Biochemical Changes and Sensory Evaluation of Soy Iru Produced Using Starter Culture

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Authors' contributions

This work was carried out in collaboration between the two authors. Author AFT designed the study. Author AM wrote the first draft of the manuscript with contribution from author AFT. Both authors managed the literature searches and analyses of the data. Authors AM and AFT managed the experimental process. Both authors read and approved the final manuscript.

Article Information

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ABSTRACT

Soybean (*Glycine max* L.) was processed into iru using *Bacillus subtilis* SBI 13 and *Leuconostoc mesenteroides* as starter culture and calabash as fermenting container. The fermenting soya beans were analysed for pH and titratable acidity, free amino acids and total soluble sugar. The Enzymatic analysis (protease, amylase and lipase) was also carried out. The combination of *Bacillus subtilis* and *Leuconostoc mesenteroides* showed higher protease and amylase activities than when they were used singly. The samples were fermented for 3 days after which were evaluated for sensory qualities. The pH of the samples ranged from 7.08 to 8.88, the titratable acidity ranged from 0.58 to 0.73, the total free amino acids ranged from 1.10 to 6.29% and the total soluble sugar ranged from 18.02 to 29.29%. All the iru samples were acceptable by the sensory panelists but the soy iru fermented with both *Bacillus subtilis* SBI 13 and *Leuconostoc mesenteroides* SBI 15 (*Bacillus* + LAB soy iru) was the most preferred.

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1. INTRODUCTION

In Nigeria, soyabean is fermented to make soy-dawadawa, a soup condiment and it is also used to make incursion into the diet of many Nigerians, particularly children and nursing mothers. Soyabean seed is rich in plant protein. Soy iru (Soy dawadawa) is a food flavouring condiment prepared by fermenting whole beans. It is widely consumed by the people of Benue and Plateau States of Nigeria and its consumption is now extending to the Southern part of Nigeria. Locust beans (*Parkia biglobosa*) have been traditional raw material for the production of iru (dawadawa). However, emphasis has been shifted to the use of soyabean (*Glycine max L.*) as a substitute [1].

The preparation of both soybean and locust bean condiments involve the natural fermentation of dehusked cooked seeds [2,3]. The traditional method of preparing soy-iru has been described [4]. The product from such fermentation is sticky with a strong ammoniacal smell [5]. Soy-iru (Soy-dadawa) is a food flavouring condiment prepared by fermenting whole soybean. It is widely consumed by the people of Benue and Plateau States of Nigeria and its consumption is now extending to the southern part of Nigeria [6]. [7] describe fermentation as a complex chemical transformation of organic substances brought about by the catalytic action of enzymes either originally present or secreted extracellularly by the microorganisms fermenting the material. The microbiology of fermentation and some biochemical changes occurring during the production of this condiment have been reviewed [8]. Fermentation is a proven method to improve flavour, texture and nutritional quality of the soybeans.

