International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Production of Non Citrinin Chinese Red Yeast Rice by Using *Monascus purpureus* Skw2 Co-cultured with *Bacillus megaterium*

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Abstract

The aim of this physiological study was to know the effectiveness of co-culture *Monascus purpureus* skw2 strain with *Bacillus megaterium* in the providing of non citrinin Chinese Red Yeast Rice (CRR) as a nutraceutical substance. This study used *B. megaterium* that are non - pathogenic to humans and animals for coculture with *M. purpureus* on rice IR46 variety medium. Analysis of citrinin content on fermentation product was carried out using high performance liquid chromatography (HPLC). Measurement of yellow and red pigments content were carried out by spectrophotometry. Results showed that the coculture between *M. purpureus* skw2 strain and *B. megaterium* inoculated simultaneously showed growth inhibition to *Monascus* therefore so there was no CRR produced, but predominantly by *Bacillus*'s growth. When *Bacillus* was inoculated three days after *Monascus* cultivation, the fungal growth was very good. The results of HPLC analysis on CRR produced by a single fungal fermentation, showed that, its citrinin content was 13.03 mg/g. Remarkably, CRR produced from the co-culture showed zero citrinin content. Spectrophotometric results showed pigment content showed slight difference between the single fungal culture and co-culture that was 2 U; 22.1 U and 2 U; 20.3 U respectively for the yellow **(**λ 390 nm**)** and red pigments **(**λ 500 nm**)** with 100x dilution factor. Lovastatin content decreased in the co-culture at 13.1µg/g, while single fungal culture was at 38.5 µg / g. These results suggested that co-culture between *M. purpureus* skw2 strain and *B. megaterium* successfully providing non citrinin CRR. Lovastatin production by co-culture method still considered high, and produced high yellow pigment as well. The yellow pigment is known to contain anti hyperlipidemic agent and enhancer high density lipoprotein cholesterol, and red pigment can be used as an alternative to the use of synthetic colorant in foodstuffs, beverages and cosmetics. Further studies are needed specifically on the health security aspects of CRR consumption even though *B. megaterium* used is known as a non-pathogenic microorganism. Moreover, the more benefits of this product by the use effect of *B. megaterium* also need consideration.

Keywords: *Monascus purpureus, Bacillus megaterium,* Chinese red yeast rice, co-culture, non citrinin, nutraceutical.

1. Introduction

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). Hawksworth *et al*. (1995) included *Monascus* in family Monascaceae, Eurotiales. There are three main species known, *M. pilosus*, *M. purpureus* and *M. ruber* (Pitt, 1997). It is common that *Monascus* strains are originated from traditional oriental food (Sabater *et al.* 1999).

Many research recently are focused on *Monascus* fungi because of its potential in producing bioactive those are secondary metabolites such as pigments, 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 responsible for cholesterol biosynthesis (Endo, Komagata and Shimada, 1986). *Monascus*-fermented products such as China red yeast rice (CRR) have been reported reduce significantly total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-C) concentrations in blood serum (Lee *et al*., 2013).

CRR is still used as traditional medicine and food and drink colorant in Asia and Chinese communities in North America (Hebber, 1999). In Indonesia, peoples commonly use this fermentation product as an alternative therapy in belief for dengue hemorrhagic fever by increasing thrombocytes in blood.

Previous study showed that this product common contains citrinin in high content in the markets. Citrinin [$C_{13}H_{14}O_5$; IUPAC, (3*R*,4*S*-*trans*)-4,6-dihydro-8-hydroxy-3,4,5- trimethyl- 6- oxo- 3 H-2-benzopyrane-7-carboxylic acid] Citrinin is a mycotoxin which is nephrotoxic and hepatotoxic (Xu *et al.* 2006) which is produced by fungi that belong to *Penicillium*, *Aspergillus*, and *Monascus* species (Blanc *et al.*, 1994). Naturally, citrinin content in the fermentation product is strain dependent. The use of fermented product by *Monascus* especially for food and drinks or as an herbal medicine or nutraceutical substances then undergoes limitation merely due to of its citrinin content (Blanc *et al.*, 1995).

