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NaCl-ADDED SOYBEAN FERMENTATION AND POST-FERMENTATION MOISTURE MODULATION: EFFECTS ON SAFETY AND SHELF LIFE OF A TRADITIONAL, NONSALTED ALKALINE FOOD

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ABSTRACT

The present study aimed to evaluate the effect of adding NaCl prior to fermentation and moisture reduction after the fermentation on the safety and shelf life of kinema, a *Bacillus*-fermented soybean food. The physicochemical parameters of kinema decreased with an increase in the concentration of NaCl up to 4 g/100 g where there was no significant change in cell count of the fermenting microorganism (*Bacillus subtilis*) and sensory score of the product but eliminated *Bacillus cereus*. *Escherichia coli* was reduced by 1 log cycle in the presence of NaCl but eliminated when the water activity (a_w) of kinema was reduced to 0.5 after fermentation. Salt had no effect on its shelf-life. Storage stability was determined in terms of its moisture sorption isotherms. Thermodynamic functions decreased with increasing moisture content. The monolayer moisture content ranged from 6.0 to 8.3 g/100 g and the optimum a_w for storage of kinema was ≤ 0.5 .

PRACTICAL APPLICATIONS

As producers do not adopt safety measures during its preparation, kinema is not always microbiologically safe for consumption. Through the present study it will be possible to eliminate pathogens like *B. cereus* and *E. coli* from kinema by the combined effect of salt and a_w . The enhanced safety level achieved opens up a future scope for exploring the possibilities of its use as a flavoring or food supplement ingredient. The sorption study revealed the ideal conditions for storage of kinema and can be utilized in the future for designing packaging materials that will ensure better food quality. The combination of salt and a_w is applicable to other traditional *Bacillus*-fermented legume foods, like Thai thua nao, Korean chongkukjang and African dawadawa.

INTRODUCTION

Kinema, a fermented food in the eastern Himalayan belt, is traditionally prepared by overnight soaking of soybean (*Glycine max* (L.) Merr.) seeds, cooking until softened, crushing to grits, wrapping and leaving at ambient temperature (25–35°C) for 2 days. *Bacillus subtilis* carries out the fermentation with the help of its strong proteolytic activity which results in the liberation of ammonia and consequent increase in pH of the beans to >8.5 (Sarkar *et al.* 1993). Completion of fermentation is indicated by the appearance

of a white viscous mass on the surface of the grits and a typical pungent odor. Kinema is cooked with salt to prepare curry which is eaten with boiled rice (Tamang *et al.* 1988).

Although the benefit of using salt as a preservative for several fermented foods is well known, salt is neither used in the preparation of kinema nor has there been any study to understand the effect of salt on kinema. Hence, our first objective was to study the effect of salt on the safety and shelf life of kinema.

Kinema is prepared at homes with little safety measures which lead to a compromised quality of the final product

(Sarkar *et al.* 1993). A survey on market samples of kinema in India revealed a high level of cells of *Bacillus cereus* and Enterobacteriaceae (Nout *et al.* 1998). Some strains of *B. cereus* produced diarrheal type enterotoxin. An improvisation in the traditional processing of kinema was achieved by the use of monoculture (*B. subtilis*) fermentation of soybeans (Sarkar and Tamang 1994), and this did not only produce organoleptically better kinema but *B. subtilis* also helped to reduce the number of *B. cereus* cells by 4 log (Nout *et al.* 1998). Although the former had an antagonistic effect on the latter, the pathogen was not eliminated and the risk of food poisoning persisted. Hence, we attempted to determine whether the survival and/or growth of foodborne pathogens could be controlled by using salt. We are aware that many governments and the World Health Organization want to reduce added salt. So, we planned not to exceed the total amount of salt that is traditionally used in cooking kinema to prepare curry.

The concept of water activity (a_w), which is defined as the ratio of the vapor pressure of water in equilibrium with a material to the vapor pressure of pure water at the same temperature, is utilized in constructing moisture sorption isotherms. The isotherms provide valuable information about the moisture-binding properties and help in designing drying processes, selecting suitable packaging materials and predicting stability and shelf life of a food (Sablani *et al.* 2007). The major concerns, while dealing with the storage and processing of various commodities, include monolayer moisture content, bound water, free energy change (ΔG), differential enthalpy and entropy. These help in a better understanding of the food microstructure, physical interactions and energy requirements for heat transfer processes occurring within a food.

Fresh kinema, being an intermediate-moisture food (62–75 g moisture/100 g), has a shelf life (2 days) which is often enhanced by drying kinema under the sun. No data on water relations of kinema are available which are required for optimization of its shelf life. Thus, the second objective of our research was to generate moisture sorption data of both fresh and dried kinema using sorption models at different temperatures, to determine various properties of sorbed water, fractionate bound water based on binding energies and estimate thermodynamic functions.

MATERIAL AND METHODS

Organisms

The microorganisms used were *B. subtilis* DK-W1 (MTCC 1747), *B. cereus* (ATCC 9139), *Escherichia coli* (MTCC 119) and *Staphylococcus aureus* (MTCC 1437). These strains were maintained on nutrient agar (M001; HiMedia Laboratories Pvt. Ltd., Mumbai, India) slants at 4°C.

Aseptic Preparation of Kinema

Small, yellow-seeded soybeans, collected from Kurseong town in the district of Darjeeling, India were washed and soaked in distilled water for 16 h at 25°C. The soaked beans were cooked in distilled water at 121°C for 15 min. On cooling to about 50°C, the water was decanted and the beans transferred to a sterile polyethylene bag and crushed with a pestle from outside to split the cotyledons and break them into grits. *B. subtilis* DK-W1, grown on nutrient agar (HiMedia M001) at 37°C for 16 h, was suspended in sterile distilled water and mixed with the grits so that the concentration of the total cells was 10^6 /g grits. About 50 g (wet wt) of inoculated grits were distributed in 250-mL sterile glass bottles. The bottles were loosely capped and incubated at 37°C (Sarkar 2000).

