

Original Research Article

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Isolation of Pigmented Yeasts, Extraction of Pigment and Study of Antimicrobial Property of its Pigment

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ABSTRACT

Keywords

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In nature, there exist a wide variety of microbes that can be of any economical importance to humans. A specialized group of microorganisms includes pigmented yeasts. They are yeasts that develop colored pigment as their secondary metabolites. In this study, pigmented yeasts were isolated from different sample (soil, leaves, fruits, coconut, and air) on Sabouraud's agar. Four strains were selected that showed pigmentation. Various morphological and biochemical tests were performed for identification of the strains. The pigments were extracted in acetone and were partitioned in petroleum ether in separating funnel. The characterization of the extracted pigments was done using UV-Visible spectrophotometer. Also the antimicrobial effect of the extracted pigments was studied against *E. coli*, *S. aureus*, *K. pneumoniae* and *C. diphtheriae*. The pigments extracted from one of the isolate showed increase in antimicrobial property against *K. pneumoniae* with increase in volume of pigment.

Introduction

Yeasts are unicellular, uninucleate eukaryotic fungi that reproduce by forming new cells serially. From the ancient times yeast particularly *Saccharomyces cerevisiae* are been used in wine or alcohol making industries. In modern sciences their uses are multifaceted such as a) in the production of SCP b) baking industries c) ethanol and acetic acid production d) biocontrol agents against many post harvest vegetable and fruit diseases e) recombinant vaccine production and understanding of cell cycle process and so on (Swapan, 2011). According to Kurtzman and Fell (1998), there exist about 100 genera and

700 species of yeasts (Kurtzman and Fell, 1998). *Rhodotorula* species are pigmented basidiomycetous yeasts in the family Sporidiobolaceae easily identifiable by distinctive yellow, orange/red colonies (Prabhu *et al.*, 2015; Kaur *et al.*, 2009). Most *Rhodotorula* species produce colonies that are pink to coral in color but can also be orange to red on Sabouraud's agar.

Most of the natural pigments are extracted from plants like annatto, beet root, marigold, grapes, carrot, paprika, etc. and microorganisms like yeast of the genera

Phaffia, *Rhodotorula*, *Cryptococcus* and *Sporobolomyces*, fungi like *Blakeslea trispora*, and algae such as *Dunaliella* and *Haematococcus* and bacteria such as *Flavobacterium* and *Micrococcus* are reported to produce carotenoids (Manimala *et al.*, 2014). Pigmented yeasts like *Rhodotorula* and *Rhodospiridium* produce the major carotenoid pigments viz. carotene, torulene, torularhodin (Kvasnikov *et al.*, 1978). Interest in carotenoids has increased considerably, due in part to the growing evidence of benefits to human health and also to the growth of certain areas of agriculture, especially aquaculture and poultry industry, the utility of carotenoids as anticancer agents, as singlet oxygen or free radical scavengers, as immune response stimulants and as colouring agents for cooked sausage, soft drinks, baked goods and as additive to cosmetics is well known (Amr Abd *et al.*, 2012).

Also, now-a-days antibiotic resistant pathogens pose an enormous threat to the treatment of a wide range of serious infections. To prevent this exponential emergence, a periodic replacement of the existing antibiotic is necessary. Currently, the greatest cause of concern is infection caused by methicillin and vancomycin resistant strains of *Staphylococcus aureus*, ESBL strains of *E. coli*, *Klebsiella sp.*, and *Pseudomonas aeruginosa*. Carotenoids are currently produced for use as nutritional supplements, food colorants, cosmetics or health purposes. Despite the availability of a variety of natural and synthetic carotenoid pigments, there is currently a renewed interest in microbial sources of pigments. There is growing interest in microbial pigments due to their natural character, medicinal properties and nutritive value. Microbial synthesis offers a promising method for production of carotenoids. This explains the increasing interest in production of microbial carotenoids as alternative for synthetic food colorants as

well as for using it as antibacterial agent (Manimala *et al.*, 2014).

Materials and Methods

Isolation of pigmented Yeast from various sources

The samples which were selected for isolation of pigmented yeasts were soil sample of rose plant and tulsi plant, leaves of tulsi and rose plant, fruits peels of pomegranate, mosambi, coconut shell, dry and wet coconut. Different samples were processed differently for isolation of pigmented yeasts. For isolation of organism from soil sample, the spread plate technique was performed. Dilution upto 10^{-8} was prepared using 0.9% saline. For isolation from leaves, fruit peel and coconut, the surface of sample was directly streaked onto the Sabouraud-Dextrose agar plate with sterile nichrome loop (Yurkov *et al.*, 2008). The inoculated plates were kept for 10 days in order to obtain pigmented yeasts.

