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Effect of Fermentation by *Rhizopus oligosporus* or *Amylomyces rouxii* on *In-Vitro* Starch and Protein Digestibility of Decorticated Red Sorghum (*Sorghum bicolor* L. Moench)

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Abstract*— Solid substrate fermentation of cooked decorticated red sorghum was carried out by using mould *Rhizopus oligosporus* or *Amylomyces rouxii*. This study aims to investigate the growth of mould biomass by determining glucosamine and investigating the effects of fermentation on the changes in proximate composition, pH and total titratable acidity, and *in-vitro* starch and protein digestibility. Red sorghum was decorticated using a carborundrum discs to remove the bran. Decorticated sorghum was soaked in water (12 h for *R. oligosporus* and 1 h for *A. rouxii* fermentation), heated up to 90^{o}C for 30 min, steamed for 30 min, and sterilized at 121^{o}C for 20 min). Fifty g of sterile sorghum was inoculated with *R. oligosporus* (6.10^{3} spore/g substrate) or *A. rouxii* spores (2.10^{3} spore/g substrate) then incubated at 30^{o}C. Samples were taken at 0, 12, 18, 24, 30, and 36 h for *R. oligosporus* fermentation and 0, 24, 48, 72, 120, and 168 h for *A. rouxii* fermentation. The results indicated that glucosamine content of fermented sorghum by *R. oligosporus* and *A. rouxii* were 4.49 and 11.72 mg/g dry matter at the end of fermentation, respectively. *R. oligosporus* hydrolyzed up to 11.4% of initial starch and 63.4% by. *A. rouxii.* There were more protein and fat losses in *R. oligosporus* fermentation than *A. rouxii*. Both fermentations produced acid and lowered pH to about 3.0, but pH went up to 4.0 at the end of *R. oligosporus* fermentation. Mould fermentation improved *in-vitro* starch digestibility, in *A. rouxii* it went up from 31.3% to 37.9% and down again to 11.4% at the end of fermentation. In *R. oligosporus* fermentation, it went up to 35.4%. *In-vitro* protein digestibility went up from about 35.0% to 37.0% and to 51.8% by *R. oligosporus* and *A. rouxii* fermentation, respectively.

***Keywords*— red sorghum; fermentation; *Rhizopus oligosporus*; *Amylomyces rouxii*; starch digestibility; protein digestibility.**

I. INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is a cereal plant, adaptable to dry land [1]. It is the potential to support food security in Indonesia, particularly in dry areas, where paddy and corn do not grow. Sorghum has been reported to be planted in Central Java, Yogyakarta, East Java, and Nusa Tenggara [2]. Sorghum contains relatively high protein compared to other carbohydrate sources consumed in Indonesia [2]. However, sorghum protein has unique characteristics; it forms disulfide bound in wet processing and becoming challenging to digest [3]. Also, sorghum protein plays a role in decreasing starch digestibility after cooking [4]. Some exogenous factors, such as polyphenol, tannin, phytic acid, starch, and non-starch polysaccharides, affect protein digestibility. Some endogenous factors, such as a non-disulfide bond, hydrophobicity, and secondary protein structure changes, have also been reported to affect this digestibility [5]. The decrease in starch and protein digestibility leads to lower nutritional value of sorghum, especially for people who consume sorghum as a main food for carbohydrate and protein sources.

The digestibility of starch and protein can be amplified by fermentation [6]. The common method is by fermenting raw sorghum using lactic acid bacteria, eventhough some yeast has been tested for protein content enrichment [6]. Meanwhile, the study on cooked sorghum fermented with *Rhizopus roux* is still limited [7]–[10]. Because the sorghum consumer in the world, from African and India, use to ferment with lactic acid bacteria rather than with fungi that is commonly practiced in South East Asia [11], [12].

In Indonesia, several fungi have been used for food processing, such as *Amylomyces rouxii* and *Rhizopus oligosporus*. *A. rouxii* which are well known to have high amylolytic activity [13] and importantly play role in tape production from cassava and glutinous rice, and their derivative product like brem [14]. *R. oligosporus* has high proteolytic activity, able to hydrolyze protein significantly [15], and has been used to ferment soybean to produce

tempe in Indonesia long ago [16]. Amylolytic of *A. rouxii* and proteolytic of *R. oligosporus* can increase starch and protein digestibility through fermentation.

Red sorghum has pericarp containing *cellulose*, *hemicellulose*, and *lignin* [17], *antifungal protein* [18], and *polyphenol* [19] that can inhibit fungal growth. Decortication can separate the pericarp part from sorghum endosperm [20], therefore, reducing the inhibition factor and negative effect of polyphenol against starch and protein digestibility [21].