Physicochemical Parameters

To measure pH, 10 g beans were mixed with 20 mL CO₂-free distilled water and the mixture was homogenized (RQ-122, Remi Laboratory Instruments, Mumbai, India) for 15 s. The pH of the slurry was measured using a CyberScan pH 510 meter (Eutech Instruments Pte Ltd, Singapore). The method described by Nout *et al.* (1998) was used to estimate the free amino nitrogen. The assay for proteolytic activity was carried out according to Sarkar *et al.* (1993) using azocasein (A2765; Sigma-Aldrich Co., St Louis, MO). For a_w , approximately 2 g beans (wet wt) were placed in a sample pan and the a_w of the beans was measured with an a_w -measuring system (Novasina model ms1-aw, Axair Ltd. Systema for Air Treatment, A WMH Company, Pfäffikon, Switzerland) and sample sensor (Novasina model enBSK, Axair).

Microbiological Safety Study

Viable Cell Count. Beans (10 g) were homogenized using a Stomacher lab-blender 400 (Seward Medical, London, UK) for 1 min at “normal” speed with 90 mL sterile peptone physiological saline (PPS). Suitable dilutions (0.1 mL) were spread plated on plate count agar (HiMedia M091) and incubated at 37°C for 16–20 h to obtain the viable cell count of *B. subtilis*.

Challenge Study. Sterile bean grits were inoculated with *B. subtilis* (10^6 total cells/g) and divided into two equal lots. Salt (4 g NaCl/100 g fresh weight) was added to one lot and mixed thoroughly. Each lot was divided into eight equal sets containing 50 g beans in 250 mL screw-capped glass bottles. Each set was inoculated separately or in combination with *B. cereus*, *E. coli* and *S. aureus* (10^4 total cells of each type/g).

The bottles were loosely capped. After incubation at 37°C for 48 h, triplicate bottles from each set were examined for the recovery of the functional microorganism as well as the pathogens.

In order to study the fate of *S. aureus* in both salt-free and salted kinema, five separate experimental set-ups containing cooked beans were inoculated with only *S. aureus* at the same inoculum level as before and incubated at 37°C for 48 h. Among the five, one set contained no added salt while the other four contained varied concentrations of salt.

Colonies of *B. cereus*, *S. aureus* and *E. coli* were counted by spread plating 0.1 mL of appropriate dilutions of the beans in sterile PPS onto plates of Bacillus Cereus Selective Agar (BCSA; HiMedia M833, FD003, FD045), Baird Parker Agar (BPA; HiMedia M043, FD003, FD047) and MacConkey agar (HiMedia M008), respectively. The inoculated BCSA and BPA plates were incubated at 30°C for 24–48 h, while the MacConkey plates for 18–24 h.

The a_w of fresh kinema samples was reduced to 0.5 by air drying it in a dehumidifying chamber (model FFB-170, Bry-Air (Asia) Pvt. Ltd., Gurgaon, India), set at 30% relative humidity ($20 \pm 2^\circ\text{C}$) for a period of 72 h.

Microbial Interference Study. To understand the effect of *E. coli* on *B. cereus*, the well diffusion method described by Sarkar and Banerjee (1996) was followed.

Sensory Analysis

Samples of kinema were analyzed by a panel of 10 trained judges on the basis of overall sensory quality consisting of flavor (50), texture (45) and color (5) using a 100-point score card (Sarkar and Tamang 1994).

Storage and Shelf Life

Monoculture kinema samples were kept in 250 mL glass bottles (50 g in each), capped tightly and stored at 20°C up to 7 days.

Moisture Sorption Study

Sorption Isotherms. Desorption isotherms were determined on fresh kinema, while adsorption isotherms were determined on kinema samples that were air dried to a constant weight in a dehumidifying chamber. Equilibrium moisture content was determined gravimetrically. The apparatus and procedure used were identical to those described earlier (Rakshit *et al.* 2014).

Sorption Models. Four mathematical models, namely Caurie, Oswin, Brunauer–Emmett–Teller (BET) and Guggenheim–Anderson–de Boer (GAB) were tried to fit the sorption data for kinema. Goodness of fit for each model

was evaluated in terms of coefficient of determination (R^2) and percent root-mean-square (%RMS):

$$\%RMS = \sqrt{\frac{1}{n} \sum_{i=1}^n \left(\frac{M_{\text{exp}} - M_{\text{cal}}}{M_{\text{exp}}} \right)^2} \times 100 \quad (1)$$

where, n denotes the number of observations. Best fit of the models was judged on the basis of higher R^2 and lower %RMS values.

Properties of Sorbed Water. (a) Number of adsorbed monolayers (N) was calculated using the formula (Caurie 1981):

$$N = \frac{M_c}{C_c} \quad (2)$$

where, M_c is the monolayer moisture content (g/100 g), calculated from Caurie's model, and C_c is the Caurie's constant.

(b) Bound water content was expressed as

$$M_c \times N. \quad (3)$$

(c) Assuming C_c to be equivalent to the density of adsorbed water in the monolayer, the surface area of adsorption (A) was calculated using the equation (Sahu and Jha 2008):

$$A = \frac{M_c}{C_c \times d \times 10^8} \quad (4)$$

where, d is the diameter of a water molecule (3.673×10^{-10} m).

Thermodynamic Properties

Free Energy Changes. ΔG occurring due to change in the moisture content of kinema were calculated by the relation,

$$\Delta G = -RT \ln a_w \quad (5)$$

where R is the universal gas constant (8.314 kJ/kmol K), T is the temperature in K and the a_w -values were obtained from the Oswin equation which showed the best fit to the experimental data for both adsorption and desorption.

