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Production and application of natural food colourant from *Thermomyces* sp

R. Poorniammal*, S. Gunasekaran[1] and A.R. Sakthi[2]

Krishi Vigyan Kendra, TNAU,
Kovilangulam, Aruppukottai -626 107, India.

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ABSTRACT

This paper presents a small scale batch technology for production of natural extract which can be utilized as food colourant. This technology was designed based on the laboratory tests performed on *Thermomyces* sp; it allows very simple way for production of valuable product for food industry which acts as an antioxidant, antimicrobial and food dye. The pigment was identified using combination of UV-visible spectral data, HPLC and FT-IR as a quinone pigment with ë max at 424nm. After extraction and purification the colourant was suspended in water for evaluation. The stability of the pigment was tested in both solutions and when incorporated in food products. *Thermomyces* sp pigments was applied to a few food products like cookies, rice wine, orange squash and guava jelly. Sensory tests inferred that orange squash, cookies and rice wine had recorded high acceptability followed by guava jelly.

Key words: Fermentation, Food products, FT-IR, HPLC, Natural dye, *Thermomyces* sp.

INTRODUCTION

With globalization in the research trends, healthier life styles, and the growing market for the natural food colourants in the economically fast-growing countries all over the world, filamentous fungi are being investigated as readily available sources of chemically diverse colourants. With two selected examples, polyketide-*Monascus*-like pigments from the new fungal production strains, and the promising and yet unexplored hydroxy-anthraquinoid colourants (Dufossé, *et al.*, 2014). Natural colourants are considered to be safer than synthetic ones, and their applications in foods, cosmetics and pharmaceuticals are growing rapidly (Cho *et al.*, 2002). There are a number of natural pigments, but only a few are available in sufficient quantities for industrial production. Production of pigments from microorganisms is advantageous over other sources because microorganisms can grow rapidly which may lead to a high productivity of the product (Kim *et al.*, 2003).The pigments from microbial sources are a good alternative that can easily be produced in high yields and capability of producing different coloured pigments. Pigment producing microorganisms and microalgae are quite common in nature. Pigment produced by microorganisms includes carotenoids, melanins, flavins, quinones and more specifically monascins, violacein, phycocyanin or indigo. However, there is a long way from the Petri dish to the market place as only five productions are operated on an industrial scale. Fungi have a higher advantage among microbial sources of pigments over the other organisms. Fungi can be scaled up in fermentor and because of higher growth rate the yield is also comparatively high and extraction of pigments from fungi is also easy whether it is extracellular or intracellualr in nature (Mapari *et al.*, 2005, 2009). The fungal pigments are intended to substitute currently used synthetic colourants and / or natural colourants derived from plant materials. In addition, some of the coloured pigments produced by the fungi can expand the current palette of colours used in various industrial applications. The present paper highlights exciting recent findings, which may pave the way for alternative and/or additional biotechnological processes for the industrial production of natural food colourants of improved functionality and an original small scale, batch type technology for production of natural pigment which can be utilized as natural food dye.

MATERIALS AND METHODS

Organism and cultivation: The microorganism used in this study was *Thermomyces*, which was isolated from soil. Stock cultures were maintained on potato dextrose agar slants, which contain potato extract and dextrose and sub cultivated periodically. The slants were incubated at 28 ± 2 ^{0}C for 7 days. After cultivation of 5-7 days, spores were collected with 5 ml sterilized water, and the spore suspension collected was

Corresponding author's e-mail r.poornii@gmai.com. [1]Dept of Agricultural Microbiology, TNAU, Coimbatore 641003, India. [2]Dept of Plant Molecular Biology and Biotechnology, TNAU, Coimbatore 641003, India.

used as inoculum preparation. 0.5 ml of spores suspension was inoculated in 50 ml of submerged culture medium in 250 ml Erlenmeyer flasks, whose ingredients include yeast extract-5.0, sucrose- 30, $NaNO_3$-3, KCl- 0.5, K_2HPO_4-1, and $MgSO_4$-1 The submerged culture medium (initial pH 6.0) was cultivated at 28 ± 2 ºC for 6–7 days in a incubator.

