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Purification and Characterization of the Pigments from *Rhodotorula glutinis* DFR-PDY Isolated from Natural Source

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Abstract: Carotenoids from some of the coloured yeasts like *Rhodotorula, Phaffia rhodozyma* have attracted commercial interest as a natural pigment for foods. Red yeast isolated from the contaminated PDA plate yeast has been found to produce carotenoids. This red was characterized and designated as *Rhodotorula glutinis* DFR-PDY. The isolated crude pigment from the red yeast *Rhodotorula glutinis* DFR-PDY was separated by column chromatography and purified by thin layer chromatography (TLC) yielding three major fractions, viz., a yellow, an orange and a red fraction). The absorption spectra of these three fractions were giving the λ_{max} at 446 nm, 479 nm and 515 nm respectively. These fractions were further purified to homogeneity through high performance liquid chromatography (HPLC) before subjecting to FT-IR and NMR spectral analysis for their structural elucidation. The spectral data of these fractions revealed that the three major fractions are β-carotene, torulene and torularhodin respectively. The red yeast pigment is pretty stable when stored in vegetable oils and it can be of use in oil based food products as foods coloured with these pigments are more appealing.

Key words: Carotenoids · Characterization · Microbial pigments · Natural food colorants · Purification · *Rhodotorula glutinis*

INTRODUCTION

Although many advances in the developments of food colours have been made over the last 25 years, particularly in terms of harmonized legislation and advances in processing and formulation technology, there is still scope for future developments. The overall forecast for colour market is to grow in line with technological and sociological changes that will lead to an overall increase in processed foodstuffs. It is thought that the natural colour market will grow on a global scale at a greater rate than synthetic colours owing to a continued consumer pressure to 'go natural' [1].

Most of the natural pigments are extracted from plants like annatto, grapes, paprika, etc. and microorganisms like *Monascus, Rhodotorula, Bacillus, Achromobacter, Yarrowia, Phaffia* etc. Pigments like carotenoids, anthraquinone and chlorophyll have been produced from yeast, fungi, bacteria and algae. There is growing interest in microbial pigments due to their natural character, medicinal properties and nutritive value; production being independent of season, geographical conditions, controllable and predictable yield and safety to use [2, 3]. Enhancement of the immune response leading to protection against bacterial and fungal infections was shown using different schedules of immunization with microbial pigments and a polysaccharide. The group of mice given carotenoids of *Rhodotorula glutinis* and polysaccharide of *Spitulina platensis* survived for 2 weeks after *Pseudomonas aeruginosa* and *Candida albicans* infection. Adding carotenoids, phycocyanin and polysaccharides to food as additives might therefore enhance the human immune response against microbial infections [4].

Among pigments of natural origin, carotenoids seem to play a fundamental role, their presence in the human diet being considered positively because of their action as pro-vitamin [3], antioxidant or possible tumor-inhibiting agents [5]. Despite the availability of a variety of natural and synthetic carotenoids, there is currently a renewed interest in microbial sources of pigments because of the problems of seasonal and geographical variability in plant

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origin [6]. Moreover, industrial interest is now gradually shifting away from the yellow carotenoids such as β–carotene and lutein towards the considerably more valuable orange-red keto-carotenoids, such as torularhodin and torulene, for which at present no commercially exploitable plant or animal sources exist [7]. Carotenoid biosynthesis of the yeast *R. glutinis* was studied by co-cultivation with *Lactobacillus helveticus* in cheese ultrafiltrate [8] and in raw materials of agro-industrial origin [9]. Earlier we have described the growth and pigmentation of a local isolate, *R. glutinis* DFR-PDY, with respect to various substrates (carbon source) utilization, optimal pH, incubation period and effect of light. Proximate analysis revealed this organism could also yield good amount of protein in addition to production of multi-component pigments [10]. In the present study we report the purification and characterization of pigments isolated from our isolate, *R. glutinis* DFR-PDY.

MATERIALS AND METHODS

Chemicals: Analytical grade chemicals and solvents were obtained from SRL, India/E.Merck/S.D. Finechemicals, India. HPLC grade solvents for spectral studies of the pigment were obtained from E.Merck and the standard β-carotene was from Sigma chemical company, U.S.A.

