20-105

Consolidated report for R&D Project

November 2011 to May 2015

Project Title

Study of the microbial diversity and biochemical characteristics of the selected non-alcoholic fermented (milk, vegetable and pulses) food product of Assam and Arunachal Pradesh

DBT Sanction Order No. & Date

BT/219/NE/TBP/2011, dated November 21, 2011

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File. Acknowledgement 10/3/16 Dean R&D(I/c)

Section A: Project Details:

A1. Project Title**:** "Study of the microbial diversity and biochemical characteristics of the selected non- alcoholic fermented (milk, vegetable and pulses) food product of Assam and Arunachal Pradesh"

A2. DBT Sanction Order No. & Date: BT/219/NE/TBP/2011, dated November 21, 2011.

A3. Name of Principal Investigator: Dr. M. Mandal

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A6. Total cost: Rs. 54.71 Lakhs (Rupees fifty four lakhs and seventy one thousand only)

A7. Duration: 3 years.

A8. Approved objectives of the Project:

- Documentation and collection of selected fermented food products and their starter culture from Assam and Arunachal Pradesh.
- Building database for commonly used fermented food products of Assam and Arunachal Pradesh.
- Making of database on bacterial species found in the selected fermented food products of Assam and Arunachal Pradesh.
- Isolation and identification of prominent/ dominant bacterial species from the fermented food products and starter culture.
- Molecular identification of the isolated species (Will be done in NEERI, Nagpur).

- Biochemical characterization of food.
- Screening of probiotic bacterial strains from the isolated strains.
- Formulation of standard starter culture for food fermentation.
- Incorporation of probiotic microorganism in the starter culture without affecting the quality of fermented food.
- Preservation of isolated strains for future use.

A9. Scientific recommendations made by task force (If any): No

Section B: Scientific and technical Progress

B1. Progress made against the approved objectives, Targets and timelines during the reporting period

Timeline:

Period of study	Achievable targets
6 Months	Procurement of instruments, consumable etc. Documentation and collection of fermented food samples from different parts of Assam and Arunachal Pradesh.
12 Months	Collection of sample, Isolation and purification of bacterial strains from different fermented foods. Analysis of proximate composition of fermented food. Identification of bacterial strains. Also identification of unculturable population from metagenome. Making of database for fermented foods
18 Months	Checking the production of extracellular enzymes by the isolated strains. Identification and biochemical characterization of fermented foods. Identification of bacterial strains and unculturable population from metagenome. Making of database for fermented foods and bacterial species available in the food samples.
24 Months	Screening of potential probiotic bacterial strains. Studying the microbial dynamics in the fermented food in laboratory condition. Sensory evaluations of fermented foods after laboratory scale preparation. Preparation of phylogenetic tree from the sequenced microbial genes.
30 Months	Optimization of fermentation process for different food products. Preparation of standard starter culture,
36 Months	Preservation of isolated strains, Writing of final report

Objective 1:

Documentation and collection of selected fermented food products and their starter culture from Assam and Arunachal Pradesh and building database.

1. Material and methods:

1.1 Sample collection:

Survey was done in the selected rural areas covering the states of Assam and Arunachal Pradesh. After thorough discussion with the local people who prepares the fermented foods process of fermentation was documented. Fermented food sample collection from the accessible parts of Assam and Arunachal Pradesh which was carried out since first year of the project throughout the project period. The indigenous methods of preparation of different types of samples and their use, sample age etc. were also documented. Table 1 illustrates different varieties of food items collected and their methods of preparations are explained by the figure 1.

1.2 Building database of fermented food:

For making the fermented food samples known to everyone, an online database was generated using the online cloud system OneDrive (https://onedrive.live.com/). It is a file hosting service that allows users to sync files and later access them from a web browser or mobile device. Users can share files publicly or with their contacts, publicly shared files do not require a Microsoft account to access. It is part of the suite of online services formerly known as Windows Live.

2. Results:

2.1 Different types of fermented food samples collected from different parts of Assam and Arunachal Pradesh are tabulated (Table 1) and their methods of preparation (as documented) are represented diagrammatically (Fig. 1).

sl no	Sample	Local name	Method of preparation	Use	Date of collection	Place	Community	Individual name	Sample age
1	Curd (Cow)	Doi	Milk kept in bamboo tubes and kept 3-4 days for fermentation by closing the mouth of the tube.	Daily consumption	05-05-2012	Tezpur	Assamese	Bazaar	7 days
2	Curd (Bufallo)	Doi			07-06-2012	Tezpur	Assamese	Bazaar	5 days
3	Curd (Cow)	Doi			27-05-2012	Kacharihat, Golaghat	Assamese	Arup Dutta	5 days
4	curd (Bufallo)	Doi			25-06-2012	Nelli, Morigaon	Tiwa	bazaar	4 days
5	Curd (cow)	Doi			14-07-2012	Khowang, Dibrugarh	Ahom	Sabitri Gogoi	4 days
6	Fermented bamboo shoot	Hennop	Young bamboo shoots cut into small pieces and kept in containers or bamboo tubes submerged in salt water (~1% wt/v) for 7-14 days.	As a side-dish	02-06-2012	Erdangte, Karbi Anglong	Karbi	Bidya Singh Teron	30 days
7	Fermented bamboo shoot	Khorisa	Young bamboo shoots grinded and kept in containers for 7 days and then mixed with salt and mustard oil (optional).	As a side-dish	02-06-2012	Nagaon, Assam	Assamese	Bazaar	20 days
8	Fermented bamboo shoot	Khorisa	Young bamboo shoots cut into small pieces and kept in containers or bamboo tubes submerged in water for 7-14 days.	As a side-dish	03-06-2012	North Lakhimpur, Assam	Assamese	B. Hazarika	6 months
9	Fermented bamboo shoot (Dry)	Xukan khorisa	Young bamboo shoots cut into small pieces and kept over fireplace for drying for about 7 days.	As a flavouring agent in food	03-06-2012	North Lakhimpur, Assam	Assamese	B. Hazarika	1 year

sl no	Sample	Local name	Method of preparation	Use	Date of collection	Place	Community	Individual name	Sample age
10	Fermented bamboo shoot	Danglong	Young bamboo shoots cut into small pieces and kept in containers or bamboo tubes submerged in water for 7-14 days.	As a flavouring agent in food	25-06-2012	Komarkuchi Gaon, Morigaon, Assam	Tiwa	Rina Patar	30 days
11	Fermented bamboo shoot (7 days old)	Khorisa	Young bamboo shoots cut into small pieces and kept in containers or bamboo tubes submerged in water.	As a flavouring agent in food	14-07-2012	Bhorali Gaon, Dibrugarh, Assam	Sonowal	Bina Bora	7 days
12	Fermented bamboo shoot (30 days old)	Khorisa	Young bamboo shoots cut into small pieces and kept in containers or bamboo tubes submerged in water for 10- 15 days.	As a flavouring agent in food	14-07-2012	Bhorali Gaon, Dibrugarh, Assam	Sonowal	Himanti Bora	30 days
13	Bamboo shoot	Ekung	Young bamboo shoot cut into small pieces and kept in containers with little water. The container is made airtight with leaves.	As a food ingredient	26-10-2012	Lichi, Arunachal Pradesh	Nishi	Taba Tegir	2 months
14	Dried bamboo shoot	Eup	Bamboo dried and pressed under stone packed in leafs of plants (banana etc.) . Local name of the preferred bamboo plant- Aye	As a food ingredient	26-10-2012	Lichi, Arunachal Pradesh	Nishi	Taba Tegir	3 months
15	Bamboo shoot	Hirring	Middle coverings of bamboo shoot are removed, covered with banana leaves and pressed under stones.	cooked with meat	27-10-2012	Ziro, Arunachal Pradesh	Nishi	Gyati Oniya	1 month
16	fermented bamboo shoot	Bastenga	Young bamboo shoot cut into small pieces and kept in containers with little water.	As a side-dish	18-08-2012	Bomdila, Arunachal Pradesh	Monpa	Tashi Chotten	2 months

sl no	Sample	Local name	Method of preparation	Use	Date of collection	Place	Community	Individual name	Sample age
17	Fermented bamboo shoot	Mesu	Young bamboo shoot cut into small pieces and kept in containers with little water.	As a side-dish	19-08-2012	Bhalukpung, Arunachal Pradesh	Nepali	S. Gurung	1 year
18	Fermented mastard	Kharoli	Black mustard ground into powder and mixed with khar Indigenous soda made from banana peels) in equal proportion and wrapped with banana leaves and kept over fireplace for fermentation.	As a side-dish	02-06-2012	Nagaon, Assam	Assamese	bazaar	7 days
19	Fermented mastard	Kharoli		As a side-dish	02-12-2012	Solmari, Nagaon, Assam	Assamese	bazaar	4 days
20	Fermented milk cheese	Churpi	Milk is churned in a large air- tight wooden vessel, steered until cheese is formed which is seperated using bamboo sieve by slow dripping of water. Then it is sun-dried and kept near fireplace putting inside Yak Calf's skin	In the preparation of chutney, vegetarian and non-vegetarian dishes, traditional chocolates	19-08-2012	Bomdila, Arunachal Pradesh	Aka	Phunsto Shongla	1 year
21	Fermented milk cheese	Churpi			18-08-2012	Bomdila, Arunachal Pradesh	Monpa	Thohe Pau	1 year
22	Fermented milk cheese	Churkham			18-08-2012	Bomdila, Arunachal Pradesh	Monpa	Tashi Chotten	
23	Fermented milk cheese	Churpi			19-08-2012	Thembang, Bomdila, Arunachal Pradesh	Monpa	Tashi Norbu	1 month
24	Fermented milk cheese	Churpi (cow milk)			18-08-2012	Sessa, Arunachal Pradesh	Nepali	Kesang Gurung	6 months

sl no	Sample	Local name	Method of preparation	Use	Date of collection	Place	Community	Individual name	Sample age
25	Fermented soybean	Kinema	Soyabeans washed, boiled till soft, excess water is drained off and boiled beans are kept in bamboo baskets lined with ginger leaves and kept near fireplace for fermentation for 3-5 days.	As a side dish with rice	18-08-2012	Sessa, Arunachal Pradesh	Nepali	Kesang Gurung	6 months
26	Fermented soybean	Libi churpi		As a side dish with rice	18-08-2012	Bomdila, Arunachal Pradesh	Monpa	Thohe Pau	4 months
27	Fermented soybean	Peruyan		As a side dish with rice	27-10-2012	Ziro, Arunachal Pradesh	Apatani	Mudan Tagin	2 month
28	Fermented lemon	Nemu	Mature lemons are at first sundried and then kept in containers filled with salt. It is usable for few years.	As a side-dish, as a medicine for stomach upset.	13-06-2012	Kekorapool, Tezpur, Assam	Assamese	Pratul Dihingia	1 year
29	Fermented mango	Aamor achar	Green mango cut into small pieces and mixed with spices before sundry. Then the sundried pieces are kept in mustard oil for about 7 days.	As a side dish.	06-08-2012	Napaam, Tezpur	Assamese	Suren Das	6 months

Young bamboo shoot

⇩

Outer layer peeled off

⇩

Washed with water and chopped in to small pieces

⇩

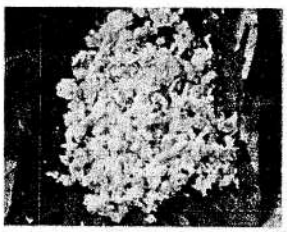
Pressed in bamboo tubes making air- tight

⇩

Fermented for 7-12 days

⇩

Mesu



Tender shoots cut in to smaller pieces

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Kept for fermentation in bamboo tubes submerged in water for 7-14 days

⇩

Henoop

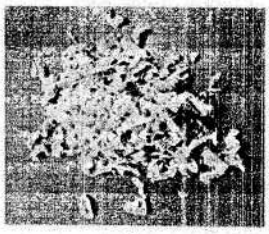
Tender bamboo shoots grinded and dried

⇩

Pressed under stone

⇩

Eup



Topmost tender portions of bamboo shoots are cut longitudinally and flattened by crushing

