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Chemical Profile of *Monascus ruber* Strains

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Summary

Chemical profile of *Monascus ruber* strains has been studied using gas chromatography--mass spectrometry (GC/MS) analysis. The colour intensity of the red pigment and secondary metabolic products of two *M. ruber* strains (AUMC 4066 and AUMC 5705) cultivated on ten different media were also studied. Metabolic products can be classified into four categories: anticholesterol, anticancer, food colouring, and essential fatty acids necessary for human health. Using GC/MS, the following 88 metabolic products were detected: butyric acid and its derivatives (25 products), other fatty acids and their derivatives (19 products), pyran and its derivatives (22 products) and other metabolites (22 products). Among these, 32 metabolites were specific for AUMC 4066 strain and 34 for AUMC 5705 strain, whereas 22 metabolites were produced by both strains on different tested substrates. Production of some metabolites depended on the substrate used. High number of metabolites was recorded in the red pigment extract obtained by both strains grown on malt broth and malt agar. Also, 42 aroma compounds were recorded (4 alcohols, 2 benzaldehydes, 27 esters, 3 lactones, 1 phenol, 1 terpenoid, 3 thiol compounds and acetate-3-mercapto butyric acid). Thin layer chromatography and GC/MS analyses revealed no mycotoxin citrinin in any media used for the growth of the two *M. ruber* strains.

Key words: Monascus ruber, GC/MS, colouring agents, anticholesterol and anticancer activities, essential fatty acids, fatty acid esters, aroma compounds

Introduction

Monascus ruber van Tieghem is a cosmopolitan fungus, found in soil, cooked potatoes, soya, sorghum, tobacco, rice, oat and silage (*1*). Increasing demand for natural products in the food industry has encouraged remarkable efforts towards the development of biotechnological processes for the production of such natural compounds (*2*). Some synthetic food colourants, like nitrosamines formed from nitrites and nitrates, applied in cured meat have carcinogenic and teratogenic effects. Also, the application of other synthetic colourants such as azorubin or tartrazin has been limited due to their possible allergenic effect. In order to avoid these problems, *Monascus* red pigment is used widely around the world on industrial scale as a natural food colourant (*3–7*), in medicinal and pharmaceutical products (*8–10*), as condiment and in cosmetic industry (*11–18*).

Red yeast rice (rice fermented with red yeast *M. purpureus*) was found to be a food product that has the ability of reducing the level of lipids in the blood of animals and humans (*12–16*). Also, red yeast rice has been used in China to improve digestion and blood circulation for thousands of years. Red pigment maintains normal cholesterol levels, supports healthy circulatory, cardiovascular and immune systems, liver function and provides antioxidant support (*17–19*).

Pigment production depends greatly on the kind of metabolites, yield, colour degree, stability, and safety, as well as on the strains, substrates and cultivation conditions (*5,10,13,20–26*). Steamed rice, bread, bran and several cereal substrates (*15,23*) as well as industrial by-products such as sugar cane molasses, corn steep liquor and cheese whey (*3*) had different influences on red pigment production due to the variation in their composition.

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Monascus has been found to be an important source of aroma compounds (*2*), such as volatile fatty acids or esters, lactones, aldehydes, alcohols and ketones, which play a significant role in the production of food to improve its flavour. They have high biological activity, low toxicity and are used in folk and classical medicine, food, perfume, cosmetic and pharmaceutical industries, as defoaming agents and to improve shelf-life and safety of minimally processed fruits (*2,27–38*).

This investigation has been designed to study the intensity of production of red colour pigment by two *M. ruber* strains cultured on inexpensive substrates and to estimate their yield of medically important metabolites.

Materials and Methods

Monascus strains

Two strains of *M. ruber*, AUMC 4066 (CBS 109.07) and AUMC 5705, were tested for red pigment production. These strains were obtained from Assiut University Mycological Centre, Assiut, Egypt. They were maintained on rice medium containing (in g/L): rice powder 50, KH_2PO_4 2.5, $NaNO_3$ 3, $MgSO_4{\cdot}7H_2O$ 0.5 and distilled water 1 L, pH=6 (*12,30*), and then autoclaved at 121 °C for 15 min. The fungus was then inoculated and incubated at 26 °C for 10 days.

Preparation of inoculum

Monascus ruber strains were grown on rice slants at 26 °C under static conditions. To the fully sporulated (10-day-old) agar slope culture, 10 mL of sterile distilled water were added and the spores were scraped under strict aseptic conditions. The obtained spore suspension was used as inoculum (10^6 spores per mL).

Fermentation media and cultivation

Semisynthetic fermentation media (malt yeast extract broth and agar), and those containing either grains, seeds, whey or molasses were used. Malt yeast extract (MYE) broth and agar contained (in g/L of distilled water): malt extract 5, yeast extract 3, glucose 5, and agar 15. Other media contained 10 g of boiled barley, corn, rice, sorghum, wheat grains or broad bean seeds, or 10 % whey or molasses (centrifuged and filtered in order to remove the solid residue), and all were supplemented with 1 g of KH_2PO_4 and 2 g/L of $NaNO_3$.

Aliquots of 50 mL of each medium were dispensed each in 250-mL flasks. After sterilization at 121 °C for 20 min, each flask was inoculated with 2-mL spore suspension and incubated at 26 °C for 10 days. Only MYE agar was inoculated and incubated for 20 days.

Assessment of growth, pigment production and metabolites of M. ruber strains

On all the above media, the growth and colour of aerial and immersed mycelia of both *M. ruber* strains were assessed visually. Colour intensity of the ethanolic pigment extracts was measured spectrophotometrically at 500 nm. Different metabolites produced by both strains were also determined by GC/MS analysis.

