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Fatty Acid Composition, Antinutritional Factors, and Oligosaccharides Concentration of *Hawaijar* (An Ethnic Fermented Soyfood of India) As Affected by Genotype and *Bacillus Subtilis* Strain

L Sophia Devi[2], Vineet Kumar[1*], Anita Rani[1], Trupti Tayalkar[1], Priyanka Mittal[1], Aseem Kumar Anshu[1], Thangjam Anand Singh[2]

[1]ICAR-Indian Institute of Soybean Research, Indore, M.P., India
[2]Central Agricultural University, Imphal, Manipur, India
*Corresponding Author: Vineet.Kumar@icar.gov.in

Abstract: *Hawaijar* is an ethnic fermented soy product from the North-East region of India. The information on the effect of soybean genotype and the *Bacillus subtilis* strain used for inoculation for preparing the product on the fatty acid composition, the level of antinutritional factors, such as Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI), and oligosaccharides is not available. In the present study, *Hawaijar* was prepared from two soybean genotypes using two different strains of *Bacillus subtilis* and investigated for the concentration of the above-mentioned biomolecules. The results showed the significant effect of genotype used on oleic, linoleic, and α-linolenic acid of the product. KTI and BBI were found absent in *Hawaijar* prepared from these two genotypes and two strains. Flatulence-inducing factors like raffinose and stachyose were found in very low concentration irrespective of genotype and strain. Retention of sweetness-imparting sucrose and maltose concentration in *Hawaijar* was genotype-dependent.

Keywords: *Hawaijar*, Fermented soybean, Fatty acid composition, Protease inhibitors

INTRODUCTION

Globally, the inclusion of soybean in daily diet is being recommended to meet the daily requirement of protein and avail numerous other health benefits derived from nutraceutical molecules reported to be present in soybean (Kumar et al., 2010a; Hendrich et al., 2017). Apart from blending soybean flour with wheat flour to prepare Indian flat bread (*chapati*), which is considered the easiest mode of incorporation of soybean in a daily diet, several unfermented soy products, namely, soy chunks, soy granules, tofu and soy milk are available in the market across the country. However, in the North-Eastern region of the country which includes eight states, namely, Nagaland, Mizoram, Manipur, Arunachal Pradesh, Meghalaya, Assam, Tripura and Sikkim, several naturally fermented soy products like *Aakhone*/*axone* (Nagaland), *Bekanthu* (Mizoram), *Hawaijar* (Manipur), *Preuyaan* (Arunachal Pradesh), *Turangbai* (Meghalaya) and *Kinema* (Sikkim) are in the diet of ethnic population of the region since several centuries (Tamang, 2015; Singh et al., 2019). *Hawaijar* is an indigenous traditional fermented soybean product of Manipur state and also a staple in every household. It is alkaline and sticky in appearance. It can be consumed directly or used as a flavoring agent in vegetables throughout the year and its addition make the vegetables soft and tasty.

Traditionally, the preparation of fermented soybean, *Hawaijar*, involves overnight soaking of soybean followed by washing under running tap water. Then, they are boiled well in a pressure cooker till the seeds become soft and washed with hot water. After draining out excess water, the boiled seeds are wrapped in healthful clean *Ficus hispida* leaves and shifted in a bamboo basket covered with lid, called '*lubak*', which is covered with clean banana leaves. Subsequently, the basket is kept under the sun for fermentation. This is to maintain the high temperature required for *Hawaijar* formation. The palatable stage of fermented soybean reaches in 3-5 days. After fermentation, the soybean seeds develop a brown colour, sticky texture and a distinctive fermented odour and taste. Unlike other fermented soy-products from East and South-East Asian countries which have been evaluated for nutritional properties (Dajanta 2011; Jing et al., 2017). *Hawaijar* has not yet been biochemically characterized.

In the present investigation, *Hawaijjar* was prepared in the laboratory from two soybean genotypes, namely, a commercial cultivar JS335 and a landrace being grown in the North-Eastern region of India, using two different strains of *Bacillus subtilis*. *Hawaijar* products prepared were subsequently analyzed for the fatty acid composition, concentration of antinutritional factors, namely, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI), and oligosaccharides such as, sucrose and maltose which impart sweetness, and raffinose and stachyose which are responsible for flatulence associated with soy products.