⇩

Put into bamboo baskets lined with leaves

⇩

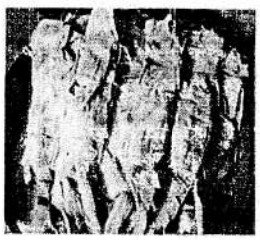
Baskets put into pits, sealed and weighed down with heavy stones

⇩

Fermented for 2-3 months

⇩

Hirring



Black mustard (*Brassica nigra*) seeds are ground

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Mixed with *kolakhar*, which is the water extract of burnt banana peel or rhizome

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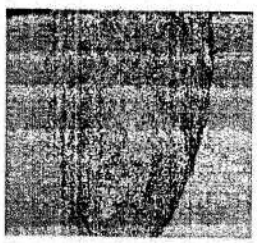
Wrapped with banana leaves and kept over fireplace for fermentation for 5- 7 days

⇩

Kharoli



Soyabean seeds are boiled and spread on bamboo mats

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After draining water kept in bamboo container covered with banana leaves

⇩

Kept near fireplace with proper rotation

⇩

When smells come out and seeds becomes thread like taken out, crushed and sun dried in the form of small balls

⇩

Libi churpi

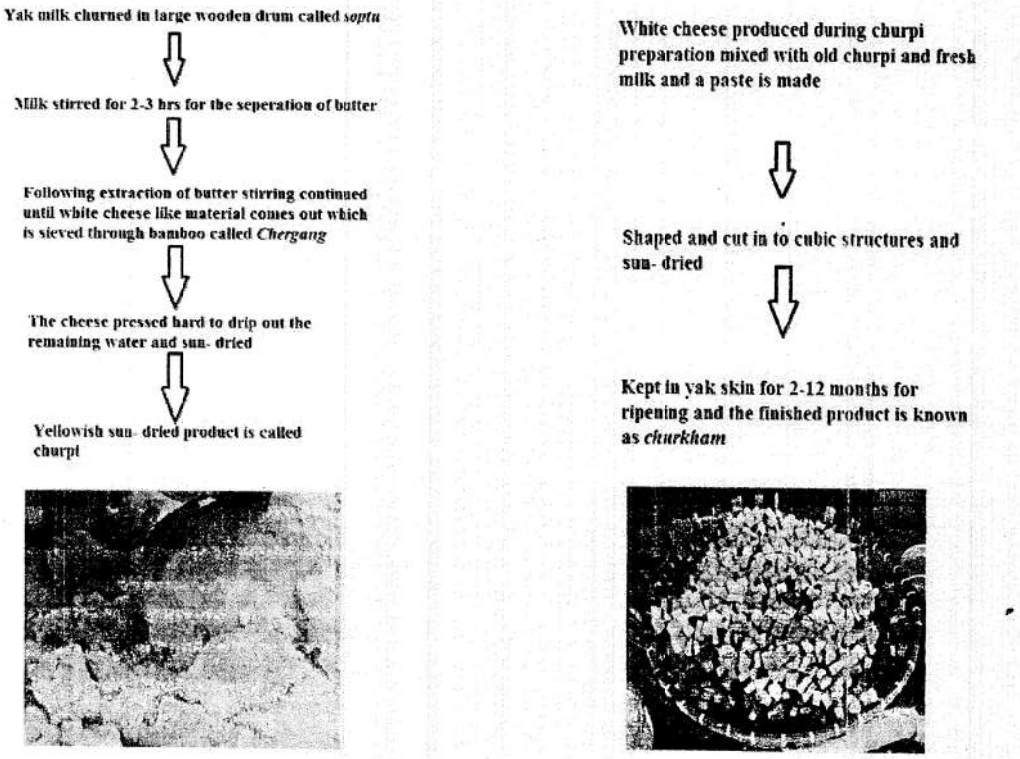


Fig 1: Schematic diagram of different fermented foods and their method of preparation

2.2 Preparation of fermented food database:

The list of different types of fermented foods and their method of preparation can be available online in the link https://onedrive.live.com/redir?resid=72472D928E2C24B4%21115 or http://1drv.ms/1OIGdA1.

Objective 2:

Isolation of prominent/dominant microbiota from fermented food and their identification:

Materials and methods:

1. Isolation of microorganisms:

The adequate amount of sample (1gm) was homogenised with 9 ml of 0.85% normal saline. The sample is diluted serially in normal saline and plated on selective media such as Rogosa and Sharpe (MRS) agar plate, nutrient agar, yeast and mould agar, plate count agar etc. In case of milk products Ringer salt solution was used for serial dilution. After proper incubation the isolated microbes were grouped according to colony morphology, gram staining and other characteristics.

In NEERI, Fermented mustard seed samples and seven samples of fermented bamboo shoot products from different locations of Assam and Arunachal Pradesh (Appendix 1) were analysed for microbiological loads and their pH value were measured using pH strips. Morphological examination showed variable colonies on different HK media plates (Appendix 2). Based on morphological variants total 377 bacterial strains were isolated as pure culture, data shown in Table 2.

Table 2: Microbial analysis done at NEERI

Sr.No.	Location	Fermented Food Sample	Local Name	pH	Total No. of Bacterial Isolates(Strains) pure cultured	Bacterial count cfu/g Sample
1	Assam	Fermented Mustard Seeds	Kharoli	6.5	205	$5x10^8$
2	Assam	Fermented Bamboo shoots	Henoop	4	21	$3x10^5$
3			Khorisa 1	3	25	$3x10^4$
4			Khorisa 2	3	20	$8x10^4$
5			Khorisa 3	4	25	$3.5x10^4$
6	Arunachal Pradesh	Fermented Bamboo shoots	Mesu	4	30	$6.8x10^5$
7			Hikung	4	31	$2.8x10^5$
8			Bastenga	4	20	$3x10^5$
			Total		377	

2. Identification of microorganisms:

3.1 Biochemical characterization:

The biochemical studies were carried out by following the standard procedure as described by Bergey's manual. The biochemical tests performed according to standard procedure are catalase activity, gas production from glucose, motility test, Indole production, citrate utilization, methyl red test, Voges- Proskauer test, oxidase test, starch hydrolysis, nitrate reduction, casein hydrolysis, utilization of different carbohydrate such as glucose, lactose, galactose, raffinose etc.

3.2 Molecular characterization:

The isolated strains were identified by 16S rDNA gene sequencing followed by phylogenetic tree construction. Universal primers analysis followed by phylogenetic studies. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′) were used for the amplification of 16S rRNA gene sequence (Guo et al., 2010). The amplified PCR product was purified and subjected to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Rotkreuz, Switzerland). The sequence was analyzed using BLAST algorithm (http:// www.ncbi.nlm.nih.gov/blast) and was submitted to the NCBI GenBank (http:// www.ncbi.nlm.nih.gov/genbank). The phylogenetic tree was constructed by neighbor- joining (NJ) method using MEGA 5.05 software (Felsenstein, 1985; Kimura, 1980; Tamura et al., 2011).

Results:

1. Isolation of microorganisms:

Different microbial isolates obtained from the fermented foods are given in the table 3.

Table 3:

sl no	Samples	Total viable count		Isolated strains	Colony morphology				Gram characteristic
		Bacterial count (CFU/gram)	Fungal count (CFU/gram)		Form	Elevation	Margin	Colour	
1	Curd (Cow)	$2.47x10^8$	0	AMD8	circular	Raised	Entire	Transluscent	+ bacillus
				AMD17	circular	Raised	Entire	White	+ coccus
				AMD20	circular	Convex	Entire	Yellow	+ coccus
2	Curd (Bufallo)	$128x10^7$	0	AMDKD1	circular	Flat	Undulate	Pale white	+coccus
				AMDKD2	circular	Raised	Entire	White	+ bacillus
				AMDKD16	circular	Flat	Entire	White	+ bacillus
				AMDKD19	circular	Flat	Entire	Pale yellow	-bacillus
3	Curd (Cow)	$94x10^6$	0	GC1	circular	Raised	Entire	White	+ bacillus
				GC2	Irregular	Raised	Entire	White	+coccus
				GC3	circular	Raised	Entire	Yellow	+ bacillus
				GC4	circular	Convex	Entire	Pale white	+coccus
4	curd (Bufallo)	$354x10^7$	0	NC1	circular	Crateriform	Undulate	Off- white	- bacillus
				NC2	circular	Convex	Entire	Pale yellow	+ bacillus
				NC3	Irregular	Flat	Entire	White	+coccus
				NC4	Irregular	Raised	Undulate	Pale white	+ bacillus
				NC5	circular	Convex	Entire	White	+ coccus
				NC6	circular	Raised	Entire	Pale yellow	+ bacillus
5	Curd (cow)	$495x10^7$	$9x10^5$	DC1	circular	Umbonate	Entire	White	+ bacillus
				DC2	circular	Raised	Entire	Light yellow	+ bacillus
				DC3	circular	Convex	Entire	Transluscent	-bacillus
				DC4	Irregular	Raised	Entire	White	Yeast- like
				DC5	circular	Crateriform	Entire	White	+ bacillus
6	Fermented bamboo shoot	$94x10^6$	0	DS1	Irregular	Umbonate	Entire	Off-white	+ coccus
				DS2	Irregular	Raised	Entire	White	+ bacillus
				DS3	circular	Convex	Entire	White	- bacillus
				DS4	circular	Raised	Entire	Yellow	+ bacillus
				DS5	circular	Convex	Entire	Yellow white	+coccus
				DS6	circular	Convex	Entire		- bacillus
7	Fermented bamboo shoot	$118x10^6$	0	NB1	Irregular	Flat	Umbonate	Pale white	+coccus
				NB2	Round	Raised	Entire	White	+ bacillus
				NB3	Round	Convex	Entire	Yellow	+ coccus
8	Fermented bamboo shoot	$120x10^7$	0	LB1	circular	Raised	Entire	White	+ bacillus
				LB2	circular	Flat	Undulate	Transluscent	+ bacillus
				LB3	Irregular	Convex	Irregular	Pale white	+ coccus
9	Fermented bamboo shoot (Dry)	$29x10^7$	$5x10^5$	NL1	Irregular	Flat	Undulate	Yellow	+ coccus
				NL2	Irregular	Flat	Filiform	White	Yeast- like
				NL3	circular	Raised	Entire	Pale white	+ bacillus
				NL4	Irregular	Convex	Undulate	White	+coccus
10	Fermented bamboo shoot	$212x10^7$	$30x10^5$	TB1	circular	Raised	Entire	Off- white	+ bacillus
				TB2	circular	Convex	Entire	White	-bacillus
				TB3	circular	Flat	Entire	off- white	+ bacillus
				TB4	Irregular	Flat	Undulate	Off- white	+coccus
				TB5	circular	Raised	Entire	White	Yeast- like
11	Fermented bamboo shoot (7 days old)	$71x10^6$	0	SK1	circular	Flat	Entire	Shiny white	- bacillus
				SK2	circular	Raised	Entire	White	+ bacillus
				SK3	circular	Flat	Entire	Transluscent	+coccus
				SK4	circular	Flat	Entire	White	+ bacillus

