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Nutritional Assessment and Molecular Identification of Microorganisms from *Akhuni/Axone*: A Soybean Based Fermented Food of Nagaland, India

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Abstract

Soybean based fermented foods are known to be highly nutritive, not just because it contains high protein content but also due to presence of polyphenols. Various microorganisms involved during the fermentation process have proven to play active role in the enhancement of the nutritional value as well as increase in phytochemicals in the product. *Akhuni/Axone* is a popular soybean product of Nagaland, India. It forms an integral part of the diet and is used as a condiment during preparation of various dishes in the Naga kitchen. This study describes the nutritional value, the antioxidant activity and the presence of polyphenols of *Akhuni/Axone,* which increase significantly in composition between the soybean seeds and the fermented product. The molecular identification of the microorganisms present in *Akhuni/Axone* product is also reported in this paper.

Indexing terms/Keywords: *Akhuni/Axone*, Microbes in fermented food, Nagaland, Nutrition value of fermented food, Soya based fermented food.

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Introduction

Soybean is known as functional food due to its high nutritional value and ability to treat various diseases like obesity, cancer, osteoporosis, cardiovascular disease and renal obstruction [1]. In parts of Asia soybeans are cooked, roasted, fermented, or sprouted and forms an integral part of diet. Soybean products are available throughout the world and fermentation of soybean by microorganisms is known to improve the bioavailability of nutrients and reduces the level of anti-nutritional factors [2]. Fermentation of soybean is one of the techniques resulting in novel foods with unique features [3-4].

Soybean in Nagaland, India is cultivated in the kitchen garden for personal consumption but in parts cultivated on commercial scale and popularly known as '*Naga*' dal. *Akhuni/Axone* is a traditional soybean (*Glycine max*.L*)* based fermented food product of Nagaland. Although advances in food science have resulted in new technologies, *Akhuni/Axone* is still prepared at the household level by traditional means. *Akhuni* is similar to other soya fermented products of other North-Eastern states of India like *Hawaijar* (Manipur), *Tungrymbai* (Meghalaya), *Bekang* (Mizoram), *Kinema* (Sikkim) and *Peruyaan* (Arunachal Pradesh) [5-6]. *Akhuni* is prepared by boiling the soybean seeds till turned soft followed by draining of excess water and packed in bamboo baskets lined with leaves *(Ficus* species) and covered. The bamboo basket is then kept near the fire place to ferment for about 3-4 days. Usually at this point most of the other fermented products are considered ready but for *Akhuni*, it is further made to paste followed by wrapping in banana leaves or *Phrynium pubinerve* leaves and kept near the fire place for 3-4 days for further fermentation. Due to this extra fermentation the shelf life of *Akhuni* increases.

Though reports are available on nutrition and microbiology of *Akhuni* [5, 7], however, thorough investigation has still not been done on the antioxidant activity as well as molecular identification of microorganisms present in *Akhuni*. Present study was under taken on the detail investigation on different fermented food of Nagaland including nutritional assessment; identification of microbes involves using molecular marker. The present communication is part of the present study.

Materials and Methods

1. Collection of Sample

Akhuni was collected from different households and markets of Nagaland. Samples were kept at 4°C until Analyzed.

2. Biochemical Analysis

Quantification of protein: Protein estimation was done using the colorimetric method of Lowry et al. [8]. One gram of oven dried sample was grounded using 20 ml of 0.1 M phosphate buffer (*p*H 7) and centrifuged at 1000 rpm for 10 min followed by filtration and filtrate was used for the analysis. To 1 ml of extract 5 ml of Lowry's solution (prepared by mixing 2% Sodium Carbonate in 0.1 NaOH, 1% Copper Sulphate in 1% Potassium Sodium Tartarate) was added. The mixture was incubated at room temperature for 10 min followed by added 0.5 ml of 1N Folin- Ciocalteau reagent and incubated in dark for 20 min. The absorbance at 660nm was measured and standard curve was prepared with 'Bovine Serum Albumin' (BSA).

Quantification of reducing sugar: Reducing sugar was estimated using 3, 5-dinitrosalicylic acid (DNSA) reagent [9]. Ethanol extraction procedure was followed for the study. To 1 ml of extract, 1 ml of DNSA reagent was added and mixture was kept in boiling water bath for 5 min followed by cooling to room temperature with 10 ml distilled water. The absorbance was measured at 540nm and glucose was taken as the standard.