MATERIALS AND METHODS

Genotypes used in this study were commercial cultivar JS335 and landrace of soybean (small seeded). Strain S and H of *Bacillus subtilis* were used in the preparation of *Hawaijar.*

Chemicals: Petroleum ether, sodium methoxide, acrylamide, bis-acrylamide, ammonium persulphate, tetramethylethylenediamine (TEMED), bromophenol blue dye, Kunitz trypsin inhibitor standard protein, coomassie brilliant blue dye, methanol, water, acetic acid, tris buffer, phosphate buffered saline, Bowman-Birk inhibitor standard protein, tween 20, bovine serum albumin, Bowman-Birk primary antibody, anti-mouse antibodies, p-nitrophenyl phosphate (pNPP), sodium hydroxide (NaOH), acetonitrile,

standards of sucrose, maltose, raffinose, and stachyose.

Preparation of Hawaijar

In the view of fact that it was difficult to maintain consistent quality control while making traditional fermented soybean - *Hawaijar*, therefore, in this study, *Hawaijar* was prepared in the laboratory. For this purpose, seeds of a commercial cultivar JS335 and land race soybean genotype (small seeded) were cleaned manually, dried in sun and stored in plastic containers for further use. Figure 1 illustrates the detailed method of preparation of *Hawaijar*. Quality fermented soybean *Hawaijar* prepared was investigated for biochemical analysis.