12	Fermented bamboo shoot (30 days old)	$50x10^7$	$2x10^5$	D1 D2 D3 D5 D6	circular circular Irregular circular circular	Umbonate Raised Flat Raised Raised	Entire Entire Entire Undulate Entire	White White White Off- white Transluscent	+ bacillus - bacillus Yeast- like + bacillus +coccus
13	Fermented Bamboo shoot	$159x10^7$	0	EKZ11 EKZ12 EKZ13	circular circular Irregular	Flat Raised Raised	Entire Entire Entire	White White Pale yellow	+ bacillus + bacillus +coccus
14	Dried bamboo shoot	$27x10^5$	$59x10^6$	EUZ11 EUZ12 EUZ13	circular circular Irregular	Raised Flat Convex	Entire Undulate Entire	cream White Grey	+ coccus + bacillus Yeast- like
15	Fermented Bamboo shoot	$214x10^7$	0	HZ1 HZ2 HZ3	circular circular circular	Raised Convex Flat	Entire Entire Entire	Off- white Pale yellow White	+ bacillus + bacillus +coccus
16	fermented bamboo shoot	$120x10^7$	$22x10^4$	BMB1 BMB2 BMB3 BMB4 BMB5	circular circular Irregular circular circular	Raised Raised Flat Raised Raised	Filiform Entire Undulate Entire Entire	Pale white yellow White White Transluscent	yeast-like + bacillus Yeast- like + bacillus - bacillus

17	Fermented bamboo shoot	$150x10^7$	0	VB1 VB2 VB3	Irregular circular circular	Flat Raised Raised	Entire Entire Undulate	Off white White Off- white	+ bacillus +coccus + bacillus
18	Fermented mustard	$113x10^5$	$25x10^5$	NK1 NK2 NK3 NK4	circular Irregular circular circular	Raised Flat Raised Raised	Undulate Entire Entire Entire	Pale white White White Pale yellow	+ bacillus Yeast- like + coccus + bacillus
19	Fermented mustard	$179x10^6$	$11x10^5$	DK1 DK2 DK3	circular Irregular circular	Raised Flat Raised	Entire Entire Undulate	yellow White White	- bacillus Yeast- like + bacillus
20	Fermented milk cheese (Churpi)	$457x10^7$	$17x10^5$	AMD1 AMD2 AMD3 AMD5 AMD6	circular Irregular circular circular circular	Convex Raised Raised Raised Raised	Filiform Entire Entire Entire Entire	White White cream White White	yeast-like + bacillus + coccus + bacillus + bacillus
21	Fermented milk cheese (Churpi)	$324x10^7$	$13x10^5$	CH251 CH252 CH253 CH254 CH255 CH256 CH257 CH258 CH259	circular circular circular circular Irregular Irregular circular Irregular circular	Raised Flat Raised Raised Flat Raised Raised Flat convex	Entire Entire Filiform Entire Undulate Entire Entire Filiform Entire	Pale white yellow White White Transluscent Shiny white Yellow Cream Pale yellow	- bacillus + bacillus yeast-like + coccus + coccus - bacillus + bacillus yeast-like +coccus
22	Fermented milk cheese, hard (Churkham)	$429x10^7$	$7x10^5$	CK51 CK52 CK53 CK54 CK55 CK56 CK57	circular circular circular Irregular circular circular circular	Raised Flat Raised Flat Convex Raised Convex	Entire Filiform Entire Filiform Entire Crateriform Entire	White White Yellow White Transluscent Yellow Pale white	+ cocci yeast-like + bacillus yeast-like + bacillus - bacillus + bacillus
23	Fermented milk cheese (Churpi)	$523x10^6$	0	CH301 CH302 CH303 CH304	Irregular circular circular Irregular	Raised Raised Convex Raised	Undulate Entire Entire Undulate	White Orange Yellow Pale white	+ bacillus + bacillus + bacillus - bacillus
24	Fermented milk cheese (Churpi)	$245x10^7$	0	ASM1 ASM2 ASM3 ASM6	circular circular circular circular	Elivated Raised Raised Convex	Entire Umbonate Crateriform Crateriform	Pale orange Pale white Yellow Pale white	- bacillus + coccus + bacillus + coccus
25	Fermented soyabean	$295x10^7$	0	LB1 LB2 LB3	circular Irregular circular	Raised Convex Flat	Undulate Entire Entire	cream White Grey	- bacillus + bacillus + bacillus
26	Fermented soyabean	$178x10^7$	$34x10^5$	FSM1 FSM2 FSM3 FSM4	circular circular Irregular Irregular	Convex Raised Raised Raised	Entire Entire Entire Filiform	White Pale white yellow White	+ bacillus +coccus + coccus Yeast- like
27	Fermented soyabean	$245x10^7$	0	AMS1 AMS2 AMS3 AMS4 AMS5 AMS6	Irregular Irregular Irregular Irregular Circular Irregular	Flat Flat Flat Flat Raised Flat	Entire Entire Entire Entire Entire Entire	Pale white. pale White White White White White	+ bacillus + bacillus +coccus + bacillus + bacillus +bacillus
28	Fermented lemon	$129x10^6$	$5x10^5$	TL1 TL2 TL3 TL4	circular circular circular Irregular	Flat Crateriform Raised Flat	Entire Undulate Entire Entire	Light orange White white White	- bacillus + bacillus - bacillus Yeast- like
29	Fermented mango	$95x10^6$	0	MT1 MT2 MT3 MT4 MT5	circular circular Irregular circular circular	Flat Raised Raised Flat Raised	Undulate Irregular Entire Undulate Enire	Yellow White White White Pale white	+ bacillus + coccus - bacillus - bacillus + bacillus

Maximum bacterial count (CFU/gm) was found in fermented soya bean ($3.09x10^9$), whereas maximum mould could was found in case of bamboo shoot ($5.9x10^7$). This is depicted in the fig 2.

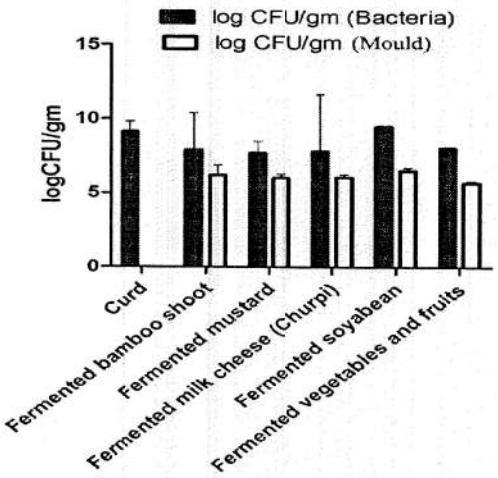


Fig2: Average microbial count (log CFU/ml) in different fermented food.

2. Identification of microorganisms:

2.1 Biochemical characterization:

On the basis of different biochemical tests, different isolates from different types of fermented food sample are presumptively identified since it is difficult to accurately identify individual strains based on utilization of different substrates. Presumptive identification implies that different fermented food based on different substrates differ in the microbial composition. For instance, fermented milk products predominantly contain *Lactobacillus* sp. (67.18%) whereas it was found that fermented bamboo and cereal products were found to be dominated by *Bacillus* sp. (Fig: 3).

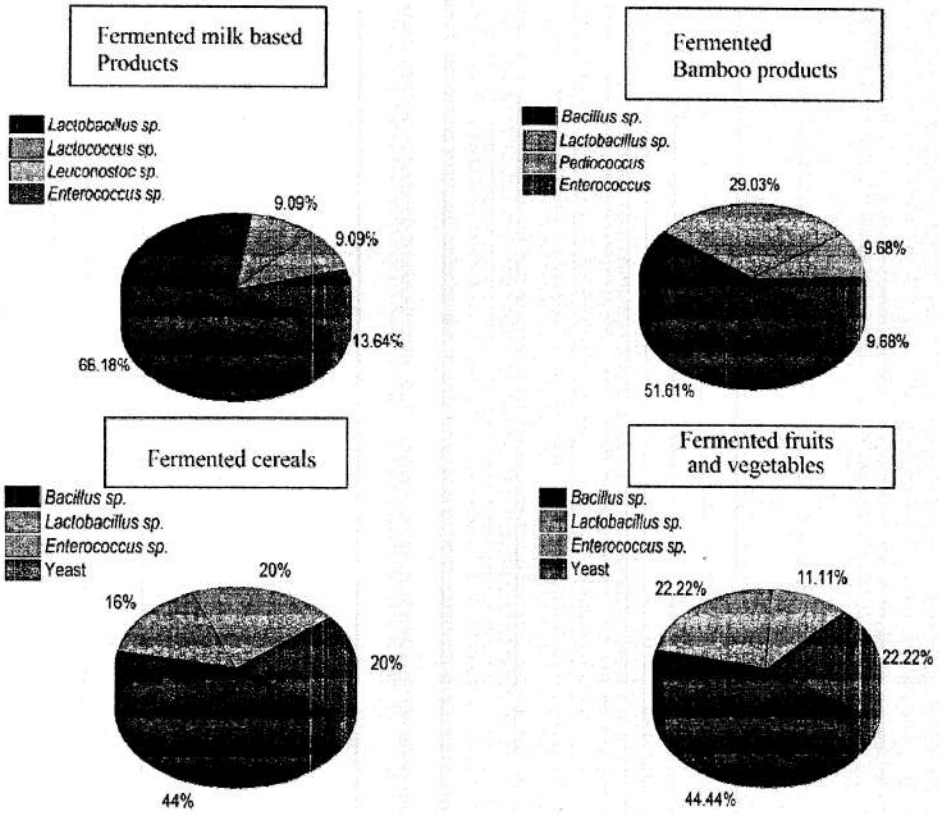


Fig 3: Microbial composition of different fermented food based on biochemical characteristics.

2.2 Molecular characterization:

RAPD Analysis:

The genotypic variation in bacterial strains was studied by RAPD analysis on the basis of different banding patterns on Agarose gel electrophoresis (Fig. 4). The isolates are shortlisted for their 16S rRNA gene PCR amplification (Fig. 5). The amplified products were sequenced and the respective isolates were identified. Sequencing results shown in Table 4.

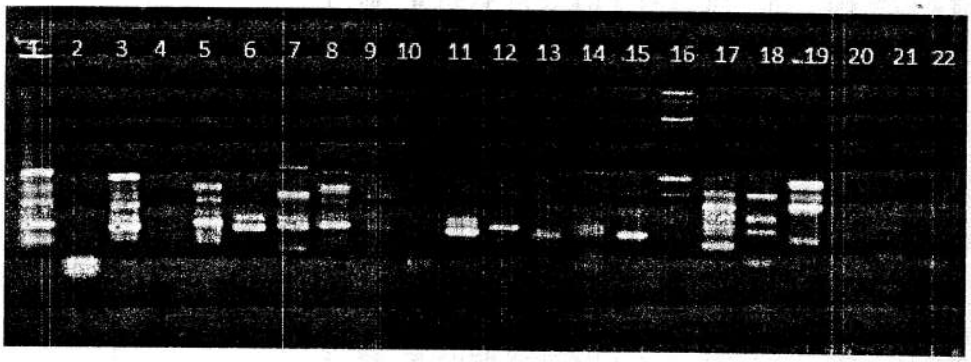


Fig 4. Agarose Gel image representing RAPD Pattern of different isolates as pure cultures from food samples

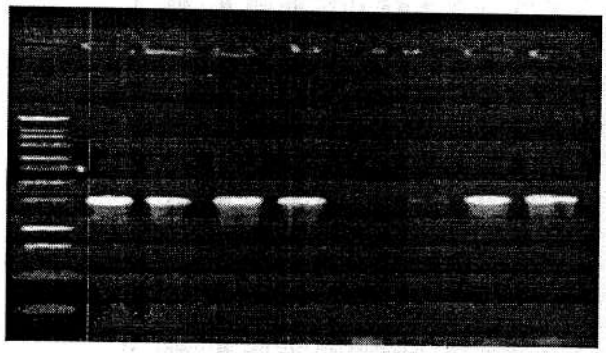


Fig 5. Agarose Gel image representing 16S r DNA PCR Amplified products (were eluted and sequenced)

Table 4. Molecular Identification of bacteria isolated from fermented Mustard seeds and Bamboo Shoots done at NEERI:

