JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

BACTERIA ASSOCIATED WITH NON-ALCOHOLIC FERMENTED BAMBOO SHOOT FOOD PRODUCT

Asifa Qureshi[1], Yogeshwari B. Itankar[1], D.Saikia[2], M.Mandal[2], Hemant. J. Purohit[1]*

Address(es): Dr Asifa Qureshi,
1.Environmental Genomics Division, CSIR-NEERI, Nehru Marg, Nagpur 20, India.
2. Tezpur University, Assam, India.

*Corresponding author: a_qureshi@neeri.res.in

doi: 10.15414/jmbfs.2016.6.1.722-729

ARTICLE INFO

Received 18. 9. 2014
Revised 5. 11. 2015
Accepted 19. 4. 2016
Published 1. 8. 2016

Regular article

OPEN ACCESS

ABSTRACT

Pure bacterial cultures were isolated from fermented products collected from two locations of North East India (Assam and Arunachal Pradesh). Forty four variants were identified by 16S rRNA gene sequencing. The dominant microbial genera found were *Bacillus, Paenibacillus* followed by *Oceanobacillus* and *Lactobacillus* in Assam and *Bacillus, Enterococcus, Lactobacillus* in Arunachal-Pradesh food products. Bacillus isolates showed extracellular enzyme production (amylases, proteases and lipases) as analyzed through plate assay. GC-MS analysis showed beneficial chemical components like organic acids, esters, aminoacids, vitamins in fermented bamboo shoot products. Probiotic attributes of culturable bacterial isolates from bamboo fermentations gives promiscuity for human consumption. Beneficial microorganisms from bamboo shoot fermented food products of North-Eastern region of India were explored and compared using bioinformatics tools.

Keywords: Non-alcoholic fermentation, bamboo shoots, bacteria

INTRODUCTION

Bamboo shoots as food products are rich source of various macro and micro nutrients but needs processing before consumption. Fermentation is one of the processing method which increases the nutritional quality of the bamboo food product in terms of its amino acids, sugars, esters and organic acid contents **(Jeyaram *et al.*, 2008)**. Although fermentation is one of the oldest and most economic methods of food preservation and ethnic people of Sub-Himalayan region of North East India, especially Assam and Andhra Pradesh prepare and consume a variety of domesticated and wild bamboo tender shoots and their fermented products, there is need to increase the probiotic attributes to the food for betterment of health (**Jeyaram *et al.*, 2008, Qureshi *et al.*, 2014**). Some popular fermented bamboo tender shoots of North East India are Mesu of Sikkim, Arunachal Pradesh, Henoop, Khorisa of Assam, Hikung ,Mesu, Bastenga, Hiring of Arunachal Pradesh, Lung-siej of Meghalaya, Soibum, Soidon, Soijm of Manipur (**Tamang *et al.*, 2008**). Henoop, Khorisa, Mesu, Hikung, Bastenga are traditional non-alcoholic fermented food products of Assam and Arunachal Pradesh consumed by Kharbi, Sonowal, Assamese community of Assam and Monpa, Nepali community of Arunachal Pradesh of North-East India (**Das *et al.*, 2012; Tamang *et al.*, 2012**). Fermented food products are not only rich in nutrients such as proteins, vitamins, essential amino acids, sugars, fatty acids but are also good for digestion (**Jeyaram *et al.*, 2008, Das *et al.*, 2012**).
Most of the bacterial species associated with the fermented food do not possess health risk, they are designated as GRAS (generally recognized as safe) organism (**Hansen, 2002, Das *et al.*, 2012**). The objective of our study was to demonstrate culturable microbial diversity of traditionally processed fermented bamboo shoot products of Assam and Arunachal Pradesh, India using molecular approach and designate probiotic attributes of each isolate as safe and healthy food for consumption.

MATERIAL AND METHODS

Total six food samples of fermented bamboo shoot products were collected from different parts of Assam and Arunachal Pradesh in India. Out of six, three samples were from Erdangte, Khowang, North-Lakimpur, Dibrugarh locations of Assam and three samples were from Ziro, Bomdila, Bhalukpung of Arunachal Pradesh. All the samples were collected aseptically into sterile containers and transferred to the laboratory for analysis. . Fermented bamboo shoot products were prepared by defoliating and finely chopping the young edible shoots of the bamboo plant. The small pieces of bamboo shoots were placed tightly into green hollow bamboo stem and were covered with leaves of bamboo. This was then allowed to ferment at room temperature for 7-14 days. These fermented bamboo shoots were extensively used as pickles, curries and as additives in various recipes

Characterisation of fermented bamboo shoot products

Microbiological analysis

Each food product (5gms) was homogenized with 45ml of 1X PBS. It was diluted serially in the same diluents. Appropriate decimal dilution (100µl) of the homogenate was spread over different media plates such as Man, Rogosa and Sharpes (MRS) agar plate (Himedia), nutrient agar, 30 different media plates having different media components and incubated at 30° C for 24-48 hrs. Morphologically different colonies were selected and pure cultured by streaking repeatedly on respective agar media plates. The isolates were grown in respective broth and pure cultures were preserved in 30% glycerol at -80°C (Sanyo, Ultralow deep freezer). The average number of microbes present per gram of different types of samples were also calculated, which was expressed in terms of CFU (colony forming units) per gram of sample (Table 1).

GC-MS based metabolite profiling of fermented bamboo shoot products

Sample preparation was carried out by modifying the method described earlier by various researchers (**Ojinnaka *et al.*, 2013, Lee *et al.*, 2007**). Fermented bamboo shoot products were finely ground in mortar and 500mg of the grinded samples were extracted with 5ml of methanol at 60°C in a heated water bath for 30 min. Samples were cooled to ambient temperature for 30 min and then vortexed for 30-60seconds prior to centrifugation at 3000rpm, at 4ºC for 10 min. Supernatant obtained was passed through the oven-dried anhydrous sodium sulfate. The filtrate obtained were used for GC-MS analysis.
GC-MS analysis (GC Clarus 500 Perkin Elmer system) of a food sample each from Assam and Arunachal-Pradesh was performed employing the following conditions: column Elite-1 fused silica capillary column (30 x 0.25 mm ID composed of 100% Dimethylpolysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml /min and an injection volume of 0.5 µl was employed (split ratio of 10:1) injector temperature 250ºC; ion-source temperature 280ºC. The oven temperature was programmed from 150ºC (isothermal for 2 min), with an increase of 6ºC/min, to 200ºC, then 5ºC/min to 300ºC, ending with a 10 min (isothermal at 300ºC). Mass

spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time of 50min. The GC-MS data was interpreted for identification of various components by comparing their retention time and mass spectra with the known component stored in the database of National Institute Standard and Technology (NIST) library having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained (Table 2).

