International Food Research Journal 22(1): 377-382 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Monascus-fermented sorghum: pigments and monacolin K produced by *Monascus purpureus* on whole grain, dehulled grain and bran substrates

[1,2*]Srianta, I. and [3]Harijono

[1]*Department of Food Technology, Faculty of Agricultural Technology, Widya Mandala Catholic University Surabaya, Jalan Dinoyo 42-44 Surabaya, East Java, Indonesia 60625*
[2]*Doctoral Program of Agricultural Product Technology, Faculty of Agriculture, Brawijaya University, Malang, East Java, Indonesia 65145*
[3]*Department of Agricultural Product Technology, Faculty of Agricultural Technology, Brawijaya University, Jalan Veteran Malang, East Java, Indonesia 65145*

Article history

Received: 27 July 2014
Received in revised form: 19 September 2014
Accepted: 27 September 2014

Keywords

Monascus
Fermentation
Sorghum
Pigment
Monacolin K

Abstract

Monascus-fermented products are widely consumed in Asian countries as food colorant, preservative, supplements and in traditional medicine. Whole sorghum grain, dehulled sorghum grain and sorghum bran are potential substrates for solid state fermentation with *Monascus purpureus*. The objective of this research was to study pigments and monacolin K production by *Monascus purpureus* using whole sorghum grain, dehulled sorghum grain and sorghum bran as substrates. *Monascus purpureus,* isolated from commercial *Monascus*-fermented rice was used in the fermentation of the whole sorghum grain, dehulled sorghum grain and sorghum bran treated with or without soaking. After sterilization, the substrate was inoculated with *Monascus purpureus* culture containing 5 x 10^5 spores/mL, then incubated at 30°C with 70% relative humidity for 14 days. The *Monascus*-fermented sorghum was dried and analyzed for the colour, biomass, pigments and monacolin K contents.The *Monascus purpureus* grew on all types of substrate with biomass content of 7.41 to 44.69 mg/g. During fermentation, the mold produced yellow, orange and red pigments; and monacolin K on all types of the substrate. Ethanol soluble pigments were in a range of 12-96.4 AU/g; 3.7-47.7 AU/g and 2.8-59.7 AU/g respectively for yellow, orange and red pigments, while water soluble pigments were in a range of 3.9-32.4 AU/g; 1.6-20.8 AU/g; and 1.3-21.5 AU/g. Sorghum bran treated by soaking were found to yield more water soluble pigments, whereas sorghum grain substrate was found to be the best substrate for ethanol soluble pigments and monacolin K production. The results of this study shows that *Monascus*-fermented sorghum is a promising source of pigments and monacolin K and that further studies on the process optimization, effect of drying method on the pigments stability, separation of the pigments and the bioactivities evaluation are being carried out.

Introduction

Monascus-fermented products, especially *Monascus*-fermented rice, have been consumed for centuries in Asian countries as food colorant, preservative, food supplement and in traditional medicine. During fermentation, *Monascus* sp. produces many secondary metabolites such as pigments and monacolins. As of June 2012, a total of 54 *Monascus* pigments compounds has been reported and more than 50 patents concerning the use of *Monascus* pigments for food have been issued in Japan, the United States, France and Germany. *Monascus* pigments have various bioactivities such as anticancer, antimicrobial, antiobesity, antiinflammation, regulation of cholesterol levels and antidiabetes (Feng *et al.*, 2012; Hajjaj *et al.*, 2012; Srianta *et al.*, 2014). Among monacolins produced by the mold, monacolin K has strongest inhibition activity on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase, a key enzyme of cholesterol synthesis in human body.

