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PROFILING OF METABOLITE PRODUCED *Monascus purpureus* FTC5391 IN TWO DIFFERENT SUBSTRATES USING HPLC- PDA AND GC-MS

Zahra Ajdari[1, 2], Musaalbakri Abdul Manan[3], Saadi Bayat[4], Mojdeh Dinarvand[5], Sahar Abbasiliasi[2], Rosfarizan Mohamad[2], Arbakariya Ariff[2*]

[1]Department of Marine Biotechnology, Iranian Fisheries Research Organization, [2]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Science
[3]Biotechnology Research Center, Malaysian Agricultural Research and Development Institute,
[4]Department of Chemistry, Faculty of Science,
[5]Department of Microbiology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia,
Email: z_azhdari@yahoo.com; musaalbakri.abdulmanan@postgrad.manchester.ac.uk; saadibayat@yahoo.com; mojdehdinarvand@yahoo.com; sahar_li_ab@yahoo.com; farizan@biotech.upm.edu.my;* arbarif@biotech.upm.edu.my

ABSTRACT

In modern pharmacology, about half of the useful drugs are derived from natural sources where drug discovery from nature is an attractive subject for researchers. *Monascus* spp. has the ability in producing a collection of useful secondary metabolite which can be applied in food, cosmetic and pharmaceutics. In this study, the potential of *Monascus purpureus* FTC5391 in producing metabolites in solid state and submerged cultivation was investigated. The ethanolic extraction of these fermented products was evaluated using HPLC-PDA and GCMS. The results showed that the profiling of metabolites produced by *Monascus purpureus* FTC5391 in two different types of cultivation employed different media was significantly varied. The number of metabolites detected in Hiroi-pd submerged and soybean solid state cultivation was 100 and 55 compounds respectively with 10% similarity. These results demonstrated that the types of substrate were greatly affected on the number and sort of metabolites produced by *Monascus purpureus* FTC5391. Biological diversity and its constituent chemical diversity have served as one of the strongest sources of bioprospecting which leads to the discovery of some of the most important bioactive molecules for mankind. Therefore, the use of different medium composition for the cultivation of microorganisms would give an extreme opportunity to achieve a broad range of metabolites with different bioactivities.

Keywords: *Monascus purpureus FTC5391; metabolite production; HPLC-PDA; GC-MS*

INTRODUCTION

Nowadays, because consumers have become more health-conscious demand for natural products is increasing. Natural products may be obtained from terrestrial or marine animal, plants or microorganisms. Among the microorganisms filamentous fungi compared with plants show a lower degree of cellular differentiation, but still they express a complex metabolism resulting in the production of a broad range of metabolites and extra and intra cellular enzymes [1]. This very high metabolic diversity has been actively exploited for many years and many metabolites produced by filamentous fungi are bioactive compounds as antibiotics, antitumour, cholesterol-lowering and immunosuppresors [2]. Filamentous fungi are important in terms of biotechnological application because of many advantages such as easy to growth in fermenters and well-suited for large-scale industrial production.

Among the natural pruducts *Monascus* fermented product (MFP) is widely used as a therapy for hyperlipidemia. MFP is unique Chinese traditional fermented cooked rice with *Monascus spp.* Although around 24 speices of *Monascus* has been identified so far, only four spices of *Monascus* including *pilosus, purpureus, ruber* and *floridanus* are extensively used in industrial. MFP consumption has been grown nearly 80% from 2005 to 2008 in the United States, with sales of $20 million in 2008 [3, 4]. Despite the fact that, *Monascus* spp. fermented has been reputed as hypocholesterolemic supplement but *Monascus* has the ability to secrete various secondary metabolites of polyketide structure. Polyketides are rich sources of pharmaceuticals, including antibiotics, anticancer drugs, cholesterol- lowering drugs, immunosuppressant and other therapeutic. So it is believed that with MFP consumption we can get a multifunction therapy beside hypercholesterolemia therapy [5-7].

