Keynote Speech

Anti-oxidant Properties of the Water-Soluble Fraction Obtained from Monascus Fermented Jatropha Seed Cake

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Abstract
A non-edible plant, Jatropha curcas, is useful for biodiesel production due to rich oil content in the seed. However, deoiled seed residues, seed cake, have not yet been effectively utilized so far, even though it is rich in protein. In this study, we explored suitable strain of genus Monascus to advance anti-oxidant capacity of the Jatropha seed cake, and investigated the chromatogram profiles to find characteristic peaks. Sun-dried Jatropha seed cake was defatted, adjusted to 70% moisture, and used as a culture substrate in a solid-state fermentation for Monascus fungi. Water-extract of Jatropha seed cake fermented with M. purpureus NBRC31842 out of 30 strains of genus Monascus showed the highest anti-oxidant activities, about 3-fold higher than those of non-inoculated control, in both oxygen radical absorbance capacity (ORAC) assay and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. The profiles of products generated by M. purpureus NBRC31842 fermentation at 30�C for 14 days were investigated using reverse-phase C18 HPLC column chromatography. Almost all peaks detected in the water-extract of non-inoculate control were confirmed in the Monascus fermented Jatropha seed cake, and we found that some of them were enhanced by fermentation. In addition, these peak areas increased in the water-extract of Monascus fermented seed cake, which means that Monascus fungus degrade abundant protein in the Jatropha seed cake during fermentation, and increase the anti-oxidant peptides. These results suggest that the fermentation of Jatropha seed cake, by-product of biodiesel production, by Monascus fungi might contribute to advance of anti-oxidant capacity, and it may be useful as a livestock feed.

Keywords: Jatropha curcas, Seed cake, Monascus, Anti-oxidant, Solid-state fermentation

Introduction
Alternative energy sources to fossil fuel are demanded in themodern society. As one of the solutions, plant resources have gained attention for bio-fuel production using fermentation technology (Zaldivar et al., 2001, Kricka et al., 2015). However, use of edible crops to make bio-fuels have raised escalating price of food crops and reduced the food supply for human consumption (Tenenbaum, 2008).

Jatropha curcas is a non-edible plant distributed widely in tropics and subtropics of Asia, America and Africa regions. Its seed has been used as a raw material for biodiesel production in many countries due to its abundant oil (Phengnuam and Suntornsuk, 2014). Up to present, the oil extracted Jatropha seed residues (seed cake) are mainly used as natural fertilizer for crop plants or as a livestock feed. Meanwhile, a considerable amount of the seed cake has been disposed as a by-product containing some toxic compounds such as phorbol esters and others. Hence, development of sustainable utilization of Jatropha seed cake is needed.

On the other hand, the seed cake contains not only toxic compounds but also abundant amount of proteins. Some microbes are reported to degrade the seed cake proteins and also detoxify the anti-nutrients including phorbol ester, lectins, phytic acid, and trypsin inhibitors (Phengnuam and Suntornsuk, 2013). In addition, it was reported that the hydrolysates produced by enzymatic reactions indicate functional properties (Selanon et al., 2014, Valdez-Flores et al., 2016). Thus it has been considered that the microbial fermentation of Jatropha seed cake is an effective means to resolve those issues.

There is no information about anti-oxidant property of solid-state fermentation of Jatropha seed cake using Monascus fungi which produce variety of hydrolytic enzymes. Monascus fungi have traditionally used in making fermentation products and folk medicine (Yasuda et al., 2012). In addition, Monascus species are reported to abundantly produce variety of proteases in the solid-state fermentation (Lakshman et al., 2010). Therefore, in this study, we investigated the

anti-oxidant properties produced by the solid-state fermentation using Monascus fungus.

Materials and Methods

Sun-dried Jatropha seed cake was defatted with three volumes of n-hexane, filtrated, and the seed cake residue was dried up in an oven at 60□C. And then it was adjusted to 70% of moisture and used as a solid-state culture substrate. Monascus strains, totally 31 strains, were maintained on potato dextrose agar (PDA) at 30°C, and then stored at 4°C until use. Autoclaved Jatropha seed cake was inoculated with each Monascus strain (1 □ 105 spores/mL) and incubated at 30°C for 14 days. After cultivation, water-soluble fraction was extracted with 3-volumes deionized water from each fermented product at 200 rpm for 1 h. The extraction procedure was repeated three times, and the filtrates were combined. Each filtrate was evaporated with rotary-evaporator, and then the concentrate was lyophilized.

Anti-oxidant activity was measured by different two methods, oxygen radical absorbance capacity assay (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPPH) radical scavenging activity assay (Cao et al., 1993, Hatano et al., 1988). The ORAC assay was performed on the water-extracts in triplicate for each strain. The diluted series of the water-extracts were analyzed in triplicate in a 96-well plate in the Synergy HT (BioTek, USA). To each well 150 µL of 90 nM fluorescein, 25 µL of diluted sample or trolox and 25 µL of 153 mM 2,2'-azobis-2-aminopropane dihydrochloride (AAPH) solutions were added. The ORAC assay quantifies the inhibition (expressed in percentage and time) of fluorescence (Ex. 485 nm, Em. 528 nm) produced by peroxyl radicals generated at a constant rate by thermal decomposition of AAPH at 37°C for 60 min. The DPPH radical scavenging activity was measured on the water-extracts in triplicate for each strain. The reaction mixture was consisted with 50 µL of 0.2 M 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0), 50 µL of 50% Ethanol, 50 µL of diluted sample extract, and 50 µL of 0.4 mM DPPH solution, totally 200 µL in each well in a 96-well plate. The plate was agitated and incubated for 30 min in the dark at room temperature. The absorbance was measured by using a Benchmark Plus micro plate reader (Bio-Rad, USA) at 520 nm. A 50% ethanol was used as the blank and control. The antioxidant capacities for ORAC and DPPH assays were expressed in µM Trolox Equivalent (TE) calculated from the Trolox standard curve.

