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CULTURAL, BIOCHEMICAL AND GENOMIC STUDIES OF STARTER CULTURES IN FERMENTED SOYBEAN CURD PRODUCTION

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ABSTRACT

Soybean is an excellent and cost-effective protein source. However, its usage is limited by anti-nutrients. Soybean curd commonly called 'awara' in Nigeria or 'tofu' in Asia is a processed cuisine that is consumed in many countries. Lactic acid bacteria (LAB) and acetic acid bacteria (AAB) that are mostly used as starter cultures in fermentation processes and have been associated with many health benefits were isolated from fresh cow milk collected from three sample areas of Nwosen, Komoto and Zaria in Rugga, a Fulani community in Wukari, Taraba State, Nigeria. A total of seven strains were isolated from the fresh cow milk post biochemical tests. Two strains with good starter culture properties were used in fermenting the soybean curds and molecularly identified thereafter. One of the strains was confirmed to be LAB (*Lactococcus lactis*) and the other was AAB (*Acetobacter tropicalis*). After forty-eight hours of fermentation, *L. lactis* and *A. tropicalis* were able to increase the protein content from 30.6% to 45.7% and 30.6% to 42.3% respectively. Decreases in anti-nutrient contents of the samples were also observed. The difference in these values proves that these *L. lactis* was the best starter that increased the nutrient and decreased the anti-nutrients via extended hours of fermentation thus making it the most preferred starter culture in fermentation of soybean curd.

Keywords: Soybean, fermentation, starter, culture, genomic, analyses

INTRODUCTION

Glycine max [L] Merr also known as soybean, is a stable food that rates well among vegetable sources in many parts of the world (Abdulkadir et al. 2020). Traditional methods for making soybean curd, popularly called "awara" in Nigeria involves soaking (submerging), wet milling and curdling with a coagulant, can be facilitated by starter cultures or back slopping (Abdulkadir et al. 2020; Adeyeye et al. 2020).

Report has shown that soybean contains anti-nutrients such as tannins, oxalates, phytate and lectins (Ojokoh and Bello 2014). Consuming soy products such as fermented soybean curds, has been linked to improved bone health and a decreased risk of cancer of the breast, prostate, colon and other cancers (Datti et al. 2019). Protein-energy malnutrition (kwashiorkor and marasmus) is a condition of malnutrition that frequently occurs due to insufficient protein consumption (Abdulkadir et al. 2020). To address this issue arising from the presence of these anti-nutrients, soybean curd fermentation is the recommended technology to either eliminate or reduce the amount of anti-nutrients thereby increasing its nutrient content. It has been shown that lactic acid bacteria (LAB) and acetic acid bacteria (AAB) are both effective fermenting organisms of cocoa and soybean (Ogodo et al. 2018; Levai et al. 2021).

The goal of this present study was to utilize LAB to increase the nutritional quality of soybean curd through fermentation, determine the proximate composition of anti-nutrients and characterize the genome of the starter cultures

used in the production of fermented soybean curds.

MATERIALS AND METHODS

Plant and animal products

Soybeans (Glycine max.) and lemon (*Citrus limon*) fruit juice were bought at the Wukari local market in Taraba State and brought to the Federal University Wukari laboratory in a clean polythene bag for analysis. Three (3) cows from three (3) sampling villages (Nwosen, Komoto and Zaria) in Rugga, a Fulani community in Wukari, provided the fresh cow milk from which the LAB used as starter cultures were isolated.

Preparation of culture media

Lactic acid bacteria were cultivated on De Man Rogosa and Sharpe (MRS) agar medium in accordance with the manufacturer's instructions (Cheesbrough, 2006).

Isolation of microorganisms

The fresh cow milk sample was serially diluted, and 10^{-4} ml of this was aseptically pipetted into adequately labelled sterile petri dishes containing the media and gently swirled in a planar circular motion to ensure homogeneous microbial growth after solidification at room temperature (Ogodo et al. 2018). The petri dishes were then incubated at 37^0C for 24 hours and bacterial isolates sub-cultured to produce pure colonies using streak plating. The latter were subsequently identified based on their colonial morphologies.

