Optimization of soybean processing into kinema, a Bacillus-fermented alkaline food, with respect to a minimum level of antinutrients

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ORIGINAL ARTICLE

Optimization of soybean processing into kinema, a *Bacillus*-fermented alkaline food, with respect to a minimum level of antinutrients

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Abstract

Aims: Optimization of traditional processing of soybeans using response surface methodology (RSM) to achieve a minimum level of antinutritional factors (ANFs) in kinema.

Methods and Results: Central composite rotatable designs were used to optimize the processing stages of kinema preparation. In each stage, the linear or quadratic effects of independent variables were significant in minimizing ANF levels. The predicted optimum condition for soaking was when the raw beans–water ratio was 1 : 10, and the soaking temperature, time and pH were 10°C, 20 h and 8·0 respectively. Here, tannins content (TC), phytic acid content (PAC) and trypsin inhibitor activity (TIA) decreased ($P < 0·05$). While haemagglutinating activity (HA) level remained unchanged ($P < 0·05$), total biogenic amines content (TBAC) increased. The optimum condition for cooking was optimally soaked beans–water ratio of 1 : 5, and cooking pressure and time were 1·10 kg cm^{-2} and 20 min respectively. Here, TC, PAC, TIA and HA decreased ($P < 0·05$), but TBAC remained unchanged compared to optimally soaked beans. TC and HA went below the level of detection. The optimum condition for fermentation was obtained when inoculum load was 10^3 total cells g^{-1} grits, and fermentation temperature and time were 37°C and 48 h respectively. Fermentation of optimally cooked beans caused a reduction ($P < 0·05$) of PAC. While TIA remained unchanged ($P < 0·05$), TBAC increased. In kinema, TC, PAC, TIA and HA decreased ($P < 0·05$) over raw beans by 100, 61, 71 and 100% respectively. Good agreement was observed between predicted values and experimental values.

Conclusions: The processing treatments significantly minimized the level of ANFs in soybeans.

Significance and Impact of the Study: RSM was successfully deployed to obtain the optimum condition for kinema-making with a minimum level of ANFs without impairing sensory attributes of the product. The results are useful for commercial production of kinema.

Introduction

Among legumes, soybean (*Glycine max* (L.) Merr.) occupies a unique position for its extensive production and consumption throughout the world. Although soybeans contain a moderately high amount of protein, calories, vitamins and minerals, the consumption preference for soybeans becomes limited due to the presence of considerable amounts of antinutritional factors (ANFs) (Bau *et al.* 1997; Egounlety and Aworh 2003). Phenolic compounds, especially condensed tannins (proanthocyanidins) with multiple phenolic hydroxyl groups having

molecular weight of 500–3000, form complexes with proteins and metal ions (El Gharras 2009). These compounds form complexes, making them less soluble and more resistant to enzymic degradation. The prevalence of phytic acid is of great concern as it is a strong chelator of mineral nutrients. The complex of phytic acid and mineral elements results in reduced bioavailability of the nutrients in foods (Kumar *et al.* 2010). Trypsin inhibitors, which are proteinaceous in nature, are likely to protect legume seeds against attack by predators. They interfere with intestinal protein digestion by inhibiting pancreatic serine proteases (trypsin and chymotrypsin) and are responsible for the inhibition of growth and intestinal digestion in animals (Guillamón *et al.* 2008). Soybean haemagglutinin (agglutinin or lectin) is a glycoprotein (MW 110 000) having mannose and glucosamine as sugar constituents of the carbohydrate moiety. In this, the carbohydrate moiety is present as a single polysaccharide unit that remains attached to the aspartic acid of the protein moiety by covalent linkage (Lis *et al.* 1966). It binds to surface glycoproteins on erythrocytes causing agglutination and anaemia. As it survives digestion by the gastrointestinal tract of consumers, it binds to glycosyl groups on the epithelial surface of the small intestine, interfering with nutrient absorption (Lajolo and Genovese 2002). Biogenic amines are nitrogenous and low molecular weight organic bases of aliphatic, aromatic or heterocyclic that are formed by decarboxylation of amino acids or amination and transamination of aldehydes and ketones (Silla Santos 1996). The formation of these amines has been reported in soybean-based fermented products such as miso (Kung *et al.* 2007), doenjang (Shukla *et al.* 2010), natto (Tsai *et al.* 2007a), tempe (Nout *et al.* 1993), douchi (Tsai *et al.* 2007b) and soy sauce (Yongmei *et al.* 2009). Consumption of these amines in a dose higher than 1000 mg kg^{-1} food leads to physiological disorders like headache, nausea, rashes, brain haemorrhage, changes in blood pressure and abdominal cramps and flushing (Ladero *et al.* 2010; Shukla *et al.* 2010). The concentration of these ANFs is reportedly reduced by the application of traditional processing treatments such as dehulling, soaking, cooking and fermentation (Khattab and Arntfield 2009; Kalpanadevi and Mohan 2013).

Kinema is a traditional soybean-fermented food of the people of Nepal, Bhutan and north-eastern Himalayan belt in India. It is a solid-state fermented product where alkaline pH favours the fermentation process. Traditionally, kinema is prepared by soaking locally grown yellow variety of soybeans overnight at ambient temperature (15–25°C), cooking the soaked beans, crushing the cooked beans to grits, wrapping the grits with fresh leaves and sackcloth and leaving them at a warm place (35–25°C) to ferment for 1–3 days. Fresh kinema is briefly fried in oil along with salt and a few vegetables to prepare a thick curry which is taken with boiled rice. The fermentation is achieved by the activities of *Bacillus subtilis* (Sarkar and Tamang 1994; Sarkar and Nout 2014).

Although there are reports on the fate of flatus-causing sugars (raffinose family oligosaccharides) in soybean during the production of kinema (Sarkar *et al.* 1997), reports on other major antinutrients, such as tannin, phytic acid, trypsin inhibitor, lectin and total biogenic amines are lacking. Hence, the objective of this study was to optimize the traditional processing stages, i.e. soaking, cooking and fermentation with respect to a minimum level of ANFs in kinema, without impairing its sensory quality.

Materials and methods

Collection of samples

Yellow cultivar soybean seeds were purchased from a local market of Kurseong town in the district of Darjeeling, India and packed in an airtight container.

Experimental design

Response surface methodology (RSM) was used to optimize numerically the three processing stages of kinema-making (soaking, cooking and fermentation) to minimize the different ANFs, namely tannins content (TC), phytic acid content (PAC), trypsin inhibitor activity (TIA), haemagglutinating activity (HA) and total biogenic amines content (TBAC). The preliminary experimental trials and literature survey data were used for the selection of processing variation levels. Central composite rotatable designs (CCRD) were used for analysing the effect of four independent variables of the soaking stage (Table 1). To study the effect of pH of soaking water on the levels of different ANFs, distilled water with adjusted pH (using 0·1 mol lactic acid l^{-1} and 0·1 mol NaOH l^{-1}) was used as a soaking medium. The soaking stage consisted of 30 experimental runs with 16 factorial points, eight axial points and six replicates at central points. Randomized experiments were conducted, and an optimized soaking condition was obtained using DESIGN EXPERT ver. 8.0 (Stat-Ease Inc., Minneapolis, MN) after setting a desired range for the independent and dependent variables. Optimally soaked beans were used for optimization of the subsequent cooking stage employing three independent variables. After cooking, pressure inside the autoclave was released immediately. Cooked water inside the bottles was drained off quickly to minimize excess heating of beans. However, to mimic the traditional procedure for obtaining a desired texture of the final product, a little free water was allowed to remain inside the bottle. The cooking stage consisted of 20 experi-

Table 1 Levels of variables in the experimental design for processing stages

Experimental parameter	Coded level*				
	−α† (augmented form)	−1 (factorial points)	0 (centre point)	1 (factorial points)	α† (augmented form)
Soaking					
Raw soybeans : water (w/w)	1 : 1	1 : 4	1 : 7	1 : 10	1 : 13
Soaking time (h)	0·5	7	13·5	20	26·5
Soaking temperature (°C)	2·5	10	17·5	25	32·5
pH	2	4	6	8	10
Cooking					
Cooking time (min)	6·59	10	15	18	23·41
Cooking pressure (kg cm^{-2})	0·5	0·7	1·0	1·3	1·5
Soaked soybeans : water (w/w)	1 : 0·98	1 : 2	1 : 3·5	1 : 5	1 : 6·02
Fermentation					
Incubation temperature (°C)	18·18	25	35	45	51·82
Incubation time (h)	5·72	18	36	54	66·27
Inoculum load (log total cells g^{-1})	1·98	3	4·5	6	7

*Low, middle and high levels of each variable were designated as −1, 0 and +1 respectively.