Traditionally, *Monascus*-fermented rice is produced by solid state fermentation with rice as the substrate. Other cereal grains i.e. corn, wheat, barley, finger millet and adlay (Tseng *et al.*, 2006; Carvalho *et al.*, 2007; Pattanagul *et al*., 2008; Venkateswaran and Vijayalakhsmi, 2010; Li *et al*., 2013; Kraboun *et al*., 2013; Kongbangkerd *et al*., 2014), and those cereal residues e.g. rice bran and wheat bran have been used as *Monascus* fermentation substrate (Adjari *et al*., 2011; Kongruang, 2011). Sorghum (*Sorghum*

*Corresponding author.
Email: *srianta_wm@yahoo.com*

bicolor (L.) Moench) is the fifth most important cereal crop in the world, after wheat, rice, maize, and barley. It is the most drought-tolerant cereal grain crops and require little input during growth. It is an underutilised resource in most developed countries, with sorghum being primarily used as animal feed. In developing countries the commercial processing of these locally grown grains into value-added products is an important driver for economic development (Taylor *et al*., 2006). Study on sorghum as *Monascus* fermentation substrate is very limited.

Each cereals substrate had different influence on *Monascus*-fermented product production due to the variation in its structure and composition. Except for its much smaller size and oval shape, the structure of sorghum grain is remarkably similar to corn grain. Sorghum contains comparable levels of starch and other major nutrients as other cereals, which are potential source for carbon and nutrients for *Monascus* growth and activities. The starch is predominantly in the endosperm as in other cereals, but among the other major cereals, sorghum is a unique grain as it has a starchy pericarp. Another uniqueness of sorghum grain is that it has a higher content of polyphenols than wheat, barley, millet, or rye. Polyphenols function of the grain is to protect the grain from insect and fungi attack. Phenolic acids, flavonoids, condensed tannins, and deoxyanthocyanidins being the predominant polyphenol compounds, which are located primarily in the bran layer of the grain (Taylor, 2001; Awika *et al*., 2005;Svensson *et al.*, 2010). The polyphenols could bind proteins and starch of the grain that cause reduced digestibility, so generally sorghum grains for consumption were dehulled by using dehulling device to remove the bran layer. This research objective was to study pigments and monacolin K produced by *Monascus purpureus* on whole sorghum grain, dehulled sorghum grain and sorghum bran substrates.

Materials and Methods

Materials

Microorganism used in this research was *Monascus purpureus* isolated from commercial *Monascus*-fermented rice (MFR). *Monascus* culture was maintained on Potato Dextrose Agar (PDA) slant and sub-cultured monthly. The *Monascus purpureus* starter was prepared by inoculating *Monascus purpureus* culture stock onto PDA slant, incubated at 30^{o}C for 7 days, then inoculated into sorghum grain and sorghum bran substrates for solid state fermentation. The starter culture contains 5 x 10^5 spores/mL. Sorghum grains variety Numbu was obtained from farmer in Atambua, Nusa Tenggara Timur, Indonesia. The sorghum grain and bran were vaccuum packed until used. All analytical and chromatography grade chemicals were purchased from local distributor.

Monascus *isolation and identification*

Monascus mold was isolated from commercial *Monascus*-fermented rice (MFR) purchased in Surabaya. Soaked- and sterilized- dehulled sorghum grain was mixed with MFR. The sorghum grain was inoculated into Potato Dextrose Agar medium, incubated at 30^{o}C for 3 days. Colony with *Monascus* characteristics was inoculated to another PDA medium repeteadly until a pure culture is obtained.

The *Monascus* isolate was identified by using molecular method as follows: the isolate was inoculated into 5 mL of Potato Dextrose Broth (PDB), incubated at 30^{o}C with shaking for 3 days. The *Monascus* DNA was extracted, isolated and purified. DNA template was amplified by using Polymerase chain reaction (PCR) with primer 18F (5'-ATC TGG TTG ATC CTG CCA GT-3') and 18R (5'-GAT CCT TCC GCA GGT TCA CC-3'). The PCR conditions of 30 cycles were denaturation at 94^{o}C for 15 seconds, annealing at 60^{o}C for 30 seconds and elongation at 68^{o}C for 90 seconds. The PCR product was visualized using 1% agarose electrophoresis and compared to a marker (Thermo Scientific Gene Ruler 1 kb DNA ladder) consist of 14 DNA fragments of 250-10,000 base pairs. The PCR product was sequenced and then the DNA sequence was analyzed using Basic Local Alignment Search Tool (BLAST) program to identify the isolate .

