J. Mycopathol, Res, ***47(2)*** *: 181—186 (2009)*

Soybean seed microbes in post harvest indigenous food (*Hawaijar*) processing

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Hawaijar is indigenously semi-fermented processed food of soybean (*Glycine max*) and is used as a flavouring item in vegetables by the valley people of Manipur throughout the season. It gives good flavour and taste when mixed with other vegetable items. The fermentation process of *hawaijar* is not a total anaerobic, it is caused by microorganisms. Assessment of these microbes in surface sterilized and unsterilized soybean seeds revealed eight fungal species, a white sterile mycelium and 2 bacterial species (*Bacillus subtilis* MTCC 9228 strain A and *Bacillus ehimenis* MTCC 7666). The bacterial species were dominated by fungi and the diminant fungi include *Aspergillus niger, A. flavus. A. ochraceus, Penicillum* sp., *Rhizopus stolonifer, Fusarium solani, Cladosporium cladosporoides* and *Curvularia lunata.* Isolation, identification, seasonal variation and their effective roles in the processing of *hawaijar* are discussed in this paper.

Key words: Hawaijar, fermentation, microflora, phylloplane, soybean, bacteria.

INTRODUCTION

Glycine max (L). Merrill is known as protein and oil rich food crop. It is used in various forms. The fermented form of soybean is used extensively in the mongoloid countries in particular and other non mongoloid communities of South East Asia. In Manipur, soybean seeds are used more in fermented form throughout the season in the preparation of *hawaijar* which is a very popular product. *Hawaijar* is either consumed directly or as a flavouring agent in vegetable items to make the vegetables more soft and tasty.

Although there are many varieties of soybean, two varieties namely the local variety with small seeds and bigger round seeded variety JS 335 are especially used in the preparation of *hawaijar.* However, *hawaijar* prepared from local variety is more preferred because of its unique and better taste. A special curry of the manipuris called *'Chagempomba'* is prepared exclusively from *hawaijar.*

In the preparation of *hawaijar,* leaves of *Ficus hispida* are used as a wrapping material to provide better taste as compared to *hawaijar* wrapped in other packing materials like cotton cloth, etc. As these leaves play an important role in the preparation of *hawaijar* they are locally known as *"hawaijar mana"* (*hawaijar* leaves). It is also known as *ashee heibong.* The fermentation occurs due to the action of microbes, and therefore, microorganisms associated with the soybean seeds and leaf surface of the wrapping materials have been studied in the present study coupled with the effects of potential bacteria in the preparation of *hawaijar.*

MATERIALS AND METHODS

Microflora of soybean seeds **:** Two varieties of soybean viz, local variety and variety JS 335 were collected in bulk (approx. 5 kg each) from *Ima* market, Imphal, during March 2005 and brought to the laboratory in sterile polythene bags for use during entire period of investigation. As per ISTA (1966), to isolate the external seed microflora, 400 seeds of each variety were randomly taken from the bulk and plated in Petridishes (9 cm diam.) containing Potato Dextrose Agar (8 seeds in each plate) and incubated for 7 days at 25±1°C. Ten replications were maitained for each variety. For the isolation of endophytically carried seed microbes, the seeds were surface sterilized with 0.1% mercuric chloride for 1–2 min and washed 3-4 times with sterile distilled water before plating (ISTA, 1966). Seasonal variation of microbes of the surface sterilized and unsterilized seeds was made during summer, rainy and winter seasons.

Phylloplane microflora of wrapping leaves : Fresh leaves of *Ficus hispida* were collected from localities in Imphal (Yaiskul, Sagolband, Uripok) where *hawaijar* is made and brought to the loboratory in sterile polythene bags. Leaf impression method (Potter, 1910) and washing leaf disc method (Dickinson, 1982) were employed for the study. In the leaf impression method the leaves were gently pressed on Potato Dextrose Agar (HiMedia M096) plates and incubated at 28±1°C for 48 hrs. In the washing leaf disc method, 100 leaf discs (5 mm diameter) were cut using sterile cork borer representing entire leaf surface and these were placed in a conical flask containing 100 ml of sterile distilled water. Flask containing the leaves was shaken for 15 minutes in order to wash away the microorganisms adhering to the leaf surfaces. Serial dilution of the washed solution was made dilution up to 10^{-4} and 1 ml from each dilution (10^{-1} to 10^{-4}) was poured in Petridishes containing PDA (20 ml). Five replications were made for each dilution and the plates were incubated for 5-6 days at 28±1°C. Microbial population/ cm sq was calculated by using the formula

$$\text{Propagules/cm}^2 = \frac{\text{Total number of spores in 1ml}}{\text{Total area of 50 discs} \times 2} \times 100$$

