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Studies and Use of Lactobacillus Isolates Obtained from Fermented Soybean Milk as Probiotics

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Abstract: ***Bacteria isolates were obtained from fermented soybean milk and screened for organic acid and antimicrobial substance production. Out of the nine isolates, three designated as ISOA1, ISOA2 and ISOA3 showed a good antimicrobial activity against the target organisms namely, Staphylococcus aureus ATCC 12600, Escherichia coli ATCC 117755, Bacillus cerus, Listeria monocytogens and Escherichia coli 0157 ATCC 438931. This study showed that ISOA1 and ISOA3 were able to tolerate 1.5 pH and salinity upto 5% w/v. But in case of high temperature tolerance, ISOA2 and ISOA3 tolerate upto 45^0C. Bacillus cerus and Listeria monocytogens were resistant to ISOA1 and ISOA2 isolates in all dilution factors while they were sensitive to ISOA3 isolate upto 10^{-2} dilution factor. ISOA1, ISOA2 and ISOA3 were identified as Lactobacillus planetarium, Lactobacillus amylolyticus and Lactobacillus sp and can be use as probiotics.***

Keywords: Antimicrobial activity, Environmental tolerance, Soybean, Probiotics and Organic acid.

1. Introduction

Bacteria, especially lactic acid bacteria play an important role in the fermentation process by rapid acidification of raw materials through the production of organic acids (Leroy and Devuyst, 2004). Suitable characteristic of lactic acid bacteria are their ability to produce antimicrobial compound such as organic acid for growth inhibition of harmful bacteria, their ability to resist high concentration of salt in food, acid and bile salt (Ammor and Mayo, 2007; Dume *et al*., 2001).

Isolation and screening of microorganism from natural occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes (Vijaipal and Kadirvelu, 2005; Coulibaly *et al*., 2008). Some bacteria produce a number of antimicrobial substances like organic acids, hydrogen peroxides, bacteriocins and bacteriocin-like substances (Kim and Worobo, 2000). Bacteriocin, organic acid and other bacteriocin-like substance exhibit inhibitory activities against sensitive strains of bacteria (Jack *et al*., 1995; Montville and Kaiser, 1993).

Bacteria are found in many nutrient environments and occur naturally in various food products such as dairy and meat product and vegetables (Carr *et al*., 2002; Stanton *et al*., 2005). Biopreservation refers to extended shelf life and enhanced safety of foods obtained by using the natural or added microflora and their antimicrobial products (Johan and Magnusson, 2005; Soomro *et al*., 2002). The use of fermentation products in terms of preservation has increased during the centuries and now includes many different kind of food and animals feed (Ross *et al*., 2002).

2. Materials and Methods

a) Soybean seed collection and processing
Soybean seeds were collected from Ogige market Nsukka, Enugu state of Nigeria. The seed were processed and fermented for 7days at 37^0C.

b) Collection of indicator organisms
The indicator organisms as Staphylococcus aureus ATCC 12600, Escherichia coli ATCC 117755, Bacillus cerus, Listeria monocytogens and Escherichia coli ATCC 43893 were obtained from the Department of Microbiology, University of Nigeria, Nsukka.

c) Isolation and screening for organic acid producing isolates from the fermented soybean
The isolation was carried out according to Nakayama and Yanoshi (1967). A 5 ml of fermented soybean was mixed with 100 ml of GYP medium containing 1% glucose (w/v), 1% yeast and 1% peptone. The sample was incubated anaerobically at 370C, after 48hrs of incubation, 100ul of the mixture was cultured onto the GYP agar containing 1% CaCO3. Acid producing bacteria were identified by the clear zone around the colonies. Those bacteria were purified by several subculturing.

d) Screening for antimicrobial activities
Antimicrobial activities to all the indicator organisms were conducted using agar spot test method described by Schillinger and Lucke (1989). Overnight cultures of the isolates were spotted (2uL) onto MRS agar containing 0.2% (w/v) glucose. Plates were incubated anaerobically for 24hrs at 370C with over layer consisting of 100ul indicator strain mixed with 7 mL soft MRS agar containing 0.2% glucose. Also aerobic incubation at 370C for 48hrs was conducted to examine for the antimicrobial activities of the isolates.