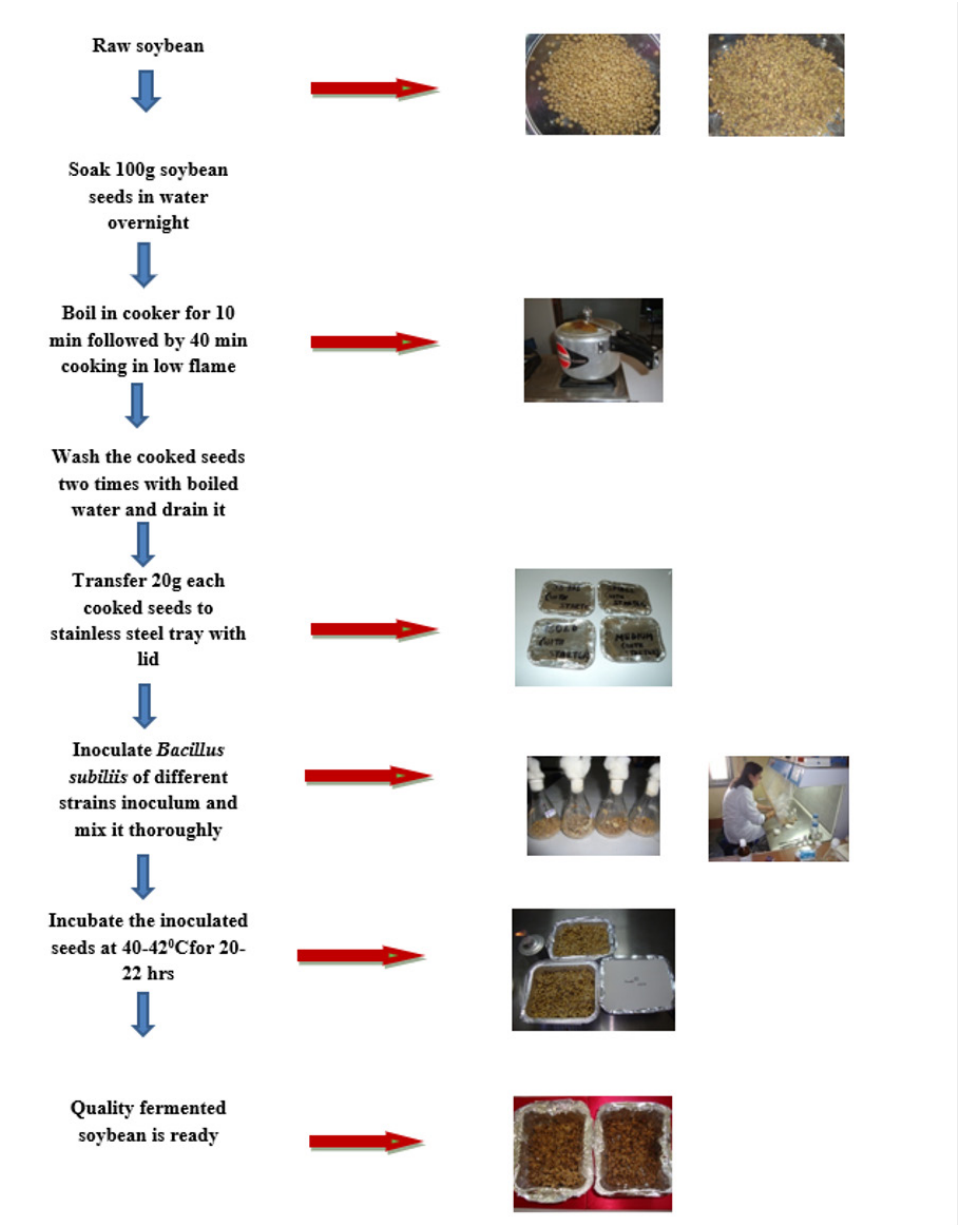

Figure 1. Flow Chart for fermented soybean- *Hawaijar* preparation using *Bacillus subtilis* starter culture.

Fatty acid composition

A small quantity (5 g) of freshly prepared *Hawaijar* from each tray was oven dried at 70°C until it became moisture free and was extracted using petroleum ether (boiling point 50°C) for obtaining oil. Fatty acid methyl esters of the oil extracted were prepared using 1N sodium methoxide and were resolved in gas chromatograph Shimadzu GC17A instrument (Kyoto, Japan), fitted with a capillary column (SGEBPX70, 30m×0.32mm×0.25µm). Programming of the oven was as follows: at 140°C for 3.6 min, then increased to 170°C at a rate of 13.5°C min^{-1} and maintained for 3.8 min, and finally increased to 182°C at a rate of 5°C min^{-1}. The flame ionization detector (detection limit: 3×10^{-12} g/s for diphenyl, dynamic range: 10^7, operational temperature range: ~ 450°C in 1°C increment) and injector (number of individually controlled zones: 2, temperature range: ~ 450°C, both heating and cooling step program) were maintained at 240°C. Nitrogen was used as the carrier gas. Peaks obtained for fatty acid methyl esters

were identified by comparing the retention times with those of standard fatty acid methyl esters (Sigma-Aldrich, Bangalore, India) (Rani et al., 2019). The quantification of fatty acid methyl ester was carried out through Class GC 10 software. The data presented in Table 1 are the average content of triplicate determinations.

KTI estimation using Densitometry

Finely ground powder (50 mg) of the product was homogenized in 30 volumes of double distilled water. The homogenized preparation was incubated for 3 h in a shaker incubator at room temperature followed by centrifugation (refrigerated benchtop centrifuge, MIKRO 22R, Hettich, dimensions H×W×D: 278×333×620 mm, running time: max. 99 min, max speed: 18000 min^{-1}, temperature: -20°C to +40°C) at 10644 g for 10 min. The supernatants were collected mixed with bromophenol blue dye (3:1) and loaded on to the 10% native polyacrylamide gel. Polypeptides, along with the standard KTI (2 µg) were resolved by applying 50 mA current for 45 min. After completing the run, the gel was stained with 0.25% coomassie brilliant blue followed by destaining using methanol/water/acetic acid in the ratio of 45:45:10. The images were captured by Bio-Rad imaging system GS-900. KTI (mg/g flour) was calculated by comparing its density with KTI standard of known concentration using software *Image Lab 5.2.1* (Bio-Rad**,** India). KTI activity (mg trypsin inhibited/g of soy flour) was calculated by multiplying KTI concentration by 2.51 as described elsewhere (Kumar et al., 2018).

Bowman-Birk inhibitor estimation using ELISA

Extraction

Finely ground powder (100 mg) of the product was suspended in 1 ml of 50 mM tris buffer (pH 8.2) and homogenized using polytron homogenizer (Kinematica, Model PT2100, Switzerland) followed by ultra-sonication of samples for 1 h. To ensure adequate suspension, samples were vortexed after every 15 min. Subsequently, the suspension was centrifuged at 20,000 g for 35 min at 4°C. The resulting supernatant was diluted 10,000 times using phosphate buffered saline. Standard curve ($y = 0.03x + 0.3146$, $R^2 = 0.996$) was generated using purified BBI. Working range for BBI standard was from 1 ng to 50 ng.

