce the delicacy *Oso* (Popoola et al, 2005).*Oso* is a staple food eaten by the people of Yewa in Ogun State. It can be eaten as snack,main meal or as condiments in soups and stews (Popoola *et al* 2005). Like most of the other alkaline fermented legumes, it is prepared by traditional uncontrolled fermentation. The mixed bacteria population involved in the spontaneous fermentation of *Oso* has been documented (Popoola *et al*, 2006). *Bacillus subtilis* and *Bacillus licheniformis* were particularly involved in the fermentation of the seeds of *Cathormion altissimum* to *Oso*. *Bacillus sp*. are the most dominant naturally fermenting organisms in alkaline fermented foods e.g. *iru* and soybean (Kolapo *et al*, 2007).They have been associated with utilization and reduction of indigestible oligosaccharides and polysaccharides (Afolabi and Abdulkadir, 2016). Starter culture are now being used to ensure consistent flavor, texture, shelf stability and improve product safety in fermented foods. However, the potential of starter cultures for fermentation on a household scale for most of the traditionally fermented foods are yet to be explored (Okorie and Olasupo, 2013).The aim of this study is to explore the potential of single strains and mixed strains of micro-organisms as starter cultures in the fermentation of the seeds of *Cathormion altissimum* to *Oso.*

Materials and methods

Isolation and identification of isolates

Spontaneously fermenting sample of *Oso* was serially diluted and plated out on nutrient agar using spread plate method. Based on cultural and morphological characteristics of the organisms, different isolates were purified by streaking on nutrient agar for *Bacillus sp*, de Mann Rogosa Sharpe for Lactic acid bacteria(LAB) and Baird Parker for *Staphylococcus sp*. Cultural and morphological features, biochemical tests and the use of the analytical profile index kit (API KIT) were used to identify the organisms. API50CHB was used for *Bacillus sp*., APISTAPH for *Staphylococcus sp*. and APICHL for LAB respectively.

Screening of isolates for enzyme production

Protease

The microbial suspension which served as

inoculums was prepared by subculturing *Staphylococcus sp* on blood agar, *Bacillus sp* were subcultured on nutrient agar while Lactic acid bacteria were subcultured on trypcase soy agar to ensure pure culture. A homogenous bacteria suspension (inoculum) from each strain was prepared using 0.5 McFarland standards. Nutrient agar was supplemented with 2% casein. It was autoclave at 121 °C for 20 minutes and distributed in petri-dish. Wells of 8 mm diameter were made in the middle of the agar in the petri-dish after solidification using a sterilized cork borer. One hundred milliliter of inoculum from each strain was transferred to the wells and then incubated at 37°C for 72 hours. At 24 hours of incubation, the clearing zone around the well was measured as an indicator of protease activity (Ouaba *et al*, 2003).

Identification of enzyme using Apizym identification kit

Micropipette was used to dispense 65microlitre of organism into each cupule which contains previously impregnated test enzymes. The enzymes were alkaline phosphatase, leucine acrylamidase, acid phosphatase, α-glucosidase and β-glucosidase. The plastic lid was placed on the tray and it was incubated for 4 hours at 37°C. After incubation, a drop of ZYM A reagent and one drop of ZYM B reagent was added to each cupule. By placing a surface-active agent (ZYM A reagent) in the cupule, solubilisation of the ZYMB reagent in the medium was facilitated. The colour was allowed to develop for at least five minutes. The reactions were read and recorded on the result sheets (API, Biomerieux, France).

Screening of selected Isolates for Haemolytic Activity

Toxigenicity of the isolates selected for optimization studies were determined by screening the isolates for haemolytic activity on blood agar. One loopful of 18 hour culture grown in tryptic soya broth (TSB) were streaked on sheep blood agar plates.The ability of cultures to induce haemolysis was determined after incubating plates for 24 hours at 37 °C. (Aderibigbe *et al*, 2014).