Besides bringing physico-chemical and sensory quality changes, fermentation contributes towards the preservation of food due to release of metabolites that discourage the growth of pathogenic bacteria in foods. Fermentation involves a range of microorganisms such as lactic acid bacteria, acetic acid bacteria, yeasts, moulds and a range of bacteria. It also covers wide range of products such as staples, adjuncts to staples, condiments and beverages that use substrates such as cereals, pulses, soybeans, flowers, milk, meat etc. [9]. There are many traditional *B. subtilis* fermented soybeans foods in various parts of the world.
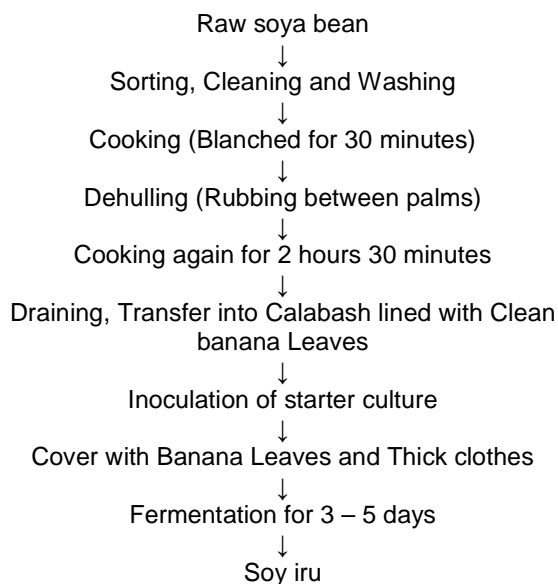
One of the common examples is *Kinema,* which is traditionally consumed by the non-Brahmin Nepali inhabiting Nepal; Darjeeling and Sikkim of India; and some parts of Bhutan. It is popular among Lepchas and Bhutias who call it *Satlyangser* and *Bari* respectively [10]. [11] reported the origin of soybean iru (*Kinema)* in Southern part of China. While it spread, this food settled into a niche as seasonings in East Nepal, North East India, Burma, Thailand and in Japan. Some *Kinema* like fermented soy foods reported in literature are: *Natto* in Japan, *Thua-Nao* in Thailand, *Douchi* in China, *Chungkook- jong* in Korea, *Tao-si* in Philippines, *Dawadawa* in Africa [12]. With its powerful odour and slimy appearance like a rotten food, large numbers of population do not like it, whereas other finds eating soy iru (and similar products) a delightful experience. These foods are eaten in the fresh form or as a fried curry dish along with boiled rice and sometime with boiled rice, and sometimes as soup, pickle or mixed with other vegetables [13].

Bacillus spp. is the most dominant naturally fermenting agents in soybeans. These hydrolytic bacteria are associated with utilization and reduction of indigestible oligosaccharides and polysaccharides. The organism has also shown to reduce the activity of anti-nutrients that hinders availability of proteins and phytochemicals present in soybeans [9]. *B. subtilis* fermentation is accompanied by covering intact granules with white-coloured viscous substance, slimy appearance, softer texture, and unique rotten flavour. It also completely removes the beany odour of raw soybeans and increases sensory quality of the product [12]. Chemical studies have been carried out to know the nutritive value of soy-iru. According to [14], fermented soybean has the following chemical composition as expressed by 100 g dry matter, crude protein 49.51, fat 31.46, crude fibre 3.49, ash 3.97, carbohydrate 15.06 and organic matter 96.03. It also contains appreciable amount of minerals. This study is focus on studying the biochemical changes in fermenting soya bean using starter culture. It will also determine the sensory pattern of the soy iru.

2. MATERIALS AND METHODS

2.1 Preparation of Inocula

The starter cultures (*Bacillus subtilis* SBI 13 and *Leuconostoc mesenteroides* SBI 15) used for this study were obtained from the Department of Microbiology, University of Ibadan, Nigeria. The representative of *Bacillus* group was grown on nutrient agar at 37°C for 18 hours, Lactic acid a bacterium was grown on MRS agar for 24 hours under anaerobic condition. Each of the culture was suspended in 10 mL sterile 1% NaCl solution, diluted to give an absorbance of 0.03 at 540 nm in a spectrophotometer, 0.5 mL of which was used to inoculate the cooked beans [15] aseptically then shake well for proper mixing (this gives about 10^4 cells g-^{1} wet wt.); the suspension was mixed for two membered mixed culture.

Raw soya bean
↓
Sorting, Cleaning and Washing
↓
Cooking (Blanched for 30 minutes)
↓
Dehulling (Rubbing between palms)
↓
Cooking again for 2 hours 30 minutes
↓
Draining, Transfer into Calabash lined with Clean banana Leaves
↓
Inoculation of starter culture
↓
Cover with Banana Leaves and Thick clothes
↓
Fermentation for 3 – 5 days
↓
Soy iru

Fig. 2.1. Flow chart of laboratory fermentation of soybeans to produce soy iru [6,15]

