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Effect of temperatures during pure culture fermentation of Kinema

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Kinema was prepared by fermenting whole cooked soybeans with pure culture of *Bacillus subtilis* KK2:B10 (MTCC 2756) strain at 35 °C, 40 °C and 45 °C for 24 h. Temperature, mesophilic plate counts, relative viscosity, water-soluble nitrogen, formol nitrogen contents and reducing sugars of fermenting soybeans were investigated during fermentation. At higher temperatures the growth rate of *B. subtilis* KK2:B10 was faster. A remarkable increase in the relative viscosity of kinema was observed at 40 °C during fermentation. Water-soluble nitrogen and formol nitrogen to total nitrogen contents increased throughout the 24 h of fermentation. Reducing sugars increased during the log phase and then decreased sharply. Kinema matured below 10 °C for 1 day after the desired fermentation showed a significant increase in relative viscosity. The quality of kinema was maintained with pure culture fermentation by *B. subtilis* KK2:B10 at 40 °C for 20 h and matured at 5 °C for 1 day.

Key words: Kinema, soybean, *Bacillus subtilis* KK2:B10, temperature.

Kinema is an indigenous traditional fermented soybean food with characteristic flavour and stickiness. It is commonly consumed in the local diet as a low-cost source of high protein food by the people of the eastern Himalayan regions of the Darjeeling hills and Sikkim in India, Nepal and Bhutan. Kinema curry is delicious eaten with boiled rice in main meals. Traditionally kinema is prepared by cooking overnight-soaked whole soybeans, wrapped in leaves and fermented naturally for 1–3 days at ambient temperature (Tamang *et al*. 1988; Tamang 1996). It is similar to itohiki-natto of Japan in respect of texture and aroma.

Bacillus subtilis was the predominant microorganism in the natural fermentation of kinema along with other accompanying microflora *Enterococcus faecium*, *Candida parapsilosis* and *Geotrichum candidum* (Tamang 1992; Sarkar *et al*. 1994). Sarkar & Tamang (1994) studied the influence of incubator temperatures of 28 °C, 37 °C and 45 °C during 48 h natural fermentation of kinema by sensory evaluation attributes, and found 37 °C as optimum incubator temperature for natural fermentation of kinema. However, the conventional method of kinema preparation by natural fermentation leads to variations in quality due to varying microbial profile, fermentation time and temperature. The best strain of *Bacillus subtilis* was selected as pure culture starter from naturally fermented kinema samples for laboratory-scale production of kinema (Tamang & Nikkuni 1996). The aim of the present research is to study the effect of incubation temperatures at 35 °C, 40 °C and 45 °C on microbial load, and selected physical and biochemical parameters during pure culture fermentation of kinema using selected starter culture of *B. subtilis* KK2:B10. The second aim is to study the effect of temperature on the maturation of freshly prepared kinema.

Materials and Methods

Starter Culture

Bacillus subtilis KK2:B10 [MTCC* 2756 (*Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India)] and *Bacillus subtilis* GK2:B10 (MTCC 2757) were isolated from naturally fermented kinema samples, and selected as best starter cultures for improved kinema production. These strains were identified as *Bacillus subtilis* (Ehrenberg) Cohn (Tamang & Nikkuni 1996).

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Kinema Preparation by Starter Culture

Yellow-seeded soybeans [*Glycine max* (L.) Merrill] were cleaned and soaked in tap water overnight at ambient temperature (10–15 °C). Soaked soybeans were steamed at 121 °C for 40 min and inoculated with cell suspension of *Bacillus subtilis* KK2:B10, harvested in nutrient broth (Difco) at 37 °C for 18 h, at 10^4 c.f.u./g of cooked soybeans while the temperature of the soybeans was above 80 °C. Approximately 80 g inoculated soybeans were put into a polystyrene paper package (Tamang & Nikkuni 1996). Packages were incubated at 35 °C, 40 °C and 45 °C with relative humidity of 90%, respectively. Samples were taken at 4 h intervals from 0 h to 24 h. The remaining sample was then frozen at −20 °C until analysis.

Mesophilic Plate Count

Ten g of sample was mixed with 90 ml of 0.85% (w/v) sterile physiological saline and shaken for 10 min. Decimal dilution series were prepared in sterile physiological saline and 1 ml of appropriate diluted suspension was mixed with molten Trypticase Soya agar (BBL 11043, Microbiology System, Cockeysville, USA) and incubated at 30 °C for 24 h. Colonies appeared were counted as colony forming unit per g (c.f.u./g) of sample.

Physical Analysis

Temperature of fermenting soybeans was recorded directly by an automatic programme pen recorder (Rikadenki Kogyo Co., HR-2300, Tokyo, Japan). The relative viscosity of fermenting soybeans was measured as described earlier (Tamang & Nikkuni 1996).