†α (1·682 for soaking and 2 for cooking and fermentation stage) is the axial distance from the central point.

mental runs with eight factorial points, six axial points and six replicates at the central points according to CCRD design. The results were used to obtain optimized cooking condition. Optimally soaked and cooked beans were crushed aseptically to grits of half to one-third of the original size. Using those grits, the fermentation stage was optimized employing three independent variables. This stage also consisted of 20 experimental runs with eight factorial points, six axial points and six replicates at the central points. The coded level parameters used for the central, factorial and augmented points of design with low and high levels of all independent variables of the three processing stages are shown in Table 1.

Bacillus subtilis DK-W1 (MTCC 1747) was used as a starter culture for fermentation (Sarkar *et al.* 1993). As per the experimental condition, the inoculum of different sizes (1·98–7·00 log total cells g^{-1}) was mixed with soybean grits to get a desired load of bacterial cells. The beans (~50 g) were then distributed aseptically into sterile 250-ml glass bottles which were capped lightly and placed in incubators, set at desired temperature levels (18·18–51·82°C). At selected fermentation times, the bottles containing beans were removed for analysis.

Sensory analysis

Kinema, prepared under optimized conditions, was subjected to sensory analysis by a panel of 10 trained judges who were frequent consumers of kinema. They evaluated the overall sensory quality consisting of flavour (50), texture (45) and colour (5), using a 100-point score card (Sarkar and Tamang 1994). All the analyses were conducted in triplicate.

Evaluation of antinutritional factors

The samples of raw soybeans were ground to powder and those of soaked, cooked and fermented beans were made to paste using a waring blender (Bajaj Electricals Ltd., Mumbai, India). The powder and pastes were kept overnight at −20°C, freeze-dried (Eyela freeze dryer, model FDU-506, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) and ground to fine powder which were used for assay.

Estimation of tannins

The method described by Price *et al.* (1978) was modified for the determination of TC. Powdered sample (200 mg) was mixed with 10 ml acidified methanol (10 ml conc. HCl (61262325001046; Merck Specialities Pvt. Ltd., Prabhadevi, Mumbai, India) l^{-1} methanol (Merck 1.06009.2500)) and shaken at 25°C for 1 h. The mixture was centrifuged (3000 *g*, 15 min) and the clear supernatant was collected. The extraction process was repeated once and pooled. Fresh extract (1 ml) was added with 5 ml of vanillin reagent (1 : 1 v/v mixture of 10 g vanillin (RM616; HiMedia Laboratories, Mumbai, India) l^{-1} methanol and 80 ml conc. HCl l^{-1} methanol). The mixture was incubated at 30°C for 20 min before taking absorbance at 500 nm using a UV–vis spectrophotometer (Type 118; Systronics, Ahmedabad, India). The TC was expressed as catechin equivalent using the standard curve of catechin (C1251; Sigma-Aldrich Inc., Shanghai, China) and the formula:

$$TC\,(mg\,g^{-1}) = \frac{C \times \text{vol. of extract}\,(10\,ml)}{\text{Sample wt}\,(g)}$$

where, C was the concentration obtained from the standard curve (mg ml^{-1}).

Estimation of phytic acid

Phytic acid was extracted and estimated following the method of Wheeler and Ferrel (1971). The sample (3 g) was mixed with 50 ml of 30 g l^{-1} trichloroacetic acid (TCA; Merck 82234205001730) and shaken (Bti-43; Bio-Technics, Mumbai, India) at 25°C for 30 min followed by centrifugation (10 000 g, 10 min). A 4 ml ferric chloride solution (5·78 mg ferric chloride (38379; S.D. Fine-Chem Ltd., Mumbai) ml^{-1} TCA solution) was added to 10 ml of the supernatant, boiled in a water bath for 45 min and centrifuged (10 000 g, 10 min). The supernatant was allowed to react with 3 ml of 1·5 mol l^{-1} sodium hydroxide (Merck 61757305001046) solution. The red precipitate formed was dissolved with hot 3·2 mol l^{-1} conc. HNO_3 and filtered using Whatman no. 2 paper. The iron content in the samples was estimated at 480 nm immediately after adding 1·5 mol l^{-1} potassium thiocyanate (SDFCL 39666) solution. Phytate phosphorus was calculated from iron results assuming 4 : 6 of iron : phosphorus molar ratio. Phytic acid was estimated by assuming that 0·282 g phosphorus was present per gram sample (Deshpande *et al.* 1982). Calculation of the iron content was made from the standard curve prepared using ferric nitrate (HiMedia RM1376) as standard.

Estimation of trypsin inhibitor activity

Beans were first defatted, based on the method described by Sarkar *et al.* (1996). Beans were blended for 1 min to powders or pastes which were frozen to −20°C, lyophilized and ground to fine powders. Approximately, 5 g of lyophilized powders were defatted using petroleum ether (Merck 61782225001730). The solvent was evaporated off at room temperature.

The procedure of Kakade *et al.* (1969) was then followed to assay TIA. About 1 g defatted powdered sample was mixed with 19 ml distilled water (pH 7·6, adjusted using 0·05 mol l^{-1} NaOH solution). The suspension was shaken at 25°C for 1 h and centrifuged (11 000 g, 20 min). The supernatant was pooled and diluted to 50 ml using phosphate buffer (0·1 mol l^{-1}, pH 7·6). An aliquot of the extract (0·2–1·0 ml) was taken into a triplicate set of test tubes (one set for each level of extract). The volume was bought to 1·0 ml with phosphate buffer (0·1 mol l^{-1}, pH 7·6). To each tube, 1 ml of stock trypsin solution (5 mg trypsin (HiMedia RM618) dissolved in 100 ml of 0·001 mol l^{-1} HCl) was added. The tubes were placed in a water bath at 37°C. One of the triplicate sets was added with 6 ml of 50 g l^{-1} TCA solution to serve as blank. To each of the other tubes, 2 ml of warmed (37°C) casein solution (2 g casein (HiMedia RM087) in 100 ml phosphate buffer) was added. All the tubes were allowed to stand at 37°C for 20 min; 6 ml of 50 g l^{-1} TCA solution

was added to terminate the reaction. After standing for 1 h at room temperature, the suspension in the tubes was filtered using Whatman no. 1 paper. Absorbance of the filtrate was measured at 280 nm against the blank.

TIA was expressed as trypsin inhibitor unit (TIU) per gram sample which was calculated from the absorbance read against the blank. One TIU was defined as a decrease in A_{280} of 0·01, relative to the blank, in 20 min using a 10 ml assay volume.

Estimation of haemagglutinating activity

HA was assayed using the method described by Liener and Hill (1953). Sample (1·0 g) was mixed with 10 ml of 9·0 g l^{-1} sodium chloride (Merck 61751905001730) solution, blended for 1 min, allowed to stand for 15 min and centrifuged (10 000 g, 20 min).

Human blood sample (B-group female), collected from the Health Centre of the University of North Bengal, was diluted four times with cold 9·0 g l^{-1} sodium chloride solution and the suspension was centrifuged (313 g, 10 min). The sediment, after washing with sodium chloride solution until the supernatant became colourless, was diluted with sodium chloride solution to obtain a final red blood cell (RBC) concentration of 4% (v/v).