2.2 Laboratory Fermentation of Soya Beans

I kg of soybeans were picked carefully then boiled for 30 minutes, and dehulled by hand under a running tap. The dehulled bean seeds were cooked in boiling water for 2 hours on electric stove. Cooking water was allowed to dry on the beans before removing the heater to retain the nutrient [10]. 50 g of cooked bean seeds was weighed and adjusted to pH 7 using phosphate buffer then transferred into calabashes lined with clean banana leaves and it

was then covered with clean banana leaves and was tightly covered with another calabash and then wrap with foil paper [3] and sterilized at 121°C for 15 minutes, then allowed to cool down. 0.5 mL of the inoculums was used to inoculate the sterile bean seeds. A control sample was set up by weighing 50 g of the cooked soybean into calabash lined with banana leaves and cover with banana leaves then cover tightly with another calabash and allowing it to ferment spontaneously at 30°C for 72 hours (3 days). The fermenting beans were labeled 0, 24, 48, 72, 96 and 120 hours in order to facilitate withdrawing of the fermenting marsh at 0, 24, 28, 72, 96 and 120 hours of incubation for microbiological, chemical and enzymatic analyses. Each experiment was carried out in duplicate.

2.3 Physico-chemical Analysis

2.3.1 Determination of pH and temperature of fermenting soy-dawadawa

The pH and temperature changes of the fermenting soybeans were measured at 1, 2, 3, 4, and 5 days of fermentation using pH meter with model number H198107. One gram of the fermenting soybeans was prepared in 10 mL of distilled water; it was mixed thoroughly and was then measured with the pH meter that was previously calibrated.

2.3.2 Determination of titratable acidity in fermenting soy-dawadawa

Titremetry method as described by [1,16] was used to determine the titratable acidity. 10 ml of the supernatant of the sample was titrated against 0.1 M of NaOH solution with phenolphthalein as indicator.

2.3.3 Determination of moisture content of fermenting soy-dawadawa

Moisture content of the fermenting soybeans was determined using the method of A.O.A.C [16].

2.3.4 Determination of free amino acids content in fermenting soy-dawadawa

The concentration of free amino acids liberated before, during and after fermentation (0, 1, 2, 3, 4 and 5 days) was determined using the colometric ninhydrin method adapted from [17] using leucine as the standard. A soy-dawadawa sample weighing 5 g after drying in the oven for

24 hours was mixed with 70% ethanol and ground in a mortal; the suspension was then washed with 5 mL n-Hexane to extract the oil, this is then filtered, using Whatman filter paper 1 and the filtrate (extract) was used for the analysis. A ninhydrin solution was prepared containing 4 g of ninhydrin, 0.6 g of hydrindantin, 150 mL of methyl celullosolve and 50 mL of sodium acetate buffer, pH 5.5, in a dark glass container, and stored in the dark. To one mL of the sample extract, 1 mL of the ninhydrin solution was added and heated in a boiling water bath for 15 min. It was diluted with 5ml of ethanol–water mixture (1:1), cooled and the extinction value read at 510 nm in a spectrophotometer. A blank determination was carried out using 1 mL of distilled water in place of the supernatant. The total concentration of free amino acids was determined from a standard curve prepared with known concentrations of leucine according to [2,17]. The data obtained were to analysis of variance (ANOVA) and Duncan's multiple range tests was used to separate the means where significant difference was obtained for sample treatments ($p \leq 0.05$).

2.3.5 Determination of reducing sugars during the fermentation of soybean dawadawa

Reducing sugars in the fermenting substrates were determined using the dinitrosalicylic reagent method. A soy-dawadawa extract was prepared as above and diluted tenfold. About 1 ml of the diluted extract was added to 2 ml of dinitrosalicylic acid reagent and heated in a boiling water bath for 5 min. After cooling, 20 ml of distilled water was added and the optical density measured at 550 nm in a spectrophotometer. The total concentration of reducing sugars was determined from a standard curve prepared with known concentrations of maltose according to [18].

2.4 Enzymatic Analysis

2.4.1 Preparation of enzyme extract

An enzyme extract of the fermenting soybeans was prepared by grinding 5 g of sample in 50 ml of an appropriate extracting buffer. For the determination of protease activity, the extracting buffer used was 0.1 M sodium hydrogen phosphate buffer, pH 6.5; for a-amylase activity, the extracting buffer used was 0.1 M potassium hydrogen phosphate buffer, pH 6.5; and for lipase activity, 0.1 M sodium acetate buffer, pH

5.5, was used. After maceration of the sample in the appropriate buffer, the suspension was washed with petroleum ether to extract the oil and centrifuged at 4000xg for 5 min. The supernatant constituting the crude enzyme extract was stored at -20℃.