Extraction and purification of yellow pigment from *Thermomyces* sp culture: Czapek yeast broth was prepared and *Thermomyces* culture was inoculated and incubated as stationary cultures for 5 days. After incubation the grown up culture was filtered to separate the fungal biomass from the broth. The supernatant broth was filtered using a sterilized muslin cloth to remove the fungal mat. To the filterate, one volume of 95% (v/v) methanol was added and kept on a rotary shaker for 30 min at 150 rpm at 35°C and was centrifuged at 5000 rpm for 15 min. The same process was repeated for removal of fungal biomass and the filterate was filtered through a preweighed Whatman filter paper (47 mm). Next, the absorption spectrum was observed at 300–600 nm using Hitachi U-2000 spectrometer (Hitachi Ltd., Tokyo, Japan.) The purified pigments were concentrated in a buchi rotary evaporator and lyophilized (Make- Lyobeta 35))to obtain the yellow pigment in a powdered form.

Purification of yellow pigment from *Thermomyces* sp culture

Absorption spectra of yellow pigment: The analysis of pigment production was done by measuring absorbance maxima of pigment extract by spectral analysis using a double beam spectrophotometer from UV to visible range to identify the components at 500nm (Shimadzu, UV1601).

HPLC analysis (Mantzouridou and Tsimidou, 2007): The analyses of the yellow colour bands were done in HPLC – Hitachi model L 6200 equipped with LC8A pump, SPD-M 10A ãp photo diode array (PDA) detector C18 analytical column (C_{18}, 5ì size, 250 x 4.6mm (Supelco)) at 40 °C in combination with class LC 10A software and Beckman Ultrasphere supelco ODS column (250 x 4.6 mm) was used. The mobile phase consisted of Acetonitrile : Water (75:25) at a flow-rate of 1.0 ml/min. Detection was performed at 400 and 500 nm, and UV–Vis absorption spectra of the pigments were recorded on-line with the photodiode array detection system.

Fourier transform infrared spectroscopy (FTIR) analysis: To confirm the structure of fungal pigments, FT-IR spectrometer (Impact 400D, Nicolet , Madison,WI) was used to measure the infrared spectra of extract solution in the wave number of 400-4000 cm^{-1} at room temperature. For each IR spectrometer samples 32 scans at 4 cm^{-1} resolution was collected in the transmittance mode.

Developing food products using fungal food pigment : For technological tests on the *Thermomyces* sp, four products were prepared with the following procedures.

Cookies: Flour and baking powder were sieved, fat and sugar creamed. Egg white was beated and added to creamed mixture along with 1% yellow colour and vanilla essence. The dough was prepared by adding required quantity of milk. After preparing dough, small balls were prepared and kept on the tray. Cashew nuts were sprinkled and kept on the top and baked at 175°C for 20 min.

Orange squash: Matured and sound fruit was selected and washed in cold water to remove dust and dirt. The fruit was cut into two halves and juice was filtered through the muslin cloth. Syrup was prepared with required amount of water and sugar and citric acid was added and filtered. The syrup was allowed to cool and mixed with juice. Preservative was dissolved in little amount of boiled lukewarm water and added to the squash. The squash was mixed thoroughly and 0.25 % yellow colour was added. The prepared squash was poured in to sterilized bottle leaving on the top and covered tightly. Labeling was done with needed information. One part of prepared squash was mixed with four parts of water before consumption.

Rice wine: Rice was weighed and washed to remove impurities and soaked in water for 15 min. Water was added twice as the volume of rice, steamed and cooled. Culture of *Aspergillus oryzae* and *Rhizopus oryzae* was inoculated and left for aerobic fermentation for 10 days. To this *S. cerevisiae var ellipisoides* was inoculated and kept for anaerobic fermentation for a week. The content was filtered to remove waste. The rice wine obtained was kept for clarification with fraction filtering. To this rice wine 0.5 % yellow colour was added and stored.

Guava Jelly: Guava fruits were washed and cut into thin slices and cooked with water until it becomes very soft. This mixture was placed in a coarse cloth and the juice was allowed to drip through into a bowl underneath and left for 12 h. The bag should not be squeezed. The juice was measured and 3 cups of sugar was added to each pint of juice. The mixture of sugar and guava juice was heated and stirred until it is dissolved. Lemon juice and 1% yellow colour were added to the strained juice and cooked on a hot fire until setting point was reached. After it becomes cool, jelly was poured into airtight jars.