Characterization of isolates : Initial characterization of the bacterial isolates included colony and cell morphology. Colony characteristics, viz. shape and size, pigmentation, surface of colony and consistency was done using a stereoscopic binocular microscope (SZ-PT Olympus). Cell morphology and motility were observed using a phase contrast microscope (Olympus BX61) (Harrigan, 1998). Bacterial cultures were sent to Institute of Microbial Technology, Chandigarh, India, for identification. Fungal isolates were identified with the help of relevant literature (Subramanian, 1971; Barnett and Hunter, 1972). The percentage frequency of occurrence of fungal isolates from soybean seeds was calculated as under Percentage Frequency of occurrence =

$$\frac{\text{Total number of individual species of fungi}}{\text{Total number of all the species of gun}} \times 100$$

Effect of microorganisms Isolated from soybean seeds and Ficus hispida leaves in the fermentation of hawaijar : Microbial isolates (both fungi and bacteria) from raw soybean seeds and *Ficus hispida* leaves were used to see their role in the fermentation of *hawaijar.* Soybean seeds of local variety were washed thoroughly and soaked overnight. They were then introduced into wide-mouthed bottles and autoclaved. On cooling down to room temperature, the autoclaved seeds were inoculated with pure cultures of microorganisms. For fungi, a loopfull of pure culture was used to inoculate the cooled autoclaved seeds. In case of bacterial cultures, they were streaked onto nutrient agar slant and incubated at 37±1°C for 16 hrs. Each of the cultures were suspended into 10 ml sterile 0.9% NaCl solution and diluted to give an absorbance of 0.03 at 540 nm in a spectrophotometer. 0.5 ml of this suspension was used to inoculate cooked soybeans for fermentation (Omafuvbe *et al.,* 2002). Qualitative characters such as taste, aroma, colour, texture and general acceptability of the fermented soybean were observed after 3 days. The organoleptic tests were carried out using a 10-man taste panellist who were very familiar with *hawaijar.* Each panellist was provided with 2 g of the test sample and asked to freely evaluate, comment and score the sample's taste, colour, aroma, texture and general acceptability using a score range of 1 (dislike extremely) to 5 (like extremely). To eliminate bias, the sample presented to a panellist at a time is not labelled and the panellists were served individually with sufficient privacy and at different times to guarantee independent judgement. Soybean seeds put in tightly capped bottles which are pasteurized but not inoculated (control) were also comparatively analyzed.

RESULTS AND DISCUSSION

From the two varieties of soybean seeds, 8 fungal species, a white sterile mycelium and 2 bacterial species (*Bacillus subtilis* MTCC 9228 strain A and *Bacillus echimensis* MTCC 7666) were recovered (Table 1). The bacterial species were dominated by fungi and the dominant fungi include *Aspergillus niger, A.flavus, A. ochraceus, Penicillum* sp., *Rhizopus stolonifer, Fusarium solani, Cladosporium cladosporoides* and *Curvularia lunata* as revealed by their occurrence during the year 2006-2007. Seasonal variation was observed for both fungal and bacterial isolates (Table 1). Total number of fungal species found in the unsterilized seeds during the summer season was 7 and 5 for local and JS335 variety respectively. From the unsterilized seeds of local variety, 8 fungal species were recovered during the rainy season

Table 1. Seasonal variation of soybean seed microbes and Percentage Frequency of Occurrence of fungal speices