The great benefits of the *Monascus's* fermentation product, it leads to numerous research at many different of scientific disciplines continuously executed mainly in effort to minimize or eliminated citrinin physiologically, biochemically or even genetically in accordance with bioactive substance such as lovastatin (Erdogrul and Azirak, 2004; Pattanagul *et al.* 2007). Many approaches have been done to overcome problem with citrinin incidence, in Chinese red yeast rice product for instance, such as making mutant, physical treatment and chemically (Lee *et al.* 2007). Mutant produced often unstable and return to initial nature to citrinin producer (Shimadzu *et al.* 2005). While physical treatment such as high temperature treatment can produce transformation product, such as citrinin H1 that is ten fold more toxic than citrinin and citrinin H2, the major product has lower toxic. Although, chemical treatment has more potential as quick result but other bioactive reduced such as lovastatin not reported (Lee *et al.* 2007).

As a major traditional fermentation product of CRR is consumed in Indonesia as nutraceutical substance so the supply this product that free of citrinin or below the standard of maximum threshold allowed is urgent.

This physiological study was aimed at to know the effectiveness of the *M. purpureus* skw2 strain which was co-cultured with *B. megaterium* to produce Chinese Red Yeast Rice with low or no citrinin content. There is no research or study before which develop method by co-culture *M. purpureus* with *B. megaterium* to produce CRR low or free of citrinin..

The use of *B. megaterium* which was based on that this *Bacillus* species is a non-pathogenic microorganism to human or animal. Analysis of citrinin content in fermentation product was accomplished by using high performance liquid chromatography. Yellow and red pigments productions were measured by spectrophotometry.

2. Materials and Methods

Monascus purpureus skw2 used in this study was a selected strain especially for its low citrinin producer. This fungus was maintained in 2% malt extract (Difco) slant agar at room temperature (4°C). Isolate of *Bacillus megaterium* originated from tap water was maintained on Luria-Bertani (LB) medium at 4°C.

2.1. Cultivation

Medium cultivation for the fungus was extract malt agar 2% (MEA) purchased from Difco) and for bacterium was LB (Difco). Rice used was IR46 variety which already known good for *M. purpureus's* growth and its lovastatin production. Other standard chemicals were purchased such as acetonitrile (Merck), citrinin (Sigma), lovastatin (Sigma), ethanol (Merck) and methanol (Merck).

2.2. Rice Fermentation

Previously, 25 g of rice was soaked in water for 8 h. After removing the excess of water, the rice was sterilized by using autoclave for 15 minute, at 121°C

at 1 atm. After excessive water was removed, the rice was then sterilized at 121°C for 15 minutes. After cooling, the sterile rice was ready for inoculation. Rice fermentation with single fungal inoculum used 2 weeks old- *Monascus* which cultivated on 2% MEA. After that a 5 ml of inoculum (10% of rice medium) was inoculated into rice which placed on Petri plate and homogenized. Rice fermentation with co-culture method was accomplished with two different time of inoculation of *Bacillus*. The first inoculation time of *Bacillus* was inoculated simultaneously with *Monascus's* inoculum. The second inoculation time *Bacillus* was inoculated at day three after inoculation of *Monascus*. The old of inoculum of *Bacillus* used was three days old which cultivated on Luria-Bertani medium at 25°C. Incubation was carried out at 30°C for 14 days. The CRR produced was oven dried at 50°C for 24 hours.

2.3. Spectrophotometric Measurement of Pigments

Analysis on red or yellow pigment was carried out spectrophotometrically by extraction of 0.05 g powdered RYR in 10 ml of methanol for 24 hours by using electric shaker, then filtered using filter paper. The filtrate was then measured for yellow pigment (λ=390nm) and red pigment (λ=500nm).

2.4. HPLC analysis of citrinin

Before analyzing citrinin content by using HPLC, extraction of sample was prepared by dissolving 1.25 g Chinese Red Yeast Rice with 50 ml ethanol 70% (pH 8.0) and homogenized by using magnetic stirrer for three hours at 15-25˚C, filtered with 0,45 μm filter paper. The 20μl of extract was injected with column C_{18} and detector UV-Vis.

2.5. HPLC analysis of lovastatin

HPLC analysis of lovastatin was initially begun by extraction of 1 gr of CRR with solution of 2 ml acetonitrile and 0,1 ml 0,1% phosphoric acid with 30 minute incubation time then the solute was centrifuge at 10,000 rpm for 10 minutes at 4°C. The supernatant was concentrated by freeze dried and then diluted by mobile phase (acetonitrile + 0.1% phosphoric acid (65:35) . HPLC analysis was accomplished by injecting 20μl and using column C_{18} and detector UV λ235 nm with flow rate 1 ml/minute at 45°C.