Culture: A new strain of *R. glutinis* designated as DFR-PDY was isolated in our laboratory by particle plating method and its identity was confirmed by IMTECH, Chandigarh. This was maintained on PD slants transferred every three weeks and stored at 4°C until needed.

Mass cultivation was carried out in 10L fermenter by BIOFLOW 110 Fermentor/Bioreactor, New Brunswick Scientific to achieve high cell densities for mass production of the crude pigment needed for separation, purification and characterization of various fractions (colourants) for their structure elucidation and to study its stability, toxicity and applicability as food colourant. The batch run was started with 8L of modified Czapek dox medium. The inoculums of *R. glutinis* DFR-PDY were grown in 250 ml Erlenmeyer flask containing 100 ml of culture medium (modified Czapek dox broth with 0.3% yeast extract at ambient temperature 29-32°C, for 48 hrs, on a rotary shaker (200 rpm) and added at 4% (v/v) to the fermenter. A 10% (v/v) suspension of antifoam (Sigma 260) in water was sterilized by autoclaving and added drop-wise to the fermenter, whenever necessary. Fermentation was carried out at 25 $\pm$ 2°C and the stirrer speed was 400 rpm. The aeration rate (0.6-1.0 VVM) and dissolved oxygen rate (10-50%) were maintained. The fermentation was continued till dissolved oxygen increased finally to almost 100% saturation.

Pigment Extraction: Freeze-dried red yeast was hydrolyzed with 1N HCl in water bath at 70°C for one and half hour [11]. After removal of excess acid by washing with water, the cells were soaked overnight in acetone: methanol (1:1). The pigment was extracted with acetone until the entire colour was removed from the cells. Acetone extracts were transferred to light petroleum (40-60°C) in a separating funnel and washed thoroughly thrice with distilled water [8]. The absorbance of the light petroleum phase was read at 474 nm (λ_{max}) and the concentration of carotenoids determined using the absorption coefficient as $A^{1\%}_{1cm} = 1600$ [12].

Separation and Purification of Various Fractions of the Crude Pigment Extract: The separation of various fractions was carried out by column chromatography, analysed and purified by thin layer chromatography (TLC) until homogeneity of the fraction achieved. The homogeneity and the purity of the different fractions were checked by HPLC.

Column Chromatographic Separation: The pigments from the red yeast *Rhodotorula glutinis* DFR-PDY were extracted with acetone, transferred to petroleum ether and fractionated on magnesium oxide-Hyflo Super Cel (1:2, w/w). A major red coloured fraction was adsorbed on the column under these conditions, while the other pigments were eluted by the developing solvents (petroleum ether, ethyl ether and methanol). The yellow fraction was eluted first by petroleum ether, followed by orange fraction by ethyl ether and methanol (10:1). The major third fraction which was retained in the column was then eluted with acetic acid- ethyl ether (1:10). The water soluble compounds were removed by water washes and the ethyl ether was dried over anhydrous sodium sulphate. The fractions were saponified by adding potassium hydroxide-methyl alcohol solution (200ml; 10% w/v) and heating for 5 min on a steam table. Petroleum ether is added to the mixture, which was washed free from water-soluble materials. The petroleum ether solution was then dried over anhydrous sodium sulphate. The red coloured fraction (fraction 3) and the orange coloured fraction (fraction 2) were taken to dryness in the rotary flash evaporator and then were dissolved in chloroform and petroleum ether respectively.

TLC Analysis and Purification of the Pigment Fractions: Thin layer chromatographic separation of the different fractions from the crude pigment of *Rhodotorula glutinis* DFR-PDY was carried out using pre coated TLC plates (silica gel 60, Merck, Germany) using petroleum ether: acetone (80:20, v/v) as a mobile phase and determined their R_f.

High Performance Liquid Chromatography (HPLC): The homogeneity and the purity of the different fractions were checked by HPLC (pump JASCO PU-1580; detector JASCO UV-1575 UV/Visible) using a RP-C_{18} column (No. 00w00218, made in Japan) and isocratic elution with. Acetonitrile: tetrahydrofuran: water (50:38.5:11.5, v/v/v) at a flow-rate of 1.0 ml/min.

