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Isolation and identification of microorgansims from fermented soybean food - prepared by Adi tribe of Arunachal Pradesh

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Abstract

Soybean fermentation is a popular fermented food that is used by Adi Tribe of Arunachal Pradesh. Soybean fermentation is popular dish which has specific flavor, aroma and taste. The local people named the fermented soybean as "*Ronyang namsing*". In this study, the process of isolation and identification of microorganisms, **VITEK® 2 GN technique** has been used for bacteria and **Lactophenol Aniline Blue technique for** fungi. This investigation trying to contributes to the current research on microbial analysis, where *Sphingomonas paucimobilis* bacteria strain has been identified and *Penicillium* spp., *Candida albicans* and *Monosporium rubrum* are fungi strains that has also been observed and identified. Soybean (*Glycine max*) is an annual legume plant that has it origin in China.

Key words : Adi tribe, Arunachal pradesh, Ronyang namsing, *Glycine max*.

Arunachal Pradesh tribe are well known for their fermentation methods, which soybean is one of the most practiced fermentated food in Arunachal state. It is studied that Soybean [*Glycine max* (L.) Merr.] is a valuable crop by its own because it consist of large reserve of proteins and oils among seasonal crops[1],And fermentation is a natural process which improve the breakdown of vitamins, essential amino acid and improving its aroma, flavor, protein, anti-nutritive values to food[14]. The first known record of soybean as a domesticated crop dates back to the eleventh century BC in China. Today the soybean crop is cultivated almost all over the globe with its major production hubs located in the North-South America region(including the USA, Argentina, and Brazil)[20]. India is also a one of the country that considered to be the a secondary center for soybean domestication for decades[11].

In India, soybean been was introduced

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during 1600's the time of Dutch Occupation. Some researcher believe it came to India through Burma (it is now call as Myanmar) traders[17]. India is now placed at 5th largest producer of soybean in the world today[18]. *Soybean* (*Glycine max* (L) Merr.) belongs to family Fabaceae[4]. Soybean are cultivated in the month of August in Arunachal Pradesh, because august is favorable condition for the growth and germination of soybean seeds. August to September is a month where soybean gets the amount of required water and minerals for their healthy seed germination. August is considered to be the growing season of the soybean plants. Soybean is a short provide a day plant, that require a short amount of sunlight for their growth, and the month of august and September receive short amount of sunlight due to rainfall and cloudiness. First week October is the end of the rainy season and also the month of soybean flower to bloom and in the end of October, flowers get fertilize. November is onset (beginning) of the winter season in Arunachal Pradesh and also the month for pods to get fully matured and the harvesting of the crops begins on the last week of November and December. For a small-scale production Arunachalee people usually grows them in a plot of kitchen gardens but for a larger scale of soybean production they prefer shifting cultivation methods.

Soybean is annual herbaceous plant with 30-40cm tall, trichomes. Leaves usually trifoliate, acute leaf, net venation, petiole 5-15cm. Flower is located at the axillary part, inflorescence-raceme, Bisexual flower, corolla dark purple with light/white purple, pistil present, stamen(filament and anther) present, bilateral. Fruit are hairy pods or legumes contain 2-6 seeds within, mature fruits are yellow or brown color, tap root system[4]. Fermentation is a traditional method to preserve a food for a longer duration and to sustain it natural sources. Fermentation can be possible because of anaerobic process (break down of food naturally by the presence of microorganisms like Bacteria, yeast, fungi etc). Fermentation process is said to have complex ecosystem which leads to changes in nutritional and biochemical properties[14]. Traditional fermentation method is mostly practices in North East States of India like Arunachal Pradesh, Meghalaya, Mizoram, Tripura, Assam etc.

Adi tribes of Arunachal Pradesh name soybean as *Peron ame* which*, Peron* means soybean and *ame* means seeds while fermented soybean is called *Ronyang namsing* (*Ronyang* – soybean*, namsing* - ferment). Arunachalee people have been practicing soybean fermentation for centuries. And they have been using their own traditional method in different ways among the 29 districts of Arunachal Pradesh. Because of its diverse culture and tradition, all the 29 districts of Arunachalee people have their own Mother tongue due to which they recite/named fermented soybean differently among all the tribes like Perupeha by Apatani tribe, *Ronyang namsing* by Adi tribe, Agya by Galo tribe, yakgya by Tagin tribe and Lip chhuro by the Monpa tribe[16].