Net Isosteric Heat of Sorption and Sorption Entropy. The net isosteric sorption heat (q_{st} , kJ/mol) was calculated using the Clausius–Clapeyron equations:

$$\left[\frac{\partial(\ln a_w)}{\partial(1/T)} \right] = -\frac{\Delta q_{st}}{R} \quad (6)$$

$$\ln a_w = \left(\frac{\Delta q_{st}}{R} \right) \left(\frac{1}{T} \right) + c \quad (7)$$

where, c is a constant. Plotting $\ln a_w$ against $1/T$ at certain moisture content gave a straight line with slope $\Delta q_{st}/R$. On multiplying each slope with the R -value, q_{st} at a given moisture content was obtained.

The differential entropy was calculated from the Gibbs–Helmholtz relationship:

$$\ln a_w = \left(\frac{q_{st}}{R} \right) \left(\frac{1}{T} \right) - \frac{\Delta S}{R} \quad (8)$$

where, ΔS is the change in differential entropy (kJ/mol K). By plotting $\ln a_w$ against $1/T$ at constant moisture content, the entropy change was calculated from the intercept value ($\Delta S/R$).

Fractionation of Bound Water. Bound water can be fractionated quantitatively based on binding energy (H_B). The H_B for primary bound water was obtained from the following equation (Brunauer *et al.* 1938):

$$\ln C_b = \ln K + \left(\frac{\Delta H_B}{R} \right) \left(\frac{1}{T} \right) \quad (9)$$

where, C_b was a H_B constant. The above equation yielded an Arrhenius plot of $\ln C_b$ against $1/T$, in which a straight line with slope $= \Delta H_B/R$ was obtained. The slope was multiplied by R to obtain the H_B for primary bound water. The H_B for secondary and tertiary bound waters was calculated using the equation:

$$\ln a_w = \left(\frac{\Delta H_B}{R} \right) \left(\frac{1}{T} \right) + c \quad (10)$$

Equilibrium Spreading Pressure. Spreading pressure was calculated from the equation (Fasina *et al.* 1999):

$$\phi = \frac{K_B T}{A_m} \int_0^{a_w} \frac{\theta}{a_w} d(a_w) \quad (11)$$

where, ϕ was the spreading energy, K_B was the Boltzmann constant (1.380×10^{-23} J/K), A_m was the area of a water molecule (1.06×10^{-19} m²) and θ (moisture ratio) was M_{exp}/M_g . By combining the equation for GAB model and Eq. (11) the above integral was expressed as:

$$\phi = \frac{K_B T}{A_m} \ln \left(\frac{1 - Ka_w + KCa_w}{1 - Ka_w} \right) \quad (12)$$

where, the K and C were GAB constants.

Statistical Analysis

All the data obtained from triplicate sets were analyzed to obtain standard error of means and subjected to one-way analysis of variance and paired t -test (SPSS for Windows v. 15.0; SPSS Inc., Chicago, IL). The data from the moisture sorption study were subjected to regression analysis.

RESULTS AND DISCUSSION

Effect of NaCl

Salt was added at the beginning and not partway through fermentation, as was done by Allagheny *et al.* (1996), so that the fermenting microorganism was not disturbed, thereby affecting the quality of the final product. With the increase in salt concentration in beans there was a significant decrease in almost all the measured parameters (Table 1). At 5 g/100 g salt, kinema had a low sensory score because of the absence of characteristic flavor and stickiness. Salt (≥ 3 g/100 g) significantly decreased the a_w -value. Dissociated Na⁺ and Cl[−] bind with the water molecules (Fennema 1996).

TABLE 1. INFLUENCE OF ADDED NaCl CONCENTRATION IN COOKED SOYBEANS ON pH, a_w , VIABLE CELL COUNT, PROTEOLYTIC ACTIVITY AND SENSORY SCORE AFTER *B. SUBTILIS* (Bs)-FERMENTATION AT 37°C FOR 48 h TO PRODUCE KINEMA

NaCl (g/100 g)	pH*	a_w *	Bst (log cfu/g)	Relative proteolytic activity†	Total sensory score (100)
0	8.06 ^a ± 0.13	0.957 ^a ± 0	8.67 ^a ± 0.28	28.93 ^a ± 0.07	78.33 ^a ± 0.33
1.0	7.09 ^b ± 0.05	0.955 ^a ± 0	8.41 ^{ab} ± 0.01	28.37 ^a ± 0.12	75.67 ^{ab} ± 0.88
2.0	6.81 ^{bc} ± 0.11	0.953 ^a ± 0	8.33 ^{ab} ± 0.18	18.20 ^b ± 0.21	74.33 ^b ± 2.19
3.0	6.46 ^{cd} ± 0.22	0.950 ^{ab} ± 0	8.35 ^{ab} ± 0.01	18.00 ^b ± 0.06	77.00 ^{ab} ± 0.58
4.0	6.07 ^d ± 0.03	0.945 ^b ± 0	8.33 ^{ab} ± 0.12	7.67 ^c ± 0.17	75.33 ^{ab} ± 0.33
5.0	5.62 ^e ± 0.19	0.933 ^c ± 0	7.77 ^b ± 0.43	4.07 ^d ± 0.69	68.67 ^c ± 1.20

Note: Values are mean ± SE of three determinations. Means within a column sharing a common superscript letter are not significantly ($P < 0.05$) different.

* The pH and a_w of cooked soybeans before addition of salt and fermentation were 6.18 ± 0.18 and 0.99 ± 0 , respectively.

† Inoculum load, 6 log cfu/g.

‡ Measured as activity on azocasein.