The inoculum or starter culture preparation

Inocula or starter cultures (SC) were created by cultivating two independent pure cultures of bacteria in 250ml Erlenmeyer flasks containing 100ml MRS broth each. The cells were separated by centrifugation, washed in sterile saline for 10 minutes at a speed of 4000 rpm and diluted using sterile saline to obtain 0.5 McFarland standard of 1.0×10^8 cells/mL at 600nm spectrophotometry (Jianming et al. 2013).

Laboratory preparation of soybean curds

The fermentation was carried out by inoculation to decrease the danger of contamination. Fermented and unfermented soybean curds were prepared following the methods of Jianming et al. (2013). However, lemon juice was used instead as SC in the production of the unfermented soybean curds. Soybean was cleaned of foreign objects, rinsed with tap water to get rid of smaller contaminants and hydrated for 12 hours at room temperature. Thereafter, it was rinsed with tap water and wet milled in a blender to create the slurry after being heated at 98^0C for 5 minutes (to sanitize the bean as well as improve its flavour and nutritional value by deactivating trypsin inhibitors).

The soymilk was extracted from the slurry by sieving it through cheesecloth. The filtrate soymilk was divided into two portions, each of which was heated at 85 to 90^0C for five minutes before being cooled to 80^0C while being constantly stirred at room temperature. Freshly made starter culture (SC1) was introduced to one portion of the soymilk while SC2 was added to the other. They were both left alone for 15 minutes to coagulate before they were transferred to different cheesecloth to drain excess water. Fermentation of the curds occurred after 24 hours and extended to 48 hours. The cheesecloth were then removed and soybean curds, cut into equally sized cubes. Thereafter, 100g each of the fermented and unfermented soybean curd, variously prepared using different starter cultures was analyzed for anti-nutritional factors and proximate composition. Experimentation was duplicated for accuracy and statistical purposes.

Proximate composition analyses

The proximate analyses of fat, moisture content, carbohydrate, ash, crude protein and fiber were variously determined using the recommended methods of the Association of Analytical Chemists (AOAC, 2019).

Determination of anti-nutrients in fermented and unfermented soybean curds

The anti-nutritional factors (tannins, phytates, oxalates and lectins) of each prepared soybean curd sample were appropriately determined (AOAC, 2019).

Statistical analyses

The results obtained were statistically analysed and expressed as mean (±) standard

derivation. The level of significance was set at p≤ 00.5.

Molecular identification of the starter cultures

Strains 1 and 3 were characterized by sequencing the 16SrDNA. The universal primers 27F and 1492R were used to amplify the 16S target region. The Sequence (5^1 to 3^1) used for both strains are AGAGTTTGATCMTGGCTCAG and CGGTTACCTTGTTACGACTT respectively (Lane et al. 1991; Altschul et al. 1997).

RESULTS

The three (3) fresh cow milk samples yielded a total of seven (7) isolates, of which five (5) were found to possess the morphological and biochemical characteristics of LAB (Table 1). They were also able to ferment different sugars, grow best and most at the temperature of 37^0C

Table 1: Morphological, biochemical and carbohydrate utilization characteristics of isolates

