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ORIGINAL ARTICLE

Screening of glucosinolate-degrading strains and its application in improving the quality of rapeseed meal

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Abstract Two myrosinase-producing fungi, *Lichtheimia* sp. JN3C and *Aspergillus terreus*, were newly isolated from decayed rapeseed meal samples obtained in Anhui Province, China. After preliminary screening, re-screening and combination of two screened strains with a yeast, an optimal composite strain to ferment rapeseed meal was obtained. Results demonstrated that the glucosinolate content of products with two molds fermentation was overall lower than that with single strain fermentation. Fermentation with composite strains containing *Candida tropicalis* CICIM Y0079(T) had a similar glucosinolate content, whereas the protein content was remarkably increased compared to two molds fermentation. Under sterile conditions, a 96-h fermentation with the composite strains resulted in the degradation of 66.2% of crude fiber, 28.3% of phytic acid, and 98% of total glucosinolates, which are responsible for goiter, and an increase of the protein and tannins content by 27.4 and 15.8%, respectively. In addition, glucosinolates and protein content under the non-sterile condition were not significantly different compared to the sterile condition. The fermentation greatly improved the nutritional quality of rapeseed meal by both degrading undesired substances and increasing protein content.

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Introduction

Each year, China produces about 7.3 million tons of rapeseed meal, only some of which qualifies as double-low rapeseed meal (i.e. low erucic acid and low glucosinolate). Ordinary rapeseed meal is often used as manure because of the presence of anti-nutritional substances such as tannins, phytic acid, crude fiber, and glucosinolates, which are responsible for goiter. However, rapeseed meal is a good protein resource for animal feed as rapeseed proteins have an excellent balance of essential amino acids. Nonetheless, the utilization of rapeseed meal as a protein resource is restricted due to its toxicity problems caused by the breakdown products from glucosinolates even though glucosinolates have been associated with beneficial anti-carcinogenic properties (Mithen et al. 2000; Fahey et al. 2003). On the other hand, the high crude fiber content in rapeseed meal makes it difficult to digest. The presence of tannins limits the utilization of essential amino acids because they form insoluble complexes with proteins, and result in a lower absorption of protein from the intestine (Hagerman and Butler 1978; Naczk et al. 1994). The insoluble phytates also tend to form complexes with proteins and therefore reduce the bioavailability of essential amino acids and proteins. Another anti-nutritional property of rapeseed meal is that phytic acid binds to multivalent cations, such as Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, etc., and reduces their bioavailability (Dvorakova 1998; Wodzinski and Ullah 1996).

The main anti-nutritional factor in the rapeseed meal is glucosinolates. Various processing techniques, such as

steam stripping, solvent extraction and leaching, etc., have been applied to minimize the deleterious effects on animals (Wallig et al. 2002; Mińkowski 2002; Das and Singhal 2005). These methods often cause protein loss, environmental pollution, and an increase of energy requirements for dry processing, although glucosinolates are eliminated. Inactivation of myrosinase may be regarded as a good method to eliminate glucosinolates, but myrosinase generated by microorganisms in the intestinal tract can also degrade the glucosinolates and produce breakdown products toxic for animals. However, microbial fermentation of rapeseed meal provides an effective approach to degrade glucosinolates without loss of proteins. The toxic breakdown products generated during fermentation can be evaporated during the drying process (Rakariyatham and Sakorn 2002).

In the present study, solid state fermentation is employed to investigate the effect of single strain, two strains and composite strains fermentation on glucosinolate and protein content. In addition to glucosinolates and proteins, degradation profiles of crude fiber, tannins and phytic acid are also assessed after fermentation.