3. Results and Discussion

Table 1 shows that co-culture with inoculation simultaneously *Bacillus* and *Monascus* did not show any growth of *M. purpureus*. The production of Chinese Red Yeast Rice was occurred when *Monascus* was co-cultured with *Bacillus* when inoculation of *Bacillus* was inoculated after three days inoculation of *Monascus*.

Citrinin analysis result by HPLC on CRR by co-culture fermentation with *Monascus* and *Bacillus* showed that no citrinin was detected, but on CRR product by single fermentation citrinin was 13 μg/g (Table 2). This result showed that co-culture *Monascus* and *Bacillus* effectively produced CRR without citrinin.

Spectrophotometric measurement showed that pigment content did not differ between single culture with *Monascus* and the co-culture that were 2 U; 22.1 U and 2 U, 20.3 U each for yellow and red pigments with 100 x dilution factor. This co- culture produced high yield of yellow pigment besides red pigment.

Lovastatin content on Chinese Red Yeast Rice from the co-culture was 1.305% lower than single fermentation that was 3.85 %. The lovastatin content was still high and it was much higher than its production report from Su*et al*. (2003). If this result was compared to 12 CRR commercial those available in the markets (Gordon *et al.*, 2010), lovastatin production was 7.8 mg, 50% much higher than the average (4.17 mg). This comparison showed that this *M. purpureus* skw2 strain was a superior strain for lovastatin production, at least at traditional fermentation production.

The yellow pigment consist two bioactive of yellow pigment, monascin and ankaflavin (Wong and Koehler, 1983; Lin & Demain, 1994*;* Hajjaj *et al*., 1997). These two bioactive were reported having better effect than lovastatin with regard to its properties as antihyperlipidemic and agent for enhancing high density lipoprotein cholesterol (Lee *et al* ., 2013). Also, the red pigment can be used as an alternative to the use of synthetic colorant in foodstuffs, beverages and cosmetics.

The phenomenon mechanism of undetectable citrinin production in its co-culture product was not analyzed yet. It is also interesting that pigments production was not change but lovastatin production decreased. Pigment and citrinin was reported originated from tetraketide in polyketide biosynthesis pathway. Pigments and citrinin are secondary metabolites derived from the polyketide pathway.

The both pigments and citrinin have a suggested tetraketide as its precursor by Hajjaj *et al*. (Ang., Ee, & An, 2004; Hajjaj *et al*. 1999; Hajjaj *et al*., 2000). This precursor was formed by condensation of one acetyl-CoA molecule and three malonyl-CoA molecules. Citrinin was formed by addition one acetyl-CoA molecule to tetraketide, followed by a sequences of reactions involving methylation, condensation, reduction, O-alkylation, cleavage between C-1 and C-2 bonding, oxidation, and dehydration. Whereas, pigments were formed by addition two malonyl-CoA molecules to tetraketide then followed by esterification. Therefore, the pigment production and the production of citrinin were synthesized alongside by the polyketide pathway (Ang., Ee, & An, 2004; Hajjaj *et al*. 1999; Hajjaj *et al*.,2000).

Although this study showed the effectiveness in producing CRR without citrinin by co-culture between *M. purpureus strain* skw2 and *B. megaterium* however more studies are needed especially at health concern consuming even though *Bacillus* species used in this study was not pathogen to human or animal. *B. megaterium* is a non pathogenic organism according to Public Health Agency of Canada (2010) (www.publichealth.gc.ca). This *Bacillus* species is a gram-positive bacterium, generally aerobic spore-forming bacterium found in widely various habitats from soil to seawater, sediment, rice paddies, honey, fish, and dried food. It can grow in simple media on more than 62 carbon sources out of 95 tested, including all tricarboxylic acid cycle intermediates, formate, and acetate (Vary *et al*., 2007). Its properties lead to an ideal industrial organism for more than 50 years. Its industrial applications are numerous that have been reviewed in detail by Vary (1992, 1994).

Due to many various benefits of use of *B. megaterium* it is possible that it may add more benefits of this CRR. Therefore, it is interesting in knowing other bioactive such as organic acid, vitamins, or other benefits secondary metabolites those might be produced by this bacterium species during co-culturing. *B. megaterium* is well known for its economic value because of its commercially important enzymes such as penicillin amidase and steroid hydrolases also it is considered the major aerobic producer of vitamin B (Vary, 1994). Therefore, this co-culture may lead to pay more attention to the bioactive value because of the use of *B. megaterium* that may enhance the benefits of CRR.