Sample A		Sample B		Sample C				
S/Nos.	Test	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7
A.				Morphological				
1.	Gram staining	+	-	+	+	-	+	+
2.	Shape	Cocci	Cocci	Rod	Coccobacillus	Rod	Rod	Cocci
3.	Pigmentation	Off-white	Cream	Cream	Off-white	Off-white	Off-white	Cream
4.	Arrangement	Clusters	Clusters	Clusters	Clusters	Chains	Clusters	Clusters
5.	Motility	-	-	-	-	-	-	-
B.				Biochemical				
1.	Catalase	-	+	-	-	+	-	-
2.	Citrate	-	-	-	-	-	-	-
3.	Oxidase	-	-	-	-	-	-	-
4.	Urease	-	-	-	-	-	-	-
5.	Indole	-	-	-	-	-	-	-
C.				Carbohydrate				
1.	Glucose	+	ND	+	+	ND	+	+
2.	Fructose	+	ND	+	+	ND	+	+
3.	Galactose	+	ND	+	+	ND	+	+
4.	Maltose	+	ND	+	+	ND	+	+
5.	Lactose	+	ND	+	+	ND	+	+
6.	Sucrose	+	ND	+	+	ND	+	+
7.	H_2S production	-	ND	-	-	ND	-	-
8.	Acid production	+	ND	+	+	ND	+	+
9.	Gas production	-	ND	+	-	ND	-	+
D.	Presumptive organism	*LacC* sp	ND	*LacB* sp	*LacB* sp	ND	*LacB* sp	*LeuC* sp

Key: H_2S = Hydrogen sulphide production + = positive reaction - = negative reaction
ND = not determined *LacC* = *LactococcusLacB* = *Lactobacillus LeuC* = *Leuconostoc*

The growth curve characteristics show that *Lactococcus* sp (strain 1) had the highest average colony count of 121 while *Lactobacillus* sp (Strains 4) had the least average colony count of 53 (Figure 1).

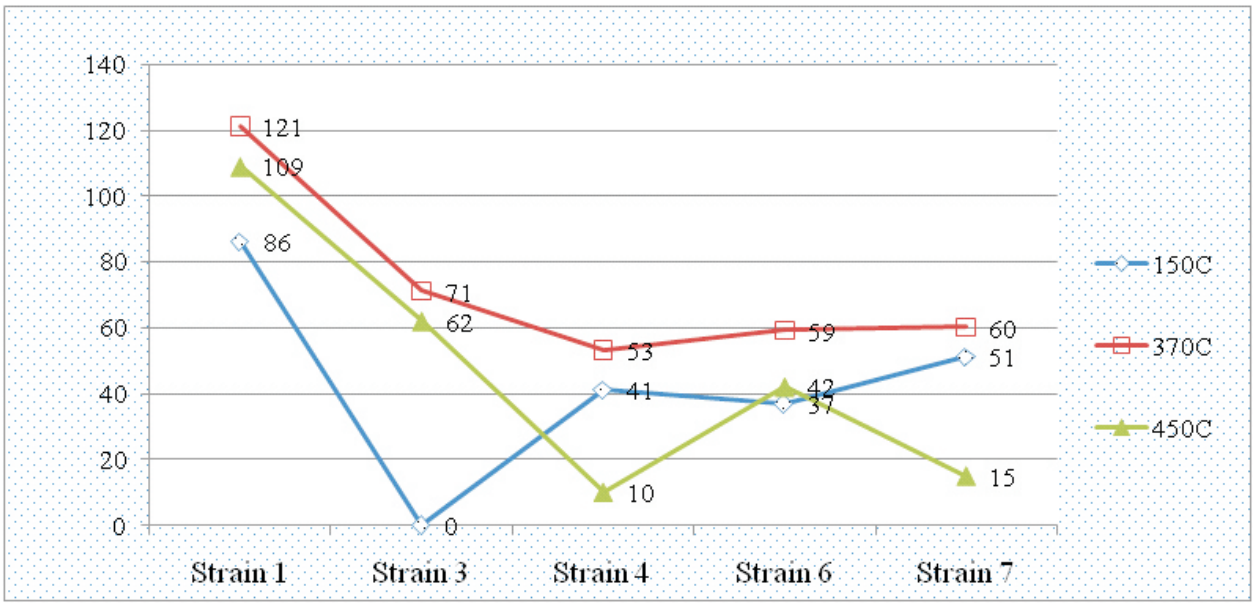

Figure 1: Growth curve of LAB isolates at different temperatures

The highest microbial growth was observed at pH 6. The *Lactoccocus* sp had an average 1.707 colony count while *Leuconostoc* sp had the least colony count of 1.382 (Figure 2).