Materials and methods

Preliminary screening of strain

Samples of 0.1 g decayed rapeseed meal were suspended in 10 ml sterile distilled water and allowed to stand at room temperature for 2–3 h. Then, 100 μl of supernatant was spread onto rapeseed meal extract agar plates. The rapeseed meal extract was prepared as follows: 200 g of finely ground rapeseed meal powder was suspended in 1 l of boiling water. The slurry was vigorously stirred for 2 h, filtered and then sterilized at 115°C for 15 min after adding agar to the slurry. Bacteria, yeast and molds was incubated at 37, 28 and 30°C, respectively. Each distinct bacteria, mold and yeast colony was picked and then plated on nutrient agar plates, malt extract agar plates and potato dextrose agar plates, respectively. Re-plating was performed until pure isolates were obtained. All strains obtained on the rapeseed meal extract agar plates were preserved at 0~4°C.

Re-screening of strain

Sixty-seven strains containing 13 yeasts, 41 bacteria and 13 molds exhibited visible growth on the rapeseed meal extract agar plates and pure colonies were obtained by re-plating. Then, solid state fermentation was employed to evaluate individual strain glucosinolate-degrading abilities. Fifteen grams (containing 11.95% moisture) of rapeseed meal was chosen as the solid state fermentation medium to assess glucosinolate-degrading abilities of 67 strains screened out from the rapeseed meal extract agar plates.

Solid state fermentation was carried out in a 150-ml triangular flask containing 15 g rapeseed meal and 18 g distilled water which was autoclaved at 115°C for 20 min before inoculation. Bacteria and yeast were inoculated from slanted to liquid seed medium, and cultivated at 37 and 28°C, respectively, and further inoculated into the solid state fermentation medium, while activation of molds was performed on agar slants at 30°C. Spore suspensions were obtained from 1-week-old cultures on agar slants by adding the sterile distilled water. Solid state fermentation medium was inoculated with a concentration of 10^7 spores or CFU per gram meal and cultured for a period of 60 h for bacteria and yeast and 72 h for molds. After fermentation, the samples were dried at 80°C, cooled, and subsequently the glucosinolates content was determined.

Strains were screened according to glucosinolates content in the rapeseed meal, a process which can degrade glucosinolates to less than 30 μmol per gram rapeseed meal. After re-screening, 4 molds from 67 strains were used for the further experiments (Table 1).

Combination and identification of strains

The combination of strains included two steps. First, the combination was preformed among the screened strains. Two of the four strains were randomly chosen, mixed at a ratio of 1:1 and then used to ferment the rapeseed meal. After the optimal combination was confirmed, the two screened strains were combined with a yeast potentially having a higher capacity to elevate protein content compared to bacteria and molds. The composite strains consisting of three strains were maintained on agar slants and subcultured once every 2 months.

The fermentation condition was described above except that the fermentation medium was supplemented with 2% $(NH_4)_2SO_4$ and 1% $C_6H_6O_{12}$ before inoculation. The inoculum concentration of yeast was about 4×10^7 CFU per gram meal. After fermentation, the glucosinolates and protein content were analyzed.

Identification of the two molds was carried out by Sangon Biotech (Shanghai) Co. Ltd., and *Candida utilis, Candida tropicalis, Geotrichum candidum* and *Saccharomyces cerevisiae* were obtained from the Culture and Information Center of Industrial Microorganisms of China Universities (CICIM-CU).

Effect of composite strains fermentation on components of rapeseed meal

Composite strains were used to investigate the effect of fermentation time on the content of glucosinolates and

Table 1 Glucosinolates degradation profiles of strains screened out from meal extract agar plates[a]

Bacteria	Strain	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
	GS (μmol/g)	43.9	58.5	47.3	32.9	86.7	54.6	75.3	51.4	61.4	30.5	85.3
	Strain	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22
	GS (μmol/g)	31.7	37.8	31.0	31.2	48.3	51.7	52.4	63.8	54.6	73.3	51.2
	Strain	B23	B24	B25	B26	B27	B28	B29	B30	B31	B32	B33
	GS (μmol/g)	56.5	32.5	54.1	78.7	57.5	68.2	32.9	46.1	53.4	67.0	58.2
	Strain	B34	B35	B36	B37	B38	B39	B40	B41			
	GS (μmol/g)	67.4	53.1	56.1	51.7	32.0	59.9	40.8	53.2			
Yeast	Strain	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	Y10	
	GS (μmol/g)	49.7	31.0	36.3	38.8	30.0	54.4	61.9	39.5	36.8	40.7	
	Strain	Y11	Y12	Y13								
	GS (μmol/g)	57.5	38.3	32.5								
Mold	Strain	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	
	GS (μmol/g)	38.3	52.6	26.9	20.0	31.7	29.0	37.8	20.5	54.4	48.3	
	Strain	M11	M12	M13								
	GS (μmol/g)	35.9	71.9	34.4								