Microbes	Summer season				Rainy season				Winter season			
	Local		JS335		Local		JS335		Local		JS335	
	Unst.	St	Unst.	St	Unst.	St	Unst.	St	Unst.	St	Unst.	St
Aspergillus niger	72.72	46.55	76.66	73.33	68.25	36.00	66.45	36.07	78.13	10.04	70.00	24.00
Aspergillus flavus	15.51	11.36	10.00	6.25	19.05	12.50	22.95	5.16	3.13	–	16.67	13.33
Aspergillus ochraceus	–	–	–	–	17.46	0.71	–	–	–	–	–	–
Cladosporium-cladosporoides	3.45	–	–	–	–	–	–	–	7.14	3.13	–	–
Curvularia lunata	1.44	–	–	–	1.20	–	–	–	–	–	–	–
Fusarium solani	6.81	5.17	3.33	3.13	6.05	-	3.87	0.28	–	–	11.11	3.33
Penicillium sp.	1.72	–	6.67	6.25	4.76	1.07	–	–	6.25	–	6.67	–
Rhizopus stolonifer	27.59	9.09	6.67	–	43.40	–	37.70	3.05	–	–	–	–
White sterile mycelium	–	–	–	–	4.76	–	–	–	14.28	9.37	7.00	5.56
Bacillus subtilis MTCC 9228 Strain A	+	–	+	+	+	+	+	–	+	+	+	–
Bacillus ehimensis MTCC 7666	+	–	–	–	+	–	+	+	+	-	+	+

Unst : surface not sterilized; St: surface sterilized; '+' present; '–' absent

whereas 4 fungal species were recovered from the unsterilized seeds of JS335 variety. During the winter season, 5 fungal species each were recovered from the unsterilized seeds of local and JS335 variety. During the summer and rainy season, the surface sterilized seeds of both varieties had 4 fungal species each. The total number of fungal species Isolated from the surface sterilized seeds of local and JS335 varieties during the winter season was 3 and 4 respectively. Variations in the occurrence of microbes were also noticed where observation was made during different seasons as well as for sterilized and unsterilized seeds. There was a variation in the occurrence of microbes for the two varieties of soybean. *A. niger* was the most dominant microbe for both the varieties on all seasons. *A.ochraceus* was found only in the local variety (both sterilized and unsterilized seeds) during the rainy season. *Rhizopus stolonifer* was found in the unsterilized seeds of both the varieties during summer and rainy season. In the sterilized seeds of local variety, *Rhizopus stolonfier* was found only during summer season whereas it was present in the sterilized seeds of JS335 variety only during the rainy season. *Fusarium solani* was isolated from the unsterilized seeds of local variety during the summer and rainy seasons and not from winter season whereas it was isolated from the unsterilized seeds of JS335 variety on all seasons. *Fusarium solani* was found in the sterilized seeds of local variety during the summer season only whereas it was present in the sterilized seeds of JS335 on all seasons. *Cladosporium cladosporoides* was present only in the unsterilized seeds of local variety during summer and winter but not during the rainy season whereas it was absent in the sterilized seeds except during winter. In the JS335 variety, whereas it was absent in the sterilized seeds except during winter. In the JS335 variety, *Cladosporium cladosporoides* was absent in all the three seasons whether the seeds are sterilized or not. *Curvularia lunata* was isolated only from the unsterilized seeds of local variety during the summer and rainy season but not during winter season. In the sterilized seeds of local variety and the sterilized and unsterilized seeds of JS335 variety, *Curvularia lunata* was absent (Table 1).

Percentage frequency of occurrence for unsterilized seeds of local variety during summer ranges from 1.44 (*C. lunata*) to 72.72 (*A. niger*) and for unsterilised seeds of JS335 variety during this season ranges from 3.33 (*F. solani*) to 76.66 (*A. niger*). For the sterilized seeds of local variety, the percentage frequency of occurrence during summer ranged from 5.17 (*F. solani*) to 46.55 (*A. niger*) and for the sterilized seeds of JS335, it ranged from 3.13 (*F. solani*) to 73.33 (*A. niger*). During the rainy season, the percentage frequency of occurrence for unsterilized seeds of local variety ranges from 1.20 (*C. lunata*) to 68.25 (*A. niger*) and that of JS335

