Effect of fermentation by different strains on the soybean oligosaccharide content in douchi

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Abstract: Douchi, a popular soybean food in China, has many prominent physiological activities. In the current research, the content changes of stachyose and raffinose during the douche processing, fermented by Aspergillus orgzae, Rhizopus oligosporus, Actinomucor elegans and Bacillus subtilis respectively were investigated. The results showed that the α-galactosidase activity of the fermented soybeans played the main role in the degradation of raffinose and stachyose during the primary fermentation, and the highest enzymatic activity reached 412.36 U/g dry weight of *A. oryzae*, 357.51 U/g dry weight of *R. oligosporus*, and 288.37 U/g dry weight of *A. elegans*. In the *B. subtilis*- fermented douchi, the main enzyme was invertase (30.05 mg/g dry weight). During the post-fermentation, the α-galactosidase activity of the fermented soybeans decreased dramatically, but the invertase activity increased. A trace amount of α-galactosidase in the *B. subtilis*-fermented douchi was detected. The sucrose content and the total amount of fructose and glucose in different douchi samples were 10.69 and 36.85 mg/g dry weight of *A. oryzae*, 7.69 and 16.23 mg/g dry weight of *R. oligosporus*, 6.05 and 14.79 mg/g dry weight of *A. elegans*, and 2.44 and 4.87 mg/g dry weight of *B. subtilis*, respectively. Our study revealed the key factors that control the soybean oligosaccharide content during fermentation, which will provide an experimental basis for producing douchi with appropriate oligosaccharide contents.

Keywords: α-galactosidase, douchi, fermentation, soybean oligosaccharides

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1 Introduction

Gastrointestinal diseases, such as diarrhea, gastritis, and colon cancer, have become common not only in the elderly, but also in children, because of changes in people's lifestyle and dietary habits (De Looze et al., 1998; Stone et al., 1990) Antibiotics, probiotics, prebiotics, and synbiotics are usually used to treat or prevent gastrointestinal problems (Collins and Gibson, 1999; Rolfe, 2000; Ziemer and Gibson, 1998). Hence, prebiotics from food sources have become an attractive therapeutic approach for preventing and treating gastrointestinal problems. Prebiotics are factors that promote the growth of bifidobacteria in human intestine. They are always in the form of oligosaccharides, which are not digested in the gastrointestinal tract. Prebiotic oligosaccharides include fructooligosaccharides, inulin, galactooligosaccharides, and soybean oligosaccharides. Soybean oligosaccharides are a general name for soluble sugars in soybean and leguminous seeds that primarily account for 7%-10% of all carbohydrates. Stachyose and raffinose account for 2-10 g/100 g soybean dry weight (Guillon and Champ, 2002).

Soybean oligosaccharides were always thought to be a cause of flatulence, because of the lack of an enzyme that metabolizes alpha-D-galactose glucoside in body, which leads to the production of carbon dioxide, hydrogen, and

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methane following fermentation (Murphy et al., 1972; Reddy et al., 1980). While stachyose and raffinose were once considered to be causes of flatulence, Edwin et al. (1981) demonstrated that they are not. These compounds did not produce any negative effects when administered in a beverage (3 g/100 mL) daily for 2 weeks (Hideo, 1994). Additionally, a study of 51 women that ate 45 g of white navy beans daily revealed that 76.5% of the women did not experience diarrhea (Song et al., 2008). These oligosaccharides can be used as probiotics when consumed in the form of 3 g of α-oligosaccharides daily for long periods, and they do not produce any negative effects. A previous study showed that regardless of the soybean variety, the degree of flatulence was so low that it was widely considered to be acceptable when the oligosaccharide contents in soybeans were 1.9, 2.6, and 3.5 g/100 g (Veenstra et al., 2010). A previous study observed that the optimal added quantity of oligosaccharides in bottle-fed infants was 4 g/L. Furthermore, the addition of oligosaccharides improved the diversity and the proportion of bifidobacteria in the gut(Holscher et al., 2012). Another study demonstrated that there were no significant differences in the number of adverse reactions in the gut between infants who consumed milk with added oligosaccharides compared with those who consumed milk alone (Mansilha, 2011). Thus, consuming a certain amount of stachyose and raffinose does not induce flatulence, and it can regulate and improve the intestinal flora of humans.