Structure Determination of the Pigments: The structure of three purified fractions was determined using absorption spectra, nuclear magnetic resonance (NMR) spectroscopy and infrared (FT-IR). The absorption spectra of the three major purified fractions were taken using Shimadzu UV-240 ultraviolet-visible recording spectrophotometer to obtain the λ_{max} of each colourant. The NMR spectra of the three samples were recorded on Bruker AMX-400 and AMX-400 instruments, both equipped probe heads for inverse detection. All measurements were performed at 23°C in $CDCl_3$ (99.95%) under argon that had been passed twice through an alumina mini-column. Prior to NMR analysis, remaining trace of solvents was removed under high vacuum. Chemical shifts of ^1H and ^{13}C resonances (δ) were related to residual solvent signals and only relevant (δ) were related to residual solvent signals and only relevant $^nJ_{HH}$ coupling constant values (J) are given. Complete proton line assignments were achieved by ^1H and H, H-COSY (correlated spectroscopy) experiments.

The chemical configuration of the pigments was determined by infrared spectroscopy. Infrared spectra of the three purified samples (fractions) were recorded using Thermo-nicolet FT-IR spectrometer using sodium chloride windows. Mid IR spectra were recorded from 4,000 cm^{-1} to 500 cm^{-1} with a resolution of 4 cm^{-1}. All the spectra were recorded in the absorbance mode.

Utilization of Waste Materials as a Cheap Carbon Source: An attempt was made to use waste materials from the food industries like whey, waste mango pulp, dry tapioca powder and ethanol as substrate (a carbon source) for the cultivation of the red yeast *R. glutinis* DFR-PDY.

Stability of Pigment: Stability of the pigment in different solvents was carried out in geometric progression for a period of 3 months. Stability was studied in acetone, a polar solvent and in petroleum ether (4-60°C), a non polar solvent in three different temperatures, 4°C, 25°C and 40°C and in presence of light and in dark. Stability of the red yeast pigment was also studied in five different types of vegetable oils namely Sunflower oil, Ground nut oil, Coconut oil, Sesame oil and Palm oil at different temperatures i.e., 4 °C, room temperature (27-34 °C) and 60 °C both in presence of light and in dark for a period of 30 days in duplicate. Similarly, stability of the pigment was also carried out in presence of added antioxidant, butylated hydroxyl toluene (BHT).

Applicability of the Pigment as Food Colourant: Carotenoid colourants are useful for margarines, oils, fats and shortenings, fruit juices, beverages, dry soups, canned soups, dairy substitutes, salad dressings, meat products, pasta, egg products, baked goods and many others [2]. Applicability of this microbial pigment extracted from *R. glutinis* DFR-PDY for colouring food stuff was studied by adding to puffed products like popcorn, in biscuit creams, in cake icing and in ice creams.

RESULTS AND DISCUSSION

In the mass cultivation of red yeast in 10 L fermenter, the pigment production was higher and faster when compared to growth in conical flasks. The pigment yield was increased to 5mg/L in 7 days when compared to conventional method in conical flasks where the pigment yield reached only to 3-3.5mg/L in 12 days. This may be due to the design of the fermenter leading to better mixing and mass transfer. Production of carotenoids from fungi/yeast such as *Rhodotorula* sp. *Phaffia rhodozyma* and bacteria such as *Brevibacterium* KY-4313 culturing in large bioreactors are some of the other examples of biotechnological production of carotenoids. Currently, the cost of natural colours in most cases is higher than synthetic colours of similar shades but this hurdle can be over come by mass production of natural colours and by increasing the demand also [13].

The crude pigment extract of *R. glutinis* DFR-PDY is fractionated in to three different major fractions by column chromatography. The first yellow fraction (fraction1) was eluted with petroleum ether followed by the second orange fraction (fraction 2) with ethyl ether and methanol. The third major red fraction (fraction 3) adsorbed to the column was then eluted with acetic acid: ethyl ether (1:10).

