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EFFECTS OF *Rhizopus oryzae* FERMENTATION OF COCOA BYPRODUCT ON CERTAIN AMINO ACID AND THEOBROMINE CONTENTS

Pengaruh Fermentasi *Rhizopus oryzae* Hasil Samping Kakao Terhadap Kandungan Asam Amino Tertentu dan Teobromin

Catur Sriherwanto*, Budhi Santoso, Anis Mahsunah, Imam Suja'i, Sarny Toelak, Mia Rusmiyati
Biotech Center BPPT, Building 630 PUSPIPTEK Area, Setu, Tangerang Selatan, Banten 15314
*E-mail: catur.sriherwanto@bppt.go.id

ABSTRACT
*Being the world's third largest producer of cocoa (*Theobroma cacao*), Indonesia provides abundant cocoa pod husk byproduct. Despite its high content of biological materials, its use as animal feed, however, has been limited due to its low nutritive values and significant content of antinutritive substances. Thus, this study was aimed to investigate the changes of selected amino acids glutamate, aspartate, valine, alanine, and proline, as well as the antinutritional compound theobromine in cocoa byproduct-rice bran mixed substrate following fermentation using* Rhizopus oryzae*. The fermented substrate obtained had its true protein content increased from 1.95% to 23.16%. After analyses using ultra-performance liquid chromatography quadrupole time of flight mass spectrometry, the following amino acids, namely: total and free glutamates, total and free valine, total proline, as well as free alanine underwent increase, while the others decreased. The concentration of the antinutritional factor theobromine was below the limit detectable by HPLC.*

Keywords: Rhizopus oryzae*,* Theobroma cacao*, theobromine, fermentation, amino acids*

ABSTRAK
Sebagai penghasil kakao (*Theobroma cacao*) terbesar ketiga di dunia, Indonesia mempunyai hasil samping melimpah berupa kulit cangkang kakao. Meskipun kandungan bahan biologisnya tinggi, penggunaan produk samping ini sebagai pakan ternak masih terbatas karena nilai gizi yang rendah serta kandungan zat antinutrisi yang tinggi. Oleh karenanya, penelitian ini bertujuan mengetahui perubahan kandungan asam amino glutamat, aspartat, valin, alanin, dan prolin, serta senyawa antinutrisi teobromin dalam campuran hasil samping coklat-dedak padi pasca fermentasi menggunakan *Rhizopus oryzae*. Substrat hasil fermentasi mengalami peningkatan kandungan protein sejati dari 1,95% menjadi 23,16%. Hasil analisis menggunakan ultra-performance liquid chromatography quadrupole time of flight mass spectrometry, menunjukkan bahwa kandungan asam amino: glutamat total dan glutamat bebas, valin total dan valin bebas, prolin total, serta alanin bebas mengalami peningkatan, sementara asam amino selainnya mengalami penurunan. Kandungan antinutrisi teobromin berada di bawah ambang batas deteksi oleh HPLC.

Kata kunci: *Rhizopus oryzae, Theobroma cacao*, teobromin, fermentasi, asam amino

INTRODUCTION

Indonesia is the third-largest cocoa producer in the world after Ivory Coast (1.45 million tons) and Ghana (0.84 million tons), with cocoa production in 2013-2014 reaching 0.78 million tons (FAO 2016). Assuming this quantity to be the total weight of the fruit during harvest, about 75% of which is the byproduct of cocoa seed processing (Fauzi et al. 2012), then 585 thousand tons of the byproduct are generated annually, consisting mainly of cocoa pod husk.

In addition to the high fibre content (36.6%), cocoa pod husk contains protein (8.6%), lipid (1.5%), total carbohydrates (32.3%), reduced sugar (10.4%), soluble phenolic compounds (4.6%), and lignin (21.4%) (Vriesmann et al. 2011). Despite this high content of organic matter, its use as a source for animal feed has been limited due to its low nutritive values and the content of antinutritive substances such as phytic acid and condensed tannins (Bonvehí and Jordà 1998). The supplementation of cocoa waste in fish feeds, for example, is limited, amongst others, by high fibre as well as the presence of antinutritional factors (Ulloa et al. 2004), although the limitation could be overcome with such pretreatment as fermentation using rumen liquor (Jusadi et al. 2014). Another cocoa byproduct, namely cacao bean shell, was fed at the level of 15% to reared chicken, resulting in a negative effect on the animal's productivity (Emiola et al. 2011). Higher inclusion level of cocoa waste might be possible when the user animals are ruminants (Puastuti and Susana 2014; Esong et al. 2015; Laconi and Jayanegara 2015).