Sr. No.	Isolates	Blast identity	% Similarity	Accession No.
1	C 25iii	*Staphylococcus fleurettii* HPCAQC-25c	99%	KC713928
2	K3-7iv	*Staphylococcus succinus* HPCAQK3-7d	100%	KC713929
3	K3-11iii	*Staphylococcus sp.* HPCAQK3-11c	99%	KC713930
4	K3-13ii	*Staphylococcus vitulinus strain* HPCAQK3-13b	99%	KC713931
5	H-24iv	*Bacillus sp.* HPCAQH24d	99%	KC713912
6	H-24ii	*Paenibacillus sp.* HPCAQH24b	94%	KC713913
7	H-23iv	*Paenibacillus sp* HPCAQH23d.	96%	KC713914
8	H-3i	*Lactobacillus brevis* HPCAQH3a	99%	KC713915
9	kh2-3i	*Paenibacillus favisporus* HPCAQKh2-3a	99%	KC713916
10	kh2-27ii	*Brevundimonas sp.* HPCAQKh2-27b	99%	KC713917
11	kh2-25ii	*Bacillus flexus* HPCAQKh2-25b	99%	KC713918
12	kh2-25i	*Oceanobacillus oncorhynchi* HPCAQKh2-25a	99%	KC713919
13	kh2-24iii	*Oceanobacillus sp.* HPCAQKh2-24c	99%	KC713920
14	kh2-23ii	*Staphylococcus pasteuri* HPCAQKh2-23b	100%	KC713921
15	kh2-23i	*Bacillus flexus* HPCAQKh2-23a	98%	KC713922
16	kh2-12ii	*Paenibacillus favisporus* HPCAQKh2-12b	99%	KC713923
17	kh2-12i	*Paenibacillus cineris* HPCAQKh2-12a	99%	KC713924
18	kh2-11i	*Paenibacillus favisporus* HPCAQKh2-11a	99%	KC713925
19	Kh1-12i	*Bacillus amyloliquefacien* HPCAQKh1-12a	100%	KC713926
20	Kh1-23iii	*Lactobacillus Plantarum* HPCAQKh1-23c	100%	KC713927

NCBI Blasts results obtained after sequencing the 16S rDNA PCR products of selected bacterial isolates (showing enzymatic activity) from Mustard seeds and Bamboo shoots showed that *Staphylococcus* species were dominant in mustard seeds samples. Diverse strains of bacteria viz; *Bacillus, Oceanobacilli, Lactobacilli, Paenibacilli* were present in Bamboo shoots sample.

The bacteria obtained from non-alcoholic fermented food samples mainly belonged to the genera *Bacillus, Oceanobacilli, Lactobacilli, Paenibacilli, Staphylococcus* etc.

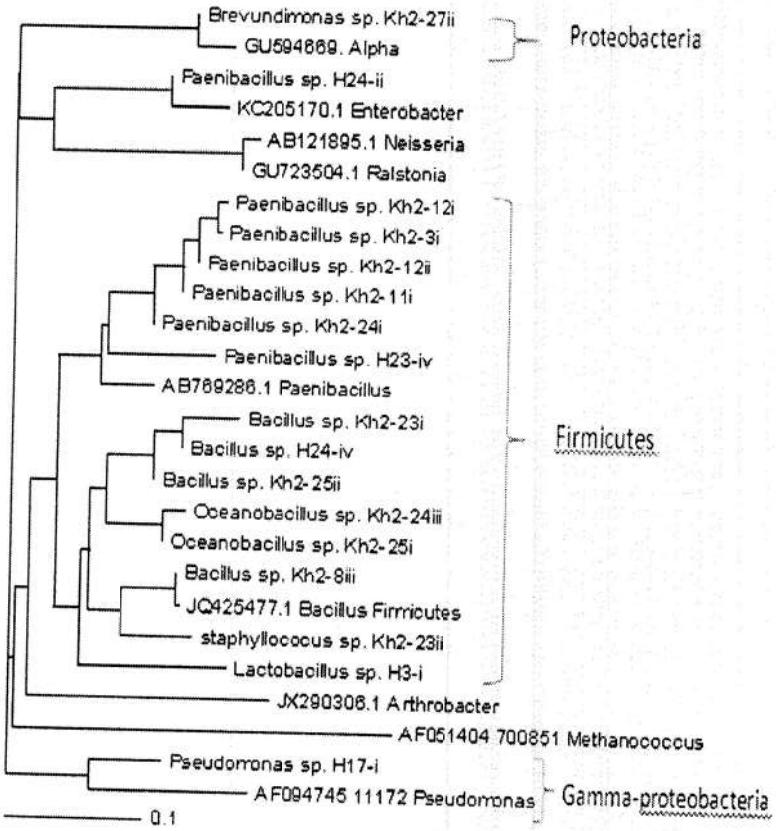


Fig 6. Phylogenetic Tree of Bacterial Isolates of fermented Bamboo Shoot products.

In Tezpur University, a total 16 strains have been sequenced and are submitted to the GenBank (Table 5 :)

Table 5: Strains identified at Tezpur University and their accession no.s:

Strain	BLAST Identity	GenBank Accession No.
AMDKD16	*Lactobacillus paracasei*	KC759402
AMD21	*Enterococcus faecalis*	KC617924
AMD20	*Leuconostoc mesenteroides subsp. mesenteroides*	KC617923
AMD17	*Lactococcus lactis*	KF113841
AMS5	*Enterococcus faecalis*	KJ162395
AMS3	*Bacillus altitudinis*	KJ162394
AMS2	*Bacillus amyloliquefaciens*	KJ162393
AMS1	*Bacillus amyloliquefaciens*	KJ162392
AMDKD19	*Enterobacter cloacae*	KC759403
DS1	*Pediococcus pentosaceus*	KP723364
D6	*Lactobacillus paracasei*	KJ867173
AMS6	*Bacillus subtilis*	KP723361
AMD6	*Lactobacillus plantarum*	KJ867175
AMD5	*Lactobacillus paracasei*	KJ867174
AMD3	*Leuconostoc mesenteroides*	KJ867171
ASM6	*Kocuria rhizophila*	KJ909534

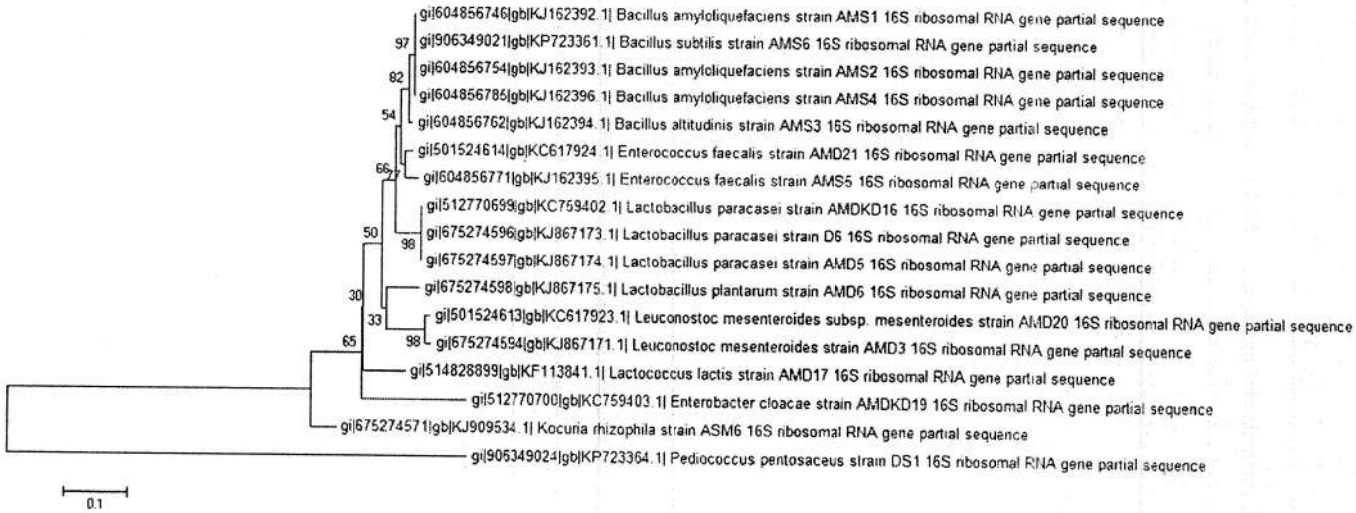


Fig 7: Phylogenetic tree constructed by Neighbor-Joining Method using MEGA 5.0 from 16S rRNA Sequences. The evolutionary distances were computed using the Kimura 2-parameter method.

Objective 3:

Biochemical characterization of food:

3.1 Materials and methods:

3.1.1 Determination of pH, water activity (a_w):

The pH was determined by pH meter and water activity was measured by water activity meter.

3.1.2 Total titratable acidity (TTA)

The total titratable acidity was determined on 10gm of sample homogenized with 90 ml of distilled water and titrated against standard solution of NaOH using phenolphthalein indicator and expressed as the g/L as equivalent of lactic acid.

Formula for determining TTA:

$$g/l = \frac{\text{ml NaOH x Normality of NaOH x 0.090 x 1000}}{\text{Sample volume (ml)}}$$

= ..... equivalent weight of tartaric acid

3.1.3 Analysis of proximate composition

Protein content will be determined by multiplying total nitrogen, estimated by micro-Kjeldahl method, by 6.25 (AOAC, 1990) and also soluble proteins will be estimated by the method of Lowry. Fat content will be determined by ether extraction using glass soxhlet (AOAC, 1990). Crude fibre content of sample will be determined following the method of AOAC (1990). Carbohydrate content will be estimated by Anthrone method.

3.2 Results:

3.2.1 Determination of pH, water activity (a_w) and moisture content:

pH, water activity (a_w) and moisture content of different fermented food products are given in the table 6.

Table 6: pH, water activity (a_w) and moisture content

Food samples	pH	Total titrable acidity (g/L as tartaric acid)	Water activity (aW) at 25°C
Fermented milk products	5.772 ± 1.221	14.86 ± 6.568	0.774 ± 0.0667
Fermented bamboo products	4.892 ± 0.573	13.30 ± 4.902	0.569 ± 0.0634
Fermented cereals	7.714 ± 0.454	12.84 ± 4.167	0.6978 ± 0.051
Fermented fruits and vegetables	3.9 ± 0.084	12.9 ± 1.272	0.682 ± 0.031

3.2.2 Analysis of proximate composition:

Proximate composition of different varieties of fermented food samples in terms of moisture content, protein content, fat content, crude fibre content and carbohydrate content (in % per gram of sample) are tabulated below:

Table 7: Proximate composition of different types of fermented foods (per 100 gm of sample)

Food samples	Moisture content (%)	Protein content (%)	Fat content (%)	Fibre content (%)	Total carbohydrate content (%)
Fermented milk products	55.87 -82.7	5.1- 31.1	1.66-15.3	0	0.5- 8.98
Fermented bamboo products	31.98- 66.45	10.44- 32.45	1.98- 4.88	8.98- 24.87	9.45- 23.78
Fermented cereals	3.45-10.56	15.67- 26.78	27.78- 40.46	2.67- 5.67	26.67- 41.34
Fermented fruits and vegetables	34.56- 45.2	1.45- 2.56	4.12- 10.45	5.67- 5.78	40.9- 42.45

Objective 4:

4.1 Probiotic characterization of isolates:

4.1.1 Acid and bile resistance

Resistance to acidic and bile conditions will be tested according to Duc et al. (2004). The pH of nutrient broth will be adjusted to pH 4.0, pH 3.0, and pH 2.0 with 1 M HCl and with pH 7.0 as control. Survival will be evaluated using the log phase cultures (8 log10 cfu mL^{-1}) by plating on respective media, after 30, 60, 90, and 120 min, of incubation at 37^0C in acidic media. Tolerance for bile acids will be tested using nutrient broth supplemented with 0.5%, 1%, 2% w/v ox bile and without supplement as a control will be inoculated with actively growing bacteria. Survival will be evaluated using log phase cultures (8 log10 cfu mL^{-1}) by plate count on respective media, after 60, 120, 180 min of incubation at 37^0C in media containing bile salts.