Table 1: TLC separation and absorption spectral analysis of the three fractions of crude pigment from *R. glutinis* DFR-PDY

Pigment fractions	R_f Value	Absorption maximum (λ_{max})
Fraction 1 (yellow)	0.92	446
Fraction 2 (orange)	0.78	479
Fraction 3 (red)	0.20	515

Thin layer chromatographic separation of the crude pigment with petroleum ether: acetone (80:20) has also revealed presence of three major pigments whose R_f and λ_{max} are presented in Table 1. The R_f of the yellow fraction is same as that of standard beta carotene and also there is a resemblance in their absorption spectra as well (data not shown). The R_f values resembles with that of torularhodin, torulene and β-carotene, as published earlier [14]. Close agreement was obtained between the absorption maxima of these fractions and published data [8]. Similar results were obtained with High Performance Thin Layer Chromatography (HPTLC) separation of the crude pigment extract from *R. glutinis* DFR-PDY. Using Petroleum-ether: Benzene (95:5), the crude pigment of red yeast separated into three major fractions-one yellow and one orange with R_f values of 0.9 and 0.7 respectively, while the red fraction was found to be retained at the application spot. Moreover, in a recent report on the chromatographic analysis of the crude extract from *R. glutinis* showed that similar three carotenoid pigments, viz., β-carotene, torulene and torularhodin were present in the extract [15].

High performance liquid chromatographic analysis of the fractions obtained from the column chromatography depicted the purity of fractions 1 and 2 (Fig. 1) except fraction 3 which was again fractionated to get a purified one. These purified fractions were subjected to NMR and IR spectroscopy.

The FT-IR spectrum of fraction 1 (Yellow solution in Petroleum Ether) is shown in Fig.2. The bands at 2955 cm^{-1} and 2920 cm^{-1} are due to asymmetrical stretching vibration of aliphatic CH_3 and CH_2 groups respectively and bands at 2869 cm^{-1} and 2851 cm^{-1} are due to symmetrical stretching vibration of same groups. The asymmetrical and symmetrical deformation vibrations CH_3 groups are observed at 1463 cm^{-1} and 1377 cm^{-1} respectively. Low intensity bands at 1713 cm^{-1} may be due to >C = O group.

The FT-IR spectrum of fraction 2 (Red solution in Benzene) is shown in Fig.2. The bands at 2954 cm^{-1} and 2921 cm^{-1} are due to asymmetrical stretching vibration of aliphatic CH_3 and CH_2 groups respectively and broad band centered around 2851 cm^{-1} is 720 cm^{-1}, (CH_2 rocking vibration), also indicates that there may be four or more CH_2 groups in a row. Low intensity bands at 3008 cm^{-1} and 1652 cm^{-1} indicate the presence of an olefinic functional group. The band at 3008 cm^{-1} is due to CH stretching vibration CH = CH and that at 1652 cm^{-1} may be due to C = C stretching vibration of the same group. Medium intensity band at 1738 cm^{-1} is due to >C = O group probably an ester. This assignment is supported by the presence of a band at 1259 cm^{-1} which could be the C – O stretching vibration of the ester. The above assignments are based on the assumptions that the samples are pure. However, IR has not been done on the fraction 3 on account of its high polar character. Elucidation of complete structures of the compound is not possible with IR spectral data alone as the amount of information from IR is limited for a molecule if it is Centro symmetric. Hence NMR was carried out for the structural elucidation of the pigment fractions.

The identification of the major carotenoid fractions isolated from the microorganism *R. glutinis* DFR-PDY was based on absorption, FT-IR and NMR spectra. According to 400 MH_z ^{1}H-NMR spectra the isolated three major fractions were identified: fraction 1 as β-carotene, fraction 2 as torulene and fraction 3 as torularhodin (Fig. 3).

Utilization of the Waste Materials for Cultivation of Red Yeast: The raw material and by-product of agro-industrial origin have been proposed as low cost alternative carbohydrate sources for microbial metabolite production [16], with the view of minimizing environmental and energetic problems related to their disposal. Starch rich raw materials of agro-industrial origin (eg. Corn, barley and other cereals, potatoes and cassava) are widely available as feed stock and could be considered as cheap sources of sugars. Several strains of *Rhodotorula* had been studied for carotenoid production on grape juice [17].