unsterilized seeds was 3.87 (*F. solani*) to 66.45 (*A. niger*). The percentage frequency of occurrence of sterilized seeds of local variety during the rainy season ranged from 0.71(*A. ochraceus*) to 36.00 (*A. niger*) and that of sterilized JS335 ranged from 0.28 (*F. solani*) to 36.07 (*A. niger*). The percentage frequency of occurrence during the winter season ranged from 3.13 (*A. flavus*) to 78.13 (*A. niger*) for unsterilized soybean seeds of local variety and for unsterilized soybean seeds of JS335, percentage frequency of occurrence ranged from 6.67 (*Penicillium* sp.) to 70.00 (*A. niger*). During the winter season, the percentage frequency of occurrence ranged from 3.13 (*Cladosporium cladosporoides*) to 10.04 (*A. niger*) for sterilized soybean seeds of local variety and for sterilized JS335 seeds it ranged from 3.33 (*F. solani*) to 24.00 (*A. niger*). As for the bacteria, *B. subtilis* was isolated from the unsterilized seeds of both varieties on all seasons (Table 1). During the summer season, *B. subtilis* was present in the surface sterilized seeds of JS335 variety but not in the local variety whereas during the rainy and winter seasons, *B. subtilis* was present in the surface sterilized seeds of local variety and not in JS335 variety. *B. echimensis* was isolated from the unsterilized seeds of both varieties on all three seasons except for the unsterilized seeds of JS335 during summer. In the sterilized seeds of local variety, *B. ehimensis* was absent in all seasons whereas it was present in the sterilized seeds of JS335 variety during the rainy and winter seasons but not in summer.

The occurrence of many fungi and *Bacillus* sp. in raw soybeans observed in this study is in agreement with the reports of Hesseltine (1983) and Singh (1991) who reported that *Bacillus subtilis* and a number of fungi are present in the raw soybean seeds. These fungi were reported to be seed borne in soybean by a number of other workers (Bhuiyan and Fakir 1993; Gupta *et al.,* 1993; Anwar *et al.,* 1995). PDA was used for isolating the microorganisms from the surface sterilized and unsterilized soybean seeds of local and JS335 varieties. It resulted in the isolation of both fungal and bacterial species. The highest number of fungal species was obtained without surface sterilization of seeds as compared to the kind and number of fungi on the surface sterilized seeds. Surface sterilized seeds yielded lesser number of fungi than from the seeds without sterilization. *A. niger* occurred for the maximum number of times in the sterilized and unsterilized seeds of both varieties on all seasons. *B. subtilis* and *B. ehimensis* were the bacterial species recovered from raw soybean seeds (Table 1). Since most of the fungal species which were found in unsterilized seeds were also present in the surface sterilized seeds it could be said that they were present endophytically in the seeds. Karmaker and Subuddhiyopaphya (1980) isolated 12 species of fungi from India, 9 species by Zad (1982) from Iran. Nasir (2003) isolated a total of 39 species of fungi belonging to 16 genera viz. *Alternaria* sp., *Aspergillus* sp., *Botryodiplodia theobromae, Cladosporium* sp., *Chaetomium globossum, Colletotricum* sp., *Curvularia* sp. *Diplodia* sp. / *Drechslera* sp. *Fusarium* sp. *Macrophomina* sp. *Nigrospora* sp. *Phoma* sp., *Penicillium* sp., *Rhizopus* sp. and *Rhizoctonia* sp., from 6 month old soybean seeds from southern region of Pakistan. Hussain *et al.* (1989) reported 15 species of fungi from North West frontier province of southern region of Pakistan. More than 40 fungi, bacteria and viruses had been reported to be actively associated with the seeds of soybean (Hartman *et al.,* 1999) ranging from major pathogens of the seed to nonpathogens.

In case of phylloplane microflora of *Ficus hispida*

Table 2. Phylloplane microflora of *Ficus hispida* leaves

Microbes	LI
Aspergillus niger	32.50
Aspergillus flavus	17.50
Rhizopus stolonifer	5.0
Penicillium sp.	10.0
Mycelia sterile (White)	12.50
Bacillus subtilis MTCC 9228 strain B	+
Macillus smithii MTCC 7665	+
Microbacterium testaceum MTCC 9230	+

LI = Leaf impression; Data represent the mean of five replications '+' present

which is a wrapping material used for the fermentation of *hawaijar,* the leaf impression method revealed the occurrence of 4 fungal species viz. *Aspergillus niger, A. flavus, Rhizopus stolonifer* and *Penicillium* sp. and 3 bacterial species viz. *Bacillus subtilis* MTCC 9228 strain B, *Bacillus smithii* MTCC 7665 and *Microbacterium testaceum* MTCC 9230 (Table 2). *Aspergillus niger* had the highest percentage frequency of occurrence among the fungal species. The washing leaf disc method also revealed the occurrence of the three bacterial

species. *Bacillus subtilis* MTCC 9228 strain B was the most predominant bacterial species among the phylloplane bacteria of *Ficus hispida* leaves (Table 3). Isolation of bacteria and fungi from the leaf surface had been done by a number of workers (Chhetry *et al.,* 1996; Mishra and Dickinson, 1981). *Bacillus subtilis* was the predominant bacteria isolated from the leaves.

