Flavoring and medicinal values of the yellow pigment produced by *Monascus ruber* 4066 strain cultivated on static malt agar medium

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The Thin Layer Chromatographic analysis (TLC) of the crude red pigment extract of *M. ruber* 4066 cultivated on the malt static medium well developed into three separate bands includes red, orange and yellow pigmented bands. Sixty four volatile metabolites are detected by GC/MS analysis which gave yellow pigment high flavoring value. The detected metabolites are classified, chemical and physical properties are characterized, and their uses are reported. The detected metabolites including fourteen aliphatic metabolites, twenty nine aromatic metabolites and terpenoids, thirteen nitrogenous metabolites and also five heterocyclic nitrogenous thiols metabolites are detected. Also they are classified into thirteen volatile and aroma groups includes one metabolite from the each "alcohol, acid, aldehyde, amine, furan and mercapto", two phenols, three amides, four from each "indoles and ketones", twelve terpenoids, fourteen alkanes & alkenes, sixteen esters and three metabolites are unknown for me.

**Keyword:** Malt medium, bioactive metabolites, *Monascus ruber*, yellow pigment, GC/MS analysis

**INTRODUCTION**

In food industry nowadays, yellow pigments production has two main approaches: chemical synthesis and natural plant and microbial extraction. The chemical synthetic dyes unsafe products such as tartrazine, due to its safety risk, has withered in use. Meanwhile the plant dyes extracts such as gardenia yellow, safflower yellow, are natural and low in toxicity, but are subject to the season change, resulting in an unstable industrial production. Comparing with these two approaches, microbiological fermentation for yellow pigments production, because its few safe hazards, low cost, and wide variety of resource choices has received increasing attention as a promising alternative. As far as industrial production is concerned, *Monascus* is one of the microbial good resources for pigment production. *Monascus* pigments in fact are mixture of red, yellow and orange pigment with different proportions but the red pigment is the major component (Zhou et al., 2009; Srianta et al., 2014).

*Monascus* yellow pigment have many advantages and ideal characters referred to customer due to naturally production, independent from weather conditions, wonderful colors with different shades, produced by simple and non expensive fermentation methods on "grains, agricultural and agro-industrial wastes", through few days (7-12 days), easily extracted, high safety, more stability and high solubility in water and alcohol. It also has high "nutritional, flavoring or aromatic, bioactive or medicinal" values without any bad impact on human health and environment (Yongsmith et al., 1993; 1994; Zhou et al., 2008; 2009, Eman 2015; Yadav et al., 2015). *Monascus* yellow pigment has many industrial applications may be wider application range than red
pigment such as a food additive and coloring agents in foodstuffs (edible oil, biscuits, bread, cakes, beverages, and dairy products specially yellow cheeses and improves the processed fruits), tissue texture, leathers, cosmetics, perfume, and soups" for centuries, folk medicine and recently it used in "pharmacology and medicine" (Shih et al., 2004; Moharrum et al., 2012; Srianta et al., 2014).

The Monascus anka mutant by UV light has high yield of yellow pigment on the different media include corn syrup, soaking rice in water and Malt Extract Agar medium. Surface cultivation of M. anka mutant MYM yellow pigment gives the maximum yield under optimizing the nutrient conditions (Zhou et al., 2009).

Monascus pigments act as the power of new pharmaceutical products and possess numerous bioactive metabolites used as anticancer, immunomodulatory, hepatoprotective, antidiabetic, antimicrobial, anti-inflammatory, anticholesterol, antacid vascular diseases, promote digestion, eliminate dampness and phlegm muscle bruises, dysentery, anthrax, and support human health agents, also they possess an antioxidant metabolites which used in prevention and treatments of warts, rheumatism, female endocrine system and neuralgia from ancient times and reported to exhibit stomachic, diuretic and antispastic (Shih et al., 2004; Radu et al., 2011; Srianta et al., 2014; Eman 2015).

When using toxigenic strains in pigment production the monascidin A or citrinin are produced with the yellow pigment under anaerobic condition. Pigment production must occur under specific nutritional and environmental condition for preventing the mycotoxins yield or occurs by using the non toxigenic Monascus strains (Eman and Abaddy 2014). The large international company must be preparing their Monascus yellow pigment under national standard with high safety without yellow citrinin mycotoxins by selecting the non toxigenic strains or improving the production conditions with citrinin level limit of 0.2μg/g. For quality, food safety must be performed on all supplements to ensure their identity, purity, strength, non toxicity and composition (Zhou et al., 2008; 2009; Srianta et al., 2014).

