GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
(**Published By**: Global Institute for Research & Education)
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PROVIDING A SINGLE STARTER FOR SUPPLYING FUNCTIONAL FOOD WITH LOW CITRININ CONTENT BY USING A SELECTED MONASCUS PURPUREUS STRAIN

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Abstract

This physiological study was aimed at providing *Monascus* starter for supplying functional food with low citrinin content. This study included microscopic observation on ascospore production and bioactive analysis by using high performance liquid chromatography (HPLC) and measurement of pigment spectrophotometrically on angkak product. Starter or angkak was made by using rice IR46 as its substrate and a selected strain of *Monascus purpureus* was used for its source of inoculum.

The highest ascospores production of angkak as starter was at 12 days of harvest (6,5 x 10^5 ascospores/g) and it reduced its production at 14 days of harvest at 3,2 x 10^5 ascospores/g. Actually, the presence of ascospores at various day of harvest was abundant in angkak. During microscopic examination there was only sexual spore (ascospores) observed under light or scanning electron microscopy. The scanning electron micrograph showed the good shape of *Monascus's* ascospores. HPLC analysis result showed that angkak was harvested at day 12 was the lowest of citrinin content at 115 ppb and the highest was at day 10 which reached at 14,181 ppb. The highest of lovastatin content of angkak was 0.32% at 10 days of harvest and reduced at 12 and 14 days of harvest. The highest absorbance of red pigment content of angkak showed at the 10 days of harvest (OD500=0.5560). The red pigment content reduced after 10 days incubation period**.** At 12 and 14 days of harvest of angkak, the OD was 0.2370 and 0.1525 respectively. The similar measurement result of yellow pigment content showed at the 10 days of incubation of angkak which the highest absorbance reached 0.555. The yellow pigment content then also reduced after 10 day incubation period**.** At 12 and 14 days of harvest of angkak, the OD was 0.1925 and 0.106 respectively.

This result indicated that the supply of starter could be achieved by high ascospore production of this selected *M. purpureus* strain used in this study. This supported potential use of the starter for providing functional food with low content of citrinin although lovastatin content was low, but retained by high content red pigment and particularly the yellow pigment.

Keywords: angkak, ascospore, citrinin, hplc, lovastatin, *Monascus purpureus*, red, pigment, yellow

1. Introduction

Monascus spp. belongs to the family of Monascaceae (Eurotiales) (Hawksworth *et al.*, 1995). The genus *Monascus* can be divided into three main species, *M. pilosus*, *M. purpureus* and *M. ruber* (Pitt, 1997). The majority of *Monascus* strains were originated from traditional oriental food (Sabater *et al.* 1999). *Monascus* spp. have been used for traditional fermentation in China for thousands years and data on special benefit of food and its application as drug substances have been in ancient records (Erdoğrul and Azirak, 2004).

The recent scientific results showed that *Monascus* spp. were well known in producing secondary metabolites including yellow, orange and red pigments (Su, Wang and Pan 2003; Lin, Li and Lai, 2005), antihypercholesterolemic agents, such as monacolin K and hypotensive agent, γ-amino butyric acid (GABA) (Lin, Li and Lai, 2005; Endo, Komagata, and Shimada, 1986) and antibacterial substances including pigment and citrinin (as monascidin A) (Blanc *et al.*, 1995a). Monacolin K (known as Lovastatin, Mevinolin and Mevacor) is a secondary metabolite produced by *Monascus* and *Aspergillus* (Endo A, 1979). Monacolin K is inhibitor to 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), an enzyme controlling in biosynthesis cholesterol (Endo, Komagata and Shimada, 1986). *Monascus*-fermented products such as angkak have been proven to significantly reduce total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) concentrations in serum (Lee *et al*., 2006)**.**