Production of carotenoids by yeast can be made economically viable by using cheap industrial by-products/wastes as nutrient sources. Out of four waste materials tried, waste mango pulp, dry tapioca powder and ethanol showed positive results. However, no growth of red yeast was observed in raw whey or in treated whey (initially inoculated with lactic acid bacteria for 24 hrs to convert lactose into lactic acid and steamed to kill the LAB and then was inoculated with red yeast) indicating the inability of the red yeast to assimilate lactose or lactic acid for its growth. Waste mango pulp as a substrate was yielding 11.4g of red yeast per litre broth, whereas in dry

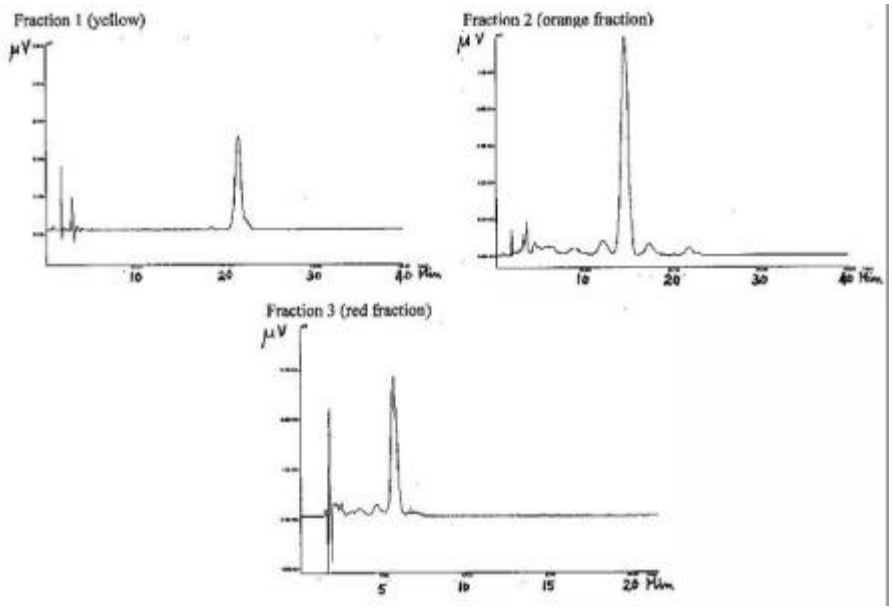

Fig. 1: HPLC analysis of the three pigment fractions from *R. glutinis* DFR-PDY

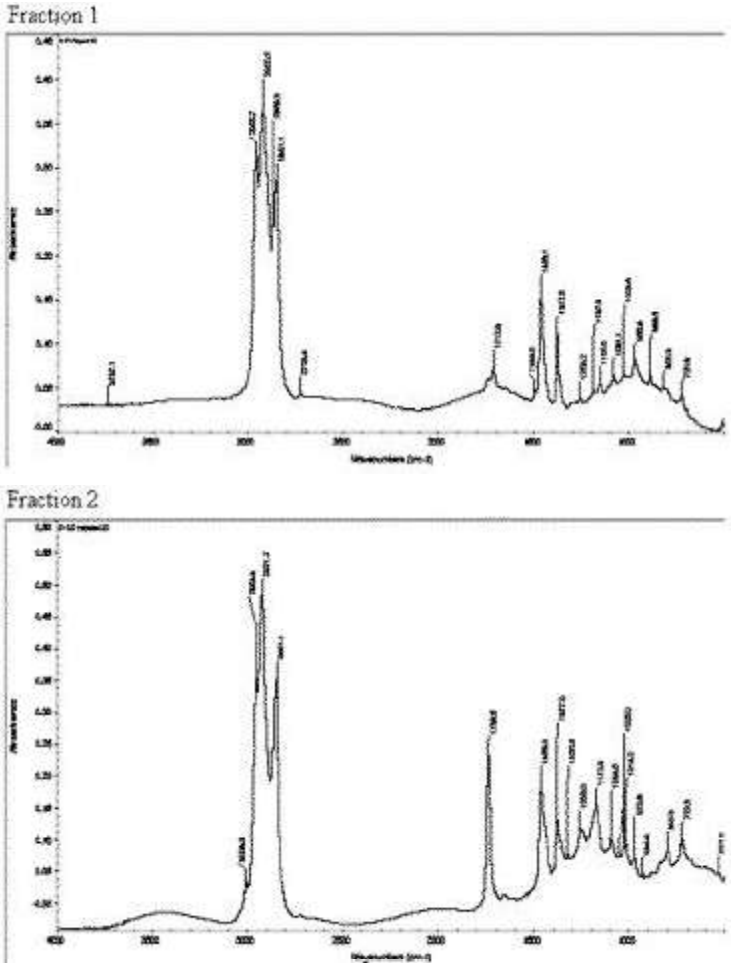

Fig. 2: FT-IR spectra of pigment fractions from *R. glutinis* DFR-PDY

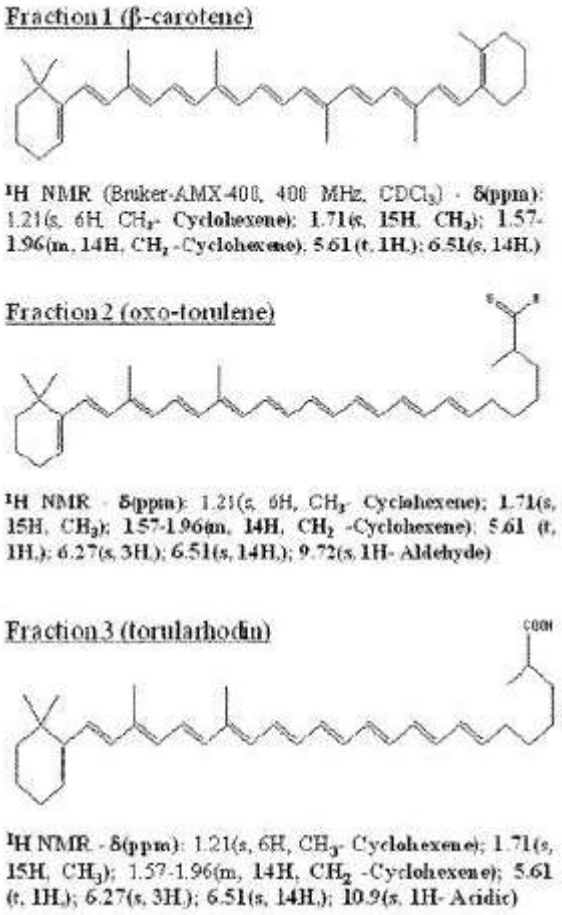

Fig. 3: Structure of the three components of crude pigment from *R. glutinis* DFR-PDY

tapioca powder the cell dry weight (CDW) obtained was 14g/L and the carotenoid yield in both the substrate was ranging from 2.5-3.0 mg/L (whereas synthetic media yielded CDW about 11-12g/L and carotenoids about 3.5mg/L).

In case of ethyl alcohol as a substrate for red yeast growth and pigmentation was observed in 2% ethanol, where CDW was 5.1g/L. There was growth but no pigmentation in 4% ethanol and there was no growth and pigmentation at and above 6% ethanol.

Storage Stability of the Pigments: Since carotenoids are sensitive to light and are less stable at higher temperature the storage stability of the red yeast pigment was initially studied in different polarity organic solvents like petroleum ether (non polar) and acetone (polar). Different conditions chosen were different temperatures (4 °C, RT and 60°C) and presence and absence of light. The spectrophotometric results revealed that after 90 days of storage 35% of the pigment remained stable in petroleum ether kept in dark at 4°C (Fig. 4) whereas only 8-10% of the pigment remained stable in acetone in dark at 4°C (Fig. 5).

Storage stability of the pigment was also studied in different vegetable oils at different temperatures (4°C, RT and 60°C) and different conditions (in presence and absence of light). The pigment was supplemented with BHT, an antioxidant in both the conditions and its effect was studied. There was a gradual decrease in the hue over