The sample extract was diluted with sodium chloride solution to get 10 different serial 2-fold dilutions of the extract (1 : 0-1 : 528). A 0·2 ml of the RBC suspension was added to each of the 10 tubes having the dimension of 10 × 75 mm containing 0·5 ml of the diluted sample extract. After incubating the mixture at 37°C for 1 h, agglutination was checked by observing settling down of the cells to the bottom of the test tube. The tubes were graded (0-4+) to measure the degree of agglutination. One haemagglutinating unit (HU) is defined as the least amount of haemagglutinin which produced positive agglutination (1+) under the condition of our experiment. HA was calculated as follows:

$$HU\,g^{-1} = \frac{D_a \times D_b \times S}{V}$$

where, D_a was dilution factor of the extract in tube 1 (which is 1), D_b was dilution factor of the tube containing 1 HU, S represented the volume of original extract per gram sample (which was 10 ml) and V represented the volume of extract in tube 1 (which was 0·5 ml).

Estimation of total biogenic amines

TBAC was assayed according to Yeh *et al.* (2006). A 5-g sample was mixed with 50 ml of 200 g l^{-1} TCA solution and homogenized using a magnetic stirrer for 10 min. The supernatant (10 ml) was diluted 10 times using distilled water and filtered using a Whatman no. 1 paper. The filtrate was adjusted to pH 9·0 with 50% potassium

hydroxide (HiMedia RM1015) and centrifuged (700 **g**, 5 min). The clear supernatant (1 ml) was mixed with 0·45 ml of colour developing reagent (four parts of 1·5 mol l^{-1} Tris buffer (204982; Sisco Research Laboratories Pvt. Ltd., Mumbai) pH 9·0, 1 part of 400 mmol l^{-1} 4-aminoantipyrine (A4382; Sigma-Aldrich, St. Louis, MO) and one part of 40 mmol l^{-1} phenol (Merck 82229605001046)), 0·5 ml of 300 mU ml^{-1} diamine oxidase (EC 1.4.3.6; Sigma-Aldrich, St. Louis, MO) and 0·05 ml of 175 U ml^{-1} horseradish peroxidase type VI-A (EC 1.11.1.7; Sigma). After incubating the mixture at 50°C for 1 h, the absorbance was read at 505 nm and compared with the standard curve prepared using histamine dihydrochloride (Sigma-Aldrich China H7250).

Statistical analysis

The DESIGN EXPERT ver. 8.0 (Stat-Ease Inc.) was used to create designs and analyse the experimental results. Response surface regression procedure was followed for analysing experimental data using second order polynomial equation.

$$Y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_D D + \beta_{AA} A^2 + \beta_{BB} B^2$$
$$+ \beta_{CC} C^2 + \beta_{DD} D^2 + \beta_{AB} AB + \beta_{AC} AC + \beta_{AD} AD$$
$$+ \beta_{BC} BC + \beta_{BD} BD + \beta_{BC} BC$$

where Y was the response (TC, PAC, TIA, HA and TBAC); A, B, C and D were independent variables, and β_0 was a constant at the centre point of the design. While β_A, β_B, β_C and β_D represented the regression coefficients for the linear effect terms, β_{AA}, β_{BB}, β_{CC} and β_{DD} represented the quadratic effect terms and β_{AB}, β_{AC}, β_{AD}, β_{BC}, β_{BD} and β_{BC} were the cross product terms. The regression coefficient produced by the software and the term combination of independent variables were selected and significance of the model was determined from the P-value given by the software. The statistical validity of the model was verified by conducting analysis of variance (ANOVA) for each response variable, and the significant ($P < 0.05$) terms were determined. The insignificant ($P > 0.05$) terms were removed to obtain a reduced equation for optimizing the processing variables. Coefficient of determination (R^2) was used to express the model significance. Higher R^2 signifies good fit for the predicted model. After model fitting, the relation between the independent and response variables was studied using 3D response surface plots.

Validation of the model equations

After numerical optimization, validation of the optimized models was carried out at the end of each processing stage. For model equation verification, deviation error in percentage was estimated by comparing the actual and predicted values at the optimum condi-

tion of each processing stage. It shows how the predicted models at optimum levels varied from the actual results.

Results

Raw soybeans

Raw soybeans contained appreciable amount of ANFs: 1·76 mg tannin, 8·41 mg phytic acid, 39900 TIU trypsin inhibitor activity, 426·67 HU haemagglutinating activity and 157·93 μg total biogenic amines per gram dry weight (Table 2).

Soaking

Under our experimental combinations of soaking (Table 3), the maximum reduction in TC, PAC, TIA and HA in soaked beans was 59·1, 27·5, 8·9 and 25·0% respectively; however, TBAC increased by 85·5–437·5%, compared to those in the raw beans.

Regression coefficient data (Table 4) indicate that the reduction ($P < 0.05$) in TC and TIA was dependent on ratio and time, and their interaction. The levels of PAC and HA remained unaffected ($P < 0.05$) by individual independent variables or their interactions. While time–temperature and temperature–pH caused an increase ($P < 0.05$) in the HA level, the interaction of time, temperature and pH together had no effect ($P < 0.05$) on HA. Although pH caused a reduction ($P < 0.05$) of TBAC, its interaction with temperature had an opposite effect. Reduced polynomial equations after removing insignificant terms for coded variables of each response were

$$TC = 0.930 - 0.114A - 0.129B - 0.062AB + 0.065A^2$$
$$TIA = 38.269 - 0.378A - 0.466B - 0.256AB$$
$$+ 0.186C^2$$
$$HA = 416.833 + 20.125BC + 26.750CD + 37.708B^2$$
$$- 27.292C^2 - 27.292D^2$$
$$TBAC = 452.175 + 73.978C - 93.805D + 36.184CD$$
$$+ 26.084C^2 + 61.031D^2$$

where, A was ratio, B was time, C was temperature and D was pH.

The response surface plots indicate that when temperature and pH were kept constant at 17·5°C and 6·0, respectively, an increase in time and ratio caused a linear reduction in TC (Fig. 1a) and TIA (Fig. 1b) to 0·71 mg g^{-1} (59·7%) and 37·08 TIU mg^{-1} (7·1%) respectively. The minimum HA (323·63 HU g^{-1}) was obtained at 25°C and pH 4·0 when the ratio and time were maintained at 1 : 7 and 13·5 h respectively (Fig. 1c). The minimum TBAC (333·95 μg g^{-1}) was

Table 2 Predicted and experimental values for the five antinutritional factors in soybeans at optimum condition for each stage of kinema-making

Parameters under different stages	Antinutritional factors *(dry wt basis)				
	TC (mg g^{-1})§	PAC (mg g^{-1})	TIA (TIU mg^{-1})	HA (HU g^{-1})¶	TBAC (μg g^{-1})
Raw beans†	1·76^a ± 0·03	8·41^a ± 0·21	39·90^a ± 0·45	426·67^a ± 0	157·93^c ± 9·36
Soaking (raw beans–water of 1 : 10, pH 8·0, 10°C, 20 h)					
Predicted values	0·79	6·22	37·06	351·33	337·66
Experimental values†	0·79^b ± 0	6·23^b ± 0·04	37·04^b ± 0·36	355·56^a ± 17·78	328·48^b ± 10·21
Error %	0	0·16	0·05	1·19	2·79
% change‡	−55·1	−26·0	−7·1	−17·7	+113·8
Cooking (soaked beans–water of 1 : 5, 1·10 kg cm^{-2}, 20 min)					
Predicted values	0·03	5·64	11·71	1·62	311·59
Experimental values	<dl	5·61^c ± 0	11·74^c ± 0·21	<dl	310·30^b ± 0·87
Error %	0	0·53	0·26	0	0·42
% change	−98·3 (−96·2)	−32·9 (−9·3)	−70·7 (−68·4)	−99·6 (−99·5)	+97·3 (−7·7)
Fermentation (inoculum load of 10^3 total cells g^{-1}, 37°C, 48 h)					
Predicted values	0	3·32	11·70	0	992·10
Experimental values	<dl	3·00^d ± 0·02	11·74^c ± 0·08	<dl	990·82^a ± 0·87
Error %	0	10·67	0·34	0	0·13
% change	−100 (−100)	−60·5 (−41·1)	−70·7 (−0·1)	−100 (−100)	+528·2 (+218·4)

*TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; HA, haemagglutinating activity; TABC, total biogenic amines content.
†Values are mean ± SE of triplicate determinations in raw/processed samples. Means followed by the same superscript in each column are not significant ($P > 0·05$) by Duncan multiple range test.
‡− and + indicates percent decrease and increase, respectively, of predicted values over raw beans (values within parentheses indicate percentages calculated over the starting material of that particular stage).
§dl, detection limit (0·004 mg g^{-1} dry wt).
¶dl, detection limit (6·67 HU g^{-1} dry wt).

obtained when the beans were soaked at 10°C in water having pH 8·0 keeping the ratio and time fixed at 1 : 7 and 13·5 h respectively (Fig. 1d). The pH caused a decrease ($P < 0·05$) in the level of TBAC (Table 4). So, the interaction of temperature and pH caused an increase ($P < 0·05$) in the TBAC of soaked beans (Table 4). Numerical optimization was carried out to determine the optimum condition to minimize the different ANFs during soaking. The predicted optimum condition for soaking of beans was 1 : 10 of beans–water ratio, and 20 h, 10°C and 8·0 as soaking time, temperature and pH, respectively, which predicted reduction ($P < 0·05$) of TC, PAC and TIA by 55·1, 26·0 and 7·1% respectively. While HA remained unchanged ($P < 0·05$), the predicted minimum increase ($P < 0·05$) of TBAC was 113·8% (Table 2). The experimentally obtained values were close to the predicated ones.

Cooking

The ANFs for each combination of independent variables of the cooking stage are shown in Table 5. Under the experimental combinations of cooking, the maximum

reduction in TC, PAC, TIA, HA and TBAC was 100, 10·1, 75·5, 100 and 11·4%, respectively, over optimally soaked beans (Table 2).

Regression coefficient data indicate that cooking time and pressure are significant processing variables for minimizing the levels of all the ANFs during cooking (Table 6). The significant ($P < 0·05$) linear terms for TC, PAC and TIA, and quadratic terms for HA and TBAC, and their interaction terms, after removing the insignificant terms for coded levels, can be written as

$$TC = 0·072 − 0·035A − 0·043B$$
$$PAC = 5·754 − 0·058A − 0·066B$$
$$TIA = 13·519 − 1·365A − 1·087B$$
$$HA = 15·663 − 5·392A − 5·392B + 5AB − 2·698A^2 − 2·698B^2 − 3·876C^2$$
$$TBAC = 323·212 − 5·970A − 5·573B − 3·825A^2 − 2·601B^2$$

where, A was cooking time; B was cooking pressure and C was ratio.

Response surface plots show a change in the contents of ANFs when cooking pressure and time were changed keeping soaked beans–water ratio fixed at 1 : 3·5 (Fig. 2). The minimum levels of ANFs were obtained at

Table 3 Design of RSM, and its experimental (Exp.) and predicted (Pred.) values of antinutritional factors for soaked soybeans

					Antinutritional factors* (dry wt basis)									
					TC (mg g^{-1})		PAC (mg g^{-1})		TIA (TIU mg^{-1})		HA (HU g^{-1})		TBAC (μg g^{-1})	
Run	Raw beans : water (w/w)	Soaking time (h)	Soaking temp. (°C)	pH	Exp.†	Pred.	Exp.†	Pred.	Exp.†	Pred.	Exp.†	Pred.	Exp.†	Pred.
1	1 : 7	13·5	17·5	6	0·94	0·93	6·30	6·36	38·35	38·27	420	416·83	441·90	452·18
2	1 : 4	20·0	10·0	4	0·92	1·04	6·28	6·28	39·04	38·70	373	378·17	609·87	605·38
3	1 : 7	13·5	17·5	6	0·99	0·93	6·38	6·36	38·04	38·27	373	416·83	466·15	452·18
4	1 : 4	7·0	10·0	8	1·06	1·17	6·32	6·38	39·04	39·15	480	447·17	424·59	369·22
5	1 : 13	13·5	17·5	6	0·82	0·96	6·20	6·09	37·23	37·57	427	397·00	534·55	559·64
6	1 : 1	13·5	17·5	6	1·62	1·42	6·28	6·23	39·10	39·08	427	432·33	410·74	456·42
7	1 : 7	13·5	17·5	6	0·90	0·93	6·34	6·36	38·58	38·27	427	416·83	446·00	452·18
8	1 : 7	13·5	17·5	2	0·99	0·94	6·63	6·59	38·47	38·73	320	289·83	848·83	883·91
9	1 : 4	20·0	10·0	8	1·09	1·08	6·28	6·32	38·19	38·37	373	382·50	421·13	375·15
10	1 : 10	7·0	25·0	4	1·05	1·08	6·22	6·28	38·79	38·81	320	362·83	773·51	745·81
11	1 : 4	20·0	25·0	8	1·02	1·08	6·28	6·29	38·85	38·38	480	440·67	533·68	575·26
12	1 : 7	0·5	17·5	6	1·30	1·26	6·32	6·19	39·23	38·78	627	592·32	400·00	473·59
13	1 : 10	20·0	10·0	4	0·79	0·75	6·25	6·29	37·30	37·34	373	373·50	678·27	626·18
14	1 : 4	20·0	25·0	4	1·02	1·07	6·25	6·29	38·87	38·96	320	329·33	446·00	452·18
15	1 : 7	13·5	17·5	6	1·00	0·93	6·50	6·36	38·09	38·27	427	416·83	500·00	452·18
16	1 : 7	13·5	32·5	6	0·89	0·92	6·32	6·24	38·81	39·09	320	298·83	749·26	704·47
17	1 : 10	7·0	25·0	8	1·16	1·08	6·14	6·20	38·93	38·76	427	394·17	593·42	600·82
18	1 : 10	7·0	10·0	4	1·12	1·10	6·25	6·31	38·72	38·67	427	438·67	688·66	650·00
19	1 : 7	26·5	17·5	6	0·76	0·74	6·14	6·11	36·35	36·92	533	543·00	509·44	506·62
20	1 : 7	13·5	17·5	10	0·95	0·93	6·66	6·54	38·30	38·35	320	325·50	459·00	452·18
21	1 : 4	7·0	25·0	4	1·24	1·25	6·30	6·33	39·03	39·01	373	367·00	584·76	597·86
22	1 : 10	7·0	10·0	8	1·16	1·13	6·20	6·26	38·76	38·87	320	363·00	400·00	360·28
23	1 : 4	7·0	10·0	4	1·06	1·13	6·30	6·33	38·98	39·01	480	496·33	648·83	593·42
24	1 : 7	13·5	17·5	6	0·95	0·93	6·31	6·36	38·22	38·27	427	416·83	459·00	452·18
25	1 : 10	20·0	25·0	8	0·72	0·66	6·10	6·17	37·04	37·21	427	463·00	640·17	621·89
26	1 : 7	13·5	17·5	6	0·80	0·93	6·30	6·36	38·33	38·27	427	416·83	400·00	452·18
27	1 : 4	7·0	25·0	8	1·20	1·25	6·30	6·36	38·76	38·91	373	424·83	540·00	518·40
28	1 : 10	20·0	25·0	4	0·72	0·65	6·27	6·27	38·35	37·73	373	378·17	714·63	772·91
29	1 : 7	13·5	2·5	6	1·02	0·93	6·37	6·29	38·89	38·94	320	316·50	292·99	408·55
30	1 : 10	20·0	10·0	8	0·76	0·79	6·19	6·22	37·55	37·06	373	351·33	340·61	330·42

*TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; HA, haemagglutinating activity; TABC, total biogenic amines content.
†Mean values of experiments carried out in triplicates.

