

Microbial Production and Characterization of 3, 4-dihydroxy-L Phenylalanine Mediated an Extracellular Heterogenic Eumelanin Pigment From Marine Actinomycetes Prauserella Sp. MAPPL 017A

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Abstract

Melanin is one of the natural biological pigment and it is derived from the enzyme tyrosinase by the oxidation of an amino acid tyrosine. The enzyme tyrosinase [EC 1.14.18.1] is a type-3 copper protein family found large amount in microorganisms and human beings involved in the biosynthesis of melanin and other polyphenolic compounds. Melanin plays an important role in all living creatures such as photo protectants against UV radiation, antioxidant, metal adsorption, electrical conductivity, camouflage, anti-aging properties, virulence and fungal pathogenesis. The above properties of melanin can be used in various application purposes which make the melanin expensive and great demand in the industry. To overcome the above situation, there is a necessity to find out the new biological sources for the maximum production of melanin. Numerous reports are available on fungal and bacterial melanin but only a few of them from actinomycetes. Based on the above view, the present investigation was aimed to purify the DOPA melanin from the novel marine actinomycetes *Prauserella* sp. (MAPPL 017A). Purified melanin was characterized using several state-of-the-art techniques and it's showed the broadband absorbance, presence of carboxylic and indolic groups, high amount of carbon, nitrogen and low level of sulfur, presence of other metal ions such as calcium, sodium and magnesium with efficient thermal properties.

1. Introduction

Melanin pigments are highly insoluble natural biological macromolecules [1] which occurs in all the animal kingdom. Based on the color and substrate sp.ecificity these macromolecules are classified into five major types such as eumelanin, pheomelanin, pyomelanin, allomelanin and neuromelanin. Among these, eumelanin is the predominant pigment synthesized in humans and microorganisms [2] which are hydrophobic, negatively charged and high molecular weight polymer [3].

In microorganisms, the melanin pigments are metabolically derived from the enzyme tyrosinase which catalyzed by the oxidation of an amino acid tyrosine [4–5] and the resultant melanin pigments are black or brown in color [3]. It is also been proved that the microbial tyrosinases are resemblance to the type-3 copper metalloprotein [6].

Melanin pigments are polymers of phenolic compounds [7] possess antioxidant and radioprotective properties which can be used as a protective shield from the harmful radiations and it is evident that the low level of melanin in the human skin has high risk of tumor [8–9]. It also plays an important role in the virulence of a broad range of phytopathogenic fungi [3, 10]. In addition, melanin has an affinity towards the metal ions and is naturally associated with them. The above characteristics made the researchers make use of it in different technologies such as metal adsorption and electric conductivity (semiconductors) and bioelectronics. Due to the above information, the present study was aimed to purify the extracellular melanin from the novel marine Actinomycetes *Prauserella* sp. and this is the first report on melanin production from the genus *Prauserella*.

2. Materials And Methods

2.1 Isolation and identification of melanin producing marine actinomycetes

Melanin producing marine actinobacterium was isolated from water collected in the marina beach, (Lat. 13° 3' 20" N and Long. 80° 17' 1" E) a region of Bay of Bengal, Chennai, Tamil Nadu, India and screened for the melanin production. The melanin positive strain was identified through polyphasic, molecular and chemotaxonomic characterization.

2.2 Test for melanin formation and tyrosinase activity

The test for melanin formation and tyrosinase activity were carried out by a simple, rapid and sensitive method proposed by Mikarni et al., [11].

2.3 Extraction and purification of melanin pigment

The extraction and purification of extracellular melanin produced by *Prauserella* sp. (MAPPL 017A) was performed by the method [12].

2.4 Purification of melanin from *Prauserella* sp. (MAPPL 017A)

For the purification of melanin pigments, different solvents and extraction procedures were carried out to remove the associated components such as protein carbohydrate in the melanin pigment. The removal of carbohydrates and proteins were carried out by acid hydrolysis which was followed by lipids extraction with the solvent chloroform and the metabolites were sequentially removed by the extraction of different solvents such as hexane, ethyl acetate, acetone and ethanol.

2.4.1 Acid hydrolysis

The crude melanin obtained from the above procedure was hydrolyzed with 10 ml of 6 M HCl for 2 h at 100°C to remove carbohydrates and proteins. The non-hydrolysable residue was collected by centrifugation at 10,000 rpm for 10 min and washed with distilled water for several times and dried under desiccation and stored in airtight containers for further use.

2.4.2 Washing with solvents

The melanin obtained after acid hydrolysis was re-dissolved in 2 M NaOH and centrifuged at 10,000 rpm for 15min. The supernatant was collected and subjected to precipitation with 1 M HCl and centrifuged to obtain the pellet. The pellet was washed several times with distilled water and dried well. The non-hydrolysable melanin was re-dissolved in 5 ml of 1M NaOH to which 2 mL of chloroform and 0.2 mL of 1-butanol were added. The mixture was shaken for 30 min and centrifuged at 10,000 rpm for 10 min. The chloroform and butanol phase was discarded and the procedure was repeated twice. The solution containing melanin was treated with organic solvent such as, hexane, ethyl acetate, acetone for the removable of secondary metabolites and finally with ethanol for the removable of water molecules bound

with the melanin pigment. The resulting content was lyophilized and stored as 'pure melanin' for further characterization.

2.5 Characterization of melanin pigment

2.5.1 Physio-chemical analysis of purified melanin

The chemical analysis of purified melanin pigment was carried out by the modified method of [13-14]. Commercially available synthetic melanin was purchased from Sigma (Catalog No. M 8631) and used as the standard for all the characterization directly without any further treatment.