The production of MFP could be improved by strain selection, substrate selection, and incubation condition modification to enhance the contents of not only the known compounds but also the novel unknown compounds [8]. *Monascus* sp. produced different secondary metabolites by different substrates [5, 9]. Most of the studies up to now have focused on quantity and quality of a particular bioactive compound produced by *Monascus* in different media. Lee et al. [10] reported that using dioscorea as the substrate can produce monacolin K at 2,584 mg/kg, which is 5.37 times to that resulted when rice is used as the substrate. In addition, more amount of yellow pigment can be found in *Monascus*- fermented dioscorea than in *Monascus*-fermented rice.

While the study of metabolites is of an immense importance as each substrate induces fungi to produce different ranges of metabolite from other substrates, therefore, changing substrates will cause different metabolites to be produced which these metabolites might be had synergic effect. As MFP has been reputed as hypocholesterolemic supplement due to its monacolins production, it can produce a wide range of metabolites with additive or synergistic effects of monacolins on serum lipid profile [7]. Therefore, in this study we have investigated the production of variety of metabolites using different media and the range of metabolites that each media is able to produce.

MATERIAL AND METHODS

Materials

Trifluoroacetic acid (TFA) analytical grade. Soybean was purchased from local markets. Yeast extract, malt extract, casamino acids, potato dextrose broth (PDB) and potato dextrose agar (PDA) were purchased from Difco (USA). Other chemicals, including methanol and acetonitrile (ACN) (HPLC grade) and ethanol (analytical grade) were obtained from Merck (Germany).

Microorganisms

The fungus, *M. purpureus* FTC5391, was isolated from local sources and maintained at the culture collection in Malaysian Agricultural Research and Development Institute. The culture was maintained on the PDA slants at 4^{o}C, and subcultured monthly.

Preparation of *Monascus* spp. fermented products

For inoculums preparation, mycelia blocks (4 × 4 mm) of *M. purpureus* were transferred into 100 mL of YMP (3 g/L yeast extract; 3 g/L malt extract; 5 g/L peptone and 20 g/L glucose) in a 250 ml flask. The flasks were incubated in rotary orbital shaker at 30^{o}C, agitated at 150 rpm for 4 days and these cultures were used as standard inoculums for fermentations. Submerged fermentation was carried out using Hiroi-PD medium [Sucrose 100 (g/L), Yeast extract 3 (g/L), Casamino acid 5 (g/L), $NaNO_3$ 2 (g/L), KH_2PO_4 1 (g/L), $MgSO_4$. 7 H_2O 0.5 (g/L), KCl 0.5 (g/L), $FeSO_4$ 0.01 (g/L), Potato starch 4 (g/L), Dextrose 20 (g/L)] in 1000 mL Erlenmeyer flasks containing 500 mL liquid medium. The medium was autoclaved for 15 min at 121^{o}C after adjusting the pH at 6.0. Glucose was autoclaved separately to avoid

the caramelization process, and then was added to the medium under sterile conditions. The flasks were inoculated with 10% (v/v) inoculums and incubated in a rotary orbital shaker at temperature 30^{o}C and agitation 150 rpm for 25 days.
Solid state fermentation was carried out using soybean. The substrate was ground and mixed with 1% (w/v) acetic acid. The 500 mL beaker containing 200 g of substrate was sterilized for 15 min at 121^{o}C. To start the fermentation, the beaker was inoculated with the standard inoculum size (10% v/w) and incubated at temperature 30^{o}C until the color of media had completely changed to red, normally about 10 days. During the fermentation, the humidity was maintained at 50 - 60%.

Extraction of metabolites

Slightly different methods for extraction of metabolites were applied to solid and liquid samples of the prepared fermented products. Solid fermented product was dried and finely ground into powder. The sample (0.5 g) was mixed with 5 mL of ethanol/water solution (75:25) for 2 h at 60^{o}C under agitation, followed by centrifugation for 10 min at 3000 x g. Liquid fermented product was homogenized to break the mycelia cells. The homogenized sample (5 mL) was extracted with 5 mL of 95% ethanol for 2 h at 60^{o}C under agitation and was subsequently centrifuged for 10 min at 3000 x g. In both cases, the supernatant (1 mL) was concentrated and dried under vacuum and then was resolved in 1 mL ACN. The mixture was filtered through a membrane filter (0.45 µm) prior to HPLC analysis, GCMS and spectrophotometer.