Blanc *et al*. (1995a) reported that almost all *M. purpureus* and *M. ruber* strains produced citrinin. Hence, the safety of angkak must be paid a critical concern because of the hepatotoxicity and nephrotoxicity of citrinin (Blanc *et al.* 1995a). Citrinin is [C13H14O5; IUPAC, (3R,4S-trans)-4,6-dihydro-8-hydroxy-3,4,5- trimethyl-6-oxo-3H-2-benzopyrane-7-carboxylic acid] is one of the mycotoxins produced by fungi that belong to *Penicillium, Aspergillus*, and *Monascus* species (Blanc *et al.* 1995b). Citrinin is also known as monascidin A (Wong and Koehler, 1981, Blanc *et al.*, 1995a) and it was firstly found in *Penicillium citrinum, Aspergillus* and *Monascus ruber* (Blanc *et al*., 1995b). Therefore, products from fermentation by *Monascus* such as angkak containing a high citrinin level are unacceptable even though it contains a higher concentration of monacolin K. Both monacolin K and citrinin are polyketide derivatives. So, they are unavoidably produced at the same time (Hajjaj *et al.*, 1999). In fact, this commercial product in market places in Indonesia is common containing citrinin at high content. Many countries including the USA, the States members of the European Union and Japan have resulted in new standards to strictly restrict the content of citrinin in *Monascus* products. Standard content of citrinin in Japan is to be lower than 200 ppb. One of approaches to provide such *Monascus* products was to have selected strain that produces no or low citrinin.

The objective of this study was to know the potential of a selected strain producing low citrinin as a starter for providing functional food production which was acceptable for consumption and market.

2. Materials and Methods

2.1. Monascus purpureus fungus.

The fungus used was *Monascus purpureus* Serasi, a selected strain. The fungus was rejuvenated before using in this study. The culture was prepared by cultivation on MEA 2% (Difco) medium at room temperature. To avoid fungal culture from bacterial contamination, 100 ug/ml of chloramphenicol was added into agar medium.

2.2. Making starter

A starter was made at laboratory by using several media and rice IR46. An amount of 25 g of rice soaked in water for overnight before sterilized by using autoclave for 15 minute, at 121°C and, 1 atm. Previously, two weeks old-*Monascus* was prepared by cultivation on MEA 2% as source of inoculum, then an amount of 5 ml of inoculum (10% of rice medium) was inoculated into rice which was earlier placed on Petri dish and then mixed well. Incubation was done at 30°C for 5, 7, 9, 12, and 14 days of period. After harvest the rice was oven dried at 50°C for 24 hours before keeping at 4°C. Starter was analyzed microbiologically for the presences of other microbes such as fungi or bacteria and also counted on density and determined type of spore produced.

2.3. Making fermentation product (angkak) by using rice medium and Monascus fungus

IR46 Rice variety was used as medium for making fermentation product (angkak) by using *M. purpureus* Serasi starter already produced at laboratory to know its efficacies for producing bioactive which analyzed by using high performed liquid chromatography for citrinin and lovastatin content.

2.4. Weighing of angkak

To know weight reduction after fermentation process, soon after harvested angkak was dried oven at 50°C for 24 hours before weighing by using digital weight balance.

2.5. Ascospore density of starter

Ascospore presence of starter was counted by using dilution method. Starter was grinded by using mortar and pestle so as to have powdered starter. Each one gram of starter then transferred into glass tube containing nine ml of sterile water then mixed carefully by using mixer for seconds. Subsequently the suspension diluted to 10 fold serial dilution. One ml of each level dilution was poured onto MEA 2% and then incubated at 30°C for several days. Every colony was assumed as one ascospore based on previous microscopic examination showed that *Monascus* starter only has one type of spores, ascospore. The ascospore presence in powdered angkak was photographed by using Scanning Microscope Electron (Hitachi TM3000).

2.6. Extraction of citrinin

Pigment content was analyzed spectrophotometry. Pigment extract was done by extraction of 0.05 gr of powdered angkak by 10 ml of methanol for 24 hours and homogenized by using electric shaker. The extract was filtered subsequently by using nylon filter paper and was then measured for yellow pigment spectrophotometrically at λ=390nm and red pigment at λ=500nm.

2.7. HPLC Analysis on Citrinin

HPLC analysis on citrinin content was performed by using HPLC according to Sakai *et al*. (2008). Standard citrinin was purchased from Sigma. Extract was prepared before by dilution of 1.25 gr of powdered angkak with 50 ml of 70% ethanol (pH 8.0) and was homogenized by using magnetic stirrer for 3 hours, at 15-25˚C, and filtered through 0,45 μm diameter of nylon filter. A 20μl of filtrate was then injected to HPLC. Colomn C_{18} and detector UV-Vis were used in this HPLC analysis. The mixture of acetonitrile/water/ trifluoroacetic acid (55 : 45 : 0.1) was used as the mobile phase at a flow rate of 1.0ml/min.