2.5.2 UV-Vis spectroscopic analysis of purified melanin

The purified melanin was dissolved in 0.1 M sodium carbonate buffer (pH 10.3) and 0.1 M sodium hydroxide solution to obtain a final concentration of 0.03g/L. The solutions were stirred by vortexing for 15 min and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant collected was subjected to UV-Vis spectral analysis at a wavelength range of 200-800 nm in a UV-Vis spectrophotometer (Hitachi U-2900, Japan).

2.5.3 FT-IR analysis of purified melanin

For FT-IR analysis, approximately 5 mg of melanin and 700 mg of KBr were thoroughly mixed and made into pellets. The pellet was used for recording the IR spectra using an FT-IR spectrometer (Perkin Elmer, USA).

2.5.4 Raman spectroscopic analysis of purified melanin

The purified melanin was subjected to Raman spectroscopic analysis (Raman 11i, Nano photon, Japan) at the range of 1000- 2500 cm^{-1} . The spectrometer has back scattering geometry for the detection of Raman spectrum with the resolution of 1 cm^{-1} and the excitation source was 532 nm.

2.5.5 ^1H NMR spectral analysis of purified melanin

The melanin pigment was dissolved in DMSO and subjected to ^1H NMR spectral analysis with Bruker instrument (400 MHz) at Sophisticated Analytical Instruments Facility (SAIF), Indian Institute of Science, Bangalore.

2.5.6 Solid state NMR analysis of purified melanin

The solid-state NMR spectral analysis of purified melanin was performed with a Bruker Avance-III HD 400 WB NMR spectrometer (9.4 T). The proton and carbon resonance frequencies were set at 400.07 and 100.61 MHz, respectively. Samples were packed in a 4-mm-diameter zirconia rotor with a Kel-F cap. ^{13}C MAS spectrum of the sample was recorded using a double resonance 4 mm MAS probe at room temperature. ^{13}C cross polarization/total sideband suppression (CP/TOSS) analysis was performed as

described by Dixon [15] at a spinning speed of 7 kHz to get sideband free spectrum. A contact time of 3 ms and a ^1H 90° pulse length of 4 μs were used. Typically, 768 scans were acquired with a relaxation delay of 4s. According to Fung et al., [16] the SPINAL-64 decoupling sequence was used to decouple protons during the carbon acquisition employing the radio frequency field strength of 83 kHz. FID was subjected to an exponential multiplication function with a line broadening value of 150 Hz prior to Fourier transform. ^{13}C chemical shifts were referenced to the carbonyl signal of glycine at 176.03 ppm as an external reference standard.

2.5.7 Quantitative elemental analysis of purified melanin.

The percentage of elements such as carbon, hydrogen, nitrogen and sulfur present in the melanin pigment was determined over a wide range of sample matrices and concentrations with TruSpec micro analyzer at Sophisticated Analytical Instruments Facility, IIT Madras, Chennai, India.

2.5.8 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analysis of purified melanin

The atomic spectrum emitted by purified melanin is used to determine its elemental composition by ICP-OES analysis (Perkin Elmer optima 5300 DV, USA). The wavelength at which emission occurs identified the element while the intensity of the emitted radiation quantifies its concentration.

2.5.8.1 Procedure for Melanin digestion for ICP-OES

Thirty milligrams of melanin was dissolved in 10 mL of deionized water which was added with 1 mL each of nitric acid (65 %, w/v) and perchloric acid (70%, w/v). The mixture was heated at 100°C for 5 min and the volume was made up to 100 mL with deionized water.

2.5.9 High Resolution Scanning Electron Microscopic and Energy Dispersive Spectrometer analyses (HRSEM-EDAX) of purified melanin

The surface topography and qualitative elemental analysis of the purified melanin was performed using HRSEM-EDAX (FEI QUANTA FEG 200 Hillsboro, USA) equipped with EDX at Sophisticated Analytical Instruments Facility, IITM, Chennai.

2.5.10 Electro paramagnetic resonance analysis of purified melanin

Electron paramagnetic resonance of the purified melanin was acquired with a Varian X-band EPR spectrometer (JEOL Model JES FA 200, Japan) with a modulation frequency of 8.75- 9.65GHz. The g-value of the melanin sample was measured at the point of zero slope of the absorption curve, and the line width (ΔH) was measured between inflection points of the resonance absorption [17].

2.5.11 X-ray diffraction analysis of purified melanin

The purified melanin obtained from *Prauserella* sp. was ground to fine powder with mortar and pestle and subjected to analysis by X-ray diffraction with the following parameters: CuK α radiation (λ -1.5406 Å),

voltage 40 kV, current 40 mA, 2 θ range 3°-80°.

2.5.12 Thermal degradation analysis of purified melanin

The purified melanin obtained from *Prauserella* sp. was weighed (3.343 mg) and analyzed with a thermal analyzer (SDTQ600v 8.0 build 95). The TGA and DSC data were recorded under nitrogen atmosphere at the flow rate of 20 mL/min. The analysis was carried out with ambient temperature to 1000°C at a heating rate of 10°C/min.

3. Results And Discussion

3.1 Isolation and identification of melanin producing marine actinomycete

Pigment producing isolate MAPPL 017A was isolated using the sea water collected from the sea shore of Marina Beach, a region of Bay of Bengal, Chennai, Tamil Nadu, India. And produced the diffusible purplish black color pigments on Czapek tyrosine (Fig. 1). The resultant pigment was confirmed as melanin through standard protocols. The melanin positive organism MAPPL 017A was identified as *Prauserella* sp. based on the morphological, physiological, biochemical, molecular and chemotaxonomic identification methods. The resultant nucleotide sequence from the above strain was deposited in the gene bank with the accession no MH341971.

3.2 Melanin Confirmation test

The qualitative and quantitative determination of melanin production by the isolate MAPPL 017A was carried out by the addition of L-DOPA to the culture supernatant. The color changes into brown confirmed the presence of enzyme tyrosinase (Fig. 2) and the amount of tyrosinase enzyme produced by the melanin positive isolate was quantitatively determined and the maximum enzyme production occurred on 15 day (Fig. 2). Similar experiments were carried out in *Streptomyces griseus* and *Streptomyces phaeochromogenes* [11].