Immunoassay for quantification

Diluted supernatants were coated on 96-well Maxisorb Nunc immunoplates in triplicate (100 µl/well) by incubation at 4°C for overnight (15-18 h) followed by 4 washing with buffer (PBS + 0.05% Tween 20) using Microplate washer (Immunowash 1575, Bio-Rad Laboratories India Private Ltd). Blocking of uncoated walls was performed using 5% bovine serum albumin + 1% Tween 20 in phosphate buffered saline for 2 h (with interval shaking) at 37°C followed by 5 washings. A fifty-microliter volume of diluted (1:1000 in 3% BSA buffer solution) Bowman-Birk primary antibody was added to each well and incubated for 2 h with shaking at an interval. After washing, 50 µl volume of diluted (1:1000 in 3% BSA buffer) alkaline phosphatase conjugated anti-mouse antibody was added to each well and incubated for 2 h at room temperature. Unbound conjugated anti-mouse antibodies were removed by repeated washing followed by addition of 100 µl of p-nitrophenyl phosphate (pNPP). The plate was immediately sealed with black polyvinyl seal and incubated at 37°C. Reaction was stopped at 30 min using 3N NaOH (100 µl/well). Absorbance was recorded using ELISA plate reader (Multiscan Go from Thermo Scientific Pvt Ltd. India) at 405 nm.

Estimation of Sugars in soy-products through HPLC

Extraction of sucrose, raffinose, stachyose, maltose from this soy based product was carried out following the method as described elsewhere (Liu and Markakis, 1987). The extracted sugars were filtered through a syringe membrane filter (0.22 µm, 13 mm diameter) and 20 µl of sample was injected into Shimadzu high performance liquid chromatography system (LC10AT *vp,* Shimadzu Analytical Pvt. Ltd. Mumbai, India). The separation of sucrose, raffinose, stachyose and maltose were achieved using a silica NH_2 column (5 µ, 250×4.6 mm, Phenomenex Luna, Hyderabad, India), preceded by a guard column, maintained at 40°C in Shimadzu CTO 10AT *vp* oven. The mobile phase, acetonitrile/water (75/25 v/v), was run isocratically at a flow rate of 1.0 ml/min and the elution was monitored by means of a refractive index detector (Shimadzu, RID10A, Shimadzu Analytical Pvt. Ltd. Mumbai, India). Identification of the peaks in the sample were done using the retention time of the peaks of different sugars, which were 7.1, 8.2, 9.7, and 13.8 min for sucrose, maltose, raffinose and stachyose, respectively (Figure 2). Quantification of these 4 sugars in the sample was done by comparing the area of the peaks of respective sugars in the sample chromatogram with that of the external standards, procured from Sigma Aldrich, using software CSW 1.7.

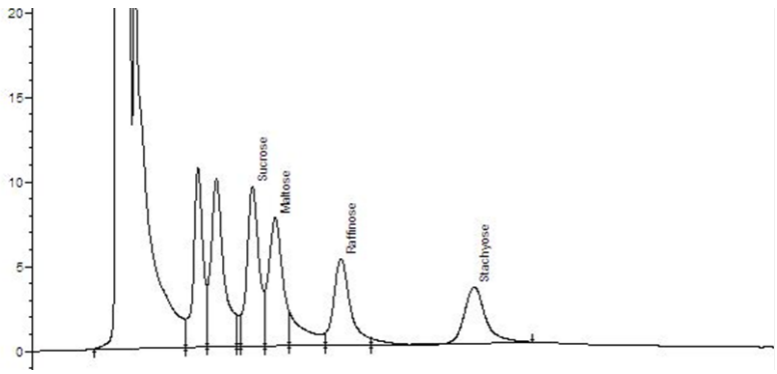

Figure 2. Chromatogram for separation of oligosaccharides, namely, sucrose, maltose, raffinose and stachyose using high performance liquid chromatography.

Statistics analysis

All the statistical analysis were carried out using SAS 9.09.

RESULTS AND DISCUSSION

Though soybean is primarily known for its high protein content, however, its oil fraction which constitutes about 20% of its dry matter and is a source of essential fatty acids is nutritionally equally important. Lipolysis of soybean grains occurring during the fermentative process may induce changes in the level of major fatty acids known to be present in soybean seeds. It may be due to the degradative/ digestive properties of enzymes present in the microbial population used during fermentation including *Bacillus subtilis* used in the present study.