2.8. Extraction of Lovastatin

A 1 gr of powdered angkak was mixed with solution of 2 ml of acetonitrile and 0,1 ml 0,1% phosphoric acid and incubated for 30 minutes and subsequently centrifuged at 10.000 rpm for 10 minutes at 4°C. Its supernatant was concentrated by freeze drying and then diluted with mobile phase (acetonitrile + 0.1% phosphoric acid (65:35)).

2.9. HPLC Analysis on Lovastatin

A 20 μl of lovastatin extract was injected to HPLC by using C_{18} column and a λ235nm UV detector with elusion rate at 1 ml/minute at 45°C.

3. Result and Discussion

Table 1 showed that weight reduction more than 50% was at 7, 9, 12 and 14 days of harvest. At two weeks, the highest loss of weight was at 62%. This result is common during angkak fermentation process as the fungus used the rice as its nutrition for its growth.

The highest ascospore production of angkak as starter was at 12 days of Harvest (6,5 x 10^5 ascospores/g) and reduced its production at 14 days of harvest at 3,2 x 10^5 ascospores/g (Table 2). Actually, the presence of ascospores at various day of harvest was abundant in angkak. Particularly, at 12 days of harvest was the best day for harvesting angkak as starter.

During microscopic examination there was only ascospores observed under light or scanning electron microscopy. Picture 1 is showing a scanning electron micrograph of ascospores of powdered angkak by using Scanning Electron Microscope (SEM) TM3000 (Hitachi). This SEM photomicrograph showed the good shape of ascospores. In general, ascospores of filamentous fungi are more heat resistant than mycelia and conidia and more resistant than yeast ascospores (Dijksterhuis and Samson, 2006). Ascospores are widely formed sexual spores within the Ascomycetes with often a high survival capability. In an investigation of 20 yeast strains from soft drinks and fruit products, mainly *Saccharomyces cerevisiae, S. bailii* (now *Z. bailii*) and *S. chevalieri* strains, the D60-values of ascospores were 25-350 times higher than those of the corresponding vegetative cells (Put and de Jong, 1980). In a pH 4,5 buffer *S. cerevisiae, S. chevalieri* and *S. bailii* ascospores exhibited D60-values of 22.5, 13 and 10 min (Put and de Jong, 1982). So, it is important the ascospores presence in *Monascus* starter so as to provide its use for making angkak.

Analysis result showed that angkak was harvested at day 12 was the lowest of citrinin content at 115 ppb and the highest was at day 10 which reached at 14,181 ppb.

The highest of lovastatin content of angkak was 0.32% at 10 days of harvest and reduced at 12 and 14 days of harvest.

The highest absorbance of red pigment content of angkak showed at the 10 days of harvest (OD500=0.5560). The red pigment content reduced after 10 days incubation period**.** At 12 and 14 days of harvest of angkak, the OD was 0.2370 and 0.1525 respectively.

The similar measurement result of yellow pigment content showed at the 10 days of incubation of angkak which the highest absorbance reached 0.5550. The yellow pigment content then also reduced after 10 day incubation period**.** At 12 and 14 days of harvest of angkak, the OD was 0.1925 and 0.106 respectively.

From the results of HPLC analysis of the content of citrinin, it was known that the 12 days of harvest is the best period time to obtain angkak with the lowest citrinin content (115 ppb). This citrinin content was lower than 200 ppb the maximum threshold allowed health security in Japan standard of citrinin allowed for health. In contrast, at the same day of harvest, lovastatin content was low (0.02%). However, spectrophotometrically measurement result showed that the red and yellow pigments yield were still very high based on the absorbance measurement result at OD 500 and OD 390.

This physiological study showed that the *M. purpureus* strain used in this study was a good strain for supplying starter for functional product with low citrinin content under maximum threshold allowed for health security according to Japan standard.

Although the angkak product had low content of lovastatin, but the yellow pigment content was very high. This was important as the recent research report indicated that two bioactive of yellow pigment, monascin and ankaflavin showed better effect than lovastatin (Lee *et al*. 2013). As it is well known that Monacolin K has long been considered a major effective component in the hypolipidemic functions of *Monascus*, this bioactive also works as hypolipidemic medication. In fact, its side effect myopathy is need of a concern. Their study indicated that monascin and ankaflavin were similar to monacolin K in significantly reducing total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) levels in serum and lipid plaque ($p < 0.05$) in the heart aorta. Significantly more effects of ankaflavin, compared to monacolin K, showed on the prevention of fatty liver and lipid plaque accumulation in heart aorta,. More essentially, monascin enhanced high-density lipoprotein cholesterol (HDL-C) concentrations significantly, while monacolin K showed the opposite effect. The side effect of monacolin K caused raise up activity of creatinine phosphokinase (CPK), which was highly linked with rhabdomyolysis development, while monascin and ankaflavin did not induce such a side effect. Lee *et al*. (2013) concluded that monascin and ankaflavin had the prospective to be developed as hypolipidemic agents without rhabdomyolysis development.

