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EFFECTS OF TEMPERATURE, pH AND LIGHT ON COLOUR STABILITY OF ETHYL ACETATE EXTRACT OF *TALAROMYCES PURPUROGENUS* LC128689 PIGMENTS

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ABSTRACT
Although natural pigments have potential applications in food, beverages, textile and pharmaceuticals industries, their instability during processing limits their applications. We have isolated *Talaromyces purpurogenus* which produces yellow, orange and red pigments in solid and submerged cultures. In the present study, the effects of temperature, pH and light on the colour stability of ethyl acetate extract of the pigments were evaluated. The results showed that the pigments were relatively stable when heated at temperatures of 60°C to 100°C for 30 minutes or autoclaved at 121°C for 20 minutes. However, during storage at room temperature, the colour intensity of heat-treated ethyl acetate extracts decreased gradually with increase in the treatment temperatures within the first three days of storage. The highest reduction in colour intensity was observed in the autoclaved pigments. Orange pigment was the most heat stable while red was the least. The yellow pigment was the most stable under yellow light while the orange and red pigments retained most of their colour under dark conditions. The yellow pigment lost 40, 42, 42 and 48% of its colour when stored under yellow, white, daylight and dark respectively while the orange and red pigments lost only 24 and 30% of their colour when stored in the dark for 12 days. The orange pigment was the most stable under all the light treatments. The pigments were very unstable at acidic pH (1~3). However, all the pigments were very stable when stored at pH values of 9 to 11.

KEY WORDS: *Talaromyces purpurogenus*, natural colourants, pigments, colour stability, fungi pigments.

INTRODUCTION

Natural pigments have been sought after as the most reliable and safe colourants for a variety of industrial applications. Natural colourants are preferred to synthetic ones for use in food, textile, pharmaceutical and cosmetics since they are safer for human health and are more environmentally friendly than the synthetic ones. Production and use of microbial pigments have continued to dominate other natural sources such as higher plants and animals because of the fast growth rates and diverse compositions and colours of pigments produced by these lower organisms. Filamentous fungi have been reported by many scientists as the leading microbial group in the production of natural colourants and dyes. As shown in Table 1, pigments produced by filamentous fungi are of diverse types with many potential applications. We have isolated a pigment producing fungus identified as *Talaromyces purpurogenus* based on the Internal Transcribed Spacer (ITS) gene sequence of the rDNA and have evaluated and confirmed its pigment production capabilities in solid state (Ogbonna *et al*., 2017), suspended shake flask (Ogbonna and Aoyagi, 2017) and liquid surface (Ogbonna, 2016) cultures. Our previous works have shown that this fungus has a great potential for large scale production of various shades of pigments, and thus has potentials for industrial applications. However, most of the industrial processes are carried out under various harsh/mild environmental conditions such as pH, temperature and light intensity. For instance in food processing industry, pasteurization and high temperature treatments are often employed at various stages of the process. Furthermore, food materials vary in their chemical compositions; some are neutral or acidic while others are alkaline and a lot of chemical reactions take place during food processing and storage. Textile dyeing is carried out at relatively acidic pH and high temperatures. Velmurugen *et al*. (2009) reported a temperature and pH of 70°C and 5.0 respectively to be the optimum for dyeing tanned leather. In pharmacy, drug formulations and processing can involve some extreme conditions. Unfortunately, the stability of most natural pigments varies with the pH and temperature of the medium where they are found. Light is another environmental condition that affects the stability of natural pigments. Many scientists have worked on the effects of light, temperature and pH on the stability of some natural pigments (Wang *et al*., 2013; Munier *et al*., 2014; HE *et al*., 2015) and some have also reported on how to stabilize some natural pigments (Cortez *et al*., 2017). However, most of the reports are on the stability of pigments extracted from higher plants and only a very few reports exist on the stability of fungal pigments. In the present study, the effects of temperature, pH and light on the stability of ethyl acetate extract of pigments produced by *Talaromyces purpurogenus* were investigated.

MATERIALS & METHODS

Microorganisms and media components

Talaromyces purpurogenus LC128689 isolated from a soil sample collected from cassava processing site in Abakaliki

Ebonyi State, Nigeria was used for pigment production. The fungus was identified based on the gene sequence of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA (Ogbonna *et al*., 2017). The fungus was maintained on PDA slant at 4^{o}C and sub-cultured once in every six weeks. All the media components used in this experiment were procured from Wako Pure Chemical Industries Ltd, Osaka, Japan unless otherwise stated.