TABLE 2. INFLUENCE OF NaCl ON FUNCTIONAL MICROORGANISM (*Bs*) AND FOODBORNE BACTERIAL PATHOGENS IN A CHALLENGE STUDY DURING FERMENTATION OF SOYBEANS TO PRODUCE KINEMA

Inoculum†	Added NaCl (g/100 g)	Recovery after 48 h on incubation (log cfu/g)			
		<i>Bs</i>	Pathogens		
			<i>Ec</i>	<i>Bc</i>	<i>Sa</i>
<i>Bs</i>	0	8.52 ± 0.02	<dl‡	<dl	<dl
	4	8.23 ± 0.01	<dl	<dl	<dl
<i>Bs</i> + <i>Ec</i>	0	8.19 ± 0.01	6.58 ± 0.14		
	4	8.16 ± 0	5.84 ± 0.02		
<i>Bs</i> + <i>Bc</i>	0	8.29 ± 0.02		6.79 ± 0.01	
	4	8.21 ± 0.01		<dl	
<i>Bs</i> + <i>Sa</i>	0	8.32 ± 0.01			<dl
	4	8.22 ± 0.01			<dl
<i>Bs</i> + <i>Ec</i> + <i>Bc</i>	0	8.16 ± 0	6.27 ± 0.34	<dl	
	4	8.12 ± 0.01	5.52 ± 0.03	<dl	
<i>Bs</i> + <i>Bc</i> + <i>Sa</i>	0	8.25 ± 0.01		6.66 ± 0.02	<dl
	4	8.19 ± 0.01		<dl	<dl
<i>Bs</i> + <i>Ec</i> + <i>Sa</i>	0	8.16 ± 0.01	6.35 ± 0.32		<dl
	4	8.12 ± 0.01	4.01 ± 0.01*		<dl
<i>Bs</i> + <i>Ec</i> + <i>Bc</i> + <i>Sa</i>	0	8.15 ± 0	6.06 ± 0.02	<dl	<dl
	4	8.09 ± 0.01	3.97 ± 0.01*	<dl	<dl

Note: Values are mean ± SE of three determinations. Means between 0- and 4%-treatments in any particular set are not significantly ($P < 0.05$) different, except the two values marked *.

† Inoculum load (log cfu/g) at 0 h: 6.31 ± 0.03, 4.04 ± 0.04, 4.15 ± 0.04 and 4.08 ± 0.04 (beans with no added salt), and 6.15 ± 0.03, 4.05 ± 0.03, 4.19 ± 0.02 and 4.1 ± 0.03 (beans added with salt) of *B. subtilis* (*Bs*), *E. coli* (*Ec*), *B. cereus* (*Bc*) and *S. aureus* (*Sa*), respectively.

‡ dl, detection limit (2 log cfu/g).

Salt-free kinema showed an alkaline pH which is a consequence of the production of ammonia due to a strong proteolytic activity of *Bacillus* during fermentation. Free ammonia concentrations of soybeans increased during the fermentation (Sarkar *et al.* 1993). Addition of salt adversely affected the proteolytic activity with a consequent reduced pungent ammoniacal odor and change in pH toward the acidic side (Table 1). The proteinases were perhaps “salted out” in the presence of salt resulting in their denaturation (Su *et al.* 2005).

Since a critical level of functional microbial cells is essential for a successful fermentation, it was of concern that addition of salt might affect the functional cell population. However, there was no significant change in either viable cell count or organoleptic attributes of kinema having up to 4 g salt/100 g (Table 1). This level of salt did not affect the taste of cooked kinema, and no further addition of salt was needed while cooking it. The observation validates the addition of salt during the kinema production stage which helps to restrict the degradation of valuable amino acids by excess of proteolytic activity without impairing the sensory attributes of the product.

A challenge study with 4 g salt/100 g added during fermentation was performed (Table 2). A challenge study on kinema conducted by Nout *et al.* (1998) revealed that some strains of *B. cereus* were capable of producing enterotoxin. Although most of the toxigenicity was inactivated while

frying the toxic kinema in oil (3 min, 180–200°C – a common culinary practice followed), there are emetic toxins which resist heating for 90 min posing a serious health hazard. They also reported *B. subtilis* DK-W1 to reduce the growth of *B. cereus* and enterotoxin level. However, at higher initial *B. cereus* levels its proliferation and toxin formation may still occur. So, it becomes necessary to eliminate the pathogen. It was achieved in the present study by adding 4 g salt/100 g prior to fermentation (Table 2); cells did not revive after withdrawal of salt. Browne and Dowds (2001) revealed that 5 g salt/100 g could induce osmotic shock in *B. cereus* cells with no adaptive response which could affect the cell metabolism.

Although *B. cereus* survived and increased by 2.5 log cycle in the absence of salt, in combinations containing *E. coli* it failed to survive. There are reports on the antimicrobial effect of *E. coli* against *Bacillus* spp. (Shah and Hasan 2012). To understand the nature of the inhibitory action of *E. coli* in the present study, a microbial interference study was performed by well diffusion assay using the cell-free culture supernatant of *E. coli* on *B. cereus*. Results (data not shown) revealed *B. cereus* to remain unaffected with both the neutralized and nonneutralized cell-free culture supernatant of *E. coli*, which proved that the death of *B. cereus* was not due to the production of any acid or antimicrobial compound by *E. coli* rather it might have occurred due to nutrient competition.

The fate of *S. aureus* appeared similar in both salt-free and salted kinema. Although known to tolerate high salt concentrations it did not survive in any of our experimental combinations of microorganisms. To determine whether the elimination was due to its inability to cope up with the other bacteria in the competition for survival or due to the presence of salt, a challenge study with only *S. aureus* was performed. The pathogen was recovered from the salt-free beans with an increase of 2.5 log cycle in viable count after 48 h-fermentation. This suggested that there was no inhibitory substance present in the beans to prevent the recovery of *S. aureus*. The recovery after 48 h of incubation at 1, 2, 3 and 4 g salt/100 g was 4.8, 3.7, 3.2 and 2.4 log cfu/g, respectively. It can be concluded that *S. aureus*, when present alone, could survive even in the presence of 4 g NaCl/100 g. However, in the presence of competitive microflora it got eliminated as was also reported in tempeh formation (Nout *et al.* 1988). The possible causes for such a decline are nutrient competition, antagonistic reactions and autolysis (Madiraju *et al.* 1987).