The inclusion level of cocoa pod husk in animal feed ration can be increased using pretreatments that can improve digestibility, enhance the nutrient content, and reduce the antinutritive substances, such as theobromine. Theobromine (3.7-dimethylxanthine) is a toxic compound contained in cocoa products which, at certain levels, may interfere with the reproduction and growth of the feeding animals (Adamafio 2013). At least three treatments that can reduce the content of theobromine in the cocoa byproduct, physically, chemically, and biologically. The first and second treatments can be done by using hot water or alkaline solution, and have disadvantages of reducing the nutrient content such as sugars, protein, and micronutrients. The third way is considered better, namely by using microorganisms, namely bacteria, yeasts, and fungi (Adamafio 2013). The use of the fungus *Aspergillus niger* in solid fermentation of cocoa byproduct, for example, was able to reduce the content of theobromine by 71.8%. In addition, solid fermentation using certain fungi improved feed quality parameters of cocoa byproduct, such as an increase in total soluble carbohydrates and crude protein, as well as decrease in crude fibre, lignin, and tannin content (Alemawor et al. 2009). Thus, the objective of this study was to select *Rhizopus* isolate best growing on cocoa pod husk containing medium, and to investigate the changes of certain amino acids and the antinutritional compound theobromine in the cocoa byproduct fermented using selected *Rhizopus* sp.

MATERIALS AND METHODS

Rhizopus isolation

Some sources known to be the habitat or to contain *Rhizopus* spores such as soybean tempeh, tempeh starter, and hibiscus (waru) leaves were collected from a number of places in Indonesia. Soybean tempeh and hibiscus leaves were sterily cut into about 1-4 cm^3 cubes and about 1-9-cm^2 explants, respectively, cultured on the middle of potato dextrose agar (PDA) plate. The cultures were incubated at ambient temperature of 25-32°C for 3 and 7 days, respectively. As for the other sample type, 1 g of tempeh starter was homogenised in 9 mL water, serially diluted 10 times, and 100 μL of which was inoculated on the middle of PDA-containing Petri-dish. Incubation was carried out at 25-32°C for 1-7 days. To purify fungal isolates, fragments of fungal colonies were transferred to PDA slants. Subsequent subcultures were carried out to further obtain purer isolates of putative *Rhizopus* strains determined based on their morphological appearance.

Rhizopus selection

Once isolated, the putative *Rhizopus* strains were then selected for its ability to grow on cocoa byproduct-containing media. Two selection media were prepared, one containing only 35 g cocoa byproduct, and

the other mixture of 31.5 g cocoa byproduct and 3.5 g rice bran, all sterilised at 121°C for 15 minutes. After being cooled to room temperature, the substrates were moisturized with 17.5 mL mineral solution This solution was made from two component solutions prepared separately: first solution was made by dissolving 18 g KH_2PO_4 and 37.8 g $(NH_4)_2SO_4$ in 300 mL demineralised water, and the second solution by dissolving 35.7 g urea in 200 mL demineralised water. After 15 minute sterilisation at 121°C and cooling down to room temperature, the two solutions were mixed homogenously.

The moisturised media (initial pH 6.25) was inoculated with *Rhizopus* spore suspension using single point inoculation method, whereby 100 μL of the suspension was pipetted onto the middle part of the petri dish. The petri dishes were wrapped tightly using plastic film around the edges on which three holes were made by stabbing using 200 μL pipette tip to facilitate limited aeration.

Incubation was carried out for 120 hours at 28°C, during which growth was determined by measuring the fungal colony diameter at 2nd, 3rd, and 4th day. Fast growing isolates with dense white morphology was considered the best one. The selected putative *Rhizopus* isolate was identified using molecular biology technique service provided by Biotech Centre, BPPT.