4.1.2 Bile salt hydrolase agar plate assay

Overnight bacterial culture will be streaked on respective media, enriched with 0.5% (w/v) taurodeoxycholic acid or oxgall and will be incubated for 48 h at 37^0C. Hydrolysis of bile salts will be indicated by altered colony morphology compared to control agar plates and by precipitation zones around the colonies. Observation will be recorded after 24 and 48 h of incubation.

4.1.3 Antimicrobial activity assay

Overnight culture of the strains under study will be centrifuged to obtain a cell-free supernatant that will be assayed for antimicrobial activity using the well diffusion assay. In brief, in freshly prepared lawns of the indicator strains in Mueller hinton Agar (MHA), wells will be punched. The cell-free supernatant will be neutralized and 20 ml will be added to each well. Incubation will be carried out at 37^0C for 48 h. Inhibition of the indicator strain's growth around the wells suggested the presence of antimicrobial activity in the supernatant used.

4.1.4 Cell surface hydrophobicity

Hydrophobicity will be determined according to the method described by Rosenberg, Gutnick, and Rosenberg (1980).

4.1.5 Antibiotic susceptibility

The isolates will be checked for antibiotic susceptibility against different antibiotics such as amoxicillin, ampicillin, cephalothin etc.

4.1.6 Production of extracellular enzymes

Each of the isolates will be grown in nutrient broth at 37°C for 20 h, and centrifuged at 9000g for 30 min. The supernatant will be filtered with 2 μm filter and will be stored in a pre-sterilized screw-capped glass tube at 4°C. A 50 μl aliquot of it will be used for determining the activities of different extra cellular enzymes using well-assay plate method in suitable media. Production of protease, lipase and amylase will be determined using milk agar, tributyrin agar base added with 1.0% v/v tributyrin, and starch agar, respectively. The incubated starch agar plates will be flooded with Lugol's iodine solution. The results will be expressed as clear zone diameter (including well diameter).

4.3 Antimicrobial activity:

Antimicrobial activity was checked for some of the isolates against different indicator strains and some of them showed positive results by showing zones of inhibition on MHA plates.

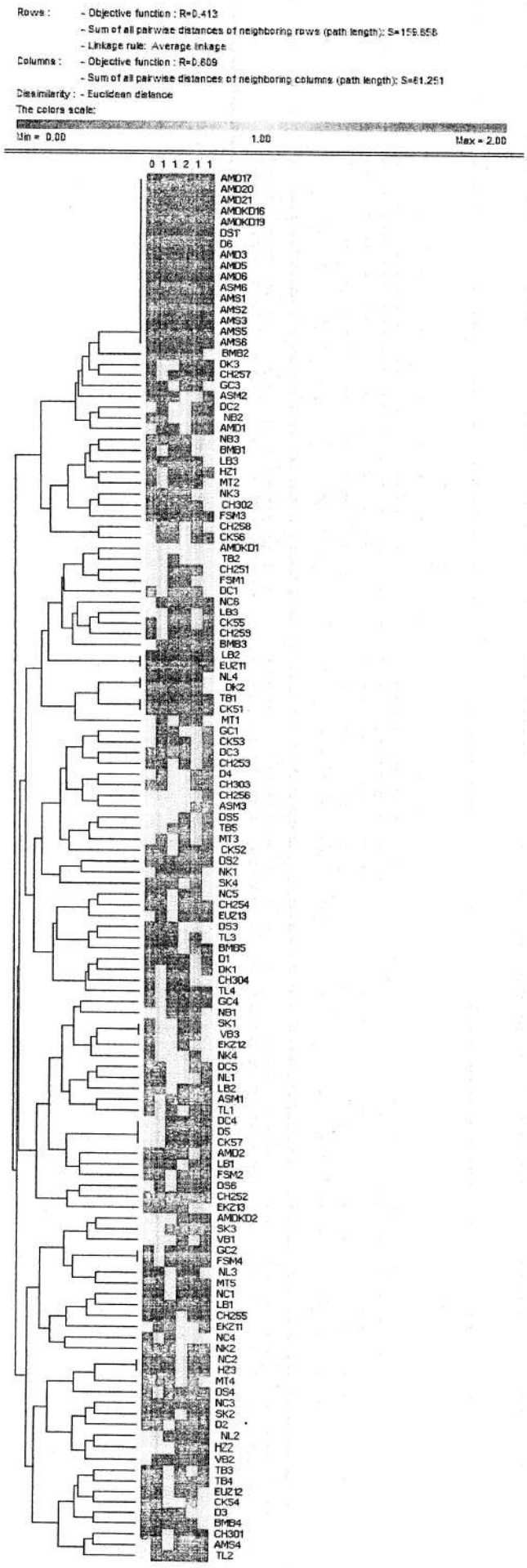


Fig 8: Matrix Hierarchical Cluster Analysis for finding most promising probiotic strains using PermutMatrix program.

Results:

In order to find out the most promising probiotic strains, strains with various properties as shown in the table 6 are separated in to different groups and were given codes 0,1, and 2. Matrix Hierarchical Cluster Analysis using PermutMatrix program v. 1.9.3 (LIRMM, France) was done in order to find out the relationship among the isolates in terms of probiotic properties (Fig. 8).

Table 8: Probiotic traits of isolated strains:

Traits	Acid tolerance			Bile tolerance			Hydrophobicity			Antimicrobial			Bile salt hydrolase (BSH)			Extracelluar enzyme production		
Groups	Viability 0-25%	Viability 26-50%	Viability 51-100%	Viability 0-25%	Viability 26-50%	Viability 51-100%	0-25%	26-50%	51-100%	No zone	<10mm	>10mm	No precipitation	<10mm	>10mm	Non-producer		
Codes	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
ISOLATES	AMD8	AMDKD1	AMD17	GC1	AMD8	AMD17	GC2	AMD8	AMD17	GC4	AMDKD1	AMD8	AMDKD2	AMD8	AMD17	AMDKD2	AMD8	AMD17
	GC2	AMDKD2	AMD20	NC1	AMDKD1	AMD20	NC2	AMDKD1	AMD20	NC1	GC1	AMD17	GC2	AMDKD1	AMD20	GC2	AMDKD1	AMD20
	NC1	GC1	AMD21	NC5	AMDKD2	AMD21	NC6	AMDKD2	AMD21	NC5	NC2	AMD20	NC1	GC1	AMD21	NC1	GC3	AMD21
	NC2	NC6	AMDKD16	NC6	GC2	AMDKD16	DC1	GC3	AMDKD16	DC3	NC4	AMD21	DS2	NC4	AMDKD16	NC2	NC4	AMDKD16
	NC3	DC2	AMDKD19	DC3	GC4	AMDKD19	DC4	NC1	AMDKD19	DC4	DC2	AMDKD2	DS6	DC3	AMDKD19	NC3	NC5	AMDKD19
	NC4	DC4	GC3	DS2	NC4	GC3	DS2	NC5	GC1	DS2	DC5	AMDKD16	LB1	DS3	GC3	DC4	DC1	GC1
	NC5	DS5	GC4	DS3	DC1	NC2	DS3	DC2	GC4	DS5	DS3	AMDKD19	LB2	DS5	GC4	DC5	DS3	GC4
	DS2	DS6	DC1	LB1	DC4	NC3	DS4	DC5	NC3	DS6	NB2	GC2	NL3	NB3	NC2	DS4	NB1	NC6
	DS3	NB1	DC3	LB2	DS4	DC2	LB2	DS5	NC4	NB1	NL1	GC3	NL4	LB3	NC3	DS6	NB3	DC2
	DS4	LB3	DC5	NL3	DS5	DC5	LB3	DS6	DC3	LB1	TB2	NC3	TB1	NL1	NC5	LB1	NL3	DC3
	LB1	NL2	DS1'	TB3	NB1	DS1'	NL4	NB2	DS1'	TB5	SK2	NC6	SK3	TB2	NC6	NL1	NL4	DS1'
	LB2	TB2	NB2	TB4	LB3	DS6	TB1	NL1	NB1	SK1	SK4	DC1	SK4	TB3	DC1	NL2	TB2	DS2
	NL3	TB5	NB3	SK4	NL2	NB2	TB2	NL3	NB3	D1	D4	DS1'	D4	TB5	DC2	SK2	SK1	DS5
	TB3	SK3	NL1	D3	TB2	NB3	SK4	TB3	LB1	D5	EKZ11	DS4	EUZ11	D1	DC4	SK3	SK4	NB2
	TB4	D4	NL4	D4	TB5	NL1	D1	TB4	NL2	EKZ12	HZ3	NB3	HZ1	D3	DC5	D2	D3	LB2
	SK2	D5	TB1	EKZ11	SK1	NL4	D5	SK1	TB5	EKZ13	BMB5	LB2	NK1	EKZ11	DS1'	D5	EKZ12	LB3
	D1	EKZ11	SK1	EUZ11	SK3	TB1	EKZ13	D2	SK2	EUZ13	NK2	LB3	NK2	EKZ12	DS4	EKZ11	EKZ13	TB1
	D2	EUZ13	SK4	EUZ12	D5	SK2	EUZ11	D4	SK3	VB3	NK4	NL2	DK2	EKZ13	NB1	HZ2	EUZ12	TB3
	D3	HZ2	D6	EUZ13	EKZ12	D1	HZ3	EKZ12	D3	NK1	AMD1	NL3	CH251	BMB1	NB2	HZ3	BMB1	TB4
	EUZ11	BMB3	EKZ12	BMB4	HZ1	D2	BMB3	EUZ12	D6	DK1	CH255	NL4	CH252	VB1	NL2	BMB5	BMB2	TB5
	EUZ12	VB1	EKZ13	BMB5	HZ2	D6	BMB5	EUZ13	EKZ11	AMD2	CH256	TB1	CH255	NK3	TB4	VB1	BMB4	D1
	HZ3	VB2	HZ1	NK1	BMB1	EKZ13	NK1	HZ2	HZ1	CH252	CH258	TB3	CH258	DK1	SK1	VB2	VB3	D4
	BMB4	NK1	BMB1	NK2	VB1	HZ3	DK1	VB1	BMB1	CH253	CK54	TB4	CK51	AMD2	SK2	AMD2	NK1	D6
	BMB5	AMD1	BMB2	NK3	VB3	BMB2	DK2	VB3	BMB2	CH254	CK56	SK3	CK52	CH253	D2	CH252	NK3	EUZ11
	NK2	CH251	VB3	AMD2	NK4	BMB3	AMD2	NK4	BMB4	CK52	CH303	D2	CH302	CH256	D5	CH255	NK4	EUZ13
	DK1	CH256	NK3	CH253	DK1	VB2	CH251	DK3	VB2	CK53	ASM2	D3	CH303	CK53	D6	CK57	DK2	HZ1
	CH254	CH258	NK4	CH254	DK3	DK2	CH252	CH253	NK2	CK57	ASM3	D6	ASM3	CK55	EUZ12	CH301	CH251	BMB3
	CH255	CK53	DK2	CH255	CH251	AMD1	CH259	CH254	NK3	CH304	LB1	EUZ11	LB3	CH304	EUZ13	ASM1	CH258	NK2
	CH259	CK56	DK3	CK53	CH256	AMD3	CK51	CH256	AMD1	ASM1	TL1	EUZ12	FSM3	ASM2	HZ2	LB1	CK54	DK1
	CK54	CK57	AMD2	CK54	CH257	AMD5	CK55	CK52	AMD3	LB2	TL3	HZ1	FSM4	FSM1	HZ3	LB2	CH302	DK3
	CK55	ASM3	AMD3	CH301	CH259	AMD6	CK57	CK54	AMD5	FSM2	MT4	HZ2	MT1	MT3	BMB2	FSM2	CH304	AMD1
	CH301	FSM1	AMD5	CH302	CK55	CH252	CH304	CH301	AMD6	TL4		BMB1	MT2		BMB3	FSM3	LB3	AMD3
	CH304	AMS4	AMD6	CH303	CK57	CH258	LB1	CH303	CH255	MT3		BMB2	MT5		BMB4	FSM4	FSM1	AMD5
	FSM4	TL2	CH252	LB1	CH304	CK51	FSM1	ASM3	CH257			BMB3			BMB5	AMS4	TL3	AMD6
	TL3	MT1	CH253	FSM2	ASM1	CK52	FSM4	LB2	CH258			BMB4			VB2	TL1	MT1	CH253
	TL4	MT3	CH257	FSM3	ASM3	CK56	TL3	FSM2	CK53			VB1			VB3	TL2	MT2	CH254
	MT4		CK51	AMS4	FSM1	ASM2	TL4	AMS4	CK56			VB2			NK4	MT5	MT4	CH256
	MT5		CK52	TL2	FSM4	ASM6	MT4	MT1	CH302			NK3			DK3			CH257
			CH302	TL3	TL1	LB2		MT3	ASM1			DK2			AMD1			CH259
			CH303	MT5	TL4	LB3		MT5	ASM2			DK3			AMD3			CK51
			ASM1		MT2	AMS1			ASM6			AMD3			AMD5			CK52
			ASM2			AMS2			LB3			AMD5			AMD6			CK53
			ASM6			AMS3			FSM3			AMD6			CH254			CK55
			LB1			AMS5			AMS1			CH251			CH257			CK56
			LB2			AMS6			AMS2			CH257			CH259			CH303
			LB3			MT1			AMS3			CH259			CK54			ASM2
			FSM2			MT3			AMS5			CK51			CK57			ASM3
			FSM3			MT4			AMS6			CK55			CH301			ASM6
			AMS1						TL1			CH301			ASM1			AMS1
			AMS2						TL2			CH302			ASM6			AMS2
			AMS3						MT2			ASM6			LB1			AMS3
			AMS5									LB3			LB2			AMS5
			AMS6									FSM1			FSM2			AMS6
			TL1									FSM3			AMS1			TL4
			MT2									FSM4			AMS2			MT3
												AMS1			AMS3			
												AMS2			AMS4			
												AMS3			AMS5			
												AMS4			AMS6			
												AMS5			TL1			
												AMS6			TL2			
												TL2			TL3			
												MT1			TL4			
												MT2			MT4			
												MT5						

