



## PRODUCTION AND APPLICATION OF NATURAL FOOD PIGMENTS BY *MONASCUS RUBER* USING POTATO CHIPS MANUFACTURING WASTES

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*This work was established for using potato chips manufacturing wastes as a substrate for the production of food colorant pigments by *Monascus ruber* Went AUMC 5705, then it was utilized individually for coloring some food products (drops sweets). The effects of different environmental and nutritional factors were studied in solid state fermentation (SSF). The maximum concentrations of red, orange, and yellow pigments produced by *M. ruber* were 147.3, 179.6 and 221.7 U/g of dry fermented materials, respectively. The optimum conditions for pigments production were determined to be 50%, 6.5, 3 % and 15 days, for the initial moisture content, pH, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and incubation period, respectively. Moreover, the optimum inoculum size, particle size of potato chips and fermentation temperature, were determined to be 81×10<sup>4</sup> spores/10g dry substrate, 1.5 mm and 30 °C under dark condition, respectively. Then, the pigments mixture was separated into red, orange and yellow water insoluble pigments that used as coloring agents of drops sweets flavoured with fruits odours. This study revealed that potato chips wastes are an innovative raw material for pigments production by *M. ruber* in SSF.*

### INTRODUCTION

Currently, there is worldwide interest in process development of pigments production from natural sources, owing to the highest used in foods as a coloring chemical compound. These synthetic compounds have been reported to have carcinogenic and teratogenic effects. This has been one of the major reasons for the increased interest in producing pigments from biological origin like microorganisms<sup>1</sup>. Microorganisms have become an important source for pigment production due to great growth rate, low-cost culture medium, and easy extraction procedure; all these offer more benefits for microorganisms than other biological resources<sup>2</sup>. The best known of the microbial pigments are produced by *Monascus* group, which belongs to ascomycetes class and monascaceae family can produce many

secondary metabolites such as pigments, monacolins,  $\gamma$ -aminobutyric acid, and dimeric acid<sup>3</sup>.

Bio-pigments produced by *Monascus* are regarded the most important, because these may be used as alternates for nitrites in meat products and for synthetic colors such as erythrosine (FD and C red No3). *Monascus* red pigments have been used as food colorant traditionally for hundreds of years predominantly in countries like China, Thailand, Korea and Japan and are produced by using long-established solid-state fermentation method<sup>4</sup>. It contributes to consumers favorite food colors, from orange-red to violet-red, nowadays used in food industry, and categorized into three main groups: yellow pigments (monascin, ankaflavin), orange pigments (rubropunctain, monascorubin), and red pigments (rubropunctamine,

monoscorubramine)<sup>5</sup>. They are synthesized as water insoluble. Aminophiles is unstable in extreme pH of the cytosol and multi-enzymatic polyketide synthase complex<sup>6</sup> becoming water soluble after being in contact with present amino acids. Their aminophile characteristic makes them to be associated with cellular proteins or with the cell wall, and this makes them difficult to be extracted. Therefore, their extraction requires cell damage and extraction in organic solvent like methanol<sup>7&8</sup>.

*Monascus* pigments can be obtained by both solid state and submerged culture. In solid state fermentation process, substrates not only supply the nutrients to the microbial culture growing but serve as harbor for the cells as well, leading to high pigment productivity. Moreover, it is cheap process uses agro-industrial residues as substrates<sup>9</sup>.

Many agricultural by-products such as corn cob<sup>10</sup>, sugarcane bagasse<sup>11</sup>, grape waste<sup>12</sup>, jackfruit seed<sup>13&14</sup>, corn steep liquor<sup>15</sup>, wheat bran<sup>16</sup>, and cassava<sup>17</sup>, were successfully used for the production of *Monascus* pigments.

Several starch by-products produced from numerous industrial processes, signify one of the most abundant carbon resources in nature. Using these resources in the cultivation process is a promising way to reduce the cost price of produced molecules particularly like antibiotics, enzymes, etc. These resources are more attractive to provide an inexpensive industrial substrate and contribute in solving pollution problems<sup>18</sup>. Many industries all over the world use potato as raw material, potato chips manufacturing industry is one of the most productions, which depends completely on potato, during chips handling, huge quantity of residue potato fragments with unsuitable size and shape are produced. These fragments consider as a waste and take away with very low price value to use as a feed component for animals and poultry<sup>18</sup>.

In this study, some of physical and nutritional factors for optimizing the pigment production from *Monascus ruber* Went AUMC 5705 were studied by using of potato chips industry wastes as a substrate in solid state fermentation (SSF) conditions. The obtained natural pigments were utilized as substitutes of synthetic pigment for coloring of some food products.