Fermentation using *Rhizopus*

Fermentation was carried out using previously obtained optimum conditions (unpublished results). The fermentation substrate, consisting of a homogenised mixture of 3 g cocoa byproduct and 27 g rice bran, which is equivalent to 10% inclusion level of the cocoa byproduct (Teguia et al. 2004), in a 9-cm diameter Petri dish bioreactor, was moisturised with 50-60% mineral solution. Onto this substrate's surface, 100μL suspension of the selected *R. oryzae* spore was inoculated using a micropipette to get final concentration of 10^2-10^4 cfu/g substrate. Incubation was carried out at 28°C for 6 days.

True protein content determination

Fermented cake was diced into 1- to 3-cm cubes, dried at 60°C for 24 hours before being milled into powder. The sample was then subjected to true protein content determination based on the albuminoid nitrogen content method (Horwitz 1965) through protein precipitation using copper (II) hydroxide, $Cu(OH)_2$.

Extraction and analysis of amino acids

To obtain free amino acids, fermented cake was firstly dried, ground into powder, then 5 g of which was added with 40 mL demineralised water, homogenised using shaker at 125 rpm for 1 hour. The resulting suspension was centrifuged at 10,000 rpm, 4°C for 10 minutes to obtain the supernatant that was transferred into a 50 mL erlenmeyer flask. Then, 0.2 mL norleucine (39 g/100 mL 0.01 N HCl), 0.5 mL Na_2CO_3 0.1 M (pH 10.5), 0.2 mL dansyl chloride sol (0.2 g/100 mL acetone), and 1 mL acetone were added. The mixture was topped up to 50 mL with demineralised water. The Erlenmeyer flask was then submerged in 100°C glycerin for 2 minutes, centrifuged, and filtered into 2 mL vials for analysis using Liquid Chromatography–Tandem Mass Spectrometry (LCMS-MS).

To obtain total amino acids, 0.5 g of powder sample was added with 200 mL HCl 0.1 M (pH 1), homogenised by autoclaving at 121°C for 30 minutes to obtain protein hydrolysate. The sample was then dried in a rotary evaporator at 45-50°C, 50 rpm, and the resulting extract was resuspended in 10 mL HCl 0.01 N, centrifuged at 10,000 rpm for 10 minutes. Finally, the suspension was filtered into a vial to get the supernatant for analysis using LCMS-MS UPLC-QTOF-MS (*Ultra-Performance Liquid Chromatography (*Acquity, Waters, USA*) Quadrupole Time of Flight Mass Spectrometry (*Xevo 62, Waters, USA*)*) run with the following parameters: scan mass 70.00-700.00 ES+, run time 12 minutes, source capillary (kV) 2.00, sampling cone 40, extraction cone 2.0, temperature 120°C (source), 350°C (desolvation), gas flow 10 L/h (cone), 600 L/h (desolvation), column acquity UPLC® BEH C18 (1.7 μm; 2.1 x 5 mm), aqueous sample solvent, injection volume 5 μl, mobile phase A (0.1% pentadecafluorooctanoic acid, 99.5% water, 0.5% acetonirile, 0.1% formic acid) and mobile phase B (0.1% pentadecafluo-rooctanoic acid, 10% water, 90% acetonirile, 0.1% formic acid) with gradient elution protocol: weak wash solvent (0.1% formic acid) and strong wash solvent (25% aceto-nitrile, 25% methanol, 50% water) (Table 1).

Table 1. Analytical gradient elution protocol of UPLC

Time (min)	*Flow rate* (mL/minute)	% A	% B
0	0.4	100	0
1	0.4	98	2
4	0.4	80	20
8	0.4	60	40
9	0.4	0	100
12	0.4	100	0

Extraction and analisys of theobromine

To extract theobromine, 5 g powder sample was added with 200 mL demineralised water, homogenised at 60°C for 30 minutes, followed by paper filtration. The obtained supernatant was added with 0.1M NaOH up to pH 8, and then with methanol at a volumetric ratio of 1:1. The mixture was homogenised using horizontal shaker for 1 hour, centrifuged at 10,000 rpm, 4°C for 10 minutes, and, finally, the precipitate was dried in rotary evaporator at 45-50°C, 50 rpm before resuspending in 2 mL demineralised water. The suspension was paper filtered into a vial to obtain the supernatant to be injected into HPLC.