GS glucosinolates determined by the palladium chloride method

[a] Each value represents the means of two independent duplicates

protein. The addition of distilled water before autoclaving helps to prevent the sterile meal from clotting after autoclaving and is beneficial for heat transfer during fermentation. Experiments were carried out according to the fermentation conditions mentioned above. Samples were taken at regular intervals from the culture flasks, dried at 80°C, cooled, and then analyzed to determine the content of glucosinolates and protein. The contents of tannins, phytic acid and crude fiber were determined after 96 h fermentation.

To compare the differences between non-sterile and sterile conditions, the glucosinolates and protein content were also analyzed without autoclaving. Experiments were conducted according to the procedures described above.

Statistical analysis

Experiments were repeated three times with three replicates for each treatment and the data were analyzed by repeated measures ANOVA for the content of glucosinolates and protein, and the differences among means were compared at $P<0.05$ using the Tukey test. However, *t* test analyses were performed to examine the difference between the means of tannins, phytic acid, crude fiber and dry weight.

Analysis method

Glucosinolates content was determined according to the palladium chloride method during the process of screening, single strain fermentation and two strains fermentation (Wathelet et al. 1988). The palladium chloride method can be described as follows: 0.1 g of rapeseed meal finely ground in a mortar was put into a 10-ml graduated test tube to which 8 ml of boiled water was added. The slurry was vigorously shaken and then heated in a boiling water bath for 30 min. Then 2 ml of rapeseed meal extract solution obtained after centrifugation was added to a graduated tube containing 6 ml of 0.1% sodium carboxymethylcellulose and 1.4 mM palladium chloride. The mixed solution was kept at 24°C for 2 h after vigorous stirring. The absorbance at 540 nm (E_1) was determined by using sodium carboxymethylcellulose and palladium chloride as a reference solution. Another 2 ml of rapeseed meal extract was added to a test tube containing 6 ml of 0.1% sodium carboxymethylcellulose. The absorbance at 540 nm (E_2) was measured by using sodium carboxymethylcellulose and distilled water as a reference solution. Glucosinolates content was assessed by the absorbance value E ($E=E_1-E_2$), which is proportionate to the content the latter being measured by a sinigrin standard curve. When the composite strains were used to ferment the rapeseed meal, the glucosinolates content was determined by a method based on a coupled enzyme assay (Smits et al. 1993; Wilkinson et al. 1984).

Rapeseed meal was analyzed for protein by the Kjeldahl method. In the fermentation medium, only part of the nitrogenous compound added as nitrogen (N) source is transformed to microbial protein, and the remaining nitrogenous compound will make the measured value higher. To solve this problem, the inorganic nitrogen (NH_4^+) was measured by formaldehyde titration to minimize the error. Crude fiber and moisture were quantified

following the method described by AOAC. Tannins were determined according to the Folin–Denis method described by China National Standards. Phytic acid was determined according to a method described previously (Haug and Lantzsch 1983).

Results and discussion

Preliminary screening

Twelve decayed rapeseed meal samples potentially containing glucosinolate-degrading microorganisms were collected in Anhui, China. After preliminary screening, 67 strains containing 13 yeast, 41 bacteria and13 molds exhibited visible growth on the glucosinolate-containing culture medium, and pure colonies were obtained by re-plating.