Analysis of extraction

The HPLC system (Waters 2695 Alliance, Waters Inc., USA) was equipped with an on-line degasser and an auto sampler was used for detection of MFP metabolites. The raw data were detected by 2996 PDA, acquired and processed by a Waters Millennium32 /Empower™ software, chromatographic workstation loaded on an IBM computer in laboratory of vaccines and immunotherapeutic program of immunotherapeutics (LIVES) Universiti Putra Malaysia. The column of Waters Symmetry C18 (150 mm × 3.9 mm i.d., 5 µm) was used as a stationary phase. A linear gradient of concentrate ACN (eluent A) and 0.1% TFA (eluent B) with a flow rate of 0.9 mL/min was performed for the detection of metabolites as the mobile phase. Eluent A was increased from 5 to 75% within 15 min, kept at 75% for 5 min, increased to 95%, and then reduced to 5% within 10 min. The PDA was used at absorbance ranging from 210 to 350 nm. The column temperature was set at 28^{o}C, and the injection volume was 20 µL.
On the other hand the ethanolic extraction of the two culture media were analysed using GCMS QP5050A SHIMADZU at the Chemistry Department, Faculty of Science, Universiti Putra Malaysia.

RESULTS AND DISCUSSION

The results clearly showed that the *Monascus purpureus* FTC5391 gained good growth on solid and submerged culture. After one week the colour changed from orange to deep red in both media. Pigment production is one of the individual properties of *Monascus* spp. which is suitable key to the classification of *Monascus* as biochemical characteristics [7]. In the current study, the red pigment was spectophotometrically determined by measuring the absorbance of culture filtrate at 480 nm. *Monascus spp.* can produce a different range of colour from yellow to purple. The bioactivity of these pigments such as anti-inflammatory, anti cancer and antibiotic activity against bacteria, yeast, and filamentous fungi have been reported [11, 12]. Among these pigments, the red pigments are regarded as the most important in the meat product industry as substitutes for nitrites and for synthetic colours like erythrosine (FD and C red no.3) [13].

Different metabolites produced by *M. purpureus* FTC5391 using different media were determined by LC/PDA and GC/MS analysis. These results demonstrated that about 100 metabolites extracted from submerged and 50 metabolites extracted from solid state (soybean) fermentation of *M. purpureus* FTC5391.