2.4.2 Determination of protease activity in fermenting soy-iru

Protease activity was determined by a modification of the assay method described by [14,19]. About 5 mL of enzyme extract was added to 10mL of 2% light soluble casein solution and incubated at 35℃ for 30 min. The reaction was terminated by adding 10 mL of 10% trichloroacetic acid. Undigested protein was removed by centrifugation at 4000xg for 15 min. The trichloroacetic acid soluble peptides in the supernatant were determined by the method of [20] using tyrosine as standard solution. One unit of protease activity was defined as the amount that produced 1.0 µmol of tyrosine in 1.0 ml of the trichloroacetic acid-soluble peptides under the assay conditions.

2.4.3 Determination of amylase activity in fermenting soy-iru

Alpha amylase activity was determined by the assay method of [21] About 2 ml of the enzyme extract was mixed with 1 ml of 1% starch solution and incubated for 1 hour at 40℃. The reaction was stopped by adding 3 ml of dinitrosalicylic acid reagent, and heated for 5 minutes. After cooling, it was diluted with 18 ml distilled water and the optical density measured at 550 nm in a spectrophotometer. In a blank determination, the dinitrosalicylic acid reagent was added before the starch solution. The amount of reducing sugars formed was calculated from a standard curve prepared with known concentrations of maltose according to [21].

2.4.4 Determination of lipase activity in fermenting soy-iru

Lipase activity was determined according to [22]. About 5 ml of the extract was added to 1 mL of olive oil, 0.4 g of sodium fluorochlorate, 1 mL of 0.1 M CaCl and 6 ml of 0.1 M sodium acetate buffer, pH 5.5. After incubation of the mixture at 35℃ for 1 h for the enzyme to liberate fatty acids from the oleic acid, the reaction was terminated by adding 40 mL of absolute alcohol and titrated against 0.02 M potassium hydroxide using phenolphthalein as an indicator. A blank

determination was carried out by using 5mL of distilled water in place of the enzyme extract. The titre, i.e. the amount of alkali required to neutralize the liberated fatty acids, was expressed as oleic acid with the unit of enzyme being the amount of enzyme required to liberate 1.0 mg of oleic acid per min.

2.5 Sensory Evaluation

Organoleptic properties of the soybean iru fermented with *Bacillus* sp, Lactic acid bacteria and the combination of the two were determined alongside with spontaneously fermented soy-iru following the method of [9]. The colour, odour, taste, mucilage and stickiness of samples were assessed by a set of 6-man panel who are regular consumers of iru, using a score range of 5 (like extremely) to 1 (dislike extremely) to grade their likeness for the odour, taste and general acceptability; while a score range 5 (strongly like iru) to 1 (not like iru at all) for colour, stickiness and mucilage.

2.6 Statistical Analysis

The data obtained were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests was used to separate the means where significant difference was obtained for sample treatments ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 Results

The changes in pH observed for the four products were similar, whilst the product in which fermentation was carried out with the combination of *Bacillus subtilis* and *Leuconostoc mesenteroides* recorded a mean lower value after 24 h of fermentation. Within 48 h, the mean pH values in all samples inoculated with starter cultures had risen from between 6.91 and 6.93 to between 8.08 and 8.88.

Fig. 3.2 shows the changes in titratable acidity the values increase steadily as fermentation time increases. The highest value obtained was 0.73 mg lactic acid/g after 72 hours of fermenting *Bacillus* + lactic acid soy iru.

In Table 3.1, the results of the release of free amino acids in the fermenting substrate resulting from the proteolytic activities of the fermenting organisms were shown. There are significant differences in the value obtained. *Bacillus* + LAB Soy iru has highest concentration of free amino

acids (6.29%) in the soybean substrate throughout fermentation.