HPLC analysis of theobromine was done using PDA (Photo Diode Array) at λ 273 nm and C-18 symmetry column (5 µm, 4.6 × 150 mm), at 25°C. Mobile phases used were solvent A (aqueous solution of 0.1% tetrahidrofuran) and solvent B (acetonitrile) mixed at a ratio of 90:10, at a flow rate of 0.8 mL/minute and sample injection volume 10 µl.

RESULTS AND DISCUSSION

From the 3 sample types which were collected from 5 different locations, 10 putative *Rhizopus* isolates were obtained (Table 2). These isolates were subsequently selected on the medium containing cocoa byproduct only and on the cocoa byproduct-rice bran mixed medium. All strains grew in the rice bran containing media, while only few fungal strains were capable of utilising the sole cocoa byproduct as growth medium. Throughout incubation period, however, the growth of all the isolates in all media was suboptimal, indicated by the sparse mycelia formed. A pungent smell characteristic of ammonia was also perceived, strongly indicating the breakdown of urea into ammonia, which is known to inhibit fungal growth (Sparringa and Owens 1999). Nowithstanding, based on its colony diameters, the best growth was demonstrated by isolate TP1 which was confirmed to be *Rhizopus oryzae* based on 16s rRNA molecular identification.

The selected *R. oryzae* strain obtained was used as fermenting agent, using previously pre-optimised fermentation condition (unpublished results), on rice bran-cocoa byproduct mixed substrate. The resulted fermented cake was found to have its true protein content increased from initially 1.95% before fermentation to 23.16% after fermentation with dry matter loss of 31-35%. Taking the dry matter loss into account, the net gain in true protein content was 14-15%. This protein increase was owing to the ability of genus *Rhizopus* to synthesize fungal protein utilising inorganic and organic nitrogen compounds such as ammonium salts and urea supplemented into the substrate (Sriherwanto 2010).

Increase in true protein content during solid state fermentation using *R. oryzae* was also reported by previous authors working on

Table 2. Putative *Rhizopus* isolates

Isolate code	Regional origin	Sample type
DWM1	Makasar, South Sulawesi	Hibiscus (waru) leaf
DWM2	Makasar, South Sulawesi	Hibiscus (waru) leaf
DWT1	Tasikmalaya, West Java	Hibiscus (waru) leaf
DWT2	Tasikmalaya, West Java	Hibiscus (waru) leaf
RTK2	Kediri, East Java	Tempeh starter
RTK4	Kediri, East Java	Tempeh starter
TB1	Bogor, West Java	Soybean tempeh
TB2	Bogor, West Java	Soybean tempeh
TP1	Jayapura, Papua	Soybean tempeh
TP5	Jayapura, Papua	Soybean tempeh

Table 3. Growth of putative *Rhizopus* isolates on the selection media of cocoa byproduct alone (CB) and of cocoa byproduct-rice bran mixture (CB + RB).

Isolate	Colony diameter (cm) during fermentation					
	at 2nd day of incubation		at 3rd day of incubation		at 4th day of incubation	
	CB	(CB + RB)	CB	(CB + RB)	CB	(CB + RB)
DWM 1	-	1.3	-	1.4	1.3	3.0
DWM 2	-	1.3	-	1.5	-	2.3
DWT 1	-	1.5	-	1.7	-	2.8
DWT 2	-	0.8	-	1.1	-	2.7
RTK 2	-	1.4	-	1.6	-	3.6
RTK 4	-	1.4	-	2.0	-	3.5
TP 1	-	1.8	1.0	2.5	1.0	4.1
TP 5	-	-	-	-	0.2	2.1
TB 1	-	1.5	-	1.6	-	3.7
TB 2	-	1.9	-	2.1	-	4