## MATERIALS AND METHODS

### Microorganism and inoculum preparation

A culture of *Monascus ruber* Went AUMC 5705 that has been used in this study was provided from Assuit University Mycological center (AUMC), Assuit, Egypt. It was maintained according to the method developed by Al-Bedak *et al.*<sup>19</sup>, and sub-cultured intermittently every three weeks. For inoculum preparation *M. ruber* Went AUMC 5705 was cultivated on YEPD slant agar in the dark at 30 °C under static conditions. To fully sporulated (6-8 days old) agar slope culture, 10 mL of sterile distilled water was added, and the spores were scrapped under aseptic conditions. The suspension spores were used as inoculum, described by Babitha *et al.*<sup>14</sup>

### Analysis of citrinin

The presence of citrinin was determined by thin layer chromatography as described by Rasheva, *et al.*<sup>20</sup>. Citrinin (Sigma-Aldrich, St. Louis, MO) was used as a standard.

### Fermentation medium:

Wastes of potato chips manufacturing (raw pieces) were obtained from a local factory of potato chips at Assuit Governorate, Egypt. After drying at 70°C, it was ground well to 1.0-2.0 mm particle size using an electrical mill and the produced potato flour (powder) was packed in polyethylene pages until use as the basal fermentation medium.

### Solid-state fermentation procedures:

Ten grams of potato powder was placed in a 250 mL Erlenmeyer flask and 2.0 mL of a nutrient salt solution (  $\text{KH}_2\text{PO}_4$ , 2;  $\text{NH}_4\text{NO}_3$ , 5; NaCl, 1; and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g/L) plus 1 mL zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.128 M) solution were added to each flask<sup>13&21</sup>. The potato powder was soaked in water at 30°C for 1h. Flask contents were mixed, then covered with two layers of aluminum foil to prevent moisture loss and autoclaved at 121°C for 15 min. After cooling to room temperature, each flask was inoculated with 1 ml spores suspension ( $7 \times 10^5$  spores/mL) and incubated at 30°C for 10 days in the dark. Unless otherwise indicated, these conditions were maintained throughout the experiment. All experiments were conducted in

triplicate and means  $\pm$  standard deviations were reported.

### Studying the optimization conditions for pigments production

The effect of initial moisture content (41%, 44%, 47%, 50%, 52% and 55% (V/W)) was adjusted by adding distilled water, incubation was for 10 days at 30°C in the dark. To study the effect of inoculation rate,  $18 \times 10^4$ ,  $27 \times 10^4$ ,  $36 \times 10^4$ ,  $45 \times 10^4$ ,  $54 \times 10^4$ ,  $63 \times 10^4$ ,  $72 \times 10^4$ ,  $81 \times 10^4$  and  $90 \times 10^4$  (Number of Spores /10gram dry substrate(gds)) spore suspension was added under aseptic conditions after sterilization and cooling. The initial pH value (4.5, 5.5, 6.5, 7.5 and 8.5) was achieved by adjusting the pH of the added sterile distilled water with 0.5M HCl or 0.5M NaOH. To study the effect of particle sizes, potato powder of different particle sizes was used to prepare different media, viz. M1 (particles  $\leq$  1.0 mm), M2 (particles  $\leq$  1.5 mm) and M3 (particles  $\leq$  2.0 mm). To study the effect of incubation period pigments were estimated after 5, 7, 9, 11, 13, 15, 17 and 19 days of incubation.

Studying the effect of different organic and inorganic nitrogen sources were evaluated such as monosodium glutamate, peptone, yeast extract, beef extract, ammonium nitrate, ammonium sulphate, ammonium chloride, potassium nitrate and urea at 1.0% concentration of each. The optimum concentration of the selected nitrogen source which stimulates pigments production was estimated by adding it at different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0%) to the production medium.

### Pigment extraction and quantification

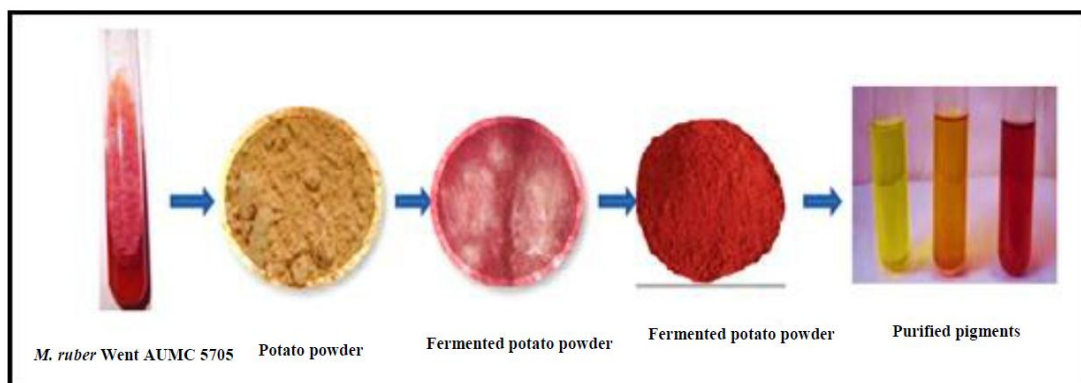
At the end of incubation period, the contents of each flask were dried on aluminum foil at room temperature and ground to a fine powder using an electrical mill. A 0.5 g of dried powdered fermented solids was extracted with 20 mL of 95% ethanol in an incubator shaker for 2 hrs at 180 rpm in a 100 mL Erlenmeyer flask. The extract was then centrifuged at 10000 rpm for 10 min to remove suspended solids. The supernatant was analyzed by a spectrophotometer using a 95% ethanol blank<sup>22&24</sup>. Pigments concentration was measured using a double beam spectrophotometer (UViline 9400-SCHOTT Instruments, EU) at 400, 470 and 500 nm for yellow, orange and red pigments, respectively, taking into consideration the dilution factor of the sample<sup>22&25</sup>. The results were expressed as absorbance unit (AU) per gram of dried solids<sup>26</sup>. The pigment absorbance was calculated using the equation:

The absorbance unit of pigments

$$(AUg^{-1}) = Abs \times \frac{20}{0.5} \times df$$

### Separation of pigments for application in the preparation of drops sweets products:

The mixture of red, orange and yellow pigments was separated and purified individually from the dried fermented potato powder as described by Abdel-Raheem<sup>27</sup>, to form separate water insoluble pigments ready to use as food colorants as shown in Figure (1).



**Fig. 1:** Separation of red, orange and yellow pigments individually from dried fermented potato powder culture

### Processing of drops sweets

Drops sweets were prepared by mixing sucrose (242.4 g/l), corn syrup (129.5 g/l), water (126.6 g/l) and citric acid (0.75g/l) then heated to 157 °C. Quickly, it was cooled to 110°C. Flavouring agent (1.05 g/l) and separated *Monascus ruber* pigments (which separated from dried solid-state culture) were added individually to produce red, orange and yellow drops sweets<sup>27</sup>. These contents should be mixed very well and put in forming blocks until it became solid, then packed in special foil.

### Sensory evaluation

Sensory evaluation was carried out by ten panelists. The panelists were asked to evaluate taste, colour, texture, odor and over all acceptability for prepared drops sweets according to the method described by Reitmeier and Nonnecke<sup>28</sup>.

### Statistical analysis

All the obtained data for the experiment were subjected to the statistical analysis of CRD (complete randomized design) according to Gomez and Gomez<sup>29</sup>. The significant means of any trait studied were compared using LSD at 5% probability level according to Waller and Duncan<sup>30</sup>

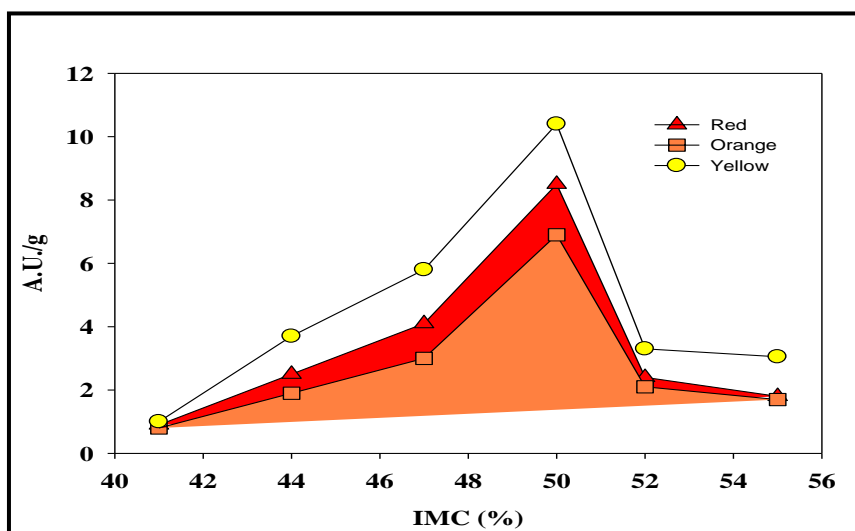
## RESULTS AND DISCUSSION

*Monascus* pigments know as traditional pigments with highly safety, richly nutrition and wide application have been widely used in Southeast Asia for thousands of years. Moreover, the application of *Monascus* pigments have been extended to modern food industry, for example, meat products, fish sauce and so on<sup>31</sup>. These pigments characterized by their wide applications, the flexibility of production and down streaming processes. In addition, it enhances the flavor of the food and acts a food preservative.