In the present investigation isolation and identification of microorganisms from the fermented soybean food was carried out – prepared by Adi tribe of Arunachal Pradesh.

Sample collection and study area :

Soybean sample was collected from

Depi Village, East Siang District of Arunachal Pradesh. Longitude (27°50'44.7 "N) and Latitude (95°13'51.0 "E).

Methods of soybean fermentation :

1. Collected soybean seeds, Wash up the soybean seeds with clean water for 2 or 3 times.
2. Take a clean multipurpose pot and add 250g of soybean seeds into it.
3. 250 grams of soybean seed was left to soak overnight in one and half liter of clean water.
4. Take the soak soybean seeds and add into any clean cooking pot. Add 2 liters / any required amount of clean water into it (cooking pot).
5. Cook the soybean seeds for 3-4 hours until seeds get soft (check with the help of skimmer/solidspoon).
6. Strain out the excess water from the hot cooking pot (with the help of strainer pot) Leave the cooked soybean seeds in strainer for 4-5 minutes (So that excess water gets vaporize).
7. Take a clean Dong leaf (*Phrynium placentarium*), Wrap the cooked soybean seeds in Dong leaf.
8. After wrapping it on a leaf it is then left to smoke just above the fire place for 3-4 days (Fermentation process started).
9. Complete fermentation can be seen in day-5-6, Fermented beans can be used more than 1 month, if it is stored in an air tide condition under control temperature (refrigerator).

Microbial analysis :

Isolationof Fungi: 100 ml of distilled water with 3.9 g (PDA) potato dextrose agar media was kept in Hot water bath until the particles get fully dissolved and was again sterilized by autoclaving at 15lb pressure at 121°C temperature for 15 minutes. Under the laminar air flow every required material were first sterilize by giving UV light for 15 to 20 minutes. Air blower is ON during the work under laminar air flow. Antibiotic called Streptomycin 400mg with 100ml of distilled water was added under aseptic condition i.e inside the laminar air flow. Then, the plating of PDA media was done and kept it for 30 minutes to solidify under the aseptic condition. After the solidifying, the sample *i.e* fermented soybean was placed over the surface of the plates by the help of forceps or tweezers. Plates was then placed inverted position inside the incubator for 5-7 days at 28±2°C to observe the colonies of the fungi. Various colonies was formed on the petriplates, and by using spore plating technique (Dr. Ingold,1949), distinguishable fungi was inoculated with the help of inoculating loop in the PAD media under aseptic condition and incubate at 28±2°C for 5-7 days to obtain the pure culture (Koch,mid-19th century).

Growth culture, fungi were identified based on their visual characteristics. By using Light microscope, the microscopic morphology characters of fungi were determined either the microorganisms are septate or non-septate hyphae and fruiting bodies

Staining techniques of fungi :

Procedure {Lactophenol Aniline Blue (LAB) technique or Lactophenol Cotton Blue technique (Dr. Claypole, 1923) is used for visualizing fungal structure such as hyphae,

spore and cell wall}. Here small portion of fungal culture specimen was taken on a slide and aniline blue (0.1% to 1%) was added on top the fungal specimen on the slide. And coverslip was placed over the slide and gently press down to spread the solution and to flatten the specimen, aniline blue solution is kept for minimum 2 minutes so it can penetrate inside for visibility of fungalstructure, and the excess aniline blue solution was dried up by using tissue or filter paper. And the slide was placed under the microscope for the identification of specific fungi.

Isolation of bacteria & media preparation:

Different kind of media were used during the bacterial culture. They are PDA and Mac Conkey. Firstly, PDA media was used for mixed culture. Secondly, Mac Conkey was used for pure culture and it inhibits the growth of unwanted bacteria.

Preparation of PDA media and MacConkey Agar:

100 ml of distilled water with 3.9 g (PDA) potato dextrose agar media was kept in Hot water bath until particle get dissolved and again sterilized by autoclaving at 15lb pressure at 121°C temperature for 15 minutes. Under the laminar air flow every required material were first sterilize by giving UV light for 15 to 20 minutes. Air blower is ON during the work under laminar air flow. Then, the plating of PDA media was done and kept it for 30 minutes to solidify under the aseptic condition. After the solidifying, the sample *i.e* fermented soybean was placed over the surface of the plates by the help of forceps or tweezers. Plates was then placed inverted position inside the incubator for 24 hours at 30°C to observe the colonies of the bacteria.