E. coli multiplied significantly in the absence of salt. In the presence of all the pathogens and the functional microorganism, in the salted beans it survived but could not grow. This is possibly due to overcrowding, competition and nutrient depletion. Survival of *E. coli* even in the presence of salt suggested halotolerance in them. Although there was a decline in the *E. coli* population in the presence of 4 g salt/100 g (where a_w reduced by 1.3%), a complete die off was not observed, and this highlights the importance of hygiene and quality control for kinema production.

This study revealed that addition of salt prior to fermentation is advantageous in the sense that the condition was capable of eliminating the presence of at least *B. cereus* cells.

A study on storage of kinema at 20C improved the quality of kinema till day 5, after which organoleptic deterioration began (Table 3). Maturation of nattō below 10C for 1–2 days after fermentation is practiced in the commercial nattō production for flavor and viscosity development (Ueda 1989). Extension of proteolysis beyond fermentation is undesirable as it leads to the loss of amino acids resulting reduction in food value. In order to ascertain whether salt had any control over proteolytic activity, it was measured on day 0 and day 5 of storage in both types of kinema. The proteolytic activity of the salted kinema was found significantly less than the nonsalted one. Thus, the addition of salt during the processing stages of kinema proved to be beneficial in decreasing the degradation levels of soy proteins at the time of storage.

Effect of Moisture Content

In a challenge study, the influence of a_w on the survival and growth of *E. coli* cells in fermented beans was studied. For

TABLE 3. SENSORY AND PHYSICOCHEMICAL CHARACTERISTICS OF KINEMA STORED AT 20C AS INFLUENCED BY SALT AND STORAGE PERIOD

Storage period (days)	NaCl (4 g/100 g)	Sensory score	Texture (45)	Color (5)	Total (100)	pH	Relative proteolytic activity*	Free amino nitrogen (mM/g)
0	-	36.93 ^c ± 0.07	37.93 ^d ± 0.18	3.47 ^c ± 0.18	78.33 ^d ± 0.33	8.06 ^e ± 0.04†	25.4 ^b ± 1.1†	0.28 ^d ± 0.03
	+	35.87 ^{dh} ± 0.13	36.27 ^c ± 0.27	3.27 ^c ± 0.07	75.33 ^{ei} ± 0.33	6.84 ^{dh} ± 0.14†	6.8 ^h ± 0.1†	0.19 ^h ± 0.02
3	-	44.00 ^b ± 1.00	42.67 ^{bc} ± 0.33	4.33 ^b ± 0.33	91.00 ^c ± 1.00	8.14 ^e ± 0.04†	nd†	0.40 ^c ± 0.03
	+	42.33 ^h ± 1.67	41.67 ^h ± 0.33	4.00 ^h ± 0	88.00 ^c ± 1.53	7.06 ^{cd} ± 0.04†	nd	0.27 ^h ± 0.02
4	-	45.00 ^{ab} ± 0.58	44.33 ^a ± 0.33	4.33 ^b ± 0.33	93.67 ^b ± 0.33	†8.27 ^d ± 0.02	nd	0.48 ^{bc} ± 0.02†
	+	47.33 ^{hi} ± 0.33	43.67 ^{ai} ± 0.33	4.00 ^h ± 0	95.00 ^b ± 0	†7.19 ^c ± 0.03	nd	0.33 ^{cd} ± 0.03†
5	-	46.33 ^a ± 0.33	44.67 ^a ± 0.33	5.00 ^a ± 0	96.00 ^a ± 0.58	8.43 ^c ± 0.02†	29.3 ^a ± 1.5†	0.55 ^b ± 0.04†
	+	48.33 ^{hi} ± 0.33	44.33 ^{ai} ± 0.33	5.00 ^a ± 0	97.67 ^{ai} ± 0.33	7.31 ^c ± 0.16†	11.5 ^h ± 0.6†	0.38 ^c ± 0.01†
6	-	30.00 ^d ± 0†	43.67 ^{ab} ± 0.33†	4.00 ^{bc} ± 0	77.67 ^d ± 0.33†	8.79 ^b ± 0.01†	nd	0.69 ^a ± 0.01†
	+	42.67 ^{hi} ± 0.33†	41.33 ^{bi} ± 0.67†	4.00 ^h ± 0	88.00 ^c ± 0.58†	7.67 ^h ± 0.08†	nd	0.50 ^h ± 0.01†
7	-	25.33 ^e ± 0.33†	42.00 ^c ± 0.58	4.00 ^{bc} ± 0	71.33 ^e ± 0.67†	9.34 ^a ± 0.03†	nd	0.76 ^a ± 0.01†
	+	39.67 ^{hi} ± 0.33†	41.33 ^{bi} ± 0.88	4.00 ^h ± 0	85.00 ^{di} ± 1.15†	8.03 ^h ± 0.07†	nd	0.57 ^h ± 0.03†

Note: Values are mean ± SE obtained from three replicates. Means within a column sharing a common superscript (^a, ^b, ^c, ^d, ^e for the salt-free kinema, and ^a₁, ^b₁, ^c₁, ^d₁, ^e₁ for the salted kinema) are not significantly ($P < 0.05$) different. Means of salt-free (-) and salted (+) kinema in a particular set are not significantly ($P < 0.05$) different, except the values marked †.

* Measured as activity on azocasein.

† nd, not determined.

FIG. 1. MOISTURE SORPTION ISOTHERMS OF KINEMA AT DIFFERENT TEMPERATURES

this, both the salted and nonsalted kinema inoculated with both *B. subtilis* and *E. coli* at the same dose as before were air dried in a dehumidifying chamber for a period of 72 h to a_w 0.5 (where a_w reduced by 48%). *E. coli* cells could not be detected in any case, and did not revive after withdrawal of salt. So, the use of salt (4 g/100 g cooked beans) prior to fermentation followed by drying of kinema led to the development of a microbiologically safer product.

