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Optimization of Pigment Production by *Rhodotorula mucilaginosa* Strain A2J2 Isolated from Soil for Promising Application in Textile and Food Industries

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Abstract Torularhodin is a β-carotene -compound from *Rhodotorula mucilaginosa*, and it not only exhibits superior antioxidant properties with strong scavenging activity of peroxyl free radicals but also has other beneficial effects, including improvement of neuroinflammation, cognitive impairment and anti-cancer activity. The present study aimed to optimize culture conditions in pigment production by *R. mucilaginosa* for potential application in textile and food industries. The yeast was isolated from soil and identified by morphological, biochemical, and sequencing of the internal transcribed sequence (ITS) region of 18S rRNA. The pigment produced was identified and characterized with a UV-visible spectrophotometer and Fourier- Transform Infrared Spectroscopy (FT-IR). The isolate was optimized with growth parameters and metals for pigment production. The isolated yeast was identified as *R. mucilaginosa* strain A2J2. The isolate produced different shades of pigment with various λmax in the presence of 4 µg/mL of $K_2Cr_2O_7$, $HgCl_2$, KCN, and $PbCl_2$, with incubation temperature of 25°C, and pH 7.0 for 4 days. The results from this study revealed the feasibility of sustainable production of different shades of pigment from torularhodin-producing *R. mucilaginosa* strain A2J2, which could be promising used in textile industries as tie and dye and in food industries as an additive.

Keywords: Optimization, *Rhodotorula mucilaginosa,* Torularhodin, Industrial applications.

I. Introduction

Pigments are chemical substances that confer colour to other substances through the optical effect of sunlight or are powders that, when mixed with liquids, can dye the surfaces of other materials. In the textile, food, cosmetic, and clinical industries, pigments are a significant raw material where they are used as additives to improve appearance of products and to be more attractive to consumers. Pigments and dyes are currently obtained from plant, animal, or mineral sources, as well as by synthetic (production of molecules using chemical reactions) and biotechnological (pigments produced by microorganisms) processes [1].

Using synthetic pigments has several benefits, including their low cost and high yield. Nonetheless, scientists are now more motivated to create novel pigment types due to the extreme toxicity of synthetic pigment to people and detrimental impacts on the environment, such as contaminating water supplies [1]. Interestingly, since microorganisms are used to synthesize colourants, the higher production costs of natural pigments can be decreased. The most effective natural sources to address cost and stability issues are microorganisms that produce pigment. The availability, greater output, cost-

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effectiveness, and downstream convenience of microbial pigments make them preferable to plant pigments [1, 2, 3].

Rhodotorula mucilaginosa is a yeast-like fungus mostly found in various environmental niches, such as water, soil, air, and plants [4]. The yeast is considered to exhibit several advantages over microalgae, bacteria, and plants as a natural producer of carotenoids, exists in unicellular form, has fast duplication periods, has the capacity to grow on a diverse range of substrates, and ease of cultivation in large fermenters [5]. *R. mucilaginosa* can generally synthesize β-carotene, torulene, torularhodin and they have the potential for producing high-value of carotenoids from waste raw substrates. The carotenoids produced by *R. mucilaginosa* play an important role in humans, such as precursors of vitamin A, antioxidants, enhancers of immunity and cancer inhibitors [6].

Torularhodin is a β-carotene -like compound with a structural formula $C_{40}H_{54}O_2$ (Figure 1) from *R. mucilaginosa*, and it not only exhibits superior antioxidant properties with strong scavenging activity of peroxyl free radicals, but also has other beneficial effects, including improvement of neuroinflammation and cognitive impairment and anti-cancer activity [5]. The present study aimed to isolate and optimize culture conditions in pigment production by *Rhodotorula mucilaginosa for potential application in textile and food industries.*

