Effect of sequential bio-processing conditions on the content and composition of vitamin K₂ and isoflavones in fermented soy food

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Abstract In the present research, effect of sequential addition of *Bifidobacterium bifidum*, *Bacillus subtilis* and *Rhizopus oligosporus* on content and composition of vitamin K₂ and isoflavones in fermented soy foods have been investigated. Initially, soybeans were fermented with *B. bifidum*; then this fermented mass was re-fermented with co-culture of *B. subtilis* and *R. oligosporus*. The evolved sequence of microbes inoculation tended towards significantly ($p < 0.5$) higher enzymes levels (126.16 ± 2.23 IU/mg lipase, 36.52 ± 1.25 IU/mg phytase and 8.52 ± 1.12 IU/mg β -glucosidase); maximum menaquinone-7 production (9.3 ± 1.27 μ g/g); and isoflavone content (84.64 ± 1.97 % daidzein, 99.29 ± 0.86 % genistein, 96.42 ± 1.32 % glycitein) after 72 h of solid-state fermentation. The study showed that co-fermentation of soybean with different microbes in a particular sequence can enhance nutritional value better than the mono-culture fermentation due to the positive correlation between enzymes (lipase, phytase, β -glucosidase) levels, menaquinone-7 and soy isoflavones content.

Keywords Soybean fermentation · Isoflavones · Menaquinone · Lipase · β -glucosidase · Phytase

Introduction

Soybean (*Glycine max* L.) is cultivated around the world due to its high protein and oil content. Now-a-days, soy isoflavones based product consumption is increasing due to their health benefits. However, widespread soy food utilization is greatly hampered due to the presence of natural anti-nutrients such as trypsin inhibitors, flatulence-causing oligosaccharides (reduces digestibility of proteins and carbohydrates), and phytic acid (interferes with the mineral bioavailability) (Urbano et al. 2000). It has been reported that different processing methods such as dehulling, soaking, moist heating (boiling, microwave cooking and autoclaving), and fermentation has been used to significantly improve the nutritional quality of legume seeds (Khattab and Arntfield 2009). Fermentation of soybeans is a common practice followed in Japan, China, Korea, Thailand and Indonesia to improve its nutritional quality.

During the fermentation process, the enzymes produced by microorganisms act on substrate and partly hydrolyse its components into small molecules. Fermentation of legumes and cereals produce enzymes such as phytase, protease, trypsin inhibitors, β -glucosidase, α -galactosidase and lipase. Endogenous phytase of seeds and enzymes produced during fermentation considerably reduces phytic acid to lower inositol phosphates and inorganic phosphates that can be absorbed through the intestine (Sandberg and Andlid 2002; Vats and Banerjee 2004). Protease degrades the protein molecules into peptides and amino acids. Trypsin inhibitors present in soybean degraded or modified during fermentation and lose its binding affinity to trypsin (Hong et al. 2004). The glycosidic soy isoflavones hydrolyse into its aglycone forms by the endogenous β -glucosidase and the enzymes produced during fermentation (Handa et al. 2014). These aglycone forms are biologically active and get absorbed faster in greater amounts

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than the glycosidic isoflavones in humans (Izumi et al. 2000; Mo et al. 2013). *Rhizopus oligosporus*, *Lactobacillus curvatus* R08, *Leuconostoc mesenteroides*, *Lactobacillus fermentum* and *Bifidobacterium bifidum* species have been reported to produce α -galactosidase that can hydrolyse the non-digestible oligosaccharides from soy foods (Egounlety and Aworth 2003; Yoon and Hwang 2008).

Lipase degrades the soy triglycerides into glycerol and lower fatty acids. The glycerol thus in the fermented media has been reported to induce *B. subtilis* growth and increase MK-7 production (Berenjian et al. 2011). MK-7 is frequently used for the treatment of osteoporosis and prevention of bone fractures and cardiovascular diseases (Gast et al. 2009; Truong and Booth 2011). The improved nutritional value of soybean, therefore, could be due to different enzymes and vitamins synthesized by the microorganisms during fermentation.