Preparation and inoculation of starter cultures

The selection of organism used as starter cultures was based on the frequency of isolation during fermentation, occurrence in final products and previous knowledge of their roles in legume fermentation process (Matilda and Sanni, 2002; Holzapfel, 2002).The selection of organisms was also based on the results of API ZYM identification table. The organisms selected were *Bacillus subtilis, Bacillus licheniformis, Leuconostoc mesenteroides* and *Staphylococcus aureus*. The selected *Bacillus* strains were grown in nutrient broth for 24 hours, while *L.mesenteroides* was grown on de Mann Rogosa Sharpe broth for 48 hours. *Staphylococcus aureus* was grown in Mannitol salt broth for 48 hours. After incubation, 0.1 ml each of the broth culture was plated on appropriate agar plates using the pour plate technique to determine cell concentrations. Broth cultures containing approximately the same concentration of viable cells in the range of 10^5 were centrifuged at 4000 rpm for 10 minutes, washed in sterile distilled water and recentrifuged. The washed cells were then used singly and as mixed combinations in the fermentation of *Oso* (Edema and Fawole, 2006).

Application of starter cultures

Cathormion altissimum seeds (1 kg) was weighed, washed and boiled for 5 hours. The boiled seeds were washed with distilled water and put in sterile wide-mouthed aluminium cups and capped tightly. It was further boiled for 2 hours. Starter (3 ml) of organisms

prepared according to the method of Edema and Fawole, 2006 was introduced into the seeds with sterilized needles and syringes. Since four multiple starter strains were used, single strain starter contained 3 ml of organisms (BS, BL, SA and LM). Starter of two different strains contained 1.5 ml each (BS+BL, BS+LM, BS+SA, BS+LM, BL+LM, BL+SA, SA+LM,) starters of three strains contained 1 ml each (BS+BL+SA, BS+BL+LM, BL+SA+LM) and starters of four different strains contained 0.75 ml each (BS+BL+SA+LM). It was then fermented for 48 hours at room temperature, thereafter; the aluminium cups with their contents were boiled for 30 minutes at 98 to 100 ^{o}C to stop the fermentation (Mbata and Orji, 2008).

Sensory evaluation of *Oso* fermented with starter culture.

Fifty-man panelists that were familiar with *Oso* were asked to assess the qualities of the spontaneously and starter fermented samples. The following sensory parameters; appearance / colour, odour / taste, texture and acceptability were assessed using 9 point hedonic scale ranging from like extremely to dislike extremely. The test for appearance / colour was by visual inspection. Testing for aroma and taste was by sniffing a spoonful or two of *Oso* and chewing the spoonful for 1-5 minutes respectively. Enough water was provided and the panelists were free to swallow or spit out the *Oso* sample and rinse his/her mouth after each test. Testing for texture was by touching and feeling in between the fingers. The final score was then awarded for overall acceptability. The final scores represent the means of the entire panelists' impression.

Statistical analysis: Data was analysed statistically by analysis of variance (ANOVA) by using Duncan's multiple range test (0.05 level) as described by Pituch and Stevens (2016).

Results

The morphology and biochemical characterisation of the thirty eight isolates involved in the spontaneous fermentation of the seeds of *Cathormion altissimum* to *oso* are shown in table1.The prominent micro-organisms were identified as ten strains of *Staphylococcus aureus*, ten strains of *Bacillus licheniformis*, fifteen strains of *Bacillus subtilis* and three strains of *Leuconostoc mesenteroides*. In Figure 1, the protease activities of all the *Bacillus subtilis* isolates were represented; Figure 2 represented the protease activities of *Bacillus licheniformis* isolates while figure 3 represented the protease activities of *Staphylococcus aureus* and *Leuconostoc mesenteroides*. The protease activities ranged from 9.5mm to 20.9mm.The enzyme activities of the four isolates selected for starter cultures were finally represented in Figure 4. Table 2 shows the result of the haemolytic test on

Table 1: Morphology and biochemical characterisation of isolates involved with spontaneous fermentation of *Oso*.

