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Rhizopus oryzae FNCC 6010, *Rhizopus oligosporus* FNCC 6011, and their hybrid lowered antioxidant capacity in velvet beans compared to germination

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Abstract

In an attempt to improve food processing for legumes, the use of commercial egg white lyzozyme in fusion technology of *Rhizopus spp*. has been investigated. The experiment was run in triplicates using 2-10 KU. The results indicated that lyzozyme lyzed cell walls of fungal mycelia better for *R. oligosporus* (0.3-1.4 x 10^6 free protoplasts/mL) than *R. oryzae* (0.09-0.6 x 10^6/mL). The fusant yields reached maximum when the use of lyzozyme was at 4 KU and the hybrid reached maximum at Rhizopus fusant ratio of 1:1 (v/v). Morphologically and physiologically, the hybrid resembled *R. oligosporus*, but enzymic activities were similar to those of *R. oryzae* characteristics, except for its very weak amylase activity. The hybrid and strain parents have been applied in producing fermented dehulled or undehulled velvet bean flour compared to germination effects at laboratory scales observed for 72 h, to explore its DPPH inhibition capacity. The inhibition capacity of methanolic hybrid flour extracts for 72 hours were higher than those of the parent strains but it was only half of the germination flours'. In conclusion, the Rhizopus spp lowered DPPH inhibition capacity compared to germination. The hybrid can be used for velvet bean flour production without starch conservations.

Introduction

The uses of microorganisms in food preservation and food processes such as fermentation of velvet beans would have been of great benefit in functional food processing because traditionally the beans have medicinal potentials e.g. as Parkinson disease drug, antidiabetic, antivenom, and neuroprotective (Lampariello *et al*., 2012). Yet, further investigation determining proper intakes of particular natural medicine are on demands e.g. L-DOPA of velvet bean and its extracts (Taylor, 2003). Thus, there is a need to find strain that capable of partially remove the natural medicinal components in the beans. Meanwhile, L-DOPA together with other bioactives in the velvet beans has been well known to scavenging radicals (Taylor, 2003; Lampariello *et al*., 2012). Hence, further investigation on application of the fungi for food processing such as flour production and determination of their inhibition capacity towards DPPH (2,2 diphenyl-1-picrylhydrazyl) become of a relevant initial study.

Improving the quality of fermented foods is a necessity and it involves deliberate investigations and efforts into areas including processing equipment, development of the commodity strain, methods of optimizing the fermentation conditions and, most importantly, microbial cultures involved in the fermentation –strain improvement (Odunfa, 1985). Microbial strain improvement for consequent application in food and food products has become of high relevant importance as beneficial features in organisms can be obtained through interbreeding from one organism to another (Verma *et al*., 2004). This simple fusion technique could be used to manipulate enzymes pack of the cultures where in the present research their applications are expected to improving velvet bean potential while preserving all beneficial compounds in the beans. Hybridization techniques using protoplasts

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fusion has become a very successful procedure in achieving this. A fungal protoplast is a cell whose entire cell wall has been completely removed so that the cytoplasmic membrane becomes the outermost layer of such a cell (Srinivas and Panda, 1997; Verma *et al*., 2004). This can be obtained simply by application of lytic enzymes which digest the cell wall (Srinivas and Panda, 1997). Although fusing the protoplasts of two Rhizopus strains could be achieved by different methods, Jogdand (2001) employed the chemically induced method in which poly ethyl glycol (PEG) causes clump formation in the protoplasts to ease protoplast collection. Some basic investigations such as counts of cell/protoplast numbers using the haemocytometer and morphological examinations and enzyme activities of the Rhizopus strains then required, as they compare the characteristics of the strain parent fungi with the new fusant, that is, hybrid as a new organism.

Both *R. oligosporus* and *R. oryzae* have been found to be particularly useful and of great economic importance, especially in the industrial production of alcohols, organic acids, and esters. *R. oligosporus* has been the important starter culture in tempeh (legumes) production and it also produces an antibiotic that inhibits Gram positive bacterial cells. *R. oryzae* shows great usefulness and economic importance in industrial enzyme production such as amylases and lipases (Kobayasi *et al*., 1992). Bearing these characteristics of the two strains in mind, hybridizing them to have a new strain appears to be a very useful effort.