Fermented profile on reverse-phase (RP) C18 HPLC column chromatography was performed using the water-extract of M. purpureus NBRC31842 after acidified ethanolic precipitation followed by anion exchange chromatography fractionation. The water-extract of non-inoculated seed cake was used as the non-inoculated control and performed as with that of Monascus fermented seed cake. The water-extract of M. purpureus NBRC31842 was acidified with addition of TFA and precipitated with 3 volumes of ethanol addition. The acidified ethanolic mixture was centrifuged at 12,000 rpm for 5 min at room temperature. The obtained supernatant was applied to an anion exchange column (HiTrap Q column, 5 mL bed volume, GE health care, USA) equilibrated with 20 m triethylammonium acetate (TEAA) buffer, pH 6.0, and then adsorbed compounds were eluted with 500 mM TEAA buffer, pH 6.0, and then further eluted with 1 M NaCl solution. The higher ORAC active fraction which was higher than that of non-inoculate control, was evaporated with rotary evaporator in vacuo and freeze-dried until weight constant. The powder of active fraction was dissolved in 0.1% (v/v) TFA solution (100 □L), and further separated by RP-C18 HPLC column (COSMOSIL AR-II C18, 4.6 mm x 250 mm, Nacalaitesque, Kyoto, Japan) chromatography with a linear gradient system from solvent A (0.1% TFA in MilliQ) to solvent B (0.1% TFA in acetonitrile) at a flow rate of 0.5 mL/min for 60 min, monitoring at 220 nm in a Shimadzu HPLC system (Shimadzu, Kyoto, Japan).

Data obtained for each set of experiments were analyzed statistically and expressed as means □ S.D. The significance of differences was expressed as $P<0.05$.

Results and Discussion

The DPPH radical is relatively stable and has been used to evaluate the anti-oxidant ability or as hydrogen donors (Hatano et al., 1988, Xie and Schaich, 2014). On the other hand, the ORAC assay system closely relates to biological system due to use peroxi radical and has been widely used as the worldwide unified index (Cao et al., 1993, Shahidi and Zhong, 2015). Jatropha seed cake is widely used not only use as a natural fertilizer but also as an animal feed because it contains good quality proteins (Makkar et al., 1997).

The anti-oxidant activity of the water-extract obtained from Jatropha seed cake fermented products using 30 strains of genus Monascus

was investigated and compared by using two methods (Fig. 1). The DPPH radical scavenging activities among most of the tested water-extracts were higher than those of the ORAC anti-oxidant activities. On the other hand, the water-extract obtained from M. purpureus NBRC31842 and M. ruber JCM22614 showed higher anti-oxidant activities for ORAC assay than those of the tested Monascus strains using DPPH assay. In addition, the ORAC anti-oxidant activity in the water-extract of M. purpureus NBRC31842 was 3-fold higher than that of control which was a non-inoculated with fungus. Although the ORAC anti-oxidant activity of the water-extract of M. ruber JCM22614 was at same level, the DPPH anti-oxidant activity of was lower than that of M. purpureus NRC31842 and was almost same with that of control. Therefore, we selected M. purpureus NBRC31842 as the suitable strain for increase of the anti-oxidant activity in the water-extracts obtaining from fermented products.

In the fermented profile of the water-extracts by RP-HPLC chromatography, the eluent was separated into six fractions, and the ORAC anti-oxidant activities of them were measured. Comparison of the ORAC anti-oxidant activities between Monascus fermented seed cake and non-inoculated seed cake showed that higher activity than non-inoculate control was found in the fraction 3, 4, and 5. In these fractions, peaks were increased in the water-extract of Monascus fermented seed cake. Of these, we found that some peaks in the fractions were remarkably increased compared to those of non-inoculate control.The result of profile suggests that these characteristic peaks generated by Monascus fermentation contribute improvement of the anti-oxidant capacity of Jatropha seed cake.

The protein hydrolysates derived from Jatropha seed cake, generated by microbial fermentation and enzymatic hydrolysis, possess functional properties such as anti-oxidative activity, growth stimulation for plant, anti-bacterial activity, and anti-fungal activity (Rahman et al., 2011, Selanon et al., 2014, Phengnuam and Suntornsuk, 2013, Valdez-Flores et al., 2016). However, in this study, Monascus fungi could grow on the sterilized Jatropha seed cake and produced the anti-oxidative activity. Thus, Monascus fermentation seems to be one of the beneficial technologies for the sustainable utilization of Jatropha seed cake.

Monascus fungi produce abundant and diverse hydrolytic enzymes in a solid-state fermentation (Lakshman et al., 2010). Although some fungi and bacteria microbes are reported to degrade proteins of Jatropha seed cake, there is no report about degradation of proteins and resulting changes in the anti-oxidant activity by Monascus fermentation. The results in this study suggest that fermentation of Jatropha seed cake by Monascus fungi might contribute to advance of anti-oxidant capacity, even though isolation and identification of the characteristic peaks found in the fermented profile in this study have remain unclear.

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