Total dna extraction from pure bacterial culture

The method used for the total genomic DNA extraction of 176 isolates was the modification of the method of (**Jeyaram *et al.*, 2010; Kapley *et al*., 2000**). A single colony from the agar medium was inoculated to 5ml of respective broth and incubated overnight at 30^o C in respective conditions. Culture volume of 1 OD at 600nm absorbance was measured (UV-1800 Shimadzu UV/Vis Spectrophotometer) and centrifuged at 8000rpm for 10min (Hettich Model: Mikro 220R, Germany). The cell pellet was washed twice with sterile distilled water (Cascada Bio water, Pall life science) and finally resuspended in 100ul of TE buffer (Sigma) with 10µl of lysozyme (1mg/ml) (Sigma). The cell suspension was incubated at 37^o C for 30min in constant temperature water bath (Cyber Lab) with intermediate mixing. It was then allowed to cool at room temperature. 25µl of 0.5 M NaOH was added. It was mixed gently and was incubated at room temperature for 30 min. Then 25µl of Tris pH 7.5 was added to it for neutralization. Finally the 345µl of sterile Milliq water was added to make up the volume to 500 µl. The content was gently mixed and allowed the debris to settle at the bottom at room temperature or at 4^0C. Then the supernatant was carefully pipette out and used for PCR analysis either immediately or can be stored at -20^0 C until required.

The variation in bacterial strains was studied by RAPD (Random Amplified Polymorphic DNA) analysis. Two primers (RAPD 58, RAPD 59) were used after screening several RAPD Primers. A 50 µl reaction mixture consisting of 5 µl of cell free lysate with 50ng DNA, 5 µl of 10X PCR Buffer, 3 µl of 25mM MgCl2, 5 µl of 2.5µM primer(IDT), 2 µl of dNTPs, 0.5 µl of Taq (5U/ µl) (Applied Bioscience).The PCR reaction was carried out in Veriti thermal cycler (Applied Biosystem) through following temperature cycles: temperature profile starts with initial denaturation cycle of 5min at 95^0 C followed by 35 cycles each consisting of denaturation at 94^0C for 1min, annealing at 40^0 C for 1 min, extension at 72^0C for 2minutes with final extension of 72^0C for 10min. followed by cooling to 4^0C. The number of bands and their migration pattern was analyzed by agarose gel (1.2%) electrophoresis. The gels were documented using gel documentation system. The biotypes of isolates were determined based on DNA band profiling. On the basis of RAPD profiles the bacterial strains showing highly similar banding patterns were grouped together and the strains showing different banding patterns were selected for sequencing.

Identification of bacterial bsolates by 16s rDNA sequence

16S rDNA PCR of the selected representative strains of each RAPD group were performed with universal primer 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). PCR was performed using the 50 µl reaction mixture consisting of 50-100ng of crude DNA, 5 µl of 10X PCR buffer,3 µl of 25mM $MgCl_2$, 2 µl of 2.5µM primer (IDT), 2ul of dNTPs, 0.5 µl of Taq (5U/ µl) under the following conditions: 4 min initial denaturation at 95°C; 35 cycles of denaturation (30s at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); a final extension at 72°C for 10 min. PCR product was purified using 1% agarose gel electrophorsis. DNA fragments were manually eluted using QIAquick PCR Purification kit (QIAGEN, Germany) and sent for sequencing using primer 27F. The resulting trimmed sequenced data were compared using BLASTN to those in the NCBI nucleotide Database. The 16S rDNA sequences of representative strains of each RAPD group were aligned using Clustal X Version 2.0. with reference strains sequences obtained from NCBI database for identifying closest strain and phylogenetic relationships (Table 3 and 4).

Enzyme assay and functional diversity analysis

The purified agar medium supplemented with 10% casein and spirit blue agar supplemented with tributyrin (0.3% lipase substrate). Inoculated plates were allow to grow for 24-48 h. the clear zone around the colonies indicate positive results for protease and lipolytic activity respectively. For amylase activity, bacterial cultures were spotted on starch agar plates and incubated for 24-48 h before being flooded with iodine solution. Production of amylase was indicated by the existence of a clear zone around the colonies, while the rest of the plates stained blue-black.

Phylogenetic tree construction

All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search. Evolutionary analyses were conducted in MEGA5.2.2 (**Tamura *et al*., 2011**). Multiple sequence alignments of partial 16S rRNA gene sequences (≥500 bp) were carried out using CLUSTAL W (**Larkin *et al*., 2007; Thompson *et al*., 1997**). Phylogenetic trees were constructed in MEGA5.2.2 software from evolutionary distances obtained using Neighbor Joining and Maximum Composite Likelihood method. The robustness of the phylogeny was tested by bootstrap analysis using 1000 iterations. Accession numbers of all isolates can be viewed in respective phylogenetic tree.

RESULTS AND DISCUSSION:

Characterization of fermented bamboo food samples from assam and arunachal pradesh, India

In the present study, six fermented bamboo shoot products collected from different locations of North-East India, three each of Arunachal Pradesh and Assam were used for exploring the diverse population of bacteria. In each fermented bamboo shoot products the total viable bacterial count were in the range of 10^4 cfu g-1 to 10^5 cfu g-1 of sample (**Table.1**).