Table 3. Bacteria isolated from phylloplane of *Ficus hispida leaves using* washing leaf disc method

Bacteria	CFU cm^{-2}
Bacillus subtilis MTCC 9228 strain B	3.3×10^4
Bacillus smithii MTCC 7665	1.13×10^4
Microbacterium testaceum MTCC 9230	8.25×10^3

Data represent the mean of five replications

Soybean seeds were fermented using pure cultures of fungal and bacterial species obtained from raw seeds and *Ficus hispida* leaves in order to establish their roles in *hawaijar* formation. The seeds which were inoculated with pure culturres of fungal species obtained from raw soybean seeds and *Ficus hispida* leaves did not have features of *hawaijar* whereas those inoculated with *B. subtilis* MTCC 9228 strain A obtained from raw seeds, *B. subtilis* MTCC 9228 strain B and *B. smithii* MTCC 7665 obtained from *Ficus* leaves had the features of *hawaijar.* The seeds inoculated with *Microbacterium testaceum* (obtained from *Ficus* leaves) and *Bacillus ehimensis* MTCC 7666 (obtained from raw seeds) showed no feature of *hawaijar.* The boiled but not inoculated seeds act as a control and did not show any microbial growth (Table 4). This indicated that *Bacillus* sp. from the raw soybean seed and phylloplane of *Ficus* leaves acted as an inoculum for *hawaijar* production and played a very significant role in the production of *hawaijar.* The organoleptic properties of *hawaijar* prepared using pure culture of *Bacillus subtilis* MTCC 9228 strain B (obtained from *Ficus hispida* leaves) compared very well with *hawaijar* prepared using the traditional methods (Table 4). *Bacillus* sp. has been reported to ferment African oil bean into *ugba,* either separately or in combination and the leaves used for packaging were considered as major sources of the organisms responsible for fermentation (Njoku *et al.,* 1990). *Bacillus subtilis* has also been isolated from *owoh,* a Nigerian fermented seasoning agent from cotton seed (Sanni and Ogbonna, 1991) and soy-*daddawa* (Omafuvbe *et al.,* 2000). *Bacillus subtilis* in a monoculture fermentation of sterile soybean had been reported to produce *kinema* (Sarkar *et. al.,* 1993) and soy-daddawa (Omafuvbe *et al.,* 2002). At the same time, the fermented product obtained by inoculating the pure culture (singly) from *Ficus* leaves did not have properties like the one obtained by using the *Ficus* leaves as a whole (Table 4). *Hawaijar* prepared using *Ficus hispida* leaves as base material was more acceptable than the other products. This study indicates that the phylloplane microbes of *Ficus hispida* might act together in producing *hawaijar* with exceptionally good quality rather than using the organisms singly. So from this study it can be derived that the soybean seed and phylloplane microbes of *Ficus* leaves have a significant contribution in the production of *hawaijar.*

Table 4. Sensory evaluation of *hawaijar* produced by the traditional and modified methods using pure cutures

Sample	Organoleptic attributes				
	Colour	Taste	Aroma	Texture	General Acceptability
With leaf	4.20 ± 0.25	4.50 ± 0.24	4.60 ± 0.26	4.20 ± 0.25	4.60 ±0.22
Without leaf	3.50 ± 0.28	3.70 ± 0.22	3.50 ± 0.28	3.20 ± 0.23	3.90 ± 0.13
BSA	3.00 ± 0.21	3.50 ± 0.28	3.10 ± 0.28	3.20 ± 0.23	3.10 ± 0.28
BSB	3.20 ± 0.29	3.70 ± 0.33	3.80 ± 0.22	3.60 ± 0.31	4.10 ± 0.23
BSm	3.00 ± 0.21	2.50 ± 0.32	2.40 ± 0.34	2.20 ± 0.23	2.40 ± 0.28
BE	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Uninoculated autoclaved seeds (control)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

Values are mean scores ±SE (n=10) BSA : *Bacillus subtilis* MTCC 9228 strain A; BSB : *Bacillus subtillis* MTCC 9228 strain B, BSm : *Bacillus smithii* MTCC 7665, BE : *Bacillus ehimensis* MTCC 7666.

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(Accepted for publication July 23, 2009)