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Characterization of carotenoid pigment production from yeast *Sporobolomyces* sp. and their application in food products

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Abstract
Carotenoid Pigment producing yeasts like *Rhodotorula* sp, *Phaffia rhodozyma* and *Sporobolomyces* sp, represent a group of valuable molecules for the pharmaceutical, chemical, food and feed industries, not only because they can act as vitamin A precursors, but also for their colouring, antioxidant and possible tumor-inhibiting activity. The carotenoid pigment producing yeast isolated from philosopher surface of rice plant was identified as *Sporobolomyces* sp. In the present study carotenoid pigment extracted from *Sporobolomyces* sp. was separated by thin layer chromatography (TLC) yielding three major fractions, viz., yellow, orange and red. These fractions were further purified through high performance liquid chromatography (HPLC) before subjecting to FT-IR spectral analysis for their structural elucidation. The carotenoid pigment from *Sporobolomyces* sp. resulted in three peaks at retention time of 3.35, 4.20 and 5.77 min respectively. Carotenoid pigment was incorporated in different concentrations in the development of few food products *viz*., ice cream, Indian milk ice cream, popcorn, almond milk and yoghurt.

Keywords: Carotenoid pigment, *Sporobolomyces* sp., Characterization, Natural food colourants.

Introduction
The synthetic colour captured the market due to ease to produce, less expensive, superior in coloring properties and only a tiny amount was needed to colour (Downham and Collins, 2000). Many colour additives at that time, had never been tested for its toxicity or other adverse effects, which ultimately lead to pose adverse effect on the health and environment with rise of diseases leading to death due to their carcinogenic nature. The growing apprehension over the eventual harmful effects of synthetic colorants on both the consumer and the environment has raised preferential interest in natural colouring alternatives (Socaciu, 2007). Natural pigments can be obtained either from plants or microorganisms. It is a well-known practice to extract the natural colours from the plant sources, but the yield is very low and they have low eco-efficiency. Extraction of colours from the microbial source is an upcoming field. Various types of microorganisms will produce pigments and natural colors from these sources can be extracted using simple and effective methods (Aberoumand, 2011).
Microorganisms provide a readily available alternative source of naturally derived pigments. The production of natural pigments utilizing microbial biosynthesis has received greater interest in recent years (Nagpal *et al*. 2011). Among the pigments of natural origin, carotenoids play primary role, since their presence in the human diet have been considered positive because of their action as pro-vitamin (Buhler *et al*. 2013), antioxidant or possible tumour-inhibiting agent (Chen, 1995). Despite the availability of a variety of natural and synthetic carotenoids from the plants, there has currently been a renewed interest in microbial sources of pigments for the problem of seasonal and geographical variability in plant origin (Edge *et al*., 1997). 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 (Frengova *et al*. 1994).
Carotenoids have the ability to act as antioxidants and thus protect cells against photo oxidation. The ability of carotenoids to quench singlet oxygen is well known and reactions with radical species have also been studied (Edge *et al*., 1997). Dietary carotenoids inhibit onset of many diseases in which free radicals are thought to play a role in initiation, such as atherosclerosis, cataracts, age-related macular degeneration, multiple sclerosis and most importantly cancer. Antioxidant properties of carotenoids in cosmetics preparations were reported to be effective in preventing various kinds of damage resulting from oxidation and

exposure to UV light. There has been a growing interest in the use of carotenoid pigment as a functional food and pharmaceutical supplement because of its proven and potent antioxidant activity [Guerin *et al*. (2003)].

Microorganism and Culture Conditions

The microorganism used in this study was isolated from phyllosphere surface of rice plant collected from wet land, Tamil Nadu Agricultural University, Coimbatore, India. Stock cultures were maintained on yeast malt extract agar slants at 4^0C after being incubated at 25-30^{0}C for 4-5 days. The basal medium for liquid culture contained 30.0 g glucose, 2.5 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 4.0 g yeast extract (per litre).

Extraction of carotenoid pigment

The isolated yeast culture was inoculated on yeast malt extract broth and incubated at 28±1^{o}C for 5 days. A known amount (500mg) of freeze-dried red yeast was hydrolysed with 1 ml of 1N hydrochloric acid in water bath at 70°C for one and half hour. After removal of excess acid by washing with water, the cells were soaked overnight in acetone: methanol (1:1) solution. The pigment was extracted with acetone until the entire colour was leached out from the cells. Acetone extracts were transferred to light petroleum (20ml) at (40-60°C) in a separating funnel and washed thrice with distilled water. The absorbance of the light petroleum phase was documented at 474 nm. The carotenoidyieldis reported on the basis of cell mass (1⁄4g g -1 dried cell weight) (Latha *et al*., 2005).