Extraction, absorbance and GC/MS analysis of M. ruber ethanolic extracts

The cultures of *Monascus ruber* strains were extracted with 95 % ethanol (by volume) in 50-mL flasks by agitation at 10 000 rpm for 24 h. The mixture was filtered through Whatman filter paper no. 2 and dried over anhydrous Na_2SO_4. The absorbance of the ethanolic extracts was measured against pure solvent (ethanol) as a blank at 500 nm, near the absorbance peak of red pigments using Spectronic 2000 spectrophotometer (Bausch and Lomb, Rochester, NY, USA) according to Kaur *et al.* (*5*).

Analysis of the extraction was preformed using Agilent GC/MS, model 6890 N/5975B (Agilent Technologies, Palo Alto, CA, USA) at the analytical Chemistry Unit, ACAL, Chemistry Department, Faculty of Science, Assiut University, Assiut, Egypt.

Detection of citrinin

A mass of 50 g of each barley, corn, rice, sorghum, wheat grains or broad bean seeds was homogenized for 5 min in a high-speed blender (at 16 000 rpm) with 100 mL of chloroform. The chloroform extracts were tested for citrinin (*8,13–15,22,27*).

The two *M. ruber* strains were grown on PDA slopes for 7 days, from which spore suspensions were prepared in 2 mL of water. The isolates were inoculated into 250--mL Erlenmeyer flasks each containing 50 mL of medium, then incubated at 26 °C for 10 days as static cultures. Cultures containing medium and mycelium were homogenized for 5 min in a high-speed blender (at 16 000 rpm) with 100 mL of chloroform. The extraction procedure was repeated three times. The chloroform extracts were combined, washed, filtered, and concentrated to near dryness and mycotoxin was detected using thin layer chromatography (*5,8,32–34*).

Results and Discussion

Table 1 clearly shows that *M. ruber* strain AUMC 5705 had good growth on most culture media. The other strain, AUMC 4066, showed good growth on barley, rice and molasses, moderate growth on corn and sorghum and failed to grow on whey.

The two strains had almost the same colour of both aerial and immersed mycelia on media enriched with natural substrates. The degree of pigmentation ranged from faint pink to deep red. Deep red colour of aerial and immersed mycelia was formed on barley after 10 days and on malt agar after 20 days by both strains. Extracellular soluble pigments were also released in all media. In this respect, Rasheva *et al.* (*16*) recorded that maximum pigment production was obtained on malt extract agar (MEA) plates supplemented with ammonium nitrate.

These results are in agreement with other findings (*12,39–46*) which stated that the biomass growth and types and production of metabolites were directly or indirectly affected by the cultivation environment and the cultivation methods. They also mentioned that solid-state cultivation results in higher pigment yield than cultivation in shake culture and this phenomenon is due to the fact

Table 1. Visual estimation of mycelial growth and colour of aerial and immersed mycelia and soluble pigment (estimated also spectrophotometrically) of the two strains of *M. ruber* (AUMC 4066 and 5705)

Medium	Visual growth estimation		Aerial mycelium colour		Immersed mycelium colour		Soluble pigment colour	$A_{500\ nm}$		No. of metabolites	
	4066	5705	4066	5705	4066	5705	4066 and 5705	4066	5705	4066	5705
barley	3	1	deep red	deep red	deep red	deep red	deep red	0.4	0.2	8	5
broad bean	1	1	faint brick red	faint brick red	red	red	light brown	0.1	0.5	10	7
corn	2	3	faint pink	faint pink	deep red	deep red	faint pink	0.3	0.7	10	5
rice	3	1	faint pink	faint pink	faint pink	faint pink	orange to red	0.8	0.1	5	3
sorghum	2	3	rose	rose	deep red	deep red	faint pink	0.4	0.5	4	6
wheat	1	3	faint pink	faint pink	deep red	deep red	faint pink	0.6	1.1	8	6
malt broth	1	3	white	faint orange	white	deep orange	colourless	0.2	3.5	15	19
malt agar	1	3	deep red	deep red	deep red	deep red	deep red	2.5	5.0	18	11
molasses	3	3	faint orange	faint orange	faint orange	faint orange	colourless	3.1	3.0	14	15
whey	0	2	–	white+red edges	–	red	colourless	0	0.2	0	17

AUMC=Assiut University Mycological Centre; visual growth estimation: 3=good growth, 2=moderate growth, and 1=fair growth
Strains were cultivated on different media at 26 °C for 10 days and on malt agar for 20 days. Number of metabolites was determined using GC/MS analysis

that pigments are released into grains under solid-state culture, whereas under submerged cultivation they are accumulated in the mycelium.

Many investigations studied the effect of using grains as a good substrate for pigment production by *Monascus* species such as wheat (*36*), corn (*3*), rice with the addition of soybean milk (*37*) and rice (*9,10,27,37,38*). Hamano and Kilikian (*3*) evaluated the production of red pigments by *M. ruber* using complex culture media (glucose or sucrose, corn steep liquor and monosodium glutamate) and found that pigment yield was higher in the complex medium than in the semisynthetic medium; however, the cell growth was similar in both media.

Spectrophometric measurements showed that AUMC 4066 strain produced the colour of highest intensity on molasses followed by malt agar, whereas strain AUMC 5705 yielded its highest intensity on malt agar followed by malt broth and molasses (Table 1).

Metabolites of M. ruber strains detected by GC/MS analysis

Significant concentrations of 88 metabolites were detected. Of these, 32 metabolites were specific for strain AUMC 4066 and 34 for strain AUMC 5705, while 22 were produced by both strains on different tested media. The detected metabolites can be classified into four categories: colouring (25 metabolites, belonging to butyric acid derivatives), anticholesterol (22), and anticancer agents, essential fatty acids (other than butyric acid) and their esters (19), and other metabolic products (22).

Table 2 shows the 25 metabolic products of butyric acid and its derivatives (colouring agents) formed by both *M. ruber* strains tested on different media. The highest concentration of metabolites was recorded for strain AUMC 5705 on molasses (17 %, by mass, of 2-ethylbutyric acid hexadecyl ester). The mass fractions of other butyric acid derivatives produced by either or both strains were between 0.1 and 9 %.