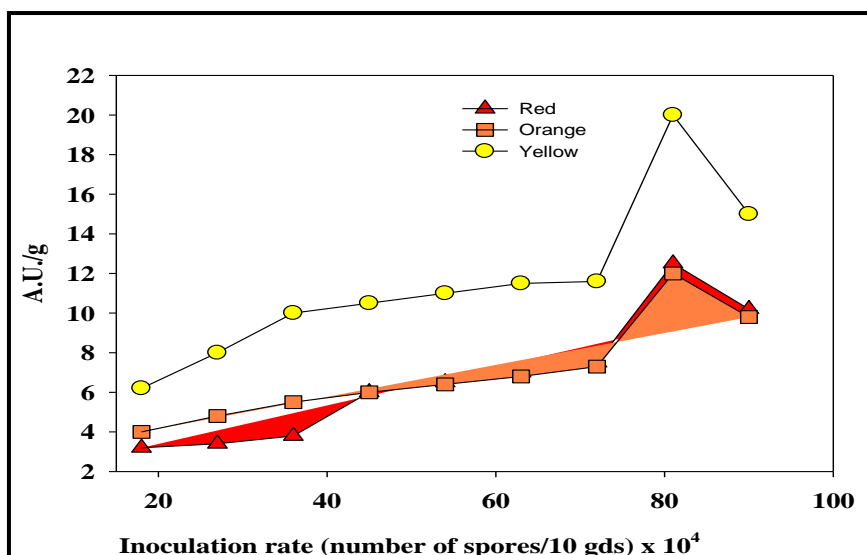
### Optimization of pigments production conditions

#### Effect of initial moisture content

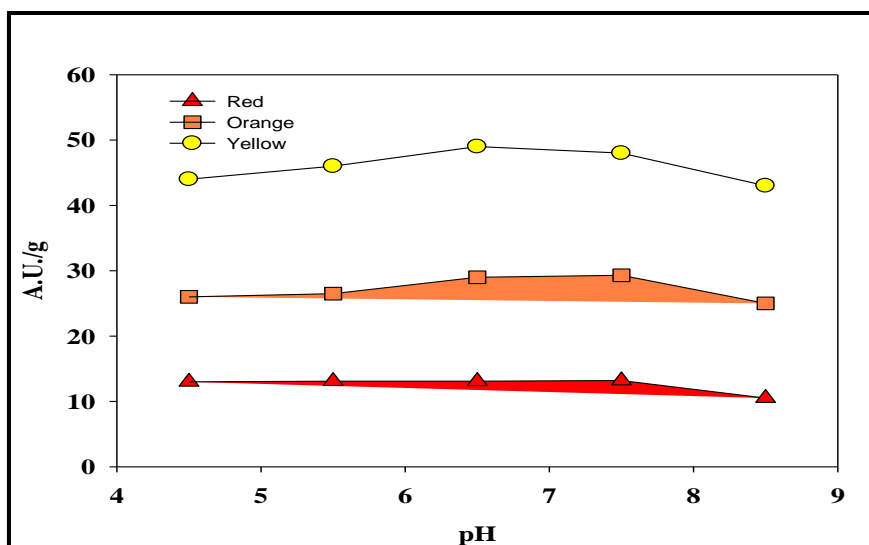
Among all 6 different moisture content tested for pigment production (Figure, 2). The results showed that *M. ruber* Went AUMC 5705 produced maximum amount of yellow, orange, and red pigments were 10.82, 7.56 and 9.14 AU/g, respectively at 50% moisture content of the substrate (Figure 2). Our results were consistent with Raimbault<sup>32</sup>, who reported that the optimal initial moisture content for pigments production tends to be in the range of 40 to 70%. Yongsmith *et al.*<sup>33</sup>, noticed that the moisture content of substrate is important in fungal growth, enzyme activity, and metabolites production in SSF this was concerned to effective utilization of sugars in the substrate.



**Fig. 2:** Effect of initial moisture content (IMC) on pigments production by *M. ruber* Went AUMC 5705 grown on potato wastes using solid state fermentation.



**Fig. 3:** Effect of inoculum sizes on pigments production by *M. ruber* grown on potato wastes using solid state fermentation.



**Fig. 4:** Effect of Initial pH on pigments production by *M. ruber* grown on potato wastes using solid state fermentation.

#### Effect of inoculation size

Figure (3) shows a peak increasing in yield of yellow pigment (20.50 AU/gds), followed by orange pigment (14.16 AU/gds), and red pigment (15.80 AU/gds) at inoculum size  $81 \times 10^4$  (spores/10 gds). The current results are widely varied from that described by other researchers<sup>10&14&34-35</sup>, they reported that, the optimal inoculum size for pigments production was 4 ml of spores ( $6 \times 10^5$  spores/ml)/g of initial dried substrate (gds). Babitha, *et al.*<sup>14</sup>, demonstrate that little inoculum caused insufficient biomass and smaller amounts of the product, whereas too much inoculum produced

excessive biomass and provided the essential nutrients for pigment formation.