The attempt to apply Rhizopus and its hybrid for velvet bean may improve either functional food potential or safety levels of the velvet bean based products. Currently, information on velvet bean flour potential as a source of functional food ingredient, not fractionated into a pure drug, is limited. Furthermore, Sardjon *et al*. (2012) found that velvet bean contains alkaloid, tannins, saponins, and steroids in shell or seed. Most bioactives in plant exist either in the vacuole or bound in the cell wall materials. On the other hand, *R. oligosporus* and *R. oryzae* release more phenolic compounds during food fermentation (McCue *et al*., 2003) and it has been reconfirmed with the research done by Schmidt *et al*. (2014). Thus, it is worthy to measure antioxidant potential of the flour products after biochemical processes (e.g. fermentation or germination). Germination is a common process to provide food materials from legumes to improve nutrients. The effectiveness of Rhizopus fermentation and germination will be compared in the present study.

Overall, research investigated fusion of Rhizopus sp. using commercial white egg lyzozyme and to characterize the hybrid prior to application. Furthermore, this research is expected to explore DPPH inhibition due to bioactive releases after biochemical processes such as fermentation and germination using velvet bean as a model after the hybrid experiments.

Material and Methods

Rhizopus oligosporus (FNCC 6010) and *R. oryzae* (FNCC 6011) were obtained from Food and Nutrition Research Center, Gadjah Mada University, Indonesia. Velvet beans were brought from rnational Institute for Tropical Agriculture, IITA, Ibadan, Nigeria.

Protoplast Fusion

The methods and procedures employed in this paper were based on the methods of Sikandar and Christos (2010) and Luciana *et al*. (2009) with a slight modification where the strains are different, magnetic stirrer was used instead of shaking water bath. *R. oligosporus*, FNCC 6010 and *R. oryzae*, FNCC 6011 were sampled and sub-cultured at 30 ± 2°C for 4-5 days. One gram of fresh 3 day old pre-treated mycelia were mixed in digestion mixture in a total volume of 10 mL using an osmotic stabilizer and lysing enzyme. The mixture was put into a 50 mL Erlen-Meyer flask and the samples were carried out in triplicates. All samples were incubated in a magnetic stirrer plate at 75 rpm at 30 ± 2°C for 24 h. Samples were taken at 6, 12 and 24 h to observe under the microscope for cell wall digestion and consequent protoplast formation. The reaction mixture containing protoplasts and cell wall debris were harvested after 24 h and filtered through sterilized cheese cloth to remove undigested materials under aseptic conditions. The filtrates were centrifuged at 3,000 rpm for 15 minutes at 15±1°C. The protoplasts were collected as pellet. The protoplasts were then washed in the same osmotic stabilizer to remove traces of the lysing enzyme. The purified protoplasts were re-suspended in the osmotic buffer and then were determined in number of protoplasts per mL of fresh weight of mycelia using haemocytometer. Five areas of hemocytometer glass were selected for each sample. The experiments were triplicates. Equation 1.1 was applied.

$$c = \frac{n \times 10^4 \, cells/mL}{\sum squares} \quad \text{.....1.1}$$

An amount of 0.5 mL of the purified protoplast suspension (10^4-10^5) of each of the Rhizopus strains, FNCC 6010 and FNCC 6011, was sampled, mixed

and centrifuged at 800 rpm for 10 min at 15±1°C. The pellet was re-suspended in a fusion medium in a total volume of 1 mL. The fusion medium was then prepared by adding 30% (w/v) poly-ethyl-glycol (PEG) 4,000 and 0.2 mM $CaCl_2$ in 1 M sucrose. The fusion mixture, containing the re-suspended pellets, was incubated for 30 min at 30 ± 2 °C. Afterwards, samples were taken from fusion mixture and spread on Petri-dishes containing PDA regeneration media.

Presentation and preparation of the enzyme

The commercial egg white lysozyme was presented as a 1 g freeze-dried powder; prepared buffered, only required to be dissolved in specific amounts of diluent (distilled, de-ionized water). Three different concentrations of the enzyme were prepared as follows: 0.01 g/10 mL, 0.02 g/10 mL, and 0.05 g/10 mL. Using available data supplied by the enzyme manufacturers (Vivantis Technology Malaysia), the enzyme activities in each concentration was calculated applying equation 1.2 below.

Specific activity at a given concentration =

$$\frac{a \times b \times c}{d} \quad \text{.....1.2}$$

a = Quantity of enzyme used;
b = Enzyme activity/mg;
c = Volume of enzyme solution used for 1 g mycelia;
d = Volume of diluent used

Morphological studies of the Rhizopus strains

Microscopic examination of the parent cells with the new hybrid was studied at the Industrial Microbiology Laboratory of Widya Mandala Catholic University of Surabaya, Indonesia. A high power microscope, (Olympus BX 41), was used along with the coupling Olympus Application Software DP 2-BSW, USA. Pictures and specific data generated were recorded.