Several researchers have found that products obtained by co-culture and mixed culture had relatively higher nutritive value than the product obtained by single culture fermentation (Penttinen et al. 2005; Oh et al. 2007; Melikoglu et al. 2013). Increased production of cellulases was found by a co-culture of *Aspergillus niger* and *Trichoderma reesei* (Ahmed and Vermette 2008). Some examples of the use of co-cultures in the food industry are the production of cheese (Martin et al. 2001), yoghurt (Sodini et al. 2000), sauerkraut, kefir (Lopitz-Otsoa et al. 2006) and cacao beans (Schwan and Wheals 2004). Previously, the effect of two-step fermentation of *R. oligosporus* and *B. subtilis* on soy protein was investigated by Weng and Chen (2011), but the effect of co-culturing using various combinations of GRAS (generally regarded as safe) fungi and bacteria on functional compounds were unknown. In the present study, steamed soybeans were fermented with different microbes; and with the aim to determine the change in nutritive value, enzyme levels and their correlation to generation of therapeutic metabolites during the fermentation were also studied.

Materials and methods

Soybean, chemicals and microorganisms

Soybean variety (KH-09-bragg) was received as a gift sample from the Pulse Laboratory of Indian Agricultural Research Institute, New Delhi, India.

Soy isoflavones (Daidzin, Genistin, Glycitin) and menaquinone-4 were procured from Sigma-Aldrich Chemicals, Germany. Phytic acid, β -D-glucopyranoside and p-nitrophenyl pyruvate were obtained from M.P. Biomedicals Pvt. Ltd., India. Vitamin K (Menaquinone-7) was obtained from MEDLEY Pharmaceutical LTD, Mumbai, India. All other microbiological media, chemicals and reagents used were

of analytical grade and were purchased from Merck, Mumbai, India.

Microbial strain of *Rhizopus oligosporus* NCIM 1215 was collected from the National Collection of Industrial Microbes (NCIM), national chemical laboratory, Pune, India. Fungal cultures were maintained on potato dextrose agar (PDA) medium. *Bifidobacterium bifidum* NCDC 320 was obtained from National Dairy Research Institute (NDRI), Karnal, India and maintained on modified MRS (De Man, Rogosa, Sharpe) agar medium. *Bacillus subtilis* MTCC 2756 and *Lactobacillus casei* MTCC 1423 were procured from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. *L. casei* cultures were maintained on MRS agar medium. *B. subtilis* cultures were maintained on nutrient agar medium. All the microbes were subcultured in every 30-day interval.

Solid state fermentation

Inoculum preparation

Inoculums of different microbial cultures were prepared by transferring one culture loop of *B. subtilis* MTCC 2756 and *R. oligosporus* NCIM 1215 to 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth and potato dextrose broth, respectively. The flasks were shaken at 150 rpm in orbital shaker at 37 °C for 16 h. *B. bifidum* NCDC 320 and *L. casei* MTCC 1423 were grown in modified MRS broth under microaerophilic conditions for 16 h at 37 °C.

Mono- culture/co-culture/two-stage/multi-stage solid state fermentation

Soybean seeds, used as substrate, were washed, soaked overnight and sterilized at 121 °C for 15 min, and then inoculated with microbial inoculums (5 % v/w) in mono-culture, co-culture, two-stage and multi-stage culture (as per the study design).

In mono-culture fermentation, culture medium was inoculated separately with a seed culture of *B. subtilis* MTCC 2756, *R. oligosporus* NCIM 1215, *B. bifidum* NCDC 320 and *L. casei* MTCC 1423, then incubated at 37 °C for 24 and 48 h.

In co-culture mode of fermentation, *B. subtilis* MTCC 2756 and *R. oligosporus* NCIM 1215 suspensions were added to soybean based culture medium at the same time.

In two-stage mode of fermentation, *R. oligosporus* (R) was inoculated in advance in the first stage of each fermentation process, then *B. subtilis* (S) in the second stage, which was expressed as R24h+S. Single microbe fermentation of soybean with *B. subtilis* and *R. oligosporus* separately were considered as a control.

After comparing the results obtained from different inoculation sequences and different microbial combinations,

experimental design with expected high enzyme activities and vitamin K content, were made and evaluated. In multi-stage solid-state fermentation, incubation of *B. bifidum* (B) for 24 h, followed by *B. subtilis* MTCC 2756 and *R. oligosporus* NCIM 1215 for 48 h, expressed as B24h+(S&R). For comparison, single culture inoculation of *B. bifidum* (B) for 48 h and *B. bifidum* (B) for 24 h, followed by *B. subtilis* (S) for 48 h, expressed as B24h+S was also included. All treatments were repeated three times.