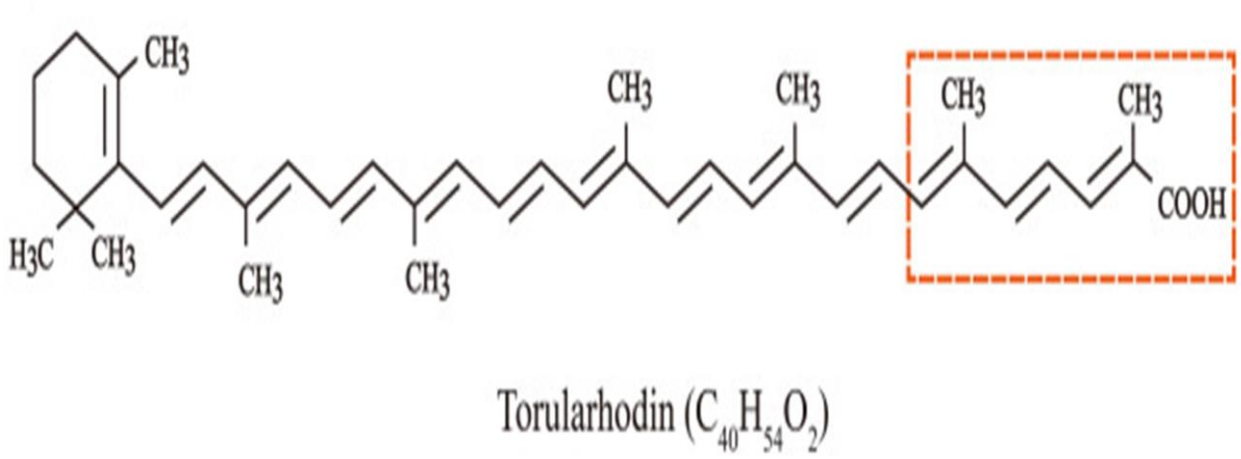

Figure 1: Structural formula of Torularhodin pigment [5].

II. Materials and Methods

A. Description of the Study Area

The study location is Zoological Garden, Osun State University, Osogbo, Nigeria, with the coordinates 7°45'20.70036" N, 4°36'17.63712" E.

B. Sample collection:

Sugarcane root soil samples were collected at the stated location and packed into sterile zip-lock bags, and transported to the Department of

Microbiology laboratory, Osun State University, for processing within 24 hours of collection.

C. Isolation of Pigment Producing Yeast

With slight modification to the method described by [7, 8, 9, 10]. The soil samples were diluted five times with sterile distilled water, and 100 µL of 10^{-3} and 10^{-5} diluent was added to Potato dextrose Agar (PDA) plates that contained 50µg/mL chloramphenicol to prevent the growth of bacteria. The samples were placed in an incubator at 25 °C for four days. Pigmented yeast colonies were noted on the plates and were sub-cultured to produce a pure culture. The pigmented yeast was stored on PDA in bijou bottles at 4 °C.

D. Molecular Identification of the Yeast Isolate

Morphology of the pigment-producing yeast and biochemical properties such as carbohydrate, nitrate assimilation, and staining of the cells with methylene blue was conducted using the method described by [9, 10, 11, 12, 13]. The isolate was identified via molecular techniques by extracting yeast DNA with Zymo-Research fungi DNA extraction kits, and PCR amplification was carried out in a thermal cycler with ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'and - ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3'primers. The PCR condition of initial denaturation at 94°C for 4min, 30 cycles of denaturation at 94°C 30 s, annealing at 55°C 45 s, elongation at 72°C 30 s, and final extension at 72°C for 10min [12, 13]. The PCR product was run on 1.5% agarose gel and visualized with an ultraviolet transilluminator to check for successful amplification. The amplified fragments were purified and sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems with manufacturer's manual, while the sequencing kit used was the BigDye Terminator v3.1 cycle sequencing kit. DNA sequences obtained were compared to sequences available online in a GenBank database (http://www.ncbi.nlm.nih.gov). A homology search was performed using online bioinformatics tools, such as BLASTn www.ncbi.nlm.nih.gov/BLA. The homology sequences obtained were aligned, and the neighbor-joining tree was obtained using Molecular Evolutionary Analysis tools (MEGA11) [9, 14, 15]. The determined sequences were submitted to GenBank for GenBank association.

E. Production and Extraction of Pigment from Yeast

To produce more pigmented yeast cells, the yeast was grown on sterile PDA and incubated for 4 days at 25 °C. The cells, 1 % (w/v), were scraped using a nichrome loop, hydrolyzed in 6M HCl with a water bath at 70°C for 15 minutes, and then extracted using chloroform (1:4) through vigorous vortexing for 5 minutes. Whatman No. 1 filter paper was used to filter the pigment-containing supernatant after the mixture was centrifuged for 10 minutes at 7000 rpm. The pigment was then stored at 4 °C in the dark to prevent photo-degradation [7, 8, 9, 10, 11].

F. UV-vis Spectrophotometer

The pigment was characterized using the Pharo 300 15320070 2.20-Merck-2.20 UV-visible spectrophotometer. The pigments were analyzed with a wavelength range of 200-700 nm to determine the highest absorption peak of the pigment, and chloroform was used as the blank [8, 9 10, 14, 16, 17, 18].

G. Fourier- Transform Infrared Spectroscopy (FT-IR) of Yeast Pigment

The SHIMADZU FTIR-8400S was the FTIR spectrophotometer used to characterize pigments. The infrared spectrum of the FTIR helps in structural analysis. The pigment was analyzed in the range of 4000 to 400cm^{-1} to identify the functional group present and their position **(OH, N-H, C=C, C-H, C-N, C-H, and C-O)** in the pigment [10, 19].