Determination of the enzyme activities of the Rhizopus strains

The enzyme activity was determined according to the methods outlined by Salle (1973). Each biochemical test was compared to control, i.e. relevant media without cultures. Protease activity substrate was milk agar (100 mL) prepared using 10 mL skimmed milk mixed thoroughly. Lipase activity was using neutral red agar (100 mL) mixed thoroughly with 5 mL fat. Amylase activity used starch agar (100 mL). Inoculated media for each enzyme activity was incubated at 30±2°C and the observations were in triplicates. Observations were made after 24 h incubation period.

Tabel 1. Haemocytometer readings of protoplast and Hybrid numbers per mL

lyzozym (U/g of mycelia)	No. of Protoplasts (x 10^6 per mL)		No. of Hybrid Cells* (x 10^6/mL)
	R. oligosporus	*R. oryzae*	
2 K	0.979	0.180	36.8
4 K	1.130	0.658	359.0
10 K	1.450	0.390	25.0

n= 15 counting areas (three replicates, counted from five squares) for each sample
* The hybrids obtained from Rhizopus spp. fusant using 0.5 mL of each free protoplast suspension

Fermentation using hybrid and Rhizopus *spp parents and germination*

Fermentation of velvet bean as a legume model using the two Rhizopus parent lines and its hybrid were according to previous work (Balogun and Olatidoye, 2012). Beans were fermented using parent microbes, hybrid, and mixture of two Rhizopus sp. Sampling was taken every 12 h for 72 h. The fermented velvet bean flour was compared to flour obtained from germination for five days in the dark room and humidified cotton beds. Each sample was analyzed triplo.

Antioxidant measurement using DPPH method

Chemical analyses included antioxidant capacity referring to standard method which has successfully been modified especially the dilution tips during sample preparation for underutilize plants (Srianta *et al*., 2012) using DPPH method. The crude extracts were reacted with 60 mM DPPH solution and analyzed in a spectrophotometer at 517 nm. Positive control was vitamin E solution prepared from vitamin E stock solution with absorbance readings of 0-1. Accepted linearity was 0.8541-0.9947 and measurements were done at least four times.

Proximate analyses for velvet beans

Proximate analyses were carried out according to routine methods used at Food Analysis Laboratory, Department of Food Technology and Human Nutrition, Widya Mandala Surabaya Catholic University, Indonesia referring to AOAC method.

Results and Discussion

The use of egg white lysozyme as a lysing agent at activity levels of 2-10 KU per gram of mycelia freed protoplasts which then fused to generate a new hybrid. However, more protoplast numbers were released from *R. oligosporus* than *R. oryzae*

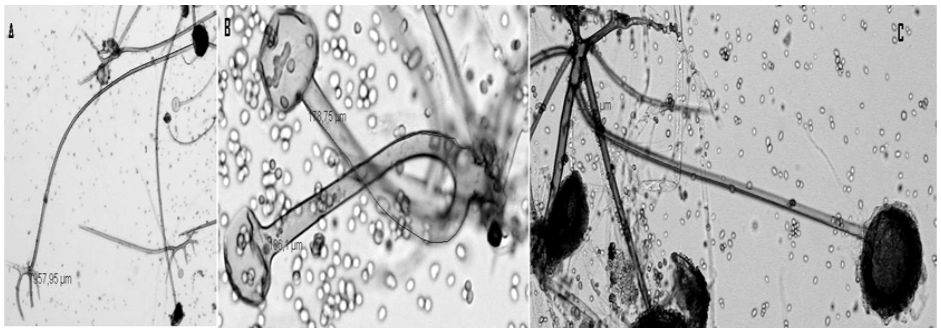


Figure 1. Length of hyphae (A) Hybrid, (±957.95 µm, at magnification of 80 x), (B) *R. oligosporus* (±182.43 µm), and (C) *R. oryzae*, (±529.20 µm at magnification of 40 x)