3.3 Purification of melanin pigments produced by *Prauserella* sp.

The purification of melanin produced by *Prauserella* sp. was done using acid precipitation method which is the familiar and specific method preferred by most of the researchers [12, 14, 18-34] and it was characterized using several state-of-the-art techniques which are described below.

3.4 Physical properties of purified melanin

The purified melanin has been found to be insoluble in water, organic solvents and partially soluble in DMSO. However, the pigment was completely soluble in ammonia water, NaOH and KOH solutions. The purified melanin has been found to be in characteristic black color, viscous in nature and susceptibility to oxidizing agents [35] as has been observed with melanin pigments from various bacterial, fungal and plant sources (Bell) [39] such as seeds of *O. fragrans* [14], *Klebsiella* sp. GSK [21], *Aspergillus nidulans*

[22], *Asp.ergillus bridgeri* ICTF-201 [24], *Pseudomonas stutzeri* [28] *Aeromonas media* [37], *Vibrio cholerae* [38], *Klebsiella pneumoniae* [39], Tea leaves [18, 40], *Escherichia coli* [41-43], Frankia strain [44], *Pleurotus cystidiosus* [45] and Black-bone silky fowl [42, 43, 46] as well as synthetic melanin.

3.5 UV-Vis Spectroscopic analysis of purified melanin

Melanin is an amorphous pigment which naturally exhibit scattering phenomenon (broadband absorption) in the UV-Vis spectrum (scattering phenomenon) and it believed to be formed by the superposition of the peaked spectra which are termed as “chemical disorder model” [47-49]. The melanin pigments show strong UV absorption in the region of 200-300 nm which could be attributed to the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ of the amino, carboxylic and aromatic moieties [50]. In the present study, the purified melanin pigment obtained from *Prauserella* sp. and synthetic melanin showed strong absorbance in the shorter wavelengths such as 211 and 213 nm, respectively (Fig. 3A) and the absorption decreased rapidly when the wavelengths increased to 400-800 nm. The absorbance of the melanin pigments were monotonically increased towards the higher energy region (200-300 nm) as it can be observed only in the range of 210-240 nm and decreased rapidly with increasing wavelength towards the visible region (400-800). The above characteristics have been considered as general features of the melanin pigments [51] which increases the protection against the most damaging high energy from harmful radiations. This phenomenon is due to the superposition of absorption of individuals, distinct chemical constituents [52] and the presence of complex conjugated structure of the melanin pigments.

3.6 FT-IR spectroscopic analysis of purified melanin

The functional groups present in the purified melanin have been characterized with FT-IR spectroscopic analysis. The broad absorption band centered around 3450 and 3445 cm^{-1} with EM and SM, respectively has been found to be characteristic of O-H or N-H stretching vibrations as has been observed by many investigators [24, 27, 53-57]. The strong characteristic band observed at 1637 and 1636 cm^{-1} in respect of EM and SM have been attributed to the bending vibrations modes of aromatic ring C=C and C=N bond of aromatic system in addition to C=O double bond (COOH) of carboxylic function involved in the bond formation with the metal ions [2, 24, 27, 53, 54, 57-60]. This may be due to the presence of carbonyl groups conjugated with a benzene ring and a similar situation has been found with quinone structure of DOPA- melanin [61]. Finally, the weak bands observed below 700 cm^{-1} have been ascribed to alkenes C-H substitution and out-of-plane bending of the aromatic carbon-hydrogen bond in the melanin pigment. The signals present at the region of 600-500 cm^{-1} (below 700 cm^{-1}) have been attributed to the out-of-plane bending of the oxygen hydrogen bonds [24, 57, 60, 62, 63] and 720 -590 cm^{-1} areas indicate the presence of aliphatic iodo group (Fig. 3C). The above observation made in the present study has been found to be similar to the eumelanin obtained from *Sepia officinalis* [55, 64-66].

3.7 Raman spectroscopy analysis of purified melanin

The molecular vibration and crystal structures of the purified melanin was analyzed using Raman spectroscopic analysis. The extra cellular melanin (EM) from *Prauserella* sp. and Synthetic melanin (SM) exhibited two prominent peaks in the ranges between 1300 cm^{-1} -1600 cm^{-1} (Fig. 3B). The presence of lower wave number centered at 1345 cm^{-1} , 1340 cm^{-1} (EM and SM) were related to the C-N stretching mode of the indole structure and peaks centered at 1557 cm^{-1} , 1576 cm^{-1} (EM and SM) were assigned to aromatic C=C stretching modes of the basic indole structure and C=N stretching/N-H bending (Matsunuma, Capozzi, 2005, Nagano, Priti Vairale) [67-70]. Similar peak were observed in cutaneous melanin [71]

3.8 Electro paramagnetic resonance spectroscopic analysis of purified melanin (EPR)

The EPR spectrum obtained with melanin pigments from *Prauserella* sp. appeared as a singlet without any hyperfine structure as found in synthetic melanin and DPPH, a well-known antioxidant compound (Fig. 4). EPR Spectroscopy analysis of purified melanin pigment produced signals at the magnetic fields 337.918 [mT], 337.919[mT], 337.881[mT], and their G- values were calculated as 1.99776, 1.99743, 1.99751 for extracellular melanin, synthetic melanin and DPPH, respectively. Similar observations were found in *Cryptococcus neoformans* [72], *Paracoccidioides brasiliensis* [73], *Histoplasma capsulatum* [74], *Sp. orothrix schenckii* [75], *Sepia officinalis* [75], *Klesiella* sp. [21]. The G-values obtained with purified melanin were found to be nearer to the value of standard antioxidant compound which indicated the presence of stable free radicals in the melanin pigments [77].