1·3 kg cm^{-2} for 20 min. Cooking lowered TC below the limit of detection (Fig. 2a). The minimum levels of PAC, TIA, HA and TBAC obtained were 5·63 mg g^{-1} (Fig. 2b), 11·08 TIU mg^{-1} (Fig. 2c), 4·87 HU g^{-1} (Fig. 2d) and 306·69 μg g^{-1} (Fig. 2e), indicating 9·5, 70·1, 98·6 and 9·2% reduction, respectively, over optimally soaked beans. The predicted optimum condition for cooking was soaked beans–water ratio of 1 : 5, and cooking pressure and time of 1·10 kg cm^{-2} and 20 min respectively. In this condition, the predicted reduction ($P < 0.05$) of TC, PAC, TIA and HA in cooked beans was 96·2, 9·3, 68·4 and 99·5%, respectively, over optimally soaked beans. TBAC remained unchanged ($P < 0.05$) (Table 2). The experimental values were in reasonable agreement with the predicted ones.

Fermentation

The response levels for each combination of independent variables are shown in Table 7. Under the experimental combinations, the percent reduction in TC, PAC and TIA were 100, 44·0 and 4·0%, respectively, over optimally soaked and cooked beans; however, TBAC increased by 91·6–320·4%.

Regression coefficient data (Table 8) show that fermentation time has a reducing effect ($P < 0.05$) on TC and PAC. Fermentation time and temperature individually shows a reducing effect on TIA; however, an increasing effect on TBAC. The reduced linear equations after removing nonsignificant terms for coded variables of the ANFs were

Table 4 Estimated regression coefficients on antinutritional factors of soaked soybeans

Factor*	Antinutritional factors† (dry wt basis)									
	TC (mg g^{-1})		PAC (mg g^{-1})		TIA (TIU mg^{-1})		HA (HU g^{-1})		TBAC (μg g^{-1})	
	Effect	*P*-value‡	Effect	*P*-value‡	Effect	*P*-value‡	Effect	*P*-value‡	Effect	*P*-value‡
Intercept	0·930	0·0006	6·355	0·0336	38·269	0·0004	416·833	0·0002	452·175	<0·0001
A	−0·114	<0·0001	−0·035	0·0749	−0·378	0·0001	−8·833	0·2366	25·805	0·0517
B	−0·129	<0·0001	−0·020	0·2872	−0·466	<0·0001	−12·333	0·1058	8·258	0·5090
C	−0·004	0·8609	−0·013	0·4958	0·038	0·6180	−4·417	0·5469	73·978	<0·0001
D	0·010	0·6278	−0·010	0·5818	−0·095	0·2183	8·917	0·2325	−93·805	<0·0001
AB	−0·062	0·0298	0·008	0·7249	−0·256	0·0124	13·250	0·1519	−8·946	0·5585
AC	−0·033	0·2182	−0·007	0·7658	0·032	0·7244	13·375	0·1483	22·841	0·1473
AD	−0·001	0·9810	−0·026	0·2759	0·012	0·8959	−6·625	0·4620	−16·381	0·2905
BC	−0·021	0·4360	0·001	0·9784	0·064	0·4868	20·125	0·0367	12·732	0·4078
BD	0·002	0·9430	−0·006	0·8073	−0·119	0·2072	13·375	0·1483	−1·509	0·9209
CD	−0·007	0·7933	−0·008	0·7249	−0·060	0·5132	26·750	0·0081	36·184	0·0287
A^2	0·065	0·0047	−0·049	0·0132	0·014	0·8412	−0·542	0·9367	13·964	0·2402
B^2	0·018	0·3798	−0·051	0·0098	−0·105	0·1487	37·708	<0·0001	9·482	0·4193
C^2	−0·001	0·9626	−0·022	0·2152	0·186	0·0166	−27·292	0·0010	26·084	0·0373
D^2	0·008	0·6970	0·053	0·0083	0·069	0·3350	−27·292	0·0010	61·031	<0·0001
R^2	0·852		0·715		0·858		0·873		0·904	
Lack of fit		0·1646		0·3263		0·0559		0·0925		0·0561

*A, Raw beans–water ratio (w/w); B, Soaking time (h); C, Soaking temperature (°C); D, pH of soaking water.

†TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; HA, haemagglutinating activity; TABC, total biogenic amines content.

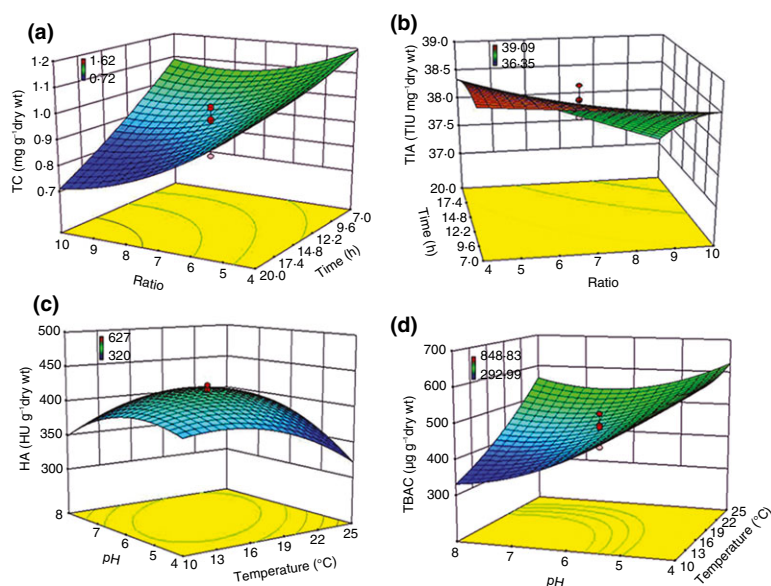‡Values of *P* > *F* < 0·0500 indicate model terms are significant.

Figure 1 Response surface 3D plots showing the influence of raw soybeans–water ratio, pH of soaking water, soaking time and soaking temperature on antinutritional factors: influence of ratio and time on tannins content (TC; a) and trypsin inhibitor activity (TIA; b) when the pH and temperature were kept constant at 6·0 and 17·50°C, respectively; influence of pH and temperature on haemagglutinating activity (HA; c) and total biogenic amines content (TBAC; d) when the ratio and time were kept constant at 1 : 7 and 13·50 h respectively.

$$TC = 0·029 − 0·031B$$
$$PAC = 3·527 − 0·256B$$
$$TIA = 11·964 − 0·232A − 0·333B$$
$$TBAC = 914·282 + 74·079A + 148·222B$$

where, *A* was fermentation temperature and *B* was fermentation time.