Colour determination: The colour of the food products were estimated from their absorption maxima. The same extracts were then used for determining CIELAB colour coordinates using Lovibond tinometer (Minolta CT 310, Konica Minolta,

Mahwah, USA). The CIELAB colorimetric system was interpreted as follows: L* indicates lightness read from 0 (black) to 100 (white). A positive a* value indicates red while a negative a* value represents green. Similarly positive and negative b* values indicate yellow and blue, respectively. Chroma values denote the saturation or purity of colour. Hue angle values represent the degree of redness, yellowness, greenness and blueness the maximum being at 0, 90, 180, and 270, respectively **(**Mapari *et al.*, 2006).

Sensory evaluation of the products: The products prepared were evaluated for the colour, appearance and flavour, texture and taste of the products.

RESULTS AND DISCUSSION

In recent years, production of natural food colourants through microbial fermentation is an extensive area of investigation, since they overcome concerns of unfavourable side effects of synthetic colours. The present study forms and proves a scientific rationale towards selection filamentous fungi as sources of natural colourants considering the enormous biodiversity of fungi. Production potential of a few of the selected pigment producers as promising fungal cell factories for the future production of natural colourants by "green chemistry" concept avoiding use of genetic manipulation

A pioneering research was carried out on the application side of an yellow pigment isolated from a fungus *Thermomyces* sp. Among the fungal cultures isolated from soil samples collected from various parts of Tamil Nadu. From the isolated cultures the *Thermomyces* sp was selected due to its attractive yellow colouration and the culture was identified and authenticated by ITCC, division of plant pathology, IARI, New Delhi. The authenticated *Thermomyces* sp culture was grown in czapeks yeast extract broth harvested to get the clear broth having yellow pigment was obtained in powder form by air drying method (Fig 1).

Based on the colour stability the *Thermomyces* sp was screened. The first and the foremost requirement towards selection of potential pigment(s) producers is that the potential fungus producing the pigment(s) is non-toxic and is non-pathogenic to humans. Toxicological studies were performed on the *Thermomyces* pigment using albino mice. The result showed that due to intake of *Thermomyces* sp pigment for 28 days did not alter the Hb, RBC, WBC, organ weight and histopathology. The *in vivo* antioxidant activity of the pigment was also confirmed. *Thermomyces* sp. can be safely applied to food and the colour remains longer (Poorniammal *et al.*, 2011, 2014).

UV-visible absorption spectra of fungal pigments are of immense importance, since they aid a great deal in determining structure of fungal pigment. The pigment was completely extractable in methanol. The UV-visible absorption spectra of the pigment showed absorption maxima at 410 nm (Fig 2).

HPLC analysis of carotenoids produced by *B. trispora* indicated the presence of three compounds, lycopene, ã-carotene and â-carotene (Mantzouridou and Tsimidou, 2007; Mapari *et al.*, 2008). Barbu *et al.* (2006) reported that HPLC analysis of *E. nigrum* contains glycosylated pigments *viz.*, glycosylated flavanoids and rhodoxanthin which were specific to polar groups and phenoilc acids which were specific to free form in retention time of 13-15 min. In the

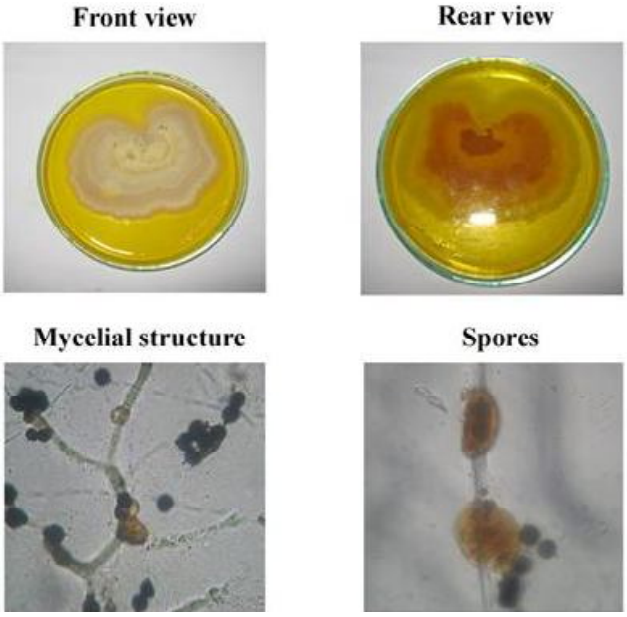

FIG 1: Growth of *Thermomyces* sp pigments in selid media.

