5

Discussion

5.1. Raw ingredients

Compared to rice, raw soybeans and dals contained much higher amounts of those antinutrients. The levels of antinutritional factors (tannins, phytic acid, trypsin inhibitor activity, haemagglutinating activity and total biogenic amines) present in raw soybeans (used for preparing kinema), blackgram dal (used for preparing idli) and bengalgram dal (used for preparing dhokla) were in close proximity to the values reported earlier (Hettiarachchy and Sri Kantha, 1981; Egounlety and Aworh, 2003; Lestienne *et al*., 2005; Alajaji and El-Adawy, 2006; Hemalatha *et al*., 2007; Guillamon *et al*., 2008). Rice (used for preparing idli and dhokla) contained no trypsin inhibitor and haemagglutinating activities. The levels of tannins and phytic acid contents were similar to the value reported earlier (Hemalatha *et al*., 2007). The levels of oligosaccharides present in raw blackgram dal during idli preparation were in close proximity to the values reported earlier by Reddy and Salunkhe (1980). All these antinutrients cause pronounced health-related abnormalities in humans (Bora, 2014).

5.2. Soaking

Soaking is considered an initial processing step which is routinely followed during the preparation of legume-based fermented foods. The response surface plots generated from the models indicate that when soaking temperature and initial pH were kept constant, increase in raw ingredients (soybeans, dal and rice) to water ratio and soaking time caused a linear reduction (P <0.05) of tannins content and trypsin inhibitor activity from raw soybeans, tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity from blackgram dal, tannins content, trypsin inhibitor activity and total biogenic amines content from bengalgram dal, and tannins and phytic acid contents from rice. As tannins and trypsin inhibitors are water-soluble (Chung *et al*., 1998; Abd El-Hady and Habiba, 2003), the increase in water proportion might have caused a reduction (P <0.05) in their values in soaked ingredients. Subsequent increase in soaking time might have provided an additional effect in getting those leached out of the ingredients. The loss of tannins content is also attributed to the binding of tannins with carbohydrates and proteins, and to the activation of polyphenol oxidase (Saharan *et al*., 2002; Sandberg, 2002; Saxena *et al*., 2003). Since, trypsin inhibitors are low-molecular weight proteins, those are likely to leach out of the seeds easily (Grewal and Jood, 2006). Hydration of seeds, activation of endogenous phytase and diffusion of products might be responsible for the reduction (P <0.05) of phytic acid content, as commented by Egli *et al*. (2002). Minimum haemagglutinating activity was obtained when soybeans and bengalgram dal were soaked in water of pH 4.0 for 20 h at 25°C and 40°C, respectively. The reduction (P <0.05) of tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity during soaking in the present study is in agreement with the earlier reports (Bansal *et al*., 1988; Vijayakumari *et al*., 1995; Egounlety and Aworh, 2003; Grewal and Jood, 2006; Shimelis and Rakshit, 2007; Khattab and Arntfield, 2009; Khandelwal *et al.*, 2010; Kalpanadevi and Mohan, 2013). The level of total biogenic amines reduced (P <0.05) with an increase in initial pH of soaking water and a decrease in soaking temperature. pH is an important factor influencing microbial decarboxylase activity; pH of 3.0-6.0 is considered optimum for bacteria to produce decarboxylase that induces formation of biogenic amines in soaked products (Silla Santos, 1996). Low temperature adversely affects proteolytic and decarboxylating reactions, resulting in a decreased (P <0.05) amine concentration (Joosten and van Boeckel, 1988).