e) Determination of the temperature, NaCl and acid tolerant on the isolates

A basal MRS medium was used in these series of studies (De man et al., 1960) without beef extract but with 0.17 g/l bromocresol purple as pH indicator. A lowering of this pH would change the medium from purple to yellow and was used to indicate cell growth because of lactic acid production. The isolates were subjected to various temperature ranges of 20, 25, 30, 37, 45 and 50 0C for incubation for 48hrs. For acid tolerance the isolates were subjected to pH values of 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5. On determining the NaCl tolerant, the isolates were subjected to 1.0, 1.5, 2.5, 5.0, 6.5, 10.0 and 12.0 (%w/v). The basal MRS medium was adjusted with 1 M HCl acid and 1M NaOH. They were incubated at 370C for 48hrs, the colour change and turbidity of each isolate was noted as a simple indication for growth or on growth. Each test was conducted in triplicate.

f) Determination of inhibitory activities of the isolates

The inhibitory activities of the isolates obtained from soybean were determined using agar diffusion method. MRS broth culture of the target organisms was prepared. Five wells were bored on each isolate using a sterile 6mm diameter cork borer to accommodate all the target organisms. A 200 ul of the target organism was added and labeled into each well. The plates were incubated at 370C for 48hrs and the diameter of the clear zone was determined.

g) Isolates identification

The isolates obtained from soybean were identified using morphological, microscopical and biochemical methods of identification.

3. Results and Discussions

Out of nine isolates obtained from fermented soybean only five showed a clear zone with $CaCO_3$ as was shown in **Table 1**. But when subjected to the target organisms, only ISOA1, ISOA2 and ISOA3 were discovered to show positive activity as was shown in **Table 1**. **Figure 1** also showed the plate's clear zone of ISOA1, ISOA2 and ISOA3 showing the organic acid production and inhibitory activities.

Table 1: Screening for organic acid production and antimicrobial activities.

TEST	ISOA1	ISOA2	ISOA3	ISOA4	ISOB1	ISOB2	ISOB3	ISOB4	ISOC1
CaCO3 (1%)	+*	+*	+*	-*	+*	-*	-*	+*	-*
S.aureus ATCC 12600	+	+	+	-	-	-	-	-	-
E.coli ATCC 117755	+	+	+	-	-	-	-	-	-
Bacillus cerus	-	-	+	-	-	-	-	-	-
Listeria monocytogens	-	-	+	-	-	-	-	-	-
E.coli 0157 ATCC 43893	+	-	+	-	-	-	-	-	-

+* = Clear zone, –* = No clear zone, + = Activity, – = No activity

Table 2: Effect of temperature on the isolates

Temp. (^{0}C)	*ISOA1*	*ISOA2*	*ISOA3*
20	-	-	-
25	+	+	-
30	+	+	+
37	+	+	+
45	-	+	+
50	-	-	-

+ = Growth – = No growth

Three of the most potent isolates (ISOA1, ISOA2 and ISOA3) were subjected to temperature variations, were no growth were observed at 20^{0}C and 50^{0}C in all the isolates, but there was growth on all the isolates between temperature 25^{0}C to 45^{0}C, except isolate ISOA3 and ISOA1 which shows no growth at 25^{0}C and 45^{0}C respectively as was shown in **Table 2**.

Table 3: Determination of acidic tolerance on the isolates

Ph	ISOA1	ISOA2	ISOA3
1.0	-	-	-
1.5	+	-	+
2.0	+	-	+
2.5	+	-	+
3.0	+	+	+
3.5	+	+	+
4.0	+	+	+

+ = Growth – = No growth

3.1 Determination of acidic tolerance on ISOA1, ISOA2 and ISOA3

There was no growth at pH1 but ISOA1 and ISOA3 the acidic environment of pH 1.5 upto 4.0. This was in consistent with Suree *et al*., (2012), who reported ph 1.5 as the lowest at which lactic acid bacteria isolated from seafoods were able to grow.