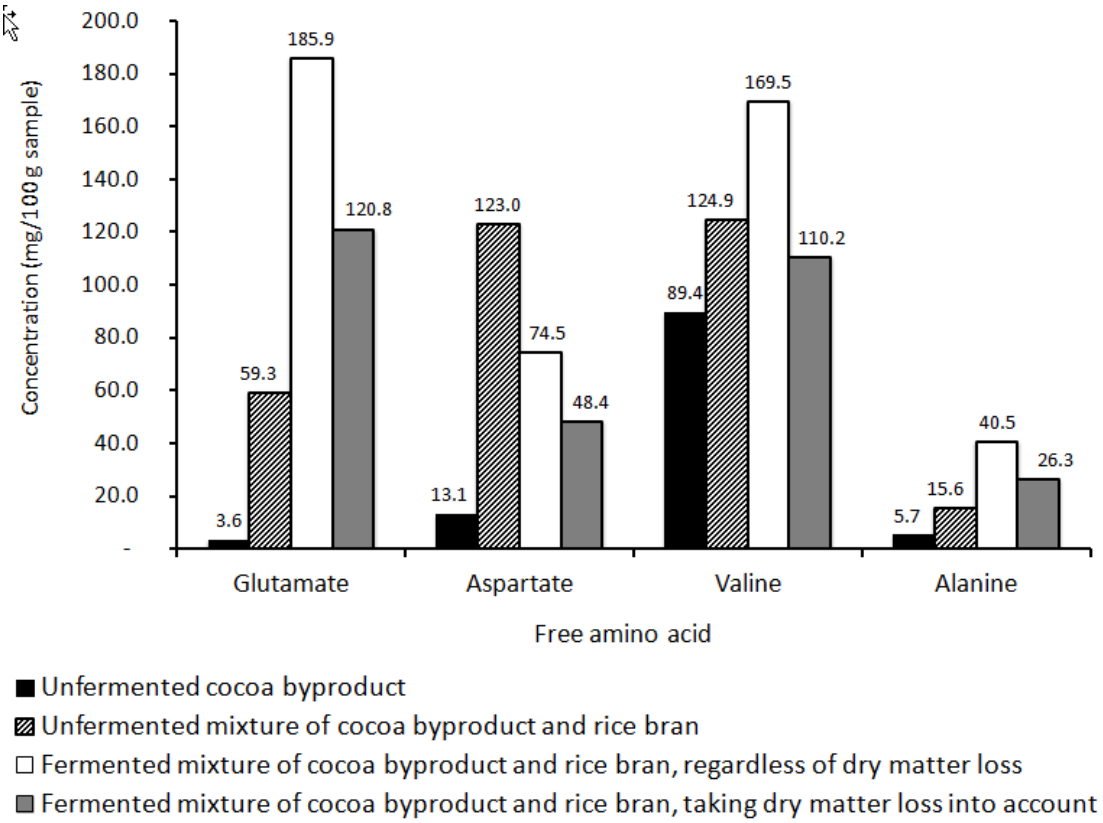

Figure 1. Free glutamate, aspartate, valine, and alanine profile in unfermented and *R. oryzae*-fermented substrates

different substrates. Initial true protein content of 1.96% in an equivalent mixture of rice bran and coconut bagasse substrate was increased to 8.39% during solid fermentation using *Rhizopus oryzae* (Umam et al. 2015) *Rhizopus* sp. fermented sago flour contained qualitatively better protein content (3.4%) than the unfermented one (1.6%) (Ab Jalil et al. 2014). Also, cassava bagasse subjected to solid fermentation using *Rhizopus* sp. caused 531% increase in true protein content (from initial value of 1.45% to 9.15% post fermentation) (Sriherwanto 2010). Two-four times increase in crude protein content was achieved when brewery spent grain was fermented using *Rhizopus oligosporus* (Canedo et al. 2016). Using the same *Rhizopus* species in 24-hour fermentation on raw and roasted buckwheat groats, significant increase in the content of

protein was also observed. (Wronkowska et al. 2015).