The fungus' growth is more precisely followed by biomass determination than colony counting because the fungus' growth is highly correlated to metabolic activity [22]. One indicator to determine the biomass of fungus is glucosamine [23], [24]. Also, the fungal metabolic activity during fermentation could be observed by dry matter loss [22]. Meanwhile, fungal biomass growth and its correlation to metabolic activity during fermentation of decorticated red sorghum by *A. rouxii* or *R. oligosporus* needs to be investigated. It is also necessary to find out the effect of fungal fermentation on the composition and starch and protein digestibility of fermented sorghum. The objectives of the study were to determine the biomass growth and metabolic activity of *A. rouxii* and *R. oligosporus* during fermentation of decorticated red *sorghum*, and to find out its effect on the changes of proximate composition, pH, acidity, and *in vitro* starch and protein digestibility.

II. Materials and Methods

A. Materials

Red sorghum was purchased from a local market in Yogyakarta. Fungi *Amylomyces rouxii* C2 was isolated from tape inoculum brand "66", Kudus and *Rhizopus oligosporus* was isolated from tempe inoculum, commercially produced by Aneka Fermentasi Industri Co. Ltd, Bandung, Indonesia. Chemical reagents used were α-amylase (Sigma A3176), pepsin (Merck), ammonium sulfamate (Fluka 09960), MBTH (3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate) (Sigma 129739), and D-glucosamine hydrochloride (Sigma G4875).

B. Sorghum Fermentation

Red sorghum was dried in a cabinet dryer to the moisture content of 11 % before the decortication process. Red sorghum was decorticated to remove pericarp part. After that, decorticated red sorghum was added with warm water (± 60°C) 8 times (v/w) and soaked at room temperature about 1 h for *A. rouxii* and for 12 h for *R. oligosporus* fermentation in order to decrease pH naturally. The different soaking times was carried out to meet the optimum growth of the respected fungus. Then, it was heated to 90 °C for 30 min. Soaked sorghum seed was then cooked for 30 min. Fifty g cooked sorghum was put on Petri dish, wrapped by plastic, and sterilized at 121°C for 20 min.

Inoculum of *A. rouxii* and *R. oligosporus* was prepared by diluting the spore from agar slant with 5 ml sterile Tween-80 of 0.05 %. One ml of spore suspension was inoculated into a Petri dish containing a sample, wrapped with perforated plastic, and incubated at 30°C for 7 day for *A. rouxii* and 36 h for *R. oligosporus*. The different incubation times was taken following the optimum growth of the respected fungus. The sampling was taken at an incubation time of 0, 24, 48, 72, 120, and 168 h for *A. rouxii* and of 0, 12, 18, 24, 30, and 36 for *R. oligosporus*. All samples were tested for proximate analysis, starch, reducing sugar. In addition, the dry matter loss, pH, titratable acidity, in-vitro starch, and protein digestibility were also determined.

C. Determination of Glucosamine and Dry Matter Loss

The sample was weighed together with the petridish. The moisture content was determined by the gravimetry method. Dry matter loss was obtained by differing the weight of dry sample between before and after fermentation, stated as g/100 g initial dry matter. Glucosamine content was determined following the method [24].

D. Proximate Analysis

Proximate composition was determined by using the method of the Association of Official Analytical Chemists [25]. Moisture content was done by drying the samples in the oven at 105°C until constant weight; protein content was carried out by using micro-Kjeldahl (% protein = % N x 6.25); lipid content was carried out by Soxhlet extraction with petroleum ether; ash content was carried out by burning the sample in the furnace at 600°C until constant weight; starch content was determined by using acid hydrolysis; non-starch carbohydrate was determined by difference. Reducing sugar was determined following the Nelson-Somogyi method [26].

E. Determination of pH and Total Acidity

The changes of pH and total acidity during fermentation of red sorghum by *A. rouxii* and *R. oligosporus* were done by homogenizing 1 g of sample with 9 ml distilled water pH 7.0. Measurement of pH was done using pH meter. In addition, the sample was then titrated with 0.02 M NaOH by drop. Phenolphthalein in ethanol was used as an indicator.

F. Analyses of Starch and Protein Digestibility

In-vitro starch digestibility was determined according to a previous method [4] with minor modification. Starch content of each sample was determined prior to analysis of starch digestibility. Two hundred milligrams of starch were added by 5 ml of distilled water, heated at 100°C for 20 min, and cooled at 40°C. Twenty-five ml of α-amylase solution (10 unit/ml in buffer [0.2 M dikalium hydrogen phospate–kalium dihydrogen phospate, pH of 6.9, 25 mM NaCl, 5 mM $CaCl_2$]) was added to the heated sample, and the suspension was incubated at 37°C. To stop the reaction, 1 ml of sample was added with 0.1 ml of 2N H_2SO_4. Reducing sugar was analyzed using Nelson-Somogyi method. The increase of reducing sugar was converted into starch in order to predict the amount of hydrolyzed starch by the enzyme.