Soybean and its products have been appreciated by people as healthy foods because of their nutritional and medicinal attributes. Specifically, long-term intake of soybean foods can prevent several diseases such as type II diabetes and cancers. Douchi, a popular soybean food in China, is fermented by different microorganisms, which have many prominent physiological activities and special flavors (Dajanta et al., 2011) Many studies have reported that douchi can decrease blood pressure and moderate blood glucose levels (Chen et al., 2007; Hiroyuki et al., 2001; Mccue et al., 2005) . After the fermentation of douchi, the contents of some active ingredients increase while inhibitory factors decrease (Cho et al., 2011; Tsai et al., 2007; Chung et al., 2011). Previous studies showed that douchi possesses anti-diabetic and anti-tumor properties, and a douchi extract exhibited significant antioxidant activities and anti-α-glucosidase activity. Douchi can be divided into different types, depending upon whether it is fermented by *Aspergillus*, *Rhizopus*, *Mucor*, *Neurospora*, or bacteria. It is accepted widely that many nutrients in douchi, such as soy isoflavone, soluble sugar, angiotensin-converting-enzyme inhibitors, vitamin B, folic acid, and short-chain fatty acids, are generated or improved after fermentation (Nakajima et al., 2005; Zhang et al., 2005).

Microorganisms and fermentation are important for improving the nutrition of soybean products; however, the changes of soybean oligosaccharides in douchi during fermentation have not yet been investigated. In this study, we analyzed the stachyose and raffinose contents in 10 douchi samples collected from various parts of China. The contents of stachyose, raffinose, fructose, glucose, and sucrose, as well as the α-galactosidase activity, of douchi prepared using four microbes (*Aspergillus oryzae*, *Rhizopus oligosporus*, *Actinomucor elegans*, or *Bacillus subtilis*) were analyzed at different fermentation time. The objective of the study was to observe the changes of oligosaccharides during fermentation, as well as the factors that are responsible for such changes.

2 Materials and methods

2.1 Materials

Ten douchi samples were purchased from douchi manufacturers in different parts of China (Table 1,). Stachyose, raffinose, fructose, glucose, and sugar standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals used in the study were of analytical grade.

2.2 Microorganisms and culture conditions

Four microbes were used: *A. oryzae*, *R. oligosporus*, *A. elegans*, and *B. subtilis*. The mold strains were cultivated and activated in potato dextrose agar culture medium at 28°C, which was made by combining wheat bran and deionized water (1:1.4) and autoclaving at 121°C for 20 min. After 72 h of cultivation, the spore concentration in each inoculum reached 1×10^7/mL. *Bacillus subtilis* was cultivated and activated in De Man,

Rogosa, and Sharpe broth at 37°C for 24 h.

2.3 Production and preparation of douchi

Fifty grams of black soybeans was washed and soaked in 200 mL of deionized water for 8 h, and then the soybeans were drained, placed into a 500 mL triangular flask, and autoclaved at 121°C for 20 min. After cooling, the soybeans were inoculated with the different strains at a 1:1 (w/v) ratio. Salt was added to douchi to a concentration of 6% (g/mL), and then douchi made by the mold strains was fermented at 28°C, while douchi made by *B. subtilis* was fermented at 37°C. Samples were removed daily until the 10th day of fermentation. Douchi was freeze-dried and stored at 4°C until use.

The pre-fermentation procedure was conducted as follows. Soybeans were inoculated with 1% (w/v) of the microbial inocula (10^7 spores/mL) as a pure culture fermentation starter. Soybeans fermented by *A. oryzae*, *R. oligosporus*, and *A. elegans* were cultured at 28°C for 16, 32, and 48 h, while those fermented by *B. subtilis* were cultured at 37°C for 16, 32, and 48 h. After the primary fermentation, salt was added to soybeans to a concentration of 6% (mL/g), and then the mold and bacterial strains were cultured at 40°C and 37°C, respectively, for 1 to 10 days to produce douchi.