Butyric acid esters have been recorded to inhibit colonic tumour cells and promote healthy colonic epithelial cells. They also have mostly pleasant aroma or taste, and are used as food, medicine, cosmetic and perfume additives, or as muscle relaxants. Among butyric acid derivatives, γ-aminobutyric acid (also called carbamate) is known as hypertensive agent, muscle relaxant, immunity system activator, it also prevents lipid peroxidation, and supports healthy liver function (*15,35,41–49*).

Both strains produced 22 metabolic products of pyran and its derivatives known as anticholesterol agents on the tested media (Table 3). Of these, only six were produced by AUMC 4066, nine by AUMC 5705, while seven were produced by both strains. Among these metabolites, methyl-β-arabinopyranoside obtained by AUMC 5705 on malt broth showed the highest peak with a mass fraction of 21 %. Mass fractions of the remaining pyran products were between 0.1 and 14 % on different media. The pyran derivative 2-(2-bromoethoxy)tetrahydro-2-H--pyran is used in the preparation of 2-hydroxyethyl derivatives of cardiolipin analogues, furan-fused compounds, indoles and pyrazoles.

Table 4 shows that 19 metabolic products of fatty acids other than butyric acid and its derivatives were produced by both *M. ruber* strains. Of these metabolic products, only four were produced by strain AUMC 4066, seven by AUMC 5705 and nine by both strains. Linoleic acid ethyl ester showed its highest peak with a mass fraction of 65 % obtained by AUMC 4066 on sorghum and of 28 % by AUMC 5705 on wheat. Also, octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester produced by AUMC 4066 showed high peaks on corn and broad bean (4 and 26 %, respectively) and by AUMC 5705 on whey (19 %).

Many investigators recorded that the red extract of *Monascus* contains numerous fatty acids that maintain

Table 2. Butyric acid and its derivatives produced by the two strains of *M. ruber* on different media at 26 °C after 10 or 20 days of incubation as determined by GC/MS analysis

Serial no.	Butyric acid derivatives	*t*(incubation)/day											
		10										20	
		Medium											
		Barley	Broad bean	Corn	Rice	Wheat	Molasses	Whey	Malt broth		Malt agar		
		M. ruber strain											
		5705	4066	5705	5705	4066	4066	5705	5705	4066	5705	4066	5705
		w/%											
1	2-ethylbutyric acid, hexadecyl ester[3]							17					
2	3-amino-4(4-methoxyphenyl)butyric acid											9	
3	(2*R*,3*S*)-1,2,3,4-butanetriol[1]						0.2	0.2			8		2
4	1,2,3,4-butanetriol, [*S*-(*R*,*R*)][1]					6	2			1			
5	butyric acid, 3-methyl-, propyl ester[3]								6				
6	4-hydroxy, 3-methyl-2-butanone									5	0.4		
7	butanedioic acid, hydroxyl, dimethyl ester[3]					4							
8	butyl-2-methylbutanoate										3		
9	butyric acid, 3-oxo-, propyl ester[3]					0.2				1	0.1		
10	butanedioic acid, 2-hydroxy-3-methyl					1							
11	butanedinitrile								1				
12	butanedioic acid, 2-hydroxy-2-methyl-, (2*S*)								1				
13	1-buten-3-one, 1-(2-carboxyl-4,4-dimethyl) cyclobutenyl				1								
14	acetate-3-mercaptobutyric acid[8]		0.4					0.1					0.1
15	2,3-butanedithiol[7]										0.2	0.4	
16	butyric acid-2-D1										0.4		
17	2-propenyl-2-ethylbutanoate										0.3		
18	1,4-bis(methylthio)butane[7]						0.3						
19	butyric acid, 3-methyl-, butyl ester[3]								0.3				
20	3-OH-4,4-2(CH_3)-γ-butanolactone-pantolactone[4]	0.3											
21	butyric acid, ethyl ester[3]		0.1										
22	2,4-dihydroxy-3,3-dimethylbutyric acid-γ-lactone[4]		0.1	0.1									
23	butyric acid, 4-(methylthio)[7]		0.1										
24	butyric acid, 3-oxo-, 1-methylethyl ester[3]								0.1				
25	butyric acid, 3-oxo-, ethyl ester[3]								0.1				
Total		1	4	1	1	4	3	3	6	3	7	2	2

[1]alcohols, [2]benzaldehydes, [3]esters, [4]lactones, [5]phenols, [6]terponoids, [7]thiols, [8]mercapto compound

the human health (*8*,*24*,*26*). The medicinally important essential fatty acids include oleic, palmitic, stearic and linoleic acids, which are essential to normal growth of children, supporting dermal integrity, skin moisturizing, renal function and parturition, and are found to be important for immune anti-inflammatory system response and therefore relevant in arthritis, lupus, asthma and cancer prevention, cardiovascular disease prevention and brain health (*47*–*52*).

Linoleic, γ-linolenic, lauric and palmitoleic acids are known as essential fatty acids playing an important role in the life of cardiac cells and in the decrease of major depression (*41*–*49*). Wan *et al.* (*49*) recorded that γ-linolenic acid has neuroprotective properties and lowers the risk of cardiovascular diseases and prostate cancer.

Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)-ethyl ester was produced at mass fractions ranging from 5–26 % on different media (Table 4). Strain AUMC 4066 yielded 26 % on molasses, 20 % on corn, 19 % on barley and 12 % on broad bean. Strain AUMC 5705 produced 16 % on broad bean, 10 % on whey and 5 % on malt agar. Hexadecanoic acid, ethyl ester of palmitic acid was produced by the two *M. ruber* strains on the majority of media in relatively high yields (11 % by AUMC 5705 on barley and 23 % by AUMC 4066 on sorghum). The mass fractions of the remaining fatty acids were between 1 and 9 % by both strains on different media. Octadecanoic acid, 2,3-dihydroxypropyl ester was also detected at mass fractions of 9–23 % and strain AUMC 4066 showed the highest productivity on barley.