The response surface 3D plots indicate that at any temperature level, the total oligosaccharides content in soaked blackgram dal decreased with an increase in dal to water ratio keeping the time and pH constant at 12 h and 6.0, respectively. An increase in the proportion of water might have caused leaching of oligosaccharides from dal. pH also had a significant effect (P <0.05) upon the response. The total oligosaccharides content after soaking dal for 12 h at 25°C decreased with an increase in pH. In fact, alkalinity favours leaching out of sugars from the beans (Kim *et al.*, 1973; Vijayakumari *et al*., 2007). In the present study, the interaction effect of soaking time and ratio indicated a non-linear relationship on the response. At 25°C and initial pH 6.0, the minimum oligosaccharides content obtained was 7.1 mg/g dry weight. A similar observation was reported by Shimelis and Rakshit (2007) and Price *et al*. (1988) where there was a reduction in the oligosaccharides content in kidney beans after 12 h of soaking in alkaline water. Further, soaking may also activate dormant enzymes, like α-galactosidase, leading to the breakdown of oligosaccharides. The absence of seed coat, which acts as a diffusion barrier, might have caused a higher rate of oligosaccharide reduction in the present study (Upadhyay and Garcia, 1988). The minimum total oligosaccharide content of 3.95 mg/g dry weight was obtained at pH 8.0 and 15°C with the ratio and time fixed at 1:6.5 w/w and 12 h, respectively. The interaction of temperature and time had a pronounced effect (P <0.05) upon the response. At a lower temperature (18°C) with the ratio fixed at 1:6.5 w/w and initial pH at 6.0, minimum oligosaccharide contents of the dal was 4.1 mg/g dry weight after 20 h of soaking. A curved response due to the interaction between pH and time was observed at all the pH levels. The reduction of individual sugar content cannot be explained solely on the basis of its solubility or molecular weight. Thus, the loss of raffinose was less than that of stachyose and verbascose, although raffinose is more soluble and is of lowest molecular weight among the oligosaccharides. This observation indicates that besides solubility and molecular weight, the location and natural bonding form of the sugar within a cell also influence both rate and extent of extraction of oligosaccharides (Sarkar *et al*., 1997a). The reduction (P <0.05) in pH values during soaking of dals and rice might be due to the growth of microorganisms and accumulation of organic acids (Ashenafi and Busse, 1991).

The predicted optimum condition for soaking of soybeans (1:10 w/w of raw beans to water, and 20 h, 10°C and 8.0 as soaking time, temperature and pH, respectively, led to reduction (P <0.05) of tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity by 55%, 26%, 8% and 18%, respectively, as compared to raw soybeans. The predicted minimum increase (P <0.05) of total biogenic amines content was 114%. Egounlety and Aworh (2003) observed that under traditional condition (1:3 w/v of beans to water, for 12-14 h) of soaking soybeans there were 55% reduction of tannins content, 35% increase in phytic acid content and 2% reduction of trypsin inhibitor activity. The optimized soaking condition of blackgram dal (1:5 w/w of dal to water, and 20 h, 16°C and pH 4.0 as soaking time, temperature and pH, respectively) caused a reduction of tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity by 49%, 25%, 35% and 26%, respectively. The predicted minimum increase (P <0.05) of total biogenic amines content was 2%. The optimized soaking condition of bengalgram dal (1:5 w/w dal to water, and 20 h, 23°C and pH 7.0) caused a reduction (P <0.05) of tannins content, phytic acid content, trypsin inhibitor activity, haemagglutinating activity and total biogenic amines by 79%, 35%, 50%, 60% and 18%, respectively. The optimized soaking condition of rice (1:5 w/w rice-water ratio and 18 h, 16°C and pH 5.6) caused a reduction (P <0.05) of the contents of tannins and phytic acid by 83% and 20%, respectively. The predicted minimum increase (P <0.05) of total biogenic amines content was 7% over raw rice. Soaking of *Vigna unguiculata* (L.) seeds having seeds to water ratio of 1:10 w/v for 12 h caused a reduction of tannins content, phytic acid content, trypsin inhibitor activity, haemagglutinating activity, raffinose, stachyose and verbascose by 26%, 31%, 8%, 11%, 21%, 46% and 37%, respectively (Kalpanadevi and Mohan, 2013).