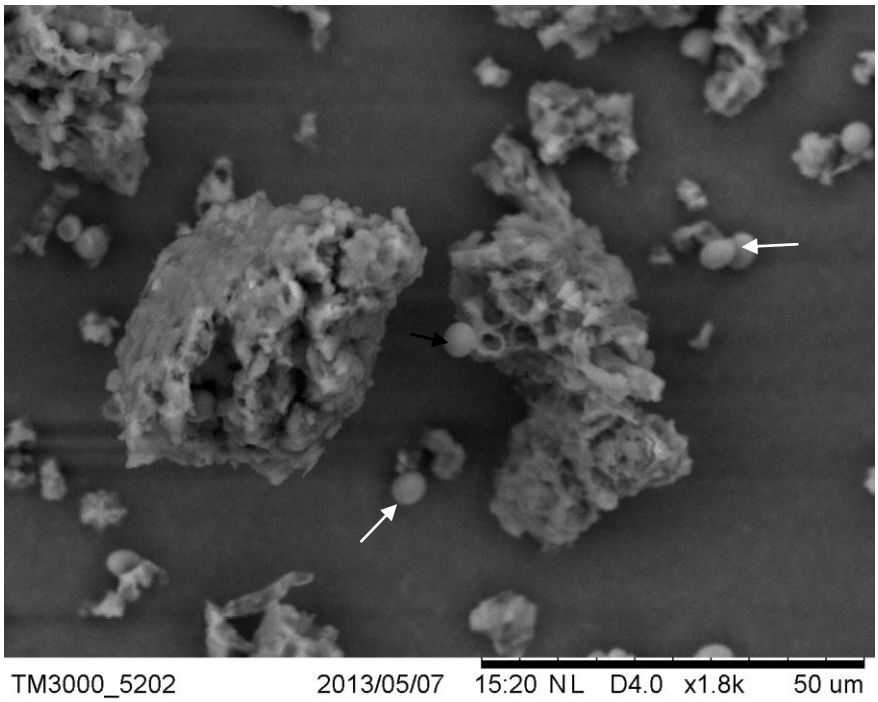
Other methods to have *Monascus* product with low or zero citrinin content such as heating method was reported improper even this was considered to be a dangerous as this treatment leading to the formation of citrinin H1 which is highly toxic. Citrinin H1 was formed upon heating citrinin at 140 °C in the presence of water. The toxic compound was isolated from heated citrinin, and the structure was determined. The toxicity evaluated by cytotoxicity assay was 10-fold higher on a weight basis than that of citrinin (Trivedi *et al.*, 1993). Whereas, several citrinin low-producing mutants of *Monascus* was created by UV/chemical mutagenesis, in fact the mutants generated revertants easily, and recovered the citrinin expressing capacity (Wang *et al.*, 2000). In contrast, this study result was an achievement of use a selected strain which produced low content of citrinin and high content of yellow pigment those were potential for its use for providing safe functional food.

Table1. Weight of angkak based on day of harvest.

Day of harvest	Weight of angkak (gram) after dried oven at 50°C for 24 hours (Initial weight was100 gram)	Weight reduction (%)
5	72	28
7	48	52
10	43.5	56.5
12	40.5	59.5
14	38	62

Table2. Ascospores density of angkak.

Day of angkak (as starter) Harvested	Ascospores density (Ascospores/g)
5	$3,6 \times 10^5$
7	$5,6 \times 10^5$
10	$6,1 \times 10^5$
12	$6,5 \times 10^5$
14	$3,2 \times 10^5$



Picture1. Photomicrograph showing ascospores of powdered angkak by using Scanning Electron Microscope TM3000 (Hitachi).

Table 3. Citrinin content of angkak.

Day of angkak harvested	Citrinin content (ppb)
5	2,730
7	7,299
10	13,394
12	115
14	14,181

Table 4. Lovastatin content of angkak.

Day of angkak harvested	Lovastatin content (%)
5	0.07
7	0.13
10	0.32
12	0.02
14	0.03

Table5. Absorbance unit of red pigment of angkak.