Table 1 Table showing total viable bacterial count, pH and water activity from food samples of Assam and Arunachal Pradesh

Location	Local Name	pH	Total viable Bacterial count cfu/g Sample	Water activity (aW)
Assam	Henoop	4.5±0.2	$3.5x10^4$	0.661
	Khorisa 1	3.8±0.1	$3x10^4$	0.595
	Khorisa 2	3.6±0.2	$8x10^4$	0.556
Arunachal Pradesh	Mesu	4.5±0.1	$6.8x10^5$	0.598
	Hikung	4.3±0.1	$2.8x10^5$	0.600
	Bastenga	4.8±0.2	$3x10^5$	0.588

The mean pH of the food sample ranged from 3 to 4.2 with Khorisa2 showing the lowest pH value (Table 1). This indicated that the fermented bamboo shoot products which we analyzed were acid fermented (**Tamang *et al*., 2008**). The water activity (a_w) of the fermented bamboo shoot products were found to be in the range of 0.556 to 0.661. Less water activity signifies less possibility of food-spoilage caused by food poisoning organisms and food-borne pathogens which requires higher water activity (a_w) for their growth (**Oyewole & Isah, 2012**). Protein and carbohydrate content of the fermented bamboo shoot products were found to be less than that of dry bamboo shoot indicating the presence of proteolytic and amylolytic activity during fermentation process. Also carbohydrates and proteins served as nutrients for bacterial biomass growth during fermentation.

Metabolite profiling of fermented bamboo shoots using gc-ms analysis

The fermented bamboo shoot samples of Assam and Arunachal-Pradesh were found to contain organic acids as dominant constituents followed by esters, amino acids and other components (**Table 2**).

Organic acids: The common organic acids in the fermented bamboo shoot products of Assam and Arunachal-Pradesh were acetic acid, hexadecanoic acid and octadecanoic acid found at retention time of 2.75min, 39.9min, and 34.5min respectively. Also butanoic acid, propanoic acids were found common in both food samples. It has been reported that butanoic acid, propanoic acid, tetrabutyl phenyl acetate compounds were determined as major aroma compounds in Korean soy sauces and barley bran sauces (**Lee *et al*., 2006; Choi *et al*., 2007; Steinhaus & Schieberle, 2007**). These compounds were used as flavor and fragrance agents in various food industries; they also provide resistance to food spoilage microorganism and thus help in preservation of food products.

Esters: During fermentation process esterification of alcohols with fatty acids leads to formation of esters. Methyl phenyl ester, linoleic acid ethyl ester, octadecanoic acid methyl ester, palmitic acid vinyl ester, ethyl esters, 2-oxo methyl ester were found to be present in the fermented bamboo shoot products of Assam and Arunachal-Pradesh. These esters are known to contribute in the characteristics pleasant aromatic and sugary flavor to the fermented food samples (**Qin & Ding, 2007; Ojinnaka & Ojimelukwe, 2013**).

Amino acids: Cystine, arginic acid, arginine, alanine, aspartic acid, aspargine, alanine were the free amino acids found in the fermented food samples. The presence of free amino acids contributes to the taste and nutritive quality of the fermented bamboo shoot products.

Other compounds found to be present in the fermented bamboo shoots are D-mannitol, β-carotene, olyl alcohol etc. which increases the nutritional quality of food (**Table 2**). Enzymatic saccharification and production of mannitol, sorbitol, xylitol etc carried out by amylase pro ducing Lactic acid bacteria have been reported in various studies (**Lee *et al*., 2012**).

Table 2 GC-MS analysis of metabolites from fermented bamboo shoot product.

	ASSAM	ARUNACHAL PRADESH
	Metabolites	**Metabolites**
Organic acids	Acetic Acid	Acetic acid
	3-Deoxy-D-Mannonic acid	N-Hexadecanoic acid
	Ethyl Malonic acid	Octadecanoic acid
	N-Hexadecanoic acid	β-carboline-3-carboxylic acid
	Octadecanoic acid	Adenosine-3-phosphoric acid
	3-Methyl Butanoic acid	
	Propanoic acid	
Esters	4-Methyl phenyl ester	4-Methyl phenyl ester
	Linoleic acid ethyl ester	9,12 Octadecanoic acid methyl ester
	9,12 Octadecanoic acid methyl ester	
	Palmitic acid vinyl ester	
	2-Oxo methyl ester	
	ethyl ester	
Amino acids	Aspargine	Arginnic acid
	Alanine	Cystine
	Glycine	
	Aspartic acid	
Other	2-3 Butanediol	Propylene glycol
	2-Amino 3-hydrooxy pyridine	2-3 Butanediol
		β-Carotene
		D-Mannitol

Table 3 Bacteria identified by 16S rDNA sequence analysis from fermented bamboo shoot products of Assam and Arunachal Pradesh