#### Effect of initial pH

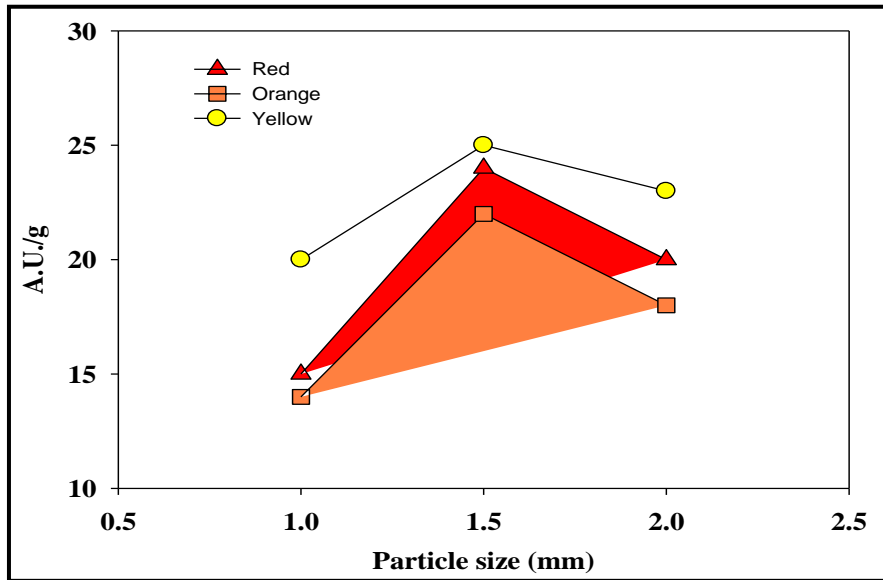
The best yield of pigments was obtained at pH 6.5 (15.12, 14.16, and 20.50 AU/g for red, orange, and yellow pigments separately). Production of yellow and orange pigments tends to fall in both acidic (pH 4.5-5.5) and alkaline (pH 7.5-8.5) values. Chen and Johns<sup>36</sup>, Lee *et al.*<sup>37</sup> and Joshi *et al.*<sup>38</sup> revealed that the suitable pH range for growth and production of *Monascus* pigments was 5.5-6.5 (figure, 4). This result was near to that of Babitha, *et al.*<sup>14</sup>, who described maximum pigments production by *M. purpureus* at pH 4.5 to 7.5, while using

jack fruit seed as substrate in solid state fermentation.

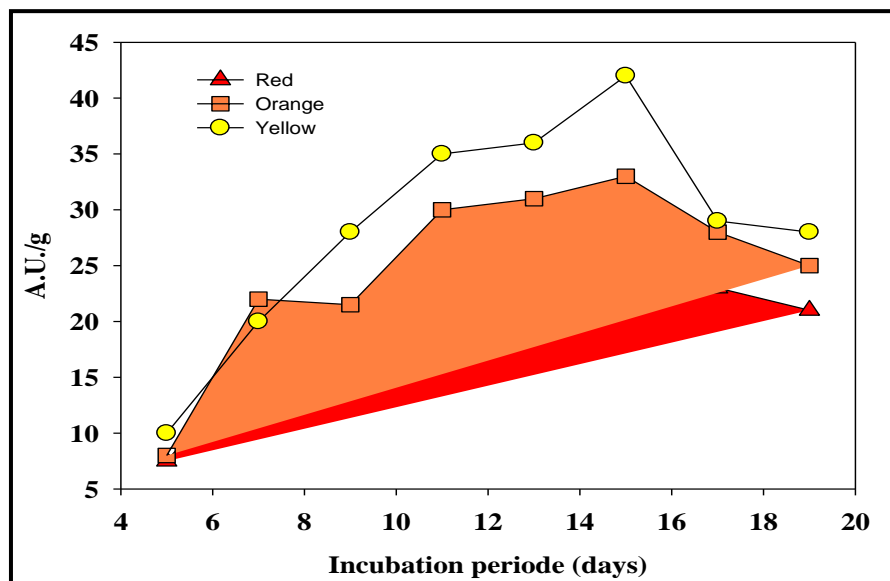
**Effect of potato particle size**

it has been noticed that 1.5 mm size of potato particles is the best size for producing yellow, orange and red pigments by *M. ruber* (Figure, 5). In the contrary of the present results, Babitha *et al.*<sup>13</sup>, found that *M. purpureus* LPB 97 produced the maximum pigments level on media consists of Jack fruit seeds with particles size between 0.4 and 0.6

mm. Usually, smaller particles of substrate offer a larger surface area for microbial dose, and thus it should be considered as a good factor. However, too small particles could result in substrate accumulation, which may affect in aeration which leads to poor microbial growth. At the same time, larger particles cause better aeration effectiveness but provide limited surface for microbial attack. Therefore, it may be necessary to provide suitable particle size<sup>34</sup>.



**Fig. 5:** Effect of particle sizes on pigments production by *M. ruber* grown on potato wastes using solid state fermentation.



**Fig. 6:** Effect of incubation period on pigments production by *M. ruber* grown on potato wastes using solid state fermentation.