Identification of yeast

For identification of yeast, morphological and biochemical tests were performed. In morphological test, colony characteristics on Sabouraud's agar and microscopic appearance were observed. For microscopic analysis, smear of isolates were Gram's stained and observed under 450 X in compound microscope. In biochemical test, Sugar fermentation test with dextrose, sucrose, mannitol, maltose and lactose as different sugars, nitrate assimilation test and urease test were performed.

Enrichment for carotenoids production

Yurkov *et al.*, (2008) used enrichment media in order to determine the content of carotenoid pigments from pigmented yeasts (Delia, 1992). So for enrichment of the

pigments and to check whether the selected yeast strain produces sufficient amount of pigment or not, the selected isolates were

The flasks were kept for six days on a rotary shaker at 260 rpm at room temperature. For taking the absorbance, λ_{\max} was determined and that wavelength was selected for taking the absorbance at every 48 hours interval. This helped in selection of those yeast strains that showed higher yield of pigment production.

Extraction of pigment

The 10 day old yeast cells grown on Sabouraud's agar plate or slants were scraped with nichrome loop and were hydrolyzed with 1 N HCl in a water bath at 70 °C for 15 minutes. The acid free cells were soaked overnight in acetone at 4° C. The pigment was extracted in acetone and transferred to petroleum ether using a separating funnel and washed thrice with distilled water. The extracted pigments were stored in petroleum ether at 4°C (Pfander, 1992).

Characterization of pigment

For characterization of pigment, its spectrophotometric analysis was performed. The model used for UV-Vis spectrophotometry was Jasco V630 spectrophotometer. Absorbance in both UV and visible range were carried out. The results were obtained using software.

Determination of antimicrobial property of pigment

The method used for this study was agar cup method/ agar well diffusion method. Bacterial cultures such as *E. coli*, *S. aureus*, *Klebsiella Pneumoniae*, *C. diphtheriae* were selected for testing their susceptibility towards the extracted yeast pigment. These bacterial

inoculated in the side arm flask having 20 ml of media. The composition of media is as follows:

Components	Gms/lit
Glucose	110
Yeast extract	2.0
Peptone	1.3
Dipotassium phosphate	0.2
Monopotassium phosphate	0.08
Magnesium sulphate	0.2
Ammonium sulphate	0.4
Sodium chloride	0.04
Calcium chloride	0.02

cultures were suspended in sterile saline to obtain O.D of 0.5 at 545 nm. Mueller Hinton agar (Hi-Media, Mumbai) plates were prepared using the sterilized molten agar medium 0.2 ml of each of above culture suspension was bulk seeded into 20 ml of sterilized molten and cooled Mueller Hinton agar medium.

After solidification, wells were punched by using sterile metal cork borer of internal diameter 10 mm. To the each well, different volumes (30, 50, 60, 80, and 100 μ l/well) of pigments were delivered into them. Control plate was maintained without the addition of carotenoids but petroleum ether alone. All the plates were incubated overnight at 37°C under static conditions. After 24 hrs, the antimicrobial activity was evaluated by measuring the diameter of inhibition zone which was expressed in millimeter (mm).

Results and Discussion

Isolation of pigmented yeast from various sources

The soil sample of the rose plant from different places gave 6 different pigmented yeasts whereas tulsi plant gave 3 yeast species. Very few pigmented yeast isolates

were obtained by streaking surface of fruits peels indicating rare occurrence of pigmented yeast in leaves. Coconut parts were also taken for isolation. Different parts of the plants along with the air sample gave pigmented yeast. Yeasts were also isolated from the air as a contaminant. 18 pigmented yeasts were obtained from 53 different samples.

The coloured colonies were streaked onto Sabouraud's slant and maintained. The 4 isolates were chosen for further analysis as they maintained their pigment colour as well as their growth rate was fast. Table 1 shows the number of sample screened for isolation of pigmented yeast and number of pigmented yeasts obtained.

In the study conducted by Amr Abd El-Rhman El-Banna *et al.*, pigmented yeast samples were isolated from different sources like flowers, trees exudates, fruits and cheese. One of which was identified as *Rhodotorula var glutinis*. Similar studies were conducted by Iriani Maldonado *et al.*, (2007) isolated pigmented yeasts from soil, leaves, fruit, flowers and from processed food.

Identification of yeast

The 18 yeast isolates were purified and 4 pigmented yeast isolates were selected. The other 14 isolates were ignored because they failed to sustain growth and maintain their colours after subculturing. The further tests were performed for the selected 4 yeast isolates. For identification, colony characteristics, morphological and biochemical tests were performed. The results are shown in table 2 and table 3. Figure 1 to 4 shows colony characteristics and microscopic observation of the isolates.

Based on the morphological characteristics and biochemical tests we can conclude that the isolated organisms were yeasts.