During and after fermentation, the changes in soybean with respect to metabolites concentration and enzyme concentrations were analysed for nutritional evaluation under different fermentation conditions.

Analysis of metabolites

Fermented soybean seeds were processed by different methods to analyse the primary and secondary metabolites of different microbes.

Determination of lipase activity

Lipase activity was assayed by spectrophotometry using p-nitrophenyl palmitate (p-NPP) as the substrate as reported earlier (Dalal et al. 2008).

Determination of phytase activity

Fermented soybean seeds (1 g) were extracted in 0.2 M citrate buffer (pH 5.5) and phytase was determined according to the method of Kim and Lei (2005). The enzyme solution (500 μ L) was mixed with 500 μ L of phytic acid (1 %v/v in 0.2 M citrate buffer) and incubated for 15 min at 50 °C, the liberated phosphate was measured according to ammonium molybdate method (Tang et al. 2010).

Determination of β -glucosidase activity

β -glucosidase enzyme activity in the fermented soybean extracts was determined as described by Hu et al. (2010).

Determination of Vitamin K (MK-7 and MK-4 content)

Vitamin K was extracted from the fermented soybean samples using solvents propan-2-ol: n-hexane (1:2v/v) as described by Berenjian et al. (2011). Menaquinone-4 (MK-4) was analysed according to the method of Wang and Huang (2002). Menaquinone-7 (MK-7) analysis was performed by HPLC (Shimadzu, Japan) using a 250 mm $\times$ 4.6 mm ID Lichrosper® 100 C18 column containing 5 mm sized particles and a 20 ml loop injector. The HPLC analysis was carried out with a multi-step gradient elution at a flow-rate of 1.2 ml/min with the following mobile phase system: B: acetonitrile, C: Mixture of water and

methanol (1:1) acidified to pH 3.0 by ortho-phosphoric acid. After sample injection (20 μ L), the mobile phase system was maintained at 80 % B for 3.5 min and then increased to 100 % in 4.5 min, held for 2 min and at the end of 10 min, mobile phase system was recycled back to 80 % B. The detection was carried out by a UV detector at wavelength 248 nm.

Analysis of soybean isoflavones

Isoflavones were extracted from fermented soybean using 80 % acidic acetonitrile (Murphy et al. 2002). The high-performance thin layer chromatographic (HPTLC) analysis was carried out using mobile phase toluene: ethyl acetate: formic acid: acetic acid in the ratio of 1:8:1:0.5, v/v/v/v. Ultraviolet detection was performed densitometrically at 260 nm (Puri and Panda 2014).

Trypsin inhibitor activity (TIA)

TIA was analysed according to Kakade et al. (1974) using benzoyl-DL-arginine-p-nitroanilide hydrochloric as the substrate.

Nutritional analysis of fermented soy food

Nutritional analysis of fermented soybean was performed according to the standard procedures of AOAC for the determination of total ash, moisture, lipid and protein content. The carbohydrate content was calculated by subtracting the total ash, moisture, lipid and protein content.

Statistical analysis

All analyses were carried out in triplicates with replication. The mean and standard deviation (SD) of the data obtained were calculated. Analysis of variance (ANOVA) ($p < 0.05$) was used for analysing the data. Pearson's correlation coefficients (r) were estimated among the metabolites produced during fermentation. All the analysis was performed using GraphPad prism 3.0 (GraphPad software; San Diego, CA).

Results and discussion

Enzymatic activities in fermented soybean

After overnight soaking, initial level of enzymatic activities was found to be 10.22 ± 1.4 IU/mg of lipase activity, 0.52 ± 0.02 IU/mg of phytase activity, 8.37 ± 1.2 IU/mg β -glucosidase activity and 20.15 ± 1.2 TIU/mg trypsin inhibitors activity. Changes in enzymatic activity (lipase, phytase and β -glucosidase) of different microbes *B. bifidum*, *B. subtilis*, and *L. casei*, *R. oligosporus* in mono-culture fermentation with soybean are shown in Figs. 1a, b and 2a. In our study, initially