3.9 ^1H Nuclear magnetic resonance (NMR) spectral analysis of purified melanin

The NMR spectral analysis performed with purified extracellular melanin from *Prauserella* sp. has shown signals in both aliphatic and aromatic regions (Fig. 5A & B). The peaks centered in the region of 0.2-2.0 ppm could be assigned to the CH_3 group of alkyl fragments such as CH_2CH_3 , $\text{CH}(\text{CH}_3)_2$ as has been observed with melanin pigments obtained from different organisms [30, 31, 54, 60, 78-79]. The peaks centered between 2.2-2.8 ppm indicated the presence of characteristic methylene group [54, 60, 78] while the peak centered at 3.5 ppm has been assigned to the presence of methyl N-CH_3 [78] which might be attached to either the pyrrole or indole ring [80]. The peaks centered in the region of 3.5-4.2 ppm have been assigned to the carbon attached to nitrogen or carbon ($-\text{CH}_2$ or $-\text{CH}_3$ group connected with N/O) as has been found in pigments of other [30, 54, 60]. The peaks observed in the region of 4.2-5.4 ppm are due to the presence of C=C-H structures in the aromatic nucleus [54, 60]. The peaks centered between 6.5-9.0 ppm have been assigned to the proton attached to the substituted aromatic and hetero-aromatic regions [30, 54, 60, 79].

3.10 Solid state ^{13}C NMR analysis of purified melanin

The solid state NMR analysis performed with purified melanin obtained from *Prauserella* sp. has shown distinctive spectral regions (Fig. 6) such as aliphatic (10-110), aromatic (110-160) and carbonyl groups (160-225) which corroborates the observation made with Sepia melanin, acid free Sepia melanin and

melanin from human hair [81]. The peaks observed in the lower region (10-95ppm) are characteristics of aliphatic carbons, which might have arisen from the proteinaceous substance as associated with pigment as has been observed in *Catharsius molossus* [79] and *Auricularia auricula* [82]. The aliphatic carbon in the lower regions centered at 10-40 ppm could be due to the presence of the alkyl group such as (-CH₂CH₃), methyl or (-CH₂-) methylene and the peaks centered in the region of 40-60 ppm are due to the presence of α -carbon or carbon in CH-N/CH-S. The results obtained in the present study corroborates with the observation made earlier by [60, 82-85]. In addition, the peaks observed in the region of 35.2 and 55.7 ppm has been found to show the presence of methyl (N-CH₃) group [86]. The peaks observed in the region 95-160 and 110-160 ppm is characteristic of aromatic carbons which indicate the presence of indole or pyrrole types of carbons in the melanin polymers. The presence of broad signals in the aromatic region (110-160ppm) is characteristic of melanin signal [83] as has been observed with natural and synthetic melanin [60, 79, 81-83, 86-89]. The peaks centered in the region 165–225 ppm are as a result of carbonyl carbon atoms derived from peptide bonds, carboxylates, amides and quinones which might be coupled with the melanin polymers or proteins. A peak observed in the region 175 ppm is characteristic of quinone moieties of the carbonyl group of the melanin pigments which has been found in other melanin pigments [60, 79, 81-83, 89-90].

3.11 Elemental analysis of purified melanin

Melanin pigments naturally have the tendency to bind to various metals and therefore the CHNS analysis has been routinely performed to identify the type of melanin and to determine the purity of the pigment. The monomeric units of eumelanin and pheomelanin respectively should have 6-9 and 8-11% nitrogen content with a sulfur content of 9-12% in the case of benzothiazine monomer [91-92]. The elemental analysis performed with the purified melanin has shown the presence of relatively higher content of carbon (41.04%) when compared to other elements such as hydrogen (8.18%) and nitrogen (7.15%; Table. 1). However, the melanin obtained from different sources have exhibited varied elemental compositions [18, 22, 31, 60, 79, 93-99]. The sulfur content found in the EM and SM in the present study has been 1.56 and 0.66%, respectively. Naturally, the subunits of eumelanin and pheomelanin are formed through similar pathways and exist in a mixed form however it can be differentiated based on the percentage of sulfur content. The sulfur content of eumelanin obtained from different sources has been found to be in the range of 0.001 to 14.83% [31, 63, 91, 98-99]. It has been suggested that the sulfur content in the melanin samples might be due to the addition of some thiol containing compounds or presence of sulfur containing aminophenol in the melano proteins during the polymerization of eumelanin.

Table 1. Quantitative elemental analysis of melanin pigments from *Prauserella* sp.. (MAPPL 017A) through CHNS analysis

S. No.	Elements	Extracellular melanin	Synthetic melanin	Sepia melanin (Magarelli, <i>et al.</i> ,2010)
1.	Carbon	41.0350	46.105	33.859
2.	Hydrogen	8.1800	8.6280	3.362
3.	Nitrogen	7.1470	5.3290	6.068
4.	Sulfur	1.5585	0.660	< 0.001

3.12 Inductively Coupled Plasma Optical Emission sp.ectrometry (ICP-OES) analysis of purified melanin

The melanin pigments isolated from a sp.ecific environment has been linked with numerous metal ions including Mg (II), Ca (II), Na (I), K (I) and almost all the first transition metals have been shown to contain Fe (III) in abundant quantity. Therefore, melanin pigments could serve as a reservoir of metal ions such as Ca (II) Cu (II) and Fe (III). In the present investigation, the quantitative determination of the abundant metal ions in the purified melanin pigments has been carried out with ICP-OES analysis which indicated the presence of Na⁺, K⁺, Mg²⁺,Ca²⁺and Fe³⁺. Both EM and SM have been found to contain the same set of metal ion with varied concentration (Table. 2). Similar observation has been made with melanin of *Sepia officinalis* [66] and *Pseudomonas stutzeri* [28].