This investigation has been designed to study the production, detection, separation of the yellow pigment from M. ruber 4066 cultivated on the statically malt medium and reported all the available information about their metabolites found in it.

MATERIALS AND METHODS

Monascus strain

Monascus ruber was obtained from Assiut University Mycological Centre, Assiut, Egypt. The strain of M. ruber, AUMC 4066 (CBS 109.07), was tested for mixture of the red and yellow pigments production and to identify their metabolites. The tested strain was maintained on rice medium containing rice powder 50, KH2PO4 2.5, NaNO3 3, MgSO4·7H2O 0.5 g/L distilled water 1 L and then autoclaved at 121°C for 15 min. The fungus was then inoculated and incubated at 30 °C and pH 6 for 10 days.

Preparation of inoculums, fermentation media and cultivation

Monascus ruber strain is grown on PDA slants at 30°C. To the fully sporulation (10-day-old) agar slope culture, were cultivated in Petri dishes under strict aseptic conditions and incubated at 28°C for 2 days. Malt medium gave the best results for pigment production and gave the highest number of detected metabolites. Also the tested strain was recorded as non toxigenic strain and completely safe, these results were recorded by (Moharram et al., 2012). Malt medium contained malt extract 5, yeast extract 3, glucose 5 g/ L distilled water (Lian et al., 2007).

Extraction of the Monascus pigment

Monascus ruber cultures were extracted with 95 % ethanol (by volume), 50 mL/ flask, while agitating at 10 000 rpm for 24 h. The mixture was filtered through Whatman filter paper No. 2 and dried over anhydrous Na2SO4 (Lee et al., 2001; de Carvalho et al., 2007).

Spectrophotometric analysis

Monascus yellow pigment was determined by spectrophotometer (Spectronic 2000 colourimeter, Bausch and Lomb) at a visible spectrum with an apex at 370-395 nm. It separated from the mixture with red and orange pigments by TLC analysis according to (Zhong et al., 2015).

TLC analysis and separation of the yellow band

The crude red ethanolic extract was dried on water path and diluted into one mL 500 μ mL were spotted on activated TLC glass plate and run in chloroform 97% and ethanol 3%. The developed plate gave two separated red and yellow bands. Yellow pigments are separated at Rf 0.73 with using ethanol aqueous solution 70% V/V. Yellow bands was crushed and dissolved again in grade Sigma Aldrich ethanol for the GC/MS analysis (Zhong et al., 2015).

Gas Chromatography/Mass Spectrometric (GC/MS) analysis

Flavouring metabolites in ethanolic extracts of yellow pigment are analyzed by GC/MS analysis. (Moharram et
Sixty four volatile metabolites are detected by GC/MS analysis. The detected metabolites including fourteen aliphatic metabolites, twenty nine aromatic metabolites and terpenoids, thirteen nitrogenous metabolites and also five heterocyclic nitrogenous thiols metabolites are detected. They are classified into thirteen chemical groups and includes one metabolite from the each "alcohol, acid, aldehyde, amine, furan and mercapto", two phenols, three amides, four from each "indoles and ketones", twelve terpenoids, 14 alkanes & alkenes and 16 esters. Also the detected metabolites are unknown (Table 1 and Figure 1, 2). All the recorded metabolites are volatile and give the Monascus yellow pigment high flavouring value according to (Moharram et al., 2012; Eman and Abbady 2014; Zhong et al., 2015).

Sixty four detected metabolites including: Fourteen aliphatic metabolites, twenty nine aromatic metabolites and terpenoids, thirteen nitrogenous metabolites and five nitrogenous thiols metabolites.

Fourteen aliphatic metabolites including one aldehyde, two alcohols, three esters and ten alkane & alkenes (Table 1 and Figure 1,2). Nonanal-dimethyl-acetal is pelargonaldehyde-dimethyl-acetal. It is a clear liquid with a crisp, citrus odor, taste and floral smell that is soluble in water and alcohol. It is used as a flavoring and fragrance agent in foods, precursor for production of other chemicals such as emollients and plasticizers. Alkanes are playing a role in fungi as a source of carbon and energy. GC/MS analysis of fungi are recorded that seventeen volatile compounds included alkenes, alkanes, alcohol, aldehyde, ketone, and other organic compounds. Cs volatiles consisted of the major volatile compositions of the fungus includes. 1-Octen-3-ol and 3-octanone are the main volatile compounds. 1-octen-3-ol and 3-octanone could be considered as biologically active compounds, which are not only an important factor in the fungal flavor but also contain crucial bio-information related to antioxidant activity (Zhang et al., 2008).