Activation of the fungus

The fungus was activated by sub-culturing into potato dextrose agar (39g/L) test tube slants and incubating at room temperature (25 ±3^{o}C) for 7 days. The fully sporulated test tube slants were used for the experiments.

Pigment production in submerged shake flask cultures

The liquid medium was prepared according to the method of Ogbonna and Aoyagi (2017). The medium was composed of the following (in grams per litre of distilled water); sodium nitrate, 0.8; magnesium sulphate, 0.4; peptone, 15.2; and glucose, 25. The culture medium was dispensed in 100 ml aliquots into 500 ml Erlenmeyer flasks and sterilized by autoclaving at 121^{o}C for 15 min. Sterilized distilled water was used to harvested *T. purpurogenus* spores from 7 days old PDA test tube slants and diluted to a concentration of 8.32x10^{7}/ml. After cooling, each flask was inoculated with 4 ml of spore suspension containing the above spore concentration and cultivated at 30^{o}C on a rotary shaker at 200 rpm for 7 days.

Extraction of the ethyl acetate soluble components of the pigment

The culture broth was removed from the rotary shaker and filtered through either sodium acetate membrane filter or No 1 Whatman filter paper and centrifuged at 1500 rpm for 10 minutes to remove every fungal propagule. The supernatant was used for ethyl acetate extraction. The filtered culture broth (350 ml) was poured into a 500 ml separation funnel and 30 ml of ethyl acetate was added. The mixture was shaken vigorously to mix and allowed to stand for 30 minutes until the upper ethyl acetate soluble pigment was clearly separated from the insoluble lower aqueous layer. The lower aqueous layer was carefully emptied into a conical flask through the lower valve while the upper ethyl acetate soluble layer was collected into another clean conical flask. The same process was repeated 5 times until most of the ethyl acetate soluble components of the pigments were extracted.

Drying of the ethyl acetate extract

A total of 150 ml of ethyl acetate soluble component was extracted from the 350 ml culture broth. The pigments soluble in ethyl acetate were recovered by evaporation in a rotary evaporator. The rotary evaporator was set at a temperature of 50^{o}C and pressure of 90mHg and the ethyl acetate was evaporated to dryness. The dry extract was stored overnight in a desiccator impregnated with silica gel to avoid moisture absorption. The recovered dry extract was used in the stability experiments.

FIGURE 1. Submerged flask cultures of *Talaromyces purpurogenus*

Effects of temperature on colour stability of the pigment extract

The dried ethyl acetate extract (3.060mg) was weighed into a 10 ml beaker and solubilized in 5 ml of absolute ethanol. The solution was transferred into a 10.0 L glass beaker and 7.495 L of distilled water was added to give a final concentration of 0.408 mg/L. The initial pH of the extract solution was measured to be 3.2 using a pH meter (Hannah Instruments) and the initial colour intensity was determined by reading the optical density at 400, 460 and 500 nm for yellow, orange and red pigments respectively (Ogbonna *et al*., 2017). The solution was divided into three equal portions of 2.5 l into 5 L glass beakers. Each portion was used for temperature, pH or light treatments.

Temperature treatment procedures

The pigment solution was dispensed in 40 ml aliquots into eighteen (18) 100 ml brown reagent bottles. The bottles were grouped into six with three bottles per group. Each group was heated for 30 minutes at 60, 70, 80, 90 or 100^{o}C in a water bath while the last set of three bottles was autoclaved at 121^{o}C for 20 minutes. After the heat treatment, they were cooled to room temperature and each bottle was wrapped in aluminium foil and placed inside a dark wooden box to avoid light penetration. The temperature was maintained at 25 ±3^{o}C. Samples were withdrawn daily from each bottle to measure optical densities at 400, 460 and 500 nm for a period of 8 to 12 days.

Effects of light treatments on colour stability of ethyl acetate extract of *T. purpurogenus* LC128689 pigments.

The pigment extract was dispensed in 40 ml aliquots into twelve (12) 100ml reagent bottles. The bottles were grouped into four with three bottles per group. Each group was exposed to a particular light treatment *viz*: darkness; daylight; white or yellow florescent light. The bottles for daylight treatment were kept at the window seal where they were illuminated by sunlight throughout the period of treatment. The bottles under white and yellow lights were

illuminated by exposing them to white and yellow fluorescent lights respectively for the treatment period. The bottles for dark treatment were wrapped with sheets of alluminium foil and stored inside a dark wooden cupboard. Two hundred microliter (200µL) samples were withdrawn from each bottle daily to determine the colour intensity using a UV visible spectrophotometer (Shimadzu Model UV-1200) for a period of 8 to12 days.