The response surface plots for TC, PAC and TIA (Fig. 3) indicate that when the inoculum load was fixed at 4·5 log total cell g^{-1} beans and fermentation condition was increased to 45°C and 54 h, the levels of ANFs reduced drastically. TC was reduced below the limit of detection (Fig. 3a). PAC was reduced to 3·21 mg g^{-1}

Table 5 Design of RSM, and its experimental (Exp.) and predicted (Pred.) values of antinutritional factors for cooked soybeans

Run	Cooking time (min)	Cooking pressure (kg cm^{-2})	Soaked beans : water (w/w)	TC (mg g^{-1}) Exp.†‡	Pred.	PAC (mg g^{-1}) Exp.†	Pred.	TIA (TIU mg^{-1}) Exp.†	Pred.	HA (HU g^{-1}) Exp.†§	Pred.	TBAC (μg g^{-1}) Exp.†	Pred.
1	10·00	0·7	1 : 5	0·12	0·15	5·84	5·84	13·46	15·89	20·00	20·53	327·62	329·38
2	15·00	0·5	1 : 3·5	0·16	0·15	5·89	5·86	16·85	15·35	20·00	17·10	324·15	325·23
3	15·00	1·5	1 : 3·5	<dl	0	5·71	5·64	11·95	11·69	<dl	0	310·30	306·48
4	6·59	1·0	1 : 3·5	0·19	0·13	5·89	5·85	17·20	15·82	20·00	17·10	328·48	322·43
5	10·00	0·7	1 : 2	0·12	0·15	5·91	5·91	13·32	16·05	20·00	23·82	327·62	329·67
6	20·00	1·3	1 : 2	<dl	0	5·66	5·66	10·83	11·14	<dl	2·25	306·84	307·02
7	20·00	1·3	1 : 2	<dl	0	5·64	5·60	10·14	10·99	<dl	0	305·97	305·86
8	15·00	1·0	1 : 0·98	0·12	0·07	5·83	5·80	12·84	13·65	13·33	7·46	324·69	323·23
9	10·00	1·3	1 : 5	<dl	0·07	5·66	5·72	13·40	13·72	<dl	0	309·44	313·47
10	15·00	1·0	1 : 6·02	0·09	0·08	5·68	5·71	12·91	13·39	<dl	1·94	323·29	322·01
11	15·00	1·0	1 : 3·5	0·09	0·07	5·76	5·75	14·84	13·52	13·33	15·66	323·56	323·21
12	23·41	1·0	1 : 3·5	<dl	0·01	5·59	5·66	9·08	11·22	<dl	0	299·05	302·35
13	15·00	1·0	1 : 3·5	<dl	0·07	5·78	5·75	14·79	13·52	20·00	15·66	323·83	323·21
14	10·00	1·3	1 : 2	<dl	0·06	5·71	5·77	13·58	13·88	<dl	3·03	313·77	317·66
15	25·00	1·0	1 : 3·5	0·16	0·07	5·76	5·75	15·02	13·52	13·33	15·66	326·69	323·21
16	20·00	0·7	1 : 2	<dl	0·08	5·76	5·79	14·12	13·32	<dl	3·03	315·50	313·40
17	15·00	1·0	1 : 3·5	0·09	0·07	5·65	5·75	12·93	13·52	20·00	13·33	324·00	323·21
18	20·00	0·7	1 : 5	0·09	0·08	5·76	5·73	13·68	13·16	<dl	0	318·09	316·14
19	15·00	1·0	1 : 3·5	0·09	0·07	5·74	5·75	14·75	13·52	20·00	15·66	320·43	323·21
20	15·00	1·0	1 : 3·5	0·12	0·07	5·65	5·75	14·69	13·52	13·33	15·66	320·29	323·21

*TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; HA, haemagglutinating activity; TABC, total biogenic amines content.
†Mean values of experiments carried out in triplicates.
‡dl, detection limit (0·004 mg g^{-1} dry wt).
§dl, detection limit (6·67 HU g^{-1} dry wt).

Table 6 Estimated regression coefficients on antinutritional factors of cooked soybeans

Factor*	TC (mg g^{-1}) Effect	P-value‡	PAC (mg g^{-1}) Effect	P-value‡	TIA (TIU mg^{-1}) Effect	P-value‡	HA (HU g^{-1}) Effect	P-value‡	TBAC (μg g^{-1}) Effect	P-value‡
Intercept	0·072	0·0066	5·754	<0·0001	13·519	0·0042	15·663	0·0007	323·212	0·0008
A	−0·035	0·0190	−0·058	0·0007	−1·365	0·0032	−5·392	0·0005	−5·970	0·0002
B	−0·043	0·0047	−0·066	0·0002	−1·087	0·0139	−5·392	0·0005	−5·573	0·0003
C	−0·002	0·8549	−0·028	0·0582	−0·078	0·8453	−1·641	0·1557	−0·363	0·7305
AB							5	0·005	1·407	0·3185
AC							0	1	0·757	0·5846
BC							0	1	−0·974	0·4838
A^2							−2·698	0·0269	−3·825	0·0033
B^2							−2·698	0·0269	−2·601	0·0263
C^2							−3·876	0·0040	−0·209	0·8379
R^2	0·524		0·735		0·552		0·899		0·895	
Lack of fit		0·6346		0·8262		0·0514		0·3026		0·0820

*A, Cooking time (min); B, Cooking pressure (kg cm^{-2}); C, Soaked beans to water ratio (w/w).
†TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; HA, haemagglutinating activity; TABC, total biogenic amines content.
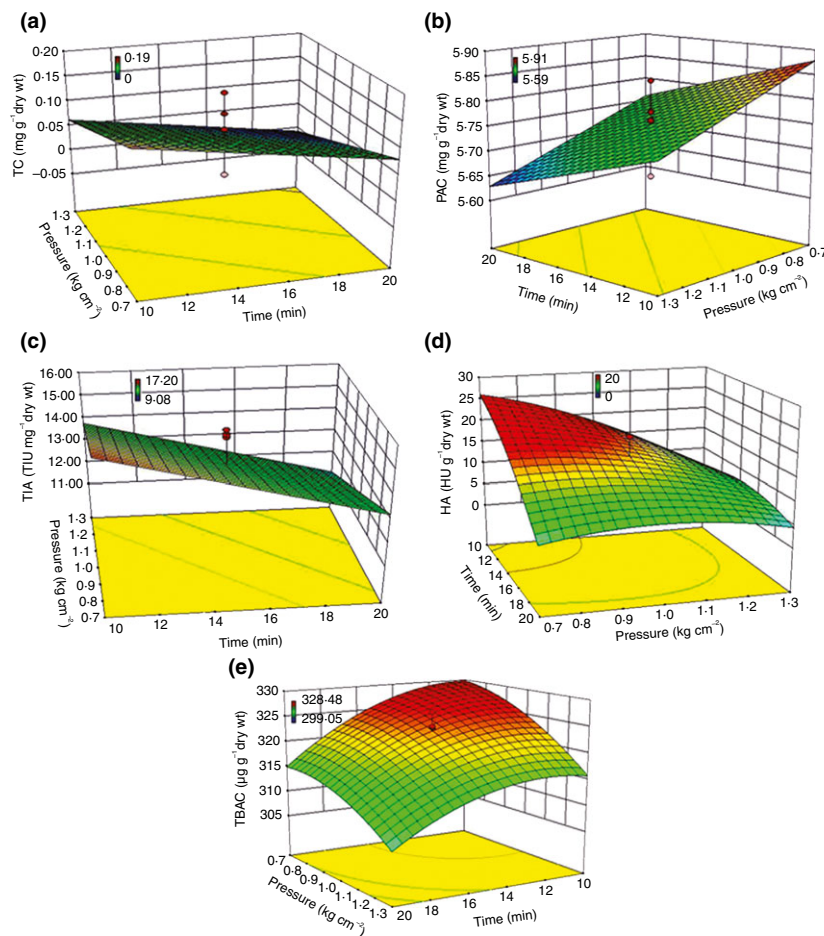‡Values of $P > F < 0.0500$ indicate model terms are significant.

Figure 2 Response surface 3D plots showing the influence of cooking pressure and time on tannins content (TC; a), phytic acid content (PAC; b) trypsin inhibitor activity (TIA; c); haemagglutinating activity (HA; d) and total biogenic amines content (TBAC; e) when the optimally soaked soybeans–water ratio was kept constant at 1 : 3·5 (w/w).

(Fig. 3b), indicating 43·1% reduction over optimally cooked beans. The lowest TIA of fermented beans was 11·40 TIU mg^{-1} (Fig. 3c), showing 2·6% reduction over optimally cooked beans. The minimum TBAC (693·9 μg g^{-1}), the value of which was 122·7% more than that of optimally cooked beans, was obtained when the fermentation temperature and time reduced to 25°C and 18 h respectively (Fig. 3d).