H. Optimization of Culture Condition for Pigment Production by *Rhodotorula mucilaginosa*

The pigment-producing yeast was streaked and incubated at different temperatures ranging from 25, 30, 37, and 42 °C for 4 days to determine the optimum temperature at which pigments from the yeast can be best produced. The intensity of the yeast pigment produced at each incubating temperature was observed and measured with a UV-visible spectrophotometer [14].Different pH ranges of 5, 7, 9, and 11 were used to optimize the production of pigment. 1M HCl and 1M NaOH were used to adjust the pH of the culture medium to attain the desired pH. The yeast was then streaked on each plate containing the selected pH and was incubated for 4 days at 25 °C. The pigment intensity produced in the presence of selected pH was measured with a UV-visible spectrophotometer [11].

With slight modification to the method of [20], the pigment-producing yeast was inoculated on potato dextrose agar containing 4 µg/mL of different metals such as Potassium dichromate ($K_2Cr_2O_7$), Mercury ($HgCl_2$), Potassium cyanide (KCN), and Lead ($PbCl_2$) and incubated at 25 °C for 4 days respectively to assess the influence of common metals on pigment synthesis. **T**he PDA without metals was used as control and the experiment was performed in triplicate. The plates were examined, and the pigment was extracted with chloroform and analyzed with 200-700 nm wavelength ranges to find the pigment's highest absorption peak.

I. Statistical Analyses

Every assay was carried out in triplicate, and the results were reported as mean ± standard deviation (SD. The data was analysed using Microsoft Excel 2016. A statistically significant difference was found using one-way Analysis of Variance (ANOVA), and $p \leq 0.05$ was used as a limit to indicate statistical significance.

III. Results and Discussion

A.

i. Isolation and Identification of Pigment-Producing *Rhodotorula mucilaginosa*

From the soil samples obtained from the Zoological Garden, Osun State University, Osogbo, Nigeria, the colony characteristic and the microscopic view (40x) of the orange pigment-producing *Rhodotorula mucilaginosa* visualized with Gram staining, Giemsa staining, Methylene blue, and Lactophenol blue staining is shown in Figure 2. It was observed that the isolate assimilated glucose, sucrose, maltose, galactose, xylose, and nitrate, and the cells were well stained. In addition, the isolate showed positive reaction to some other biochemical tests carried out in Table 1.

ii. Molecular identification of *Rhodotorula mucilaginosa* Isolate

The DNA partial sequence of the yeast obtained was subjected to multiple alignment algorithms. It was compared to the nearest reported sequences using BLASTn and identified as *Rhodotorula mucilaginosa* strain A2J2 (OR225816.1) with 99.68% similarity. Using MEGA 11 software, a phylogenetic tree was constructed Figure 2. The most adjoining yeast strain was