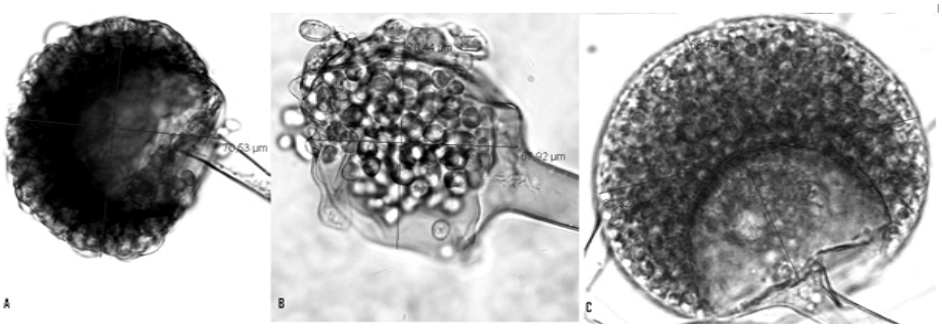


Figure 2. Diameter of Sporangium: (A) Hybrid, 70.53 µm horizontal, 81.00 µm vertical, (B) *R. oligosporus*, 67.92 µm, 70.44 µm, and (C) *R. oryzae*, 81.32 µm, 68.74 µm and average spore sizes: Hybrid,7.89 µm, *R. oligosporus*, 4.97 µm, and *R. oryzae*, 8.75 µm at magnification of 80 x (The digital measurements were five times each)

(Table 1). Apparently, the cell wall of *R. oryzae* was stronger than *R. oligosporus* resulting in lower free protoplasts.

Fusing the free protoplasts in the suspension obtained from each lysing yields was carried out at a ratio of *R. oligosporus*: *R. oryzae* of 1:1 (v/v); the hybrids obtained are presented in Table 1. The highest numbers of hybrid (3.590 x 10^8 fusants/mL) was from lysing activity using 4 KU white egg lyzozyme. Overall, the numbers increased around 100-1,000 cells possibly due to fusant multiplied during incubation (counted based the lowest numbers of protoplast from *R. oryzae* in each lyzing activity). Again, the maximum numbers of fusants was obtained from lyzing activity of white egg lyzozyme at 4 KU where the highest yield of *R. oryzae* protoplasts was obtained.

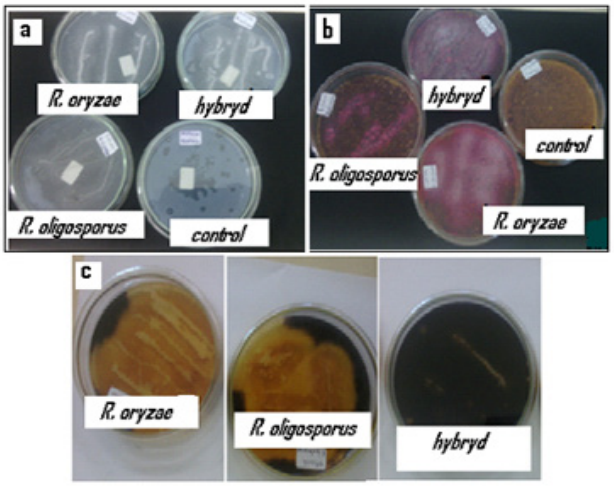
Morphological studies of the Rhizopus cells

Microscopic examination of the parent cells with the new hybrid are shown in Figure 1. It is clearly seen that the longer mycelia was obtained from the hybrid (A) relative to the Rhizopus strain parents (B and C). It was recorded up to four times longer (using digital measurement of the microscopic DP 2-BSW software). The morphological of the sporangia were marked clearly: *R. oryzae*'s sporangiophor is shorter with a flatten-ellipse sporangium while *R. oligosporus*' is long with a round sporangium (Figure 2). The hybrid has a longer sporangiophor than the *R. oligosporus* (Figure 2A). However, the general morphology is quite similar. Figure 2 also shows the differences of mycelia lengths, sporangia sizes, and spore sizes of the three strains. The hybrid has longer mycelia, sporangium shape resembled to *R. oligosporus* but bigger spores (average 7.89 µm). The sporangia of hybrid fell in the range closer to those of *R. oryzae* parent. The spore sizes were in between that of *R. oligosporus* (average 4.97 µm) and *R. oryzae* (average 8.75 µm) parents.

The biochemical tests for protease, amylase, and lipase activities were qualitatively observed to see the differences. *R. oryzae* has been recognized to produce amylase and lipase. It is expected to have hybrid with better activities of both enzymes so that it is useful for tempeh making from other beans excluded soya as well as starchy legumes. There are many leguminoceae members that are high in either carbohydrates or proteins.

Qualitatively, the order of high protease activity tested were *R. oryzae* >hybrid >*R. oligosporus*. The transparent areas of plate were relatively wider than that of control media. *R. oryzae* showed complete transparency media followed by quite comparable transparent areas for both *R. oligosporus* and hybrid. Hence, the hybrid's protease activity was closer to that of *R. oryzae*.