The moisture sorption results revealed that the isotherms of kinema were type II sigmoid showing three regions (Fig. 1). In case of both adsorption and desorption, region I (a_w , 0–0.11) corresponds to the monolayer moisture content where water is strongly bound to the polar groups of the food components. Region II or multimolecular phase in which additional layers of water accumulate over the monolayer has a gentle slope. In adsorption it ranged from a_w 0.11 to 0.75, and in desorption it was from a_w 0.11 to 0.84. In this region the bound water gradually converts into free water. Region III shows a steep rise in the isotherm. Here the water exists in the free state and condenses into the pores and spaces of the food (Kinsella and Fox 1987). Availability of free water in which the food components begin to dissolve and various chemical reactions which began in Region II ultimately leads to product deterioration (Chetana *et al.* 2005). Pan (2003) observed that soy protein products when exposed to high moisture contents for even a short period of time could adsorb a significant amount of moisture leading to microbial growth and degradation of product quality. Kinema, being a soybean food, exhibited a similar sorption pattern. The equilibrium moisture content (EMC) decreased progressively with an increase in temperature at a particular a_w because temperature helped to activate the

water molecules which tended to make free from their binding sites (McMinn and Magee 2003). Thus, at a_w 0.43 the EMC of fresh kinema decreased from 10.86 g/100 g at 15C to 9.93 and 7.67 g/100 g at 25 and 35C, respectively, during desorption. A similar trend was observed for dried kinema during adsorption as well. On the other hand, EMC at a particular temperature increased with an increase in a_w . This occurs due to a rise in the internal vapor pressure of water within the food as a result of an increase in the vapor pressure of the external environment (Shivhare *et al.* 2004).

The moisture sorption data obtained for kinema at three different temperatures are presented in Table 4. The Oswin model exhibited the best fit over the entire range of a_w at all the test temperatures. Caurie, BET and GAB also showed good fit for the experimental data with the GAB model giving the best fit among the three. The BET model was applicable in a_w range of 0.11–0.55.

Caurie's model was used in evaluating the different properties of sorbed water in kinema as it contains the parameters such as M_c and C_c required for the calculation (Table 4). Although the Oswin model gave the best fit, it was not within the scope of this model to determine the various properties as it does not have these parameters. The monolayer moisture (M_c) refers to the amount of water tightly bound to the sorption sites and is the safe lower limit of moisture levels for foods. With the rise in temperature, there is loss of sorption sites, and the hydrophobic hydration also begins to melt leading to reduced monolayer values at higher temperatures. This explains why the storage of kinema at a higher temperature may lead to its spoilage. Surface area of the sorption sites also affects the rate of sorption. A larger surface area means more polar hydrophilic groups are exposed leading to increased sorption. The area decreased with the increase in temperature (Table 4). Bound water in dried kinema which is directly related to the monolayer moisture content decreased with the rise in temperature. However, density of the sorbed water increased with the increase in temperature (Table 4). Intermingling of food components with sorbed water at higher temperatures may be the reason for higher densities, as was observed in wadi (Rakshit *et al.* 2014).

ΔG occurring in sorption were calculated (Table 5). The ΔG decreased with an increase in temperature at a particular moisture content and also with an increase in moisture content at all the temperatures. Furthermore, the ΔG -values obtained for desorption process were higher than those obtained in the adsorption process. Similar observations have been reported in red chillies (Kaleemullah and Kailappan 2007). Both net isosteric heat (Fig. 2) and differential entropy (Fig. 3) for kinema decreased with an increase in moisture content due to reduced availability of active sorption sites. This indicated a strong interaction between water and food components and hence a greater

TABLE 4. MOISTURE SORPTION ISOTHERM MODELS FOR FITTING EXPERIMENTAL SORPTION DATA OF KINEMA, PARAMETER VALUES AND PROPERTIES OF ADSORBED WATER AT DIFFERENT TEMPERATURES

Model	Equation	Reference	a_w	Parameter*	Adsorption temp. (C)			Desorption temp. (C)		
					15	25	35	15	25	35
Caurie	$\frac{1}{M} = \frac{1}{C_c M_c} \left(\frac{1-a_w}{a_w} \right)^{\frac{2C_c}{M_c}}$	Caurie 1981	0.11–0.91	M_c	8.27	6.55	6.00	9.28	7.17	5.82
				C_c	1.24	1.30	1.39	1.40	1.79	1.85
				N	6.67	5.04	4.34			
				%BW	55.16	33.00	26.25			
				A	181.58	137.18	118.30			
				R^2	0.93	0.97	0.94	0.94	0.98	0.91
				%RMS	9.45	5.98	7.33	8.36	7.10	9.97
Oswin	$M = a \left(\frac{a_w}{1-a_w} \right)^b$	Chirife and Iglesias 1978	0.11–0.97	a	9.38	8.51	6.84	9.86	9.07	7.40
				b	0.29	0.29	0.32	0.82	0.66	0.42
				R^2	0.99	0.98	0.98	0.96	0.95	0.98
				%RMS	2.95	5.14	4.71	5.24	5.36	3.26
BET	$M = \frac{M_b C_b}{(1-a_w)(1+(C_b-1)a_w)}$	Caurie, Lee, Salomon and Chichester 1976	0.11–0.55	M_b	4.61	3.41	1.07	7.08	5.91	2.72
				C_b	9.99	13.40	21.32	9.06	9.49	10.17
				R^2	0.96	0.97	0.98	0.98	0.92	0.96
				%RMS	8.42	4.80	4.68	5.35	10.20	7.73
GAB	$\frac{M}{M_g} = \frac{C_g k a_w}{(1-ka_w)(1-ka_w + C_g k a_w)}$	Bizot 1983	0.11–0.91	M_g	4.65	3.40	2.92	5.92	5.06	3.44
				C_g	53.69	41.06	30.07	97.43	62.17	45.28
				k	0.80	0.90	0.95	0.65	0.72	0.79
				R^2	0.90	0.95	0.95	0.91	0.94	0.95
				%RMS	10.53	5.53	4.24	9.11	6.31	5.43

* M , equilibrium moisture constant; a_w , water activity; M_b , M_c and M_g , monolayer moisture content (g/100 g); a , b , C_b , C_c (density of sorbed water, g/mL), C_g and k , model constants; N , number of adsorbed monolayers; %BW, percent bound water; A , surface area of adsorption (m^2/g).

stability during storage at lower moisture content. The desorption heat or the energy required for the desorption process was higher than the adsorption heat over the entire range of moisture content, as observed in case of Chilean papaya (Vega-Gálvez and Lemus-Mondaca 2008).