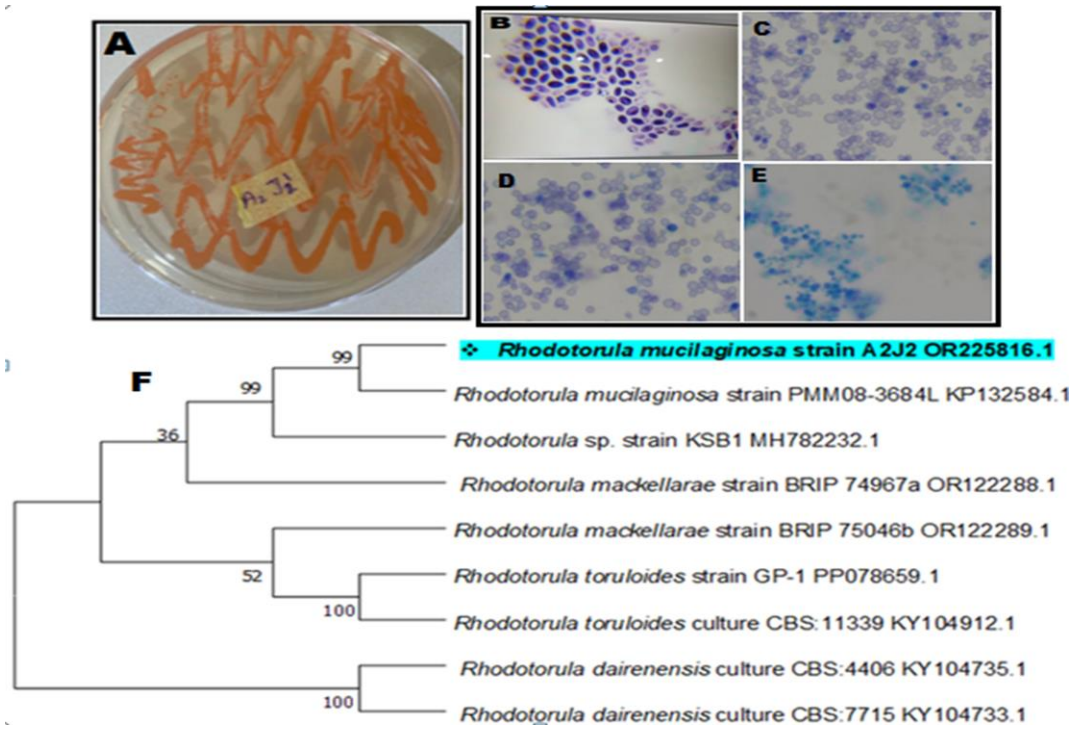

Figure 2: (A) Isolated *Rhodotorula mucilaginosa* on PDA (B) Microscopic view of the yeast cell visualized under 40x with Gram staining (C) Giemsa staining (D) Methylene blue staining (E) Lactophenol blue staining (F) Neighbor-joining tree based on 18S rRNA (partial sequence)-ITS1-5.8S rRNA-ITS2-28S rRNA (partial sequence) showing the phylogenetic position of the isolates among closely related taxa at 1000% bootstrap replicates. Evolutionary analyses were conducted in MEGA11.

Table 1: **Morphological and biochemical characteristics of** *Rhodotorula mucilaginosa* strain A2J2

Colony	**Appearance**	**Biochemical characteristics**			
Pigment	Orange	**Carbon assimilation**	**Reaction**	**Other**	**Reaction**
Cell shape	Ovoid	Glucose	+	Citrate	+
Colony shape	Round	Sucrose	+	Oxidase	-
Elevation	Raised	Fructose	-	Catalase	+
Margin	Entire	Lactose	-	Urease	+
Appearance	Smooth	Maltose	+	Indole	+
Texture	Mucoid	Galactose	+	Nitrate	+
Spore formation	Negative	Mannitol	-	Glycerol	+
Size	2-3 mm	Xylose	+	Urea hydrolysis	+

Rhodotorula mucilaginosa strain PMM08-3684L (KP132584.1). The obtained sequences were submitted to Genbank.

iii. Extraction and UV-vis Spectrophotometer Characterization of Pigment

Chloroform was observed to extract the pigment after acid hydrolysis of the cell efficiently. The extracted pigment was orange in colour, which is similar to carotenoid pigment. The UV-visible study of the extracted pigment from the *Rhodotorula mucilaginosa* showed several peaks in the spectra's UV region, and the peak with the highest λmax was selected as the pigment from the yeast. It was observed that the pigment absorbed at a λmax of 490 nm with an absorbance of 2.866 abs, showing the presence of torularhodin Figure 3.

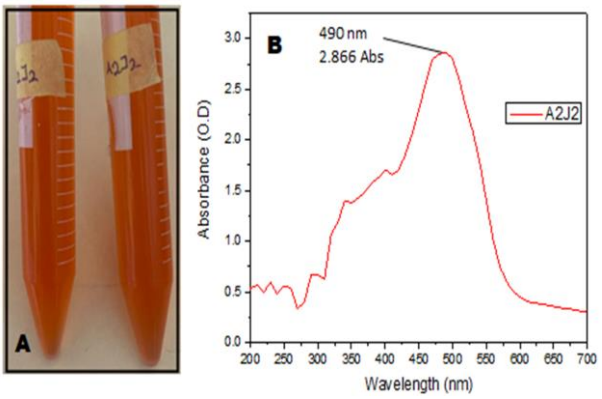

Figure 3: (A) Chloroform extracted pigment (B) UV-visible Spectrophotometer characterization of the extracted pigment. Key: A2J2 (*Rhodotorula mucilaginosa*)

iv. Fourier-transform infrared spectroscopy (FTIR) of extracted pigment

The FTIR spectrum of the pigment from *Rhodotorula mucilaginosa* is shown in Figure 4 and Table 2. The broad and long peak at 3468 cm^{-1} is due to Dimeric O-H stretch. The peak at 2924 cm^{-1} and 2854 cm^{-1} is due to N-H stretching of amine salt. The peak at 2681 cm^{-1} is due to C-H stretching of aldehyde. Also, the peak at 2364 cm^{-1} is due to O=C=O stretching of carbon dioxide. The peak at 1747 cm^{-1} is due to C=O stretching of ester. The peak at 1639 cm^{-1} is due to C=C stretching of alkene. The peak at 1543 cm^{-1}, 1508 cm^{-1}, and 1458 cm^{-1} is due to N-O stretching of nitro compound. The peak at 1373 cm^{-1} is due to O-H bending of alcohol. The peak at 1234 cm^{-1} is due to C-O stretching of alkyl aryl ether, and the peak at 1168 cm^{-1} is due to C-O stretching of, ester. The peak at 1072 cm^{-1} is due to C-O stretching of primary alcohol, and the peak at 1033 cm^{-1} is due to S=O stretching of sulfoxide. The peak at 964 cm^{-1}, 875 cm^{-1}, and 721 cm^{-1} is due to C=C bending of alkene. The peak at 609 cm^{-1} is due to C-Br stretching of halo compound.