ID	GR	SP	CA	GE	MO	O	CI	HS	MR	VP	G	GA	L	M	AR	ORG
C1	+	-	++	-	-	FA	-	+	-	+	A/G	A	-	A	A	A*staphylococcus aureus*
C2	+	+	+	+	+	FA	-	+	-	+	A/G	A	-	A	-	*Bacillus licheniformis*
C3	+	+	+	+	+	FA	-	+	-	+	A	SA	-	A	A	*Bacillus subtilis*
C4	+	-	-	-	-	FA	–	0	-	+	A	0		A/G	A0	*Leuconostoc mesenteroides*

ID-identification, GR-Gram reaction, SP-Spore formation, CA-Catalase, GE-gelatin, MO-Motility, O-Oxygen relationship, CI-Citrate utilization, HS-hydrogen sulphide, MR-Methyl red, VP-Vogeus proskauer, G-Glucose, GA-Galactose, L-Lactose, M-Maltose, AR-Arabinose.+-positive,--negative, FA-Facultative anaerobe, A/G-Acid and gas, A-Acid, SA-Slightly acidic

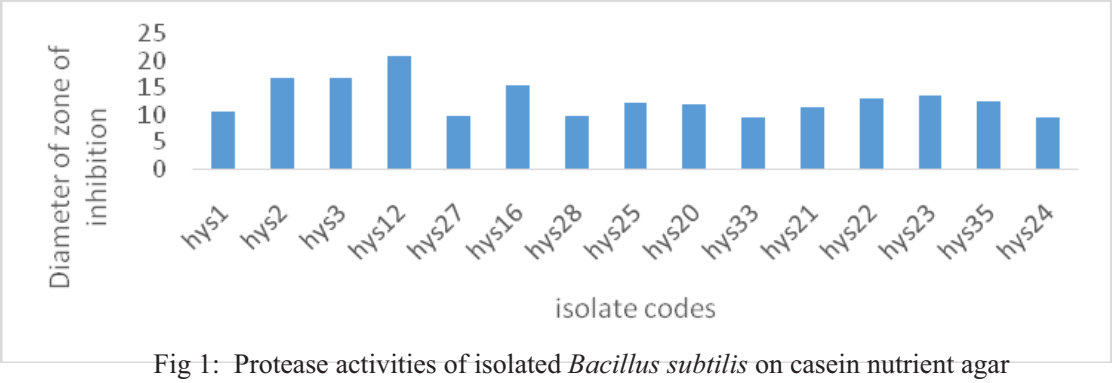

Fig 1: Protease activities of isolated *Bacillus subtilis* on casein nutrient agar

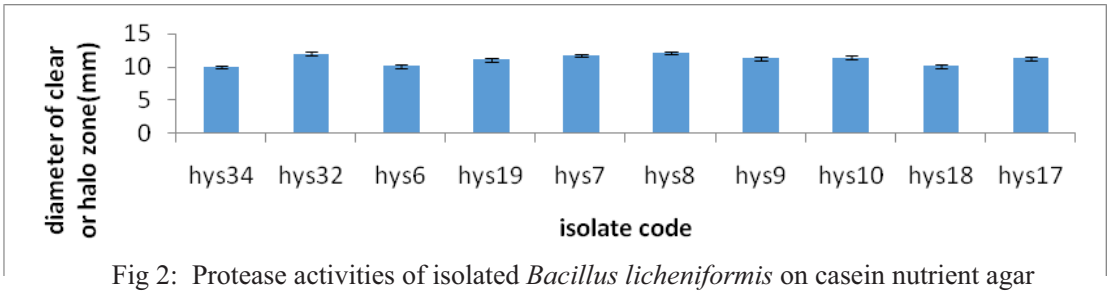

Fig 2: Protease activities of isolated *Bacillus licheniformis* on casein nutrient agar

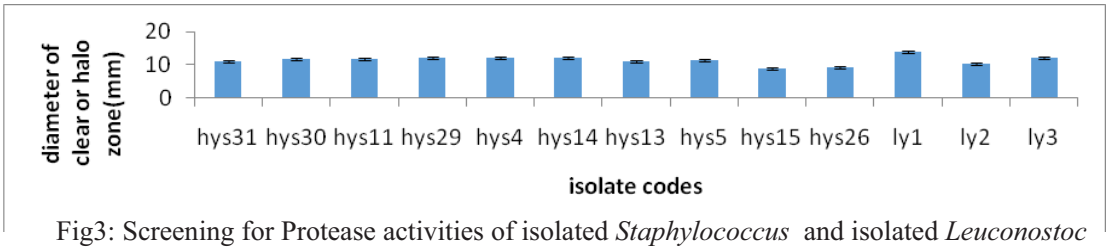

Fig3: Screening for Protease activities of isolated *Staphylococcus* and isolated *Leuconostoc species* on casein nutrient agar