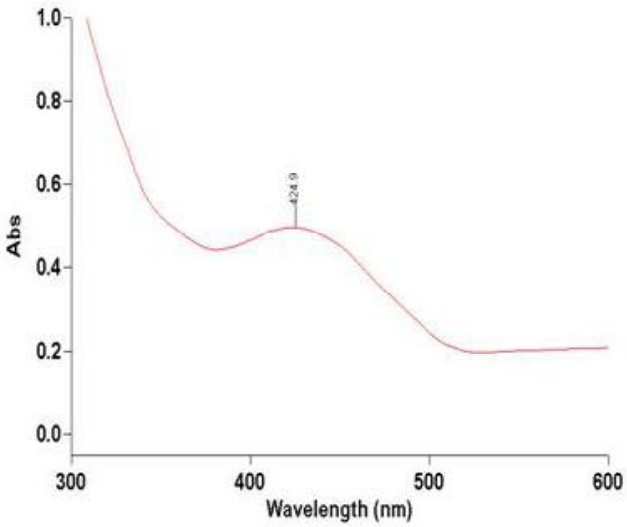

FIG 2: UV-Visble spectrometnic scanning of *Thermomyces* sp pigments

present study, using HPLC the methanolic pigment extract of *Thermomyces* sp was analyzed. The HPLC profile of Thermomyces sp pigment can be ascertained from Fig 3. All major peaks in fig 2 could be tentatively identified on the basis of their absorption spectra. The principal pigment of *Thermomyces* sp was found to be quinone pigment.

FT-IR spectroscopy has widely been used for the characterization and identification of fungi, bacteria and yeasts which are hydrophilic microorganisms and can easily be suspended in water for sample preparation (Fischer *et al.*, 2006). In this study, the infra red spectra showed the presence of hydrogen bonded OH groups (3300 – 3400 cm^{-1}) and of the carbonyl function (Fig 4). The carbonyl stretching vibration frequency of the pigments is in the region 1635 - 1639 cm^{-1}. The observed stretching frequencies are however close to phenol and quinones. Similar results were also reported by Somasundaram *et al.* (1986) in *Thermomyces langinosus.* The chemical properties of the pigments in the thermophilic fungi were similar to those of aphins (Thomson, 1992). The hydroxylated quinoid pigments were found in some insects (aphids). The protaphin and xanthophin were yellow (lambda max, 270, 360 and 460 nm) with infra red bands around 3470, 1670, 1613 and 1590 cm^{-1}. It is readily transformed to xanthophin which was also yellow in colour with absorption bands in the 320 - 450 nm region.

Product development with *Thermomyces* sp: Concentrated yellow pigment extracted from *Thermomyces* sp. was utilized as colour additive for the development of food products. To enhance the appearance and acceptability of foodstuffs yellow pigment was added. The products namely cookies, rice wine, jelly and orange squash were developed by adding the pigment. The colour value of food products was analyzed. In the current study, *Thermomyces* sp pigment was added to the common food products such as cookies were prepared