The predicted optimum condition of fermentation was l0^3 total cells g^{-1} beans, fermentation temperature and time of 37°C and 48 h respectively (Table 2). The percent reduction ($P < 0.05$) of TC and PAC during fermentation was 100 and 41·1% respectively.

The optimally processed product was finally subjected to sensory analysis. Kinema of good quality should have nutty flavour with mild ammonia smell, a greyish brown colour and highly sticky or mucilaginous texture (Sarkar and Tamang 1994). The sensory analysis was carried out to evaluate the organoleptic quality of kinema produced through optimized processing stages. Overall sensory quality of kinema was evaluated by the judges based on 100-point score card (Table 9). The sensory score of optimized kinema in this study was 90·7, justifying an 'excellent' quality.

Discussion

Raw soybeans

The ANF levels in raw soybeans obtained were close to the ranges as reported earlier (Egounlety and Aworh 2003; Lestienne *et al.* 2005).

Soaking

Soaking is an initial processing step which is routinely followed during the preparation of all legume-based fermented foods. The response surface plots generated for the model of soaking stage indicate that when temperature and pH were kept constant at 17·5°C and 6·0, respectively, an increase in time and ratio caused a linear reduction in TC and TIA. As tannins and trypsin inhibi-

Table 7 Design of RSM, and its experimental (Exp.) and predicted (Pred.) values of antinutritional factors for fermented soybeans

Run	Fermentation temp. (°C)	Fermentation time (h)	Inoculum load (log total cells g^{-1})	TC (mg g^{-1})		PAC (mg g^{-1})		TIA (TIU mg^{-1})		TBAC (µg g^{-1})	
				Exp.†‡	Pred.	Exp.†	Pred.	Exp.†	Pred.	Exp.†	Pred.
1	45	18	6	0·06	0·05	3·50	3·75	12·17	12·07	898·18	875·94
2	35	36	4·5	0·03	0·03	3·55	3·53	11·74	11·96	1002·08	914·28
3	25	54	6	<dl	0	3·36	3·35	11·77	11·86	934·55	1024·24
4	25	54	3	<dl	0	3·41	3·31	11·68	11·86	926·75	952·62
5	35	36	4·5	<dl	0·03	3·41	3·53	12·44	11·96	917·55	914·28
6	35	36	4·5	0·03	0·03	3·21	3·53	12·21	11·96	978·70	914·28
7	35	36	7·02	<dl	0·03	3·85	3·56	11·94	11·96	989·09	974·51
8	35	5·73	4·5	0·12	0·08	4·80	3·96	12·41	12·53	596·88	665·00
9	25	18	6	0·09	0·07	3·55	3·86	12·74	12·53	698·18	727·79
10	35	36	4·5	<dl	0·03	3·60	3·53	11·61	11·96	925·35	914·28
11	35	66·27	4·5	<dl	0·02	3·16	3·09	11·40	11·40	1309·90	1163·56
12	45	18	3	<dl	0·05	3·55	3·70	11·90	12·07	840·17	804·33
13	35	36	4·5	0·09	0·03	3·65	3·53	12·24	11·96	1009·87	914·28
14	25	18	3	0·06	0·07	3·45	3·82	12·63	12·53	725·02	656·17
15	35	36	4·5	0·03	0·03	3·41	3·53	11·60	11·96	930·30	914·28
16	51·82	36	4·5	<dl	0·01	3·21	3·43	11·24	11·57	999·48	1038·87
17	35	36	1·98	0·03	0·02	3·70	3·49	12·25	11·96	735·87	854·06
18	18·18	36	4·5	0·03	0·05	3·60	3·63	12·17	12·36	742·34	789·69
19	45	54	6	<dl	0	3·31	3·23	11·59	11·40	1074·81	1172·39
20	45	54	3	<dl	0	3·26	3·19	11·55	11·40	1050·56	1100·77

*TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; TABC, total biogenic amines content.
†Mean values of experiments carried out in triplicates.
‡dl, detection limit (0·004 mg g^{-1} dry wt).

Table 8 Estimated regression coefficients on antinutritional factors of fermented soybeans

Factor*	Antinutritional factors† (dry wt basis)							
	TC (mg g^{-1})		PAC (mg g^{-1})		TIA (TIU mg^{-1})		TBAC (µg g^{-1})	
	Effect	P-value‡	Effect	P-value‡	Effect	P-value‡	Effect	P-value‡
Intercept	0·029	0·0054	3·527	0·0341	11·964	0·0004	914·282	<0·0001
A	−0·010	0·1904	−0·059	0·4659	−0·232	0·0048	74·079	0·0025
B	−0·031	0·0009	−0·256	0·0052	−0·333	0·0002	148·222	<0·0001
C	0·003	0·7053	0·022	0·7873	0	0·9978	35·811	0·1028
R^2	0·537		0·409		0·673		0·807	
Lack of fit		0·7866		0·0621		0·9557		0·0533

*A, Fermentation temperature (°C); B, Fermentation time (h); C, Microbial load (log total cells g^{-1}).
†TC, tannins content; PAC, phytic acid content; TIA, trypsin inhibitor activity; TABC, total biogenic amines content.
‡Values of $P > F < 0.0500$ indicate model terms are significant.

tors are water-soluble (Kumar *et al.* 1979; Afify and Shousha 1988), the increase in ratio might have caused a decrease in their values in soaked beans. A simultaneous increase in time might have provided an additional effect in getting them leached out into the soaking medium. The loss of TC may also be attributed to its binding with other organic substances such as carbohydrates or protein (Saharan *et al.* 2002) or degradation due to an activation

of polyphenoloxidase (Saxena *et al.* 2003). There are reports for the reduction in TC and TIA during soaking of beans (Egounlety and Aworh 2003; Khattab and Arnt-field 2009). The loss of HA in the blood group 'B' when subjected to soaking is in line with the earlier reports in chickpea (Bansal *et al.* 1988), cowpea (Kalpanadevi and Mohan 2013), lentil (Batra 1987), kidney bean (Shimelis and Rakshit 2007) and lablab bean (Vijayakumari *et al.*
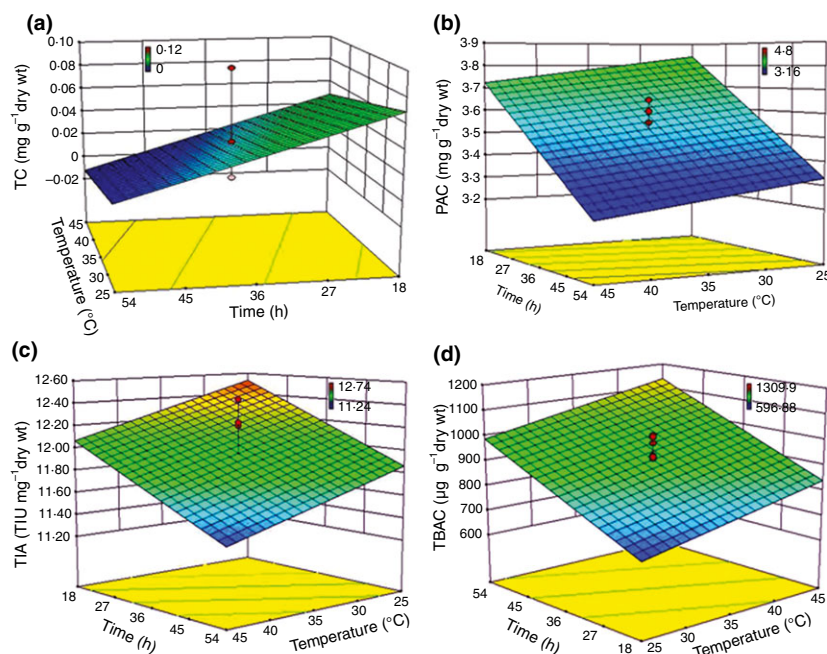
Figure 3 Response surface 3D plots showing the influence of fermentation temperature and time on tannins content (TC; a), phytic acid content (PAC; b), trypsin inhibitor activity (TIA; c) and total biogenic amines content (TBAC; d) when the inoculum load was kept constant at 4·5 log total cells g^{-1} optimally soaked and cooked soybeans.