Thin Layer Chromatography (TLC) and HPLC analysis for separation and purification of the pigment fractions
Thin layer chromatography

Thin layer chromatographic separation of the different fractions from the carotenoid pigment of *Sporobolomyces* sp. was carried out using TLC plates using benzene and petroleum ether (85:15, v/v)as a mobile phase and determined their Rf values. The samples were identified by comparing the distance travelled by the standard to the distance travelled by the test sample β-carotene. The RF values is a mathematical representation of the ration of the distance travelled by the solvent (Park *et al*. 2007).

HPLC-High Performance Liquid Chromatography

The purity of the different fractions was checked by HPLC using a reverse phase-C18 column. For mobile phase (HPLC grade solvents were used) and samples were filtered through 0.251⁄4m membrane filter, C18 column consists of acetonitrile, isopropanol and ethyl acetate (40:40:20, v\v\v) with flow rate at 1 ml/min (Park e*t al*. 2005).

Structure determination of carotenoid pigments

The structure of three fractions were determined using FT-IR absorption spectra. 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.

Applicability of the pigment as food colourant

The carotenoid pigment extracted from *Sporobolomyces* sp. was applied to different food items as colour additive and added antioxidant properties for the development of food products. To enhance the appearance and acceptability of foodstuff, carotenoid pigments was added in ice cream, Indian milk ice cream, popcorn, almond milk and yoghurt.

Results and Discussion

TLC, HPLC analysis and FT-IR spectra of carotenoid pigment extracted from *Sporobolomyces* sp.

In the present study, the different solvent systems ranging from low polar to high polar were tested for effective separation of carotenoid pigment compounds from acetone extracts of *Sporobolomyces* sp. Best separation of the *Sporobolomyces* sp. pigment compounds were separated using hexane: acetone (70:30) as mobile phase. Three bands (pink, orange and yellow) were separated from the pigment fractions Fig 1. and the Rf values of the TLC bands were listed in Table 1.The Rf value of the yellow fraction is similar as that of standard β-carotene spot and also there is a resemblance in their absorption spectra as well. Close agreement was obtained between absorption maxima of these fractions and Rf values which resembles with that of orange, yellow and red fractions as torulene, β-carotene and torularhodin was published earlier. (Frengova *et al*. 1994; Park *et al*. 2007; Perrier *et al*. 1995)

RP-HPLC studies were performed for the determination of compounds present in the pigment of *Sporobolomyces* sp.The standard β-carotene was used as reference (Fig 2). In the present study data indicated the presence of three peaks in *Sporobolomyces* sp. pigment at retention time of 3.35, 4.20 and 5.77 min respectively (Fig 3). Bhosale and Gadre (2001) reported that the carotenoids, β-carotene, torulene and torularhodin eluted at 2.39, 3.39 and 3.49 min respectively. The retention times of β-carotene extracted from *Sporobolomyces* sp. observed were similar to that of standard graph. In agreement with the results of Bhosale and Gadre (2001) identified pigment was confirmed as carotenoid compounds. The retention time of β-carotene extracted from yeast was similar to the standard β-carotene. Therefore our study demonstrated that the extracted pigment of yeast is a carotenoid compound.

The identification of the major carotenoid fractions extracted from *Sporobolomyces sp.* was based on absorption. FT-IR spectra (Fig.4) showed the band at 2931 $cm^1$are due to symmetrical or asymmetrical stretching vibration of aliphatic CH_3 group and bands at 2862 cm^{-1} are due to symmetrical stretching vibration of same groups. The band observed at 1427 cm^{-1} was due to C–C stretch and 1365 cm^{-1} was due to symmetrical stretching vibrations of CH_3 groups. Low intensity band at 3008cm^{-1} is due to CH stretching vibration CH = CH and 1705 cm^{-1} may be due to C=O stretch ketone group. Presence of a band at 1226cm^{-1} due to the C – O stretching vibration of the ester. Similar results were also reported by Latha and Jeevaratnam (2010) in *R. glutinis*. Marshell (1998) reported that FT-Infra red spectrum of freshly isolated β-carotene bands around 2930, 1720, 1450 and 1370 cm^{-1} respectively.

An attempt was made to see the applicability of the pigment to different food items. To enhance the appearance and acceptability of foodstuff, carotenoid pigments extracted from *Sporobolomyces* sp. were added to food products like ice cream, Indian milk ice cream, popcorn, almond milk and yoghurt. The fortified food preparations received very high acceptability.