MacConkey agar (5.5g) was treated with 100ml of distilled water and was mixed thoroughly with the help of glass rod. And the mixture of solution was kept in hot water bath for atleast 10 to 15 minutes so that the small particles get fully dissolved. And again, it was transfer into the autoclave for sterilization at 15lb pressure at 121°C temperature for 15 minutes.

And under the aseptic condition the cultured bacteria from PDA media was transferred to MacConkey agar.

*Streak plate method (*Robert Koch, *1881):*

Streaking method was used under the aseptic condition with the help of sterilized inoculating loop or needles for transferring of bacterial cultural from PDA media to MacConkey agar media. And for second time use inoculating loop were heated 3 to 4 times under the Bunsen burner for sterilization. Then the Petri plates were placed inverted position inside the incubator for 24 hours at 30°C.

{Gram Staining of Bactrial Technique (Hans Christian Gram, 1884) was used for the Identification of Bacteria}

Identification of Bacteria :

Specimen was identified as Gram-negative rod shaped bacteria.

Confirmation of bacteria by using VITEK® 2 GN techniques (author name) :

The VITEK® 2 Gram-Negative

identification card (GN) is intended for use with VITEK® 2 Systems for the automated identification of most clinically significant fermenting and non-fermenting Gram-negative bacilli. The Gram-Negative card is based on established biochemical methods 1,2,4,8,9,10, 11,12,17,18,20,21,24,25,27 and newly developed substrates measuring carbon source utilization, enzymatic activities, and resistance.

Results of Sample found :

1) Result of identified fungi :

Sample I: From singly mycelium, conidiophores are arising,branched like brush structure can be seen near the apex, conidiophores bearing conidia. It is identified as *Penicillium* spp.

Sample II: Mycelium septate, closely submerged in culture. Ovoid to fucoid structure have been seen. Forming short chain of budding,conidiophores lacking, mostly cover with conidia and conidia hyaline. It is identified as *Candida albicans*.

Sample III: Conidiophores dendroid with numerous branched,erect and hyaline. Conidia borne single at the apex of branches It is identified as *Monosporium rubrum*. Those speciemens were able to characterize and identified by the help of **Illustration genera of IMPERFECT FUNGI**[2].

2) Result of identified bacteria :

The bacteria specimen was identified as Gram-negative bacteria, *Sphingomonas paucimobilis*.

Identification Information	**Analysis Time: 7.80 hours**	**Status:** Final
Selected Organism	87% Probability	***Sphingomonas paucimobilis***
	Bionumber:	0601512050000000
ID Analysis Message		

Result of biochemical test of bacteria:

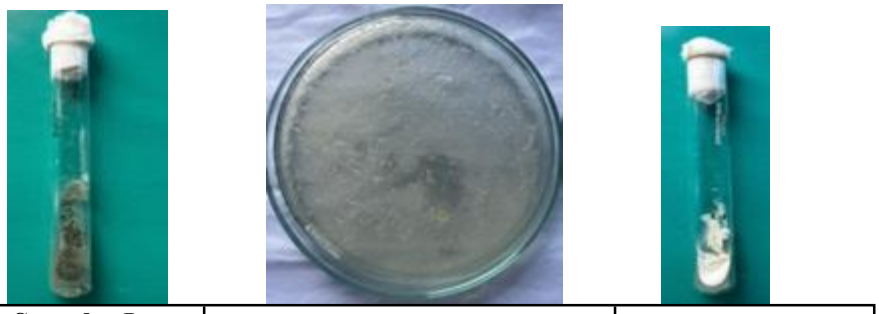
Biochemical details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	1ARL	-	7	dCEL	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	15	OFF	-
17	BGLU	+	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	22	BAlap	-
23	ProA	-	26	LIP	+	27	PLE	-	29	TyrA	-	31	URE	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	39	5KG	-
40	1LATK	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	57	BGUR	-
58	O129R	-	59	GGAA	-	61	1MLTa	-	62	ELLM	-	64	1LATa			

Gram negative (GN Well contents table) :