Table 2. Quantitative elemental analysis of melanin pigments from *Prauserella* sp.. (MAPPL 017A) through ICP-OES analysis

S. No.	Elements	Wavelength (nm)	Extra	Synthetic melanin	Sepia melanin
			cellular melanin		(Magarelli <i>et al.</i> , 2010)
			Concentration (mg/g)		
1.	Ca	317.933	1.393	3.789	47.302
2.	Fe	238.204	0.189	1.569	0.100
3.	K	766.490	0.090	0.140	1.300
4.	Mg	285.213	0.648	1.714	17.310
5.	Na	589.592	1.948	5.144	5.500

3.13 HRSEM–EDAX analysis of purified melanin

In the present study the morphological characteristics and quantitative determination of the elements present in the melanin pigments have been carried out with SEM-EDAX analysis (Fig.7 & 8). The obtained biopolymer appeared to be in amorphous nature as has been observed in synthetic melanin as well as the melanin obtained from a mushroom, *Auricularia auricula* ([82], silky fowl [99] however the melanin

pigments obtained from Jurassic period have been found to be spherical in shape [89]. The elemental composition in respect of EM and SM has been found in the following orders C, O, N, S, Cl, Fe, K, Na and C, O, N, Fe, Cl, S, K, Na. It has been observed that carbon, oxygen and nitrogen have been found to be the most abundant elements while sulfur, chlorine, iron, potassium and sodium are the least elements (Table. 3). A higher quantity of carbon, nitrogen and lower quantity of oxygen was observed in the purified melanin when compared to the synthetic melanin which indicated the density of the melanin layers. A traces of sulfur content was also found in the purified melanin which could be due the presence of copolymer of eumelanin and pheomelanin or aminophenol in the melanoprotein.

Table 3. Quantitative elemental analysis of extracellular melanin from *Prauserella* sp.. (MAPPL 017A) through EDAX analysis

S. No.	Element	Extracellular melanin		Synthetic melanin	
		Wt%	At%	Wt%	At%
1.	CK	61.47	69.47	60.66	68.58
2.	NK	09.12	08.84	07.19	06.97
3.	OK	22.28	18.90	26.76	22.71
4.	NaK	00.08	00.05	00.22	00.13
5.	SK	03.46	01.47	00.58	00.25
6.	ClK	02.81	01.07	01.64	00.63
7.	KK	00.11	00.04	00.22	00.08
8.	FeK	00.68	00.17	02.73	00.66

3.14 X-Ray Diffraction Analysis (XRD) of purified melanin

The XRD spectra of melanin pigments obtained from *Prauserella* sp. has shown broad peak on the 2θ scale of 20°C in all the diffractograms (Fig. 9). The broad band peaks observed are characteristic of amorphous materials like melanin polymers appeared in the region of 20°C which might be due to the parallel planar layers found in melanin structure [100]. Melanin pigments obtained from *Catharsius*

molossus [79] and *Pseudomonas stutzeri* [28] have exhibited similar peaks.

3.15 Electron Spray Ionization Mass Spectroscopic (ESI-MS) analysis of purified melanin

Due to the remarkable structural diversity and amorphous nature, melanin pigments fail to dissolve in organic solvents. Therefore, investigations to study their structural property and functions have become difficult task however are considered essential for application purposes. Therefore, the characterization of melanin pigments has been performed with Mass Spectrometry (MS) such as ESI-MS and MALDI-MS [101-104]. In the present study, the ESI-MS analysis has revealed the molecular masses of 341.0523 m/z and the molecular formula was derived as $\text{C}_{18}\text{H}_{10}\text{N}_2\text{O}_4\text{Na}$, which fell in the lower molecular mass range (Fig. 10) as has been observed earlier in LEM404 [31].

3.16 Thermal properties of purified melanin

3.16.1 Thermal gravimetric analysis

The extracellular melanin from *Prauserella* sp. underwent two stages of thermal degradation. The initial degradation occurred between 75.40 - 97.47°C which was treated as the glass transition temperature where the compound lost a weight of 0.394 mg (11.79 %) as a result of loss of free and bound water molecules present in the melanin pigment. The second degradation occurred at 236.53 - 369.80°C which resulted in the loss of 1.252 mg (37.44 %) due to the destruction of advanced and sub-structure of melanin pigments, non-covalent bonds present between layers of the structural units, covalent bonds of the monomer units such as indole and pyrrole group. The major weight losses occurred at 88.21 and 302.01°C and their derived weight were 0.1458 mg/ 3.74 min and 0.1946 mg/ 14.31 min, respectively. The complete weight loss occurred at 979.00 °C (Fig. 11A and Table. 4). Similar observations have been made for the melanin obtained from *Catharsius molossus* [79].

Table 4. Thermal gravimetric analysis of extracellular melanin from *Prauserella* sp.. (MAPPL 017A)

S. No.	Temperature	Weight	Weight
	Degree Celsius	Mg	%
Step transition			
1.	75.40 - 109.86	0.3941	11.79
2.	236.53 - 369.80	1.252	37.44
Residue			
3.	979.00	0.8506	25.44
Point value			
4.	88.21	3.74	0.1458
5.	302.01	14.31	0.1946

3.16.2 Differential Scanning Calorimetric analysis of purified melanin

It was observed that there was a difference in the heat flow at three different temperature ranges such as 85.04 - 111.22, 210.61 - 247.57 and 272.79 - 309.80°C and the maximum heat energy absorption were at 91.47/0.06059 area j/g, 229.61/86.44 area j/g and 282.65 /23.62 area j/g, respectively (Fig. 11B and Table. 5). Similar results were obtained for the melanin obtained from *Catharsius molossus* [79].