Measurement of colour stability of the pigment extract at various pH

The pigment solution with an initial pH of 3.2 was dispensed in 40 ml aliquots into 36 100ml beakers. They were grouped in threes into 12 groups and the pH of each group was adjusted differently from 1.0 to 6.0 with 1N HCl and pH 7.0 to 13.0 with 1N NaOH. After the pH adjustment, they were transferred into labeled 100ml reagent bottles. The bottles were wrapped with sheets of aluminium foil and stored inside dry dark cupboard. Samples were withdrawn daily to determine colour intensity for a period of 8 to 12 days.

Data Analysis

All the experiments were done in triplicates (or else stated) and the results were expressed as the mean ±standard deviation. The data were also subjected to Analysis of Variance (single classification) and where there were significant differences at 5% probability, Least Significant Difference (LSD) was used to separate the means.

RESULTS & DISCUSSION

Effect of temperature on pigment colour stability

Figure 1 shows the changes in the colour intensity of the culture broth during sub-merged cultivation of *T. purpurogenus.* As shown in Figure 2, the intensity of the three colour components of *Talaromyces* pigment decreased slightly following the treatment temperatures (60-121^{o}C). There was a progressive decrease in colour intensity with increase in temperature. This was in agreement with the work of Devi *et al*. (2011) who reported that the colour of anthocyanins extracted from red sorghum bran decreased with increase in storage temperature. The highest percentage reduction in colour intensity for *T. purpurogenus* LC128689 pigments was recorded in the batch of pigments exposed to 121^{o}C (autoclaved). Among the three colour components, orange was the most heat stable while red was the least. It is interesting, however, to note that after the initial decrease, all the colour components remained relatively stable during storage at room temperature (Figure 2). This shows that the pigments can be used for pigmentation processes that involve heat treatment for about one hour. Poorniammal and Gunasekaran (2015) reported that the yellow pigment from *Thermomyces* was stable at very high temperatures ranging from 100 to 300^{o}C while according to Munier *et al*. (2013) two algal pigments (B-phycoerythrin and R-phycoerythrin) responded differently to temperature. R-phycoerythrin was more sensitive to temperature than B-phycoerythrin when stored at 60^{o}C.

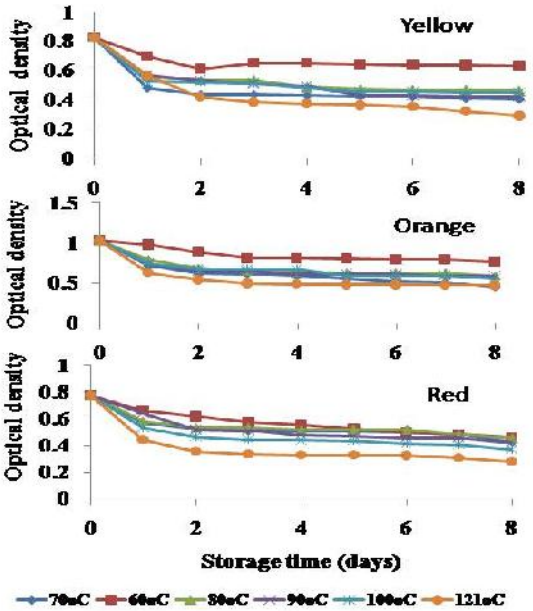

FIGURE 2. Effects of heat treatment on stability of yellow orangeand red pigments produced by *Talaromyces purpurogenus*. The pigments were extracted with ethyl acetate heated to the indicated temperatures for 30 minutes and stored at room temperature. The colour intensity was measured spectrophotometrically every day for a period of 8 days.

Effects of exposure to light on pigment colour stability

The results of the effects of storage at different lights on the colour stability of the *T. purpurogenus* LC128689 pigments are shown in Figure 3. The initial optical densities of the yellow, orange and red pigments were 1.031, 0.653, and 0.776 UOD, respectively. There were initial decreases in the intensity of the yellow, orange and red pigments during storage after which the colours stabilized. Most of the decreases in the colour intensity were observed within the first three days after which the colours remained relatively stable. At the end of storage, there were 42%, 48%, 40% and 42% loss in the intensity of the yellow component of the pigment when stored in day light, dark, yellow and white lights respectively. This

shows that storage under yellow light was the best for the yellow pigments while the decrease was most pronounced when stored in dark. In the case of orange and red pigments, the stability was highest when stored in dark, resulting in only 24% and 30% loss, respectively at the end of the storage. On the whole, the orange pigment was the most stable under all the types of light studied. This is in contrast to the findings of Mappari *et al*. (2009) who reported that the yellow component of an orange-red pigment produced by *Epicoccum* was more photostable than the orange and red components. The red pigment was more stable when stored in dark than under lights. Jung *et al*. (2005) reported that the colour of *Monascus* pigment obtained by fermentation using different amino acids showed equal stability under UV light (365 nm) and after exposure to sunlight. On the other hand, Poorniammal and Gunasekaran (2015) reported that a yellow pigment produced by *Thermomyces* sp. was more stable under fluorescent light than sunlight.