Glucosinolates are the secondary metabolites in Brassicaceae and related plant families. The glucosinolate core consists of a sulfonated oxime and a β-thio-glucose moiety. This core structure is linked to assorted side chains that are derived from diverse amino acids. Due to the variations in the side chain or R-group, at least 120 different glucosinolates have been identified in plants (Wu et al. 2004). Myrosinase-catalyzed degradation of glucosinolates results in the production of glucose. Thus, a myrosinase-producing strain can grow on the culture medium containing glucosinolates as a sole carbon source (Sakorn et al. 2002). Therefore, the strain exhibiting invisible growth on the plate can neither produce myrosinase nor degrade the glucosinolates, while the strain with visible growth may have the ability to consume glucosinolates.

Re-screening

Among the 67 strains, 4 molds can degrade glucosinolates to less than 30 μmol per gramin rapeseed meal according to the secondary screening experiments using 15 g rapeseed meal as the solid state fermentation medium to measure the glucosinolate-degrading ability (Table 1). Yeast and bacteria showed a lower ability to reduce glucosinolates compared to molds. In contrast, some yeast and bacteria fermentations elevated the glucosinolates level, probably because carbohydrate, which can be transformed to CO_2 by fermentation, was consumed during the process of strain growth. The glucosinolate-degrading profiles of four molds are listed in Table 1 and more than 50% of glucosinolates could be eliminated after fermentation.

Most research in the past employed liquid state fermentation to degrade glucosinolates in order to readily control the fermentation conditions. However, studies on glucosinolate degradation with solid state fermentation is also prevalent due to both low energy requirements for downstream processing and low operating costs needed for effluent treatment. Since fermentation technology generally decreases glucosinolates content to zero at a set level regardless of liquid or solid state fermentation, it has been applied to degrade glucosinolates for decades (Rakariyatham and Sakorn 2002; Bau et al. 1994; Sakorn et al. 1999; Smits et al. 1993; Rakariyatham et al. 2006; Rakariyatham et al. 2005; Butrindr et al. 2004; Palop et al. 1995).

In the present study, although the glucosinolate-degrading ability of a single strain is lower than that of the reported strains, glucosinolates content in fermented rapeseed meal may be significantly reduced by combining two strains. It is well established that a myrosinase generated by strains just hydrolyzes a glucosinolate due to the enzyme specificity towards the substrate or R-group. Because of the variations in the R-group, there are many kinds of glucosinolates present in rapeseed meal. Thus, complete degradation needs the generation of multiple myrosinases by the strains. Based on this idea, we hypothesize that multiple strains fermentation may produce more species of myrosinases and result in a higher level of glucosinolates degradation compared with single strain fermentation. Therefore, in the next experiment, we combined two screened strains to observe whether a lower glucosinolates content would be seen in the fermented rapeseed meal after combination.

Strain combination

Glucosinolate-degrading profiles of single strain fermentation and two strains fermentation are outlined in Table 2. The results showed that the degradation ability of all combined strains was better than that of a single strain except for the sixth group where the glucosinolates content was 21 μmol/g after fermentation. Single strain fermentation resulted in the degradation of less than 70% glucosinolates, whereas glucosinolates were consumed by 92% when M4 and M6 were cultured simultaneously. The results confirmed our previous hypothesis. Therefore, the eighth group was selected as an optimal combination for further experiments.

In order to elevate protein content in the fermented rapeseed meal, M4 and M6 were combined with a yeast. The results are shown in Table 3. Protein increment in the fourth group was the lowest among all groups. However, fermentation with *Saccharomyces cerevisiae* CICIM Y0086 (T) only resulted in an increase of 19% protein. Fermentation with M4 and M6 showed that protein increment was limited in the absence of yeast in spite of better glucosinolate-degrading ability compared to other groups except the second one. In comparison, protein was elevated by 27% and glucosinolates were reduced by 96% in the

Table 2 Effect of fermentation of single strain and two strains on glucosinolates degradation (dry matter basis)[a]