v. Optimization of growth condition in production of pigment

The result of the optimization of growth conditions in the production of pigment by *Rhodotorula mucilaginosa* is shown in Figure 5. It was observed that at an incubating temperature of 25°C, an absorbance of 2.856 and 1.685 at 30°C was recorded, respectively. However, no production of pigment was seen at 37°C and 42°C. In addition, 2.189, 2.849, 2.479, and 2.256 absorbance of the pigment was recorded in the 5.0, 7.0, 9.0, and 11.0 pH, respectively.

vi. Optimization of metals in the production of pigment

The optimization of metals in the production of pigment showed that growth of the *Rhodotorula mucilaginosa* strain A2J2 was observed in the presence of all the metals, which can be regarded as metal tolerant. When the pigment produced in the culture medium containing each metal was

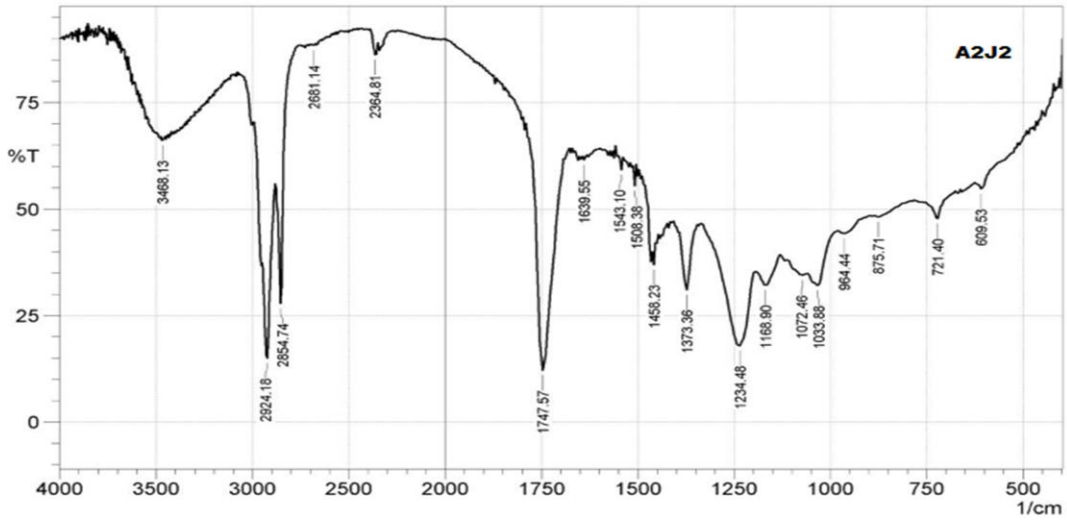

Figure 4: FTIR spectra of extracted pigment from yeast isolate
Key: A2J2 (*Rhodotorula mucilaginosa*)**.**

Table 2: Spectrum range of FTIR result of pigment from *Rhodotorula mucilaginosa*

Absorption (cm^{-1})	Group	Compound Class
690-515	C-Br stretching	halo compound
980-960	C=C bending	alkene
1070-1030	S=O stretching	sulfoxide
1085-1050	C-O stretching	primary alcohol
1210-1163	C-O stretching	ester
1275-1200	C-O stretching	alkyl aryl ether
1420-1330	O-H bending	alcohol
1550-1500	N-O stretching	nitro compound
1648-1638	C=C stretching	alkene
1750-1735	C=O stretching	esters
2400-2000	O=C=O stretching	carbon dioxide
2830-2695	C-H stretching	aldehyde
3000-2800	N-H stretching	amine salt
3550-3200	O-H stretching	alcohol

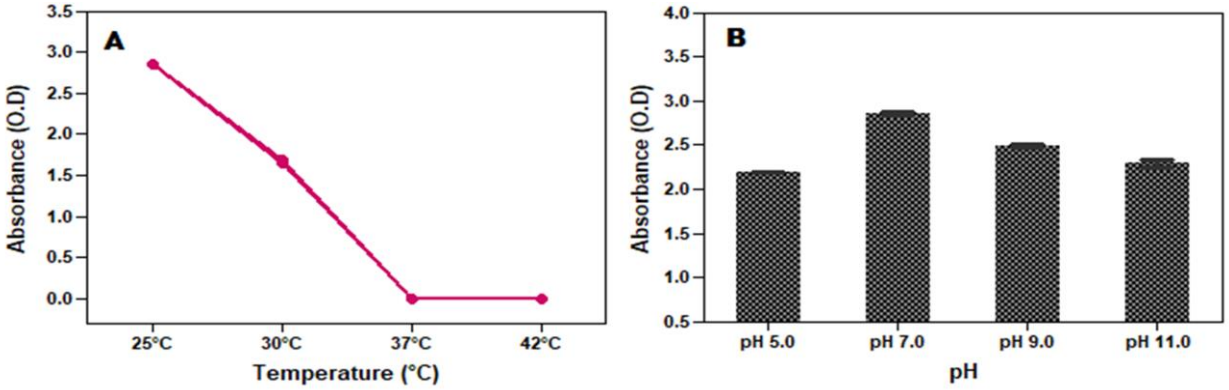

Figure 5: Intensity of pigment produced at various (A) temperature (B) pH
Key: Optical density (O.D)