In-vitro protein digestibility was determined based on previous studies [27],[28]. A sample of 60 g was added with 1 ml of pepsin (20 mg/ml buffer 0.1 M KH2PO4; 0.7-unit FIP/mg), incubated at 37°C for 120 min. The reaction was stopped by adding 100 µl of 2 M NaOH and immediately cooled with ice. The sample was filtered using Whatman No. 4 paper, the residue was rinsed with 1 ml distilled water, and

nitrogen in the filtrate was determined using the micro-Kjeldahl method.

III. Results and Discussion

A. Glucosamine and Dry Matter Loss

Figure 1 shows changes of glucosamine content and dry matter loss during fermentation of decorticated red sorghum. The highest glucosamine production rate was obtained after 24-48 h and after 18-24 h for *A. rouxii* and *R. oligosporus*, respectively. The highest glucosamine content of *A. rouxii* reached 11.72 mg/g dry matter for 168 h. Meanwhile, highest glucosamine content of *R. oligosporus* reached 4.49 mg/g dry matter for 36 h. Dry matter loss at the end of fermentation for *A. rouxii* was 22.63 g/100 g of initial dry matter, whereas dry matter loss of *R. oligosporus* was only 5.87 g/100 g of initial dry matter.

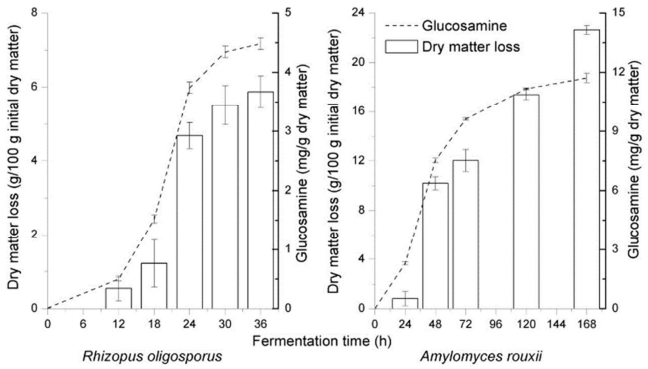

Fig. 1 Change of glucosamine and dry matter loss of fermented sorghum by Rhizopus oligosporus and Amylomyces rouxii

Figure 1 shows changes in glucosamine content and dry matter loss during fermentation of decorticated red sorghum. The highest glucosamine production rate was obtained after 24 h-48 h and after 18-24 h for *A. rouxii* and *R. oligosporus*, respectively. The highest glucosamine content of *A. rouxii* reached 11.72 mg/g dry matter for 168 h. Meanwhile, highest glucosamine content of *R. oligosporus* reached 4.49 mg/g dry matter for 36 h. Dry matter loss at the end of fermentation for *A. rouxii* was 22.63 g/100 g of initial dry matter, whereas dry matter loss of *R. oligosporus* was only 5.87 g/100 g of initial dry matter.

A correlation between accumulation of CO_2 and dry matter loss in solid state fermentation using *Aspergillus oryzae* has been reported by Sardjono [22]. In this current work, biomass was determined by glucosamine content and metabolic activity was analyzed indirectly using dry matter loss. As shown in Fig. 1, glucosamine production has similar trend with dry matter loss profile during fermentation, especially for fermentation using *R. oligosporus*. The result showed that the dry matter loss rate was still high although the glucosamine production rate has decreased in 72-168 h for *A. rouxii* fermentation. It was due to enzymatic hydrolytic by amylase from *A. rouxii* that still occurred. Fungi degraded macromolecules using an extracellular enzyme. They utilize starch and fat as carbon sources, whereas protein was used as nitrogen source.