Sample Location	**16S rDNA sequence analysis**			
	Bacterial Isolates	**Accession No.[a]**	**Identification[b]**	**Similarity(%)**
Assam:	HPCAQKh1-12a	KC713926	*Bacillus amyloliquefaciens*	99%
	HPCAQKh2-25b	KC713918	*Bacillus flexus*	99%
	HPCAQKh2-23a	KC713922	*Bacillus flexus*	98%
	HPCAQKh1-4b	KC899351	*Bacillus licheniformis*	99%
	HPCAQH24d	KC713912	*Bacillus sp.*	99%
	HPCAQKh2-8c	KC899350	*Bacillus thuringiensis*	100%
	HPCAQKh2-27b	KC713917	*Brevundimonas sp.*	99%
	HPCAQH3a	KC713915	*Lactobacillus brevis*	99%
	HPCAQKh3-M6	KF574823	*Lactobacillus brevis*	100%
	HPCAQKh1-23c	KC713927	*Lactobacillus plantarum*	100%
	HPCAQKh2-25a	KC713919	*Oceanobacillus oncorhynchi*	99%
	HPCAQKh2-24c	KC713920	*Oceanobacillus sp.*	99%
	HPCAQKh2-24a	KC899349	*Paenibacillus cineris*	99%
	HPCAQKh2-12a	KC713924	*Paenibacillus cineris*	99%
	HPCAQKh2-3a	KC713916	*Paenibacillus favisporus*	99%
	HPCAQKh2-12b	KC713923	*Paenibacillus favisporus*	99%
	HPCAQKh2-11a	KC713925	*Paenibacillus favisporus*	99%
	HPCAQH24b	KC713913	*Paenibacillus sp.*	94%
	HPCAQH23d	KC713914	*Paenibacillus sp.*	96%
	HPCAQH-17a	KC899352	*Pseudomonas aeruginosa*	99%
	HPCAQKh2-23b	KC713921	*Staphylococcus pasteuri*	100%
Arunachal Pradesh:				
	HPCAQM-6d	KC899354	*Bacillus amyloliquefaciens*	100%
	HPCAQM-10b	KC899357	*Bacillus amyloliquefaciens*	100%
	HPCAQHi-2a	KC899363	*Bacillus amyloliquefaciens*	100%
	HPCAQHi-6b	KC899365	*Bacillus amyloliquefaciens*	99%
	HPCAQM-1c	KC899353	*Bacillus licheniformis*	99%
	HPCAQM-23b	KC899362	*Bacillus licheniformis*	99%
	HPCAQM-25d	KC899359	*Bacillus methylotrophicus*	98%
	HPCAQHi-5a	KC899364	*Bacillus methylotrophicus*	99%
	HPCAQM-25a	KC899360	*Bacillus methylotrophicus*	99%
	HPCAQM-8a	KC899355	*Bacillus sp.*	99%
	HPCAQM-5	KF574822	*Bacillus sp.*	99%
	HPCAQHi-7c	KC899366	*Bacillus subtilis*	99%
	HPCAQHi-23b	KC899368	*Bacillus subtilis*	99%
	HPCAQHi-14a	KC899367	*Bacillus tequilensis*	99%
	HPCAQM-8	KF574830	*Enterococcus casseliflavus*	99%
	HPCAQM-13	KF574826	*Enterococcus casseliflavus*	100%
	HPCAQM-14	KF574827	*Enterococcus casseliflavus*	100%
	HPCAQM-15	KF574828	*Enterococcus casseliflavus*	100%
	HPCAQM-1	KF574825	*Lactobacillus brevis*	99%
	HPCAQM-2	KF574829	*Lactobacillus brevis*	99%
	HPCAQM-16	KF574824	*Lactobacillus rhamnosus*	99%
	HPCAQM-24b	KC899361	*Paenibacillus dendritiformis*	99%
	HPCAQM-11b	KC899358	*Staphylococcus pasteuri*	99%

Legend [a] Accession numbers of the sequences obtained after submitting the partial 16s sequences of bacterial isolates to NCBI Genebank . [b] Identification obtained on NCBI BLAST analysis of 16S rDNA sequence of bacterial isolates

Bacterial identification

Based on morphological examination a total of 140 variable bacterial colonies from six fermented bamboo shoot products were isolated at random. Based on the RAPD DNA profiling and phenotypic variations 21 strains from fermented bamboo shoot products of Assam and 23 from Arunachal Pradesh were selected for further study. Total 44 isolates were subjected to 16S rRNA gene sequencing and identified by BLAST analysis. 16S rRNA gene sequencing and BLAST analysis of selected strains revealed that the bacteria present in the fermented food samples of Assam belongs mainly to genera *Bacilli, Lactobacilli, Staphylococcus, Oceanobacilli, Paenibacilli, Brevundimonas* and that of Arunachal Pradesh belongs to *Bacillus, Staphylococcus, Lactobacillus, Enterococcus, Paenibacillus* (**Table. 3**).

It was also observed that all six food samples were predominated by Gram-positive bacteria, with the most abundant phylum being Firmicutes **(Table 4)**. In earlier studies, dominant species associated with fermented bamboo shoots were identified as *Lactobacillus brevis. L Lactobacillus lactis, Lactobacillus fallax, Lactobacillus plantarum* (**Tamang et al., 2008, Qureshi *et al*., 2014**). Our study revealed the presence of *Paenibacillus cineris, Staphylococcus pasteuri, Oceanobacillus oncorhynchi, Paenibacillus favisporus, Brevundimonas sp*. along with *Lactobacillus brevis and Lactobacillus plantarum*. The fermented bamboo shoot products of Assam showed the presence of *Bacillus* and *Paenibacilus* as the predominant bacterial genus followed by *Lactobacillus*. Presence of *Bacilli and Paenibacilli* were also reported in fermented cassava, maize, peanuts (**Namrata *et al*., 2004**). Also reports of presence of *Bacillus licheniformis* being a predominant species found in fermented Cauim are available (**Almeida *et al*., 2007**). Our finding also showed presence of *Bacillus licheniformis* with probiotic attributes (Table 5) in both Assam and Arunachal Pradesh food samples.

Table 4 Taxonomic distribution of culturable bacterial isolates associated with fermented bamboo shoots of Assam and Arunachal Pradesh.

Phylogenetic group	Class	Genus	A(21)	AP(23)	Species	Assam	Arunachal Pradesh
						(strains)	(strains)
Firmicutes	*Bacillus*	*Bacillus*	*6*	*14*	*B. amyloliquefaciens*	*HPCAQKh1-12a*	*HPCAQM-6d*
					B. amyloliquefaciens		*HPCAQM-10b*
					B. amyloliquefaciens		*HPCAQHi-2a*
					B. amyloliquefaciens		*HPCAQHi-6b*
					B. flexus	*HPCAQKh2-25b*	
					B. flexus	*HPCAQKh2-23a*	
					B. licheniformis	*HPCAQKh1-4b*	*HPCAQM-1c*
					B. licheniformis		*HPCAQM-23b*
					B. methylotrophicus		*HPCAQM-25d*
					B. methylotrophicus		*HPCAQM-25a*
					B. methylotrophicus		*HPCAQHi-5a*
					B. sp. (bataviensis)	*HPCAQH24d*	
					B. sp. (tequilensis)		*HPCAQM-8a*
					B. subtilis		*HPCAQHi-7c*
					B. subtilis		*HPCAQHi-23b*
					B. tequilensis		*HPCAQHi-14a*
					B. thuringiensis	*HPCAQKh2-8c*	
					Bacillus sp.		*HPCAQM-5*
		Enterococcus		*4*	*E. casseliflavus*		*HPCAQM-8*
					E. casseliflavus		*HPCAQM-13*
					E. casseliflavus		*HPCAQM-14*
					E. casseliflavus		*HPCAQM-15*
		Lactobacillus	*3*	*3*	*L. brevis*	*HPCAQH3a*	*HPCAQM-1*
					L. brevis	*HPCAQKh3-M6*	*HPCAQM-2*
					L. plantarum	*HPCAQKh1-23c*	
					L.rhamnosus		*HPCAQM-16*
		Oceanobacillus	*2*		*O. oncorhynchi*	*HPCAQKh2-25a*	
					O. sp.	*HPCAQKh2-24c*	
		Paenibacillus	*7*	*1*	*P. cineris*	*HPCAQKh2-24a*	
					P. cineris	*HPCAQKh2-12a*	
					P. dendritiformis		*HPCAQM-24b*
					P. favisporus	*HPCAQKh2-3a*	
					P. favisporus	*HPCAQKh2-12b*	
					P. favisporus	*HPCAQKh2-11a*	
					P. sp.	*HPCAQH24b*	
					P. sp.	*HPCAQH23d*	
		Staphylococcus	*1*	*1*	*S. pasteuri*	*HPCAQKh2-23b*	*HPCAQM-11b*
Proteobacteria	*Alpha Proteobacteria*	*Brevundimonas*	1		*B. sp.*	HPCAQKh2-27b	
	Gamma Proteobateria	*Pseudomonas*	1		*P. aeruginosa*	HPCAQH-17a	