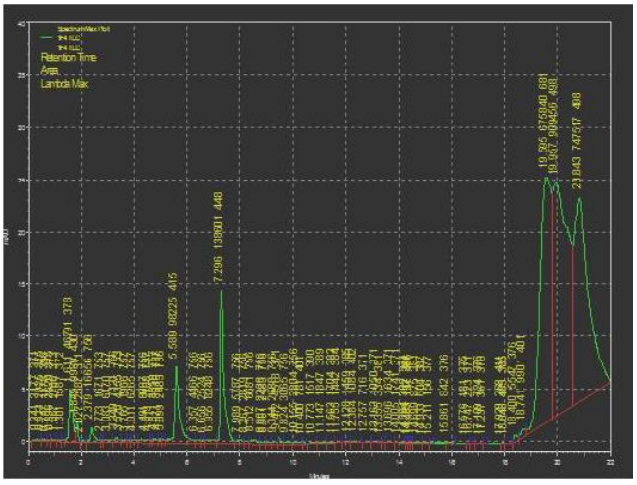

FIG 3: HPLC fractionation of crude extract of *Thermomyces* sp

TABLE 1: Application of *Thermomyces* sp pigment to different foods

Colour values	Cookies		Rice wine		Orange squash		Guava jelly	
	Control	Coloured product	Control	Coloured product	Control	Coloured product	Control	Coloured product
L^*	81.59	89.19	91.90	104.91	89.99	98.39	77.67	79.81
a^*	-1.56	0.97	-3.88	-7.73	-7.37	-9.95	-1.8	-4.14
b^*	11.34	17.77	19.76	46.07	14.41	44.25	9.86	13.79
C^*	nd	nd	20.13	46.72	16.19	45.35	9.99	14.4
H^*	nd	nd	101.12	99.53	117.07	102.67	nd	nd
% Relative colouration	nd		86.00		44.00		nd	

% Relative colouration = (1 - (tan h after treatment)$^{-1}$ – (tan h before treatment)$^{-1}$ / (tan h before treatment)$^{-1}$) nd – not determined

TABLE 2: Colorimetric values for the storage stability of natural colourants added food products as a function of time under the light exposure

Colour coordinates	Cookies (Storage period (Days))			Rice wine (Storage period (Days))			Squash (Storage period (Days))		
	30	60	90	30	60	90	30	60	90
L^*	93	95.95	95.38	104.51	104.50	100.30	98.93	85.11	90.09
a^*	-1.64	-2.61	-1.51	-7.41	-7.53	-7.66	-11.37	-5.51	-8.19
b^*	15.05	14.99	14.20	45.98	45.56	43.82	43.89	22.64	15.13
C^*	15.14	15.21	14.37	46.58	46.18	44.48	45.34	23.30	17.21
H^*	96.22	99.99	96.02	99.39	99.15	99.92	101.66	101.52	100.24
% Discolouration	nd	nd	nd	2.25	3.99	4.24	1.67	9.3	11.00

% Discolouration = (tan h after treatment)$^{-1}$ – (tan h before treatment)$^{-1}$ / (tan h before treatment)$^{-1}$

TABLE 3: Organoleptic evaluation of food products coloured with yellow pigment from *Thermomyces* sp

Characters	Sensory scores							
	Cookies		Rice wine		Orange squash		Guava jelly	
	A	B	C	D	E	F	G	H
Colour and appearance	9.2	7.5	9.2	4.6	9.5	5.1	7.8	5.6
Flavour	9.0	9.0	8.7	8.6	8.7	7.8	8.5	8.1
Texture	9.3	9.2	8.8	8.7	9.0	9.0	9.0	8.8
Taste	9.4	9.2	8.6	7.8	9.1	8.9	9.1	8.2
Overall acceptability	9.3	8.5	8.6	7.5	9.5	8.4	8.5	8.0

*Scores are the means of five member judges' panel

***Thermomyces* pigment added**	**without pigment**
A Cookies	B Cookies
C Rice wine	D Rice wine
E Orange Squash	F Orange Squash
G Guavajelly	H Guavajelly

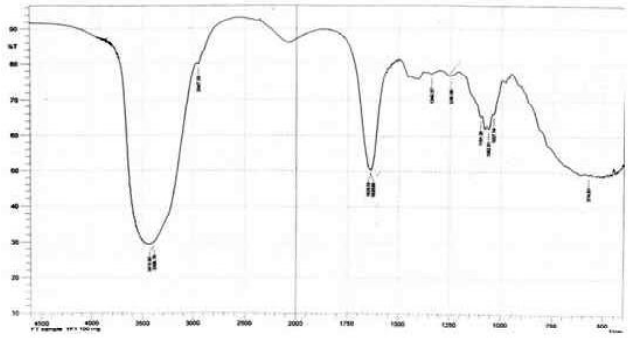

FIG 4: FT-IR spectrum of *Thermomyces* sp pigment extract.