Chemical Analysis

Total nitrogen and water-soluble nitrogen of samples were determined by the micro-Kjeldahl method (AOAC 1990). Formol nitrogen of samples was determined by formaldehyde titration. Homogenized samples were mixed with distilled water and centrifuged at 20,000 × ***g*** for 15 min, 10 ml supernatant was mixed with 20 ml of previously neutralized formaldehyde and titrated against 0.1 N NaOH using an auto-titrator (TOA, TSB-10a, Tokyo, Japan). Reducing sugar of the sample was estimated by a colorimetric method (Somogyi 1945) using glucose as standard.

Effect of Maturation

Samples after desired fermentation were matured at 5 °C and 10 °C for 1 day. The relative viscosity, water-soluble nitrogen and formol nitrogen contents were estimated as described above.

Statistical Analysis

Data obtained were analysed statistically by determining standard errors of the mean and analysis of variance (Snedecor & Cochran 1989) and the SALS software package (version 2.5).

Results and Discussion

During fermentation, the temperature of the fermenting soybeans began to rise after 4–8 h and became markedly higher than the respective incubation temperatures due to microbial growth (Table 1). In natto, the temperature of fermenting soybean should not rise above 50 °C during fermentation (Nikkuni 1997). The growth rate of viable cells of *Bacillus subtilis* KK2:B10 was faster at 45 °C upto 16 h (Figure 1). However, mesophilic plate counts increased exponentially during fermentation at 35 °C and 40 °C till the end of fermentation. The surface of the fermenting soybeans changed to a whitish colour due to growth of *B. subtilis* on or in between the soybeans during fermentation. Kinema prepared at 40 °C showed a remarkable increase in relative viscosity from 16 h to 20 h compared with kinema prepared at 35 °C and 45 °C (Figure 2). The unique feature of *B. subtilis* KK2:B10 was the formation of sticky viscous materials which started from 8–12 h at all incubation temperatures. Stickiness is an important criterion for judging quality of kinema by consumers (Sarkar & Tamang 1994).

Water-soluble nitrogen and formol nitrogen to total nitrogen contents of kinema increased rapidly during fermentation (Figures 3 and 4). This was due to high proteolytic activity of *B. subtilis* KK2:B10 (Tamang 1995).

Table 1. Changes in temperature (°C) of fermenting soybean during kinema fermentation by *B. subtilis* KK2:B10 at different incubation temperatures.

Fermentation time (h)	Incubation-temperature		
	35 °C	40 °C	45 °C
0	82.0	82.0	82.0
4	34.4	39.5	46.5
8	34.7	44.3	51.0
12	38.5	49.1	53.0
16	41.4	49.0	52.0
20	38.8	47.7	51.2
24	38.8	47.0	50.0

Data represent the means of triplicate sets.

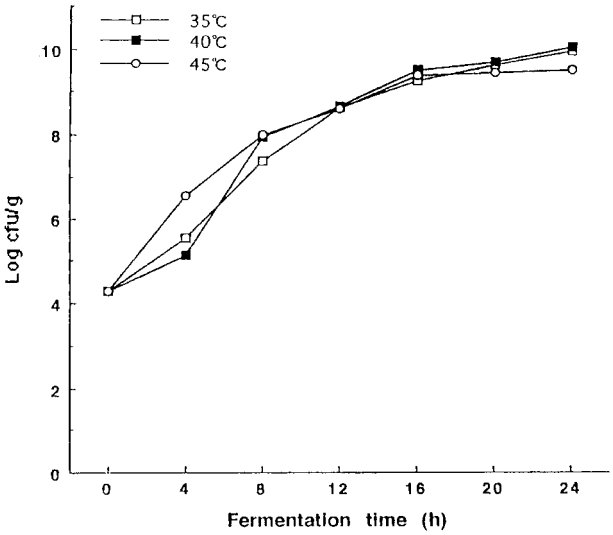

Figure 1. Changes in mesophilic plate counts of *B. subtilis* KK2:B10 during kinema fermentation at different incubation temperatures. Data represent the means of triplicate sets.

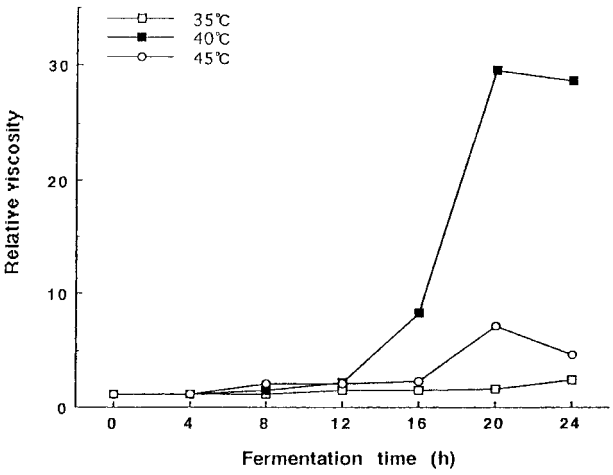

Figure 2. Changes in relative viscosity during kinema fermentation by *B. subtilis* KK2:B10 at different incubation temperatures. Data represent the means of triplicate sets.

