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Production of Natural Pigments from *Monascus ruber* by Solid State Fermentation of Broken Rice and its Application as Colorants of Some Dairy Products

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ABSTRACT

The present study was aimed to maximize the production of natural pigments from *Monascus ruber* Went AUMC 5705 by solid state fermentation of broken rice to be a safe and healthy substitute of the harmful chemical dyes in the coloring of some flavored dairy products. Maximum pigments productivity was achieved under the following conditions: initial substrate moisture content 39%, substrate particle size 2-2.5 mm, PH 6.5, inoculums size 126×104 spores per 10 g dried rice and incubation period of 15 days at 30°C. The favorable nitrogen source was monosodium glutamate at 2% concentration. The obtained pigments mixture was extracted with 95% ethanol and further separated into red, orange and yellow water soluble pigments. These pigments were applied as coloring agents of flavored yoghurt and milk beverages. On the sensory evaluation, application of these natural colorants in a preparation of flavored dairy products was found to be highly acceptable.
Keywords: Monascus - pigments - broken rice- dairy products.

INTRODUCTION

Recently, coloring of food with natural pigments is of worldwide attention and is gaining concern. These pigments are looked upon for their safe use as natural food colorants in replacement of synthetic dyes which exhibited unfavorable toxic effects including mutagenicity and potential carcinogenicity. Though many natural colors are obtainable, microbial colorants play an important role as food coloring agents because of its profuse production and easy down streaming process. Among the various pigment-producing microorganisms, *Monascus* was proved to produce non-toxic pigments, which can be employed in food industries. Besides a coloring agent, it enhances the flavor of the food and acts a food preservative (Vidyalakshmi *et al.,* 2009).

The genus *Monascus* belongs to the family Monascaceae of the phylum Ascomycecota, based on the cultural characteristics, nine *Monascus* species are internationally acknowledged. These are *M. ruber*, *M. pilosus*, *M. purpureus*, *M. floridanus*, *M. eremophilus*, *M. pallens*, *M. sanguineus*, *M. lunisporas*, and *M. argentinensis*. However, over 20 species of *Monascus* have been recorded in the literature (Shao *et al*., 2010).

The choice and preparation of the raw substrate can significantly affect a solid-state fermentation. Moreover, the used substrates are commonly agro-industrial wastes, insoluble in water and are made of easily digestible particles (Raimbault, 1998; Manpreet *et al.,* 2005). Growth of the fungus is controlled often by its ability to digest the substrate. A majority of solid- state fermentations utilize Starchy materials as substrate. Examples of starchy materials that are commonly used are rice, cassava, and sweet potato (Prado *et al*., 2004). Further supplementation of substrate with other nutrients or growth factors may be required to achieve the desired fermentation and enhance metabolite production. In addition to its chemical composition, the physical properties of the substrate (i.e. particle size, shape, porosity, consistency) may affect its utility in solid-state fermentation. Particle size and shape appeared to be the most important characters that influence the consumption of substrate (Mitchell et al., 1992a; Manpreet *et al.,* 2005).

For economical production of the bio products, it is important to select cheap and efficient substrates. Various agricultural wastes and by-products such as wheat bran (Dominguez-Espinosa and Webb, 2003), jackfruit seed (Babitha *et al*., 2006), corn steep precipitate (Hamano and Kilikian, 2006), grape waste (Silveira *et al.,* 2008), sugarcane bagasse (Silveira *et al.,* 2011), corn cob (Velmurugan *et al.,* 2011), and potato wastes (Abdel-Raheam, 2016) were successfully used for the production of *Monascus* pigments. Rice broken represent a useful and inexpensive substrate for the production of highly valued microbial metabolites at an economic manner and can be applied to varying food products (Abdel-Raheam, 2016).

Accordingly, this work was conducted to maximize and sustain the productivity of *M. ruber* pigments from rice broken under solid state fermentation. Therefore, the effects of various fermentation conditions such as the initial substrate moisture content, incubation temperature and period, pH, inoculums size, substrate particle size, and nitrogen supplements were studied. The obtained pigments mixture was extracted from the fermented rice and separated into red, orange and yellow colors. These natural colors were applied individually as coloring agents during processing of flavored yoghurt and milk beverages which were sensorial evaluated.