Table 9 Sensory scores of kinema obtained through optimized process conditions

Attribute	Score*
Flavour (maximum score, 50)	45·37 ± 0·42
Body texture (maximum score, 45)	41·37 ± 0·22
Colour and appearance (maximum score, 5)	3·97 ± 0·11
Overall quality (total maximum score, 100)	90·70 ± 0·33

*Values are mean ± SE (n = 30).

1995). The incomplete destruction of HA might be due to the presence of high levels of other ANFs, such as tannin, phytic acid and trypsin inhibitor which may interfere with the lectin destruction (Kalpanadevi and Mohan 2013). In this study, pH caused a decrease ($P < 0.05$) in the level of TBAC. The pH is an important factor influencing decarboxylase activity, and low pH (3·0–6·0) is optimum for bacteria to produce decarboxylase (Silla Santos 1996). Lower temperature adversely affects proteolytic and decarboxylating reactions, resulting in a decreased amine concentration (Joosten and van Boeckel 1988).

The predicted optimum condition for soaking of beans (1 : 10 of beans–water ratio, and 20 h, 10°C and 8·0 as soaking time, temperature and pH respectively) led a reduction ($P < 0.05$) of TC, PAC and TIA by 55·1, 26·0 and 7·1%, respectively, as compared to raw soybean. While HA remained unchanged ($P < 0.05$), the predicted minimum increase ($P < 0.05$) of TBAC was 113·8%. Egounlety and Aworh (2003) reported 54·6 and 2·4% reduc-

tion of TC and TIA, respectively, as compared to raw soybean, under the traditional condition (1 : 3 w/v of beans–tap water ratio, for 12–14 h). While they observed 34·6% increase in PAC over raw soybean, under optimized soaking condition in this study PAC reduced by 26%.

Cooking

This study was carried out using optimally soaked beans. As boiling improves the protein quality of beans by destruction or inactivation of heat-labile ANFs (Vijayakumari *et al.* 1997), optimization of this stage is worthwhile. Complete removal of tannins in cooked beans signifies an extensive leaching due to the combined effect of initial soaking followed by cooking. Shimelis and Rakshit (2007) reported that the combined effect of soaking and cooking was more effective than either soaking or cooking alone. Also, cooking can destroy heat-labile tannin molecules causing a qualitative change in its molecular structure and makes them less extractable and detectable during the assay (Rakic *et al.* 2007). Complete removal of tannins in soybeans during cooking was reported by Egounlety and Aworh (2003). PAC showed 9·5% reduction over optimally soaked beans. The reduction of PAC in legumes during cooking has also been reported earlier (Shimelis and Rakshit 2007; Embaby 2010). The degradation during cooking under pressure can be due to the formation of insoluble complexes of phytate-protein and phytate-protein-minerals or leaching

into the cooking medium or degradation of inositol hexaphosphate into pentatetraphosphate (Vijayakumari *et al.* 1997, 2007). In this study, TIA reduced by 70·1% over optimally soaked beans. The reduction in TIA during cooking might be due to the destruction of disulphide bonds or hydrolysis of peptide bonds or splitting of covalent bonds (Adams 1991). The thermolabile nature of trypsin inhibitors in legumes was reported earlier (Egounlety and Aworh 2003; Shimelis and Rakshit 2007; Embaby 2010). Haemagglutinin, due to its heat-sensitive nature, was reduced to below the limit of detection by the heating processes (cooking and autoclaving), and the combination of soaking and cooking was more pronounced for inactivation of the lectins (Shimelis and Rakshit 2007). Elimination of HA by autoclaving was observed in faba bean (Khalil and Mansour 1995), chick pea (Alajaji and El-Adawy 2006) and cow pea (Kalpanadevi and Mohan 2013). The reduction in lectin activity during cooking may be due to the breakdown of haemagglutinins into their subunits or to other unknown conformational changes in their native (Batra 1987).

The predicted optimum condition for cooking (soaked beans–water ratio of 1 : 5, and cooking pressure and time of 1·10 kg cm^{-2} and 20 min respectively) predicted a reduction ($P < 0.05$) in TC, PAC, TIA and HA in cooked beans by 96·2, 9·3, 68·4 and 99·5%, respectively, over optimally soaked beans. TBAC remained unchanged ($P < 0.05$). The cooking of soaked soybeans had no effect ($P < 0.05$) on TBAC during the production of tempe (Nout *et al.* 1993). Boiling soybean (1 : 6 w/v soaked beans–water ratio) for 30 min caused a reduction in TC, PAC and TIA by 100, 10 and 81·7% (Egounlety and Aworh 2003).

Fermentation

Optimally soaked and cooked beans were utilized in the final processing stage. Fermentation is an important microbial and enzymic method of food processing so as to achieve products having improved organoleptic quality and prolonged shelf life. After fermentation, TC went below the limit of detection. An activity of polyphenol oxidase by the growth of microbiota during fermentation might be a cause of reduction of TC (Reddy and Pierson 1994). In this study, PAC and TIA reduced by 43·1 and 2·6%, respectively, over optimally cooked beans. The reduction in PAC might be due to phytase activity shown by the inoculated culture of *B. subtilis*. It is already known that *B. subtilis* contains phytase-encoding gene and can degrade phytate during growth through production of extracellular phytases (Kumar *et al.* 2010). Shimizu (1992) reported phytase activity of *B. subtilis* in natto samples. The reduction in PAC and TIA during

legume seed fermentation has been reported earlier (Egounlety and Aworh 2003; Khattab and Arntfield 2009).

The minimum TBAC (693·9 μg g^{-1}), the value which is 122·7% more than that of optimally cooked beans, was obtained when the fermentation temperature and time reduced to 25°C and 18 h respectively. While the TBAC of traditional doenjang was 2121·1 μg g^{-1}, that of its modern version was 304·9 μg g^{-1} (Cho *et al.* 2006). Natto and chungkukjang had TBAC of 138·3 and 334·6 μg g^{-1} respectively (Cho *et al.* 2006; Tsai *et al.* 2007a). Biogenic amines are formed in fermented soybean products by micro-organisms during fermentation, and high levels of them have been reported for soy products (Yen 1986; Nout *et al.* 1993). *Bacillus* spp. isolated from various fermented foods were found to be weak biogenic amine-formers (Silla Santos 1996; Tsai *et al.* 2007a,b). Kinema contains relatively high amount of free amino acids (Sarkar and Nout 2014), which could be a potential source of biogenic amine formation. Decarboxylase activity has been described in several microbial groups, including *Bacillus* (ten Brink *et al.* 1990). The use of short fermentation with carefully selected active starter cultures instead of wild fermentations will help to prevent the formation of toxic amines (Shukla *et al.* 2010).

The predicted optimum condition of fermentation (l0^3 total cells g^{-1} beans, fermentation temperature and time of 37°C and 48 h respectively) caused a reduction ($P < 0.05$) of TC and PAC during fermentation by 100 and 41·1% respectively. The value of R^2 (0·409) in PAC (Table 8) shows that 40·9% of the variations in PAC are influenced by changes in the fermentation time. However, the remaining 59·1% variations can be attributed to other factors. While TIA was not influenced ($P < 0.05$) by fermentation, TBAC increased ($P < 0.05$) by 218·4% over optimally cooked beans. Although TBAC increased ($P < 0.05$) in optimally processed kinema, the content remained below the hazardous level of 1000 μg g^{-1} food (Silla Santos 1996) and, so, can safely be consumed by humans.

Kinema produced through optimized processing stages scored 'excellent' in terms of overall quality. Hence, the response surface optimization of soybean processing significantly minimized the level of ANFs which enhanced the nutritional status of kinema.

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Conflict of Interest

No conflict of interest declared.

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