The predicted optimum condition for soaking blackgram dal was: dal-water ratio of 1:10 w/w, and soaking temperature, time and pH of 16°C, 21 h and 6.0, respectively. Under this condition, the minimum total oligosaccharide content predicted was 1.97 mg/g dry weight and the corresponding experimental value was 2.02 mg/g dry weight. The percent decrease of raffinose, stachyose, verbascose and ajugose in dal was 94%, 93%, 97% and 92%, respectively; the total oligosaccharide content decreased by 95%.

5.3. Cooking of soybeans

Since boiling improves the protein quality of beans by destroying or inactivating heat-labile antinutritional factors (Vijayakumari *et al*., 1997), optimization of cooking stage is worthwhile. In the present study, the complete removal of tannins in cooked soybeans signifies an extensive leaching due to the collective effect of soaking and cooking. A complete removal of tannins during cooking was also reported by Egounlety and Aworh (2003). The effect of soaking plus cooking is more effective than either soaking or cooking (Shimelis and Rakshit, 2007). Furthermore, cooking destroys molecular structure of tannins, causing those less extractable and detectable during the assay (Alonso *et al*., 1998). In this study, the phytic acid content of cooked soybeans reduced (P <0.05) by 10% over optimally soaked ones. The reduction of phytic acid content in legume seeds during cooking has also been reported earlier (Shimelis and Rakshit, 2007; Embaby, 2010). The degradation of phytic acid during autoclaving might 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 the present study, trypsin inhibitor activity was reduced by 70% over optimally soaked soybeans. This reduction (P <0.05) 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 various legumes was reported earlier (Egounlety and Aworh, 2003; Shimelis and Rakshit, 2007; Embaby, 2010). Haemagglutinin or lectin, due to its heat-sensitive nature, was reduced (P <0.05) below the limit of detection by autoclaving. Similar reduction of haemagglutinating activity during cooking has been reported in faba bean (Khalil and Mansour, 1995), chick pea (Alajaji and El-Adawy, 2006) and cow pea (Kalpanadevi and Mohan, 2013). The reduction (P <0.05) in activity during cooking may be due to the breakdown of haemagglutinins into their subunits or to other unknown conformational changes in their native form (Batra, 1987).

The predicted optimum condition for cooking was soaked beans-water ratio of 1:5 w/w, and cooking pressure and time of 1.10 kg/cm^2 and 20 min, respectively. Under optimum condition, the tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity reduced (P <0.05) by 96%, 10%, 68% and 99%, respectively, over optimally soaked beans. Boiling soybeans (1:6 w/v of soaked beans to water) for 30 min caused a reduction of tannins content, phytic acid content and trypsin inhibitor activity by 100%, 10% and 82%, respectively (Egounlety and Aworh, 2003). Total biogenic amines content remained unchanged (P <0.05). Cooking had no effect (P <0.05) on total biogenic amines content of soaked soybeans during the production of tempe (Nout *et al*., 1993).

5.4. Unfermented mixed batter

Unfermented mixed batters, prepared by mixing dal paste and rice slurry in a ratio of 1:2 v/v and 3:1 v/v, were used for the preparation of idli and dhokla, respectively. Compared to dal, rice contained a negligible amount of antinutritional factors. Since, in the mixed idli batter, dal constituted one-third by volume, the level of antinutrients in mixed batter was approximately one-third of the level of that in soaked dal. Similarly, in the mixed dhokla batter, dal constituted three-fourth by volume; hence, the level of antinutrients in mixed batter was correspondingly lower than that in soaked dal.