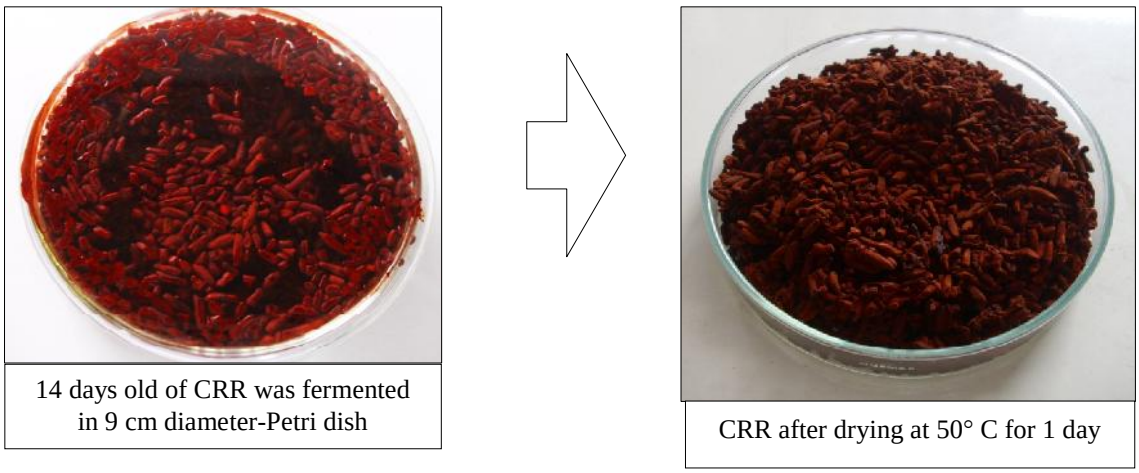

Figure 1. Photography of Chinese Red Yeast Rice (CRR), a co-culture fermentation product between *M. purpureus* skw2 with *B. megaterium*

Table 1. The growth of *Monascus purpureus* skw2 and *Bacillus megaterium* on IR46 Rice variety medium.

Fermentation method	**Growth of *Monascus purpureus* SKW2**		
	Without inoculation of *Bacillus*	**Simultaneously inoculation of *Bacillus* (at 0 day)**	**Inoculation of *Bacillus* three days after *Monascus* inoculation**
Single Fermentation by *M.purpureus* skw2	+++++	x	x
Coculture *M. purpureus* skw2 with *B. megaterium*	x	-	+++++

Note: +++++ = very good growth, - = no growth, x = not present

Table 2. HPLC analysis of Citrinin Content in Chinese Red Yeast Rice

Fermentation Method	**Concentration (ppm)**		**Average (ppm)**	**Concentration «g/g**
	Repetition 1	**Repetition 2**		
Single Fermentation by *M. purpureus* skw2	0.253	0.398	0.326	13.033
Co-culture *M. purpureus* skw2with *B. megaterium*	-0.159	0.000	-0.079	-3.175

Table 3. HPLC analysis Lovastatin Content in Chinese Red Yeast Rice.

Fermentation Method	**Lovastatin Concentration (ppm)**		**Average(ppm)**	**Concentration mg/g**	**Lovastatin Concentration (%)**
	Repetition 1	**Repetition 2**			
Single Fermentation by *M. purpureus* skw2	29696.871	6971.483	18334.177	38501.772	3.850
Co-culture Fermentation by *M. purpureus* skw2 with *B. megaterium*	6841.6118	5591.131	6216.371	13054.380	1.305

Table 4. Chinese Red Yeast Rice pigment production by spectrophotometric measurement.

Fermentation Method	**Yellow Pigment (λ390nm)** Absorbance Unit (U)		**Average λ390nm**	**Red Pigment (λ500nm)** Absorbance Unit (U)		**Average λ500nm**
	1	2		1	2	
Single Fermentation by *M. purpureus* skw2	23.4	20.7	22.1	12.8	9.9	11.4
Co-culture Fermentation by *M. purpureus* skw2with *B. megaterium*	19.6	21	20.3	9.7	13.7	11.7

Conclusion

These results showed that this co-culture between *M. purpureus* skw2 strain and *B. megaterium* successfully produced non citrinin CRR. Other bioactive such as lovastatin and pigment were produced high.

Acknowledgments

I would thank Dr. Novik Nurhidayat, a researcher staff from Research Center for Biology, Indonesian Institute of Sciences for *Bacillus megaterium* isolate supply, Mrs. Ratih Mellina Dewi and Mr. Acun Samsuri for their helpful support.

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