Twenty nine aromatic metabolites including one ketone, two phenols, three alkane, eleven esters and twelve terpenoids contains or includes six mono-cyclic terpenoids fenchol, eucalyptol or cineole, borneol, endoborneol, camphor and farnesane; five sesquiterpene cedrol, chrysorhedral, artemisinin, chromolaena and seychellane and one steroid androstenolone or prasterone. One ketone, two phenols, three and eleven esters (Table 1 and Figure 1, 2). 2,6-diisopropynaphthalene acts as plant growth regulator and is used in the manufacturing of pesticide. It has clear yellowish brown colour with a faint sweet odor. Six mono-cyclic terpenoids metabolites are detected in yellow band including fenchol, eucalyptol is a monoterpenoid cyclic ether, it is a colorless liquid with flavorings, fragrances and taste which used as a flavoring agent for baking, confectionery, meat products and beverages, fragrances, cosmetics, medicinal products, insecticide, insect repellent, brands of mouthwash, cough suppressant and body powder. Borneol and endo-borneol are bicyclic monoterpenes and used in traditional Chinese medicine as moxa insect repellent. Camphor is a terpenoid. It used in cooking; as a plasticizer for nitrocellulose; ingredient for fireworks and explosive munitions; antinsects, used to make mothballs, antisnakes and other reptiles; antimicrobial agent; anti-itch gels and cooling gels cough suppressant and as a decongestant. Camphor is used for minor heart symptoms and fatigue. treat sprains, swellings, and inflammation. antimania, anticholera. Farnesane; 2,6,10-trimethyl-dodecan is responsible for the characteristic green apple odor. Its oxidation by air gives compounds that are harmful the fruit. The oxidation products injure the cell membranes which eventually causes cell death in the outermost cell layers of the fruit, resulting in a storage disorder known as scald. α-Farnesene is also used as insect repellent. Five sesquiterpenes which are as antioxidant, aroma agent includes cedrol alcohol, chrysorhedral has pungent aldehyde, artemisinin has lactone and acts as antimalaria, anticancer and antisweet wormwood. Chromolaenin is used for treatment of skin diseases such as wounds, rashes, antimicrobial, anti-inflammatory, insect repellent, fungicidal, nematocidal and soil fertilizer agent. In general terpenes are large class of bioactive 2ry fungal metabolites used as fragrance and flavor industries. The terpenoids group show significant pharmacological activities, such as antiviral, anti-bacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities. Antioxidant compounds are therapeutics agents used for prevention and treatment of oxidative diseases such as cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/ reper fusion injury, diabetes mellitus, neurodegenerative diseases "Alzheimer and Parkinson diseases", rheumatoid arthritis, ageing, anti-inflammatory, antitumor, antimutagenic, anticancer, antimicrobial activities. Antioxidants agents such as phenols, flavonoids and terpenes (Pimentel et al., 2011; Smith 2014; Eman 2015; Yadav et al., 2015).

Fourteen nitrogenous metabolites are detected and...
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Legend: All the chemical structure and formula are collected from different internet sites includes NIST (Notional Institute of Standards and Technology); Chem-Spider Search and share chemistry and Wikipedia, free encyclopedia https://en.wikipedia.org/wiki/DIPN.

Note: Three metabolites are detected by GC/MS and I can’t to draw their chemical structure which includes [spiro(cyclopropane-1,3-tricyclo-(3.2.(2,7)heptane)] alk; 2-benzoyl-3-formyl-6-methyl-4,5-diphenyl-O-diaicylbenzole K and dimethyl(1,1,2-trimethyl) –silanol-Alc].
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### Table 1. Classification of the GC/MS detected metabolites into different flavouring groups in the yellow M. ruber strain