Well	Test	Mnemonic	Amount/Well
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	0.0384 mg
3	ADONITOL	ADO	0.1875 mg
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018 mg
5	L-ARABITOL	lARL	0.3 mg
7	D-CELLOBIOSE	dCEL	0.3 mg
9	BETA-GALACTOSIDASE	BGAL	0.036 mg
10	H2S PRODUCTION	H2S	0.0024 mg
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	0.0408 mg
12	Glutamyl ArylamidasepNA	AGLTp	0.0324 mg
13	D-GLUCOSE	dGLU	0.3 mg
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0228 mg
15	FERMENTATION/ GLUCOSE	OFF	0.45 mg
17	BETA-GLUCOSIDASE	BGLU	0.036 mg
18	D-MALTOSE	dMAL	0.3 mg
19	D-MANNITOL	dMAN	0.1875 mg
20	D-MANNOSE	dMNE	0.3 mg
21	BETA-XYLOSIDASE	BXYL	0.0324 mg
22	BETA-Alanine arylamidasepNA	BAlap	0.0174 mg
23	L-Proline ARYLAMIDASE	ProA	0.0234 mg
26	LIPASE	LIP	0.0192 mg
27	PALATINOSE	PLE	0.3 mg
29	Tyrosine ARYLAMIDASE	TyrA	0.0276 mg
31	UREASE	URE	0.15 mg
32	D-SORBITOL	dSOR	0.1875 mg
33	SACCHAROSE/SUCROSE	SAC	0.3 mg
34	D-TAGATOSE	dTAG	0.3 mg
35	D-TREHALOSE	dTRE	0.3 mg
36	CITRATE (SODIUM)	CIT	0.054 mg
37	MALONATE	MNT	0.15 mg
39	5-KETO-D-GLUCONATE	5KG	0.3 mg
40	L-LACTATE alkalinization	lLATk	0.15 mg
41	ALPHA-GLUCOSIDASE	AGLU	0.036 mg
42	SUCCINATE alkalinization	SUCT	0.15 mg
43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.0306 mg
44	ALPHA-GALACTOSIDASE	AGAL	0.036 mg

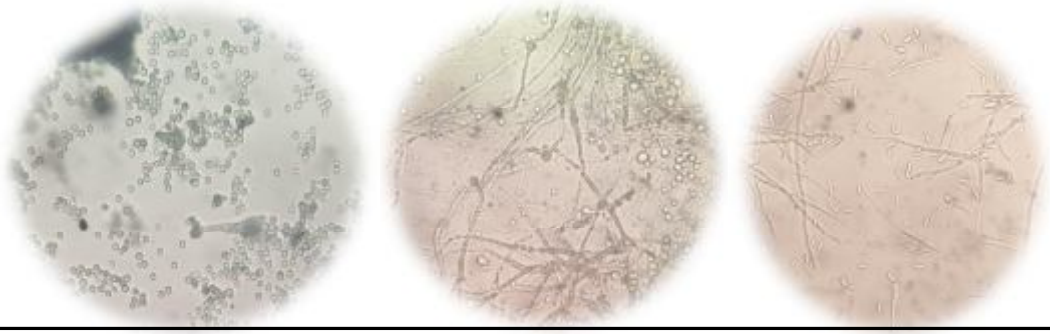
45	PHOSPHATASE	PHOS	0.0504 mg
46	Glycine ARYLAMIDASE	GlyA	0.012 mg
47	ORNITHINE DECARBOXYLASE	ODC	0.3 mg
48	LYSINE DECARBOXYLASE	LDC	0.15 mg
52	DECARBOXYLASE BASE	0ODEC	N/A
53	L-HISTIDINE assimilation	IHIHa	0.087 mg
56	COUMARATE	CMT	0.126 mg
57	BETA-GLUCURONIDASE	BGUR	0.0378 mg
58	O/129 RESISTANCE (comp.vibrio.)	O129R	0.0105 mg
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	0.0576 mg
61	L-MALATE assimilation	IMLTa	0.042 mg
62	ELLMAN	ELLM	0.03 mg
64	L-LACTATE assimilation	ILATa	0.186 mg

Photoplates : 1. Photoplates of isolated and identified Fungi



Sample. I | **Sample. II** | **Sample.III**

A. Pure culture



Sample. I *Penicillium* spp. Sample. II *Candida albicans.* Sample. III *Monosporium rubrum*

B. Microscopic View

Fig. 4. Fungal colony isolated from fermented soybean food.