5.5. Fermentation

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 of optimally cooked soybeans and mixed dhokla batter, the tannins content went below the limit of detection. Fermentation of mixed idli batter caused 56% reduction of tannins content. An activity of polyphenol oxidase by the growth of microorganisms during fermentation might be a cause of reduction of tannins content during fermentation (Reddy and Pierson, 1994). Phytic acid content and trypsin inhibitor activity reduced by 43% and 5%, respectively, over optimally cooked soybeans. The reduction (P <0.05) of phytic acid content in kinema might be due to phytase activity shown by the inoculated culture of *B. subtilis. Bacillus subtilis* contains phytase-encoding gene and can degrade phytate during its growth through production of extracellular phytases (Kumar *et al*., 2010). Phytase activity of *B. subtilis* in natto has been reported by Shimizu (1992). During idli and dhokla batter fermentation too, there was a reduction (P <0.05) of phytic acid content. This reduction might be due to phytase activity of the fermenting microorganisms (Kumar *et al*., 2010). Bacteria produce lactic and acetic acids and lower the pH, thereby favouring phytase activity, causing the reduction of phytic acid content (Lopez *et al*., 1983). Lactic acid fermentation is effective in reducing trypsin inhibitors in legumes (Khetarpaul and Chauhan, 1989; Holzapfel, 2002). A drastic reduction (P <0.05) of haemagglutinating activity in the present study signifies its hydrolysis into simpler and more soluble products. *Leuconostoc mesenteroides*, isolated from idli batter, has the potentiality to hydrolyze lectins (Rao, 1978). The bacterium secretes a mixture of proteases, such as β-N-acetylglucosaminidase and α-D-mannosidase, which are involved in the hydrolysis of lectin. The effect of fermentation in reducing tannins and phytic acid contents, and trypsin inhibitor and haemagglutinating activities during legume seed fermentation has been reported earlier (Reddy and Pearson, 1994; Holzapfel, 2002; Egounlety and Aworh, 2003; Khattab and Arntfield, 2009). The minimum level of total biogenic amines content in kinema was 166 μg/g (= 694 μg/g dry weight), the value which was 122% more than that of optimally cooked soybeans. Similarly, the total biogenic amines contents in fermented idli and dhokla batters were 493 μg and 282 μg/g dry weight, respectively, which are almost 288% and 4% more than those of unfermented mixed batters. While the total biogenic amines content of traditional doenjang was 2121 μg/g , that of its modern version was 305 μg/g (Cho *et al*., 2006). Natto and chungkukjang had total biogenic amines content of 138 μg and 335 μg/g, respectively (Cho *et al*., 2006; Tsai *et al*., 2007a). Biogenic amines are formed in fermented soybean products by microorganisms during fermentation, and high levels ofthose have been reported in 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, 2007b). Kinema contains relatively high amount of free amino acids (Sarkar *et al*., 1997b; 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). This is because biogenic amines are formed by several microbial groups possessing decarboxylase activity during fermentation (Majjala *et al*., 1993; Holzapfel, 2002). Interestingly, in this study, a reduction (P <0.05) in the level of total biogenic amines content was observed when salt concentration was increased. Similar results were reported by Chander *et al.* (1989) while studying the fermentation of soybeans by *Lactobacillus delbrueckii* susp. *bulgaricus*. A high salt concentration leads to reduced cell yield and progressively disturbs the membrane-located microbial decarboxylase (Sumner *et al.*, 1990). The use of short fermentation time 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 soybean fermentation was 3 log total cells/g beans as inoculum load, and fermentation temperature of 37°C and time of 48 h, causing a reduction (P <0.05) of the contents of tannins by 100% and phytic acid by 41%. While trypsin inhibitor activity was not influenced (P <0.05) by fermentation, total biogenic amines content increased (P <0.05) by 218% over optimally cooked beans. Although total biogenic amines content increased (P <0.05) in optimally produced kinema (to 238 μg/g), it remained much below the hazardous level of 1000 μg/g food (Silla Santos, 1996), and so, it can safely be consumed by humans. The optimum idli batter fermentation condition having 16 g/kg added salt at 35°C for 19 h caused a reduction of tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity by 44%, 53%, 36% and 65%, respectively. The total biogenic amines increased by 436% over unfermented mixed batter. The optimum dhokla batter fermentation condition having 8 g/kg added salt at 32°C for 18 h caused a reduction of tannins content, phytic acid content, trypsin inhibitor activity and haemagglutinating activity by 57%, 64%, 50% and 70%, respectively. The levels of total biogenic amines increased by 32% over unfermented mixed batter. The total biogenic amines contents in fermented batters of idli and dhokla were 681 μg and 355 μg/g dry weight, respectively. Natural fermentation of Roba variety of dry common bean (*Phaseolus vulgaris* L.) at 25°C for 24 h caused a reduction of 15% tannins content, 18% phytic acid content and 9% trypsin inhibitor activity (Shimelis and Rakshit, 2008).