Observations on media plates during lipase



957.95 µm, at
(±182.43 µm),
cation of 40 x)

Figure 3 Culture plates of the Rhizopus strains showing result of (a) protease, (b) lipase, and (c) amylase activities

investigation qualitatively indicated that hybrid's media becomes very transparent in the growth region, this is not so in other regions where no cell growth was observed; also, agar appears to have been utilized in these regions downwards and upwards. *R. oryzae*'s agar was not observed to have been consumed downwards; no significant transparency was observed in the growth region. *R. oligosporus*' agar was observed to have been consumed downwards; significant transparency was observed in the growth region. Those all observations were contrasted to control media which did not change. Therefore, it was concluded that *R. oryzae* grew well in the media and used lipid in the media so that it turned media color to red. The lipase activity of hybrid was lower compared to that of *R. oryzae* parent, but the hybrid has higher activity compared to *R. oligosporus* parent indicating transparency in agar media.

Amylase activity was apparently lost in hybrid (Figure 3C), although it was strongly observed in parent *R. oryzae* followed by *R. oligosporus*. Batagglia *et al*. (2011) found that genome of *R. oryzae* 99-880 has 116 glycoside hydrolases; polysaccharide lyases at a common level for other microbes; carbohydrate esterases and carbohydrate binding modules (CBM); as well as 21 candidate of cellulolytic enzymes. None of the genome shows potential α-glucosidases as well as cellulase booster, so that *R. oryzae* does not use cellulose as a carbon source in its growth. However, it has four candidates glucoamylase or related enzymes, and one of them has been assigned as AmyA; RO3G_00082.3; a well known enzyme of *R. oryzae*'s applied in starch industries. Hence, this is the cause why in this present study we found transparent areas were only in the growth areas (Figure 3). Evolution of *R. oryzae* 99-880 results in the disappearance of CBM20 (generally exist in eukaryote and bacteria) but a lot of CBM21 (existing only among eukaryote) (Batagglia *et al*., 2011). In line with this, therefore, the hybrid lacking of amylase activity or weaken amylase activity is possible happening during fusion (recessives genes). It warrants genomic study in the future. Nevertheless, when the hybrid is applied for fermented flour production, it will be capable of preserving starch left intact during fermentation.

The uses of *R. oligosporus* and *R. oryzae* in food fermentation have shown the increase in phenolic compounds as metabolites (McCue *et al*., 2003; Schmidt *et al*., 2014). Thus, flour rich in starch and bioactives such as phenolic metabolites would contribute to better healthy food materials in the future. Such product development may be valuable for velvet bean because of its dark kernel as a potential antioxidant source. Furthermore, it is quite interesting that the hybrid lost the amylase activity to benefit flour industry.

Comparison of hybrid performance on velvet bean flour

The result of approximate analyses of velvet bean including seed coat as a raw material of the flour production was as follow: 10.33 ± 0.07% of moisture, 2.12 ± 0.11% fat, 22.67 ± 0.33% proteins, 60.23% carbohydrate, and 3.94 ± 0.02% ash. The velvet bean used in the present study were obtained from Nigeria, the proximate analyses of the kernel obtained by Balogun and Olatidoye (2012) are 6.02 ± 0.11% of moisture, 14.52 ± 0.05% fat with calculated fatty acid of 12.49%, 25.65 ± 0.14% proteins, 42.98% carbohydrate with crude fiber of 7.23 ± 0.05%, and 3.60 ± 0.01% ash. The differences are at acceptance levels of variations. Meanwhile, the moisture content is relatively similar to those of shell and seeds found by Sardjon *et al*. (2012) use velvet bean obtained from Yogyakarta, Indonesia. The shell contains moisture and ash 9.14% and 2.18%, respectively; whereas the kernel has 10.80% and 3.04% (Sardjon *et al*., 2012). Since the present study intended to compare dehulled and undehulled velvet bean thus it is worthy to mention that more mineral, moisture, protein, and fat are contributed from the kernel. Compared to Indonesian velvet beans conducted by Sardjon *et al*. (2012), the ash content in the present research is higher (3.94% compared to 3.04%) which is more likely due to different annually environmental conditions during its growth. Furthermore, the velvet bean also contains various bioactive compounds especially tannins, alkaloids, saponins, and steroids Sardjon *et al*. (2012) by which antioxidant capacity would give useful information to support scientific data for improving velvet bean images as a functional