extracted, it was seen that in the presence of $HgCl_2$, $K_2Cr_2O_7$, there was a change in the colour of the pigment to wavelength 380nm, 1.438 abs and 400nm, 2.943 abs respectively. In the presence of $PbCl_2$, a more intense pigment with 500nm and 3.772 abs was observed compared to the initial colour without metals.

However, in a medium containing KCN, there was production of different pigment colours with 370nm, 1.034 abs compared to the pigment obtained in the presence of other metals Figure 6. The shades and the intensity of each pigment produced are shown in Figure 7.

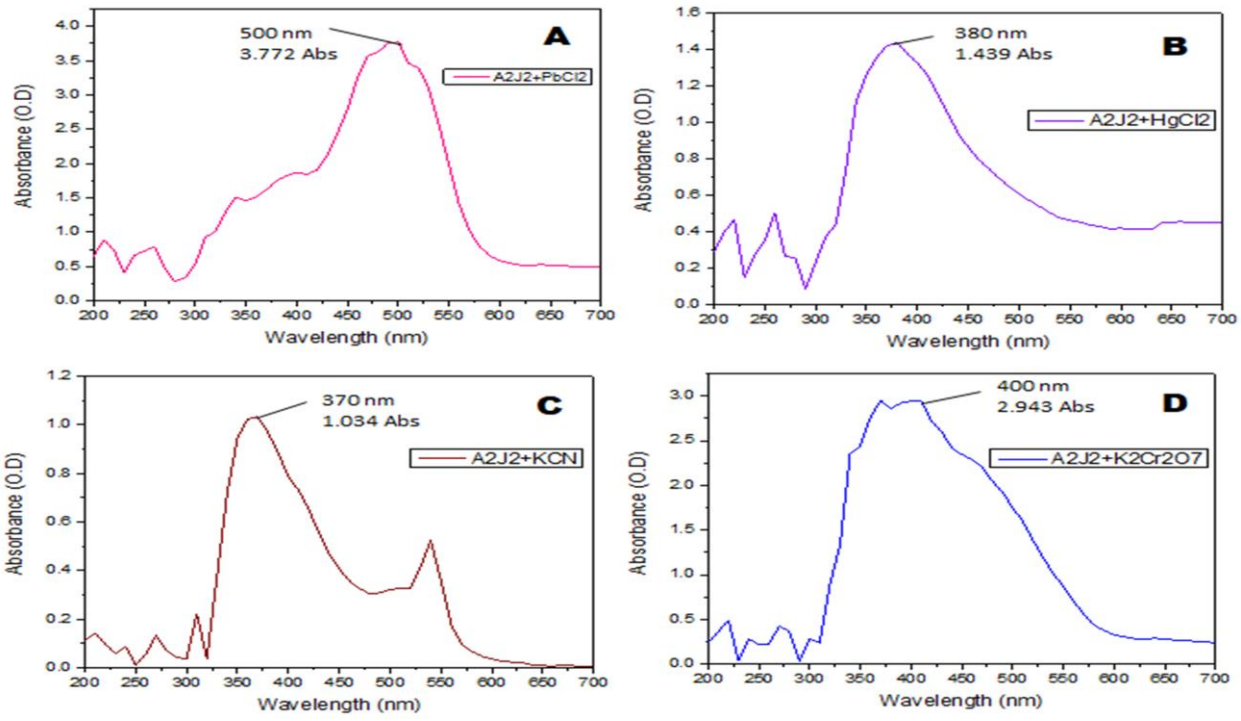

Figure 6: UV-vis Spectrophotometer characterization of pigment produced by yeast isolate in the presence of (A) $PbCl_2$ (B) $HgCl_2$ (C) KCN (D) $K_2Cr_2O_7$ Key: A2J2 (*Rhodotorula mucilaginosa*), Potassium dichromate ($K_2Cr_2O_7$), Mercury ($HgCl_2$), Potassium cyanide (KCN), and Lead ($PbCl_2$)