Table 4: Effect of salinity on the isolates

NaCl (% w/v)	ISOA1	ISOA2	ISOA3
1.0	+	+	+
1.5	+	+	+
2.5	+	+	+
5.0	+	-	+
6.5	-	-	+
10.0	-	-	-
12.0	-	-	-

+ = Growth – = No growth

3.2 Determination of salinity tolerant rate on the isolates

The three isolates grew at NaCl concentration of 1 to 2.5% w/v but ISOA3 grew upto 6.5% w/v of NaCl. This made ISOA3 to be the most tolerant isolates followed by ISOA1 that tolerates upto 5% of the same salt as shown in table 4. But none of the isolates were able to grow at 10% and 12% w/v salinity and this might be the same reason why bacterial cells cultivated in a very high salt concentration would

experience a loss of turgor pressure, which would then affect the physiology, enzyme activity, water activity and metabolism of the cells (Kashket, 1987). Yet some cells still overcome as in ISOA3.

Table 5: Determination of inhibitory activity of ISOA1

Target Organisms	Inhibitory zone diameter (mm)			
	Dilutions of the isolate ISOA1			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
S.aureus ATCC 12600	9.5 ± 0.5	8.3 ± 0.3	7.4 ± 0.2	-
E.coli ATCC 117755	11.0 ± 0.6	10.6 ± 0.3	10.2 ± 0.4	9.1 ± 0.5
Bacillus cerus	-	-	-	-
Listeria monocytogens	-	-	-	-
E.coli 0157 ATCC 43893	8.5 ± 0.5	8.1 ± 0.4	7.6 ± 0.2	-

Table 6: Determination of inhibitory activity of ISOA2

Target Organisms	Inhibitory zone diameter (mm)			
	Dilutions of the isolate ISOA2			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
S.aureus ATCC 12600	6.6 ± 0.3	6.2 ± 0.2	-	-
E.coli ATCC 117755	7.3 ± 0.4	6.5 ± 0.2	5.8 ± 0.2	-
Bacillus cerus	-	-	-	-
Listeria monocytogens	-	-	-	-
E.coli 0157 ATCC 43893	7.9 ± 0.3	5.2 ± 0.1	-	-

Table 7: Determination of inhibitory activity of ISOA3

Target Organisms	Inhibitory zone diameter (mm)			
	Dilutions of the isolate ISOA3			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
S.aureus ATCC 12600	9.4 ± 0.5	8.8 ± 0.4	5.6 ± 0.2	-
E.coli ATCC 117755	11.7 ± 0.5	10.6 ± 0.5	10.2 ± 0.4	9.4 ± 0.2
Bacillus cerus	7.8 ± 0.3	7.2 ± 0.3	-	-
Listeria monocytogens	8.5 ± 0.4	8.2 ± 0.3	7.3 ± 0.3	-
E.coli 0157 ATCC 43893	11.8 ± 0.6	11.2 ± 0.5	10.7 ± 0.5	9.5 ± 0.3

– = No inhibition

Figure 1: ISOA1, ISOA2 and ISOA3 showing the inhibitory activities

3.3 Determination of the inhibitory activity of ISOA1, ISOA2 and ISOA3

Table 5 shows the inhibitory activity of different dilution factors of the ISOA1 with the target organisms. ISOA1 showed no inhibitory tendency on *Bacillus cerus* and *Listeria monocytogens* in all dilution factors of ISOA1. And also in **Table 6**, ISOA2 followed the same trend with ISOA1, where *Bacillus cerus* and *Listeria monocytogens* were not inhibited. But ISOA1 inhibits *E. coli* ATCC 117755 upto dilution factor 10^{-4}, attaining 9.1±0.5 as diameter zone of inhibition. In Table 7, all the target organisms were sensitive to ISOA3 upto 10^{-2} dilution factor. ISOA3 seems to have broader spectrum than the other two isolates and are likely to produce more antimicrobial substances than ISOA1 and ISOA2. Eklund (1989) in his work stated that bacteriostasis and death of susceptible bacteria is as a result of antimicrobial substances such as organic acids or/and bacteriocins produced by the probiotic organisms. This attribute is consistent with our work, which makes ISOA3 a better probiotic isolate and making fermented soybean recommendable for consumption. In conclusion the results of this research clearly suggested that fermented soybean was a source of Lactobacillus isolates and can serve as a probiotic.

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