2.4 Determination of oligosaccharides in douchi

Briefly, freeze-dried douchi was crushed and sieved through a 100-mesh screen. After degreasing in a hexane solution, 1.5 g of douchi powder was mixed with 15 mL of 80% ethanol at 70°C for 1 h in a shaking water bath, and then it was subjected to three rounds of centrifugation at 8,000 *g* for 20 min in a refrigerated centrifuge at 0°C to 5°C to remove the insoluble fraction; the soluble supernatant was collected after each round of centrifugation. Each supernatant was rotary evaporated to 2 mL and diluted with water to 5 mL; all the supernatants were filtered through a 0.45 μm filter using a Membranes-aquo system. The filtrate was analyzed using high-performance liquid chromatography with an evaporative light scattering detector using an amino column (4.6 mm × 250 mm, 5 μm, Prevail Carbohydrates ES). The mobile phases were 30% water and 70% acetonitrile. The temperatures of the column and drift tube were 30°C and 82.3°C, respectively. The oligosaccharides were identified based on their retention times after comparison with corresponding standards purchased from Sigma-Aldrich.

2.5 Determination of the fructose, glucose, and sugar in douchi

The fructose, glucose, and sugar in the aforementioned filtrates were analyzed using high-performance liquid chromatography with a refractive index detector using an amino column (4.6 mm × 250 mm, 5 μm, Sugar-D). The mobile phase was 30% water and 70% acetonitrile. The temperature of the column was 30°C. Fructose, glucose, and sugar were identified based on their retention times after comparison with corresponding standards purchased from Sigma-Aldrich.

2.6 Determination of α-galactosidase activity of douchi

The α-galactosidase activity was analyzed by a microplate spectrophotometer at 605 nm, after modifying the method described by Alazzeh et al. (2009) The fermented douchi (2 g) was dissolved in 40 mL of acetate buffer and ground for 1 min. α-galactosidase was extracted by magnetic stirrers at room temperature for 40 min, and then the solution was subjected to three rounds of centrifugation at 8000 r/min at 4°C for 20 min to remove the insoluble fraction; the soluble superanatant was collected after each round of centrifugation. Each supernatant (10 μL) was added to 80 μl of acetate acid buffer and 10 μL of *p*-nitrophenol glucopyranoside in 96-well plates. Additionally, 10 μL of crude enzyme was added to 80 μL of acetate acid buffer and 100 μL of a sodium carbonate solution as a control. Then, two experimental replicates were shaken in a water bath at 40°C for 10 min, and then 100 μL of a sodium carbonate solution was added rapidly to terminate the reaction, while 10 μL of *p*-nitrophenol glucopyranoside was added to the control. The α-galactosidase activity was based on the optical density value according to the *p*-nitrophenol standard curve.

2. 7 Determination of sucrose-metabolizing enzymatic activity of douchi

The sucrose-metabolizing enzymatic activity was determined according to the method described by Yanase

with modifications (Yanase et al., 1991). Two grams of fermented douchi was mixed with 40 mL of sodium acetate buffer (0.2 mol/L, pH 4.6), ground for 40 min, and then it was subjected to three rounds of centrifugation at 8000 r/min for 20 min at 4°C to remove the insoluble fraction; the soluble supernatant was collected after each round of centrifugation. The sucrose-metabolizing enzymatic activity was determined based on the reducing sugars released from the sample.

2.8 Statistical analysis

All data analyses were performed using Origin 8.0 software, and structural formulas were generated by ChemDraw 7.0. All experiments were performed at least twice.

3 Results and discussion

3.1 Stachyose and raffinose contents in commercial douchi samples

Ten douchi samples were collected from various parts of China. Their stachyose and raffinose contents, brand names, origins, and type of strains used for their preparation are listed in Table 1. Among these samples, the stachyose and raffinose contents of douchi fermented by *B. subtilis* were significantly higher than those of the others. Thus, the stachyose and raffinose contents differed because of the use of different fermentation strains. However, the stachyose and raffinose contents even differed when the same strain was used. These differences most probably arose from variations in processing techniques.