<table>
<thead>
<tr>
<th>Groups</th>
<th>Alcohol</th>
<th>Aldehyde</th>
<th>Acid</th>
<th>Amines</th>
<th>Furan</th>
<th>Mercapto</th>
<th>Phenols</th>
<th>Amines</th>
<th>Ketones</th>
<th>Indoles</th>
<th>Terpenoids</th>
<th>Alkanes</th>
<th>Esters</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>12</td>
<td>14</td>
<td>16</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Flavouring and medicinal metabolites of *Monascus ruber* 4066 strain in yellow pigment analyzed by GC/MS

including one from each of acid, amine, ester and three ketones. Levomethamphetamine is a sympathomimetic vasoconstrictor which is the active ingredient used in some over-the-counter nasal decongestants including the formulation of Vicks Vapo Inhaler. It is useful for nasal decongestion. Five heterocyclic nitrogenous thiols metabolites are detected. *Monascus ruber* strains have been found to be an important source of aroma compounds, such as volatile fatty acids, esters, lactones, aldehydes, alcohols and ketones, which play a significant role in the production and improve the aroma flavor. Malt extract medium has a high nutritional and aromatic values; it contains enzymatic modifying complex macromolecules specially starch malt grains and proteins. The modifying starch includes glucose, maltose, maltotriose, maltodextrines and small amounts of sucrose and fructose; also proteins converted to amino acids, vitamin B complex, minerals, fragrance and aroma enhancer metabolites, enzymes and prevention of crystal formation agents. Malt medium acts as an excellent substrate for production of the deep red pigment of the *M. ruber* and their bioactive metabolites. Esters are responsible for the fruity fragrance. The ethanolic extract of the red pigments have 27 esters including eight butyric acid esters 2-ethylbutyric acid, hexadecyl ester. Different fruity fragrance compound such as methyl butyrate "apple fragrance", methyl butanoate pineapple, ethyl butyrate orange, ethyl butanoate pineapple, pentyl butyrate pear and pentyl butanoate apricot (Moharram et al., 2012).

Nemours endophytic fungi are produced many bioactive metabolites with pharmacological activities which act as a health-promoting benefits, antimicrobial, anticholesterols, antiviral, immune suppressants and immune enhancer agents. These metabolites include vitamins, organic acids, β-glucans, antibiotics, polyketides, terpenoid, and trace elements "selenium", antioxidant metabolites such as phenols, and terpenoids which are used as anticancer, anti-microbial, anti-inflammatory and cytotoxic agents (Govindappa et al., 2013; Ruma et al., 2013;
Sadrati et al., 2013; Kandasamy and Kandasamy 2014; Smith 2014; Kumaresan et al., 2015).

*Monascus* bioactive metabolites are classified into eight categories including anticancer agents "ergosterol, ergothioneine, essential fatty acids, eicosanoids, β-glucan, glycoproteins, lectins, monacolin K, pyran derivative, phenols and triterpenoids; Anticardiovascular disease; human health supporting agent's and health care; terpenoids "ergosterol and flavonoids"; immune enhancer; fragrances and flavor compounds "alcohols, benzaldehydes, esters, lactones, phenols, terpenoids, thiols and mercapto compounds"; antioxidant and hepatoprotective agents; anticholesterol. Also the bioactive metabolites of the yellow mutant of the *M. pilosus* strain on the fermented rice (Eman & Abaddy 2014).

Two *Monascus ruber* strains are cultivated on four kinds of media and recorded 142 flavour and antioxidant metabolites by GC/MS analysis including 48 phenolic compounds, 39 esters, 23 alkanes and alkenes, 10 ketons, 5 from each of azole and indoles compounds, 4 terpenoids, 2 for each of amides and amines, one from each of aldehyde, free fatty acids, pyran and cycloribitol-alcohols (Eman 2015).

**CONCLUSION**

Yellow pigment has high flavoring and medicinal value due to the presence of 64 volatile and flavors metabolites which are classified into 13 aroma groups including acid, alcohol, aldehyde, alkanes, amids, amine, furan, indoles, ketones, mercapto, nitrothiol compounds, phenols, terpenoids and esters. Fourteen metabolites related two chemical groups (two phenols and twelve terpenoids) have antioxidant activity and reported by many Authors and includes Two metabolites have floral odor including nonanal "green apple odor". Also three new yellow metabolites are detected and include 2,6-diisopropyl naphthalene, 4-phenylquinoline and 2,4-dimethyl-benzo[1]quinoline which reported as a food coloring agent.

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