Table 5. Differential Scanning calorimetric analysis for extracellular melanin from *Prauserella* sp.. (MAPPL 017A)

S. No.	Start and end temperature	Maximum	Area
	Degree Celsius	Degree Celsius	J/g
1.	85.04 - 111.22	91.47	0.06059
2.	210.61 - 247.57	229.61	86.44
3.	272.79 - 309.80	282.65	23.62

4. Conclusion

Melanin the natural biopolymer which plays an important role in most living organisms. Based on the physical and chemical composition it has been isolated and purified from different sources and the exact structure of the melanin was not yet identified. Among the different types of melanin, eumelanin is the predominant one. Sepia melanin is well-known eumelanin purified from cuttlefish (*Sepia officinalis*) which was completely characterized by many of the researchers and it occupies a foremost position in melanin industries. The second position was occupied by fungi followed by bacterial melanin but only a few reports are available on actinomycetes. In the present study, the melanin pigment purified and characterized from the novel actinomycetes *Prauserella* sp. and it was confirmed as eumelanin based on the color and chemical nature. For the additional support, the enhanced production of melanin by the isolate *Prauserella* sp. was observed only in the presence of tyrosine through DOPA pathway thus confirmed that the resultant eumelanin was DOPA type. The various biological activities of the melanin from *Prauserella* sp. can be used various fields like cosmetics, bioelectronics, semiconductors, anticancer drugs, and protective equipment.

Declarations

Conflict of Interest

We know of no conflicts of interest with this publication and there has been no significant finance support for this work that could have influenced its outcome.

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Figures

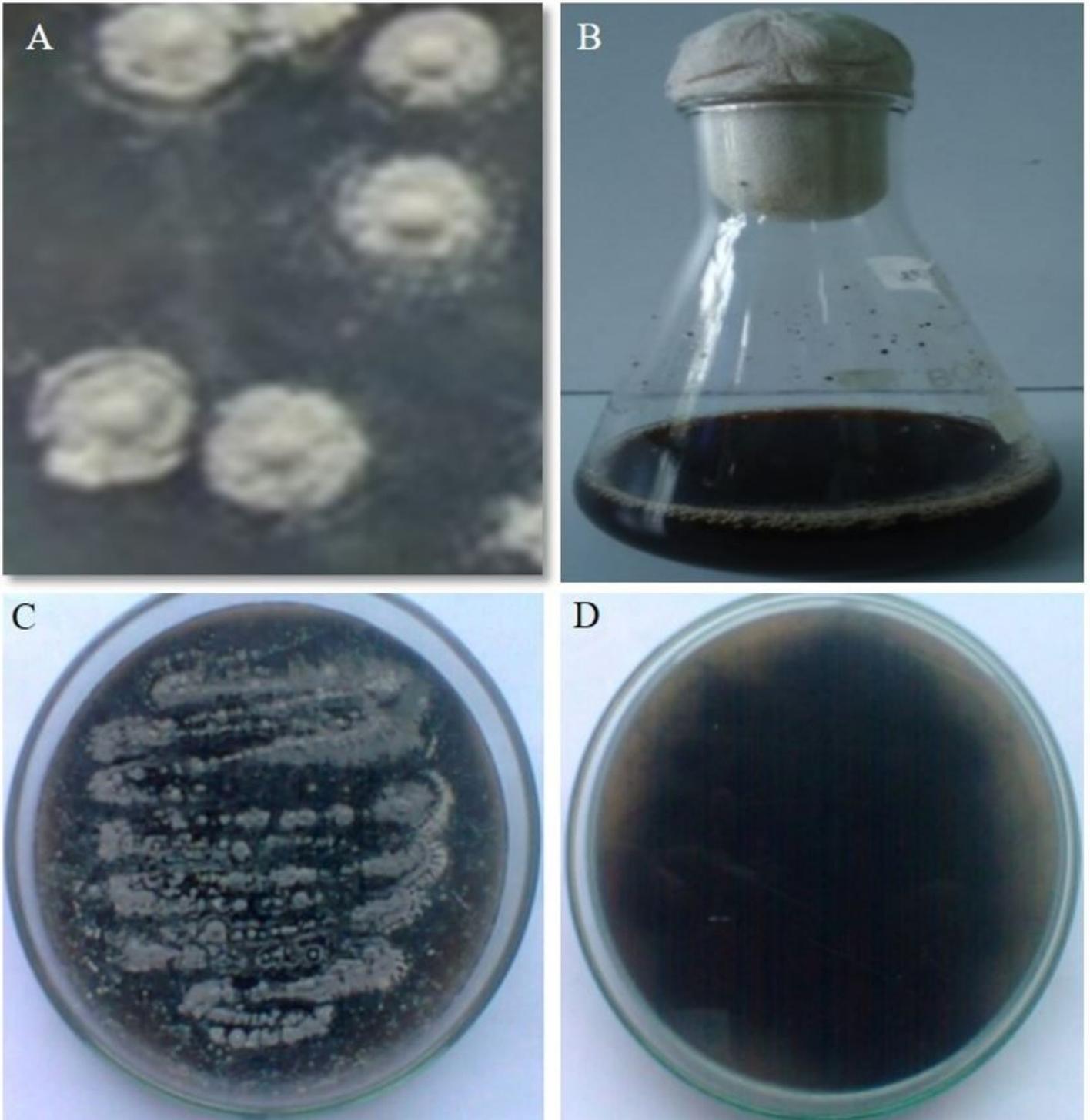


Figure 1

Colony morphology and production of melanin in/on liquid and solid medium of Czapek tyrosine. A: colony morphology of MAPPL 017A; B: Production of melanin in Czapek tyrosine broth; C&D: Aerial (C) and substrate (D) view of melanin production on Czapek tyrosine agar.