Table 1 Contents of raffinose and stachyose in commercial douchi samples

Sample No.	Brands	Origins	Tape of strains	Raffinose, mg/g	Stachyose, mg/g
1	YC	Sichuan	Hair mold	0.653	1.998
2	TC	Sichuan	Hair mold	0	2.583
3	CN	Sichuan	Hair mold	0.437	1.47
4	SDC	Sichuan	Bacterial	2.012	2.183
5	WYZ	Shandong	Aspergillus	0.208	1.825
6	TH	Yunnan	Aspergillus	0.198	1.798
7	LJ	Hunan	Aspergillus	1.258	0
8	YPX	Hunan	Aspergillus	0.611	1.923
9	LYP	Hunan	Aspergillus	0.298	1.753
10	YJ	Guangdong	Aspergillus	0.292	1.592

3.2 Determination of oligosaccharides in douchi

The oligosaccharide contents in douchi during the fermentation are shown in Figure 1. Generally, the stachyose and raffinose contents at the beginning of the fermentation were 44.1 and 26.0 mg/g dry weight, respectively. After soaking and boiling, the oligosaccharide contents decreased slightly. During fermentation, the stachyose and raffinose in soybeans are hydrolyzed by microorganisms (Mital and Steinkraus, 1975; Prashanth and Mulimani, 2005). Aworh found that the stachyose and raffinose contents decreased by 83.9% and 50%, respectively, after douchi was fermented by *R. oligosporus*. The stachyose and raffinose in cowpea were eliminated after 15 h of fermentation by *Rhizopus microspores*. However, changing trends of the stachyose and raffinose contents in douchi fermented by different microorganisms have not been reported until now. As can be seen in Figure 1, before fermentation, the stachyose and raffinose contents were 20.39 and 24.56 mg/g dried weight, respectively, whereas at the end of the pre-fermentation, the contents were 5.23 and 6.03 mg/g dry weight of *A. oryzae*, 6.48 and 6.59 mg/g dry weight of *R. oligosporus*, 11.75 and 10.72 mg/g dry weight of *A. elegans*, and 12.81 and 8.64 mg/g dry weight of *B. subtilis*, respectively. The results showed that the hydrolysis of stachyose and raffinose at 16-32 h was higher than that at 0-16 h and 32-48 h; this is because of the enzymatic activity in the fermentation (date not shown). At the beginning of the fermentation, protease, α-D-galactose glucoside enzyme, α-D-glycosidase, and amylase in the soybeans were secreted, and the microorganisms used the resulting nutrients, including proteins, glucose, fructose, sucrose, and oligosaccharides, as carbon sources, but α-galactosidase was not the dominant enzyme. With increasing fermentation time, the enzymatic activity increased until maximum activity was observed at 32 h. This was the result of the oligosaccharides becoming the main carbon source at 16-32 h, when starch and other carbon sources had been degraded, and the stachyose and raffinose contents in douchi declined significantly when fermented by *A. oryzae*, *R. oligosporus*, or *B. subtilis*. At 32-48 h, the stachyose and raffinose contents stabilized because of an increase in secondary metabolites, which may affect microbial growth.