A combination of BET and Clausius–Clapeyron equations was used for determining the H_B at different moisture contents and quantitative fractionation of bound water. The H_B of primary bound water was calculated as -27.87 MJ/mol. The plot of ΔH_B against the moisture contents showed a curvature and a break point (Fig. 4) which signified the end of the secondary bound water. After this, there was a linear and gentle decrease in ΔH_B . When this line was extended to intersect the abscissa ($\Delta H_B = 0$), the moisture content at which the tertiary bound water ended was obtained. Beyond 49 g moisture/100 g, free water existed that could be easily removed or used for any process. The primary H_B , -27.87 MJ/mol, was plotted on the ΔH_B versus moisture content graph by drawing a horizontal line corresponding to this value on the curve. The point of intersection gave the moisture content level for the end point of the primary bound water.

The spreading pressure of dried kinema increased with an increase in moisture content up to 50 g/100 g after which there was no increase (Table 5). Temperature had a very

little effect upon it. Hence, the presence of moisture increased the surface tension of the sorption sites due to adsorbed molecules.

CONCLUSION

An attempt to improve the traditional method of kinema preparation with added salt (4 g/100 g) was found to be organoleptically acceptable. The significant achievement was the elimination of *B. cereus* cells without adversely affecting the functional microorganism, *B. subtilis*. Salt reduced but could not eliminate *E. coli* from kinema. However, it was achieved by reducing the a_w of kinema. This opens up a future scope for exploring the possibilities of using kinema as a flavoring or food supplement ingredient. The sorption study demonstrated the effect of moisture and temperature on the safety and quality of the food. Lower moisture content and temperature proved to be better for storage, as less energy was required to remove moisture from both fresh and dried kinema.

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TABLE 5. EQUILIBRIUM SPREADING PRESSURES AND FREE ENERGY CHANGES (ΔG) DURING SORPTION PROCESSES OF KINEMA AT DIFFERENT TEMPERATURES

Temperature (C)	Moisture content (g/100 g)	Equilibrium spreading pressure (J/m ²)	Adsorption		Desorption	
			a_w	ΔG (kJ/kmol)	a_w	ΔG (kJ/kmol)
15	10	0.1210	0.5554	1408.09	0.5043	1639.19
	20	0.1903	0.9332	165.54	0.7032	843.11
	30	0.1986	0.9828	41.54	0.7952	548.71
	40	0.2005	0.9936	15.37	0.8465	399.02
	50	0.2012	0.9970	7.19	0.8786	309.90
	60	0.2014	0.9984	3.83	0.9004	251.21
	70	0.2015	0.9990	2.40	0.9160	210.08
	80	0.2016	0.9994	1.44	0.9277	179.69
	90	0.2017	0.9996	0.96	0.9368	156.32
	100	0.2017	0.9997	0.72	0.9440	137.99
25	10	0.1555	0.6344	1127.48	0.5364	1543.22
	20	0.2117	0.9492	129.17	0.7669	657.54
	30	0.2231	0.9868	32.92	0.8584	378.29
	40	0.2260	0.9950	12.42	0.9034	251.70
	50	0.2022	0.9976	5.95	0.9291	182.20
	60	0.2273	0.9987	3.22	0.9452	139.63
	70	0.2275	0.9992	1.98	0.9560	111.48
	80	0.2276	0.9995	1.49	0.9637	91.61
	90	0.2276	0.9996	0.99	0.9695	76.74
	100	0.2277	0.9997	0.74	0.9738	65.78
35	10	0.1766	0.7645	687.64	0.6713	1020.54
	20	0.2345	0.9654	90.17	0.9135	231.67
	30	0.2494	0.9899	25.99	0.9651	90.97
	40	0.2538	0.9958	10.78	0.9820	46.51
	50	0.2555	0.9979	5.38	0.9893	27.55
	60	0.2562	0.9988	3.07	0.9930	17.99
	70	0.2566	0.9993	1.79	0.9951	12.58
	80	0.2568	0.9995	1.28	0.9964	9.24
	90	0.2570	0.9997	0.77	0.9973	6.92
	100	0.2571	0.9998	0.51	0.9979	5.38

FIG. 2. NET ISOSTERIC HEAT OF SORPTION AT DIFFERENT MOISTURE CONTENTS FOR KINEMA **FIG. 3.** DIFFERENTIAL ENTROPY OF SORPTION AT DIFFERENT MOISTURE CONTENTS FOR KINEMA