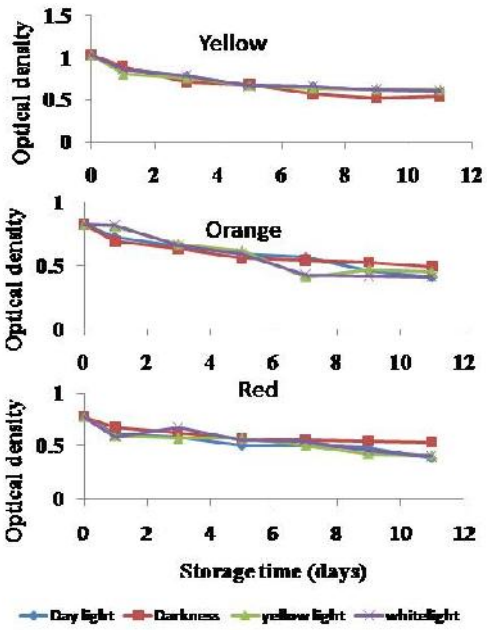

FIGURE 3. Effects of storage under light or dark condition on the stability of ethyl acetate extracts of yellow, orange and red pigments produced by *Talaromyces purpurogenus.* The ethyl acetate soluble components of the pigments were extracted, dissolved in distilled water and stored under various light and dark conditions.

HE *et al*. (2015) reported that anthocyanins extracted from purple sweet potato were stable at pH values ranging from 2.0 - 6.0 when stored in dark for 30 days at 20^{o}C while the color of purple sweet potato (PSP) extract remained stable during 30 days of storage at 20°C in dark. HE *et al.* (2015) reported that anthocyanins extracted from purple sweet potato were unstable when exposed to UV and florescent lights. However, UV light had a more drastic effect on the colour of the pigments than fluorescent light. Woo *et al.* (2011) reported that light was a major factor that brought about degradation of betalain pigments extracted from red dragon fruits. They further reported that the colour of betalain pigment could be maintained for three weeks if stored at 4^{o}C without exposure to light. Munier *et al*. (2014) studied the effects of light on the stability of two pigments produced by two different red algae. The pigments (B-phycoerythrin and R-phycoerythrin) responded differently when exposed to light for 48h. After 48h exposure to light only 30% of the B-phycoerythrin concentration was lost while R-phycoerythrin lost as much as 70 ±1% of its concentration. These results show that the stability of pigments during storage depends on both the type of pigment and on the nature of light.

Effects of pH on the stability of *Talaromyces* pigments

The pigments, especially the yellow and orange components were very unstable at acidic pH (1~3) as shown in Figure 4. However, all the pigment components were very stable when stored at alkaline pH (9-11). Increasing the alkalinity above 11 resulted in decrease in the stability of the pigments. This is in agreement with the work of Poorniammal and Gunasekaran (2015) who reported that the yellow pigment produced by *Thermomyces* sp. lost most of its colour when stored at pH 3.0. According to Poorniammal and Gunasekaran (2015) the yellow pigment from *Thermomyces* had its optimum storage pH between 5.1 and 8.0. Wongjewboot and Kongruang (2011) reported that the yellow, orange and red pigments produced by mutation induced Thai isolate of *Monascus purpurogenus* TISTR 3002 were most stable at pH8.0. They further reported that the pigments produced by all the strains of *M. purpureus* TISTR 3002 generated through mutation were more stable at alkaline than at acidic pH values. However, our result on pH stability differs from the results of a work done by Jenshiroobha *et al.* (2011) who reported that the optimum stability of anthocyanin extracted from *Musa acuminata* bract was between pH5.1 and 6.0 when stored at the temperature of 20^{o}C and 30^{o}C respectively both in the presence and absence of light. On the other hand, HE *et al.* (2015) reported that anthocyanins extracted from purple sweet potato were stable at pH values ranging from 2.0 to 6.0 when stored in dark at 20^{o}C for 30 days. Wang *et al.*