The data presented in Table 1 shows the presence of 5 major fatty acids, namely, palmitic acid, stearic acid, oleic acid, linoleic acid, and α-linolenic acid in the fermented soy product prepared from two soybean genotypes, one of them is landrace while the other is a commercial cultivar of soybean, using two different strains of *Bacillus subtilis*. No significant ($P < 0.05$) differences were noted for oleic acid content of *Hawaijar* prepared in both landrace and commercial cultivar JS335 of soybean due to different strains (S and H). However, oleic acid content was significantly ($P < 0.05$) high in the soy products prepared from JS335 (irrespective of the strain used) compared to the product prepared from landrace.

Table 1. Palmitic, stearic acid, oleic acid, linoleic acid and α-linoleic acid content (%) of oil fraction extracted from *Hawaijar* prepared from two soybean genotypes using *Bacillus subtilis* strains

***Hawaijar* prepared from soybean genotype and *Bacillus subtilis* strain**	**Palmitic acid**	**Stearic acid**	**Oleic acid**	**Linoleic acid**	**α-Linolenic acid**
Land race + strain S	11.24±1.02^{b}	2.24±0.18^{a}	14.06±1.24^{a}	67.33±4.98^{b}	5.10±0.42^{a}
Land race + strain H	9.90±0.82^{a}	1.81±0.12^{a}	13.31±1.29^{a}	69.21±5.01^{b}	5.75±0.39^{a}
JS335 + strain S	13.82±1.03^{c}	1.50±0.14^{a}	20.61±1.89^{b}	58.20±5.04^{a}	5.80±0.50^{a}
JS335 + strain H	12.20±0.99^{b}	1.91±0.17^{a}	20.77±2.03^{b}	58.32±4.89^{a}	6.78±0.62^{b}

Values given are mean of triplicate determinations ± standard deviation. Values given with different superscripts are significantly ($P < 0.05$) different from each other.

With regard to linoleic acid, irrespective of the starter culture used, this essential fatty acid was significantly ($P < 0.05$) higher in the *Hawaijar* prepared from landrace than from the product prepared from the commercial variety JS335. With regard to α-linolenic acid, this essential fatty acid was found to be significantly ($P < 0.05$) higher in the *Hawaijar* prepared from both small seeded landrace and commercial variety JS335 using H strain of the *Bacillus subtilis* than the S strain. With regard to saturated fatty acids, palmitic acid, irrespective of the soybean genotype used as raw material, was found to be significantly ($P < 0.05$) higher in the *Hawaijar* prepared from the strain S than strain H. For stearic acid, no significant differences were noted across the variety and the strain used for preparing the *Hawaijar*. The results showed significant ($P < 0.05$) effect of variety × starter culture strain interaction on all the fatty acids except stearic acid of the soy products prepared.

Cho et al., (2017) investigated Korean fermented soybean paste from high protein cultivar '*Saedanbaek*' and oil rich cultivar '*Neulchan*' using *Bacillus subtilis* CSY191 and reported 12 fatty acids through GC-MS, though the study showed same five major fatty acids as observed in our results. Further, the presence of eicosanoic acid has also been reported besides palmitic acid, stearic acid, oleic acid, linoleic acid in a fermented soybean prepared using *Bacillus subtilis* (Kanghae et al., 2017). As evident from our results, eicosanoic acid was not detected in *Hawaijar* prepared in our study. On the other hand, instead of eicosanoic acid, *Hawaijar* showed the presence of α-linolenic acid, which is a well-known essential unsaturated fatty acid of soybean seeds. Palmitic acid, stearic acid, oleic acid, linoleic acid and α-linolenic acid reported in fermented soybean *okara* prepared using Bacillus species in an earlier study also supports our results (Mok et al., 2019).

One of the major concerns in inclusion of unfermented soy products in the daily diet in several countries where fermented products have not been able to permeate the cuisine of the masses, is the presence of antinutritional factor trypsin inhibitor. This antinutritional affects the protein digestibility (Gumbmann et al., 1986). The heat inactivation of trypsin inhibitor incurs extra cost, and the residual activity of this antinutritional factor lurks in the final product depending upon the temperature and the duration of heating applied. Further, two polypeptides, namely, Kunitz trypsin inhibitor (20 kDa) and Bowman-Birk inhibitor (8 kDa) constitute total trypsin inhibitor activity, though the former has been reported to contribute primarily to total trypsin inhibitor activity (TIA) (Kumar et al., 2019). Kunitz trypsin inhibitor and Bowman-Birk inhibitor vary in their heat labile property due to the different number of disulfide linkages, the former possesses only 2 disulfide linkages while the latter 7 disulfide linkages. With regard to KTI and BBI content, irrespective of genotype used as raw material and *Bacillus subtilis* strain deployed as an inoculant for preparing *Hawaijar*, both these protease inhibitors were in the undetectable range. Figure 3 and Table 2 shows the absence of Kunitz trypsin inhibitor (KTI) polypeptide corresponding to the lanes of *Hawaijar* product prepared from landrace + strain S, landrace + strain H, JS335 + strain S and JS335 + strain H.