ISOLATE CODES:
Hys31, hys30, hys11, hys29, hys4, hys14, hys13, hys5, hys15, hys26 are all *Staphylococcus sp.*
Ly1, lys2, lys3 are all *Leuconostoc sp.*

sheep blood agar. The result showed that none of the selected isolates was haemolytic in nature.

The isolates subjected to haemolytic test (Table 2) were reassigned isolate numbers for easy identification.They were HYS12 reassigned as BS (*Bacillus subtilis*), HYS8 as BL (*Bacillus licheniformis*), HYS30 as SA (*Staphylococcus aureus*) and LY1 as LM (*Leuconostoc mesenteroides*) respectively and subjected to sensory evaluation test after fermentation with single or mixed strains of selected micro-organisms.

Figure 5 shows the sensory evaluation of Oso produced by mono and mixed starter cultures as well as the natural fermentation. In terms

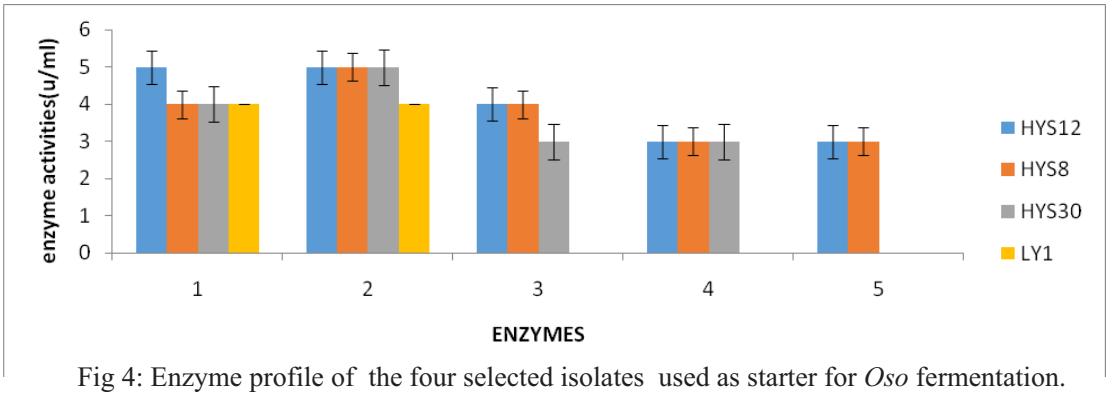

Fig 4: Enzyme profile of the four selected isolates used as starter for *Oso* fermentation.

Enzymes; 1. alkaline phosphatase 2. leucine acrylamidase 3. Acid phosphatase
4. α-glucosidase 5. β-glucosidase

Table 2: Haemolytic test of selected isolates on sheep blood agar

Isolate code	Organisms	Haemolysis
HYS16	*B.subtilis*	+ve (positive control)
HYS4	*B.licheniformis*	-ve(negative control)
HYS12	*B.subtilis*	-ve
HYS8	*B.licheniformis*	-ve
HYS30	*S.aureus*	-ve
Ly1	*L.mesenteroides subsp. cremoris*	-ve

+ve indicates haemolytic reaction
-ve indicates non-haemolytic reaction

of general acceptability; the starter culture fermented samples and the spontaneously fermented sample were not significantly different ($P > 0.05$). They were both rated the same and the best. The sample fermented with only the *Bacillus subtilis* and the one fermented with only *Bacillus licheniformis* were not significantly different ($P > 0.05$). The sample fermented with mixed culture of *Bacillus licheniformis* and *B.subtilis* were also accepted. Some mixed culture fermentation had the same average scores and were not significantly different ($P > 0.05$). They were BS + BL + LM, BS + SL + SA, BL + SA + LM and SA + LM + BS. Other starter combinations were not acceptable to the panelists and were rated below average.