MATERIALS AND METHODS

Substrate:

Broken rice was obtained from a local mill at Assiut Governorate, Egypt. It kept at 4°C in polyethylene pages until used as substrate of solid state fermentation.

Fungal culture:

Monascus ruber Went AUMC 5705 obtained from Assuit University Mycological center (AUMC), Assuit, Egypt, was utilized in this study. It was cultivated on yeast extract- peptone- dextrose medium held at 4°C. The culture was tested for citrinin formation and found to be non-producing.

Inoculums preparation:

M. ruber Went AUMC 5705 was grown on YEPD slants at 30°C. Moreover, 10 ml of sterile distilled water was added to the grown culture and the spores were rubbed off and collected in a sterilized flask. The obtained spore suspension (36×10^4 spores/ml) was used as inoculums (Abdel-Raheam, 2016).

Solid-state fermentation procedures:

Ten grams of broken rice were placed in a 250 ml Erlenmeyer flask and 1.0 ml zinc sulfate ($ZnS0_4.7H_2O$, 0.128 M) solution was added to each flask. (Nimnoi and Lumyong, 2009). Calculated amounts of distilled water were added to obtain initial moisture contents ranging from 31% to 43% (V/W). Broken rice in each flask was thoroughly mixed for 1h at room temperature, covered with double layers of aluminum foil to avoid moisture loss and sterilized at 121°C for 15 min. After cooling, each flask was inoculated with 1 ml spores suspension (36×10^4spores / ml) and incubated at 30°C for 10 days. All experiments were conducted in triplicates.

Optimization of the pigments production conditions:

Broken rice was fermented with *M. ruber* Went AUMC 5705 using solid-state fermentation technique. The effect of initial moisture content (31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, and 43%) was adjusted by adding calculated amounts of distilled water to a constant weight of broken rice. To study the effect of inoculation rate, 180×10^3, 270×10^3, 36×10^4, 45×10^4, 54×10^4, 63×10^4, 72.5×10^4, 81×10^4, 90×10^4, 99×10^4,108×10^4, 117×10^4, 126×10^4, 135×10^4, 144×10^4, 153×10^4, and 162×10^4 spores /10-gram dry substrate (gds) were added to different flasks. The initial pH value (4.5, 5.5, 6.5, 7.5 and 8.5) was achieved by adjusting the pH with 0.5M HCl or 0.5M NaOH. To study The influence of particle sizes, broken rice of various particle sizes was used for preparing of different media, viz. M1 (particles ≤ 1.0 mm), M2 (particles 1.1 -1.5 mm), M3 (particles 1.6 - 2.0 mm) and M4 (particles >2.0 mm). To study the effect of the incubation period, pigments were estimated after 5, 7, 9, 11, 13, 15, 17 and 19 days of incubation. Experiments were also performed to evaluate the effect of different external nitrogen sources (peptone, yeast extract, beef extract, monosodium glutamate, ammonium nitrate, ammonium sulfate, ammonium chloride, potassium nitrate and urea) at 1% concentration pigments production. The effect of different concentrations of the selected nitrogen source (monosodium glutamate) was also studied. All experiments were conducted in triplicate.

Pigment extraction and quantification

After the incubation period, the contents of each flask were dried at 50˚C and ground to a fine powder using an electrical mill. 0.5 g of fermented rice powder was extracted with 20 ml of 95% ethanol in on a rotary shaker for 2 h at 180 rpm in a100 ml Erlenmeyer flask. The obtained extract was centrifuged at 10000 g for 10 min to discard suspended solids. The supernatant was analyzed by spectrophotometer (UViline 9400 – SCHOTT Instruments, EU) using ethyl alcohol 95% as control. Pigments concentration was measured at 500, 470 and 400 nm for red, orange, and yellow colors, respectively with considering the dilution factor of the sample. The data were expressed as absorbance unit (AU) /g dried substrate (Carvalho *et al*., 2003).

Absorbance unit (AU g^{-1}) =

$$\frac{(\text{OD} \times \text{Total volume of Solvent} \times \text{Dilution factor})}{\text{Dried sample (g)}}$$

Analysis of citrinin

Presence of the mycotoxin citrinin (if any) was estimated by Thin Layer Chromatography according to Rasheva *et al*., (2003). Authentic sample of Citrinin (Sigma) was used as a standard.