Table 2 also shows the absence of BBI, as determined by ELISA, in the *Hawaijar* prepared from both the genotypes and both the strains. In the literature, we did not come

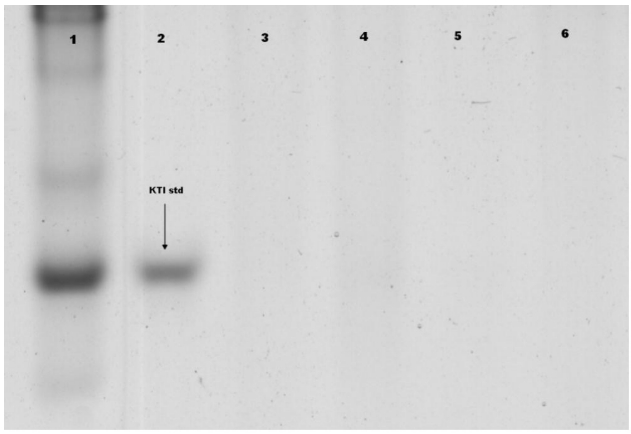

Figure 3. Native PAGE analysis of Kunitz Trypsin Inhibitor in *Hawaijar* prepared from two soybean genotypes using *Bacillus subtilis* strains. Lane 1-JS335, lane 2-KTI Std (2 ug), lane 3-Landrace +strain S; lane 4- Landrace +strain H; Lane 5- JS335 +Strain S; lane 6- JS335 +Strain H.

across any reference wherein the level of these two protease inhibitors have been investigated separately in any of the fermented soy product, and also no study available wherein trypsin inhibitor reduction has been reported in any fermented soy product wherein Bacillus culture has been used for carrying out fermentation. About 90% reduction in trypsin inhibitor activity has been reported in soybean through indirect method of urease index in a fermentative medium using *Lactobacillus* supplemented with saccharose (5%) (Victoria et al., 2016). However, urease method indicates only the degree of heat applied. It is not only the indirect method of estimation of trypsin inhibitor activity (TIA) but also does not distinguish KTI from BBI. Significant reduction in antinutritional factor in soybean meal after application of exogenous proteases in combination with *Bacillus subtilis* fermentation has been reported in a recent study (Cheng et al., 2019), and bioprocessed soymeal so obtained was reported to enhance the growth performance of broilers in this study. These authors reported reduction in α and β subunits of β-conglycinin fraction (7S) but did not specify the reduction of 20 kDa and 8 kDa protein which correspond to KTI and BBI, respectively.

Furthermore, significant reduction in trypsin inhibitor of bio-modified soymeal due to the fermentation carried out by *Aspergillus* + *Bacillus subtilis* culture has also been

Table 2. Sucrose, maltose, raffinose, stachyose, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI) content (mg/g of product) of *Hawaijar* prepared from two soybean genotypes using *Bacillus subtilis* strains

***Hawaijar* prepared from soybean genotype and using *Bacillus subtilis* strain**	**Sucrose**	**Maltose**	**Raffinose**	**Stachyose**	**KTI**	**BBI**
Land race, strain S	0.57 ±0.02^{a}	0.05±0.01^{b}	0.10±0.02^{b}	1.27±0.12^{b}	n.d.	n.d.
Land race, strain H	0.80±0.04^{a}	0.06±0.01^{b}	0.07±0.01^{b}	0.24±0.03^{a}	n.d.	n.d.
JS335, strain S	1.51±0.12^{b}	0.60±0.05^{a}	0.04±0.01^{a}	1.87±0.18^{c}	n.d.	n.d.
JS335, strain H	1.62±0.15^{b}	0.40±0.09^{a}	0.04±0.01^{a}	1.87±0.17^{c}	n.d.	n.d.