Strain	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
M3	√				√	√	√			
M4		√			√			√	√	
M6			√			√		√		√
M8				√			√		√	√
GS (μmol/g)	26.9	20.0	29.0	20.5	6.7	21.0	16.4	4.9	7.6	13.0

GS glucosinolates determined by the palladium chloride method, √ strain(s) selected to ferment the rapeseed meal in this group

[a] Each value represents the means of two independent duplicates

presence of *Candida tropicalis* CICIM Y0079(T) in the composite strains. Taking the glucosinolates degradation and protein increment into account, the second group was selected as an optimal combination due to its high glucosinolate-degrading ability even though the protein content was just 48.2% in this group compared with 49% in the first group. As a result, M4, M6 and *Candida tropicalis* CICIM Y0079(T) were used to conduct the next study.

M4 and M6 were first identified by Sangon Biotech (Shanghai) Co. Ltd. The identification results showed that M4 and M6 were *Lichtheimia* sp. JN3C and *Aspergillus terreus,* respectively. *Lichtheimia* sp. JN3C is a new strain that can not be obtained from the publicly available culture collection. Therefore, *Lichtheimia* sp. JN3C has been submitted to the China Center for Type Culture Collection (CCTCC); the corresponding serial number is CCTCC M 2010114.

In the previous study, it has been reported that *Aspergillus* sp. NR-4201 degraded 100 μmol/g of glucosinolates in 48 h (Rakariyatham and Sakorn 2002). In addition, under a liquid state fermentation condition, mutant strains *Aspergillus* sp. NR4617E1 and NR4617MG3 degraded 10 mM glucosinolates in 36 h (Rakariyatham et al. 2006). Rakariyatham et al. (2005) isolated 28 strains from soil and *Aspergillus* sp. NR463, *Aspergillus* sp. NR465, *Aspergillus* sp. NR468, *Aspergillus* sp. NR4617 and *Aspergillus* sp. NR4621 could consume 10 mM glucosinolate in 36 h under liquid state fermentation condition. The strains reported here are all related to *Aspergillus* sp. in the current study, both *Lichtheimia* sp. JN3C and *Aspergillus terreus* were found to be able to consume glucosinolates. To the best of our knowledge, no report that has used composite strains to degrade glucosinolates and also increase the protein content has been published.

Table 3 Effect of two molds fermentation with or without yeast on glucosinolates and protein level (dry matter basis)[a]

Strain	Group 1	Group 2	Group 3	Group 4	Group 5
M4	√	√	√	√	√
M6	√	√	√	√	√
Candida utilis ATCC 8206	√				
Candida tropicalis CICIM Y0079(T		√			
Geotrichum candidum CICIM Y0080(T)			√		
Saccharomyces cerevisiae CICIM Y0086(T)				√	
GS (μmol/g)	20.8	2.8	9.6	15.9	4.5
Protein content (%)	49.0	48.2	48.4	45.4	45.8

Fermented medium was supplemented with 2% $(NH_4)_2SO_4$ and 1% $C_6H_6O_{12}$

GS glucosinolates determined by the palladium chloride method, √ strain(s) selected to ferment the rapeseed meal in this group

[a] Each value represents the means of two independent duplicates

Effect of composite strains fermentation on the components of rapeseed meal

The contents of glucosinolates, protein, crude fiber, phytic acid, tannins and dry weight were found to be 63.5 μmol/g, 38%, 14.2%, 5.3%, 0.95% and 13.2 g, respectively, in the unfermented rapeseed meal, while in the 96-h fermented rapeseed meal, those contents were found to be 1.3 μmol/g, 48.4%, 4.8%, 3.8%, 1.10% and 11.7 g, respectively.. The glucosinolates were degraded almost to zero at the 84-h time point. Glucosinolates content at individual time points was significantly lower than that in the control group and it also significantly decreased from 24 to 60 h. No significant differences were observed between the 72-, 84- and 96-h time points. Protein content was significantly higher than the control group with extended duration of fermentation except for the 24-h time point. No statistical differences were observed during the period of 48–96 h even though the protein content increased slowly overall. The crude fiber, phytic acid content and dry weight in the control group were significantly higher than those in the 96-h fermented group while the tannins content was about 16% lower in the control compared to the fermented group. The results are shown in Table 4.