(2013) studied the effects of pH, light, temperature, metal ions and ascorbic acid on the stability of anthocyanins from the skin of black peanuts and reported that the appropriate conditions for extracting anthocyanin from the skin of black peanuts (*Arachis hypogaea* L.) were pH 2.0 at a temperature of 60°C. Some pigments are more stable at low (acidic pH) than under alkaline condition and the colour of such pigments is red at such low pH (Wahyuningsih *et al*., 1997**).** Munier *et al.* (2014) also reported that the algal pigments (Phycoerythrins) were stable between pH 4- 10 but the pigments were degraded at a very alkaline (pH 12). One of the algal pigments (R-phycoerythrin) lost 90% of its concentration at pH 2 while B-phycoerythrin lost only 40% of its concentration at the same pH. The above results have also shown that the effects of pH on the stability of pigments depend to a very great extent on the nature of the pigment.

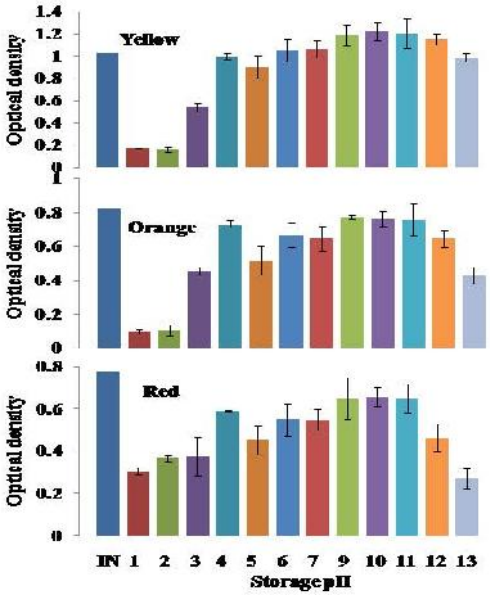

FIGURE 4. Effects of p H on stability of yellow, orange and red pigments produced by *Talaromyces purpurogenus.* The pigments were extracted with ethyl acetate, the pH adjusted to 1~13, stored for 8 days and the optical densities measured. IN denotes the initial optical density before pH adjustment and storage.

TABLE 1: Some Genera and species of filamentous fungi and the type of pigments they produce

Strain	Type of pigments	Uses	References
Talaromyces purpurogenus	Yellow, orange and red	Food processing, textile dyeing	Ogbonna *et al*., 2017,
Talaromyces spp	N-threoninerubropunctamine,	General purpose	Lebeau *et al.*, 2017
Talaromyces verruculosus	Not stated	Textile dyeing	Chadni *et al*., 2017
Talaromyces verruculosus			Devi *et al*., 2017
Talaromyces atroroseus CBS 133442	red diffusible pigments	Not stated	Frisvad *et al*., 2013
Monascus spp.	Not stated	Not stated	
Neurospora intermedia	Not stated	Not stated	Gmoser *et al*., 2018
Lecanicillium aphanocladii (CML2970)	Oosporein	Not stated	Souza *et al*., 2016
***Aspergillus terreus* KMBF1501.**	Not stated	Industrial application	Akilandeswariand Pradeep, 2017
Fusarium spp.	Not stated	Industrial application	Souza *et al*., 2016
Penicillium flavigenum (CML2965)	dihydrotrichodimerol	Industrial application	Souza *et al*., 2016
Epicoccum nigrum (CML2971	Orevactaene	Industrial application	Souza *et al*., 2016
Penicillium aculeatum ATCC 10409	Yellow pigments	Not stated	Afshari *et al*., 2015
Monascus purpreus ATCC1603	Pigments	Not stated	Baneshi *et al*., 2014
Monascus purpreus ATCC1603	Pigments	Not stated	Baneshi *et al*., 2014
Monascus ruber	Pigments	Not stated	(Bühler *et al*., 2015
Monascus sp strain M9	Pigments	Not stated	Wang *et al*., 2015
Monascus ruber	red pigments	Not stated	Meinicke *et al*.,(2012)
Monascus ruber CCT 3802	Orange pigments	Not stated	Vendruscolo *et al*., (2017)

CONCLUSION

Ethyl acetate extracts of *Talaromyces purpurogenus* pigments are relatively stable when heated at temperatures of 60 to 100^{O}C for 30 minutes or autoclaved at 121°C for twenty minutes, showing that the pigments can be used as colourants for food that requires heat treatment. They were also relatively stable under light and dark condition, and were very stable at a wide range of pH but most stable at alkaline pH values. Thus, *Talaromyces purpurogenus* pigments have wide potential applications.

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