Discussion

In the present study, the following micro-organisms are prominent in *Oso* fermentation. *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus aureus* and *Leuconostoc mesenteroides*. *Bacillus sp* has been found to be prominent in most oil seed fermentation like African oil bean seed, soybean seed (Ogueke and Aririatu, 2004). In accordance with the result of the present study, *Staphylococcus sp*. has been implicated in Ugba fermentation (Okorie and Olasupo,

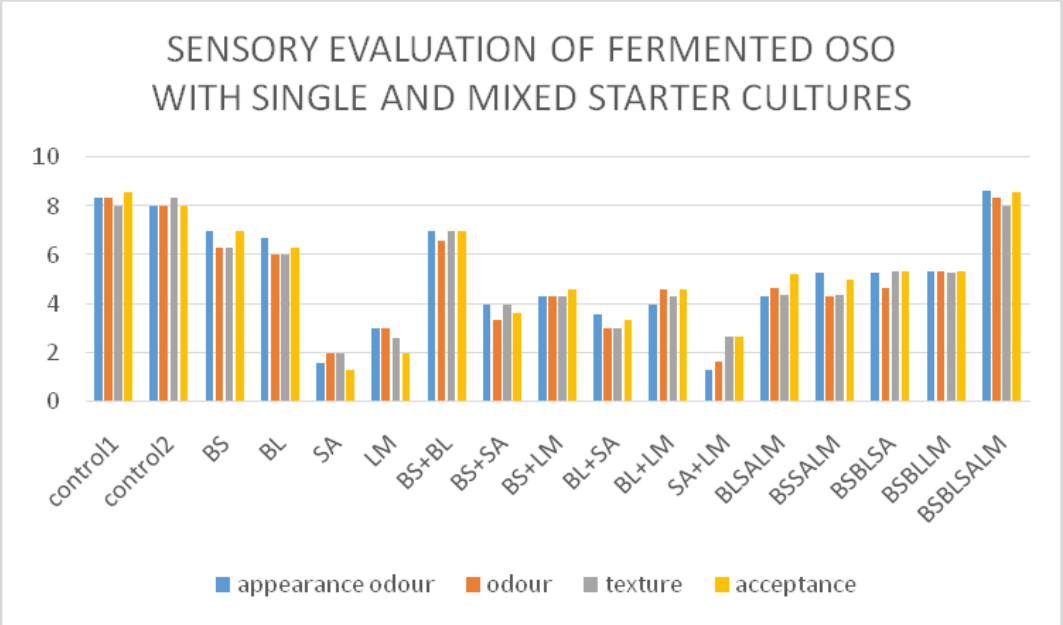

Fig 5: Sensory Evaluation Tests

2014) and from African oil bean seed (Ogueke and Aririatu, 2004).The screening of the thirty-eight isolates (Figures1,2,3) for protease activity showed that all the micro-organisms obtained were able to exhibit proteolytic activity. Isolate HYS12 (BS) and HYS8 (BL) were *Bacillus sp.* strains and they recorded the highest activity. This observation is similar to the reports of Oguntimehi (1993). Okorie and Olasupo (2013) also identified *Bacillus sp* as proteolytic micro-organisms. Endospores of the *Bacillus sp*. has been associated with cotyledon of most oilseed fermentation (Isu and Ofuya, 2000; Sanni *et al*, 2002). High levels of hydrolytic enzymes have been associated with *Bacillus sp*. during oilseed fermentation (Oguntoyinbo *et al*, 2007). However, in other legume/oilseed fermentation, *B.subtilis* has been identified as the most adapted and most dominant with properties such as higher protease and amylase production, production of polyglutamic acid (Oguntoyinbo et al, 2007). Extracellular proteases hydrolyse protein into mono or oligomers mainly peptides and amino acids (Jones and Lock, 1998). The Leucine acrylamidase enzyme included in the API kit is a protease and the level of activity of this enzyme is a good measure of the proteolytic activity of bacteria as it is a peptide bond hydrolyzing enzyme (Jones and Lock, 1998). This showed the contribution of this microflora to the properties of legume products which included the degradation of carbohydrates and proteolytic activities. Nutritional benefits are derived from fermentation where micro-organisms breakdown the flatulence-causing indigestible oligosaccharides into absorbable and monosaccharides and then into organic acids (Granito, *et al* 2003). *Bacillus* species have been reported as producers of certain enzymes such as amylase, galactanase, gluconidase and fructofuransidase which are involved in the degradation of carbohydrates (Aderibigbe and Odunfa, 1990; Omafuvbe *et al* 2000).This is in line with the result of the