Enrichment for carotenoids production

It was observed that isolate no 1 produced more pigments as compared to isolate no 2, isolate no 3 and isolate no 4. Isolate no 2 and isolate no. 3 produced in more or less same quantity. Isolate no 4 produced less pigments than all 4 samples. Isolate 1 was found to be high pigments producing strain as compared to the other 3 strains. The results are shown in table 4:

Extraction of pigment (Pfander, 1992)

The pigments from the yeast isolates were extracted in acetone and partitioned into petroleum ether in a separating funnel. The spectrophotometric results conducted by Latha and Jeevaratnam (2005) revealed that after 90 days of storage, 35 % of the pigments remained stable in petroleum ether kept in dark at 4°C. Therefore, pigments were kept in dark condition and were stored at 4°C under refrigeration condition. These pigments were further used for characterization and for antimicrobial assay. Pigment extracted from isolate no. 1 was labeled as sample 1 and so on.

Characterization of pigment

For characterization of pigments, the spectra of the carotenoids were analysed at UV-Vis range. For Sample 1, the maximum absorption (λ_{max}) was observed at 465.5 nm, sample 2 has 450.8 nm whereas Sample No. 3 and Sample No. 4 had 550.9 nm and 396.3 nm respectively. Based on the spectral analysis (λ_{max}) and the literature available it can be concluded that the pigments of the sample 1 might be β - carotene while that of sample 2 might be capsorubin. Sample 3 has far less resembles to any carotenoids in available literature whereas pigment of isolate no 4 i.e. sample 4 resembles ζ - carotene. The exact characterization of the pigments was not possible as the peaks were merged i.e. it has

less resolution. The reason for such low resolution is the carbonyl group in conjugation with the series of conjugated bonds extends the chromophore which is the actual reason for shape of spectra and hence it caused bathochromic shift or loss of spectral fine structure, to the extent that the three-maxima spectrum is replaced by a single broad curve. Also the extraction procedure or that pigments being extracted in petroleum ether might also have hindered the spectral

line curve (Iriani *et al.*, 2007), Latha and Jeevaratnam (2005) characterized and separated the pigments by column chromatography and purified by thin layer chromatography (TLC). The homogeneity and the purity were checked by HPLC. The structure of carotenoid was determined by taking absorption spectra Shimadzu UV-240 ultraviolet-visible spectrophotometer, nuclear magnetic resonance (NMR) spectroscopy and infrared (FT-IR).

Table.1

Sr. No.	Samples processed	No. of samples	No. of pigmented yeast obtained
1.	Soil sample		
	<ul style="list-style-type: none"> • Rose plant • Tulsi plant 	10 10	6 3
2.	Leaves of plant		
	<ul style="list-style-type: none"> • Rose plant • Tulsi plant 	5 5	2 2
3.	Fruits peels		
	<ul style="list-style-type: none"> • Mosambi • Pomegranate 	5 5	0 1
4.	Coconut		
	<ul style="list-style-type: none"> • Peels of wet coconut • Dried coconut • Wet coconut 	3 3 3	1 0 1
	Air	2	2
Total Sample:		53	18

Table.2 Shows the colony characteristics of the isolated yeasts

Characteristics	Isolate no 1	Isolate no 2	Isolate no 3	Isolate no 4
Size	2 mm	2 mm	3 mm	3 mm
Shape	Round	Circular	Round	Round
Colour	Orange	Pink	Orange	Orange
Appearance	Smooth	Rough	Smooth	Smooth
Margin	Entire	Irregular	Entire	Entire
Elevation	Raised	Slightly raised	Raised	Raised
Gram nature	Positive	Positive	Positive	Positive
Cell shape	Oval	Elliptical	Round/oval	Round/Oval

Table.3 Results of the biochemical tests of all the four isolates wherein “+” sign indicate positive reaction and “-” sign indicate negative reaction

Tests	Isolate 1	Isolate 2	Isolate 3	Isolate 4
1. Sugar fermentation /gas production				
• Dextrose	+ / +	+ / -	+ / -	+ / -
• Sucrose	+ / +	+ / -	+ / -	+ / -
• Mannitol	+ / +	- / -	- / -	- / -
• Maltose	+ / +	+ / -	+ / -	+ / -
• Lactose	+ / +	+ / -	+ / -	+ / -
2. Nitrate assimilation	+	-	-	-
3. Urease test	+	-	+	+

Table.4 Shows absorbance of the growth of cells in liquid media used for enrichment, at 515 nm (λ_{max}) at every 48 hours interval

Organism	48 hours.	96 hours	144 hours	192 hours
Blank	0.0	0.0	0.0	0.0
Isolate 1	0.94	1.54	1.64	1.29
Isolate 2	0.03	0.09	0.23	0.25
Isolate 3	0.03	0.11	0.20	0.36
Isolate 4	0.02	0.06	0.10	0.10