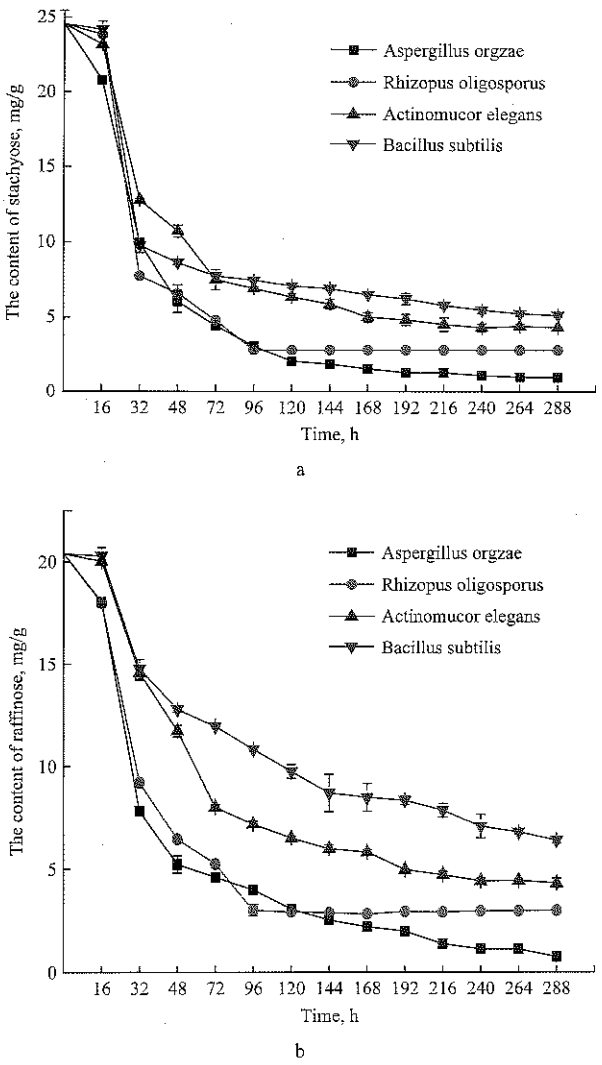

Figure 1 Effects of different microorganisms on the contents of stachyose (a) and raffinose (b) in douchi during fermentation

During the post-fermentation, salt was added to the soybeans at a concentration of 6%, and stachyose and raffinose contents of douchi decreased to 0.73 and 0.98 mg/g dry weight of *A. oryzae*, 3.01 and 2.83 mg/g dry weight of *R. oligosporus*, 6.42 and 5.14 mg/g dry weight of *A. elegans*, and 11.75 and 10.72 mg/g dry weight of *B. subtilis*, respectively. The downward trends decreased slightly during processing, which may be associated with the oxygen level, temperature, and salt concentration. When the fermentation began, the oxygen level declined sharply and the temperature increased in the culture medium. Salt can destroy the charge balance on the surface of cells, which resulted in a decrease in enzymatic activity. Meantime, short-chain fatty acids and organic acids were produced during the fermentation; thus, the total acid content in douchi increased, and the pH of the culture medium decreased, which restrained the growth of *B. subtilis*. Zhang et al. (2007) reported that organic acids are mainly produced during post-fermentation. The change in the pH affects the dissociation of charged groups and microstructures on the cell surface, thus affecting the ratio of absorbed nutrients and secreted metabolic substances. This phenomenon is consistent with the observations of Sims et al.

The decreases in the stachyose and raffinose contents during the fermentation are shown in Table 2, and all the microorganisms showed the ability to hydrolyze raffinose and stachyose. Among the four stains, *A. oryzae* showed higher hydrolytic activities than the others, especially during the pre-fermentation. In contrast, the decreases in the stachyose and raffinose contents in douchi fermented with *B. subtilis* were significantly lower than those of the others.

Table 2 Decrease of raffinose and stachyose content in douchi during the pre-fermentation and total fermentation

Microorganism	Stage	Decrease ratio of Raffinose, %	Decrease ratio of Stachyose, %
Aspergillus orgzae	Pre-fermentation	74.35	75.44
	Total-fermentation	96.41	96.01
Rhizopus oligosporus	Pre-fermentation	68.22	73.17
	Total-fermentation	85.24	88.48
Actinomucor elegans	Pre-fermentation	42.37	56.35
	Total-fermentation	78.67	82.33
Bacillus subtilis	Pre-fermentation	37.17	64.82
	Total-fermentation	68.46	79.07

3.3 Contents of sucrose, glucose, and fructose during fermentation

Soybean oligosaccharides include raffinose (4%), stachyose (1%), and sucrose (5%). Stachyose is a tetrasaccharide, which comprises two α-D-galactose units, one α-D-glucose unit, and one β-D-fructose unit. However, raffinose is a trisaccharide, which comprises galactose, glucose, and fructose, in which the galactose and sucrose moieties are linked by α-(1,2) galactosidase. Stachyose, raffinose, and galactose are non-reducing sugars. Raffinose and stachyose can be hydrolyzed into galactose and sucrose, and the sucrose can be hydrolyzed into glucose and fructose, but glucose and fructose are the reducing sugars. Figure 2 shows the sucrose content during the fermentation, and Figure 3 shows the glucose and fructose contents during fermentation.