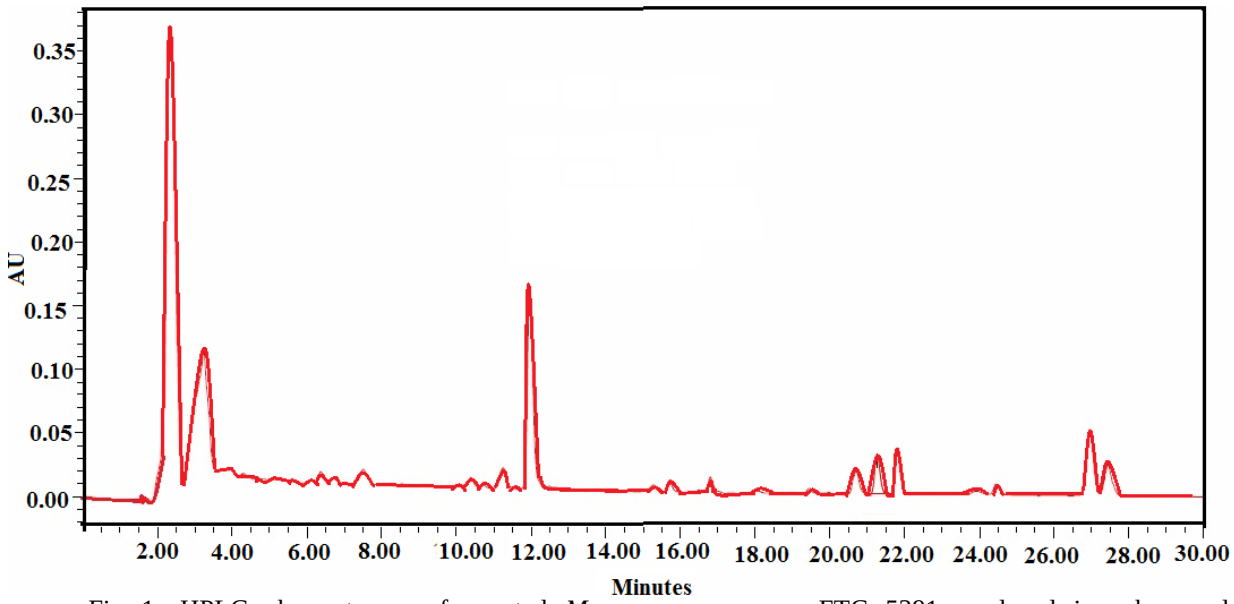

Fig 1: HPLC chromatogram fermented *Monascus purpureus* FTC 5391 produced in submerged fermentation.

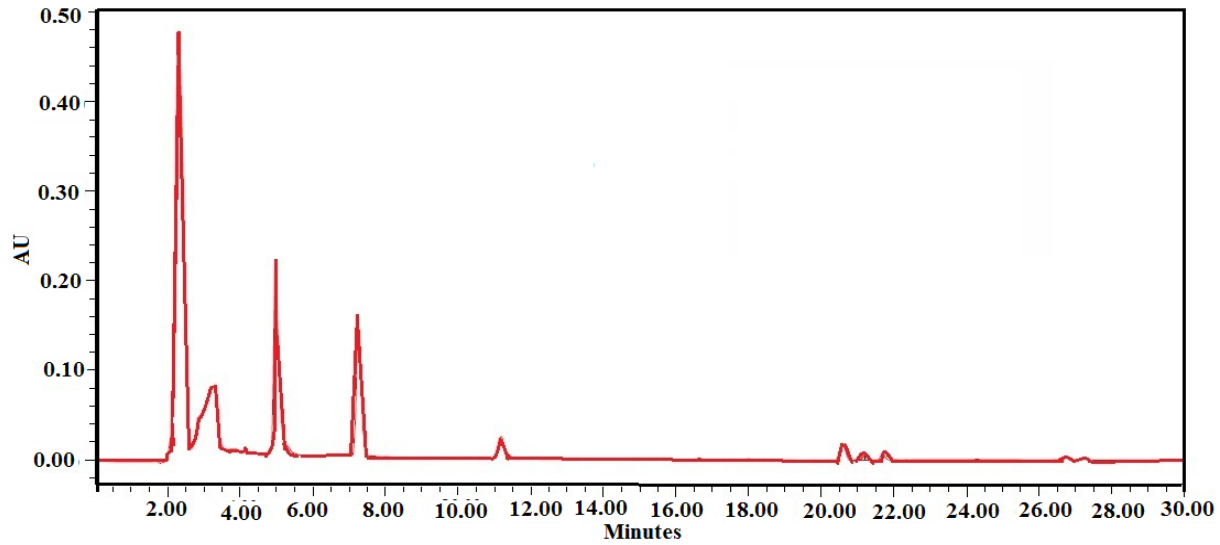

Fig 2: HPLC chromatogram fermented *Monascus purpureus* FTC 5391 produced on solid state (soybean) fermentation.

Figures 1 and 2 show the chromatograms of soybean fermented product and submerged fermented product of *M. purpureus* FTC5391 at 238 nm. In this wavelength the most metabolites of *M. purpureus* FTC5391 fermented products as number and amount were identified. The ethanolic extract of *M. purpureus* FTC5391 fermented products were analyzed by GCMS and subsequently the structural information of the detected molecular ions compared with instrument on-line library NIST 08S for further identification. Although, the metabolic products extracted from both media included acids, esters, free fatty acids, amins, pyrans, phenols, glycerin, amino acids and alcohol, the similarity between metabolites in two different media was 10%. Nevertheless *Monascus spp*. has been reputed as monacolins producer, in the pervious study of *Monascus* purpureus FTC 5391 indicated that citrinin and monacolins have not been produced by this strain [14].