Conclusion

In the present study identifying the presence of commercially important carotenoid compounds viz., β-carotene, torulene and torularhodin were confirmed and also indicated the importance of pigment applications in food products enriched with antioxidant properties. The commercial production of the carotenoid pigments using yeast has gained more importance owing to its highly efficient and easy manipulation. It is fervently hoped that in the foreseeable future, the yeast carotenoid pigment could receive greater attention.

Table 1: TLC of acetone extracts of carotenoid pigment from *Sporobolomyces* sp.

Band No.	Colour of the band	Distance travelled (cm)	Rf value (cm)
1	Pink	9.6	0.41
2	Orange	10.4	0.74
3	Yellow	12.5	0.88
4	Yellow (Standard β-carotene)	12.7	0.89

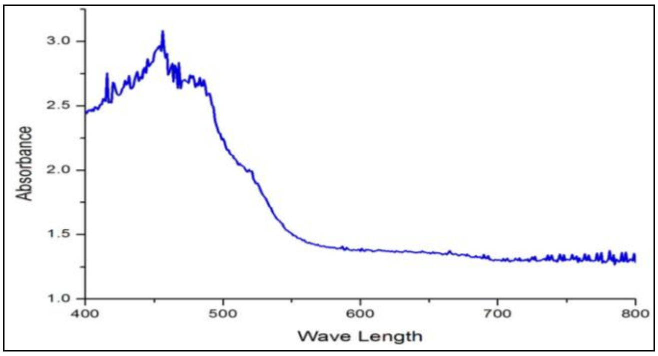

Fig 1: Spectrometric scanning of yeast carotenoid pigments

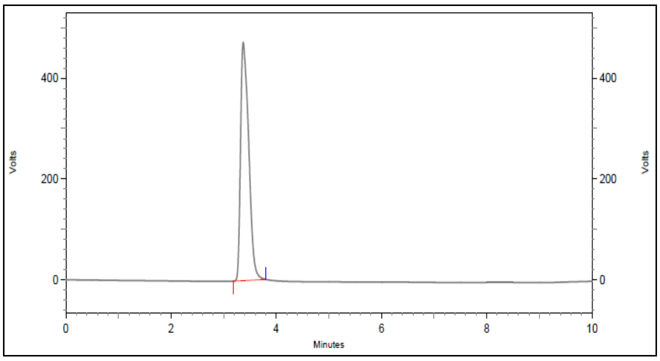

Fig 2: HPLC analysis of standard β-carotene compound

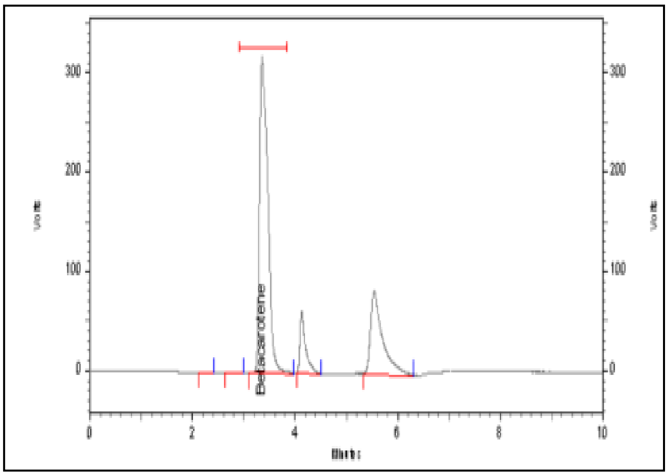

Fig 3: HPLC analysis of carotenoid pigment extract from *Sporobolomyces* sp.

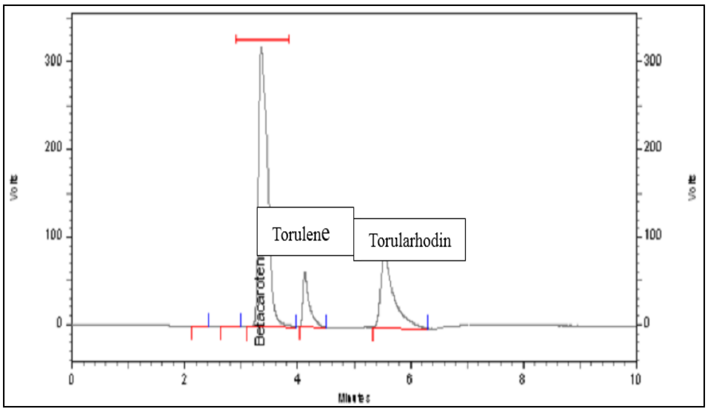

Fig 4: FT-IR spectrum of pigment extracted from *Sporobolomyces* sp.

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