Table 3. Pyran and its derivatives produced by the two strains of *M. ruber* on different media at 26 °C after 10 or 20 days of incubation as determined by GC/MS analysis

Serial no.	Pyran derivatives	*t*(incubation)/day																	
		10															20		
		Medium																	
		Barley		Broad bean		Corn		Rice	Sorghum		Wheat		Molasses		Whey	Malt broth		Malt agar	
		M. ruber strain																	
		4066	5705	4066	5705	4066	5705	4066	4066	5705	4066	5705	4066	5705	5705	4066	5705	4066	5705
		w/%																	
1	β-arabinopyranoside, methyl																21		
2	β-D-glucopyranose, 4-O-β-D-galactopyranosyl												0.2	0.3	1	11	1	14	1
3	α-1-sorbopyranose												10						
4	ethyl-α-D-glucopyranoside				0.3		7												
5	methyl-β-1-arabinopyranoside											7							
6	β-D-ribopyranoside, methyl									7									
7	β-D-glucopyranoside, methyl								0.4	3									
8	3-benzyl-2-oxo-2-H-pyrido[2,1-b][1,3]oxazin--5-lum-4-olate	3																	
9	4-H-pyran-4-one, 2,3-dihydroxy-6-methyl												2		1			1	
10	4-H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl										2	2	1						
11	2,3-dihydro-3,5-dihydroxy-6-methyl-4-H-pyran-4-one													0.3			1	1	0.3
12	methyl-β-D-arabinopyranoside	1				0.4													
13	tetrahydro-4-H-pyran-4-ol[1]														1				
14	α-D-galactopyranoside, methyl																1		
15	β-D-glucopyranose, 1-thio-, 1-(*n*-HO-5-(methylthio)pentanimidate													0.4			0.1	1	0.3
16	octanoic acid, 8-[(tetrahydro-2-H-pyran-2-yl)oxy														0.4				
17	1,6-anhydro-β-D-telopyranose		0.3																
18	2-H-pyran, tetrahydro-2-methoxy																0.2		
19	methyl-4-azido-4-desoxy, β-1-arabinopyranoside															0.2			
20	6-(2,3-dideuteriopent-2-en-1-yl)-3,5,6-trideuterio--tetrahydro-pyran-2-one																0.2		
21	*cis*-spiro[cyclopenta[c]pyran-7(1-HO,2-[1,3] dioxide)--7α-4α-HO-carboxylic, 1-oxo, methyl ester[3]							0.1											
22	1-(*p*-toluidino)-1-deoxy-β-D-idopyranose			0.1															
Total		2	1	1	1	1	1	1	1	2	1	2	4	3	4	2	7	4	3

Legends as those in Table 2

Table 4. Fatty acids other than butyric acid and their derivatives produced by the two strains of *M. ruber* on different media at 26 °C after 10 or 20 days of incubation as determined by GC/MS analysis

Serial no.	Fatty acids other than butyric acid derivatives	*t*(incubation)/day																	
		10																20	
		Medium																	
		Barley		Broad bean		Corn		Rice	Sorghum		Wheat		Molasses		Whey	Malt broth		Malt agar	
		M. ruber strain																	
		4066	5705	4066	5705	4066	5705	4066	4066	5705	4066	5705	4066	5705	5705	4066	5705	4066	5705
		w/%																	
1	linoleic acid, ethyl ester[3]	17		22	10		15	17	65	8		28	4	3	4		6		
2	octadecanoic acid, 2-OH-1-($OHCH_3$)ethyl ester[3]			26		41									19				
3	hexadecanoic acid, 2-OH-1-($OHCH_3$)ethyl ester[3]	19		12	16	20							26		10				5
4	hexadecanoic acid, ethyl ester[3]	4	11	5	3	2	3	7	23	2	2	7	1	1	1		2	2	1
5	octadecanoic acid, 2,3-dihydroxypropyl ester[3]	23			21									16					9
6	octadecanoic acid, ethyl ester[3]	2	22	1	1	2		1	5			3		1		1	1		1
7	9,12-octadecanoic acid, (Z,Z)=linoleic acid					4					4		7	4	2	5	1	7	
8	9,12-octadecanoic acid, ethyl ester[3]		7			2					9							5	3
9	*n*-hexadecanoic acid=palmitic acid					4							2	1	3	8		4	
10	hexadecanoic acid, 2,3-dihydroxypropyl ester[3]													8					
11	decanoic acid, ethyl ester[3]									3									
12	nonadecanoic acid, ethyl ester[3]											3							
13	pentadecanoic acid=pentadecyclic acid															3			
14	heptadecanoic acid, 15-methyl-, ethyl ester[3]														3				
15	octadecanoic acid															3			
16	octadecanoic acid, 2-methyl, 1-methyl ester[3]												2						
17	*n*-decanoic acid=carpic acid									2									
18	pentadecanoic acid, ethyl ester[3]						2												
19	9-octadecenal, (Z)					2													
Total		5	3	5	5	8	3	3	3	4	3	4	6	7	7	5	4	4	5

Legends as those in Table 2

As shown in Table 5, 22 other metabolic products were produced, most of them by one of the two strains and only one product (3-deoxy-5-mannoic lactone) by both strains. Glycerol at 65 % mass fraction was produced only on rice by strain AUMC 4066. Only oxalic acid, monoamide, *n*-propyl, decyl ester at 27 % mass fraction was produced by strain AUMC 5705 on molasses. 3-Deoxy--5-mannoic lactone was produced at the mass fraction of 17 % by strain AUMC 4066 on malt and at 11 % by strain AUMC 5705 on molasses. Octane-2,5,6-trimethyl was produced at 16 % by AUMC 4066 on molasses.