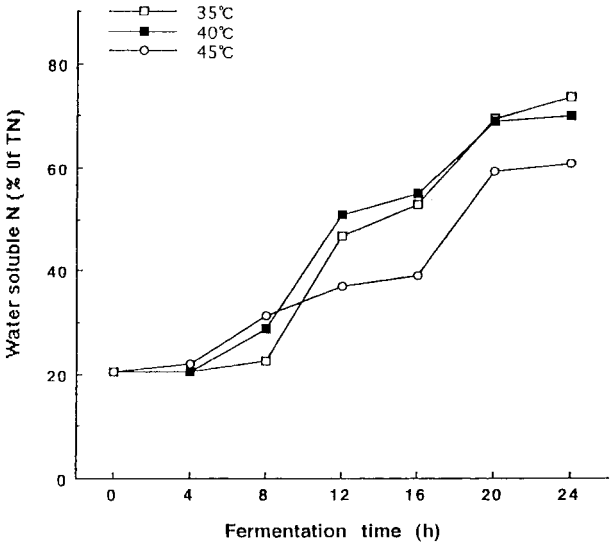

Figure 3. Changes in water-soluble nitrogen contents during kinema fermentation by *B. subtilis* KK2:B10 at different incubation temperatures. Data represent the means of triplicate sets.

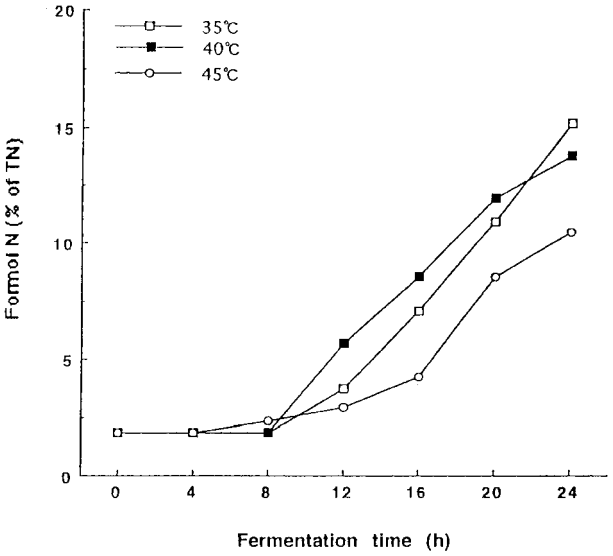

Figure 4. Changes in formol nitrogen contents during kinema fermentation by *B. subtilis* KK2:B10 at different incubation temperatures. Data represent the means of triplicate sets.

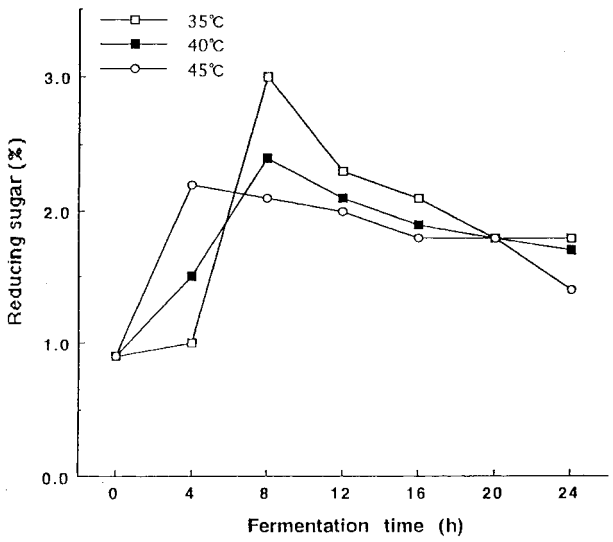

Figure 5. Changes in reducing sugars during kinema fermentation by *B. subtilis* KK2:B10 at different incubation temperatures. Data represent the means of triplicate sets.

Reducing sugars increased at log phase and then decreased sharply during kinema fermentation (Figure 5). This indicates that the reducing sugars of fermenting soybeans were used by *B. subtilis* for its metabolism, since *B. subtilis* KK2:B10 produces high activity of α-amylase (Tamang & Nikkuni 1996). Kanno *et al.* (1982) reported that glucose and fructose increased upto 8 h and then decreased rapidly during natto fermentation by *B. subtilis* (*natto*).