Legend :Number indicates number of isolates assigned to particular genus.
: Number in bracket indicates number of isolates identified from fermented Bamboo shoots of Assam(A) and Arunachal Pradesh(AP)

The presence of *Lactobacilli* in bamboo shoot food samples were responsible for acidic fermentation. *Lactobacillus plantarum*, a known facultative heterofermenter and *Lactobacillus brevis*, an obligate heterofermenter produces acetic acid along with lactic acid, were found to be present in food sample of Assam and Arunachal-Pradesh. The results indicated that these food samples were mixed acid fermented product. Also the pH values of these food samples were in acidic range (Table. 1). The production of both the end products can prevent a food from spoilage and extend the shelf life. Acidic fermentations generally offer cost effective methods of preserving food for people in developing countries, where more sophisticated means of preservation are unaffordable and could not be used.

Screening of isolates for extracellular enzymes by plate assay

Strains of *Bacilli, Staphylococcus, Lactobacilli, Oceanobacilli, Paenibacilli, Brevundimonas,* and *Enterococcus* were screened for their extracellular amylase, protease and lipase activity. A total of 19 isolated strains i.e. 7 from Assam and 12 from Arunachal Pradesh showed their ability to hydrolyze starch on agar plate, 20 isolates were found to show protease activity and 17 isolates showed lipase activity.

Among isolated strains of Assam, *Brevundimonas sp. HPCAQKh2-27b, Paenibacillus favisporus HPCAQKh2-12b, Lactobacillus plantarum HPCAQKh1-23c,* and *Bacillus amyloliquefacian HPCAQKh1-12a* showed highest amylolytic activity and from Arunachal Pradesh, *Bacillus licheniformis HPCAQM-1c, Bacillus amyloliquefaciens HPCAQM-10b,* showed highest amylolytic activity (**Table. 5**). Amylolytic strains of bacterial genera *Lactobacillus plantarum*, *Bacillus licheniformis* were also isolated from Brazilian fermented food (**Almeida *et al*., 2007**) and Nigerian fermented food (**Johansson *et al*., 1995; Sanni 2002**). The amylolytic activity was responsible for hydrolysis of starch during Bamboo shoot fermentation.

Table 5 Amylase, Protease and Lipase activity of bacterial species isolated from fermented bamboo shoot products of Assam and Arunachal Pradesh.

Location	*Bacterial Isolates*	Amylase	Protease	Lipase
Assam	*Bacillus amyloliquefaciens HPCAQKh1-12a*	++	-	-
	Bacillus flexus HPCAQKh2-23a	-	+	-
	Bacillus flexus HPCAQKh2-25b	-	+	-
	Bacillus thuringiensis HPCAQKh2-8c	+	+++	+
	Brevundimonas sp. HPCAQKh2-27b	++	-	+
	Lactobacillus brevis HPCAQH3a	-	++	+
	Lactobacillus plantarum HPCAQKh1-23c	++	+	-
	Paenibacillus cineris HPCAQKh2-12a	+	-	-
	Paenibacillus favisporus HPCAQKh2-12b	++	++	++
	Paenibacillus favisporus HPCAQKh2-3a	+	-	-
	Pseudomonas aeruginosa HPCAQH-17a	-	-	+
Arunachal Pradesh				
	Bacillus amyloliquefaciens HPCAQHi-6b	++	++	+
	Bacillus amyloliquefaciens HPCAQHi-2a	++	+++	+
	Bacillus amyloliquefaciens HPCAQM-1-b	+++	+++	++
	Bacillus amyloliquefaciens HPCAQM-6d	++	+++	+
	Bacillus licheniformis HPCAQM-1c	+++	++	-
	Bacillus licheniformis HPCAQM-23b	+	+	+
	Bacillus methylotrophicus HPCAQHi-5a	++	+++	+
	Bacillus methylotrophicus HPCAQM-25a	+	+	+
	Bacillus methylotrophicus HPCAQM-25d	-	++	-
	Bacillus sp. HPCAQM-8a	++	++	+
	Bacillus subtilis HPCAQHi-23b	+	++	+
	Bacillus subtilis HPCAQHi-7c	++	++	+
	Bacillus tequilensis HPCAQHi-14a	++	+++	+
	Enterococcus casseliflavus HPCAQM13	-	-	+
	Staphylococcus pasteuri HPCAQM-2b	-	+	-

Legend: + ─ zone of clearance ≤ 10mm
++ ─ zone of clearance >10mm ≤ 15mm
+++ ─ zone of clearance > 15mm

Proteolytic enzyme assay revealed that there was appreciable difference in protease activity among the strains identified. The protease secreting ability of genus *Bacillus* have been well known (**Nascimento & Martin 2004; Beg and Gupta 2003**) and we found total six strains showing highest protease activity indicated by a zone of clearance of more than 150mm. In general *Lactobacilli* showed low protease activity. Low protease activity of *Lactobacilli* have been reported in fish fermentation also by Namrata et al., 2004. Reports showing both amylase and protease activity of *Lactobacilli* was also available (**Thapa *et al*., 2006**).