Separation of pigments for application in the preparation of dairy products:

The mixture of orange, red and yellow pigments was separated and purified individually from the wet fermented broken rice to form separate water soluble pigments according to the method described by Lakshmi and Selvi, (2011).

Application of the separated pigments for the coloring of dairy products:

Yoghurt and milk beverages were manufactured from Buffalos milk by the traditional methods. Red pigment was added to both yoghurt and milk beverage flavored with strawberry, while yellow pigment was added only to banana-flavored milk beverage. View drops of each pigment extract were required to give a color degree similar to that of the commercial corresponding products. All products were kept at 4°C for 3 days and then subjected to sensory evaluation by fifteen panelists. Attributes of color, taste, odor, texture and overall acceptability were tested using slandered score card.

RESULTS AND DISCUSSION

Optimization of pigments production:

Effect of initial moisture content:

The effect of different moisture contents in broken rice medium on pigments production was examined and the results are illustrated in Figure (1) indicate that production of red, orange and yellow pigments was maximal at 39% moisture content (91.80, 72.72and 144.84AU/g dry fermented substrate (DFS), respectively). These results are in consistence with that reported by Thunnaree, *et al*., (2014) who found optimum moisture content of 38% for *Monascus* sp.KB9, while using non-sticky rice as substrate in solid state fermentation. On the other hand, the present results are somewhat different from those described by Babitha *et al*., (2007). They reported that lower concentration of M. rubbe's pigments was obtained from jack fruit seeds containing moisture content below 40%. The lower pigments yield at the high levels of moisture content may attribute to agglomeration of the substrate and reducing oxygen supply for the fungus. On the other hand, the decreased pigments production at low moisture contents may be attributed to low nutrient availability as well as less efficient heat exchange and oxygen transfer (Carrizales and Rodriguez, 1981; Babitha, *et al*., 2007).

Effect of incubation temperature

Incubation temperature affects the rate of all biological activities of microorganisms, nutrient uptake, enzymes synthesis and metabolites production. So, an experiment was carried out to explore the influence of different incubation temperatures (20, 25, 30, 35and 40˚C) on production of pigments by *M. ruber* Went AUMC 5705. Results illustrated in figure (2) showed that 30˚C was the optimal temperature giving maximal red, orange and yellow pigments productivity. Production of red, orange and yellow pigments were gradually enhanced by increasing of the fermentation temperature from 20 to 30˚C and then sharply decrease with increasing the incubation temperature from 30 to 35˚C.These results were in agreement with those reported by Park *et al*., (2005) and Jeon *et al*., (2006). They found that, the optimum temperature for red pigment production by *M. purpureus*MMK2, *Monascus ruber* KCTC 6122 and *M. purpureus* P-57 was 30 °C. More recently, Padmavathi and Prabhudessai (2013) reported that mycelium growth and pigments production by *Monascuss anguineus* and *M. purpureus*MTCC410 were optimum at fermentation temperature of 30°C.

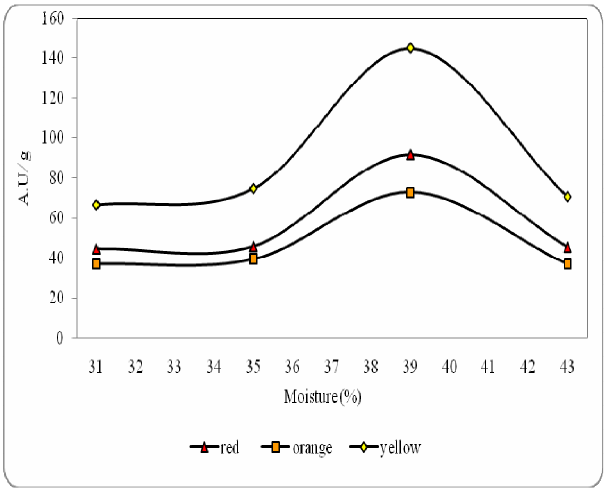

Figure 1. Effect of initial moisture content on pigments production by *M. ruber* Went AUMC 5705 after 10 days of incubation at 30°C.