Solid state fermentation

Solid state fermentation was carried out by inoculation 3 mL *Monascus purpureus* culture containing 5 x 10^5 spores/mL into 25 g of sterilized substrate of whole sorghum grain with soaking (WSWS), whole sorghum grain not soaking (WSNS), dehulled sorghum grain with soaking (DSWS), dehulled sorghum grain not soaking (DSNS), sorghum bran with soaking (SBWS) and sorghum bran not soaking (SBNS). The substrates were analyzed for the moisture, starch, tannin and phenol contents. After inoculation, it was incubated at 30^{o}C with 70% of relative humidity for 14 days. Control of uninoculated whole grain sorghum (WSC), dehulled grain sorghum (DSC) and sorghum bran (SBC) were also done. The fermentation product was then dried at 45^{o}C for 24 hours. The products were analyzed for the colour (L^*,a^*,b^* were measured by using Konica Minolta CR-10), biomass, pigments and monacolin

Table 1. Moisture, starch, tannin and phenol contents of sorghum fermentation substrate

Substrate	Moisture (%)	Starch (% db)	Tannin (% db)	Phenol (% db)
DSWS	66.20±0.02	64.08±0.00	0.11±0.002	0.06±0.002
DSNS	62.59±0.29	62.21±0.45	0.26±0.002	0.20±0.000
WSWS	49.34±0.20	59.63±0.09	0.11±0.001	0.08±0.001
WSNS	43.23±0.13	56.80±0.31	0.12±0.000	0.08±0.001
SBWS	75.79±0.31	29.39±0.54	0.42±0.004	0.32±0.003
SBNS	11.11±0.04	34.98±0.05	0.64±0.001	0.53±0.001

Note: % db is % dry basis

K contents.

Biomass estimation

The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released by acid hydrolysis of chitin, present in the mycelia cell wall. One gram of dried sample was washed with 50 mL of 5 M H_2SO_4 under agitation for 15 min. The mixture was then centrifuged at 2500 g for 10 min and was rinsed twice with distilled water. For chitin hydrolysis to N-acetyl glucosamine, the washed sample was incubated with 10 mL of 10M HCl for 16 h. After dilution with 40 mL distilled water, the hydrolysis proceeded during autoclaving at 130°C for 2 h. The hydrolysate was neutralised to pH 7.0 with 10M NaOH and subsequently with 0.5M NaOH. The neutralised sample of 1 mL was mixed with 1 mL acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, 6 mL of ethanol was added and followed by the addition of 1 mL of Ehrlich reagent and incubated at 65°C for 10 min. The optical density was read at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma) was used as a standard (Kraboun *et al*., 2013).

Pigments analysis

Ethanol soluble pigments were analyzed according to Babitha *et al*. (2007) with slight modification. One gram of fermented matter was transferred in a 250-mL conical flask and mixed with 70% ethanol at a ratio of 5 mL of ethanol per gram of fermented matter on dry basis. The content was mixed by agitation for 1 hour, followed by centrifugation at 3000 rpm for 10 minutes, and then filtered through Whatman No 1 filter paper. The filtrate was measured using spectrophotometer at 400 nm for yellow pigment, 470 nm for orange pigment and 500 nm for red pigment. Pigment yield was expressed as Absorbance at corresponding wavelength per gram of dry substrate (AU/g). Water soluble pigments were analyzed with the same procedure to ethanol soluble pigments analysis except the solvent was changed by distilled water.