Crude fiber: Crude fiber was determined following Maynard [10] with modification. One gram of dried samples was boiled with 200 ml of 0.25N Sulphur acid (H_2SO_4) for 30 min. It was then filtered with No. 1 Whatman filter paper. The filtrate was then again boiled with 200 ml of 0.313N NaOH solution for 30 min followed by filtration and washed subsequently with 25 ml of boiling 1.25% H_2SO_4 and thrice with 50 ml distilled water and 25 ml of

alcohol. The residue was removed and transferred to pre-weighed aching dish (W_1g). The filtrate was then dried for 2 h at 130±2ºC and then cooled. The aching dish was cooled and weighed (W_2g). It was ignited for 30 min at 600°C. After cooling in desiccator, it was again reweighed (W_3 g). The crude fiber content was determined using the formula:

$$\text{Crude fiber (g/100g)} = \frac{\text{Loss in weight on ignition } (W_2\text{-}W_1) - (W_3\text{-}W_1)}{\text{Original weight of sample}} \times 100$$

Moisture content: Moisture content was estimated by taking five g of sample in a pre-weighed dish plate and placed in the oven for ~16 h at 70±1°C till a constant weight was achieved. After drying, samples were weighed again and the moisture content was determined by using the formula:

$$\text{Moisture content (\%)} = \frac{\text{Loss of weight} \times 100}{\text{Weight of the sample}}$$

Determination of pH: Five gram of sample was blended with 10 ml of distilled water in a homogeniser and the *p*H of the slurry was determined directly using a digital pH meter.

Preparation of Methanol Extract

Ten grams of dried sample was ground and extracted in 100 ml of 80% (v/v) methanol by shaking for 24 h at room temperature. The extraction procedure was repeated until the extraction solvent became colorless. The extract was then filtered over Whatman No. 4 filter paper. The filtered obtained was directly used for antioxidant analysis.

Determination of Antioxidant Activity:

DPPH radical scavenging assay: The scavenging activity of stable 2,2-Diphenyl-1-picrylhydazyl (DPPH) free radical was determined following Aoshima et al. [11] with modification. To 100µL of methanol extract, 2.9 ml of DPPH reagent (0.1 mM in methanol) was added followed by vigorous shaking and incubated in dark and room temperature for 30 min before reading the absorbance at 517nm in spectrophotometer (Multiskan Go, Thermo Scientific). Standard curve was calculated using Trolox and inhibition percentage was calculated using the formula:

$$\text{\% inhibition} = \frac{\text{OD control-OD sample} \times 100}{\text{OD control}}$$

Determination of total phenolic content (TPC): Total phenol content was determined following Folin-Ciocalteau method [12]. About 0.1 ml extract was added to 1 ml Folin-Ciocalteau reagent and 0.9 ml of distilled water and allowed to stand for 5 min followed by mixing of 2 ml of saturated sodium carbonate (75 gL^{-1}) and 2 ml of water. The absorbance was measured at 765 nm after incubating at 30°C for 1 h with intermittent shaking. Gallic acid was used for making the standard graph and expressed as mg Gallic acid equivalents (GAE) / g of extract.

Determination of total flavonoid content (TFC): Total flavonoid content was determined following technique of Sahreen and Khan [13] with slight modification. To 0.3 ml of extract, 3.4 ml of 30% methanol, 0.15 ml of 0.5M sodium nitrite and 0.15 ml of 0.3M aluminum chloride were added. The mixture was then allowed to stand for 5 min and then added 1 ml of 1M NaOH. The absorbance was measured at 510 nm and standard curve was prepared using Quercetin and expressed as mg Quercetin equivalents (QE) / g of extract.

3. Statistical Analysis

The experiments were done in triplicate (n=3) and expressed as mean ± standard deviation.

4. Microbiological Study

Ten grams of sample was homogenised in 90 ml sterile physiological saline (0.1% w/v, peptone, 0.85% w/v, NaCl). Serial dilutions of 10^{-1} to 10^{-8} was made in the same diluents and appropriate decimal dilutions (0.1 ml) of the homogenate was spread on Nutrient Agar and Tryptone Soya Agar and incubated at 37°C for 24 h. For Lactic acid bacteria, MRS agar supplemented with 1% (w/v) calcium carbonate was used and incubated anaerobically in an Anaerobic Gas-Bag system at 30°C for 72 h. Plate count agar was used to determine the total viable counts and incubated at 28°C for 72 h. Members of Enterobacteriaceae were enumerated using Violet red bile glucose agar and incubated at 30°C for 48 h. The presence of fungi, yeasts and moulds were enumerated using potato dextrose agar and yeast malt agar, supplemented with 12µg ml^{-1} Streptomycin and incubated at 28°C for 72 h. Morphologically different colonies were isolated and purified cultures were grown on slants of the same medium and stored at 4°C. Purified isolates were checked for gram stain and for catalase production.