Values given are mean of triplicate determinations ± standard deviation. Values given with different superscripts are significantly ($P < 0.05$) different from each other, n.d. represents undetectable.

reported (Teng et al., 2012). The concentration of BBI in raw seeds of landrace and JS335 was 6.5 and 6.9 mg/g defatted flour, respectively. With respect to concentration of KTI, raw seeds of landrace and JS335 contained 6.36 and 11.4 mg/g flour, respectively. Undetectable concentration of KTI and BBI in the *Hawaijar* products in the present study may be attributed to the pressure cooking for 10 min on high flame followed by 40 min heating on low flame in addition to the *Bacillus subtilis* fermentation, which is known to release microbial proteases, which might have led to efficient degradation of both the polypeptides of trypsin inhibitor. Complete inactivation of KTI of soybean seeds due to boiling for 15 min as documented in our recent study (Kumar et al., 2019) supports these results.

Table 2 also presents the concentration of sucrose, maltose and flatulence-inducing factors, such as raffinose, stachyose in *Hawaijar* prepared from two genotypes and two strains of *Bacillus subtilis*. Sucrose content was very low in the *Hawaijar* prepared from both JS335 and landrace compared to the concentration of this oligosaccharide in soybean seed which is about 4% (40 mg/g) as reported elsewhere (Kumar et al., 2010b). However, sucrose content in the product prepared from JS335 showed slightly higher concentration than the landrace, though the effect of strain on the retention of sucrose content was not observed in both the genotypes. Jing et al., (2017) reported sucrose content to the magnitude of 2.44 mg/g dry weight in *Douchi* sample using *Bacillus subtilis* culture. In comparison to this value, sucrose value was slightly less than half in *Hawaijar* prepared from JS335 and about one third in *Hawaijar* from landrace. Like sucrose, maltose is also sweetness imparting molecule in soybean and soy product. Regarding maltose content, though no significant differences were noted due to different strains across both the genotypes, the concentration of this oligosaccharide was significantly ($P < 0.05$) higher in the *Hawaijar* prepared from JS335 than landrace. With regard to sucrose-derived flatulence inducing factors, such as raffinose and stachyose content, raffinose concentration (0.10, 0.07 mg/g) was statistically same in *Hawaijar* prepared from landrace using both the

strains. However, this value was significantly ($P < 0.05$) higher than in the product prepared from both the starter culture from cultivar JS335.

With regard to stachyose, its concentration in *Hawaijar* prepared from both the strains from genotype JS335 was same (1.87, 1.87 mg/g), and was significantly ($P < 0.05$) higher in *Hawaijar* prepared from landrace than commercial cultivar JS335. The results showed that in case of landrace the strain H was significantly ($P < 0.05$) more efficient in degrading the stachyose compared to the strain S as with the former strain barely 1.27 mg/g stachyose content was noted in the product while the later strain retained 0.24 mg/g stachyose in the product. The concentration of raffinose and stachyose content in the *Hawaijar* prepared from both the genotypes and strains are very low compared to their values to the magnitude of 0.5-1.0% and about 4%, respectively, in seed as reported in our earlier study (Kumar et al., 2010b). Jing et al., (2017) investigated the effect of fermentation by *Bacillus subtilis*, *Aspergillus oryzae*, *R. oligosporus* and *Aspergillus elegans* on the concentration of oligosaccharides in *Douchi*. These authors reported α-galactosidase activity in all the fermented soybean products, though invertase was the major enzyme responsible for the degradation of oligosaccharides in *Bacillus subtilis* fermented *Douchi*. Very low concentration of raffinose and stachyose in *Hawaijar* in the present study may be attributed to α-galactosidase and invertase activity released by *Bacillus subtilis* during the fermentation.

CONCLUSION

The study showed the significant effect of not only genotype used as raw material but also of *Bacillus subtilis* strain used on the fatty acid composition of *Hawaijar*. Both the protease inhibitors i.e., KTI and BBI were found to be absent in *Hawaijar* prepared from both the genotypes and both the strains of *Bacillus subtilis*. Raffinose and stachyose content were found to be in very low concentration in *Hawaijar* prepared irrespective of genotype and *Bacillus subtilis* strain used. The results also showed that selection of suitable genotype to be used as raw material is equally important for the sucrose and maltose content of the fermented soybean product.

Conflict of Interest

The authors declare no competing interest in this manuscript.

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