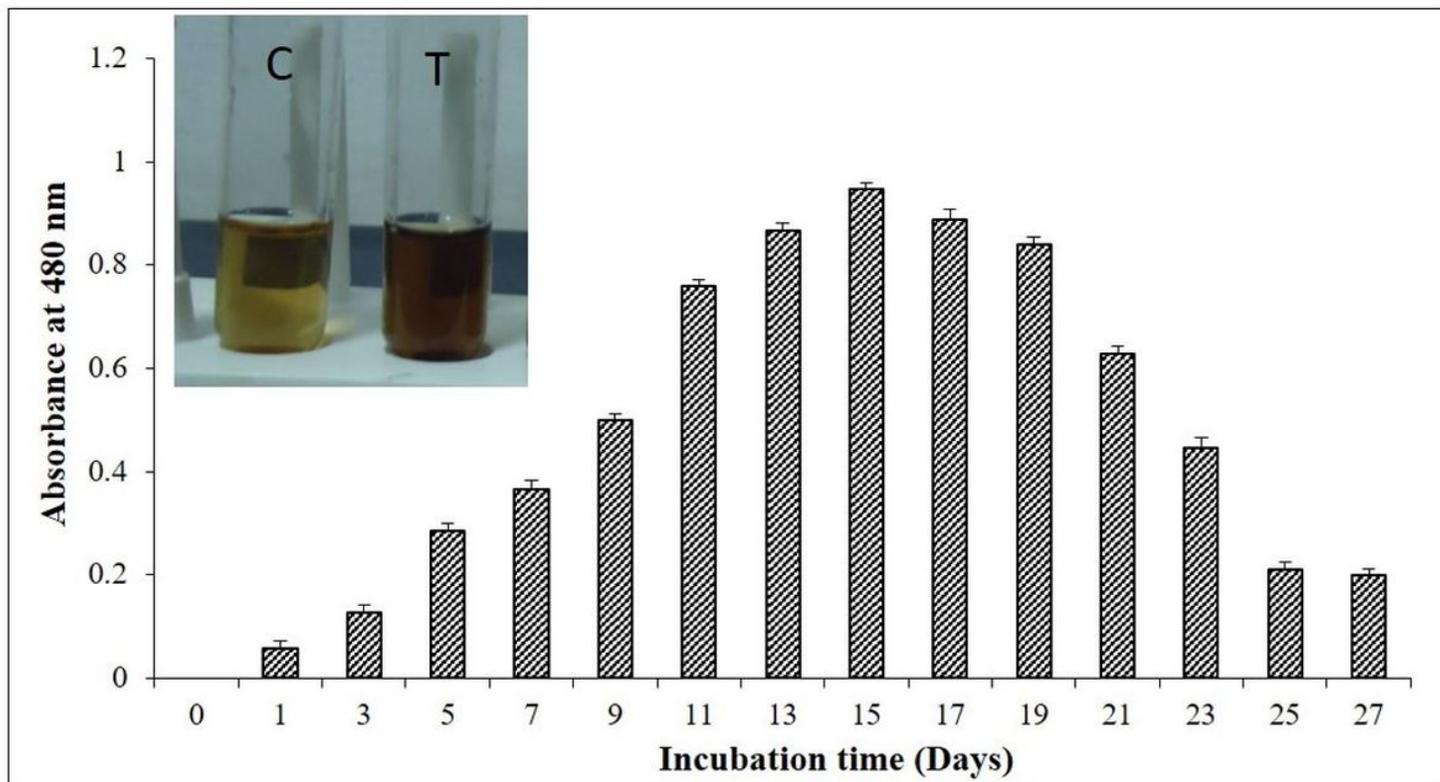


Figure 2

Qualitative and quantitative determination of tyrosinase activity by *Prauserella* sp. at different time intervals