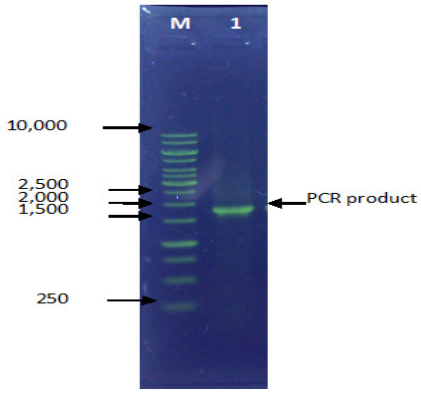


Figure 1. Visualization of PCR product of the *Monascus isolate.* (M=Marker, 1=PCR product) on 1% agarose electrophoresis

Monacolin K analysis

Monacolin K content was analyzed according to Wang *et al.* (2006) with slight modification. One gram of product was extracted with 5 mL of ethanol 70% with agitation for 1 hour, and then filtered through filter paper, followed by filtration through a 0.45 µm pore size PTFE filter and analyzed by High Performance Liquid Chromatography (HPLC, model LC-20A Prominence, Shimadzu, Japan). Chromatographic separation was conducted on a Shim-Pack ODS C18 column (250 mm x 4.6 mm i.d.). Acetonitrile-phosphoric acid (pH 2.5)= 65:35 v/v was used as the mobile phase. The eluent was pumped at a flow rate of 1.0 mL/min. Monacolin K was detected by SPD 20-A UV-Vis detector at 238 nm. Monacolin K from Sigma-Aldrich was used as standard.

Data analysis

All the obtained data of moisture, starch, tannin, phenol, colour, biomass, pigments and monacolin K were calculated for the mean and standard deviation.

Results and Discussion

Isolation and identification of Monascus purpureus

The *Monascus* isolate has been identified using molecular method. Figure 1 is the visualization of PCR product of the isolate DNA template using 1% agarose electrophoresis. Position of single band of the PCR product is between 1,500 and 2,000 base pairs of the marker. Result of the PCR product sequencing with 1,701 nucleotide bases sequence has been analyzed using BLAST program from NCBI, the isolate was identified as *Monascus purpureus*.

Solid state fermentation

Solid state fermentation were carried out on

Table 2. L*, a* and b* values of the *Monascus*-fermented products

Substrate	L* value	a* value	b* value
WSWS	43.93±0.40	19.90±0.44	5.97±0.15
WSNS	45.07±0.49	19.87±0.25	6.23±0.12
DSWS	40.57±0.35	16.17±0.40	4.17±0.23
DSNS	45.30±0.20	20.53±0.15	6.20±0.10
SBWS	44.93±0.45	11.43±0.12	4.60±0.32
SBNS	68.50±0.17	5.40±0.10	18.30±0.15

the various substrates with moisture, starch, tannin and phenol contents presented in Table 1. Dehulled sorghum grain contains higher starch than the whole grain. Dehulling process removes the bran layer, so the starch concentrated in the dehulled grain as the starch is mainly located in the endosperm than in the other parts of the sorghum grain structure. However, the sorghum bran contains starch in relatively high level because sorghum has a starchy pericarp, besides the outer part of the starchy endosperm which could be bound on the bran layer during dehulling process. The endosperm cell walls contain non-starch polysaccharides rich in water insoluble glucuronoarabinoxylans. Those compounds may cause lower water absorption during the whole grains soaking, furthermore produced lower moisture content than the dehulled sorghum. Starch and moisture are very important compounds for *Monascus* growing and metabolic activities. Sorghum bran consist of testa and pericarps layers which are rich in the polyphenols, thus the sorghum bran contains higher tannin and phenol than the whole and dehulled grains. Those compounds may affect the *Monascus purpureus* growth and activities. Figure 2 present photograph of sterilized fermentation substrates and the powdered *Monascus*-fermented products. It could be observed visually that the mold produced pigments. The Lightness (L*), redness (a*) and yellowness (b*) values of the *Monascus*-fermented products presented in Table 2. The positive a* and b* values of all the fermented-products reflected that the fermented-products colour is combination of red and yellow.