Both amino acid profiles (Figure 1 and 2) prior to fermentation showed that adding cocoa byproduct with rice bran at 1:9 ratio increased both free and total amino acids, with exception of total valine, which was present in a very low quantity (less than 5 mg/100g) in the rice bran. This amino acid enrichment through mixing with rice bran prior fermentation might explain the growth of all the putative *Rhizopus* isolates on the rice brain containing medium being better than that on the cocoa byproduct only medium (Table 3). Indeed, the inclusion of rice bran in fermentation substrates in a number of studies related to *Rhizopus* solid fermentation by different authors testified its nutrient suitability for the growth of the *Rhizopus* fungi (Abd Razak et al. 2015a; Kaur et al. 2015; Abd Razak et al. 2015b; Massarolo et al. 2016).

All of the amino acids analysed underwent increase after fermentation, regardless of the dry matter loss, with the exception of aspartate, both total and free forms. Taking the dry matter loss into consideration in order to get the real net increase in the amino acids studied, it is the total valine, free glutamate, and free alanine contents which increased during fermentation (Figure 1 and 2). Changes in amino acid profiles during solid state fermentation using *Rhizopus* fungi were reported in previous studies as well. For example, increase in free and essential amino acids were observed in *Rhizopus* fermented soybeans (Koh et al. 2016). Changes in amino acids contents were also observed when pulverized seed of baobab (*Adansonia digitata*) was fermented using *Rhizopus stolonifer*, where the contents of some amino acids increased while the others decreased after the fermentation (Adedayo and Sani 2015). Interrestingly, similar to this study, relative to the unfermented baobab seed, the fermented product contained higher contents of glutamate and valine, and lower content of aspartate (Adedayo and Sani 2015). However, in a study involving fermentation of green coffee beans using *Rhizopus oligosporus*, no significant differences were observed in the net total free amino acids concentrations present in the blank, fermented and unfermented substrates (Lee et al. 2016).