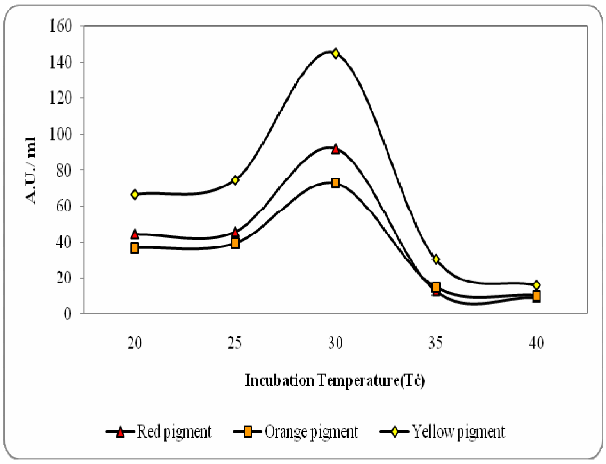

Figure 2. Effect of incubation temperature on pigments production by *M. ruber* Went AUMC 5705.

Effect of substrate inoculum size:

Results represented in Fig. (3) indicated that production of all pigments was increased gradually with increasing the inoculum size. The highest concentrations of red, orange and yellow pigments (452.5, 420.8 and 316.2AU/g DFS, respectively) were obtained at inoculum rate of 126×10^4 spores /10 g of fermented dried substrate. The present results are rather differed from this described by previous investigators. Lee *et al*., (2002) reported that production of red pigment was maximized at inoculum size of about 1x10^4 spores /gram of dry solid substrate while using SSF for production of pigments by *M. purpureus* ATCC 16362 from Long grain rice. It is advantageous to achieve high level of the product by small inoculums size as obtained from our results. This may reflect a high potentiality of the studied fungal strain.

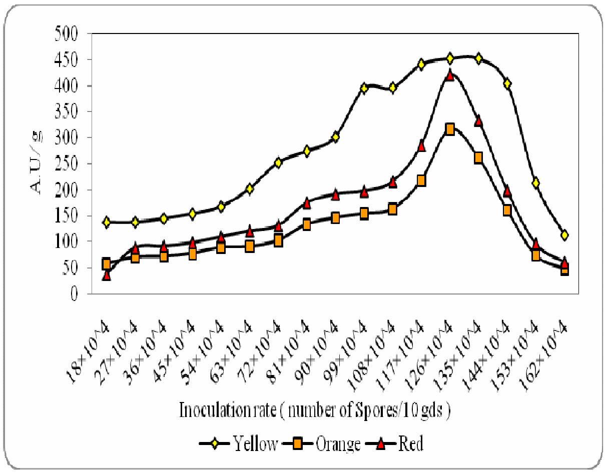

Figure 3. Effect of inoculum rate on pigments production by *M. ruber* Went AUMC 5705 after 10 days of incubation at 30°C.

Effect of initial substrate pH:

M. rubber pigments yield was determined at different initial substrate pH values (4.5 to 8.5). The maximum yield of pigment was obtained at pH 6.5 (452.5, 420.8, and 316.2 AU/g for yellow, red, and orange, respectively), Results in Fig. (4) also showed that pigments production was almost constant within the acidic PH range (4.5-6.5) especially orange and red pigments. Meanwhile, the alkaline PH range exhibited strong inhibitory effect on pigments formation. Production of all pigments was markedly reduced at PH 7.5 and totally suppressed at PH 8.5, especially the red pigment.