Biomass, pigments and monacolin K contents

Monascus purpureus growth and its activities produced pigments and monacolin K presented in Table 3. The growth is indicated from the biomass content. The mold grew and produced pigments and monacolin K on all types of the substrate. It grew well on dehulled grain, whole grain and bran with soaking treatment. Soaking process made the substrates more susceptible for the mold to utilize the starch and other nutrients because enough water amount is more available than the non soaking grains and bran. As

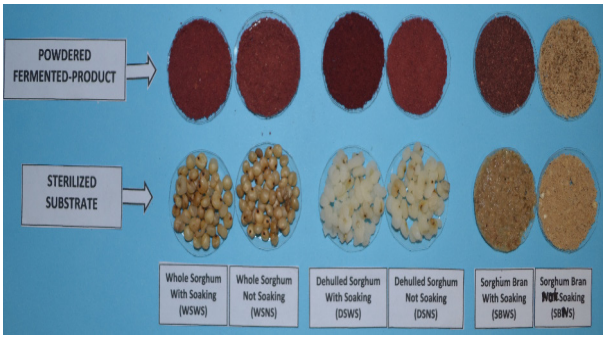
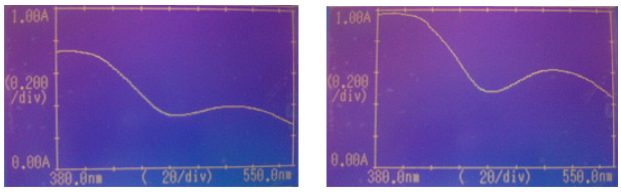


Figure 2. Photograph of sterilized substrate and powdered Monascus-fermented product



(a) (b)

Figure 3. Representatives of scanning photographs of ethanol soluble pigments on DSWS (a) and WSWS (b) substrates

reported by Feng *et al*. (2012), moisture content is a critical factor in *Monascus* solid state fermentation. It play an important role in the substrate nutrients utilization and all the living activities of the mold. The presence of water is the key for both intracellular as well extracellular biochemical reactions. The results indicated that substrate with higher moisture content produced higher content of biomass. SBNS substrate has the lowest moisture content and affected the growth and thus yielded the lowest biomass of 7.41 mg glucosamine/g. Higher starch content in the grains produced higher biomass than the bran. Starch is the main carbon source in those substrates which were hydrolized first prior to be transported into the mold cells. It seems moisture and starch contents and their interaction are critical factors in the mold growth and secondary metabolites production.

The data obtained show that *Monascus purpureus* produced yellow, orange and red pigments with the yellow pigment being the predominant. Two representatives scanning photographs were presented in Figure 3. The ethanol soluble pigments scanned with spectrophotometer in a spectrum range of 380 nm to 550 nm showed that 2 peaks were detected, the left peak is of yellow pigment at around 400 nm and the right peak is of the red pigment at aroud 500 nm. The left peak was higher than the right one.

In general, the results presented in Table 2 indicated that ethanol soluble pigments contents were higher than those of water soluble pigments similarly as reported by Carvalho *et al.* (2007) that the *Monascus* pigments are slightly polar so they are more soluble in ethanol than in water. However,

Table 3. Biomass, pigments and monacolin K contents of Monascus-fermented sorghum

Substrate	Biomass (mg/g)*	Ethanol soluble pigment (AU/g)			Water soluble pigment (AU/g)			MK** (mg/g)
		Yellow	Orange	Red	Yellow	Orange	Red	
DSWS	36.70±0.59	72.40±3.50	32.70±1.80	37.80±2.10	18.80±0.10	13.30±0.20	14.10±0.20	1.04±0.02
DSNS	26.64±0.89	61.00±2.02	29.10±0.90	35.60±2.20	16.30±0.30	12.40±0.10	13.30±0.10	0.66±0.01
WSWS	44.69±1.78	96.40±3.10	47.70±1.10	59.70±3.20	24.30±0.10	19.00±0.20	21.50±0.10	1.23±0.01
WSNS	20.92±0.17	45.70±1.00	24.60±1.20	31.6±1,50	18.70±0.29	13.90±0.20	15.60±0.20	0.46±0.01
SBWS	21.02±0.59	27.90±0.80	17.40±0.40	22.90±0.80	32.40±0.10	20.80±0.10	20.80±0.20	0.10±0.00
SBNS	7.41±0.30	12.00±0.20	3.70±0.10	2.80±0.10	3.90±0.10	1.60±0.10	1.30±0.10	0.00±0.00