5. Molecular Identification

DNA isolation: Extraction of genomic DNA was done using CTAB protocol [14] with slight modification. About 5 ml bacterial broths was centrifuged at 10,000 rpm for 5 min at 4°C followed by suspended in 500µL of TE buffer and thoroughly mixed with 200µL of Lysozyme. The mixture was incubated for 45 min at 37°C water bath. To this 10µL of proteinase K and 50µL of SDS were added and mixed thoroughly and incubated at 37°C until the solution became clear and viscous. Subsequently, 100µL of 5M NaCl was then added and incubated at 65°C for 5 min. It was again incubated at 65°C for 10 min after addition of 100µL CTAB solution. The suspension was extracted with equal volumes of phenol: chloroform: iso-amyl alcohol (25:24:1) and centrifuged at 10,000 rpm for 10 min. The upper phase was transferred and to it equal volume of chilled isopropanol was added and mixed thoroughly by inverting the tubes. Aqueous phase was recovered by centrifugation at 10,000 rpm for 15 min. Isopropanol was removed and the pellet was washed in 70% ethanol by centrifugation at 10,000 rpm for 15 min. The pellets were then allowed to stand for 5-10 min and then re-suspended in 50µL of TE buffer. The extracted genomic DNA was tested qualitatively on 1% (w/v) agarose gel electrophoresis and quantified using Nanodrop Spectrophotometer.

16S rDNA sequencing: The 16S rDNA gene sequences were amplified using universal primers 9F (5'-CGCGGGATCCGAGTT TGATCCTGGCTC-3') and 1492R (5'-GGCCGTCGACACGGA TACCTTGTTACGACTT-3') [15]**.** About 25µL of PCR mixture containing 2.5µL of PCR buffer (10X) with 15 mM $MgCl_2$**,** 0.5µL of 10mM dNTP, O.5µL of each primer, 5.0 µL of DNA template, 0.2µL of T*aq* DNA polymerase and 15.8 µL of pure water was amplified in a PCR programmed with the following temperatures: 94°C for 5 min then 35 cycles at 94°C 1 min, 60°C for 1 min and 72°C for 30 sec. The final extension was at 72°C for 5 min and stopped at 4°C.

Amplified products were separated by electrophoresis in 1.2%, w/v agarose gel and were purified using a commercial kit (HiPura PCR Product Purification Kit, Make: HiMedia, India). Sequencing was done at 1st Base, Singapore. Search for homologous nucleic acid sequences was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/) and nucleotide sequences with highest percent of similarity were submitted at NCBI GenBank and accession numbers were obtained. Multiple sequence alignment was done using the CLC sequence viewer. Phylogenetic tree was constructed using the neighbour- joining method and the stability determined by a thousand bootstrap replications in CLC sequence viewer.

Results and Discussion

1. Proximate Analysis

Nutritional value of soybean is known to improve during fermentation process. Studies have also confirmed the availability of nutrients and degradation of anti-nutritive compounds during the fermentation of soybean [16]. The most important activity that occurs during fermentation process is proteolysis leading to unique flavour and taste. Nutritional profile is an important parameter as guideline for food quality reference and to help improve product formulation. Present study revealed that there is significant increase in protein, reducing sugar and crude fiber in the fermented product compared to raw ingredient (Table 1).

Table1: Comparison of nutritional quality of soybean seeds and fermented product

	Soybean seeds (Dry weight basis)	Fermented product (Dry weight basis)
Moisture (%)	11.2(0.02)	50.0 (0.01)
***p*H**	6.8 (0.003)	8.0 (0.001)
Protein (g/100g)	41.8 (0.004)	42.1 (0.03)
Reducing sugars (%)	27.6 (0.013)	29.7 (0.01)
Crude fiber (g/100g)	1.04 (0.03)	1.61 (0.01)

2. Antioxidant Activity

The antioxidant activity of fermented soybean has been reported due to presence of polyphenols and phytochemicals in soya fermented foods [2, 17-18]. In *Axone*, there is increase in antioxidant content. Free radical scavenging activity for DPPH radical was expressed as IC50 value (the concentration required to scavenge 50% of DPPH). By increasing the plant extract concentration there was a corresponding continuous increase in scavenging activity. Table 2 shows the free radical scavenging ability of *Akhuni* as compared to that of the non-fermented product.