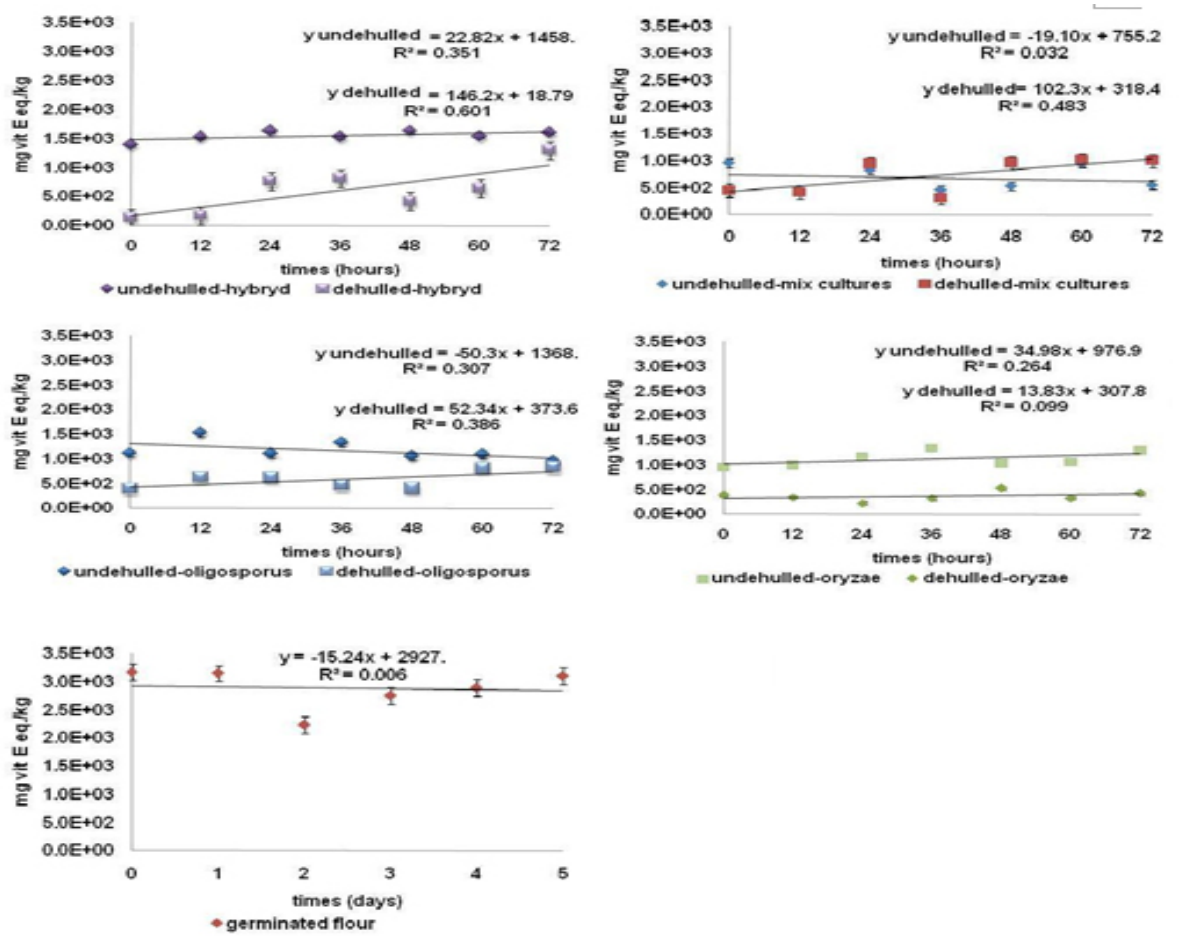


Figure 4. The antioxidant activities expressed as meq vitamin E/kg flour during fermentation using hybrid, and mixed culture of *R. oryzae* and *R. oligosporus*, Rhizopus sp. parents, and germination. Analyses were in more than triplicates

food product.

The activity of antioxidant either for dehulled or undehulled fermented velvet bean flours as well as germinated bean flour are apparently affected by the whole processing steps. The inhibition capacities of fermented flours obtained from the dehulled and undehulled beans using all Rhizopus sp. and its hybrid are shown in Figure 4.

The inhibition activities of the fermented velvet bean flours in the present study ranged from 219.5004-1,633.3436 meq vitamin E/kg for undehulled samples to 133.3368-1,331.5036 meq vitamin E/kg for dehulled samples during fermentation up to 72 h using the three microbes observed every 12 h. Prior to fermentation, the beans were soaked 24 h and then boiled for 5 minutes. Certainly, such processing steps could affect the level of scavenging compounds loss either from soaking or boiling, in which the compounds may degrade into inactive scavenging compounds. Those DPPH inhibition capacities were much lower than those due to germination activities (i.e. 2,234.1703-3,115.7153 meq vitamin E/kg germinated). The germinated bean flour only experienced soaking step without boiling.