Lipolytic activity was found comparatively less than the amylase and protease activity. Total 17 isolated strains showed liypolytic activity. *Paenibacillus favisporus HPCAQKh2-12b* strain showed highest lipolytic activity (zone of clearance more than 100mm).

Amylolytic, proteolytic and lipolytic properties of fermenting microorganism may be important for degradation of starch (which determines the availability of free sugars), proteins(which determines the availability of essential amino acids) and lipids (which determine the availability of short-chain fatty acids in particular). These properties may have considerable effects on the taste and flavor of fermented food products. Strains isolated from Arunachal Pradesh showed comparatively higher enzyme activity than that of Assam although both the regions belong to North-East India (**Table. 5**).

Bacteria isolated in this study were found to possess extracellular enzyme activity (amylases, proteases, lipases) which were responsible for the breakdown of raw organic molecules (polymers) resulting into accumulation of certain byproducts of smaller molecular weights, which may improve the organoleptic and nutritional quality of food products.

Phylogenetic analysis

Phylogenetic tree based on 16s rRNA gene sequences placed the 21 isolates of Assam into 2 major groups viz; Firmicutes and Proteobacteria and Arunachal Pradesh into single group Firmicutes. The major cluster Firmicutes were further divided into smaller sub-clusters of *Paenibacilli, Bacilli, Oceanobacilli, Staphylococcus, Lactobacilli*. The genus *Bacillus* (Firmicutes) represented the major group in food samples of both the location (**Fig 1, Fig 2**). In Assam sample dominant population was represented by the genus *Paenibacillus* followed by the genus *Bacilli* and then by *Lactobacilli* and *Oceanobacilli* while in Arunachal Pradesh food samples *Bacillus* was the only genus found to be dominant.

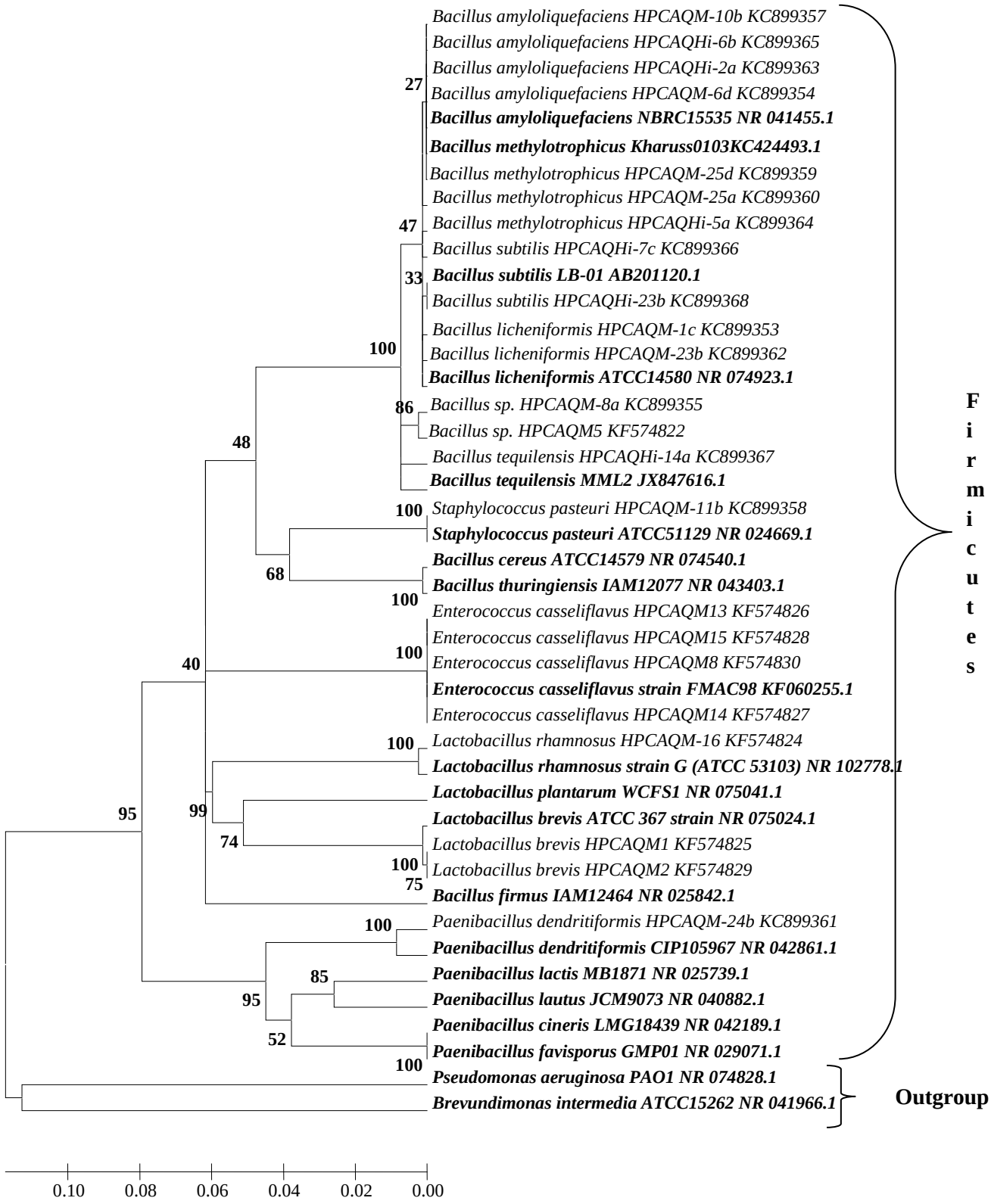

Figure 1 Phylogenetic relationship of Arunachal-Pradesh isolates: based on the partial 16S rDNA sequences of representative isolates generated using the Neighbor-Joining method in MEGA5. The reference sequences were obtained from NCBI database are indicated in bold