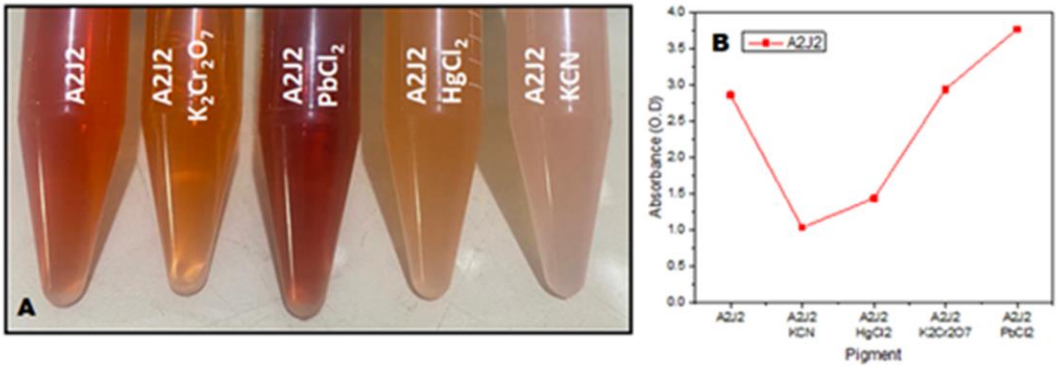

Figure 7: (A) Shades of pigment (B) Intensity of pigment produced by *Rhodotorula mucilaginosa* strain A2J2 in the presence of metals
Key: A2J2 (*Rhodotorula mucilaginosa*), Potassium dichromate ($K_2Cr_2O_7$), Mercury ($HgCl_2$), Potassium cyanide (KCN), and Lead ($PbCl_2$).

vii. Optimization of metal concentrations in the production of pigment

The results of the different concentrations of metals in the production of pigment revealed the potential of the *Rhodotorula mucilaginosa* strain A2J2 to tolerate and produce pigmentation in the presence of the selected metals Figure 8. Intense pigmentation was observed in concentration ranges from 8 to 0.5 µg/mL of $K_2Cr_2O_7$. No growth of the *Rhodotorula mucilaginosa* was recorded in 8 µg/mL, and intense pigmentation was seen in concentration ranges from 4 to 0.5 µg/mL of the $PbCl_2$. In addition, slight pigmentation was observed in the presence of 8 to 0.5 µg/mL of KCN and $HgCl_2$, respectively.

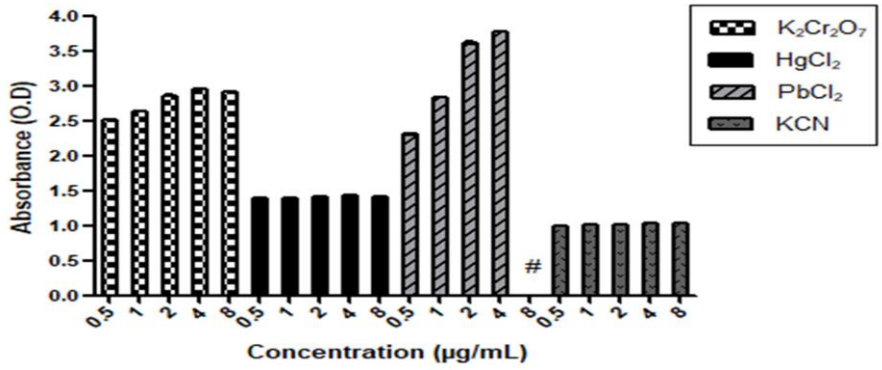

Figure 8: Optimization of metal concentrations in pigment production.
Key: Potassium dichromate ($K_2Cr_2O_7$), Mercury ($HgCl_2$), Potassium cyanide (KCN), and Lead ($PbCl_2$) No microbial growth (#)

B. Discussion

From the soil samples obtained from Osun State University Biological Garden, pigment-producing *Rhodotorula mucilaginosa* was isolated on potato dextrose agar supplemented with Chloramphenicol 50μg/mL for 4 days. The morphological identification of the isolate on the plate, revealed that the yeast is Citrate positive, Oxidase negative, Catalase positive, orange in pigment colour. Similar characteristics were reported by [21] from *Rhodotorula mucilaginosa* isolated and identified from mangrove sediments of various sites along North Kerala. In malt extract medium, the smooth and mucoid colonies of *R. mucilaginosa* showed an orange to red colouration. Under a microscope, the cells were found to be oval-shaped, hyphated, and capable of asexual reproduction through multilateral budding. In addition, the result of this study also corresponds to the colony characteristic and carbohydrate assimilation of the red pigment-producing *Rhodotorula* sp. RY1801 isolated from the sediment of the marine environment [12].

The molecular identification of the isolate using the ITS region primers confirmed the identity of the isolate as *Rhodotorula mucilaginosa* strain A2J2 (OR225816.1) with 99.68% similarity when subjected to an algorithm for multiple alignments to the closest published sequences using BLASTn. The neighbor-joining tree constructed with MEGA 11 revealed the closely related taxa to be *Rhodotorula mucilaginosa* strain PMM08-3684L (KP132584.1). [12] also reported the identification of marine yeast strain *Rhodotorula* sp. RY1801 using the sequencing of the ITS region in the yeast isolate. In addition, [21] utilized the sequencing of the ITS region of the yeast DNA to confirm the species as *R. mucilaginosa* isolated from the mangrove sediments in North Kerala when compared with the GenBank database with 100% sequence homology.