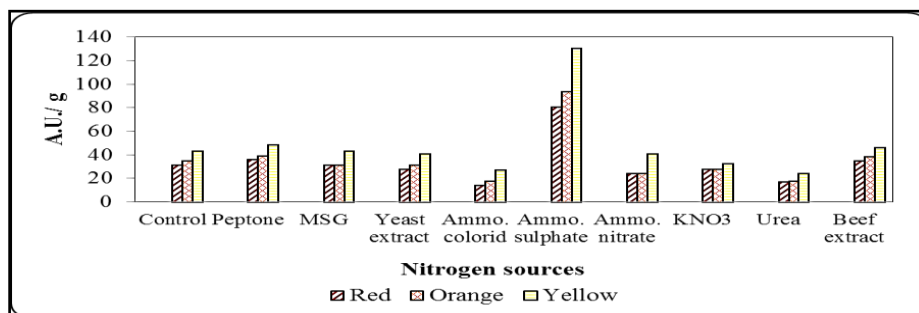
### Effect of incubation period

The concentration of pigments produced were increased gradually with increasing the incubation time (Figure, 6). Maximum yellow, orange and red pigments production was obtained after 15 days (43.48 AU/g, 34.94 AU/g and 31.24 AU/g, respectively). Results are in harmony with Emon *et al.*<sup>39</sup>, who noticed that the maximum concentration of pigments was obtained after 2 weeks of incubation by *M. purpureus* CMU001, grown on Korkor 6 white glutinous rice as substrate in solid state fermentation. The current results are rather differed from this described by Velmurugan, *et al.*<sup>10</sup>, they reported that the maximum pigments production was achieved by *M. purpureus* KACC 42430 within 7 days by using corn cob as substrate in solid state fermentation.

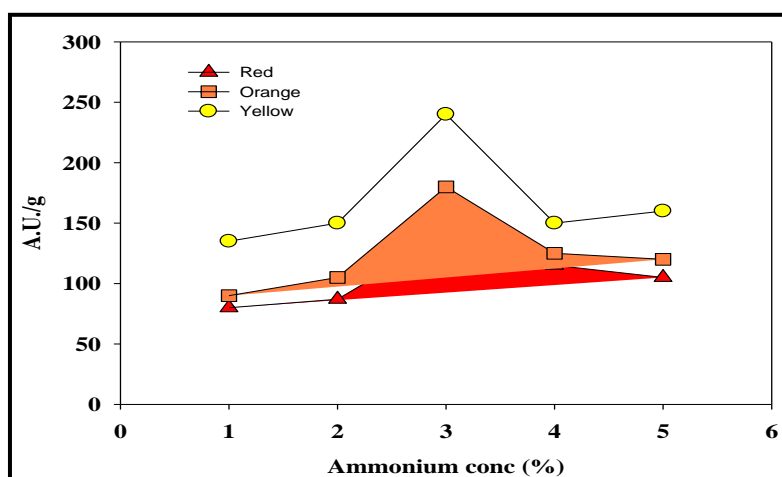
### Effect of nitrogen source

Nine sources of organic and inorganic nitrogen were separately studied at concentration of 1% to fermentation media. From the spectral analysis it has been observed that only ammonium sulphate showed a peak

effect which duplicated the producing amount of yellow, orange, and red pigments to reach 130.4, 93.72 and 80.36 AU/g, respectively when compared with control (Figure, 7). To estimate the optimum concentration of ammonium sulphate that was the best nitrogen source. We tested different concentrations of it (1, 2, 3, 4 and 5%). Data illustrated in figure (8) indicated that 3% ammonium sulphate proved to be the optimum concentration for maximum pigments production. The obtained color intensity of red, orange, and yellow pigments increased from 80.36, 93.72 and 130.40 AU/g to 147.30, 179.6 and 221.7 AU/g, respectively by increasing ammonium sulphate concentration from 1.0 to 3.0%. The higher concentrations (4 and 5%) of ammonium sulphate haven't the same stimulatory effect. Vidyalakshmi *et al.*<sup>40</sup>; Rashmi and Padmavathi<sup>41</sup>, reported that by using rice as production medium, addition of mono sodium glutamate (MSG) was the best nitrogen source, and the optimum concentration was 5%.



**Fig. 7:** Effect of nitrogen sources on pigments production by *M. ruber* grown on potato wastes using solid state fermentation



**Fig. 8:** Effect of different concentrations of ammonium sulphate on pigments production by *M. ruber* grown on potato wastes using solid state fermentation.

### Qualitative analysis of citrinin using thin-layer chromatography (TLC):

TLC analysis showed that no citrinin was detected in solid state culture extract. Hence the *Monascus ruber* used in this study was found to be safe for food use.

### Sensory evaluations of some food products

Produced pigments were extracted and separated from SSF *Monascus ruber* culture after optimization and utilized as colorants additives for the development of food products as described by Abdel-Raheam<sup>27</sup>. The food products namely red, orange, and yellow drops sweets were developed by adding the separated *Monascus* pigments individually. In the current study, *Monascus ruber* pigments directly mixed with the food products during their preparation to impart yellow, orange, and red pigments individually to these products (Figure 9) and improving the aesthetic value. The food products prepared using *Monascus* pigments as colorants were sensory evaluated for taste, color, texture, odor and over all acceptability by ten panelists.