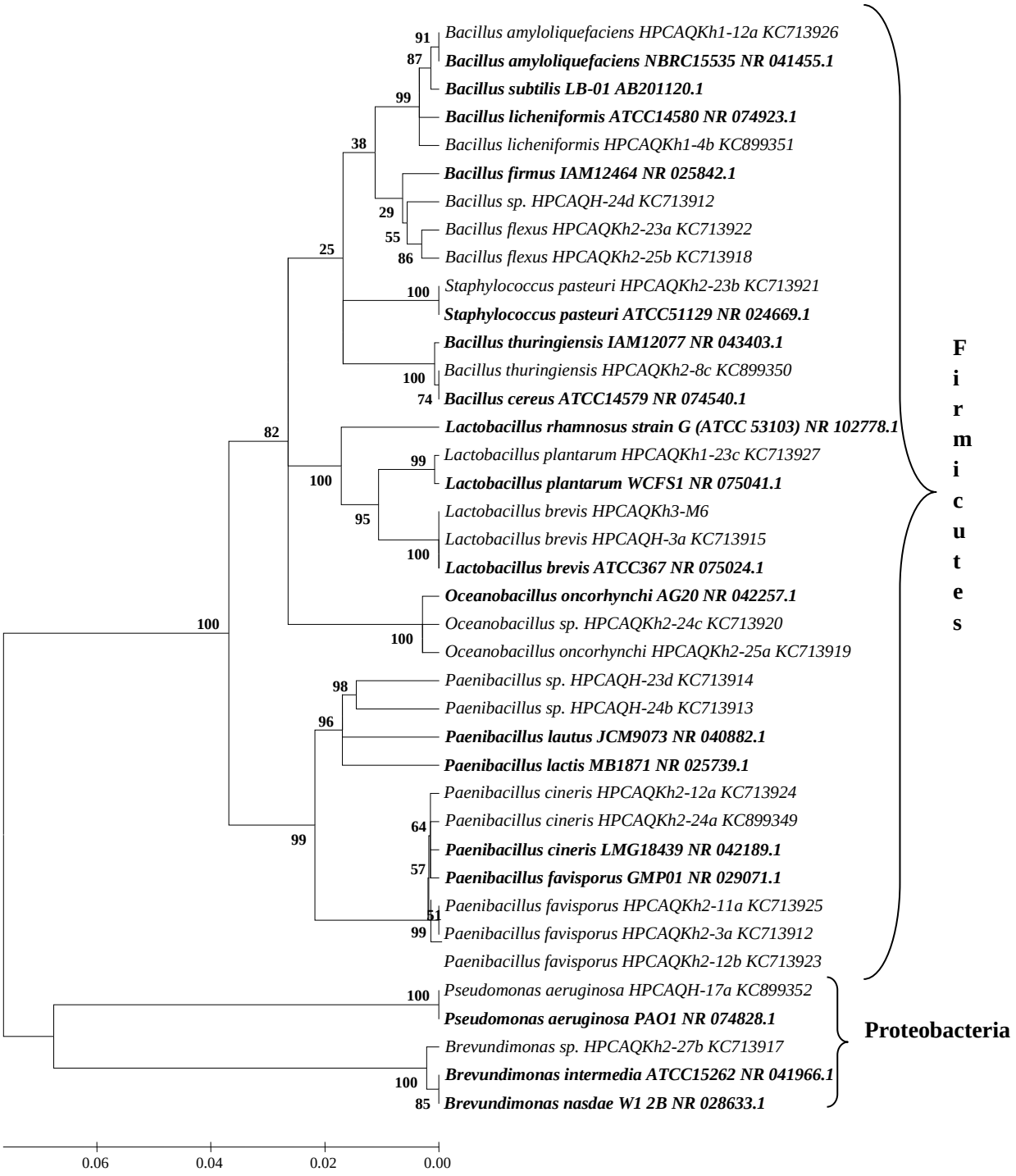

Figure 2 Phylogenetic relationship of Assam isolates: based on the partial 16S rDNA sequences of representative isolates generated using the Neighbor-Joining method in MEGA5.2.2. The reference sequences were obtained from NCBI database are indicated in bold.

CONCLUSION

The culturable bacterial diversity of non-alcoholic bamboo shoot fermented food products of North East India (Assam and Arunachal Pradesh) by using molecular tool like 16S rDNA sequencing were envisaged. The dominant microbial genera found in Arunachal-Pradesh food products were *Bacillus*, *Paenibacillus*, *Oceanobacillus* and *Lactobacillus*. However in Assam *Enterococcus* were present additionally. Mainly *Bacillus* species from both the locations showed extracellular enzyme activity (amylases, proteases and lipases) which added to the probiotic attribute of the food along with the beneficial metabolites like esters and amino acids. The results infer to provide idea for formulating the functional starter cultures for large scale bamboo fermentations for food industries.

Acknowledgement: Authors of the manuscript thank Director, CSIR-NEERI (Council of Scientific and Industrial Research - National Environmental Engineering Research Institute), Nagpur, India for providing the required facilities for carrying out this work and DBT (Department of Biotechnology) for funding under DBT's Twinning Program for North Eastern Regions (NER) of India.

REFERENCES

ALMEIDA, E.G., RACHID, C.C., SCHWAN, R.F. 2007. Microbial population present in fermented beverage 'cauim' produced by Brazilian Amerindians. *International Journal of Food Microbiology*, 120, 146-51. http://dx. doi.org/ 10.1016/j.ijfoodmicro.2007.06.020

BEG, Q.K., & GUPTA, R., 2003. Purification and characterization of an oxidation-stable, tiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme and Microbial Technology, 32*, 294–304

CHOI, U. K., KIM, M. H., KWON, O. J., LEE, T. J., & LEE, N. H. 2007. Characterization of aroma components in barley bran sauce using statistical analysis. *Food Science Biotechnoogy*, 16, 23-28.

CHOUDHURY, D., SAHU, J.K., SHARMA, G.D. 2012. Bamboo shoot: Microbiology, Biochemistry and Technology of fermentation - a review. *Indian Journal of Traditional Knowledge*, 11, 242-249.

DAS, A. J. AND DEKA, S. C. 2012. MiniReview: Fermented foods and beverages of the North-East India. *International Food Research Journal*, 19 Issue 2, 377.