Table.5 Antimicrobial property of pigments extracted from isolate no. 1 i.e. sample 1

Bacterial culture	Zone of inhibition (mm)					
	Blank (P.E)	30 μ l	50 μ l	60 μ l	80 μ l	100 μ l
<i>E. coli</i>	0.0	0.0	0.0	0.0	0.0	0.0
<i>S. aureus</i>	0.0	0.0	0.0	0.0	0.0	12
<i>Klebsiella pneumonia</i>	0.0	12	14	18	22	26
<i>C.diphtheriae</i>	0.0	0.0	0.0	0.0	0.0	0.0

Fig.1



Fig.2



Fig.3



Fig.4

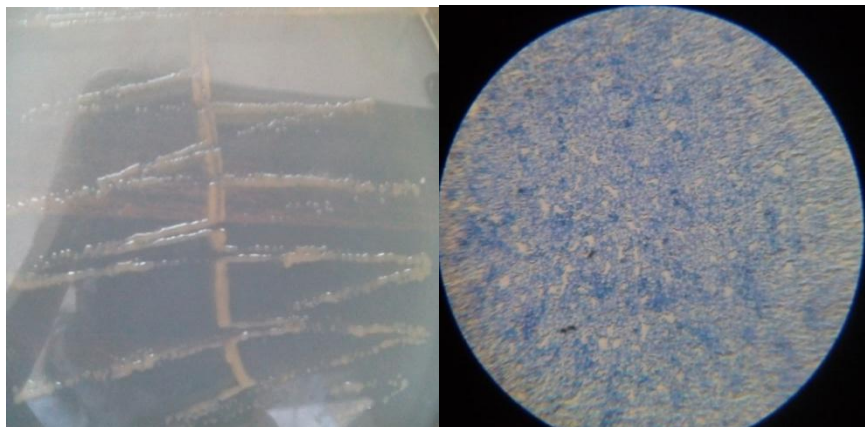
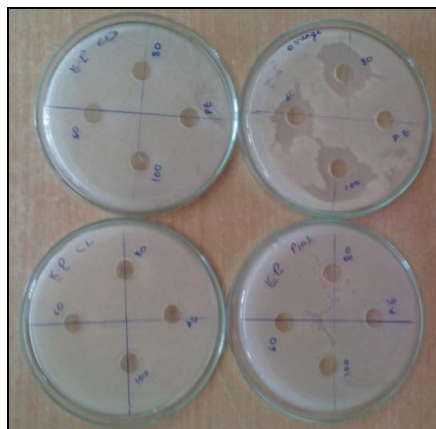


Fig.5



Determination of antimicrobial property of pigment

In order to test the antimicrobial activity of the extracted pigments, agar cup diffusion method was performed. The pigments were added in different volumes to check the antibacterial effect. The bacterial cultures used for the assay were *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *C. diphtheriae*. Table 5 shows the result for the antimicrobial activity of pigment extracted from isolate no. 1. Figure 5 shows the zone of inhibition by pigment isolated from isolate 1 against *Klebsiella pneumoniae*.

Rest all of the pigments did not show any antimicrobial effect on any of the bacterial culture. Also there was no growth seen in the blank/ negative control (petroleum ether) suggesting that zone of inhibition was purely by the pigments and not because of any other factor. Patil (2013) evaluated antimicrobial activity of methanolic extract of *Punica granatum* linn and *Cymbopogon* against gram positive and gram negative bacteria and obtained good results.

In conclusion, totally 58 samples were processed which included soil of rose and tulsi plant, leaves of rose and tulsi plant, fruit peels, coconut peels and air sample. Out of

these, 18 pigmented yeasts were isolated. Out of 18 pigmented yeasts isolated, 4 strains of yeasts were selected as they showed distinct pigment production, faster growth as compared to other isolates and ability to grow on Sabouraud's agar.

Pigmented yeasts were identified on the basis of colony characteristics, morphological and biochemical tests and one isolates (isolate no 1) was found to be *Rhodotorula* species. Isolate no. 2, isolate no 3, isolate no 4 could not be identified just on the basis of these tests.

All the pigments extracted were stored in petroleum ether at 4°C. The pigment from isolate no 1 i.e., sample 1 was found to be more stable as compared to sample 2, sample 3 and sample 4.

Antimicrobial activity of extracted pigments was tested by agar cup method on organisms such as *E. coli*, *S. aureus*, *K. pneumoniae*, *C. diphtheriae*. It was observed that pigment from isolate no 1 which was identified as *Rhodotorula spp* showed antimicrobial activity against *Klebsiella pneumoniae*. It could be more interesting to check the antimicrobial activity of the pigments on the isolates obtained from clinical samples.

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