The obtained results were similar to that observed by Chen and Johns (1993), Lee *et al*., (2002) and Joshi *et al*., (2003). They found that production of Monascus pigments was optimum at pH value ranging from 5.5 to 6.5. Also, Musaalbakri, *et al*. (2005) found that the suitable initial PH for red pigment production was varied from 5.5 to 9.0, but the optimum was stated at 6.5. Babitha, *et al*., (2007) obtained highest pigments yield by *M. purpureus* at pH 4.5 to 7.5

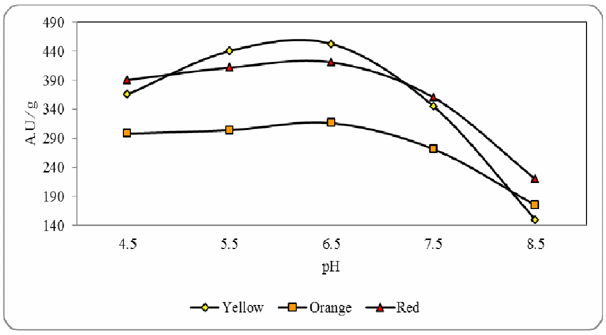

Figure 4. Effect of initial pH values on pigments production by *M. ruber* Went AUMC 5705 after 10 days of incubation at 30°C.

Effect of substrate particle size:

Results illustrated in Fig. (5) Indicated that pigments production by *M. ruber* Went AUMC 5705 increased gradually with increasing the rice particle size. Maximum concentrations of yellow, red and orange pigments (452.5, 420.8 and 316.2 AU/ g. dried fermented rice, respectively) were obtained on medium with particle size larger than 2.0 mm. The present results are rather differed from this described by Babitha *et al*., (2006) who found the highest pigments yield from *M. purpureus* LPB 97 when used jack fruit seeds at particles size between 0.4 and 0.6 mm. This variation may be attributed to the difference in the fermented substrate between broken rice and jack fruit seed or difference between the employed fungal strains. However, Pandey *et al*., (2000) indicated that very small particles may interfere with the aeration process as a result of substrate agglomeration and consequently lead to poor microbial growth. At the same time, larger particles can improve aeration efficiency but provide limited surface for fungal growth. So, it may be essential to provide an appropriate particle size.

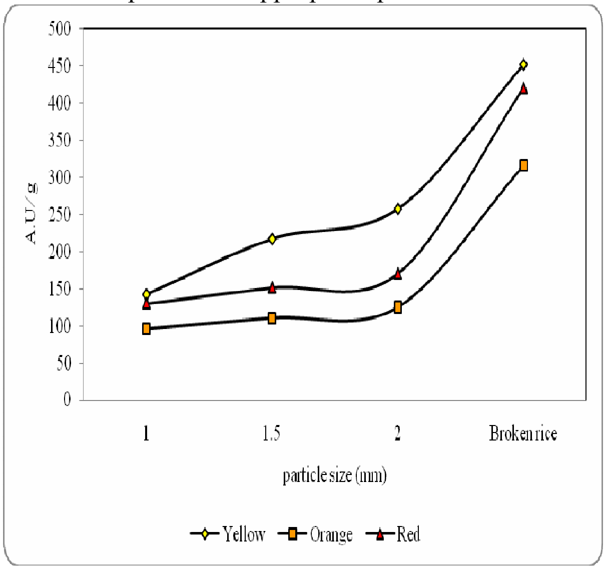

Figure 5. influence of different substrate particle sizes on pigments production.

Effect of Fermentation time:

The amount of pigments produced by *M. ruber* Went AUMC 5705varied greatly with the incubation period. Data represented in Figure(6) clearly showed that pigments production was commenced at the 5th day and progressively increased with increasing the incubation time up to15 days, then began to decline. Maximum yellow, orange and red pigments concentrations obtained at the15th day were 906.5, 837.4and 637.8AU/ gdfs, respectively. These results are consistent with the findings of Emon *et al*., (2007) that maximum pigments production by *M. purpureus* CMU001 was obtained after 2 weeks of incubation while using Korkor 6 white glutinous rice as fermentation medium. However, Velmurugan *et al*., (2011) observed that highest pigments production by *M. purpureus* KACC 42430 within 168 h, while using corn cob as a basal medium for fermentation.