Strain and substrate specificity for secondary metabolite production

Out of the 88 metabolic products detected, 32 were specific for strain AUMC 4066: seven of them are related to butyric acid derivatives, six to pyran derivatives, four to fatty acids and their derivatives, in addition to 15 other metabolites (Table 6). On the other hand, 34 metabolites were produced only by strain AUMC 5705. Of these, 13 are related to butyric acid, nine to pyran derivatives, six to other fatty acids and their derivatives, and six to other metabolites. Twenty-two metabolites were detected in cultures of both strains: five metabolites related to butyric acid, seven to pyran, and nine to other fatty acids, in addition to one other metabolite.

When substrate containing only barley was used, 13 metabolites were produced: eight by AUMC 4066 and five by AUMC 5705 (Table 7). On substrate containing only broad bean, 17 metabolites were detected among which acetate-3-mercaptobutyric acid; butyric acid, ethyl ester; octadecanoic acid, 2-hydroxy-1-($OHCH_3$)ethyl ester; hexadecanoic acid, 2-hydroxy-1-($OHCH_3$)ethyl ester; hexadecanoic acid, ethyl ester and octadecanoic acid ethyl ester

Table 5. Other metabolites produced by the two strains of *M. ruber* on different media at 26 °C after 10 or 20 days of incubation as determined by GC/MS analysis

Serial no.	Other fungal metabolites	*t*(incubation)/day										
		10									20	
		Medium										
		Barley	Broad bean	Corn	Rice		Molasses		Malt broth		Malt agar	
		M. ruber strain										
		4066	5705	4066	4066	5705	4066	5705	4066	5705	4066	5705
		w/%										
1	glycerol[1]				65							
2	oxalic acid, monoamide, *n*-propyl, decyl ester[3]							27				
3	3-deoxy-5-mannoic lactone[4]							11	17			
4	octane-2,5,6-trimethyl						16					
5	thiocyanic acid, 2-(2-butoxyethoxy)ethyl ester[3]									11		
6	*n*-methoxy-*n*-acetyl-2-carbomethoxyethylamine										11	
7	3-methoxy-4-(trimethylsilyl)oxy-benzaldehyde--O-methyloxime[2]					9						
8	E,Z-1,3,12-nonadecatriene		8									
9	4-nitrobenzaldehyde[2]								2		5	
10	quinazoline, 2-methyl								5			
11	2-furancarboxaldehyde, 5-(hydroxymethyl)								5			
12	methyl-β-D-ribofuranoside										4	
13	3-benzyl-2-oxo-2-H-pyrido(2,1-β-)[1,3]oxazin--5-lum-4-olate	3										
14	benzofurazan-1-oxide, 6-cyano										2	
15	benzoic acid, 4-OCH_3-2-(2-furoythydrazono--CH_3)phenyl ester[3]										2	
16	1,2-benzenedicarboxylic acid, diisooctyl ester[3]										2	
17	4(1-H)-pyrimidinone, 2,6-diamino										1	
18	1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester[3]											1
19	benz(α)anthracene-7-carbonitrile										1	
20	4-diethylaminomethyl-2,5-dimethylphenol[5]			1								
21	limonene[6]					1						
22	nonane, 5-methyl								1			
Total		1	1	1	1	2	1	2	5	1	8	1

Legends as those in Table 2

Table 6. Summarized results indicating the number of different metabolite derivatives recorded from each or both strains (derived from Tables 2–5)

Categories	Strain 4066	Strain 5705	Both strains	Total
butyric acid	7	13	5	25
pyran	6	9	7	22
fatty acids other than butyric	4	6	9	19
other metabolites	15	6	1	22
Total	32	34	22	88

were produced in relatively high mass fractions (10–26 %) by AUMC 4066 and seven by AUMC 5705. Three products were produced only on corn and these include 4-(methylthio)butyric acid by AUMC 4066, and 2,4-dihydroxy-3,3-dimethylbutyric acid γ-lactone and 1-(*p*-toluidino)-1-deoxy-β-idopyranose by AUMC 5705. Four products were produced on rice only, including 1-buten-3-one, 1-(2-carboxyl-4,4-dimethylcyclobutenyl); *cis*-spiro[cyclopenta[c]pyran-7(1-hydroxy,2-[1,3]dioxane)]-7-α(4α-hydroxycarboxylic, 1-oxo, methyl ester); glycerol and 3-methoxy-4-(trimethylsilyl)oxy-benzaldehyde-O-methyloxime.

Only sorghum supported the production of methyl-β--D-ribopyranoside, whereas three metabolic products were specific to wheat (3-oxo-1-methylethyl ester of butyric acid; 2-hydroxy-3-methyl-butanedioic acid and methyl-β--1-arabinopyranoside). Molasses supported the production of seven products and these include 1,4-bis(methylthio)-butane; α-1-sorbopyranose; oxalic acid, monoamide, *n*-propyl, decylester; octan-2,5,6-trimethyl, and others. On whey only, seven metabolic products were produced and these include butanedinitrile; butanedioic acid, 2-hydroxy-2-methyl-, (2*S*); butyric acid, 3-methybutyl ester and others. Ten metabolites were detected on malt broth only and these include butyric acid-2-D1; 2-propenyl-2--ethylbutanoate; methyl-β-arabinopyranoside; 2-tetrahydro-2-methoxy-H-pyran and others. Nine products were specific to malt agar and these include methyl-α-D-galactopyranoside; *n*-methoxy-*n*-acetyl-2-carbomethoxyethylamine; 1,2-benzenedicarboxylic acid, mono(2--ethylhexyl) ester and others.

Generally, of the 88 compounds detected, 42 are aroma compounds, which include 4 alcohols, 2 benzaldehydes, 27 esters, 3 lactones, 1 phenol, 1 terpenoid, 3 thiols and 1 mercapto compound (Tables 2–5). Four alcohols were formed: (2*R*,3*S*)-1,2,3,4-butanetriol, 0.2 % on molasses and tetrahydro-4-H-pyran-4-ol, 1 % on whey by AUMC 5705; 1,2,3,4-butanetriol, [*S*-(*R*,*R*)], 6 % on wheat and 2 % on molasses and glycerol 65 % on rice by AUMC 4066.