B. Proximate Composition

Figure 2 presents the profile of starch, protein, and fat during fermentation. The result showed that starch decreased by 50.1 g/100g of initial dry matter corresponding to losses of 63.4% for fermentation by *A. rouxii* for 168 h. In the case of fermentation using *R. oligosporus*, the starch reduced to 8.9 g/100g of initial dry matter corresponding to losses of 11.4% for 36 h fermentation. Starch was hydrolyzed into dextrin as non-starch carbohydrate and short chain sugars such as maltose and glucose, representing reducing sugar. Non-starch carbohydrate increased to 17.96 g/100 g initial dry matter and 4.4 g/100 g initial dry matter for *A. rouxii* and *R. oligosporus*, respectively. Reducing sugars at the end of fermentation reached 13.5 g/100 g initial dry matter and 0.3 g/100 g initial dry matter for *A. rouxii* and *R. oligosporus*, respectively. The highest reducing sugar content of *A. rouxii* reached 14.3 g g/100 g initial dry matter at 48 h and slightly decreased to 13.5 g/100 g/100 g initial dry matter at the end of fermentation.

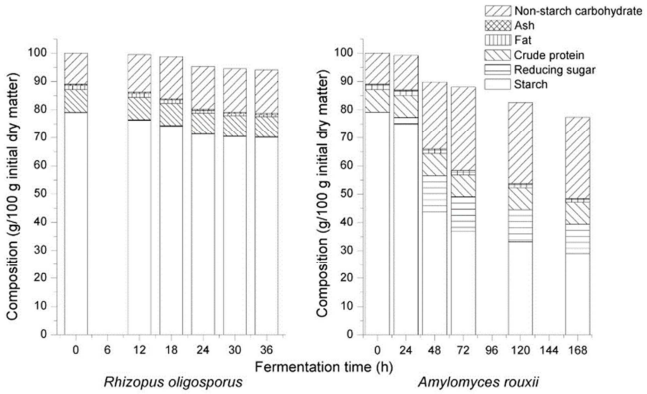

Fig. 2 Proximate composition change during fermentation by *Rhizopus oligosporus* and *Amylomyces rouxii*

Hydrolysis by amylolytic enzyme led to degradation of starch and loss of water holding capacity. This might be explained by fermentation using *A. rouxii* resulting in a moister product than that of by *R. oligosporus*. However, the result of this work showed that *A. rouxii* could not degrade the starch completely [14] on the fermentation of glutinous rice. This might be due to the interaction between protein and starch during heating, making the starch difficult to hydrolyze by enzymes [5].

The crude protein decreased to 0.18 g/100 g initial dry matter and 0.74 g/100 g initial dry matter for fermentation using *A. rouxii* and *R. oligosporus*, respectively which correspond to the conversion of 2.3 % and 9.3% of initial protein. Fermentation using *R. oligosporus* reduced more fat (0.79 g/100 g initial dry matter) than that of using *A. rouxii* (0.62 g/100 initial dry matter). The losses of crude protein and fat using *A. rouxii* contributed to 0.84 and 2.74 % of dry matter loss. Meanwhile, the loss of crude protein and fat using *A. rouxii* contributed to 12.6 and 13.4 % of dry matter loss.

Fermentation of soy using *R. oligosporus* NRRL 2710 at 30°C for 72 h resulted in fat oxidation of 13.5 g/100 g initial dry cotyledon and protein of 2 g/100 g initial dry cotyledon [29]. The fat oxidation contributed to 80% of dry matter loss, and 2.5% of hydrolyzed protein by the fungi was oxidized.

In this work, the dry matter loss was mostly caused by oxidation of fat and protein rather than by starch degradation since *R. oligosporus* might have low amylolytic activity.

C. pH and Total Acidity

The change of pH and titratable acid during fermentation is presented in Fig. 3. *A. rouxii* produced acid up to 11.7 mg lactic acid/g dry matter at 48 h and started to decline until 10.7 mg lactic acid/g dry matter at the end of fermentation. The pH of the sample during fermentation using *A. rouxii* depended on the acid content, and the lowest pH was reached at 48 h and slightly increased up to 3.3 at the end of fermentation. In the case of fermentation using *R. oligosporus*, the pH started to decrease after 12 h and reached the lowest pH of 3.1 at 24 h, subsequently, the pH increased to 4 at the end of fermentation. Total acid concentration reached 9.2 mg lactic acid/dry matter at 30 h and declined to 9.2 mg lactic acid/dry matter at the end of fermentation.