R. oligosporus reduced antioxidant capacity of the undehulled flours (DPPH inhibition from 1,112.9516 to 954.4241 meq vitamin E/kg), but not *R. oryzae* (from 383.3869 to 423.2149 meq vitamin E/kg). This is also supported by the observation on the fermented flour obtained from mixed cultures that the undehulled flours obviously decreased (962.9284-556.1756 meq vitamin E/kg) suggesting dominant activities of the *R. oligosporus* in the mixed cultures. In contrast, all flour samples obtained from dehulled beans indicated increasing trends even when only *R. oligosporus* was used. The increased inhibition of flours fermented by *R. oryzae* support the finding by McCue *et al*. (2003) and Schmidt *et al*. (2014) that fermentation by Rhizopus could increase more bioactives. Why did *R. oligosporus* could lower inhibition capacity only for undehulled beans?

The untreated seed coat inhibited DPPH at 350.5861 meq vitamin E/kg whereas that of germinated seed coat was at 2,753.7667 meq vitamin E/kg. It suggests that the seed coat matrices may hardly be extracted during sample preparation. On the contrary, germination made extraction better so that the inhibition capacity soared, which were

relatively stable at interval levels of 2,234.1703-3,115.7153 meq vitamin E/kg along the germination periods studied. This happened mainly because the velvet bean should be soaked into water making them soft and the black color in the seed coat was easily dissolved prior to germination. On the second day of germination, it was observed that the capacity to inhibit DPPH from germinated flour dropped and then increased gradually up to day 5. It is more likely due to survival demand against environmental conditions. There was no boiling step for the bean at this germination process. Therefore, the unheated seed coat of velvet bean is the major contributor of the inhibition activity of flours producing from undehulled.

It is more likely that fungal capability to digest the matrices of untreated seed coat become obstacles for *R. oligosporus*. However, the strains are capable of digesting kernel matrices indicated by increasing inhibition capacity due to biochemical processes during fermentation of the dehulled beans. Thus, the enzyme pack of *R. oligosporus* may poorly digest the seed coat matrices resulting in the lowest scavenging compound releases. Yet, the released compounds tend decreasing instead of increasing. The most possible explanation for that is *R. oligosporus* strain consumed the released compounds. Thus, Rhizopus species not only affect bioaccessibility of bioactives but they could consume the bioactives for their growths. Finally, this opens up a window opportunity to use the *R. oligosporus* for controlling safe levels of particular compounds in food processing technology. *R. oligosporus* has been proven to reduce ca. 80-90% of L-DOPA from velvet bean during 72 h fermentation that we have presented in HerbFest 2015 in Abuja

The main finding in the present research is that the seed coat is a very promising source for antioxidant substances where germinated seed coat contributed higher bioactive released giving more DPPH inhibition than the dried untreated seed coat and fermented undehulled beans aforementioned. This implies that extraction of germinated seed coat is much more effective than untreated one. Another alternative processing adopted from this finding is fermentation ultimately using the hybrid or by *R. oryzae* when the flour required to have scavenging radicals. The other way processing for functional food ingredient is velvet beans flour with controlled scavenging activity thus fermentation using *R. oligosporus* is recommended. However, it still requires a proper processing and a method to incorporate them into food products without affecting the consumer acceptance.

Conclusion

The uses of egg lyzozyme in fusion of Rhizopus spp. have been done and it shows that lyzozyme lyzed cell wall of fungal mycelia better for *R. oligosporus* than *R. oryzae*. The hybrid is a new organism with slightly different characteristics from the parent strains. This hybrid will be useful for producing modified legume flour without changing the starch or carbohydrates in the legumes which usually good for diabetes mellitus diets. We have done an initial experiment to produce flour in the present research using the Rhizopus spp studied. It shows that the Rhizopus FNCC 6010 and 6011 and their hybrid affected the available levels of compounds capable of scavenging electron from DPPH. Generally, the inhibition activity of the methanolic extract of the fermented flour using Rhizopus is lower than those from germinated flour. These lowering effects probably due to different extractability of the compounds or, specifically for *R. oligosporus*, the bioactives are digestable. There is a window opportunity to adopt this lowering effects only if fermentation uses *R. oligosporus*. The second option would be benefiting food processing to obtain proper levels of alkaloids, or other substances capable of scavenging electron in the velvet beans. It is recommended to investigate legume flour production, toxicity, and digestion profile of the flour produced both for germination and fermentation for noncommunicable diseases.