Table 4 Effect of composite strains fermentation on components of rapeseed meal (dry matter basis)

Component	Control (unfermented meal)	Fermented meal						
		24 h	36 h	48 h	60 h	72 h	84 h	96 h
GS (μmol/g)	63.5±1.3 a	40.3±1.0 b	28.7±1.5 c	19.1±4.7 d	8.4±1.3 e	3.5±0.9 e,f	1.4±0.35 f	1.3±0.8 f
Protein (%)	38.0±0.4 a	41.0±1.7 a,b	43.9±0.8 b,c	46.1±1.8 c,d	47.7±1.1 d	48.5±1.3 d	49.3 ±1.6 d	48.4 ±0.7 d
Crude fiber (%)	14.2±0.3 a	ND	ND	ND	ND	ND	ND	4.8 ± 0.5 b
Tannins (%)	0.95±0.06 a	ND	ND	ND	ND	ND	ND	1.10±0.06 b
Phytic acid (%)	5.3±0.2 a	ND	ND	ND	ND	ND	ND	3.8±0.3 b
Dry weight (g)	13.2±0 a	ND	ND	ND	ND	ND	ND	11.7±0.4 b

The meal was autoclaved at 115°C for 15 min, supplemented with 2% $(NH_4)_2SO_4$ and 1% $C_6H_6O_{12}$

Results represent means ± SD of three independent experiments. Means with a different letter in a row significantly differ at $P<0.05$

GS glucosinolates content determined by the method based on the coupled enzyme assay, *ND* not determined

In previous research, Vig and Walia (2001) investigated the effect of single strain fermentation on the components of rapeseed meal. The results showed that glucosinolates, phytic acid and crude fiber were reduced by 43.1, 42.4 and 25.5%, respectively, after fermentation. In comparison, in the present study they were reduced by 98, 28.3 and 66.2%, respectively (Table 4).

It has been reported that a 40-h fermentation with *Rhizopus oligosporus* sp-T3 caused degradation of 84% of carbohydrates, 30% of lignin and 47% of total glucosinolates, but protein content was increased by 8.7% without significant reduction of phytic acid, tannins and crude fiber (Rozan et al. 1996). To our surprise, when Bau et al. (1994) used the *Rhizopus oligosporus* sp-T3 to eliminate the anti-nutritional substances in rapeseed meal, the results were significantly different and the content of aliphatic glucosinolates and indol glucosinolates were reduced by 57.7 and 97.3%, respectively. In addition, fermentation during 24 h induced degradation of 73% of ethanol-soluble sugars.

Crude fiber content is often elevated or reduced slightly after fermentation (Rajesh and Imelda-Joseph 2010; Ezekiel et al. 2010; Oboh 2006; Joseph et al. 2008). The most effective degradation of crude fiber was reported by Lateef et al. (2008) where the fermentation with *Rhizopus stolonifer* LAU 07 reduced the crude fiber content by 44.5% in a palm kernel cake group, whereas it was raised to 66.2% in the present study. In addition, compared to the fermented group, the weight also declined sharply and tannins content was significantly lower in the control, presumably because of the absence of tannase in the process of fermentation and substrates loss after fermentation. In general, composite strains fermentation greatly improves the quality of rapeseed meal.

It is well documented that relevant enzymes generated by microorganisms are responsible for the degradation of glucosinolates, phytic acid, tannins and crude fiber (Travers-Martin et al. 2008; Ebune et al. 1995; Bhat et al. 1998). Therefore, elimination of anti-nutritional components in the rapeseed meal needs multiple enzymes. Solid state fermentation with a single strain may have difficulty hydrolyzing the diverse anti-nutritional factors. Low degradation of the crude fiber, tannins and phytic acid in previous studies may be explained by the lack of enzymes. Composite strains fermentation may be a good approach to reduce the multiple anti-nutritional substances simultaneously.