Objective 5:

Incorporation of probiotic bacteria in fermented food:

5.1 Materials and methods:

5.1.1 Sample preparation:

For the preparation of fermented milk or *doi*, 1 L of buffalo milk was collected from nearby area of Napam, Tezpur and heated for 15 min at 90°C with intermittent stirring and fortified with honey at levels of 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v). The *L. lactis* AMD17 starter culture was prepared in autoclaved skimmed milk by sub-culturing once for maintaining its potential activity. It was inoculated with at 7.4 log CFU/mL and incubated at 37°C until fermentation is complete (14 h). By the time, the milk curdled and became semi-solid and the preparation was considered as *doi*. It was firm and of uniform consistency with a smooth and glossy surface. The set *doi* samples were stored aseptically in sterile earthen pot at 4 °C until use.

5.1.2 Sensory evaluation

Sensory evaluations were privately conducted after 1 day while participants were seated in a quite area behind a privacy divider in the milk processing lab (Department of Food Engineering and Technology, Tezpur University). A nine-point facial hedonic scale in which 9 = "liked extremely", 5 = "neither liked nor disliked" and 1 = "disliked extremely" was used by each participant for sample evaluation. A control sample of plain probiotic yogurt was offered and then the remaining five samples were served in a random order.

5.1.3 Texture Profile of probiotic *doi* during one month storage:

Texture evaluation of the extrudates was performed weekly with texture analyzer (TA-HD-plus, Stable Micro Systems,UK). The pre- and post-test speed of the probe was 2 mm/s, the test speed was 0.2 mm/s during measurements. The distance covered in the sample was 30 mm, using a cylindrical probe of 20 mm diameter. The results were presented as the average of three measurements. Texture properties such as Hardness (N), Springiness (dimensionless), Cohesiveness (dimensionless), and Gumminess (N) were considered.

Results and discussion:

5.2.1: Survivability of *L. lactis* AMD17 in honey-enriched *doi*

Preparation of fermented milk is illustrated in the fig 5. The changes in the viable colony count of *L. lactis* AMD17 in storage conditions is depicted in the fig 6. It was observed that in case of control the viability of the bacteria decreases significantly with increase in the storage time. Conversely the addition of honey retains its viability since the viability did not change significantly as compared to the initial viability. viability was observed with 3% (w/v) honey followed by 4% and 5% (w/v) honey. It was suggested that probiotic products should contain lactic acid bacteria count of at least 10^7 CFU/mL (Ishibashi and Shimamura 1993). Our findings are in agreement with this suggestion. During first week, the increase in cell count was observed in control whereas *doi* enriched with honey showed no significant growth in cell viability. After two weeks, the decrease in cell viability was observed in control experiment whereas; *doi* fortified with honey showed lesser reduction in cell count.

5.2.2 Sensory evaluation

Increase in the amount of added honey (1%-5%) contributed to the increase in sweetness of all samples. In addition, honey has the ability to decrease the sourness of solutions and hence can serve to increase consumer acceptability (Varga 2006). The fermentation required for buffalo yoghurt takes longer respect to bovine yoghurt (Nguyen et al. 2014). Honey, a prebiotic source may recompense extended fermentation time and the taste of the *doi*. The tastes of *doi* were found to increase significantly with 3-5% honey as compared to control. Lisak et al. 2012 had also reported better taste score for yoghurt with added sweetener at highest concentration (5%). Sensory scores for colour and texture were found to be different for honey incorporated samples as compared to control. Scores for overall acceptability of *doi* ranged between 5.75 and 7.66 (Table 3). The overall scores showed that the best evaluated samples were those with added honey (3-5%).

5.2.3 Texture Profile

The texture profile of *doi* during refrigerated storage is shown in Fig. 9. Comparing days 1 to 28 for all *doi* formulations during storage, the addition of increased concentration of honey (1% - 5%) resulted in firmer and gummy products ($P < 0.05$) and had no effect on the cohesiveness and springiness of the product as compared to first day

Interaction between the ingredients present in*doi*formulation continued to occur duringrefrigerated storage, which could explain the gradual increase in hardness for these products throughout the storage period. Earlier reports suggested that exopolysaccharides (EPS) produced by probiotic cultures could increase the viscosity, water retention and interaction with other ingredients of milk lead to firmness of the casein matrix in the final product (Duboc and Mollet 2001). The augmented firmness is interrelated to an improvement of the texture since firm dahi is less susceptible to rearrangements within its network and hence less susceptible to shrinkage and serum expulsion (Oliveira et al. 2011). Besides the storage period, the presence of honey might also have contributed to the significant changes in the texture profile of the products. With the increased levels of firmness during storage, gumminess (multiplication of firmness and cohesiveness) also increased. The cohesiveness and springiness during storage did not differ significantly to which gel could be deformed while eating the product.

Fig 9: Preparation of fermented milk (*doi*)

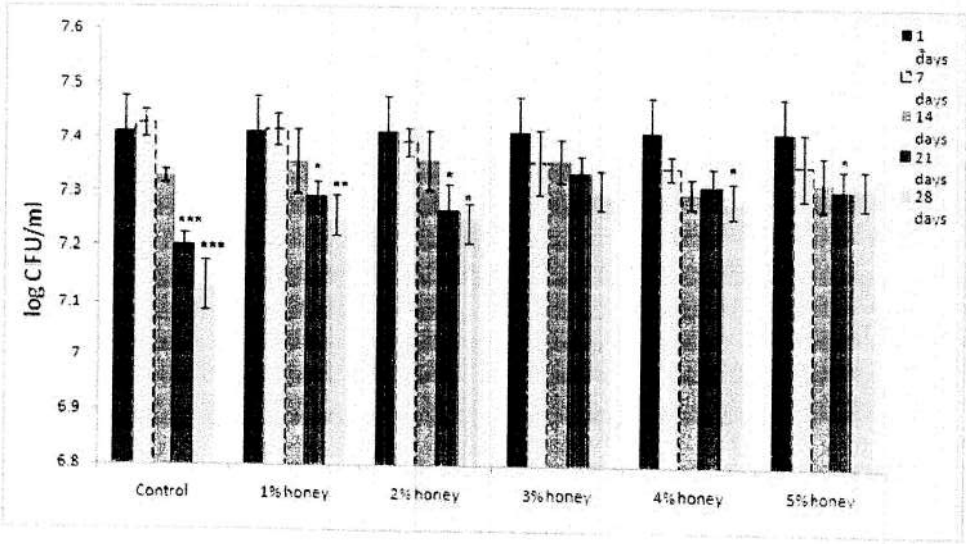
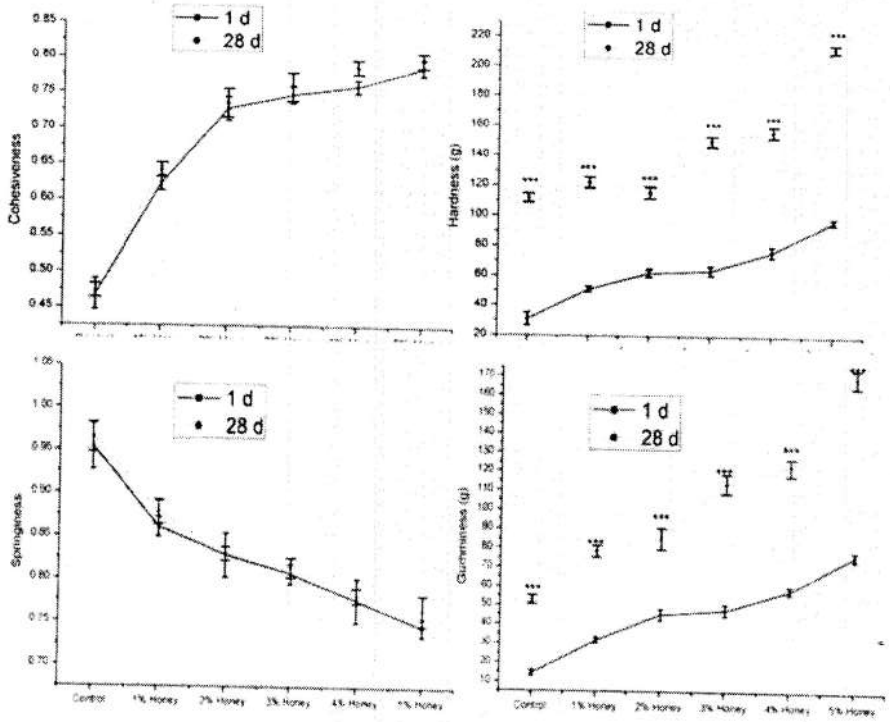


Fig 10: Viability of *L. lactis* AMD6 at different concentrations of honey

Table 9: Sensory evaluation of *doi* formulations

Attributes	Control	1% Honey	2% Honey	3% Honey	4% Honey	5%Honey
Taste	3.5 ± 0.925^{b}	4.37 ± 1.026^{b}	4.75 ± 1.133^{b}	7.3 ± 1.33^{a}	6.8 ± 1.131^{a}	7.3 ± 0.744^{a}
Colour	4.75 ± 0.755^{bc}	6.50 ± 1.414^{ac}	6.93 ± 0.776^{a}	7.31 ± 0.593^{a}	7.25 ± 0.707^{a}	7.37 ± 1.060^{a}
Texture	3.25 ± 1.035^{b}	6.125 ± 1.827^{a}	7.0 ± 1.603^{a}	7.5625 ± 0.979^{a}	6.81 ± 1.307^{a}	7.25 ± 1.069^{a}
Overall acceptability	5.75 ± 1.195^{bc}	6.51 ± 1.626^{ac}	6.97 ± 0.928^{ac}	7.66 ± 0.843^{a}	7.375 ± 0.942^{a}	7.43 ± 0.821^{a}

Results are expressed as Mean ± S.D, $^{a\text{-}c}$ different superscript letters represent significant different ($p<0.05$)



*p<0.05, **P<0.01 ***p<0.001

Fig 11: Texture profile at different time interval

Uses of fermented food isolate *Bacillus amyloliquefaciens* AMS1 as a feed additive:

Materials and methods:

Probiotic Characteristics:

Done as mentioned earlier in this report (Objective 4)

Cellulolysis: The assessment of cellulolytic potential of the isolate was routinely done on carboxymethylcellulose (CMC) agar plate (Kasana et al., 2008). Briefly, wells were prepared on CMC plate containing culture and incubated for 18 h at different temperatures and were flooded with Gram's iodine.