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References

Balogun, I. O. and Olatidoye, O. P. 2012. Chemical Composition and Nutritional Evaluation of Velvet Bean Seeds (Mucuna utilis) For Domestic Consumption and Industrial Utilization in Nigeria. Pakistan Journal of Nutrition 11(2): 116-122

Battaglia, E., Benoit, I., van den Brink, J., Wiebenga, A., Coutinho, P. M., Henrissat, B. and de Vries, R. P. 2011. Carbohydrate-active enzymes from the zygomycete fungus Rhizopus oryzae: a highly specialized approach to carbohydrate degradation depicted at genome level. BMC Genomics, 12: 38 (17 January 2011) DOI: 10.1186/1471-2164-12-38 Retrieved on January 15, 2015 from Springer Website: *http://www.biomedcentral.com*/1471-2164/12/38.

Jogdand, S. N. 2001. Protoplast Technology, Gene Biotechnology. 3rd ed. Himalaya: Publishing House.

Kobayasi, S., Okazaki, N. and Koseki, T. 1992. Purification and characterization of an antibiotic substance produced from Rhizopus oligosporus IFO 8631. Bioscience Biotechnology and Biochemistry 56(2): 94-8.

Lampariello, L. R., Cortelazzo, A., Guerranti, R., Sticozzi, C. and Valacchi, G. 2012. The Magic Velvet Bean of Mucuna pruriens. Journal of Traditinal Complementary Medicine 2(4): 331-339.

Luciana, F., Haroldo, Y. and Helia, H. 2009. Production, purification and application of extracellular chitinase from cellulosinocrobium cellulans 191. Brazilian Journal of Microbiology 40(3): 623-630.

McCue, P., Horii, A. and Shetty, K. 2003. Solid-state bioconversion of phenolic antioxidants from defatted soybean powders by Rhizopus oligosporus: Role of carbohydrate-cleaving enzymes. Journal of Food Biochemistry 27: 501–514.

Odunfa, S. A. 1985. African fermented foods. In Wood, B. J. B. (Ed.) Microbiology of Fermented Foods. Vol. 2, p. 155–191. London and New York: Elsevier Applied Science Publishers.

Salle, A. J. 1973. Laboratory Manual on Fundamental Principles of Bacteriology. 7th ed. New York: McGraw-Hill Book Company.

Sardjon, R. E., Musthapa, I., Sholihin, H. and Ramdhani, R. P. 2012. Physicochemical composition of Indonesian velvet bean (*Mucuna pruriens* L.) Global Journal of Research on Medicinal Plants and Indigenous Medicine 1(4): 101-108

Schmidt, C. G., Gonçalves, L. M., Prietto, L., Hackbart, H. S. and Furlong, E. B. 2014. Antioxidant activity and enzyme inhibition of phenolic acids from fermented rice bran with fungus Rizhopus oryzae. Food Chemistry 146: 371–377.

Sikandar, H. and Christos, C. 2010. Isolation and Fusion of protoplasts from the Phytopathogenic Fungus Sclerotium rolfsii (sacc.) Brazilian Journal of Microbiology 41: 253-263.

Srianta, I., Patria, H. D., Arisasmita, J. H. and Epriliati, I. 2012. Ethnobotany, nutritional composition and DPPH radical scavenging of leafy vegetables of wild Paederia foetida and Erechtites hieracifolia International Food Research Journal 19(1): 245-250

Srinivas, R. and Panda, T. 1997. Localization of carboxymethyl cellulase in the intergeneric fusants of Trichodermma reesei QM 9414 and Saccharomyces cerevisee NCIM 3288. Bioprocess Biosystems Engineering 18: 71-73.

Taylor, L. 2003. Technical Data Report for Mucuna pruriens. Preprinted from Herbal Secrets of the Rainforest, 2nd ed. Austin: Sage Press, Inc. Retrieved on January 9, 2016 from Raintree Website: *http://www.rain-tree.com/reports/velvetbean-techreport.pdf*

Verma, N., Bansal, M. C. and Vivek, K. 2004. Protoplast Fusion Technology and Its Biotechnological Applications. Retrieved on May 6, 2015 from The Italian Association of Chemical Engineering Biotech Working Group Website: *http://www.aidic.it/IBIC2008/webpapers/96Ver ma.pdf*