The value of the percentage soluble sugar increased greatly with fermentation in all the samples fermented. soluble sugar in *Bacillus* + lactic aci soy iru increased from 18.01 mg/g dry wt to 29. 09 mg/g dry wt.

All the iru samples have no divided opinion in respect to the colour and stickiness but *Bacillus* + LAB soy iru was the most preferred in terms of aroma and general acceptability.

3.2 Discussion

The pH and titratable acidity of the iru samples fall within the alkaline pH range of 6.91 and 8.88, they still fall within the normal pH range of soy iru [23]. The increase in pH towards alkalinity is probably due to proteolysis and release of ammonia following the utilization of amino acids by fermenting microorganisms (Fig. 3.1). The release of ammonia is responsible for the ammoniacal odour characteristic of most vegetable protein fermentation [24]. The titratable acidity in the fermenting soya bean mash range within 0.53 and 0.73 mg lactic acid/g (Fig. 3.2). Both the pH and titratable acidity of the products signifies its quality and they were still within the pH and titratable acidity range in soybean iru fermented for 72 hours [14].

In Table 3.1, the results of the release of free amino acids in the fermenting substrate resulting from the proteolytic activities of the inocula are shown. The results of the concentration of free amino acids in the fermenting substrate generally reflected the levels of protease activity assessed during fermentation. This is because the combination of *Bacillus subtilis* and *Leuconostoc mesenteroides*, which showed the highest level of protease activity, also recorded the highest concentration of free amino acids of 6.29% in the soybean at 72 hours of fermentation. These results show that there are differences in the ability of the fermenting organisms to ferment soybeans into soy iru and that the organisms in combination had an advantage than when they are used singly as starter culture to ferment soybeans with respect to their ability to break down proteins. Protein hydrolysis is reported to be the major biochemical change which occurs during iru fermentation [8]. [25] found the production of extracellular proteases by seven strains of *Bacillus subtilis* isolated from African locust bean dawadawa and cultured in Nutrient

Broth containing 0.8% African locust bean to differ significantly. They characterized the proteases as serine protease, neutral protease and an esterase. [2,14] also reported a steady increase in the concentration of free amino acids in fermenting soybean iru attaining a mean value of 5.32% at the end of fermentation. In the present work, the concentrations of the free amino acids measured in the different fermented samples were in the range of 4.82 to 6.29% whilst the control sample recorded a level of 4.82%.

Another important biochemical activity which occurs during soya bean fermentation is the hydrolysis of carbohydrates. Both [14,26] have shown that amylase activity increases in soybeans during fermentation into soy iru. In the present work, there was increase in the concentration of total soluble sugars in all the samples over the fermentation periods. The total soluble sugar varies from 18.01 to 29.09%. *Bacillus* + LAB soy iru recorded the highest percentage total soluble sugar of 29.09% at 72 hours of fermentation (Table 3.2). Biochemically,
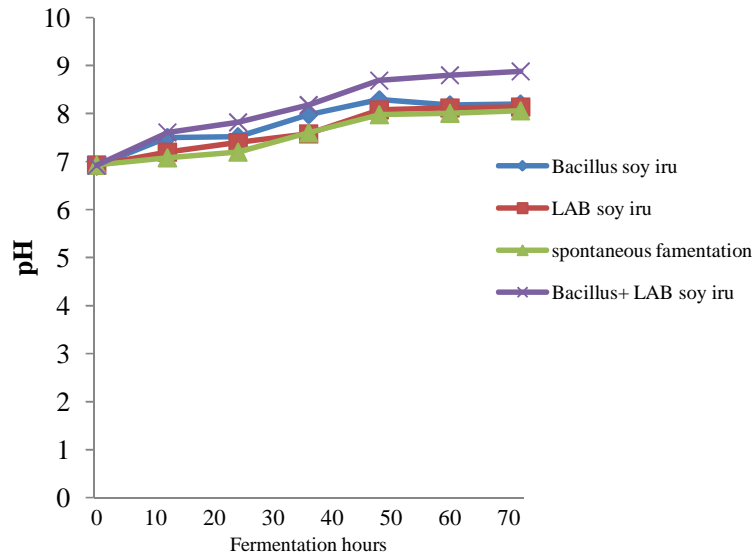