FIG. 4. WATER-BINDING ENERGY OF KINEMA DURING THE ADSORPTION PROCESS AT EACH MOISTURE CONTENT

REFERENCES

- ALLAGHENY, N., OBANU, Z.A., CAMPBELL-PLATT, G. and OWENS, J.D. 1996. Control of ammonia formation during *Bacillus subtilis* fermentation of legumes. *Int. J. Food Microbiol.* 29, 321–333.
- BIZOT, H. 1983. Using the G.A.B. model to construct sorption isotherms. In *Physical Properties of Foods* (R. Jowitt, F. Escher, B. Hallström, H.F.T. Meffert, W.E.L. Speiss and G. Vos, eds.) pp. 27–41, Elsevier Applied Science Publishers, London, UK.
- BROWNE, N. and DOWDS, B.C.A. 2001. Heat and salt stress in the food pathogen *Bacillus cereus*. *J. Appl. Microbiol.* 91, 1085–1094.
- BRUNAUER, S., EMMETT, P.H. and TELLER, E. 1938. Adsorption of gases in multimolecular layers. *J. Am. Chem. Soc.* 60, 309–319.
- CAURIE, M. 1981. Derivation of full range moisture sorption isotherms. In *Water Activity: Influences on Food Quality* (L.B. Rockland and G.F. Stewart, eds.) pp. 63–87, Academic Press, New York, NY.
- CAURIE, M., LEE, T.-C., SALOMON, M. and CHICHESTER, C.O. 1976. A rearranged B.E.T. plot for a more direct estimation of B.E.T. constants. *J. Food Sci.* 41, 448.
- CHETANA, R., SRINIVASA, P.C. and YELLA REDDY, S.R. 2005. Moisture sorption characteristics of *milk burfi*, an traditional Indian sweet, using sugar substitutes. *Eur. Food Res. Technol.* 220, 136–141.
- CHIRIFE, J. and IGLESIAS, H.A. 1978. Equations for fitting water sorption isotherms of foods: Part I – A review. *Int. J. Food Sci. Technol.* 13, 159–174.
- FASINA, O.O., AJIBOLA, O.O. and TYLER, R.T. 1999. Thermodynamics of moisture sorption in winged bean seed and gari. *J. Food Process Eng.* 22, 405–418.
- FENNEMA, O.R. 1996. Water and ice. In *Food Chemistry* (O.R. Fennema, ed.) pp. 17–94, Marcel Dekker, New York, USA.
- KALEEMULLAH, S. and KAILAPPAN, R. 2007. Monolayer moisture, free energy change and fractionation of bound water of red chillies. *J. Stored Prod. Res.* 43, 104–110.
- KINSELLA, J.E. and FOX, P.F. 1987. Water sorption by milk proteins. *Bull. Int. Dairy Fed.* 209, 12–40.
- MADIRAJU, M.V., BRUNNER, D.P. and WILKINSON, B.J. 1987. Effects of temperature, NaCl, and methicillin on penicillin-binding proteins, growth, peptidoglycan synthesis, and autolysis in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 31, 1727–1733.
- MCMINN, W.A.M. and MAGEE, T.R.A. 2003. Thermodynamic properties of moisture sorption of potato. *J. Food Eng.* 60, 157–165.
- NOUT, M.J.R., NOTERMANS, S. and ROMBOUTS, F.M. 1988. Effect of environmental conditions during soya-bean fermentation on the growth of *Staphylococcus aureus* and production and thermal stability of enterotoxins A and B. *Int. J. Food Microbiol.* 7, 299–309.
- NOUT, M.J.R., BAKSHI, D. and SARKAR, P.K. 1998. Microbiological safety of kinema, a fermented soya bean food. *Food Control* 9, 357–362.
- PAN, Z. 2003. Adsorption characteristics of functional soy protein products. *J. Food Process Eng.* 25, 499–514.
- RAKSHIT, M., MOKTAN, B., HOSSAIN, S.A. and SARKAR, P.K. 2014. Moisture sorption characteristics of *wadi*, a legume-based traditional condiment. *J. Food Sci. Technol.* 51, 301–307.
- SABLANI, S.S., KASAPIS, S. and RAHMAN, M.S. 2007. Evaluating water activity and glass transition concepts for food stability. *J. Food Eng.* 78, 266–271.
- SAHU, J.K. and JHA, A. 2008. Storage stability of sandesh – An Indian milk sweet. *Agric. Eng. Int.* 10, 1–12.
- SARKAR, P.K. 2000. Ecology of soybean acidifications and some biochemical changes during controlled kinema production. *J. Food Sci. Technol.* 37, 477–483.
- SARKAR, P.K. and BANERJEE, S. 1996. Antibacterial activity of lactic acid bacterial isolates obtained from natural habitats. *J. Food Sci. Technol.* 33, 231–233.
- SARKAR, P.K. and TAMANG, J.P. 1994. The influence of process variables and inoculum composition on the sensory quality of kinema. *Food Microbiol.* 11, 317–325.
- SARKAR, P.K., COOK, P.E. and OWENS, J.D. 1993. *Bacillus* fermentation of soybeans. *World J. Microbiol. Biotechnol.* 9, 295–299.
- SHAH, M.S. and HASAN, F. 2012. Selection and optimization of bacteriocin production from *Escherichia coli* and *Pseudomonas aeruginosa*. *Int. J. Biosci.* 2, 156–163.
- SHIVHARE, U.S., ARORA, S., AHMED, J. and RAGHAVAN, G.S.V. 2004. Moisture adsorption isotherms for mushroom. *Lebensm. Wiss. Technol.* 37, 133–137.
- SU, N.-W., WANG, M.-L., KWOK, K.-F. and LEE, M.-H. 2005. Effects of temperature and sodium chloride concentration on the activities of proteases and amylases in soy sauce koji. *J. Agric. Food Chem.* 53, 1521–1525.

- TAMANG, J.P., SARKAR, P.K. and HESSELTINE, C.W. 1988. Traditional fermented foods and beverages of Darjeeling and Sikkim – A review. *J. Sci. Food Agric.* **44**, 375–385.
- UEDA, S. 1989. Utilization of soybean as natto, a traditional Japanese food. In *Bacillus subtilis: Molecular Biology and Industrial Application* (B. Maruo and H. Yoshikawa, eds.) pp. 143–161, Elsevier, Tokyo, Japan.
- VEGA-GÁLVEZ, A. and LEMUS-MONDACA, M.P.R. 2008. Moisture sorption isotherms and isosteric heat determination in Chilean papaya (*Vasconcellea pubescens*). *Quim. Nova* **31**, 1417–1421.