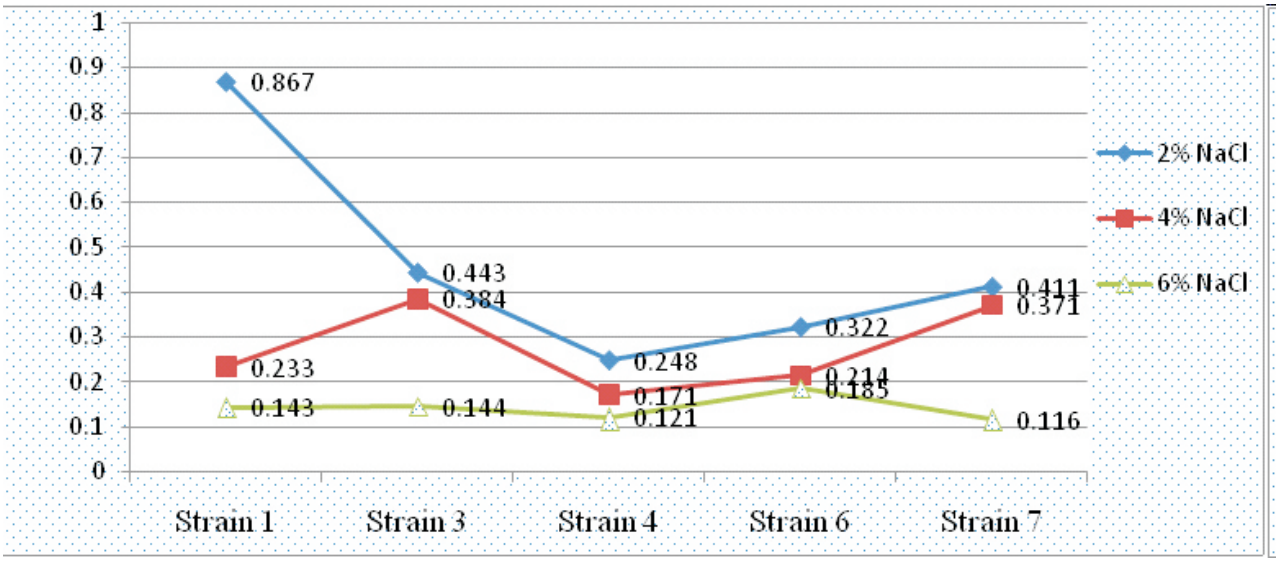

Figure 2: Growth curve of LAB isolates at different NaCl concentrations

As indicated in Figure 3 below, when grown at various sodium chloride (NaCl) concentrations, at 2%, the growth of *Lactococcus* sp was highest (0.867) while that of *Lactobacillus* sp was least (0.248). Strains 4 (*Lactobacillus*) and 6 (*Lactobacillus*) thrived at all temperatures but did not produce gas while strain 7 (*Leuconostoc*) grew more at pH of 6 and was able to produce gas (Figures 1 and 3).