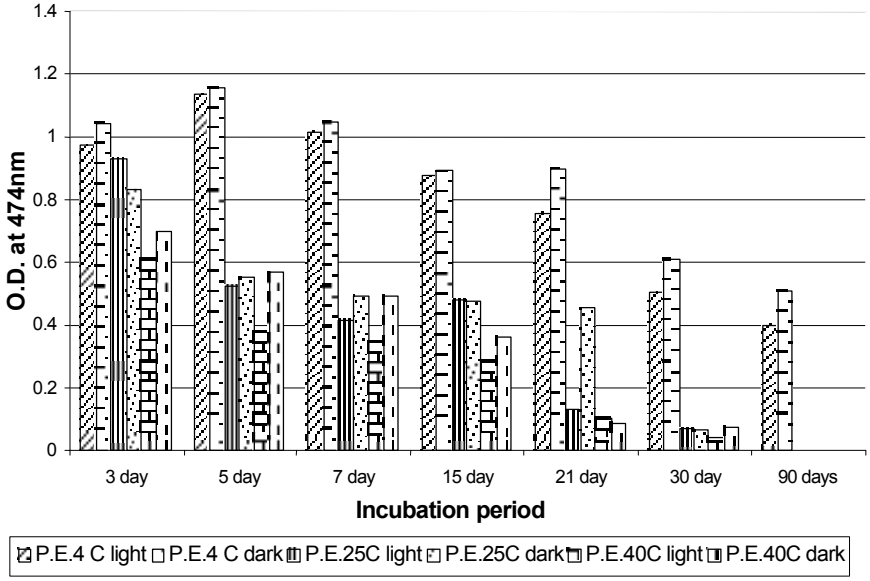

Fig. 4: Stability of the PDY pigment in petroleum ether at different temperatures (4, 25 and 40 ^{0}C) and in presence and absence of light. Values are mean of four independent observations

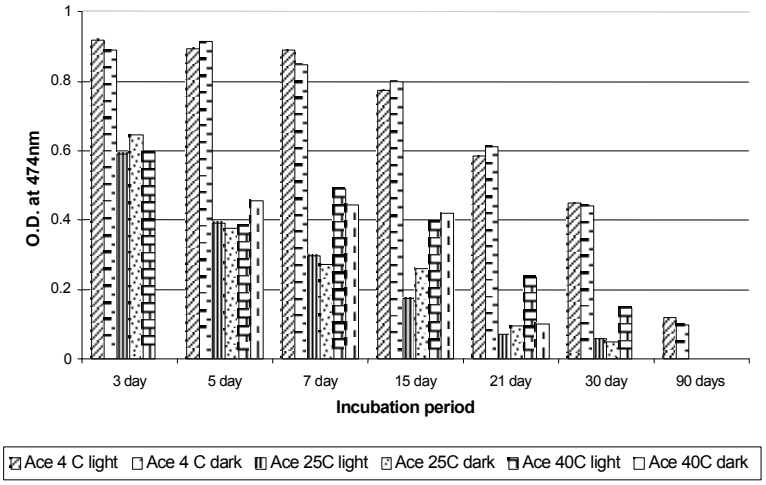

Fig. 5: Stability of the PDY pigment in acetone at different temperatures (4, 25, 40 ^{0}C) and in presence and absence of light. Values are mean of four independent observations.

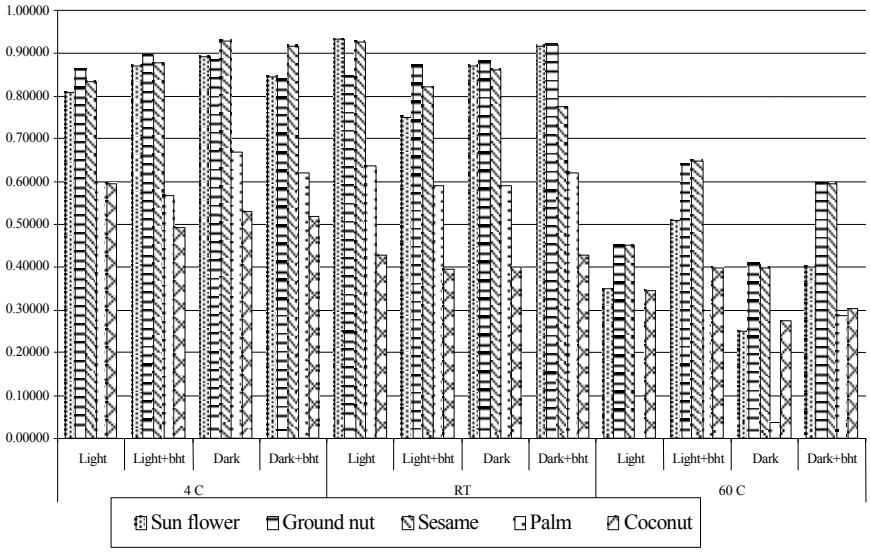

Fig. 6: Stability of the pigment after 30 days of storage in different vegetable oils and conditions. Values are mean of four independent observation