Data in Table (1) shows the average sensory analysis scorecard and all scores for the separated *Monascus ruber* pigments as natural colors for the butterscotch flavored drops sweets. The overall average score for the red, orange, and yellow drops sweets samples were 45.9, 45.3 and 46.3, respectively. In general, all prepared products were recorded highly scores

in all sensory evaluated tested parameters as shown in table (1).

The range score for taste, color, texture, odor and over all acceptability were between 8.6 to 9.8 scored which describes as 'like extremely' for all studied food samples as described by Wang and Zhao<sup>42</sup>. The result shows that using of *Monascus ruber* pigments for coloring tested food products has proved to be excellent. The pigment dispersed evenly in the food samples which giving an attractive appearance. Besides the application of the natural pigment encourages consumers, health protection<sup>43</sup>.

These results were near to those reported by Blanc *et al.*<sup>44</sup>, who noticed that food samples show more powerful and stable red colour and improved organoleptic characteristics when *Monascus purpureus* pigment was used. Usually, red rice, red wine, sausages, fish sauces, meat products and soybean curd were prepared with these pigments. Vidyalakshmi *et al.*<sup>40</sup>, reported that *Monascus* fermented rice (MFR) when used as colorants in the preparation of food product (kesari), it displayed very good color and appearance. They also calculated the incorporation of MFR for colouring flavored milk, which showed an attractive color and appearance with better acceptability. Also, these results agree with the reports of other reserachers<sup>45-48</sup>.

**Table (1):** Mean sensory scores of red, orange, and yellow drops sweets samples colored with *Monascus ruber* pigments

No.	Name of product	Taste (10)	Colour (10)	Odor (10)	Texture (10)	Overall Acceptability (10)	Total score (50)
1	Red drops sweet	9.0 <sup>a</sup>	9.2 <sup>a</sup>	8.8 <sup>a</sup>	9.8 <sup>a</sup>	9.1 <sup>a</sup>	45.9 <sup>a</sup>
2	Orange drops sweet	8.6 <sup>a</sup>	9.4 <sup>a</sup>	9.0 <sup>a</sup>	9.0 <sup>a</sup>	9.3 <sup>a</sup>	45.3 <sup>a</sup>
3	Yellow drops sweet	9.5 <sup>a</sup>	8.9 <sup>a</sup>	9.1 <sup>a</sup>	9.3 <sup>a</sup>	9.5 <sup>a</sup>	46.3 <sup>a</sup>
L.S.D		NS	NS	NS	NS	NS	NS

Means with the same letters in the same column are not significant different at < 0.05 level of probability. NS = non-significant.



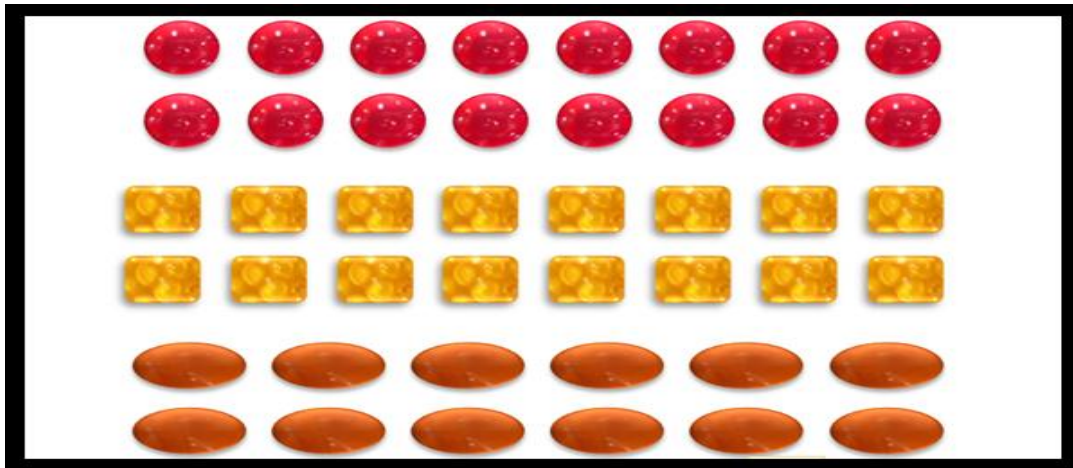


Fig. 9: The red, yellow, and orange drops sweets prepared by separated *M. ruber* pigments