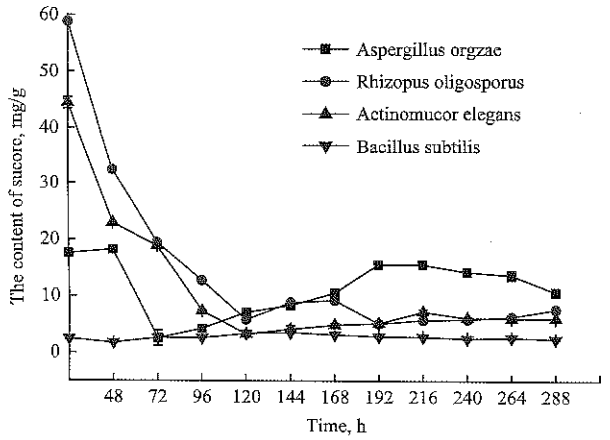

Figure 2 Effects of microorganisms on the sucrose content of douchi during fermentation

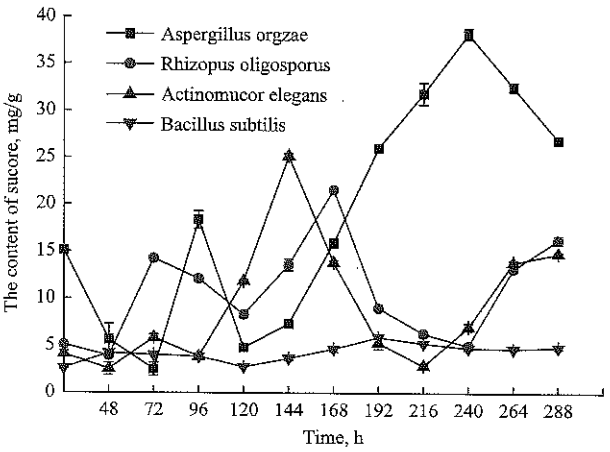

Figure 3 Effects of microorganisms on the glucose and fructose contents of douchi during fermentation

During the pre-fermentation, the sucrose content of douchi fermented by *A. oryzae* increased slightly, while the total fructose and glucose contents decreased significantly. Meanwhile, the sucrose, total fructose, and glucose contents of douchi fermented by *R, oligosporus* or *A. elegans* decreased. In contrast, in douchi fermented by *B. subtilis*, the sucrose content declined slightly, while the total fructose and glucose contents were steady. During the post-fermentation, the sucrose content of douchi fermented by *A. oryzae*, *R. oligosporus*, or *A. elegans* showed a similar trend, which declined rapidly at the beginning, rose slightly later, and then stabilized. In contrast, the sucrose content in douchi fermented by *B. subtilis* increased initially, followed by an additional slight increase and a subsequent stabilization. The total fructose and glucose contents in douchi fermented by *B. subtilis* decreased until 120 h (Figure 3), and this was followed by a relatively low increase and then a subsequent stabilization.

3.4 α-galactosidase activities of douchi during fermentation

α-galactosidase is distributed widely in microorganisms. It hydrolyzes a variety of simple α-D-galactosides and more complex polysaccharides. Figure 4 shows the results of the α-galactosidase production by the four microorganisms.

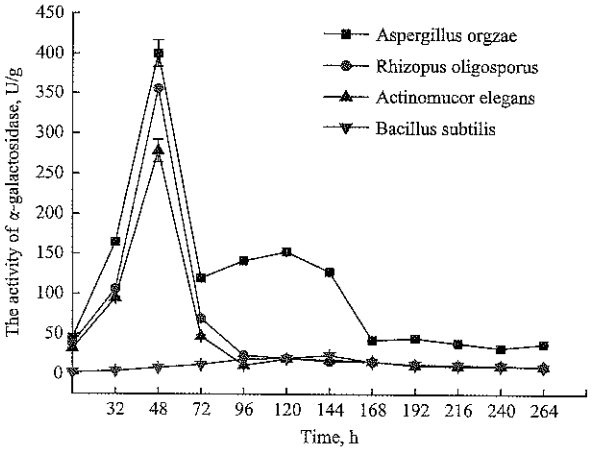

Figure 4 Production of α-galactosidase by *A. orgzae*, *R. oligosporus*, *A. elegans*, and *B. subtilis* in douchi