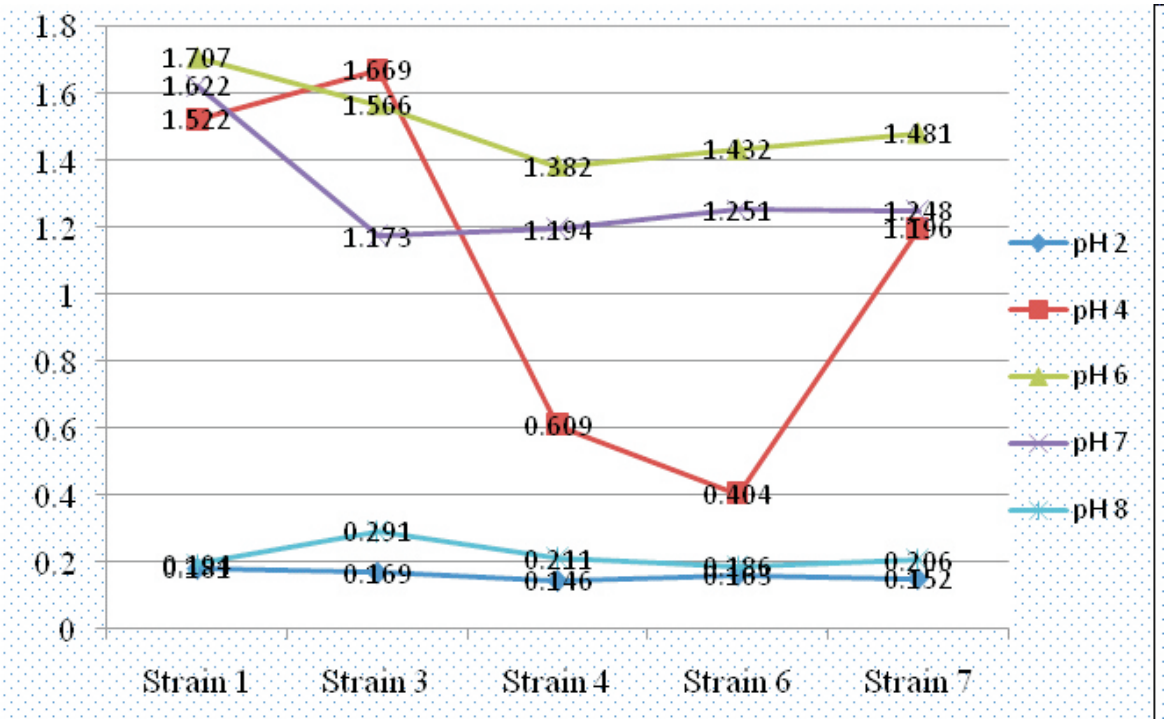

Figure 3: Growth curve of LAB isolates at different pH levels

The molecular basic local alignment search tool (BLAST) confirmed the starter cultures that were presumed to be *Lactococcus lactis* (strain 1) and *Lactobacillus* sp(strain 3), as *Lactococcus lactis* and *Acetobacter tropicalis* respectively with a corresponding percentage identification of 99.95% and 92.27% (Table 2).

Table 2: Molecular identification of bacterial isolates

Name of isolates	Strain 1	Strain 3
Percentage ID	99.95%	92.27%
Predicted organism	*Lactococcus lactis*	*Acetobacter tropicalis*
Gen Bank accession number	NR_113960.1	NR 036881.1

A 48-hour fermentation of soybean curds caused significant increases in moisture, protein and fibre contents and decreases in ash, lipid and carbohydrate contents (Table 3). Decreases in anti-nutrient factors were also recorded (Table 4).

Table 3: Proximate values of fermented and unfermented soybean curds

Starter Culture	Time (hours)	Percentage composition (%) Moisture	Ash	Protein	Lipid	Fiber	Carbohydrate
USB	0	11.64±0.00	3.11±0.00	30.64±0.00	21.78±0.02	4.92±0.04	27.91±0.00
Ll	24	10.43±0.02	2.98±0.02	37.06±0.04	18.11±0.05	5.23±0.02	26.19±0.02
Ll	48	11.88±0.03	1.28±0.04	45.72±0.03	14.78±0.03	6.71±0.00	19.63±0.01
At	24	8.82±0.01	2.92±0.00	34.08±0.01	24.18±0.01	5.06±0.05	24.94±0.02
At	48	12.36±0.05	2.74±0.05	42.34±0.05	26.54±0.02	5.33±0.01	10.69±0.05

Values are mean ± standard deviation of duplicate determinations

Key: Ll = *Lactococcus lactis* At = *Acetobacter tropicalis* USB= Unfermented soybean curd

Table 4: Concentration of anti-nutrient factors in soybean curd samples

Starter culture	Time (h)	Phytate (mg/g)	Lectin (mg/g)	Oxalate (mg/g)	Tannin (mg/g)
USB	0	1.20±0.02	2.32±0.02	0.60±0.04	1.98±0.02
Ll	24	0.07±0.02	0.10±0.00	0.36±0.03	0.86±0.03
Ll	48	0.06±0.02	0.03±0.02	0.10±0.03	0.07±0.02
At	24	0.09±0.01	0.11±0.01	0.38±0.00	0.95±0.04
At	48	0.07±0.00	0.05±0.02	0.16±0.04	0.10±0.02