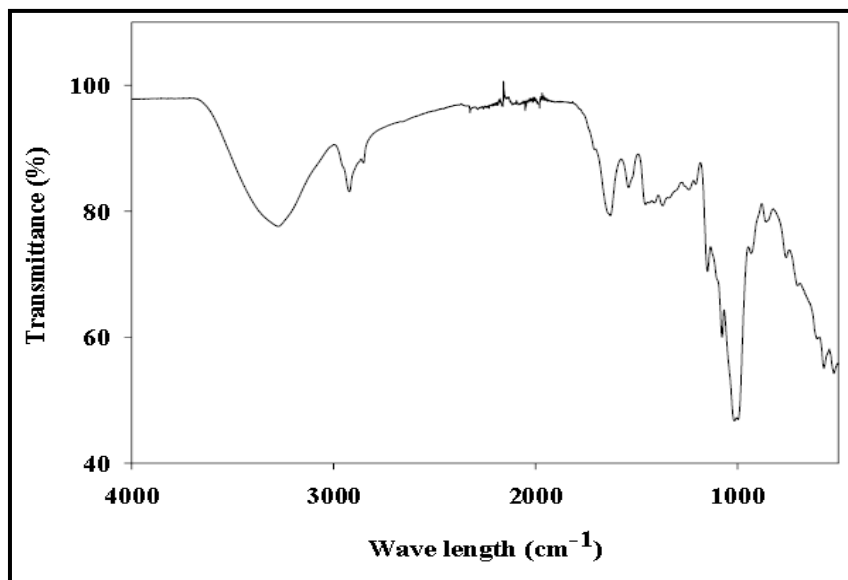


Fig. 10: FT-IR spectrum of *M. ruber* pigments

#### FTIR of *M. ruber* pigments

Fourier transform infrared (FTIR) for the *M. ruber* pigments was characterized by FTIR spectroscopy according to Hassan *et al.*<sup>49</sup>. The spectrum was illustrated in Figure (10). The absorption bands characterizing hydroxyl  $\text{OH}^-$  was detected at  $3200\text{--}3600\text{ cm}^{-1}$ , alkyl chains and CHO detected in the range  $2921\text{--}2851\text{ cm}^{-1}$ ,  $\text{C}=\text{O}$  at  $1648\text{--}1546\text{ cm}^{-1}$ , the band located at  $1100\text{--}1200\text{ cm}^{-1}$  may indicate to phosphate group  $\text{P}=\text{O}$ , the spectrum  $1210\text{--}1320\text{ cm}^{-1}$  strong intensity was attributed to the C-O link of the acidic groups. The bands in the range between  $1034$  and  $1075\text{ cm}^{-1}$  may be indicate to organic phosphate C-  $\text{PO}_2^{-3}$  groups affinity.

#### Conclusion

Overall, the best concentration of red, orange and yellow pigments were 147.3, 179.6 and 221.7 AU/g dry fermented material, respectively. The results revealed that potato chips wastes are an innovative raw material for pigments production by *M. ruber* Went AUMC 5705 in SSF. In addition, it is economic and environment friendly. In this study, optimization of nutritional and environmental conditions had a significant effect on the pigment production which raised *M. ruber* pigments concentration to 19.5, 22.8 and 15.1 times more than basal medium for yellow, orange, and red, respectively. The pigment was applied as coloring agent for conventional sugar confectionary (red, orange, and yellow drops sweets) it found to be greatly acceptable.

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## نشرة العلوم الصيدلانية جامعة أسيوط



### إنتاج واستخدام أصباغ الطعام الطبيعية التي تنتجها فطره *Monascus ruber* باستخدام مخلفات تصنيع رقائق البطاطس

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الغرض الأساسي من هذا البحث هو استخدام مخلفات تصنيع رقائق البطاطس كركيزة لإنتاج أصباغ تلوين الطعام بواسطة فطره *Monascus ruber* Went AUMC 5705، ثم تم استخدام هذه الصبغات بشكل فردي لتلوين بعض المنتجات الغذائية (الحلويات). تمت دراسة تأثيرات العوامل البيئية والتغذية المختلفة في حالة التخمير الصلبة (SSF). كانت التركيزات القصوى لإنتاج الأصباغ الحمراء والبرتقالية والصفراء بواسطة *M. ruber* ١٤٧.٣ و ١٧٩.٦ و ٢٢١.٧ وحدة/جرام من المواد المخمرة الجافة على التوالي. تم تحديد الظروف المثلى لإنتاج الأصباغ بنسبة ٥٠٪، ٦.٥، ٣٪ و ١٥ يوماً، للمحتوى الرطوبي الأولي، الأس الهيدروجيني،  $(NH_4)_2SO_4$ ، وفترة الحضانة على التوالي. علاوة على ذلك، تم تحديد حجم اللقاح الأمثل وحجم حبيبات البطاطس ودرجة حرارة التخمير لتكون ٨١ × ١٠<sup>٤</sup> جراثيم / ١٠ جم ركيزة جافة و ١.٥ مم و ٣٠ درجة مئوية تحت ظروف الظلام، على التوالي. ثم تم فصل خليط الأصباغ إلى أصباغ حمراء وبرتقالية وصفراء غير قابلة للذوبان في الماء تستخدم كموامل تلوين لقطرات الحلوى المنكهة برائحة الفواكه. كشفت هذه الدراسة أن مخلفات رقائق البطاطس هي مادة أولية مبتكرة لإنتاج الأصباغ بواسطة *M. ruber* في SSF.