*mg glucosamine/g substrate dry weight
**MK=Monacolin K

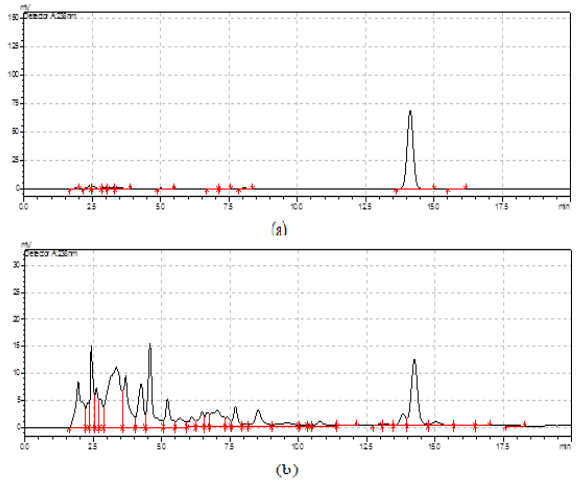


Figure 4. Chromatograms of monacolin K standard (a) and a representative of *Monascus*-fermented sorghum (b)

in contrast our results shows that sorghum bran substrate with soaking (SBWS) contain higher amount of water soluble pigments than that soluble in ethanol solvent. Interestingly, among the six types of subtrate, SBWS contain the highest water soluble pigments evenly on whole grain substrates which the bran layer was still presence. Hajjaj *et al*. (2012) reported that *Monascus* pigments could react with amino group containing compounds such as proteins, amino acids and nucleic acids to form water soluble pigments. The *Monascus* pigments solubility in water could be promoted by adding glutamate, leucine, glycine to the media of *Monascus* or by chemical modification through introducing –COOH or $–NH_3$ groups of amino acids into the *Monascus* pigments (Feng *et al*., 2012). Chemical compounds in the sorghum bran substrates may be more exposed, as a result of dehulling process, to interact with the pigments.

Monascus purpureus produced monacolin K on all the types of substrate, except on sorghum bran without soaking (SBNS). Figure 4 show the chromatograms of monacolin K standard and representative of *Monascus*-fermented sorghum samples. The monacolin K was detected at time retention of 14.2 minutes. The monacolin K contents were in a range of 0.10 and 1.23 mg/g, higher than that of reported by Venkateswaran and Vijayalakhsmi (2010). The *Monascus purpureus* produced higher monacolin K on the whole and dehulled grains substrates than on the bran. Those *Monascus*-fermented sorghum grains both whole and dehulled forms are potential as sources of monacolin K, an antihypercholesterol compound. Further studies on the process optimization, effect of drying method on the pigments stability, separation of the pigments and the bioactivities evaluation are being carried out.

Conclusions

The *Monascus purpureus* isolate could grow and produce yellow, orange and red pigments; and monacolin K on all types of the whole sorghum grain, dehulled sorghum grain and sorghum bran substrates with soaking. Starch and moisture contents of the substrates affect the mold growth and those secondary metabolites production. *Monascus*-fermented whole sorghum grain contains the highest biomass, ethanol soluble pigments and monacolin K, while *Monascus*-fermented bran with soaking contains the highest water soluble pigments contents. *Monascus*-fermented sorghums have good potency as functional compounds. Further studies on the process optimization, effect of drying method on the pigments stability, separation of the pigments and the bioactivities evaluation are being carried out.

Acknowledgement

Thanks to Directorate General of Higher Education, Ministry of National Education, Republic of Indonesia for financial supporting to this research through BPP-DN scholarship with contract number of 1014/UN10.14/KU/2013

References

Ajdari, Z., Ebrahimpour, A., Manan, M.A., Hamid, M., Mohamad, R. and Ariff, A.B. 2011. Assessment of Monacolin in the Fermented Products Using *Monascus purpureus* FTC5391. Journal of Biomedicine and Biotechnology, vol. 2011, Article ID 426168, 9 pages. doi:10.1155/2011/426168.