Optimization of cellulase production at different temperatures and pH:

Production medium at pH 7.0 was inoculated with overnight grown selected bacterial isolate. The broth was incubated at different temperatures viz. 15, 30, 37, 40, 50, and 60 °C for 24 h. At the end of incubation period, the cell-free culture filtrate was obtained, dialyzed (HiMedia Dialysis membrane-110) and used as enzyme source. Erlenmeyer flasks with broth containing the optimum concentration of substrate and carbon source was taken and the pH of the broth was adjusted to 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 in different flasks using 1N HCl and 1N NaOH and sterilized. The cultures were inoculated with the selected isolate and incubated at 37 °C. At the end of incubation period, the cell-free culture filtrate was obtained, dialyzed and used as enzyme source.

Cellulase activity assay:

Total cellulase activity was determined by measuring the amount of reducing sugar formed from filter paper (Ghose, 1987). Briefly, 0.5 ml of culture supernatant was incubated with 1.0 ml of 0.05M sodium citrate buffer (pH 4.8) containing Whatman no. 1 filter paper strip (1.0 × 6.0 cm). After incubation for an hour at 50 °C, the reaction was terminated by adding 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent to 1 ml of reaction mixture. The reducing sugars were estimated spectrophotometrically with DNS reagent (Miller, 1959) using glucose as standard. One unit of enzyme activity was defined as the amount of enzyme that released 1µmol of glucose per minute.

SEM analysis of the degradation of maize straw:

The maize straw used in this study was obtained locally, washed, and dried. The dried maize straw was chopped into small pieces and then ground into smaller homogenous particles

using grinder mixer. SEM analysis of the degradation of maize straw by incubating the maize straw powder [2% (w/v)] with dialyzed enzyme for 6 h was done. Samples incubated at 37 and 60 °C were centrifuged for 2500× g for 10 min and supernatant was collected to estimate the amount of reducing sugars released from treated sample. The tested maize straw was fixed with 2.5% glutaraldehyde for 6 h and washed twice with 1X PBS, pH 7.4. Further samples were dehydrated in graded concentration of ethanol.

Results and discussions:

(Fig. 12).

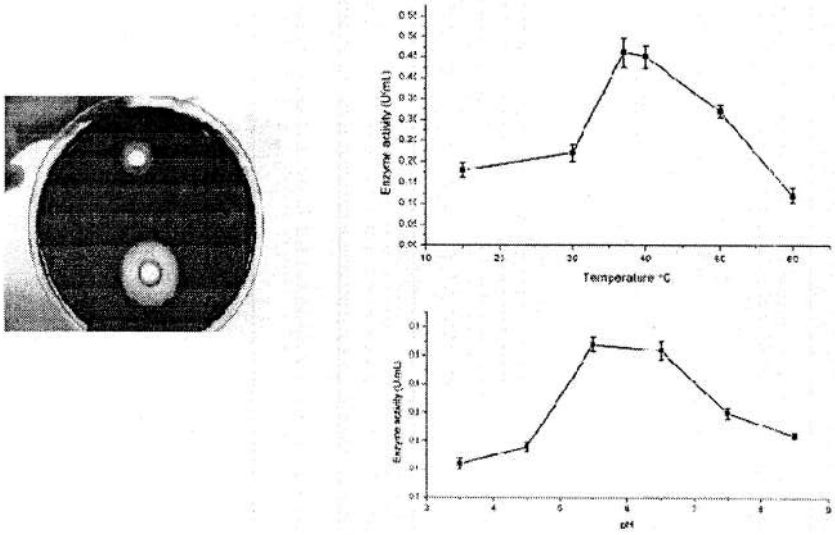


Fig 12 Cellulolytic activity of crude enzyme produced by *B. amyloliquifaciens* AMS1

SEM analysis of the degradation of maize straw:

The morphological changes of the maize straw powder after treatment with dialyzed supernatant containing cellulase were recorded by SEM. As shown in Fig. 13, structure of the guard and subsidiary cells in corn leaves is shown in the control figure whereas treated sample was more disordered and the peeling off of the linters was clearly observed.

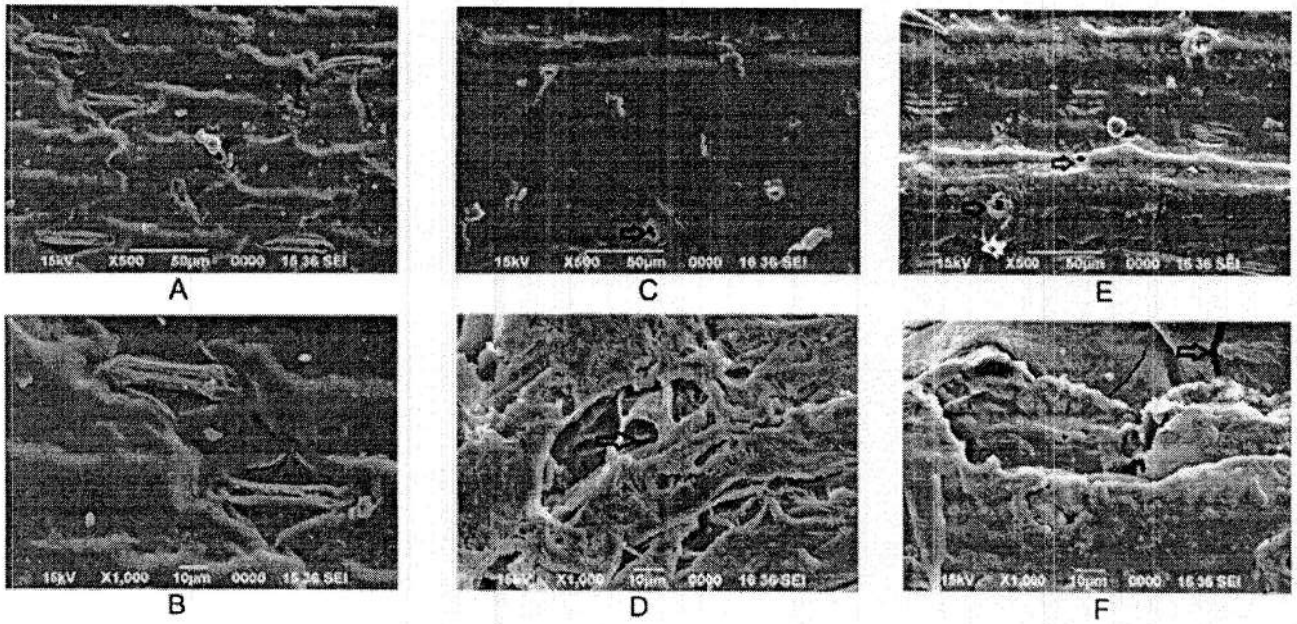


Fig.13 Scanning electron micrograph showing cellulose degradation of maize straw at different temperatures. (A) Control contains only maize straw at 500×. (B) Control contains only maize straw at 1000× (C) Maize straw incubated for 37 °C with dialyzed culture supernatant at 500×. (D) Maize straw incubated for 37 °C with dialyzed culture supernatant at 1000×. (E) Maize straw incubated for 60 °C with dialyzed culture supernatant at 500×. (F) Maize straw incubated for 60 °C with dialyzed culture supernatant at 1000×.

B. amyloliquefaciens AMS1 showed potential probiotic characteristics as well as a significant cellulolytic activity in vitro. It proved to be sufficiently robust to survive the harsh physico-chemical conditions present in the gastrointestinal tract. The ability to degrade CMC, maize straw and filter paper conferred cellulolytic potential on the bacterium isolated from a fermented food, Soybean. Generally the animal feeds of plant origin have higher cellulose contents which could be hydrolyzed by cellulolytic probiotics in conjunction with the rumen microbes, forming a source of energy for the animal rather than it being passed in the undigested feces.

Objective 7:

Preservation of isolated strains for future use:

Isolated microbial strains were preserved in 20% (w/v) glycerol and kept at -80^{0}Cfor future use.

Major findings of the Project (Project Summary):

1. Total four different types of fermented foods based on substrates utilized were studied from Assam and Arunachal Pradesh; these are fermented milk based products, fermented bamboo shoots, fermented cereals and fermented fruits and vegetables.
2. A total of 503 + 480 different microbial strains were isolated from different types of fermented food and are preserved at -80°C.
3. Fermented milk products were found to be dominated by *Lactobacilli* whereas fermented cereals were found to be dominated by *Bacillius spps.*
4. Total 36 isolates were identified and their sequences were successfully submitted to GenBank.
5. Database of the fermented food was prepared and uploaded online as orhttp://1drv.ms/1OIGdA1.
6. Total 16 isolates were found to possess good probiotic traits.
7. Strain *L. Lactis* AMD17 which showed promising probiotic characteristic was found suitable for preparation of fermented milk (Dahi). The texture and sensory profiles of Curd prepared with this single strain shows that it can be used as ready to use starter culture for preparation of Dahi with functional properties.
8. A potential probiotic *B. amyloliquifaciens* AMS1 isolated from fermented soybean found to have very good probiotic and cellulolytic activity. The bacterial strain was found suitable for improving cellulosic feed conversion rate.

Future work: Checking probiotic and other useful functional characteristics in all the uncharacterised (~480) microbial strains.

Publications during the project period:

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2. Manhar, A. K., Saikia, D., Bashir, Y., Mech, R. K., Nath, D., Konwar, B. K., & Mandal, M. (2015). In vitro evaluation of celluloytic *Bacillus amyloliquefaciens* AMS1 isolated from traditional fermented soybean (Churpi) as an animal probiotic. *Research in Veterinary Science*, 99, 149–156.
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1. Bergey, D. H., Buchanan, R. E., Gibbons, N. E., & American Society for Microbiology. (1974). Bergey's manual of determinative bacteriology. Baltimore: Williams & Wilkins.
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10. Varga L (2006) Effect of acacia (Robinia pseudo-acacia L.) honey on the characteristic microflora of yogurt during refrigerated storage. Int J Food Microbiol 108, 272-275.
11. Lisak K., Lenc M., Jelicic I., Bozanic R. (2012) Sensory evaluation of the strawberry flavored yoghurt with stevia and sucrose addition, Croatian Journal of Food Technology, Biotechnology and Nutrition. 7, 39-43.
12. Duboc P. & Mollet B. (2001) Applications of exopolysaccharides in the dairy industry. Int Dairy J 11, 759-768.

13. Oliveira RPS, Perego P., Oliveira, M.N., Converti A. (2011) Effect of inulin as prebiotic and synbiotic interactions between probiotics to improve fermented milks firmness. J Food Eng 107, 36-40.