Chloroform efficiently extracted the intracellular carotenoid pigment from the *Rhodotorula mucilaginosa* strain A2J2. This could be due to the hydrolysis of the yeast cells with 6M HCl followed by the chloroform penetrating the cell to extract the pigment contents. This extraction procedure conforms with the report of [11] on carotenoid pigment from *Rhodotorula, Sporobolomyces,* and *Cystobasidium* extracted with chloroform/methanol (2:1 v/v) mixture. A similar report was seen by [21] on extracting carotenoid pigment from *R. mucilaginosa* with DMSO: acetone solvent from frozen pelleted cultures.

In this study, the major carotenoid in the extracted pigment from the *Rhodotorula mucilaginosa* strain A2J2 was identified with a sharp peak at 490nm and an absorbance of 2.866 Abs to be torularhodin. According to other findings, the 400–500 nm range is thought to be the fingerprint region of carotenoids. The yeast *Rhodotorula mucilaginosa* present*s* three significant carotenoids in the extracted pigment, indicated in the major sharp peaks at 450 nm and 480-490 nm. These carotenoids exhibited λmax at 490 nm (Torularhodin), 484 nm (Torulene), and 450 nm (β-carotene) [2]. Similarly, [22] reported the λmax at 490 nm to estimate carotenoid content from *Rhodotorula mucilaginosa* extracted pigment.

The FTIR analysis showed the presence of the pigment belonging to functional groups such as aliphatic amines, alkyl halides, alcohols, alkanes, esters, carbonyl groups, and nitro. This is in line with the findings of a similar report from [19] on the presence of alkane, alkene, alkynes, alcohols,

esters, and sulfate in the pigment produced by *Brevundimonas olei* strain RUN-D1.

The optimization conditions for the production of pigment by the *Rhodotorula mucilaginosa* strain A2J2 revealed an intense pigmentation at 25°C, a temperature lower than the environmental temperature at which the yeast was isolated. Slight pigmentation occurred at 30°C, and no pigment production was produced at 37°C and 42°C. This could result from the temperature above the environmental temperature at which the yeast was isolated or from the fact that it is not favourable for pigment production by the isolate. In addition, the *Rhodotorula mucilaginosa* strain A2J2 produces pigmentation in all the tested pH. However, high intensity was recorded at pH 7.0. This indicated that the optimum temperature at which the *Rhodotorula mucilaginosa* strain A2J2 could produce this pigment is 25°C, and the optimum pH is 7.0. This is in agreement with the optimization study from [21] on *R. mucilaginosa* when the pH of the media was pre-adjusted from 5-9, and maximum growth was observed at pH 7. The optimum temperature required for the maximum growth of *R. mucilaginosa* was found to be 25°C.

The optimization of metals in the production of pigment showed the *Rhodotorula mucilaginosa* strain A2J2 tolerated the metals. Diver peaks on the UV-visible spectrophotometer were recorded based on the pigment intensity. In the presence of $K_2Cr_2O_7$, $HgCl_2$, KCN and $PbCl_2$, *Rhodotorula mucilaginosa* strain A2J2 produced different shades of pigment with λmax of 370nm, 380nm, 400nm, and 500nm. The production of the different shades of pigment by the *Rhodotorula mucilaginosa* strain A2J2 may be due to the 4 μg/mL of the metals being high enough to induce expression and change the metabolic pathways in the yeast isolate. The *Rhodotorula mucilaginosa* strain A2J2 showed tolerance at different concentrations of 0.5 – 8 μg/mL of the metals except in the presence of 8 μg/mL of $PbCl_2$, which showed no growth of the *Rhodotorula mucilaginosa* strain A2J2. This could be because the high concentration of the $PbCl_2$ inhibited the growth of the *Rhodotorula mucilaginosa* strain A2J2. A similar study on the tolerance of *S. aureus, Bacillus* sp., and *P. aeruginosa* to chromium (Cr), silver (Ag), and mercury (Hg) in pigment production was reported by [20].

IV. Conclusion

Our study isolated pigment-producing *Rhodotorula mucilaginosa* strain A2J2 from soil sample, and identified the pigment as torularhodin with UV-visible mass spectroscopy at 490 nm. The optimization study revealed that *Rhodotorula mucilaginosa* strain A2J2 synthesized several shades of pigment in the presence of $K_2Cr_2O_7$, $HgCl_2$, KCN, and $PbCl_2$, with an optimum concentration of 4 μg/mL, incubation temperature of 25°C, and pH 7.0 for 4 days. The pigment produced can be purified and use as alternative to conventional pigment used in the textile industry for tie and dye, and in food industry as an additive agent.

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Declarations of competing interests

All authors declare that there are no competing interests.

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