Values are mean ± standard deviation of duplicate determinations.
Key: Ll = *Lactococcus lactis* At = *Acetobacter tropicalis* USB= Unfermented soybean curd

DISCUSSION

Fermentation can be carried out utilizing starter culture or naturally (Adeyeye et al. 2020). However, though the latter is the most commonly employed method of fermentation in developing countries, it is less effective and unpredictable because of its non-specificity (Abdulkadir et al. 2020). The abilities of strain 1 to grow best at 37^{0}C but not produce gas and strain 3 to grow at 15^{0}C and produce gas classifies them as homofermenters and heterofermenters respectively (Cheesbrough, 2006). These and the results of other preliminary tests presumed strains 1, 3, 4, 6 and 7 to be *Lactococcus*, *Lactobacillus*, *Lactobacillus*, *Lactobacillus* and *Leuconostoc* species respectively (Wassie and Wassie, 2016; Cheesbrough, 2006).

The choice of strains used as starter cultures in the fermentation of soybean curd in this present study, was because they had the highest growth values when cultured at various temperature, pH, and NaCl concentrations. The reduction of anti-nutrients including tannins, phytates, lectins, and oxalates could result from the degradation by microbial enzymes released during fermentation (Adeyeye et al. 2020; Babalola and Giwa 2012).

Increments in protein, moisture and fibre values obtained in this present study as a result of fermentation, supports similar previous observations (Datti et al. 2019; Iheukwumere and Iheukwumere, 2022). Fermentation is known to improve nutritional content of cereals through activation of endogenous enzymes (Nkhata et al. 2018). Therefore, specific rise in protein values may partly be due to the degradation of complex proteins by these organisms thereby releasing peptides and amino acids. Reduction with time in values of fibre and moisture could be attributed to loss of dry matter especially carbohydrates while reduced carbohydrate and ash contents was because these organisms were hydrolyzing and utilizing them as energy sources during the fermentation process (Nkhata et al. 2018).

The reduced quantity of tannin (0.07mg/g) and phytate (0.06mg/g) obtained in this present study after 48 hours of fermentation was significantly different from, and lower than, reduced values of tannin (0.6932mg/g) and phytate (0.8365mg/g) reported byAdeyeye et al. (2020). The depletion of anti-nutrients in fermented samples could be due to the production of β-glucosidase by the fermenting bacteria. This enzyme is highly efficient in degrading cyanogenic glucosides present in these anti-nutrients causing their total removal or reduction (Li et al. 2018). Although both starter cultures were able to reduce the anti-nutrition contents in the fermented soybean curd, *Lactococcus lactis* had a higher fermenting ability than *Acetobacter tropicalis* in soybean curd.

BLAST is used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families (Altschul et al. 1997). The BLAST results used in the identification of the starter culture strains with a percentage ID of almost 100% proved that it is one of the best common tools in bioinformatics that can be employed to examine DNA and protein sequences. The programme is an algorithm that compares primary biological sequence information, such as the amino acid sequences of

proteins or the nucleotides of DNA and/or RNA sequences and calculates the statistical significance of matches.

CONCLUSION

The present findings shows that identifying a good SC, using a genome database search tool such as BLAST and, utilizing such in the fermentation of soybean curds, will result in a better product with reduced anti-nutrient content and improved nutritive value. Though LAB SCs have not been commercially adopted for industrial use in soybean curd fermentation, these present findings, obtained under laboratory settings, suggests that Lactococcus lactis when used as a SC is preferably more efficient and productive than natural fermentation and therefore should widely be used in curd production. The continued consumption of fermented soybean curds is also recommended as a protein dietary supplement to improve health.

COMPETING INTERESTS

The authors declare no conflict of interest.

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