Aspergillus oryzae was the best strain for α-galactosidase production, as it gave the highest enzyme activity (412.36 U/g). This contrasts with earlier reports, which showed that *R. oligosporus* and *A. elegans* yielded the highest α-galactosidase production. Maximum α-galactosidase activities of 210 and 215 U/g were produced with *R. oligosporus* and *A. elegans* in 48 h(Han et al., 2003). Another study showed that *A. oryzae* is the best fungus for α-galactosidase production in soymilk (Shankar and Verappa, 2010). Ouoba et al. (2004) reported that *B. subtilis* is less effective for α-galactosidase production. During the post- fermentation, the α-galactosidase activity produced by the three molds declined dramatically, and this might be associated with the salt added to medium, as well as the negative effects of increased temperature and an exhaustion of oxygen, which inhibited their growth. These results are in accordance with a previous study, Sun., 2008 demonstrated that molds cannot grow well during post-fermentation, while fungi, yeast, and lactic acid bacteria can. Another study reported that the number of mold colonies decreased from 7.08×10^{10} colony-forming units/g to 4×10^{10} colony-forming units/g during post-fermentation (Hu et al., 2012). Fungi favor a moist

environment for their growth, and the initial moisture content is a critical factor for growth and enzyme production. A lower moisture easily reduces substrate stability and swelling, while higher moisture levels can cause particles to agglomerate, thereby resulting in a gas transfer limitation and competition with bacteria (Gowthaman et al., 2001). During the post-fermentation, enzyme production did not increase, which might be due to increased competition for carbon sources and nutrients. This could lead to nutrient exhaustion, which could result in reduced enzyme production.

3.5 Invertase activities of douchi during fermentation

Invertase can hydrolyze sucrose into D-glucose and D-sucrose because it can aid raffinose and stachyose degradation by hydrolyzing α-1, 2-glucosidic bonds. The results showed that *B. subtilis* was the best strain for invertase production during the pre-fermentation, as it yielded the highest enzymatic activity (30.1 U/g). *Aspergillus oryzae* (2.6 U/g), *R. oligosporus* (1.8 U/g), and *A. elegans* (1.2 U/g) were less effective for enzyme production. During the post-fermentation, the invertase activity of *A. oryzae* increased to 7.48 U/g initially, and then it decreased to 3.66 U/g; however, the invertase activities of the other strains decreased during the fermentation.

During the preparation of enzyme-ripened douchi, it is reasonable to expect that the hydrolytic enzymes that leach from microorganisms may catalyze the hydrolysis of sugar components in the douchi substrate. It was reported that *A. oryzae* produces α-galactosidase in addition to β-glucosidase, protease, amylase, and lipase (Hernaiz and Crout, 2000). α-galactosidase may catalyze the hydrolysis of α-1,6-linked α-galactoside residues. Thus, the catalytic action of α-galactosidase may lead to the reduced stachyose and raffinose contents in douchi during fermentation. During the pre-fermentation, stachyose and raffinose contents declined significantly as the activity of α-galactosidase increased. The sucrose from these oligosaccharides was hydrolyzed into glucose and fructose by invertase, which was due to the high rate of microbial growth at the beginning of the fermentation. However, in the *B. subtilis*-fermented douchi, the α-galactosidase activity was lower than that of the other strains, while the invertase activity was the highest; thus, the fructose and glucose contents increased during the fermentation. During the post-fermentation, the stachyose and raffinose contents of douchi fermented by *A. oryzae*, *R. oligosporus*, or *A. elegans* decreased steadily, and this was associated with environmental changes, such as temperature and pH. In contrast, the α-galactosidase activity in the *B. subtilis*-fermented douchi increased at the beginning of the fermentation and then decreased. These differences occurred because the growth of the molds was inhibited during the fermentation.

4 Conclusions

This study determined the stachyose and raffinose contents in commercial douchi. The stachyose and raffinose contents in the *B. subtilis*-fermented douchi were higher than those of the others. Moreover, the effects of fermentation by *A. oryzae*, *R. oligosporus*, *A. elegans*, or *B. subtilis* on stachyose and raffinose metabolism in douchi were also investigated. The degradation of raffinose and stachyose during the pre-fermentation was higher than that during the post-fermentation, and the highest degradation rate was observed in douchi fermented with *A. oryzae*. Higher α-galactosidase activity during the fermentation was associated with a higher oligosaccharide degradation rate, and *B. subtilis* yielded the high degradation rate during the post-fermentation.

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