Fermentation of the mixed batter of idli, produced by using optimally soaked dal, reduced the contents of each of the oligosaccharides below their respective limits of detection. This highlights the importance of optimizing the soaking condition of blackgram dal. The decrease in the oligosaccharide contents during fermentation is due to the action of α-galactosidase produced by the lactic acid bacteria (Reddy and Salunkhe, 1980). They reported that the fermentation of blackgram-rice blends for 45 h decreased oligosaccharides to 28% of the original value. Akinyele and Akinlosotu (1991) showed that 16 h-fermentation of cowpea led to a decrease of 38.4% raffinose, 35.4% stachyose and 49.9% verbascose. Reduction of total oligosaccharide content in the fermented dough of wari, a blackgram dal-based fermented food, has been reported by Tewary and Muller (1992).

5.6. Microbiological analysis

The viable counts of total aerobic mesophilic bacteria, lactic acid bacteria and yeasts for both unfermented and fermented mixed batters of idli and dhokla were studied under different experimental conditions. The increase (P <0.05) in titratable acidity and decrease (P <0.05) in pH from mixed batter during fermentation indicates the growth of lactic acid bacteria. A similar situation was observed during lactic acid fermentation of various food grains (Agarwal *et al.*, 2000; Shimelis and Rakshit, 2008). It was further observed that lactic acid bacteria outnumbered other, non-lactic acid bacteria causing reduction (P <0.05) in pH of fermented batter. Lactic acid bacteria readily grow in most food substrates and can lower the pH rapidly to a point where competing microorganisms are no longer able to grow (Shimelis and Rakshit, 2008). This lowering of pH favours yeasts (Soni and Sandhu, 1991), which is evident from their growth (P <0.05) during fermentation.

5.7. Steaming of fermented products

The complete reduction of tannins during steaming might be due to degradation at high temperatures (Nithya *et al*., 2007) or interaction with other seed components, such as proteins, to form insoluble complexes (Luo and Xie, 2013). During steaming of fermented idli batter, phytic acid content increased initially. Such a time-dependent change in phytic acid content during heating at 100°C has been reported earlier (Kim and Kim, 1998). The decrease (P <0.05) in phytic acid content with a further increase of the duration of steaming might be due to its degradation into penta- and tetra-phosphates (Vadivel and Biesalski, 2012). Trypsin inhibitor activity decreased with the increase in steaming time. Reactions involving deamidation and splitting of covalent bonds, such as hydrolysis of peptide bonds, and interchange or destruction of disulphide bonds, might be involved in the thermal inactivation of trypsin inhibitors (Alonso *et al*., 1998). Since, haemagglutinin is heat-sensitive, it reduced (P <0.05) below the limit of detection during steaming. Lectin activity can be eliminated during steaming (Reddy and Pierson, 1994). Total biogenic amines content in idli reduced by 7%, and its content in dhokla was 269 μg/g dry weight. Therefore, steaming for 20 min is effective in reducing total biogenic amines content in the final product. Steaming had no effect (P <0.05) on the oligosaccharide content of fermented idli batter. Cooking alone is not sufficient to bring about any significant reduction in the flatulence-inducing activity of cowpeas (Price *et al*., 1988). The experimental values were in a reasonable agreement with the optimum values during each processing stages of kinema, idli and dhokla preparation. Overall, it can be stated that these foods produced through optimized processing stages successfully minimized the levels of antinutritional factors with enhanced organoleptic attributes, attaining 'excellent' score in terms of overall sensory quality. The outcome can be the basis for their commercial production.