The effect of temperature on the maturation of freshly prepared kinema was studied (Table 2). There was a significant increase in the relative viscosity of kinema during maturation at 5 °C and 10 °C over kinema without maturation. However, no significant differences ($P < 0.05$) in water-soluble nitrogen and formol nitrogen content of kinema were observed during maturation at low temperatures compared with kinema kept at the control temperature. Keeping freshly prepared kinema below 10 °C for 1 day stabilized the quality of the product by preventing the further biological activity of microorganisms and showed better stickiness which is very important sensory property of kinema. This

Table 2. Effect of temperature on maturation of kinema.

Sample	Relative viscosity	WSN/TN (%)	FN/TN (%)
KK2:B10 Kinema			
Control	30.2[a]	74.5[a]	11.6[a]
Matured at 5 °C	32.0[b]	76.5[a]	12.0[a]
Matured at 10 °C	31.8[b]	76.0[a]	12.0[a]
GK2:B10 Kinema			
Control	30.6[a]	62.1[a]	7.1[a]
Matured at 5 °C	34.1[b]	62.5[a]	7.6[a]
Matured at 10 °C	33.7[b]	63.5[a]	7.7[a]

WSN, water-soluble nitrogen; FN, formol nitrogen; TN, total nitrogen. KK2:B10 Kinema, kinema prepared with *B. subtilis* KK2:B10 (MTCC 2756) at 40 °C for 20 h; GK2:B10 Kinema, kinema prepared with *B. subtilis* GK2:B10 (MTCC 2757) at 40 °C for 20 h; Control, kinema without maturation. Data represent the means of triplicate sets. Values bearing different superscripts in column in each sample differ significantly ($P < 0.05$).

maturation process may improve the unique flavour of kinema. Maturation of natto below 10 °C for 1–2 days after fermentation is one of the major steps in commercial natto production for flavour and viscosity development (Ueda 1989).

The present results revealed that the quality of kinema prepared by using starter culture of *B. subtilis* KK2:B10 (MTCC 2756) was maintained around 40 °C for 20 h fermentation period followed by maturation at 5 °C for 1 day. Kinema prepared under these optimized conditions has more advantages over the conventional method due to shorter fermentation time, better hygienic conditions, maintaining consistency, and increased levels of soluble protein.

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References

AOAC 1990 Official Methods of Analysis 15th edn. Virginia: Association of Official Analytical Chemists. ISBN 0-93558442-0.

Kanno, A., Haruki, T., Takano, N. & Akimoto, T. 1982 Change of saccharides in soybean during manufacturing of natto. *Nippon Shikuhin Kogyo Gakkaishi* **29**, 105–110.

Nikkuni, S. 1997 Natto, Kinema and Thua-nao: traditional non-salted fermented soybean foods in Asia. *Farming Japan* **31**, 27–36.

Sarkar, P.K. & Tamang, J.P. 1994 The influence of process variables and inoculum composition on the sensory quality of kinema. *Food Microbiology* **11**, 317–325.

Sarkar, P.K., Tamang, J.P., Cook, P.E. & Owens, J.D. 1994 Kinema – a traditional soybean fermented food: proximate composition and microflora. *Food Microbiology* **11**, 47–55.

Snedecor, G.W. & Cochran, W.G. 1989 *Statistical Methods* 8th edn. Ames: Iowa State University Press. ISBN 0-81381561-4.

Somogyi, M. 1945 A new reagent for the determination of sugars. *Journal of Biological Chemistry* **160**, 61–62.

Tamang, J.P. 1992 *Studies on the microflora of some traditional fermented foods of Darjeeling hills and Sikkim*. Ph.D. Thesis. Darjeeling: North Bengal University.

Tamang, J.P. 1995 *Study of traditional fermented foods production in Darjeeling hills and Sikkim, with emphasis on Kinema*. Postdoctoral dissertation. Tokyo: The United Nations University.

Tamang, J.P. 1996 Fermented soybean products in India. In *The proceedings of the Second International Soybean Processing and Utilization Conference*, ed Buchman, A. pp. 189–193. Bangkok: Kasetsart University.

Tamang, J.P. & Nikkuni, S. 1996 Selection of starter culture for production of kinema, a fermented soybean food of the Himalaya. *World Journal of Microbiology and Biotechnology* **12**, 629–635.

Tamang, J.P., Sarkar, P.K. & Hesseltine, C.W. 1988 Traditional fermented foods and beverages of Darjeeling hills and Sikkim – a review. *Journal of Science of Food and Agriculture* **44**, 375–385.

Ueda, S. 1989 Utilization of soybean as natto, a traditional Japanese food. In *Bacillus subtilis: Molecular Biology and Industrial Application*, eds Maruo, B. & Yoshikawa, H. pp. 143–161. Tokyo: Elsevier. ISBN 4-06201777-6.

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