Awika, J.M., McDonough, C.M. and Rooney, L.W. 2005. Decorticating sorghum to concentrate healthy phytochemicals. Journal of Agricultural and Food Chemistry 53: 6230-6234.

Carvalho, J.C., Oishi, B.O., Woiciechowski, A.L., Pandey, A., Babitha, S. and Soccol, C.R. 2007. Effect of substrates on the production of *Monascus* biopigments by solid-state fermentation and pigment extraction using different solvents. Indian Journal of Biotechnology 6: 194-199.

Feng, Y., Shao, Y. and Chen, F. 2012. *Monascus* pigments. Applied Microbiology and Biotechnology 96:1421–1440.

Hajjaj, H., François, J.M., Goma, G. and Blanc, P.J. 2012. Effect of amino acids on red pigments and citrinin production in *Monascus* ruber. Journal of Food Science 77:M156–M159.

Kongbangkerd, T., Tochampa, W., Chatdamrong, W. and Kraboun, K. 2014. Enhancement of antioxidant activity of monascal waxy corn by a 2-step fermentation. International Journal of Food Science and Technology, doi:10.1111/ijfs.12479

Kongruang, S. 2011. Growth kinetics of biopigment production by Thai isolated *Monascus purpureus* in a stirred tank bioreactor Journal of Industrial Microbiology and Biotechnology 38:93–99

Kraboun, K., Tochampa, W., Chatdamrong, W. and Kongbangkerd, T. 2013. Effect of monosodium glutamate and peptone on antioxidant activity of monascal waxy corn. International Food Research Journal 20(2): 623-631.

Li, P.C., Tsai, W.H. and Chien, C.T. 2013. Dietary Monascus adlay supplements facilitate suppression of cigarette smoke-induced pulmonary endoplasmic reticulum stress, autophagy, apoptosis and emphysema-related PLGF in the rat. Food Chemistry 136(2): 765-774.

Pattanagul, P., Pinthong, R., Phianmongkhol, A. and Tharatha, S. 2008. Mevinolin, citrinin and pigments of adlay angkak fermented by Monascus sp. International Journal of Food Microbiology 126:20-23.

Srianta, I., Ristiarini, S., Nugerahani, I., Sen, S. K., Zhang, B. B., Xu, G. R. and Blanc, P. J. 2014. Recent research and development of *Monascus* fermentation products. International Food Research Journal 21(1): 1-12.

Svensson, L., Sekwati-Monang, B, Lutz, D.L, Schieber, A. and Ganzle, M.G. 2010. Phenolic acids and flavonoids in nonfermented and fermented red sorghum *(Sorghum bicolor* (L.) Moench). Journal of Agricultural and Food Chemistry 58: 9214–9220.

Taylor, J.R.N. and Dewar, J. 2001. Developments in sorghum food technologies. In: Taylor, S. (Ed.), Advances in Food and Nutrition Research, vol. 43. Academic Press, San Diego, CA, USA. pp. 217–264.

Taylor, J.R.N., Schober, T.J. and Bean, S.R. 2006. Review: Novel food and non-food uses for sorghum and millets. Journal of Cereal Science 44: 252-271.

Tseng, Y.H., Yang, J.H., Chang, H.L., Lee, Y.L. and Mau, J.L. 2006. Antioxidant properties of methanolic extracts from monascal adlay. Food Chemistry 97: 375–381.

Venkateswaran, V. and Vijayalakshmi, G. 2010. Finger millet *(Eleusine coracana)* – an economically viable source for antihypercholesterolemic metabolites production by Monascus purpureus. Journal of Food Science and Technology 47(4): 426-431.

Wang, J.J., Pan, T.M., Shieh, M.J. and Hsu, C.C. 2006. Effect of red mold rice supplements on serum and meat cholesterol levels of broilers chicken. Applied Microbiology and Biotechnology 71: 812–818.