present study in which the enzyme α-glucosidase and β-glucosidase have been found in abundance in the four isolates, hys12 (BS), hys8 (BL), hys30 (SA), ly1 (LM) used as starter cultures. Carbohydrates are hydrolysed into sugars which are then readily digestible to humans. Similarly galactanase soften the texture of the seeds and liberate sugar for digestion (Omafuvbe *et al* 2000). Involvement of *Staphylococcus* species and *Bacillus* species has been attributed to their proteolytic activities (Ouaba *et al* 2003; Popoola *et al*, 2006).This also conforms with the present study which reports the proteolytic activities of *Bacillus sp* and *Staphylococcus sp* (Figures 1 and 2). Alkaline and acid phosphatases have the same functions. Alkaline phosphatase has been found in all the isolates selected for starter cultures. Phosphatase occurs in acid and alkaline forms and is able to hydrolyse phosphoric esters (Browne and Goulder, 1996). An essential property that all starter cultures involved in food production must have is to be non-toxigenic (Aderibigbe *et al* 2014).All the isolates used in this study were negative to haemolytic tests (table 2). Ostensvik *et al* (2004) reported the presence of cytotoxic *Bacillus* species belonging to the *B.cereus* and *B.subtilis* groups in Norwegian surface waters which was used in food processing. The use of starter culture is an appropriate approach for the control and optimization of fermentation process in order to alleviate the problems of variations in organoleptic quality and microbiological instability observed in most African indigenous fermented foods (Sanni, 1993). The sensory evaluation results showed that products of fermentation of monocultures of *Bacillus subtilis and B. licheniformis* were generally accepted. The predominance of *Bacillus* species had been demonstrated in other legumes (Achi, 2005). Similarly mixed culture fermentation by *Bacillus subtilis and B. licheniformis* also churned out an acceptable product. A pure starter culture is essential for controlled experimental fermentation and for this purpose, a number of *Bacillus species, Staphylococcus* have been explored (Suberu and Akinyanju, 1996). Controlled fermentation of soybeans was achieved by using pure single cultures of *Bacillus subtilis*, *B.licheniformis* or in combination (Suberu and Akinyanju, 1996). Most researchers now agree that there is a predominant development of *Bacillus* species during various legume fermentations (Aderibigbe and Odunfa, 1990; Ouaba *et al* 2003).The use of a single strain would seem too restrictive for the production of condiments with a generous range of organoleptic characteristics. Mixed culture of *B.subtilis* and *B.licheniformis* were recommended by the same authors and fermentation was achieved in 72 hours. Omafuvbe *et al* (2003) on the other hand tested three *Bacillus* species namely *B.subtilis, B.licheniformis* and *B.pumilus* singly and in their combination for their ability to ferment soybean for the production of *Dawadawa*. In the present study, the use of mixed cultures of *B.subtilis, B.licheniformis*, *S.aureus* and *L.mesenteroides* produced the best fermented *Oso* as shown by its highest level of acceptability. This further asserts the potentials of starter culture to produce acceptable *Oso*.

Conclusion

This study has shown the ability of single and combined starter strains of *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus aureus* and Leuconostoc *mesenteroides* isolated from fermenting seeds of *Cathormion altissimum* to produce enzymes such as amylase and protease which are important in the fermentation of all legume seeds, *Oso inclusive* and the ability of single and combined strains to produce controlled and consistent fermented product.

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