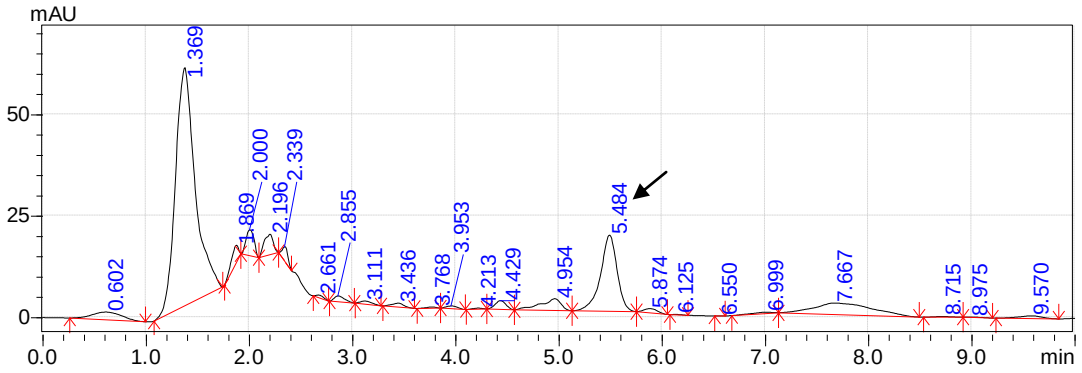
Day of angkak harvested	Red pigment (λ500nm) Optical density
5	0.1885
7	0.2270
10	0.5560
12	0.2370
14	0.1525

Note: OD : 10x Dilution Factor

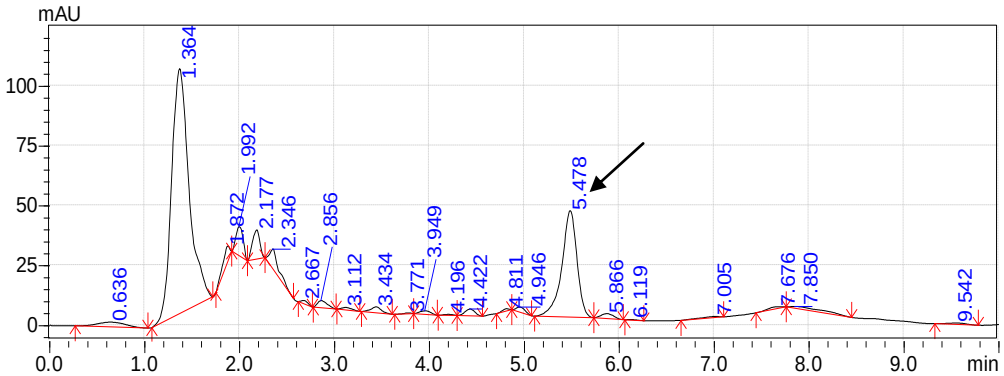
Table 6. Absorbance unit of yellow pigment from angkak.

Day of angkak harvested	Yellow pigment (λ390nm) Optical density
5	0.2220
7	0.2085
10	0.5550
12	0.1925
14	0.1060