Fig. 3.1. Changes in pH of soy iru produce with different inocula at different fermentation hours
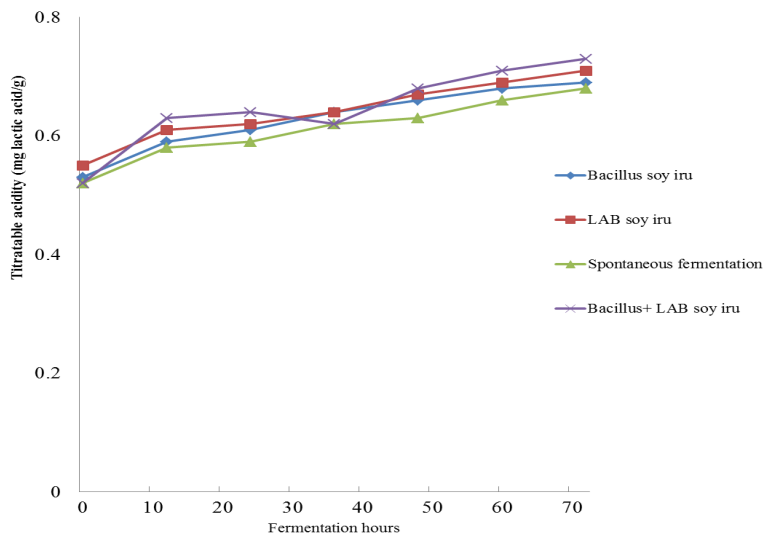

Fig. 3.2. Changes in titratable acidity of soy iru produce with different inocula at different fermentation hours

these results could be interpreted as breakdown of starch due to microbial enzymatic activity and utilisation of the simpler sugars produced for microbial growth. Indeed, a measure of the a-amylase activity in the fermenting substrates showed that the sample inoculated with both *Bacillus subtilis* and *Leuconostoc mesenteroides* showed a steady rise in the concentration of total soluble sugars throughout fermentation, also showed the sharpest rise in amylase activity during fermentation and recorded the highest level of amylase activity after 72 hours (Table 3.3). All the other samples, including the spontaneously fermented sample, also showed a steady rise in amylase activity throughout fermentation. Since soya beans contain high amounts of fat, about 20% (dry matter basis), the ability of the isolates to hydrolyse the fat present into fatty acids during fermentation is of considerable importance just as in the traditional African locust bean dawadawa which contains about 31.2% fat [27]. In the present work, the lipase activity in the fermenting substrate, determined as the amount of enzyme required to liberate 1.0 mg of oleic acid from olive oil in 1 min. was determined and all samples, including the control, showed a steady increase in lipase activity during the 72 h of fermentation. At the end of fermentation, the highest lipase activity was recorded in the control sample with a value of 2.60 U/g dry wt., whilst in the inoculated samples, the lipase activity fell within a range of between 1.31 and 2.53 units/g dry wt (Table 3.3). During fermentation, the measured range of lipase activity did not vary much between all the samples including the control, but the steady rise in lipase activity in all samples confirmed hydrolysis of fat as a significant activity in the fermentation of soybeans into soy iru. These results are similar to the study of [28] in which they compare ability of four isolates of *Bacillus subtilis* to ferment soybeans into dawadawa.

The sensory evaluation of the products is shown in Table 3.4 There was no significant difference ($p<0.05$) in the taste of all the iru samples. Although all the iru samples were no divided opinion the colour and stickiness but *Bacillus* + LAB soy iru was the most preferred in terms of aroma and general acceptability. This study showed that soy-iru could be processed using *Bacillus subtilis* and *Leuconostoc mesenteroides* either singly or in combination at pH7 and 37°C for 72 hours of fermentation in calabash. The combined use of the two organisms proved to be the most appropriate starter culture for fermentation of soy-iru as it contained and recorded the highest protein content. It is also the most preferred by the sensory panelists when evaluated. [6] also recorded the similar results on sensory evaluation of soy iru.