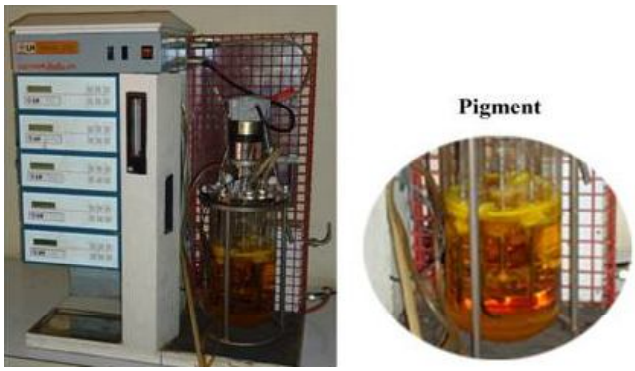

FIG 5: Mass preduction of *Thermomyces* sp for pigment production.

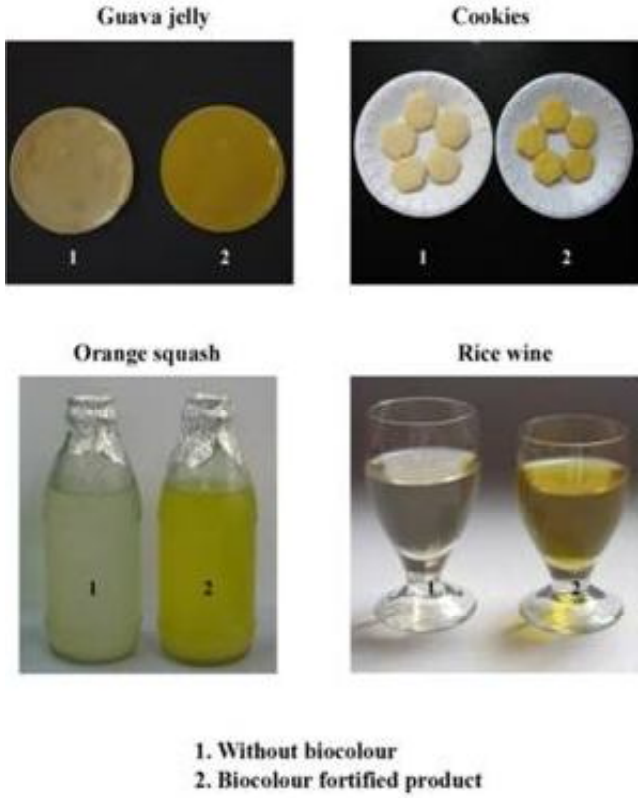

FIG 6: Natural feed dye fertified feed products

with colour for improving the aesthetic value (Table 1 and 2). The pigment was directly mixed with dough and baked. The pigments formed a complex with the foodstuff. The other food products like orange squash, guava jelly and rice wine were prepared with *Thermomyces* sp pigment to impart yellow colour to these products (Fig 5 and 6).Food products gain more intense and stable red colour and improved organoleptic characteristics when *Monascus purpureus* pigment was used (Blanc *et al.*, 1995). Moreover, application of the natural pigment promotes consumers´ health protection by decreasing the intake of salt and allows manufacturing fully natural food without any synthetic additives (Su *et al.*, 2005). Traditionally, red rice, red wine, sausages, fish sauces, meat products, soybean curd were prepared with these pigments (Anonymous, 1999). Vidyalakshmi *et al.* (2009) reported that *Monascus* fermented rice (MFR) when used as colourants in the preparation of food product (kesari), it showed very good colour and appearance. They also studied incorporation of

MFR for colouring flavored milk, which showed appealing colour and appearance with better acceptability. The pigment distributed evenly in the food product giving a pleasing appearance. The present study has also revealed that *Thermomyces* sp pigment fortified food preparations received very high acceptability. It is fervently hoped that in the future, these fungal pigments could receive greater attention (Table 3).

CONCLUSION

Application of new technologies in the food industry requires untraditional processing of foodstuffs with the aim to improve their quality, durability, storage, nutritional value and visual attraction. Application of the *Thermomyces* sp natural pigment promotes not only fulfilment of / or all these requirements, but also the positive health aspects from and with *Thermomyces* sp prepared food.

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