The two *M. ruber* strains produced 27 esters, including eight butyric acid esters, of which 2-ethylbutyric acid, hexadecyl ester produced by strain AUMC 5705 on molasses had the highest mass fraction of 17 %. Different fruity fragrance compounds such as methyl butyrate (apple fragrance), methyl and ethyl butanoate (pineapple), ethyl butyrate (orange), pentyl butyrate (pear), and pentyl butanoate (apricot) are reported elsewhere (*2*,*28*–*38*).

Five other esters including oxalic acid, monoamide, *n*-propyl, decyl ester (27 % mass fraction) were produced on molasses; thiocyanic acid, 2-(2-butoxyethoxy)ethyl ester (11 %) on malt by strain AUMC 5705; benzoic acid, 4-methoxy-, 2-(2-furoylhydrazonomethyl)phenyl ester (2 %); 1,2-benzenedicarboxylic acid, diisooctyl ester (2 %) and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (2 %) by strain AUMC 4066 on malt agar.

Three lactones (3-hydroxy-4,4-dimethyl-γ-butanolactone-pantolactone; γ-butyrolactone and 2,4-dihydroxy-3,3--dimethylbutyric acid γ-lactone) were detected. One phenol 4-diethylaminomethyl-2,5-dimethylphenol, one cyclic terpene limonene, which gives the orange fragrance, three thiol compounds (2,3-butanedithiol; 1,4-bis(methylthio)-butane and 4-methylthiobutyric acid) and acetate-3-mercaptobutyric acid (grapefruit fragrance) were determined. *Monascus* and other fungi play an important role in the enhancement of aroma compounds in many foods as indicated by Daigle *et al.* (*39*).

Chromatographic analyses for detection of citrinin in the cultivated media

TLC and GC/MS analyses revealed that no citrinin (nephrotoxic or hepatotoxic) was detected in all media on which the two *M. ruber* strains were cultivated (data not shown). These results are in agreement with the results found by Chen *et al.* (*51*), who investigated the distribution of mycotoxin citrinin biosynthesis-related genes in 18 *Monascus* strains. The results show that the acyltransferase and ketosynthase domains of the *pksCT* gene encoding citrinin polyketide synthase were found in *M. purpureus*, *M. kaoliang* and *M. sanguineus*. Furthermore, the *ctnA* gene, a major activator of citrinin biosynthesis, was found in *M. purpureus* and *M. kaoliang*, but was not present in *M. sanguineus*. The *orf3* gene encoding oxygenase, located between *pksCT* and *ctnA,* was also present in *M. purpureus* and *M. kaoliang*. The *pksCT* gene was highly conserved in *M. purpureus*, *M. kaoliang* and *M. sanguineus*, while the *ctnA* and *orf3* genes were shown to be highly homologous in *M. purpureus* and *M. kaoliang*. In contrast, the PCR and Southern blot analyses suggest that *pksCT*, *ctnA*, and *orf3* were absent from or significantly different in *M. pilosus*, *M. ruber, M. barkeri*,

Table 7. Summarized results showing the number of different metabolites detected by *Monascus* strains on different types of media

	Medium																			
	Barley		Broad bean		Corn		Rice		Sorghum		Wheat		Molasses		Whey		Malt broth		Malt agar	
Strain no.	4066	5705	4066	5705	4066	5705	4066	5705	4066	5705	4066	5705	4066	5705	4066	5705	4066	5705	4066	5705
No. of detected metabolites	8	5	10	7	10	5	5	3	4	6	8	6	14	15	0	17	15	19	18	11

M. floridanus, *M. lunisporas* and *M. pallens.* A citrinin-producing phenotype was detected only in *M. purpureus* and *M. kaoliang* using high-performance liquid chromatography (HPLC). These results clearly indicate that the highly conserved citrinin gene cluster in *M. purpureus* and *M. kaoliang* carries out citrinin biosynthesis. In addition, according to the phylogenetic subgroups established with the β-tubulin gene, the citrinin gene cluster can group the species of *Monascus*.

Pisareva and Kujumdzieva (*52*) studied the influence of different carbon and nitrogen sources on the pigment biosynthesis by *Monascus pilosus* C_1 during batch cultivation under the following conditions: pH=6.0, 30 °C, 300 rpm for 7 days and found that the pigment production was strongly stimulated by glucose and sodium glutamate. None of the investigated carbon or nitrogen sources stimulated citrinin biosynthesis.

Conclusions

TLC and GC/MS analyses showed that the ethanolic extracts of two *M. ruber* strains cultivated on different growth media were free of citrinin. According to Chen *et al.* (*52*) the gene responsible for citrinin biosynthesis has not been found in *Monascus ruber* so the red pigments produced by these two strains can be safe for biotechnological applications. The colour intensity of the pigment and the number of metabolites were markedly high on malt agar, followed by malt broth and molasses. Eighty-eight metabolites including 25 derivatives of butyric acid, other fatty acids and their derivatives (19), pyran and its derivatives (22) and other metabolites (22) were detected. Many of them are useful products for applications in medicine, food and cosmetic industries. Some metabolites were strain- or medium-dependent. Strain AUMC 5705 revealed good production of butyric acid, pyran, fatty acids and their derivatives. Several studies reported possible anticancer effects of fatty acids (particularly breast, colon and prostate cancer). Therefore, this strain is recommended for further studies aiming at its application in biotechnology. Forty-two aromatic metabolites were detected (4 alcohols, 2 benzaldehydes, 27 esters, 3 lactones, 1 phenol, 1 terpenoid, 3 thiols and 1 mercapto compound). These metabolites give desirable fruit fragrances in food, perfume, cosmetic and pharmaceutical industries, they are used as defoaming agents, to improve shelf life and safety of minimally processed fruits, and also in folk and classical medicine. Also, strain AUMC 4066 is a high producer (65 % by mass) of glycerol, a compound of many significant applications in food, pharmaceutical and other industries.