Table 3.1. Change in percentage free amino acids (mg/g) of soy iru production with different inocula at different fermentation hours

Products	Hours of fermentation						
	0	12	24	36	48	60	72
Bacillus soy iru	0.21[a]	1.55[b]	2.57[c]	2.96[c]	3.93[d]	4.32[e]	5.14[f]
LAB soy iru	0.21[a]	1.18[b]	1.94[b]	2.92[c]	3.27[d]	3.48[d]	4.90[e]
Bacillus + LAB soy iru	0.22[a]	1.50[b]	3.50[d]	3.80[d]	4.60[e]	5.23[f]	6.29[g]
Spontaneous fermentation	0.21[a]	1.10[b]	2.20[bc]	2.82[c]	3.03[cd]	4.12[e]	4.82[e]

Mean values followed with different lower case letters are statistically significantly different at p <0.05

Table 3.2. Change in percentage total soluble sugar (mg/g dry wt) of soy iru production with different inocula at different fermentation hours

Products	Hours of fermentation						
	0	12	24	36	48	60	72
Bacillus soy iru	18.03[a]	19.08[b]	20.32[bc]	21.02[c]	21.72[c]	22.21[cd]	23.46[d]
LAB soy iru	18.01[a]	18.82[a]	21.30[c]	22.21[cd]	23.71[d]	24.02[de]	25.86[e]
Bacillus + LAB soy iru	18.01[a]	20.21[bc]	21.73[c]	24.52[de]	25.64[e]	27.53[f]	29.09[g]
Spontaneous fermentation	18.02[a]	18.89[b]	19.02[b]	19.72[b]	20.22[bc]	20.61[bc]	21.52[c]

Mean values followed with different lower case letters are statistically significantly different at p <0.05

Table 3.3. Enzyme production during fermentation of soy iru sample at different fermentation hours

Products	Period of fermentation (hrs)/enzyme concentration (unit)	Amylase	(units/g dry wt) protease	Lipase
Bacillus iru	0	4.20[a]	18.44b	1.22[a]
	24	11.09[b]	40.19[cd]	2.10[b]
	48	15.22[d]	46.18[e]	2.50[b]
	72	15.21[d]	41.22[cd]	2.53[b]
LAB iru	0	4.32[a]	10.21[a]	1.01[a]
	24	5.18[ab]	19.82[b]	1.31[a]
	48	6.01[b]	26.10[bc]	1.51[a]
	72	6.31[b]	32.29[c]	1.53[a]
Bacillus + LAB iru	0	4.82[a]	18.81[b]	1.22[a]
	24	12.29[c]	41.2[cd]	1.86[a]
	48	17.32[e]	46.53[e]	2.31[b]
	72	19.28[f]	48.23[e]	2.48[b]
Control	0	4.28[a]	10.48[a]	1.10[a]
	24	6.53[b]	18.24[b]	2.00[b]
	48	7.18[c]	40.13[cd]	2.21[b]
	72	7.52[c]	41.10[cd]	2.60[b]

Mean values followed with different lower case letters are statistically significantly different at p <0.05

Table 3.4. Sensory evaluation of soy iru produced using different inocula

Products	Taste	Aroma	Colour	Mucillage	Stickiness	General acceptability
Bacillus soy Iru	4.22[b]	3.67[a]	3.33[a]	4.22[b]	4.44[a]	3.89[b]
LAB soy iru	4.50[b]	3.50[a]	3.44[a]	3.33[a]	3.67[a]	3.25[a]
Bacillus + LAB Soy Iru	4.78[b]	4.44[b]	3.33[a]	4.33[b]	4.44[a]	4.78[c]
Spontaneous fermentation	3.40[a]	3.60[a]	3.67[a]	4.22[b]	4.11[a]	3.60[ab]

Mean values followed with different lower case letters are statistically significantly different at p <0.05

4. CONCLUSION

From this research, it has been confirmed that *Bacillus subtilis* and *Leuconostoc mesenteroides* used as starter culture in the fermentation of Soya beans to produce Soy Iru can cause useful biochemical changes in fermenting soya beans. Their use in combination produced improved and more acceptable sensory quality soy iru.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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