the period of 30 days (Fig. 6). The pigment was found to be more stable in sesame oil which was followed by ground nut oil, sunflower oil, palm oil and coconut oil. The stability is more at 4°C and also even at room temperature (RT) in some cases and very poor stability was observed at 60°C. Addition of BHT to the red yeast pigment was found to increase the stability of the pigment to certain extent. Significance was observed between different vegetable oils, between different temperatures, between different conditions of storage, interaction between different oils and different temperatures, interaction between different oils and different conditions and interaction between different temperatures and conditions by repeated ANOVA (Table 2).

Table 2: Repeated ANOVA of storage stability of the red yeast pigment in different vegetable oils at different temperatures and conditions

Source	Sum of Squares	df	Mean	F Square	Significance
Change	4.812	5	0.962	13363.874	.000
Change*Oil	2.099	20	0.105	1457.087	.000
Change*Temp	2.459	10	0.246	3415.101	.000
Change*Condi	0.0837	15	0.00558	77.482	.000
Change*Oil*Temp	0.960	40	0.02401	333.386	.000
Change*Oil*Condi	0.159	60	0.002649	36.783	.000
Change*Temp*Condi	0.355	30	0.01184	164.354	.000
Error (Change)	0.0216		300	0.000072	

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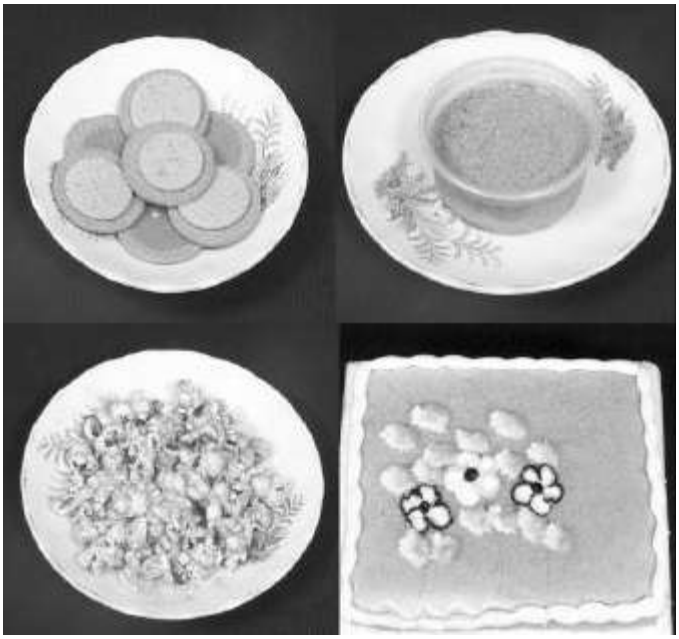

Fig. 7: Food products coloured with the pigments from *R. glutinis* DFR-PDY

Since carotenoids are light sensitive the pigment here is less stable when exposed to light during the storage whereas it is stable when stored in dark. In a recent report, the carotenoids, the main pigment produced by *Rhodotorula* was shown to get denatured on exposure to the light, heat and oxygen [18]. However, we found that the red yeast pigment is pretty stable when stored in vegetable oils and it can be of use in oil based food products. These characteristics of red yeast pigment give them considerable potential for development for use in the food industry.

Applicability of the Pigment as Food Colourant: An attempt was made to see the applicability of the pigment to different food items like popcorn, ice-cream, biscuits and in cake icing. The pigment was mixed in different concentration and these were seen more appealing (Fig. 7). The fungi *Thamnidium elegans*, as a

producer of ã-linolenic acid (GLA) and the yeasts *Rhodotorula glutinis* and *Sporobolomyces roseus*, as producers of ß-carotene, torulene and torularhodin were tested for their ability to utilize cereal substrates during solid state fermentations (SSF). Depending on the strain and conditions, the cereal materials were effectively enriched with polyunsaturated fatty acids (PUFAs) or carotenoids. These naturally prepared bioproducts could find applications in food, feed, biomedical, pharmaceutical and veterinary fields [19].

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