Fig. 1 Lipase (a) and phytase (b) activity of processed soybean fermented with *B. bifidum*, *B. subtilis*, *L. casei* and *R. oligosporus* under mono-culture conditions after 24 and 48 h of solid-state fermentation. All values are statistically significant ($p < 0.05$)

microbes were mono-cultured with soybean and screened on the basis different enzyme production level. Soybean fermented with *B. subtilis* MTCC 2756 showed a considerably higher lipase activity of 131 ± 1.67 IU/mg and phytase activity of 40.77 ± 2.12 IU/mg at 48 h compared to 24 h fermentation time. However, β -glucosidase activity in *B. subtilis* MTCC 2756 fermented soybean increased until 24 h and decreased slightly to 3.17 ± 0.42 IU/mg at 48 h fermentation time. *B. bifidum* NCDC 320 showed significantly ($p < 0.05$) higher β -glucosidase enzyme activity, i.e. 5.62 IU/mg after 48 h of fermentation time than other microbes (Fig. 2a). Soybean fermented with *R. oligosporus* had higher lipase and phytase activity than other three microbes viz *B. bifidum* NCDC 320, *B. subtilis* MTCC 2756, and *L. casei* MTCC 1423. Therefore, *R. oligosporus* NCIM 1215 was selected to improve the nutritional quality of soybean by fermentation process. *R. oligosporus* NCIM 1215 and *B. subtilis* MTCC 2756 was found to be better, because of overall enzymes and metabolite productivity, historical backgrounds and traditional uses of this microbe in soybean fermentation.

Next, investigations on different inoculation time of *B. subtilis* MTCC 2756 and *R. oligosporus* NCIM 1215 was conducted to determine the effect on enzymes and metabolites production. The change in enzymatic activity of fermented soybean during two-stage and co-culture fermentation has been shown in Figs. 3a, b and 4a. Co-culture of *R. oligosporus* NCIM 1215 and *B. subtilis* MTCC 2756, expressed as (S+Rco-culture), had significantly ($p < 0.01$) higher enzymatic activities than soybean fermented under mono and two-stage fermentation. The higher enzymatic

activities were observed after 48 h of incubation, when *B. subtilis* and *R. oligosporus* were inoculated at the same time in the soybean medium. Co-culture of *B. subtilis* and *R. oligosporus* was found to produce a considerably higher amount of enzyme such as lipase 56.17 ± 2.15 IU/mg, phytase 108.18 ± 1.34 IU/mg and β -glucosidase 4.57 ± 0.87 IU/mg at 48 h. The enzymatic activities during the fermentation process improve nutritional and functional properties of soybeans due to increased generation of small bioactive compounds. During initial stages of fermentation, lipids and carbohydrates degrade rapidly, since these are used as the major energy sources for microorganisms (Yamabe et al. 2007). Fermentation of soybean breaks down protein, lipid, and carbohydrate molecules by enzymatic hydrolysis to small molecules such as peptides, amino acids, fatty acids, and sugars, which are responsible for the unique sensory and functional properties of the final products (Lee et al. 2007; Jang et al. 2006).

Isoflavone content in fermented soybean

The higher hydrolysis rate of glycosidic isoflavones (daidzin, genistin, glycitin) to aglycone form (daidzein, genistein, glycitein) during fermentation was correlated with the higher amount of β -glucosidase activity. The reduction of glycosidic isoflavones by fermentation in the present study is in the agreement with the findings of Chun et al. (2008); who reported that this was due to the production of β -glucosidase, which causes hydrolysis and subsequent formation of the bioactive aglycone isoflavones. The changes in isoflavone content during mono-culture, two-stage and co-culture fermentations are

Fig. 2 β -glucosidase activity (a) and % aglycone isoflavone (daidzein, genistein, glycitein) (b) in processed soybean fermented with *B. bifidum*, *B. subtilis*, *L. casei* and *R. oligosporus* under mono-culture conditions after 24 and 48 h of solid-state fermentation. All values are statistically significant ($p < 0.05$)

Fig. 3 Lipase (a) and phytase (b) activity in soybean fermented with *B. subtilis* and *R. oligosporus* under two-stage (R24h+S) and co-culture (S+R co-culture) conditions after 24 and 48 h of solid-state fermentation. All values are statistically significant ($p<0.01$) compared with the values of two-stage fermentation