HANSEN, E. B. 2002. Commercial bacterial starter cultures for fermented foods of the future. *International Journal of Food Microbiology*, 78, 119-131..

JEYARAM, K., MOHENDRO SINGH, W., PREMARANI, T., DEVI, A.R., CHANU, K.S., TALUKDAR, N.C., SINGH, M.R. 2008. Molecular identification of dominant microflora associated with 'Hawaijar' - a traditional fermented soybean (Glycine max (L.)) food of Manipur, *India. International Journal of Food Microbiology*, 122, 259-68.

JEYARAM, K., ROMI, W., TH. ANAND SINGH, RANJITA DEVI, A., SONI DEVI, A. 2010. Bacterial species associated with traditional starter cultures used for fermented bamboo shoot production in Manipur state of India. *International Journal of Food Microbiology*, 143, 1-8. http://dx. doi.org/ 10.1016/j.ijfoodmicro.2010.07.008

JOHANSSON, M.L., SANNI, A., LONNER, C., MOLIN,G. 1995. Phenotypically-based taxonomy using API 50 CH of Lactobacilli from Nigerian Ogi, and the occurrence of starch fermenting strains. *International Journal of Food Microbiology*, *25*, 159–168. http://dx.doi.org/10.1016/0168-1605(94)00096-O

KAPLEY, A., LAMPEL, K., PUROHIT, H.J., 2000. Development of duplex PCR for Salmonella and Vibrio. *World Journal of Microbiology Biotechnology*, 16, 457– 458. http://dx.doi.org/ 10.1023/A:1008924119825

LARKIN, M.A., BLACKSHIELDS, G., BROWN, N.P., CHENNA, R., MCGETTIGAN, P.A., MCWILLIAM, H., VALENTIN, F., WALLACE, I.M., WILM, A., LOPEZ, R., THOMPSON, J.D., GIBSON, T.J. AND HIGGINS, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics, 23* (21): 2947-2948.

LEE, S. M., SEO, B. C., & KIM, Y. S. 2006. Volatile compounds in fermented and acid-hydrolysed soy sauces. *J. Food Sci., 71*, C146-C156. http://dx. doi.org/ 10.1111/j.1365-2621.2006.tb15610.x

LEE, YUN, S.,, KIM, H. Y., LEE, S., LEE, J. M., MUTHAIYA, M. J., KIM, B. S., OH, J. Y., SONG C. K., JEON, E. J., RYU, H. S., LEE, C. H. 2012. Mass Spectrometry-Based Metabolite Profiling and Bacterial Diversity Characterization of Korean Traditional Meju During Fermentation. *J. Microbiol. Biotechnol., 22(11)*, 1523–1531. http://dx.doi.org/10.4014/jmb.1207.07003

NAMRATA, T., PAL, J., TAMANG, J. P. 2004. Microbial Diversity in Ngari, Hentak and Tungtap, Fermented Fish Products of North-East India. *World Journal of Microbiology and Biotechnology, Volume 20*, Number 6, 599-607(9). http://dx. doi.org/ 10.1023/B:WIBI.0000043171.91027.7e

NASCIMENTO, W.C.A., MARTINS, M.L.L. 2004. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Brazilian Journal of Microbiology*, 35, 91–96. ISSN 1517-8382

OJINNAKA M.T. C., OJIMELUKWE, P. C. 2013. Study of the Volatile Compounds and Amino Acid Profile in *Bacillus* Fermented Castor Oil Bean Condiment. *Journal of Food Research, 2*, 1. doi:10.5539/jfr.v2n1p191. http://dx.doi.org/10.5539/jfr.v2n1p19

OYEWOLE, O.A. AND ISAH, P. 2012. Locally Fermented Foods in Nigeria and their Significance to National Economy: a Review. *J Rec Adv Agri*, 1(4): 92-102.

QIN, L., & DING, X. 2007. Formation of taste and odour compounds during preparation of *douchiba*, a Chinese traditional soy-fermented appetizer. *J. Food Biochem.*, 31, 230-251. http://dx. doi.org/ 10.1111/j.1745-4514.2007.00105.x

QURESHI, **A.**, ITANKAR. Y., OJHA. R., MANDAL, M., KHARDENAVIS, A., KAPLEY. A., PUROHIT. H.J. 2014. Genome Sequence of Probiotic *Lactobacillus plantarum* EGD-AQ4, *Genome Announcement*, January/February, 2, Issue 1 e01122.13

SANNI, A.A.I., MORLON-GUYOT, J., GUYOT, J.P. 2002. New efficient amylase-producing strains of *Lactobacillus plantarum* and *L. fermentum* isolated from different Nigerian traditional fermented foods. *International Journal of Food Microbiology, 72*, 53–62.

STEINHAUS, P., & SCHIEBERLE, P. 2007. Characterization of the key aroma compounds in soy sauce using approaches of molecular sensory science. *J. Agric. Food Chem.*, 55, 6262-6269.

TAMANG, B., TAMANG , J. P., SCHILLINGER, U., FRANZ, C.M.A.P., GORES, M., HOLZAPFEL, W. H. 2008. Phenotypic and genotypic identification of lactic acid bacteria isolated from ethnic fermented bamboo tender shoots of North East India. *International Journal of Food Microbiology*, 15; 121, 35-40.

TAMANG, J. P., TAMANG, N., THAPA, S., DEWAN, S., TAMANG, B. YONZAN, H., RAI, A. K., CHETTRI, R., CHAKRABARTY, J., & KHAREL, N. 2012. Microorganisms and Nutritional value of Ethnic fermented foods and alcoholic beverages of North East India. *Indian Journal of Traditional Knowledge*, 11, 1.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M., AND KUMAR, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods, *Molecular Biology and Evolution* 28: 2731-2739. http://dx. doi.org/ 10.1093/molbev/msr121.

THAPA, N., PAL, J., TAMANG, J.P. 2006. Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. *International Journal of Food Microbiology*, 107, pp. 33–38. http://dx. doi.org/ 10.1016/j.ijfoodmicro.2005.08.009

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F., HIGGINS, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25, 4876-4882.