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References

1. P.D. Bridge, D.L. Hawksworth, Biochemical tests as an aid to the identification of *Monascus* species, *Lett. Appl. Microbiol. 1* (1985) 25–29.
2. S.G. Dastager: Aroma Compounds. In: *Biotechnology for Agro-Industrial Residues Utilisation*, P.S.N. Nigam, A. Pandey (Eds.), Springer, Kerala, India (2009) pp. 105–127.
3. P.S. Hamano, B.V. Kilikian, Production of red pigments by *Monascus ruber* in culture media containing corn steep liquor, *Braz. J. Chem. Eng. 23* (2006) 443–449.
4. C.W. Hesseltine, A millennium of fungi, food and fermentation, *Mycologia, 57* (1965) 149–197.
5. B. Kaur, D. Chakraborty, H. Kaur, Production and evaluation of physicochemical properties of red pigment from *Monascus purpureus* MTCC 410, *Internet J. Microbiol.* 7 (2009) 1–7.
6. C.F. Lin, Studies on the *Monascus* isolated from the starter of kaoliang brandy, *Chinese J. Microbiol. 8* (1975) 152–160.
7. R. Vidyalakshmi, J.R. Paranthaman, S. Murugesh, K. Singaravadivel, Stimulation of *Monascus* pigments by intervention of different nitrogen sources, *Global J. Biotechnol. Biochem. 4* (2009) 25–28.
8. J.C. de Carvalho, B.O. Oishi, A. Pandey, C.R. Soccol, Biopigments from *Monascus*: Strains selection, citrinin production and color stability, *Braz. Arch. Biol. Technol. 48* (2005) 885–894.
9. D. Heber, I. Yip, J.M. Ashley, D.A. Elashoff, R.M. Elashoff, V.L.W. Go, Cholesterol-lowering effects of a proprietary Chinese red-yeast-rice dietary supplement, *Am. J. Clin. Nutr. 69* (1999) 231–236.
10. M.Y. Hong, N.P. Seeram, Y. Zhang, Y. Chin, D. Heber, Anti-cancer effects of Chinese red yeast rice beyond monacolin K alone in colon cancer cells, *J. Nutr. Biochem. 19* (2008) 448–458.
11. H.C. Wong, Y.S. Bau, Pigmentation and antibacterial activity of fast-neutron- and X-ray-induced strains of *Monascus purpureus* Went, *Plant Physiol. 60* (1977) 578–581.
12. H. Hajjaj, A. Klaebe, M.O. Loret, T. Tzedakis, G. Goma, P.J. Blanc, Production and identification of *N*-glucosylrubropunctamine and *N*-glucosylmonascorubramine from *Monascus ruber* and the occurrence of electron donor-acceptor complexes in these red pigments, *Appl. Environ. Microbiol. 63* (1997) 2671–2678.
13. H. Hajjaj, P. Blanc, E. Groussac, G. Goma, J. Uribelarrea, P. Loubiere, Improvement of red pigment/citrinin production ratio as a function of environment conditions by *Monascus ruber, Biotechnol. Bioeng. 64* (1999) 497–501.
14. H. Hajjaj, P. Blanc, E. Groussac, J. Uribelarrea, G. Goma, P. Loubiere, Kinetic analysis of red pigment and citrinin production by *Monascus ruber* as a function of organic acid accumulation, *Enzyme Microb. Technol. 27* (2000) 619–625.
15. H. Hajjaj, A. Klaébé, G. Goma, P.J. Blanc, E. Barbier, J. François, Medium-chain fatty acids affect citrinin production in the filamentous fungus *Monascus ruber, Appl. Environ. Microbiol. 66* (2000) 1120–1125.
16. T. Rasheva, J.N. Hallet, A. Kujumdzieva, Taxonomic investigation of *Monascus purpureus* 94-25 strain, *J. Cult. Collect. 2* (1998) 51–59.
17. C. Li, Y. Zhu, Y. Wang, J. Zhu, J. Chang, D. Kritchevsky, *Monascus purpureus*-fermented rice (red yeast rice): A natural food product that lowers blood cholesterol in animal models of hypercholesterolemia, *Nutr. Res. 18* (1998) 71–81.
18. X. Lian, C. Wang, K. Guo, Identification of new red pigments produced by *Monascus ruber*, *Dyes Pigments, 73* (2007) 121–125.
19. C.F. Lin, H. Iizuka, Production of extracellular pigment by a mutant of *Monascus kaoliang* sp. nov., *Appl. Environ. Microbiol. 43* (1982) 671–676.

20. S. Babitha, J.C. Carvalho, C.R. Soccol, A. Pandey, Effect of light on growth, pigment production and culture morphology of *Monascus purpur*eus in solid-state fermentation, *World J. Microbiol. Biotechnol. 24* (2008) 2671–2675.

21. M.H. Chen, M. R. Johns, Effect of pH and nitrogen source on pigment production by *Monascus purpureus*, *Appl. Microbiol. Biotechnol. 40* (1993) 132–138.

22. C.C. Chung, T.C. Huang, H.H. Chen, The optimization of *Monascus* fermentation process for pigments increment and citrinin reduction, *BIBE, '09 proceedings of the 2009, Ninth IEEE International Conference on Bioinformatics and Bioengineering*, IEEE Computer Society, Washington, DC, USA (2009) pp. 77–83.

23. T.F. Lin, A.L. Demain, Effect of nutrition of *Monascus* spp. on formation of red pigments, *Appl. Microbiol. Biotechnol. 36* (1991) 70–75.

24. T.F. Lin, K. Yakushijin, G.H. Buchi, A.L. Demain, Formation of water-soluble *Monascus* red pigments by biological and semisynthetic processes, *J. Ind. Microbiol. Biotechnol. 9* (1992) 173–179.

25. S.I. Udagawa, H. Baba, *Monascus lunisporas*, a new species isolated from mouldy feeds, *Cryptogamie Mycol. 19* (1998) 269–276.