The results from HPLC analysis with photo diode array detection provide a wealth of information; in the retention time domain chromatograms can be extracted at different wavelengths. At each retention time point, the UV spectrum can be extracted with the UV spectral properties of the eluting component. Under optimized gradient elution conditions, the chromatogram profile of metabolites could be divided into three groups. The hydrophilic compounds were in the first 10 min, within 10 to 15 min the moderate polar components and finally hydrophobic components were washed out. The metabolites extracted from soybean fermented products included 27% hydrophilic, 19% moderate polar and 53% polar while the pattern of metabolites extracted from submerged fermented products were 28%, 13% and 58% for hydrophobic, moderate polar and polar components respectively. Although, these results demonstrated that the most number of the components extracted of *M. purpureus* FTC5391 were polar in both media, the hydrophilic components were the most as amount (Figurs 1 and 2).

It is a fact that all functions of organisms are under genes expressions, linking gene function to a specific metabolic capabilities or metabolite production is crucial for understanding and development of biotechnological processes. So far, the genome has only been sequenced for a few species of filamentous fungi [15], and there has not been a detailed metabolic reconstruction of any filamentous fungi. Most of the studies of genome have been done on *Aspergillus sp.and Penicillumn sp*. [1, 16]. These studies illustrated about more than 10000 genes in filamentous fungi. Although the 'blueprint' of a given organism is represented by the genome, its behaviour is expressed as its phenotype, i.e. growth characteristics, cell differentiation, response to the environment, the production of secondary metabolites and enzymes. It has been estimated that more than 10 000 secondary metabolites might be present in *Aspergillus and Penicillium* [1] where perhaps less than 10% of these metabolites are known. However, it is still not always clear whether a given gene may exert several functions or if it is inactive. A large number of genes may be involved in the production of one metabolite. So, it is important to realize that there is not always a one-to-one relationship between a gene and a metabolite, and the metabolite levels are therefore usually a complex result of the expression of many genes and the function of many enzymes. However, it is a fact that filamentous fungi are great and wonderful source of metabolic diversity which would have been induced to produce using different physic o chemical conditions. As noted above, up to today only 10% of 10000 metabolites that can be produced by fungi have been discovered, which more probably would be valuable as bioactive compounds usage in food and pharmacy industry. It is therefore necessary to find efficient methods to induce filamentous fungi to produce and secret metabolites as much as possible. The results of this study revealed that the substrate is one of the significant factors on metabolite production by *Monascus purpureus FTC5391*. Optimization and improvement of media such as mixing substrate, causes *Monascus* to produce some new metabolites with higher bioactivity or synergic effect. It is known that MFP is the best alternative natural supplement to cure number of illnesses today. However, the new metabolites found in MFP might provide a more desirable cure for the patients.
On the other hand, based on similarity of filamentous fungi, using different media might be an efficient method to induce these organisms to produce remarkable array of metabolites. Hence, further studies should be considered to verify effect of substrates on metabolites secretion by filamentous fungi in the future research.

CONCLUSION

This study indicated that usage of different media induced production of different metabolites using *Monascus purpureus FTC5391* with 90% variation. *Monascus spp*. like other filamentous fungi can produce a wide range of metabolites. The most of the identified metabolites of *Monascus* up to now have been valuable as bioactive compounds usage in food and pharmacy industry. Regarding this potential of *Monascus* as nontoxic beneficial fungi encouraged to design further study to find the new method to gain and improve new metabolite production. Culture media modification would have been significant parameter to induce *Monascus* to secret a broad range of metabolites.

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Conflicts of interest

The authors declare that there are no conflicts of interest. None of the authors had any direct financial relation with the trademarks mentioned in our paper that might lead to conflict of interest for author or any of the co-authors.

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