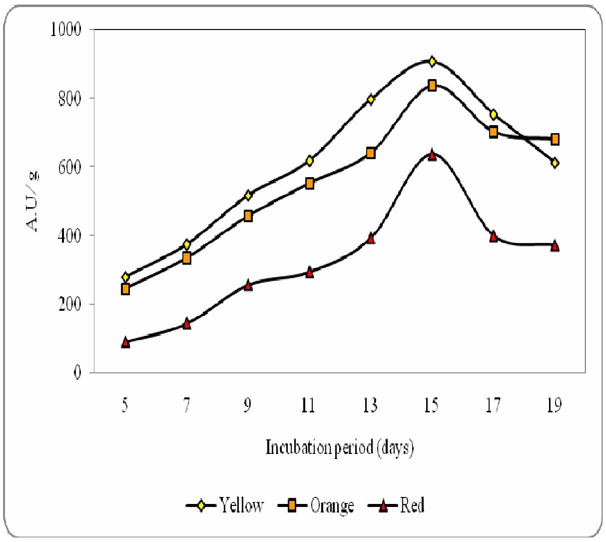

Figure 6. Effect of Incubation period on pigments production by *M. Ruber* Went AUMC 5705 at 30°C.

Influence of various external sources of nitrogen pigments formation:

Nine external sources of nitrogen were separately added at 1% concentration to the broken rice medium to assess its effect on pigments production. Data illustrated in Fig. (7) indicated that monosodium glutamate (MSG) acted as the best nitrogen source for pigments production by *M. ruber*. Concentrations of red, orange and yellow pigments reached to 1190.08, 1283.7and 1189.04 AU/gdfs, respectively in the presence of MSG, followed by1038.92, 848.08 and 1012.52 AU/gin the presence of peptone. Compared to the control treatment, addition of MSG increased the yield of red, orange and yellow pigments by about46.4%, 34.8% and 23.8%, respectively (Fig. 6). These results are closely similar to those reorted by Vidyalakshmi, *et al*., (2009). They demonstrated that, monosodium glutamate gave superior growth and pigments production by *M. ruber*, it increased the pigments yield by about 56% when added to rice grains during fermentation.

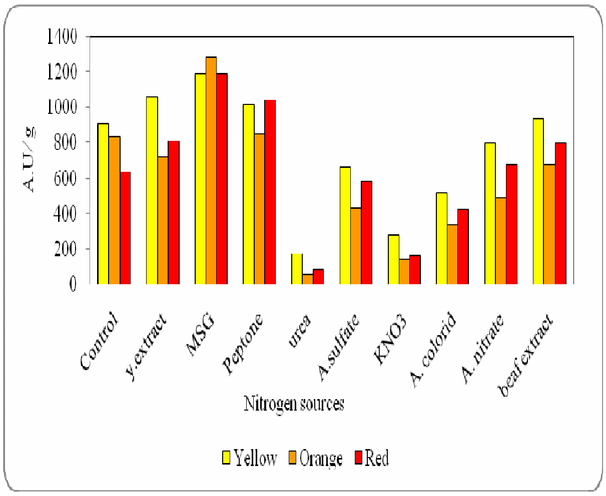

Figure 7. Influence of various nitrogen sources on pigments production by *M. ruber* Went AUMC 5705 after 15 days of incubation at 30°C.

Influence of monosodium glutamate concentrations:

The time course of pigments fermentation by *M. ruber* Went AUMC 5705 at different MSG concentrations is shown in Figure (8). However, 2 % concentration of MSG was optimal for the formation of red, orange and yellow pigments (1424.8, 1692.4and 1442.4 AU/gdfs, respectively). Pigments production was gradually decreased with increasing of MSG concentration, this phenomenon may be due to that the excess level of MSG enhanced mycelium growth rather than pigments formation. However, Rashmi and Padmavathi, (2011) reported that maximum pigments production were observed by *M. purpureus* MTCC 410 when rice supplemented with 5% monosodium glutamate in solid state fermentation.

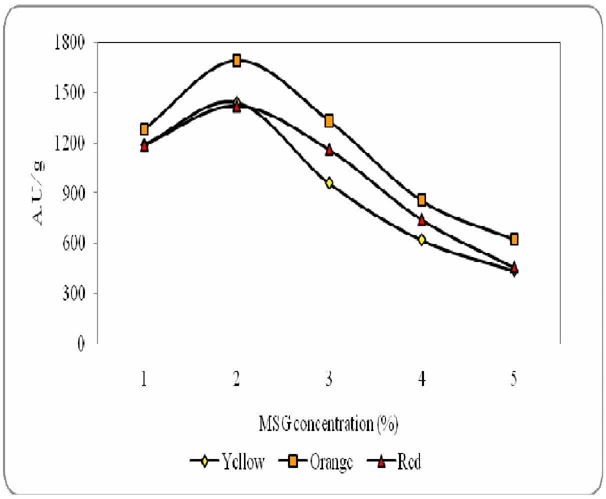

Figure 8. Effect of different concentrations of MSG on pigments production by *M. ruber* Went AUMC 5705 after 15 days of incubation at 30°C.