26. H.C. Wong, Y.C. Lin, P.E. Koehler, Regulation of growth and pigmentation of *Monascus purpureus* by carbon and nitrogen concentrations, *Mycologia, 3* (1981) 649–654.

27. F. Chen, X. Hu, Study on red fermented rice with high concentration of monacolin K and low concentration of citrinin, *Int. J. Food Microbiol. 103* (2005) 331–337.

28. M.R. Johns, D.M. Stuart, Production of pigments by *Monascus purpureus* in solid culture, *J. Ind. Microbiol. Biotechnol. 8* (1991) 23–38.

29. P. Juzlová, L. Martinková, V. Kren, Secondary metabolites of the fungus *Monascus*: A review, *J. Ind. Microbiol. Biotechnol. 16* (1996) 163–170.

30. M. Anese, M. Manzano, M.C. Nicoli, Quality of minimally processed apple slices using different modified atmosphere conditions, *J. Food Qual. 20* (1997) 359–370.

31. R. Lanciotti, A. Gianotti, F. Patrignani, N. Belletti, M.E. Guerzoni, F. Gardini, Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits, *Trends Food Sci. Technol. 15* (2004) 201–208.

32. R.A. Samson, E.S. Hoekstra, J.C. Frisvad: *Introduction to Food and Airborne Fungi*, Centralbureau voor Schimmelcultures, Utrecht, the Netherlands (2004).

33. C.C. Ng, F. Sheu, C.L. Wang, Y.T. Shyu, Fermentation of *Monascus purpureus* on agri-by-products to make a colorful and functional bacterial cellulose (nata), *Food Processing and Post-Harvest Techonology*, Food & Fertilizer Technology Center, Taipei, Taiwan ROC (2004).

34. B.A. Roberts, D.S. Patterson, Detection of twelve mycotoxins in mixed animal feedstuffs using a novel membrane cleanup procedure, *J. AOAC, 58* (1975) 1178–1181.

35. S. Babitha, C.R. Soccol, A. Pandey, Jackfruit seed – A novel substrate for the production of *Monascus* pigments through solid-state fermentation, *Food Technol. Biotechnol. 44* (2006) 465–471.

36. R.M. Dominguez-Espinosa, C. Webb, Submerged fermentation in wheat substrates for production of *Monascus* pigments, *World J. Microbiol. Biotechnol. 19* (2003) 329–336.

37. E. Chairote, G. Chairote, S. Wongpornchai, S. Lumyong, Preparation of red yeast rice using various Thai glutinous rice and *Monascus purpureus* CMU001 isolated from commercial Chinese red yeast rice sample, *KMITL Sci. Technol. J.* (Suppl. 1), *7* (2007) 28–37.

38. J. Chayawat, S. Jareonkitmongkol, A. Songsasen, B. Yongsmith, Pigments and anti-cholesterol agent production by *Monascus kaoliang* KB9 and its color mutants in rice solid cultures, *Kasetsart J. (Nat. Sci.), 43* (2009) 696–702.

39. P. Daigle, P. Gélinas, D. Leblanc, A. Morin, Production of aroma compounds by *Geotrichum candidum* on waste bread crumb, *Food Microbiol. 16* (1999) 517–522.

40. C. Herbaut, ω-3 and health, *Revue Médicale De Bruxelles* (Suppl.), *27* (2006) 355– 360 (in French).

41. E. Honoré, J. Barhanin, B. Attali, F. Lesage, M. Lazdunski, External blockade of the major cardiac delayed-rectifier K+ channel (Kv 1.5) by polyunsaturated fatty acids, *Proc. Natl. Acad. Sci. USA, 91* (1994) 1937–1944.

42. K. Landmark, C.S. Alm, α-Linolenic acid, cardovascular disease and sudden death in Norwegian, *Tidsskr. Nor. Laegeforen, 126* (2006) 2792–2794.

43. J.A. Reiffel, A. McDonald, Antiarrhythmic effects of ω-3 fatty acids, *J. Am. Card.* (Suppl. 1), *98* (2006) 50–60.

44. A.C. Logan, ω-3 fatty acids and major depression: A primer for the mental health professional, *Lipids Health Dis. 3* (2004) 25–32.

45. A.M. Rees, M.P. Austin, G. Parker, Role of ω-3 fatty acids as a treatment for depression in the perinatal period, *Aust. N.Z. J. Psychiatry, 39* (2005) 274–280.

46. P. Juzlová, L. Martinkkova, J. Lozinski, F. Machek, Ethanol as substrate for pigment production by the fungus *Monascus purpureus*, *Enzyme Microbiol. Tecbnol. 16* (1994) 996–1001.

47. J. Ma, Y. Li, Q. Ye, J. Li, Y. Hua, D. Ju *et al.*, Constituents of red yeast rice, a traditional Chinese food and medicine, *J. Agric. Food Chem. 48* (2000) 5220–5225.

48. E.J. St. Martin, P.R. Kurek, E.F. Schumacher, Water-soluble red pigments from monascorubrin and rubropunctatin as food colorant. *US patent 5013565* (1991).

49. X. Wan, Y. Zhang, P. Wang, F. Huang, H. Chen, M. Jiang, Production of γ-linolenic acid in *Pichia pastoris* by expression of a Δ-6 desaturase gene from *Cunninghamella echinulata, J. Microbiol. Biotechnol. 19* (2009) 1098–1102.

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

51. Y.P. Chen, C.P. Tseng, I.L. Chien, W.Y. Wang, L.L. Liaw, G.F. Yuan, Exploring the distribution of citrinin biosynthesis related genes among *Monascus* species, *J. Agric. Food Chem. 56* (2008) 11767–11772.

52. E.I. Pisareva, A.V. Kujumdzieva, Influence of carbon and nitrogen sources on growth and pigment production by *Monascus pilosus* C_1 strain, *Biotechnol. & Biotechnol. EQ. 24/2010/SE 2nd Balkan Conference on Biology*, University of Plovdiv, Plovdiv, Bulgaria (2010) pp. 501–506.