shown in Figs. 2b and 4b. The content of daidzin, genistin and glycitin in unfermented soybean were 276.90 ± 2.21 , 597.54 ± 1.32 , 109.40 ± 1.67 ng/mg, respectively. When the unfermented soybean was heat-treated at high temperature (121°C , 15 min or at autoclaving conditions) the content of these glycosidic isoflavones decreased to 137 ± 1.56 , 403 ± 2.67 , 69.29 ± 1.78 ng/mg, respectively. This indicates that during medium preparation step, glycosidic isoflavones content decreased due to high-temperature treatment. Similarly, the soaked soybean showed higher level of β -glucosidase activity (8.37 ± 1.2 IU/mg) and after autoclaving lower level of β -glucosidase was observed probably because of heat damage. This observation is in agreement as previously described by Kao et al. (2004) in that the process of soaking and heating soybeans in water led to a decrease of glycosides. Carrao-Panizzi et al. (2003) observed that glycosidic isoflavone content was decreased by soaking soybeans for 12–18 h at room temperature, which was attributed to the activation of β -glucosidase enzymes. However, sterilization step is necessary for fermentation process and cannot be over ruled. Co-culture fermentation (S+R co-culture) was found to produce significantly ($p<0.01$) higher amount of β -glucosidase and aglycone isoflavones (daidzein, genistein, glycitein) than two-stage fermentation (R24h+S) after 48 h of fermentation (Fig. 4a, b). From the result, it was clear that the loss of bioactive soy isoflavones

was better compensated by co-culture fermentation compared to two-stage fermentation.

Vitamin K₂ content in processed soybean

The level of MK-7 and MK-4 in soybean fermented with *B. subtilis* MTCC 2756 under mono-culture fermentation, two-stage fermentation (R24h+S), and co-culture fermentation (S+R co-culture) is shown in Fig. 5. MK-7 content of fermented soybeans produced during two-step fermentation was considerably higher ($6.41 \mu\text{g/g}$) than the content of soybean fermented under mono-culture of *B. subtilis* MTCC 2756. However, fermented soybean had a significantly ($p<0.05$) higher MK-7 content ($8.16 \mu\text{g/g}$), when microbial strains were inoculated at the same time i.e. during co-culture fermentation for 48 h compared to two-step fermentation. The MK-4 level was hardly affected by the fermentation conditions.

Based on the foregoing account, the combination of S+R (co-culture) was chosen as the final optimum microbial combination, in terms of increasing MK-7 and enzyme content in the fermented soybean.

Further in our experiments, we found that in comparison with other microbes, *B. bifidum* produced significantly higher amount of β -glucosidase under mono-culture mode.

Fig. 4 β -glucosidase activity (a) and % aglycone isoflavone (daidzein, genistein, glycitein) (b) in soybean fermented with *B. subtilis* and *R. oligosporus* under two-stage (R24h+S) and co-culture (S+R co-culture) conditions after 24 and 48 h of solid-state fermentation. Co-

culture fermentation showed significantly ($p<0.01$) higher amount of β -glucosidase and aglycone isoflavones (daidzein, genistein, glycitein) compared with the two-stage fermentation after 48 h of fermentation

Fig. 5 levels of vitamin K₂ [menaquinone-7 (MK-7) and menaquinone-4 (MK-4)] in soybean fermented with *B. subtilis* and *R. oligosporus* under two-stage (R24h+S) and co-culture (S+R co-culture) conditions after 24 and 48 h of solid-state fermentation. Values of MK-7 content produced during co-culture fermentation was significantly ($p < 0.05$) higher compared with the values of two-stage and mono-culture fermentation

B. bifidum NCDC 320 could produce maximum content of daidzein, genistein and glycitein (64.12 ± 1.54 %, 94.448 ± 1.87 %, 86.30 ± 2.42 %, respectively) after 24th h of fermentation without any significant ($p > 0.05$) change in aglycone content in soybean after 48 h (Fig. 2b). Therefore, *B. bifidum* NCDC 320 was selected to start the fermentation process in soybean for 24 h to hydrolyse the glycosidic isoflavone (B24hr), to convert into active aglycone forms. Subsequently, *R. oligosporus* and *B. subtilis* were inoculated simultaneously for co-culture in the *B. bifidum* fermented soybean, and incubated for next 48 h. Our studies clearly indicated that inoculation scheme of different microbes was paramount to achieve higher enzymatic activities and better MK-7 production.

Solid-state fermentation of soybean with the screened microbial combination sequence, B (24 h) and S+R (co-culture) was conducted, and analysed. The kinetics of enzyme activities and nutritional level of the fermented soybean during the total fermentation period (from 24 to 72 h) have been shown in Fig. 6a, b and c.