Application of *M. ruber* pigments as colorants of some Dairy products:

Attempts were carried out to investigate the applicability of the isolated red, orange and yellow pigments for coloring of flavored yoghurt and milk beverages. The red color was added to yoghurt and milk beverage flavored with strawberry, while the yellow color was added to milk beverage flavored with a banana during preparation of these products by the traditional methods. All dairy products were kept at 4°C for 3 days and then subjected to sensory evaluation by fifteen panelists. Texture, color, taste, odor and overall acceptability were evaluated using score card. Data in Table (1) indicated that all prepared dairy products colored with *M. ruber* pigments recorded high scores for all sensory evaluated parameters. The average total scores of yoghurt and milk beverage colored with red pigment recorded 87.84% and 87.44%, respectively. At the same time, the average total score of banana flavored milk beverage colored with yellow pigment was 83.88%. The average score of color property in all the studied dairy products as calculated percentages, ranged between 89.6 and 94.0 % which indicated that incorporation of the pigments into yoghurt and milk beverage showed uniform an d appealing color, appearance and distribution within the product. These results are in close agreement with that reported by Balnc, *et al.* (1995) who demonstrated that incorporation of Monascus pigments as coloring agent of food products improved the sensory attributes and increased the intensity and stability of the product. Also, Vidyalakshmi, *et al.* (2009) reported that utilization of red fermented rice in the preparation of Kesari showed a very good appearance and color. Furthermore, application of Monascus pigments promotes consumer's health benefits by decreasing the intake of salts (Su *et al.,* 2005).

Table 1. Mean sensory scores of dairy products colored with *M. ruber* pigments

No.	Name of product	Taste 20	Color 20	Odor 20	Texture 20	Total Acceptance 20	Total score 100
1	Strawberry flavored yoghurt (Red)	18.40	18.12	17.60	16.12	17.60	87.84
2	Strawberry flavored milk beverage (Red)	16.92	18.80	17.60	17.60	18.92	87.44
3	Banana flavored milk beverage (Yellow)	16.52	17.92	16.26	17.06	18.12	83.88

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انتاج صبغات طبيعية من فطر *Monascus ruber* بالتخمر الصلب لكسر الارز واستخدامها كمواد ملونة لبعض منتجات الالبان

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تهدف هذه الدراسة الى تعظيم انتاج الصبغات الطبيعية من فطر ***Monascus ruber* Went AUMC 5705** بواسطة تخمر الحالة الصلبة لكسر الارز لأجل إستخدامها فى تلوين بعض منتجات الالبان المنكهه كبديل آمن وصحى للصبغات الكيميائية الضارة بالصحة، وقد تحققت أقصى انتاجية من الصبغات باستخدام ظروف التخمر التالية: الرطوبة المبدئية لمادة التخمر 39% ، حجم جزيئات مادة التخمر 2- 2.5مم ، درجة الأس الهيدروجينى 6.5 ، حجم اللقاح 126× 10^4جرثومه/ 10 جم مادة جافة ، مدة التخمر 15 يوما ، درجة حرارة التخمر30 مْ مع استخدام احادى جلوتامات الصوديوم بتركيز 2% كأفضل مصدر للنيتروجين. مخلوط الصبغات الناتج تم استخلاصه من بيئة التخمر بواسطة الايثانول 95% وأمكن فصله الى ثلاث صبغات قابلة للذوبان فى الماء هى الصبغات الحمراء والبرتقالية والصفراء. وقد تم تطبيق استخدامها كعوامل تلوين عند انتاج اليوغورت ومشروبات اللبن المنكهه وأظهرت نتائج التقييم الحسى أن استخدام هذه الملونات الطبيعية قد أعطى درجة تقبل عالية لتلك المنتجات.