Table 2: Comparison of antioxidant activity of soybean seeds and fermented product

	IC50 (µg/ml)
Soybean seeds	186.75
Fermented product	98.79

3. Total Phenolic and Flavonoid Content

Besides antioxidant, soybean is also known for rich isoflavones and phenolic compounds, which increase further during fermentation [1, 19]. Soya isoflavones like genistein and daidzein have been reported to have inhibitory ffect on the breakage of DNA induced by hydrogen peroxide [20]^{0}. Reports are available on increase in polyphenols of soya fermented foods [2, 18, 20-21]. In the present study it was found that there was increase in both TPC and TFC in the fermented food as compared to raw materials (Table 3).

Table 3: Comparison of total phenolic and flavonoid of soybean seeds and fermented product

	TPC at 765nm (mg GAE/g)	TFC at 510nm (mg QE/g)
Soybean seeds	0.2	0.46
Fermented product	0.86	0.64

Molecular identification: The total microbial loads were in the range of 10^7 cfu/ml and yeasts and moulds were not detected in any of the samples. Isolates were differentiated based on their grams staining and catalase activity. Most of the isolates were gram negative, spore formers and catalase positive belonging to the genus *Bacillus*. The different groups of *Bacillus* sp. identified by sequencing the partial 16S rDNA were *Bacillus subtilis*,

Bacillus licheniformis and *Bacillus cereus*. *Bacillus* species was found to be dominant in other soya fermented foods [22-25] and their role being to accelerate the hydrolysis of protein, thus releasing ammonia [26]. The release of ammonia is responsible for the ammoniacal odour characteristic of most soybean based fermentations [27]. The prevalence of *Bacillus* species in the fermented product may be due to the alkaline condition (*p*H 7.6-8) that occurs during the fermentation process leading to favorable condition for some bacteria to grow, but also causing unfavourable condition for other microbes to grow. Use of *Bacillus subtilis* as a pure starter culture reported high increase in total amino acids and isoflavones in soybean fermented product [28]. Apart from *Bacillus subtilis* strain, *Bacillus licheniformis* strain was also reported to produce good quality *chongkukjang* fermented product [29-30]. The presence of *Staphylococcus epidermis* and *Bacillus cereus* which are considered as food pathogens may have entered the food from unhygienic use of tools and hands during its preparation process. *Alcaligenes* sps. was also identified from the samples, which was also reported from Hawaijar [23]. Table 4 shows the different bacterial strains isolated from *Akhuni/Axone* with GenBank accession numbers.

Table 4: 16S rRNA sequence based identification of microbes from *axone/akhuni* with GenBank accession numbers

Sl. No.	Isolates	Closest related microorganism	Max. score	Query (%)	E value	Similarity (%)	Gene bank accession No.
1.	BJ-DEBCR-2	*Bacillus licheniformis*	2455	100	0.0	99	KU301334
2.	BJ-DEBCR-33	*Bacillus licheniformis*	1700	99	0.0	99	MF487831
3.	BJ-DEBCR-3	*Bacillus subtilis*	2407	99	0.1	99	KU301335
4.	BJ-DEBCR-24	*Bacillus subtilis*	1251	100	0.0	99	MF487822
5.	BJ-DEBCR-22	*Bacillus cereus*	1094	99	0.1	99	KX364205
6.	BJ-DEBCR-29	*Bacillus cereus*	1146	99	0.0	99	MF487826
7.	BJ-DEBCR-1	*Staphylococcus epidermis*	2615	100	0.0	99	KU301333
8.	BJ-DEBCR-21	*Alcaligenes faecalis*	2536	100	0.0	100	KX364204

Phylogenetic tree constructed by the neighbour-joining method showed similarities with the reference strains taken from NCBI gene bank. The strains BJ-DEBCR-33 and BJ-DEBCR-2 grouped with *Bacillus licheniformis* reference strains. Strains BJ-DEBCR-3 and BJ-DEBCR-24 grouped with *Bacillus subtilis* reference strains and the other two strains BJ-DEBCR-22 and BJ-DEBCR-29 were grouped with *Bacillus cereus* reference strains. Phylogenetic tree constructed for the strain BJ-DEBCR-1, showed forming a group with *Staphylococcus* sp. reference strain. Another strain BJ-DEBCR-21 was found to form a group with *Alcaligenes faecalis* reference strain (Figure 1 a-c).