Lipase activity in soybean fermented by *B. bifidum* NCDC 320 was 20 IU/mg at 24 h of fermentation. The lipase activity was analysed in the fermented medium after the addition of *B. subtilis* MTCC 2756 and *R. oligosporus* NCIM 1215 to the *B. bifidum* NCDC 320. The lipase activity significantly increased after 36 h, and highest level (128 IU/mg) was achieved at 54 h. Figure 6a shows the co-relationship between the lipase enzyme and MK-7 production. Lipase activity correlated positively with MK-7 production ($r = 0.873$) and with MK-4 ($r = 0.641$) at a 0.01 significance level. The MK-7 content was significantly increased from 15 h of fermentation and was maximum (9.30 μg/g) after 72 h of fermentation. This implied that lipase enzyme helps in the MK-7 biosynthesis (Mahanama et al. 2012). Figure 6b shows a co-relation between the β-glucosidase activity and aglycone isoflavone production. β-glucosidase activity was positively correlated

Fig. 6 Kinetic of lipase activity, menaquinone-7 (MK-7) and menaquinone-4 (MK-4) (a), β-glucosidase activity and % aglycone isoflavone (daidzein, genistein, glycitein) β-glucosidase activity was positively correlated with aglycone isoflavones like daidzein ($r = 0.795$), genistein ($r = 0.725$) at 0.01 significance level and with glycitein ($r = 0.682$) at a 0.05 significance level. b phytase activity c in soybean fermented with *B. bifidum* and with *B. subtilis* and *R. oligosporus* under co-culture (S+R co-culture) conditions from 24 to 72 h. Lipase activity correlated positively with MK-7 production ($r = 0.873$) and with MK-4 ($r = 0.641$) at a 0.01 significance level

with daidzein ($r = 0.795$) and genistein ($r = 0.725$) at 0.01 significance level, and with glycitein $r = 0.682$) at a 0.05 significance level. Figure 6c) shows the change in phytase level

during the soybean fermentation with optimum microbial combination.

After 72 h of fermentation, fermented soybean showed considerably higher lipase activity of 126.16 IU/mg, phytase activity of 36.52 IU/mg and β -glucosidase activity of 8.52 IU/mg and 3.51 ± 0.22 TIU/mg trypsin inhibitor activity. This inoculation scheme also led to the highest MK-7 production (9.3 μ g/g) and aglycone content (daidzein, genistein, glycitein, 84.64 %, 99.29 %, 96.42 %, respectively). MK-7 produced under different fermentation condition (mono- and co-culture fermentation). Soybean fermented under co-culture condition produced 8.16 ± 0.37 μ g/g of MK-7 which was significantly ($p < 0.05$) higher than the MK-7 produced under monoculture fermentation (2.3 ± 0.04 μ g/g). MK-7 content of fermented soybeans produced during co-culture fermentation was increased by 3.5 fold i.e. 8.16 μ g/g as compared to soybean fermented under mono-culture of *B. subtilis* MTCC 2756. The final soya fermented product is found to contain 0.932 mg of MK-7, 0.0014 mg of MK-4, 87 mg of phytic acid, 24.69 mg of daidzein 50.75 mg of genistein and 9.94 mg of glycitein per 100 g. Nutritional analysis showed that fermented soybean contained 38.25 % of crude protein, 15.4 % of crude fat, 22.1 % moisture, 4.7 % crude ash and 19.55 % carbohydrate. The total energy value of fermented soy food was found to be 369.8 Kcal/100 g of fermented soy food. In summary, a particular inoculation sequence of microbes (*B. bifidum* NCDC 320, *B. subtilis* MTCC 2756 and *R. oligosporus* NCIM 1215) was identified as an important factor to enrich the soybean with different therapeutic metabolites during solid-state fermentation.

Conclusions

Results obtained from the present study demonstrate that the inoculation sequence of different microbes (*B. bifidum*, *R. oligosporus* and *B. subtilis*) produced enzymes that were recognized as an important feature to achieve more nutritious soy product for human consumption. Combination of *B. bifidum* with incubation of 24 h, followed by fermentation with co-culture of *R. oligosporus* and *B. subtilis*, showed the higher nutritional content in fermented soybean. Co-culture fermentation can be utilized in the production of fortified food, food additives, pharmaceuticals and enzymes. The current work also paves a way for the identification/screening of new microorganisms for co-cultures/mixed culture fermentation to achieve the production of new substances with industrial, food and pharmaceutical interest such as antibacterial substance and other secondary metabolites.

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