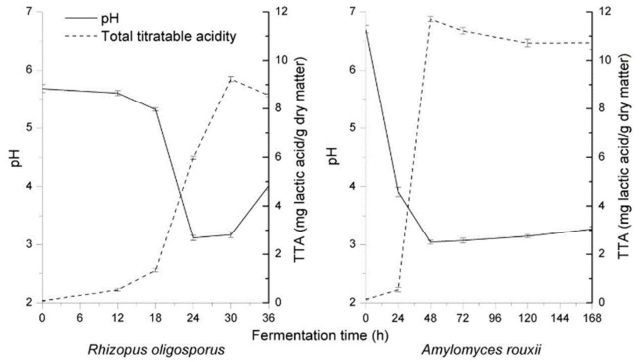

Fig. 3 The change of pH and total acidity during fermentation by *Rhizopus oligosporus* and *Amylomyces rouxii*

The ability of *A. rouxii* for producing lactic acid and decreasing pH is in accordance to previous reports [30], [31]. The combination of low pH and acid during fermentation minimizes the growth of the pathogen. Fermentation using *Rhizopus oligosporus* on various substrates mostly increases pH; however, in this work the pH decreased in the beginning and increased until the end of fermentation. Acid formation and decrease of pH could be due to the metabolism of R. oligosporus that produced lactic acid or the acid-producing microorganism was still alive during fermentation.

D. Starch and Protein Digestibility

In-vitro starch and protein digestibility are presented in Fig. 4. The result showed that fermentation using *A. rouxii* increased protein digestibility from 34.9% to 51.8%, while the starch digestibility increased from 31.3% to 37.9% for 48 h and decreased to 11.4% at the end of fermentation. Similarly, the protein digestibility increased from 35.1 to 37.1 % and starch digestibility increased from 31.3 to 35.4% for 36 h fermentation using *R. oligosporus.*

Fermentation could increase the digestibility due to enzyme productions that can hydrolyze macromolecules. *A. rouxii* dan *R. oligosporus* produce protease which makes starch granule more accesible to amylase enzyme [32]. The decrease of starch digestibility of fermentation using *A. rouxii* after 48h might be explained by the limited acces of the enzyme due to the formation of kafirin polymer during cooking that covers the starch surface [33]. Another reason could be an increase of resistant starch during heating process [34]. In the case of fermentation using *R. oligosporus*, starch digestibility did not decrease until the end of fermentation might be due to the amount of hydrolyzed starch was lower than that of fermentation using *A. rouxii*.

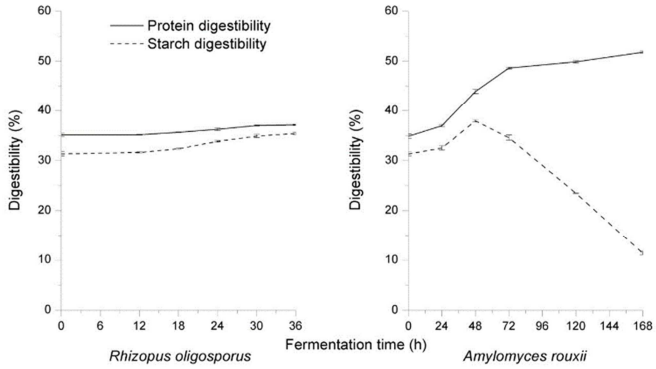

Fig. 4 Change of *in-vitro* starch and protein digestibility during fermentation *Rhizopus oligosporus* and *Amylomyces rouxii*

Protein digestibility of brown sorghum using *R. oligosporus* increased from 51.91 % to 79.13% for 72 h [10]. This increase of protein digestibility (27.2 %) is higher than in the current work (2%). This might be explained by different subtrate, fermentation process and analysis of *in-vitro* protein digestibility. *A. rouxii* has higher capability to increase protein digestibility compared to *R. oligosporus*, that could be due to its higher amylolytic activity. According to Duodu et al. [28], digestion using α-amilase on cooked sorghum increased *in-vitro* protein digestibility compared to control. In addition, intensive digestion of *A. rouxii* produces water that increase moisture content and enzymatic hydrolysis activity.

IV. Conclusions

Rhizopus oligosporus and *Amylomyces rouxii* were able to grow in cooked decortitated red sorghum. The biomass reached 4.49 mg glucosamine/dry matter for 36 h and 11.72 mg glucosamine/g dry matter for 168 h by *R. oligosporus* and *A. rouxii*. The dry matter loss at the end of fermentation was 5.87 g/100 g initial dry matter and 22.63 g/100 g initial dry matter by *R. oligosporus* and *A. rouxii*. The change of glucosamine content was in line with dry matter loss. *A. rouxii* hydrolyzed starch up to 63.4 % and produced reducing sugar of 10.4 g/100 g initial dry matter. *R. oligosporus* oxidized fat and protein up to 0.74 g/100 g dry matter and 0.79 g/100 g dry matter, respectively. Starch digestibility of fermentation using *A. rouxii* increased then decreased to 11.4% at the end of fermentation. In case of *R. oligosporus*, starch digestibility increased up to 4.1%. *A. rouxii* increased protein digestibility to 16.9%, whereas *R. oligosporus* increased protein digestibility up to 2%.

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