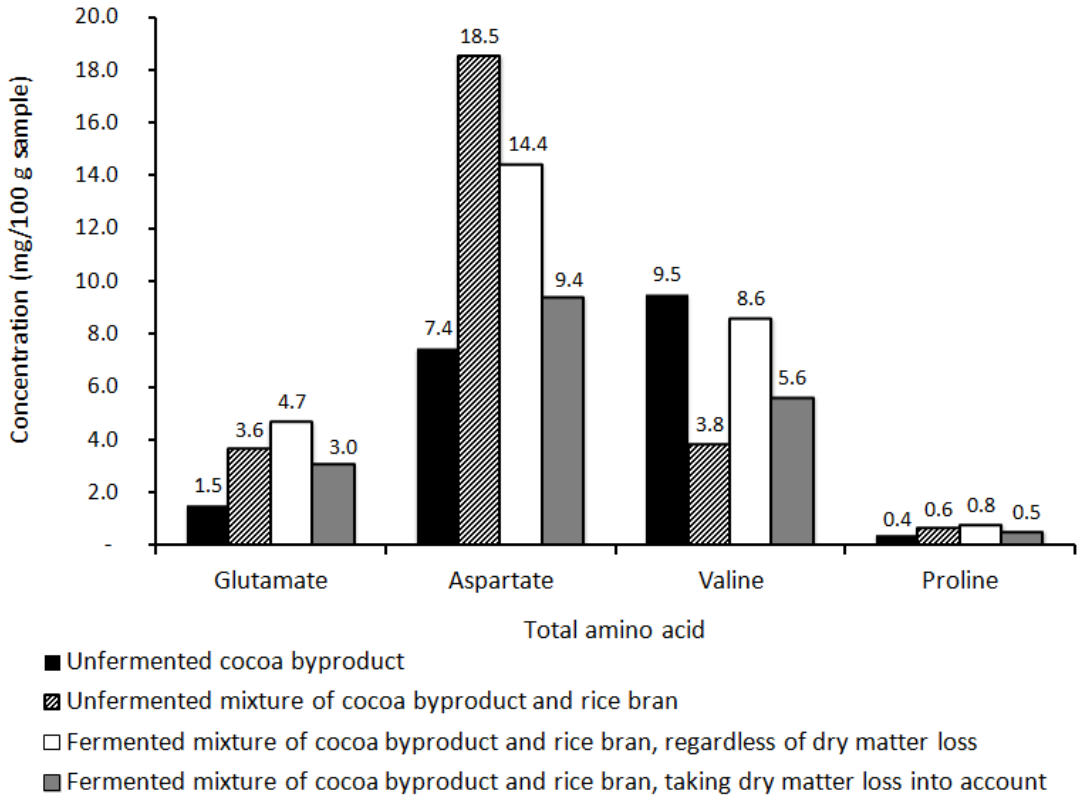

Figure 2. Total glutamate, aspartate, valine, and proline profile in unfermented and *R. oryzae*-fermented substrates

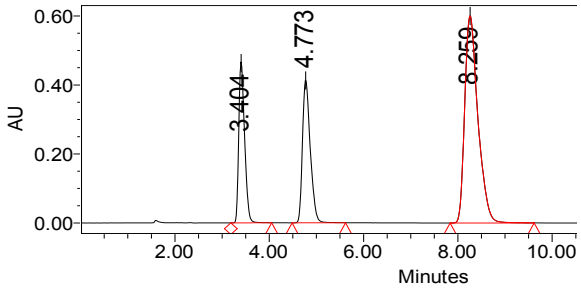

Figure 3. The HPLC chromatogram of the antinutrition standards with their corresponding retention time (minutes): theobromine (3.404), theophylline (4.773), and caffeine (8.259)

To determine the content of the antinutrition theobromine in the substrate before and after fermentation, standard graphs were prepared based on the chromatograms of the standard solutions of theobromine, as well as its polymorphs theophylline and caffeine, each of which showed clearly distinct peak having separate retention time (Figure 3). The same was not true for the samples, however, in which peaks corresponding to the standards' retention times were hardly observed or having quantities below the range of the standard values (Figure 4). This indicates that the measured antinutritional substances contained in the cocoa byproduct might have been significantly reduced due to the mixing with much higher proportion of rice bran. The loss might also have occured during cocoa byproduct pretreatment including drying and milling. Another explanation could be the poor extraction process which had not yet been optimized, resulting in the theobromine concentration in the samples being lower than the HPLC detectable limits. Thus, the role of *Rhizopus* fermentation in reducing the cocoa byproduct's theobromine could not be demonstrated, unlike in other previous studies in which, for example, *Aspergillus* spp. fermentation of cocoa pod husk was shown to have lowered its theobromine content (Munier and Hartadi 2012).

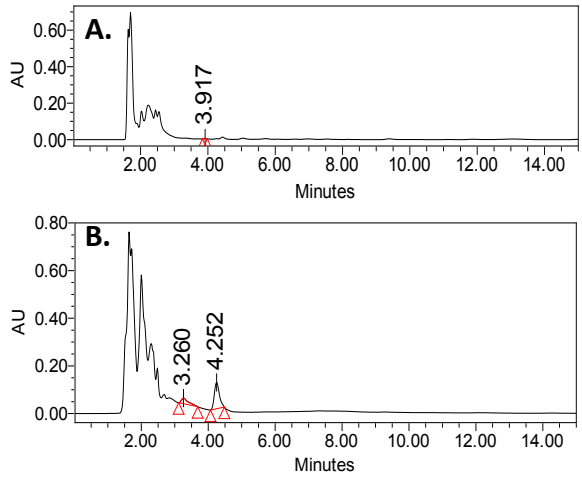

Figure 4. HPLC chromatogram of theobromine extracted from cocoa byproduct-rice bran mixed substrate (A) before and (B) after fermentation using *R. oryzae*.

CONCLUSION

Rhizopus oryzae solid-fermentation of a mixed substrate consisting of cocoa byproduct and rice bran in a 1:9 mass ratio increased the true protein content from 2% to 23%. The amino acids analysed were total and free glutamates, total and free aspartates, total and free valines, total proline, and free alanine. Of the amino acids analysed, total and free glutamates, total and free valine, total proline, as well as free alanine underwent increase, while the others decreased. The antinutrional factor theobromine was not detected, probably having been considerably reduced during cocoa processing.

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