Appendix: 1

Fermented food samples for Diversity Study (at NEERI, Nagpur)

Sr. No.	Local Name	Fermented Food samples	Location	Date	Individual Name	Community
1	Kharoli 1	Mustard chutney	Nagaon, Assam	13/2/2012	Bazaar	Assamese
2	Kharoli 2		Nagaon, Assam	13/2/2012	Bazaar	Assamese
3	Kharoli 3		Solmari, Assam	13/2/2012	Bazaar	Assamese
4	Henoop	Bamboo shoot	Erdangte, Karbi Anglong, Assam	6/2/2012	Robin Ingti	Karbi
5	Khorisa		Khowang, Dibrugarh, Assam	7/14/2012	Himanti Bora	Sonowal
6	Khorisa		North- lakhimpur, Assam	6/3/2012	Jiten Bora	Assamese
7	Akone	Fermented Soyabean	Merapani,Assam	2/6/2012	Akho Bhese	Naga
8	Kinema 1		Chariduar,Assam	2/7/2012	Raju Dhungana	Nepali
9	Kinema 2		Sungajan,Assam	21/8/2012	P. Lama	Nepali
10	Doi 1(Curd)	Milk Product	Tejpur, Assam	5/6/2012	H. Sutardhar	Assamese
11	Doi 2(Curd)		Golaghat, Assam	21/6/2012	Anima Sonowal	Sonowal
12	Doi 3(Curd)		Nagaon, Assam	1/7/2012	Bina Patar	Tiwa
13	Hikung	Bamboo shoot	Ziro,Arunachal Pradesh	19/10/2012		
14	Bastenga		Bomdila, Arunachal Pradesh	8/18/2012	Bomdila bazar	Monpa
15	Mesu		Bhalukpung, Arunachal Pradesh	8/18/2012	Sabita Gurung	Nepali
16	Libi 1	Fermented Soyabean	Bomdila, Arunachal Pradesh	8/19/2012	Mon Merak	Monpa
17	Libi 2		Bomdila, Arunachal Pradesh	18/8/2012	Pem Dolma	Monpa
18	Peruyani		Ziro,Arunachal Pradesh	19/10/2012	Gyati Onya	Apatani
19	Churpi(Yak milk cheese)	Milk Product	Bomdila, Arunachal Pradesh	8/19/2012	Bomdila bazar	Monpa
20	Dudh Churpi(Cow milk cheese)		Sessa, Arunachal pradesh	8/18/2012	H. Gurung	Nepali
21	Churkham(Hard cheese)		Bomdila, Arunachal Pradesh	8/18/2012	Bomdila bazar	Monpa

Appendix: 2

List of HK media with their respective components

Hk media	Media components
1	Yeast Extract, $CaCl_2$
2	Yeast Extract, Casein Hydrolysate,K_2HPO_4,D-Sorbitol
3	Glucose, Yeast Extract, Malt Extract
4	Salts, Trace Element solution
5	Casein Soyabean Meal,Dextrose,NaCl
6	Lactose, Bile Salt, NaCl, Neutral red
7	Soil Extract, Malt Extract
8	L-Aspargine, Sodium Caseinate, Sodium Propionate, $FeSO_4$, Mgso4
9	Salts-NaCl,MgCl,$MgSO_4$,,KCl,$NaHCO_3$,NaBr,Glucose
10	NH_3SO_4,$MgSO_4$,$FeCl_3$,Cellulose,Cellobiose
11	Tryptone,Sodiun acetate,Soil Extract
12	Peptone,Meat Extract
13	Glucose, Malt Extract,$CaCO_3$
14	L-aspargine,$FeSO_4$,$ZnSO_4$,$MgCl_2$,K_2HPO_4.
15	peptone,beef extract,glucose,MgSO4,MnSO4
16	Soyabean,Mannitol.
17	yeast extract,Mannitol,peptone.
18	peptone specific ,chromogenic mix
19	Mannitol,yeast
20	Malt extract,Ox-bile,Tween 40.
21	Casein enzyme hydrolysate,Yeast Extract.
22	Tryptone,Protease peptone,K2HPO_4,$MgSO_4$.
23	Peptone from casein,Peptone from Soyameal,NaCl.
24	peptic digest of animal tissue,Beef extract,Yeast extract,NaCl.
25	NH4So_4,K2HPo4,Fumaric acid,Yeast extract,$MgCl_2$,$FeSO_4$,Sodium Formate,Yeast extract,Resazurin.
26	Tryptone,Protease peptone,K_2HPO_4,$MgSO_4$.
27	Sodium aspartate,Yeast extract,$MgSO_4$,$CaCl_2$,K_2HPO_4
28	K2PO_4,$NH4SO_4$,MgSO4infusion broth,dextrose,Soluble starch,Yeast extrat,Pancreatic digest of Casein.
29	Peptone,NaCl,$CaCl_2$
30	Sucrose,Casein enzyme hyolyse, Yeast extract,Pancreatic digest of Casein

Appendix-B

FINAL UTILIZATION CERTIFICATE

(For the entire project period 2011-2016)

(Rs. in Lakhs)

1.	Title of the Project/Scheme: "Study of the microbial diversity and biochemical characteristics of the selected non- alcoholic fermented (milk, vegetable and pulses) food product of Assam and Arunachal Pradesh".	
2.	Name of the Organization:	Tezpur University
3.	Principal Investigator:	Dr. Manabendra Mandal
4.	Deptt. of Biotechnology sanction order No. & date of sanctioning the project:	BT/219/NE/TBP/2011, dated November 21, 2011
5.	Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given:	Nil
6.	Amount received from DBT during project period (*please give No. and dates of sanction orders showing the amounts paid)*:	Rs. 22,39,000.00 Lakhs (Order No.s BT/219/NE/TBP/2011, dt 3rd Nov 2011 & BT/219/NE/TBP/2011, dt 3rd Sept 2014
7.	Interest earned, if any, on the DBT grants:	0.46895 Lakhs
8.	Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):	Rs. 22.85895 Lakhs
9.	Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed):	Rs. 21.90935 Lakhs
10.	Unspent balance refunded, if any *(Please give details of cheque No. etc.)*:	No
11.	Balance amount available at the end of the financial year:	Rs. 0.94960 Lakhs
12.	Amount allowed to be carried forward to the next financial year vide letter No. & date:	Nil

1. Certified that the amount of **Rs. 21.90935 Lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 0.94960 Lakhs** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

7.11.15

(FINANCE OFFICER)
Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix- C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred for the entire project period 2011-16

		Year- wise release made (₹ in Lakhs)					Interest earned (₹ in Lakhs)	Total (₹ in Lakhs)	Year-wise expenditure (₹ in Lakhs)					Total expenditure (₹ in Lakhs)	Balance (₹ in Lakhs)
Heads	Sanctioned cost	1st year (2011-12)	2nd year (2012-13)	3rd year (2013-14)	4th year (2014-15)	5th year (2015-16)			1st year (2011-12)	2nd year (2012-13)	3rd year (2013-14)	4th year (2014-15)	5th year (2015-16)		
1. Non- recurring															
(i) Equipments	12.69000	12.69000	0.00000	0.00000	0.00000	0.00000			0.00000	8.70978	3.88890	0.00000	0.00000		
2. Recurring															
(i)Manpower	6.60000	2.11000	0.00000	0.00000	1.40000	0.00000			0.00000	1.33600	0.77400	1.38600	0.00000		
(ii)Consumables	6.00000	2.00000	0.00000	0.00000	1.50000	0.00000			0.00000	1.99926	0.00000	0.00000	1.48451		
(iii)Travel	1.70000	0.70000	0.00000	0.00000	0.24000	0.00000			0.00000	0.40950	0.14775	0.02400	0.00000		
(iv)Contingency	1.30000	0.50000	0.00000	0.00000	0.30000	0.00000			0.12222	0.37185	0.00000	0.26493	0.04065		
(v)Overheads (if applicable)	1.50000	0.75000	0.00000	0.00000	0.20000	0.00000			0.37500	0.00000	0.00000	0.37500	0.20000		
Total	**29.79000**	18.75000	0.00000	0.00000	3.64000	0.00000	0.46895	**22.85895**	0.49722	12.82639	4.81065	2.04993	1.72516	**21.90935**	**0.94960**

(PROJECT INVESTIGATOR)

7.11.15

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure A

Manpower Staffing Details (For the period 2011-2016)

(₹ in Lakhs)

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY	TOTAL SALARY PAID DURING PROJECT PERIOD
Mr. Devabrata Saikia	JRF	27/04/2012	1/1/15	0.12	3.49600

(Signature of Principal Investigator)

7.11.15

(Signature of Accounts Officer)

Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure B

Manpower Expenditure Details (For the period 2011-2016)

(₹ in Lakhs)

Sanctioned posts	Number	Scale of pay	Annual outlay	Outlay for the entire period	Revised scale, if any	Revised annual outlay	Revised project outlay	Actual releases by DBT	Actual expenditure	Balance
JRF	1	0.12	2.11	6.60	Nil	Nil	Nil	3.51	3.49600	0.014

(Signature of Principal Investigator)

7.11.15

(Signature of Accounts Officer)

Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Tezpur University

* Details of manpower salary/ fellowship revision along with due- drawn statement and arrears requested should be given separately, if applicable.

Due- Drawn Statement

Name of the Project Staff	Month and Year	Due	Drawn	Difference
		(₹)	(₹)	(₹)
	Apr-12	1600	1600	
	May-12	12,000	12,000	
Devabrata Saikia	Jun-12	12,000	12,000	
	Jul-12	12,000	12,000	
	Aug-12	12,000	12,000	
	Sep-14	12,000	12,000	
	Oct-12	12,000	12,000	
	Nov-12	12,000	12,000	
	Dec-12	12,000	12,000	
	Jan-13	12,000	12,000	
	Feb-13	12,000	12,000	
	Mar-13	12,000	12,000	
	Apr-13	12,000	12,000	
	May-13	12,000	12,000	
	Jun-13	12,000	12,000	
	Jul-13	12,000	12,000	
	Aug-13	12,000	12,000	
	Sep-13	12,000	12,000	
	Oct-13	12,000	12,000	
	Nov-13	12,000	12,000	
	Dec-13	12,000	12,000	
	Jan-14	12,000	12,000	
	Feb-14	12,000	12,000	
	Mar-14	12,000	12,000	
	Apr-14	12,000	12,000	
	May-14	12,000	12,000	
	Jun-14	12,000	12,000	
	Jul-14	12,000	12,000	
	Aug-14	12,000	12,000	
	Sep-14	12,000	12,000	

(Signature of Principal Investigator)

(Signature of Accounts Officer)
Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)
Registrar
Tezpur University

Appendix-A

Details of Assets acquired wholly or substantially out of Govt. grants

Register to be maintained by Grantee Institution

Name of the Sanctioning Authority:		Department of Biotechnology (DBT), Government of India, New Delhi
1.	Sl. No.	54
2.	Name of the Grantee Institution	Tezpur University
3.	No. & Date of sanction order	BT/219/NE/TBP/2011, dated November 21, 2011
4.	Amount of the sanctioned grant	Rs. 22.39 Lakhs
5.	Brief purpose of the grant	To carry out the project "Study of the microbial diversity and biochemical characteristics of the selected non- alcoholic fermented (Milk, Vegetable and pulses) food product of Assam And Arunachal Pradesh."
6.	Whether any condition regarding the right of ownership of Govt. in the Property or other assets acquired out of the grant was incorporated in the grant-in-aid sanction order:	Yes
*7.	Particulars of assets actually credited or acquired.	Attached below
8.	Value of the assets as on 21.05.2015	**Rs. 12,59,868.00**
9.	Purpose for which utilised at present	To meet the objectives defined in the project.
10.	Encumbered or not	Not applicable
11.	Reasons, if encumbered	Not applicable
12.	Disposed of or not	No
13.	Reasons and authority, if any, for Disposal	Not applicable
14.	Amount realised on disposal	Not applicable
15.	Remarks	No

List of Expenditure under non- recurring head:

Sl. No.	Instruments	Status	Cost (in Rupees)
1	Deep freezer (-80°C)	Installed	4,41,000.00
2	Shaking incubator	Installed	1,95,601.00
3	Laminar air flow	Installed	72,072.00
4	Trinocular microscope	Installed	1,62,305.00
5	UV- Vis spectrophotometer	Installed	3,88,890.00
		Total	12,59,868.00

(PROJECT INVESTIGATOR)

(FINANCE OFFICER)
Finance Office
Tezpur University

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

* List of equipment purchased indicating the item wise costs may please be provided.