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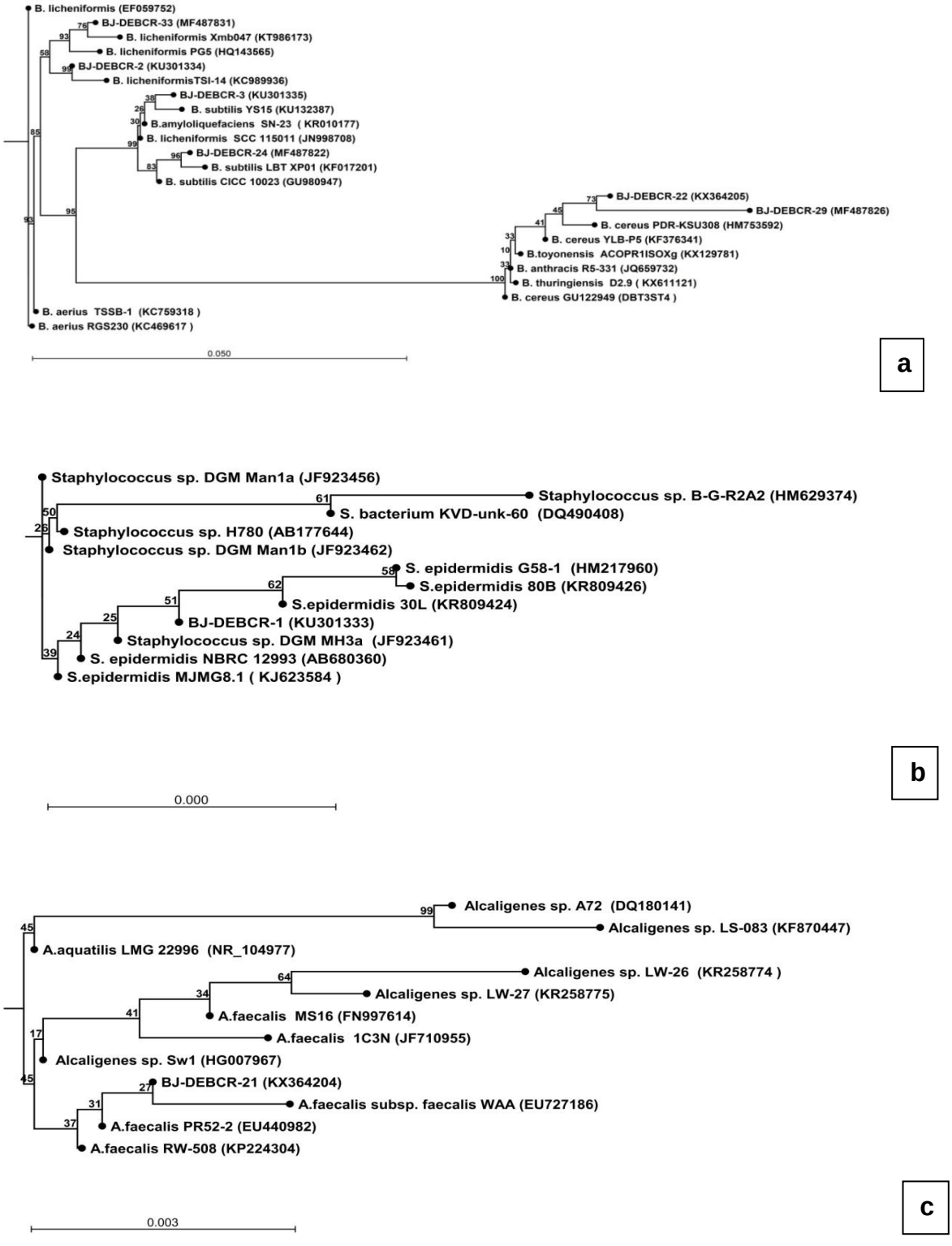

Figure 1: Phylogenetic tree showing relationships of isolate and related species from NCBI. a) *Bacillus* species. b) *Staphylococcus epidermis*. c) *Alcaligenes faecalis*

Conclusion

Numerous reports available on microbiology and biochemistry of soybean fermentation however, *Akhuni/Axone* has not been studied comprehensively for its nutritional value addition and microorganisms associated. *Bacillus species* was found to be dominant microorganisms while, presence of polyphenols in *Akhuni/Axone* have been the ever first report which increases significantly. The isolation of antioxidant components and factors responsible for enhancement of the antioxidant activity, quality control for popularizing *Akhuni* need to be further investigated.

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Author Disclosure Statement

No competing financial interests exist.

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Author' Biography With Photo

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