Our aim is to obtain the fermented rapeseed meal product with low glucosinolate content and high protein content. Thus, in addition to degrading the undesirable substances, an increase of protein content by fermentation is also desired. Yeast is often considered as single-cell protein-producing strain because of its high protein content (Anupama and Ravindra 2000). *Candida utilis* had a higher ability to elevate the protein content versus other yeasts when it was combined with two molds (Table 3). Our results were in agreement with previous studies (Xiao et al. 2009; Barrette and Gélinas 2007), but the composite strains containing *Candida utilis* showed a lower ability to consume glucosinolates than that containing *Candida*

Table 5 Effect of non-sterile and sterile conditions on the glucosinolates and protein content after 84-h fermentation (dry matter basis)

Component	Sterile condition	Non-sterile condition
Protein (%)	49.3±1.2 a	48.8±0.7 a
GS (μmol/g)	1.4±0.35 a	1.1±0.4 a

Fermented medium is supplemented with 2% $(NH_4)_2SO_4$ and 1% $C_6H_6O_{12}$

Results represent means ± SD of three independent experiments. Means with the same letter in a row are not significantly different at $P<0.05$

GS glucosinolates content determined by the method based on the coupled enzyme assay

tropicalis, probably because the presence of *Candida utilis* inhibited the growth of glucosinolate-degrading molds. When molds were cultured with yeast, the molds hydrolyzed the cellulose or hemicellulose component of the rapeseed meal by secreting extracellular enzymes. The released sugar was then used by the yeast. The higher protein content with the composite strains fermentation compared to two molds fermentation probably results from the enzymatic hydrolysis of crude fiber and carbohydrate by molds. The released hexoses and pentoses can be efficiently metabolized by yeast. These might explain the reasons why the protein content was higher after combination and why crude fiber was reduced dramatically after 96-h fermentation (Tables 3 and 4).

Fermentation with *Aspergillus* sp. reported by other studies cannot eliminate some anti-nutritional substances or increase the protein content in spite of a higher glucosinolate-degrading ability compared to composite strains in the present study, whereas fermentation with the composite strains resulted in a significant increase of 29.7% of protein (38 to 49.3%) after 84-h fermentation compared to an increase of 7% (45.97 to 49.32%) for fermentation of soybean meal and an increase of 14% (35.04 to 39.95%) for fermentation of mixed ingredients reported by Joseph et al. (2008). Lateef et al. (2008) pointed out that the protein content of the products was increased by 33.3% (19.7 to 26.3%), 55.4% (12.3 to 19%), and 94.8% (8.2 to 16%) in palm kernel cake, cassava peel and cocoa pod husk, respectively. Rajesh and Imelda-Joseph (2010) also reported that fermentation with *A. niger* S14 and *A. niger* NCIM 616 resulted in a significant crude protein increase of 37.2% (20.62 to 28.29%) and 29.1% (21.81 to 28.15%), respectively. Fermentation with composite strains also resulted in a similar protein increment. The significant ($P < 0.05$) increase in protein content during fermentation may be attributed to the efficient bioconversion of highly polymerized carbohydrates into microbial protein, and to the production of different types of enzymes hydrolyzing both crude fiber and phytic acid.

Comparison of non-sterile and sterile conditions

Content of glucosinolates and protein under non-sterile and sterile conditions were determined after 84-h fermentation. The results demonstrated that fermentation under sterile conditions increased protein and glucosinolates content by 1 and 27%, respectively, compared to non-sterile conditions. Other than that, no significant differences were observed between the two conditions (Table 5). However, glucosinolates content of unfermented rapeseed meal was significantly reduced after autoclaving than that before autoclaving. This is presumably because autoclaving breaks down glucosinolates (data not shown). Our results suggest that the sterilization step is not essential.

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