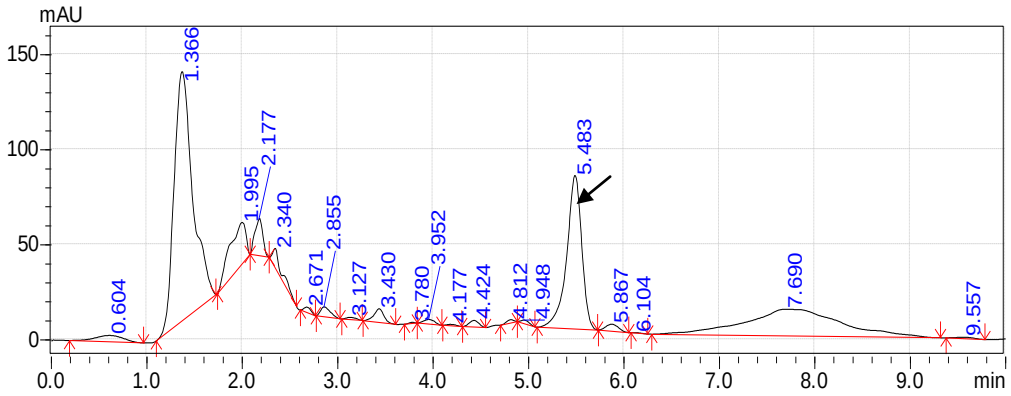
Note: OD: 10x Dilution Factor



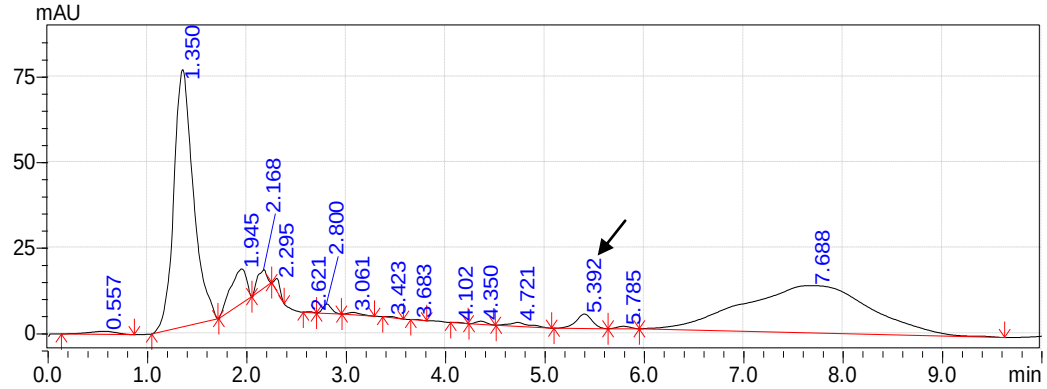
Picture 2. HPLC chromatogram of citrinin (RT value was indicated by arrow sign) analysis from angkak extract harvested after 5 days.



Picture 3. HPLC chromatogram of citrinin (RT value was indicated by arrow sign) analysis from angkak extract harvested after 7 days.



Picture 4. HPLC chromatogram of citrinin (RT value was indicated by arrow sign) analysis from angkak extract harvested after 10 days.



Picture 5. HPLC chromatogram of citrinin (RT value was indicated by arrow sign) from angkak extract harvested after 12 days.

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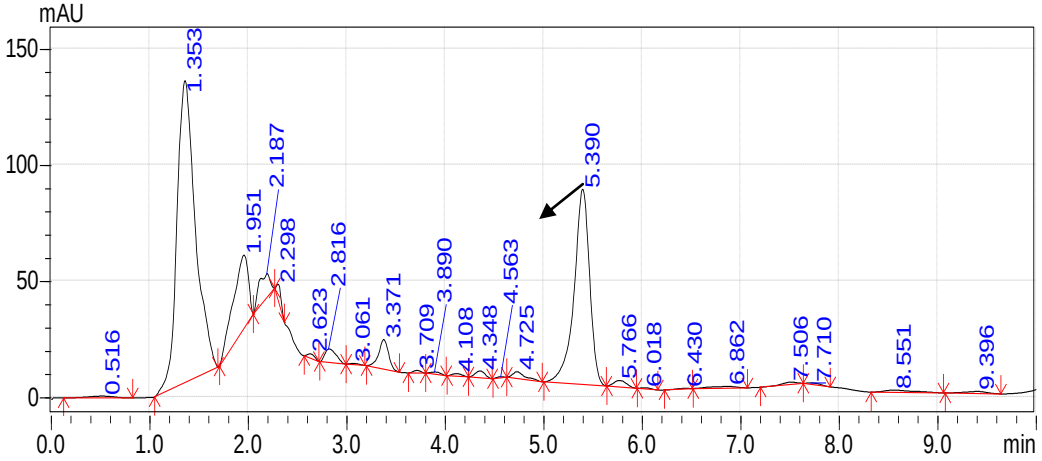

Picture 6. HPLC chromatogram of citrinin (RT value was indicated by arrow sign) analysis from angkak extract harvested after 14 days.

4. Conclusion

The supply of starter could be fulfilled by high ascospore production of this selected *M. purpureus* strain used in this study. This was supported by its potential use for providing functional food with its low production of citrinin. Although it produced lovastatin at low content, but it was retained mainly by its high production of the yellow pigment and red pigment as well.

5. Acknowledgment

This research was funded under the incentives of PKPP (Engineers and Researchers Capacity Development) from The Ministry of Research and Technology (RISTEK). The author wishes to thanks to Mrs. Ratih Mellinadewi SSi and Mr. Acun Samsuri Amd for their technical assistances and also to PT. Fajar Mas which kindly provided SEM Hitachi T3000 for photography of ascospores.

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