2. Photoplates of isolated and identified Bacteria :

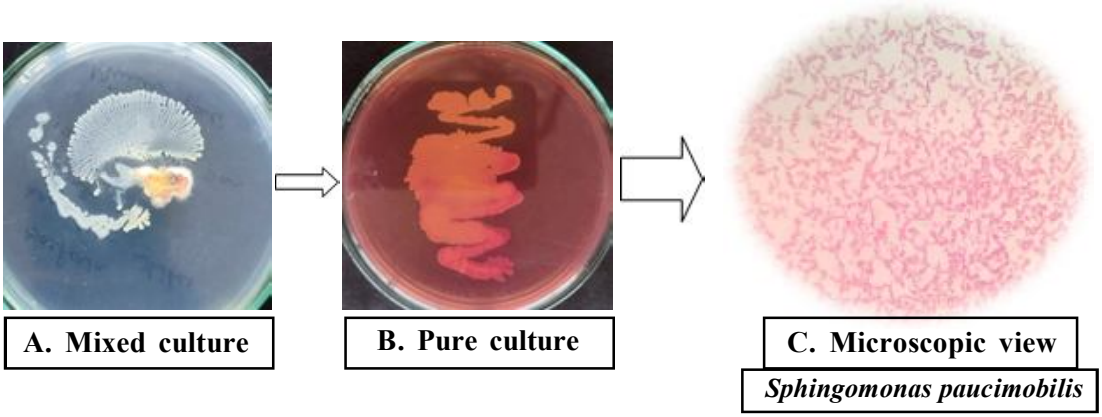


Fig. 5. Bacterial isolated from fermented soybean food.

In their work it is stated that Soybean is important source of protein for both humans and animals consumption. Soybean is said to have lowering blood cholesterol and it reduce the risk of having breast cancer and athero-sclerosis. And also stated that soybean contain anti-nutritional compound which is specially harmful for infant. Having uncooked with improper process of soybean is harmful which trigger the anti-nutritional compound i.e trysin inhibitors, lectics, flatulence[7].

In other state of North East India like Meghalaya also practices soybean fermentation. Garo and Khasi tribes of Meghalaya region practices tungrymbai food fermentation which a ethnic fermented indigenous soybean food in their region. They consume it as a side dish. And this soybean provides high amount of proteins in their daily local diet. And it is said that 45.9g % protein is contain in tungrybai on dry weight basis, where fat (30.2g %), fibre (12.8g %) and ash(5.5g%) contain have been found[12].

Fermentation of soybean degrade the allergenicity food by microbial proteolytic enzymes. Fermentation has the ability to hydrolysis soybean protein into smaller peptides, example-soybean allergen *i.e* Glym Bd 30K can be break into peptides and amino acids[5]. Figure 2, shows the traditional ways of preparation among the Adi tribes of Arunachal Pradesh. This traditional technique is mostly used among all the Arunachalipeople,such as Apatani, Galo, Tagin, Monpa etc. And it is mostly seen that fermented soybean are only used as a side dish in locality of Arunachal Pradesh but in compare to other country like Korea, soybean are commercially use in a large quantity for the production of Ganjang and Doenjang which means Soybean sauce and Soybean paste respectively[9]. And in China, soybean are used for the production of Sufu or Furu which is a soft creamy chesse[6].

In the study soybean (*Glycine max.* L) is naturally rich in nutritional values. Soybean have high protein content, minerals, vitamins and bio-actives. Soybean is poorly digestible, due to which fermentation is technique proven to improve flavor, texture and nutritional

quality of the soybean and also fermentation is said to be a contributor towards the preservation of food for the long period of time [15].

According to them several species of *Bacillus* were found to be predominant in the fermented soybean [13,3,19,8,12,14]. In microbial analysis of fungi- *Penicillium* ssp., *Candida albicans* and *Monosporium rubrum* were obtained. And these result were quite similar with the result of [9,14]. In fungal species like *Mucor, Penicilium, Scoplariopsis, Candida* and *Saccharomyces as been* oberseved[9]. And in *Penicilium, Aspergillus, Trichothecium, Paecilpmyces, Fusarium, Candida* and *Monosporium* etc. are said to be found after complete food fermentation[14].

In Sufu (Chinese fermented soybean food) consider to have antibacterial properties *i.e* NaCl (5-15%) and ethanol (1-7%). *Bacillusspp and Clostridium* spp. aresaidto be grow as salt tolerant bacteria[6]. In Doenjang (Traditional Korean soybean fermented food), *Bacillus* spp. *and Aspergillus oryzae* used to play the major role in the fermentation process[9].

The isolated and identified bacteria is yellowish in color and has Gram negative rod shape, this statement match with work were it is stated that *Sphingomonas paucimobilis* is a strictly aerobic Gram negative rod shape bacteria, non-fermented and have yellow pigmentation[10].

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