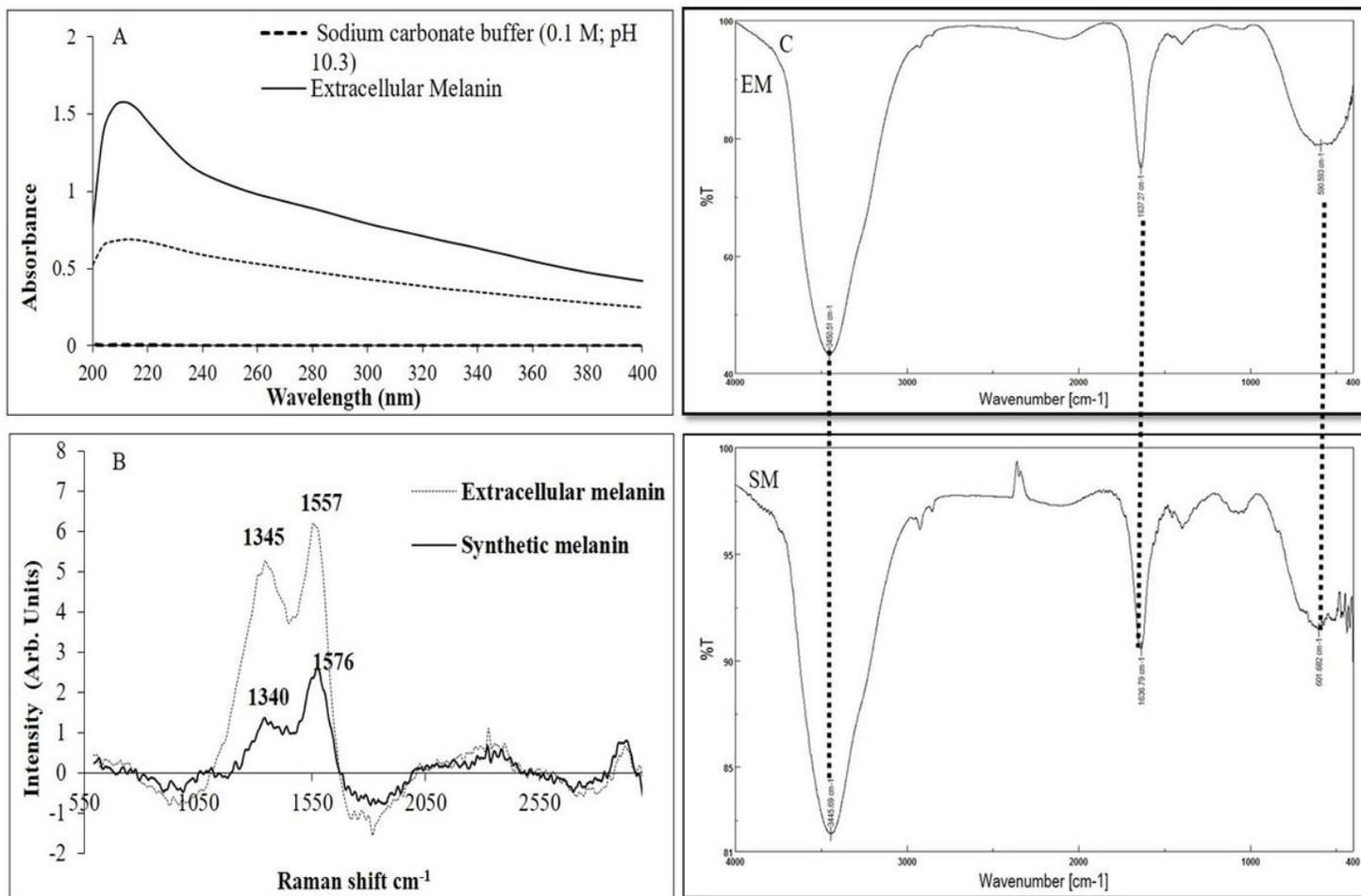


Figure 3

Sp.ectroscopic analysis of extracellular melanin from *Prauserella* sp. A: UV-VIS sp.ectroscopic analysis; B: Raman Sp.ectroscopic analysis; C: FTIR Sp.ectral analysis of purified and Synthetic melanin.

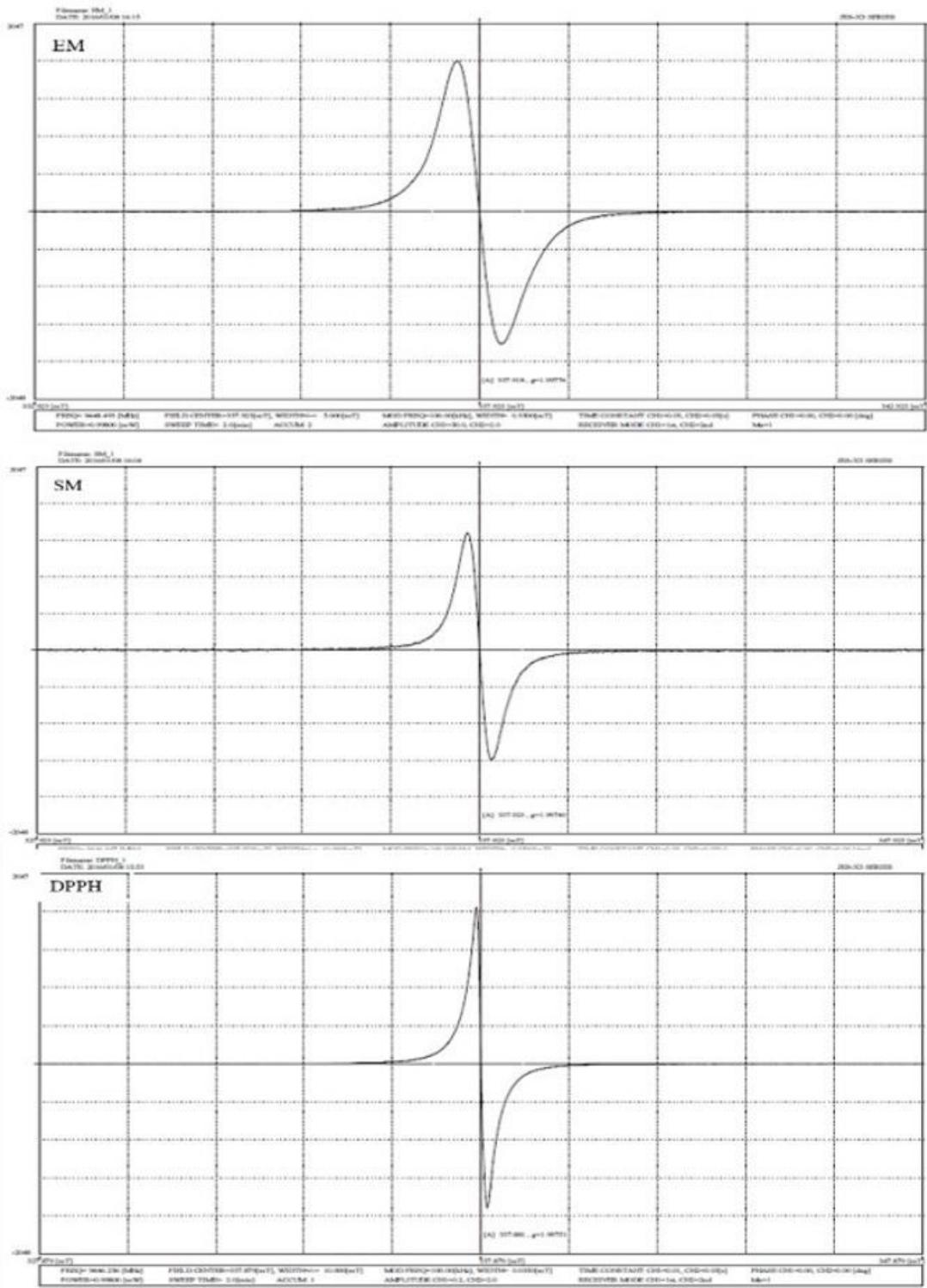


Figure 4

Electro paramagnetic resonance spectroscopic analysis of extracellular melanin of *Prauserella* sp.. (MAPPL 017A)

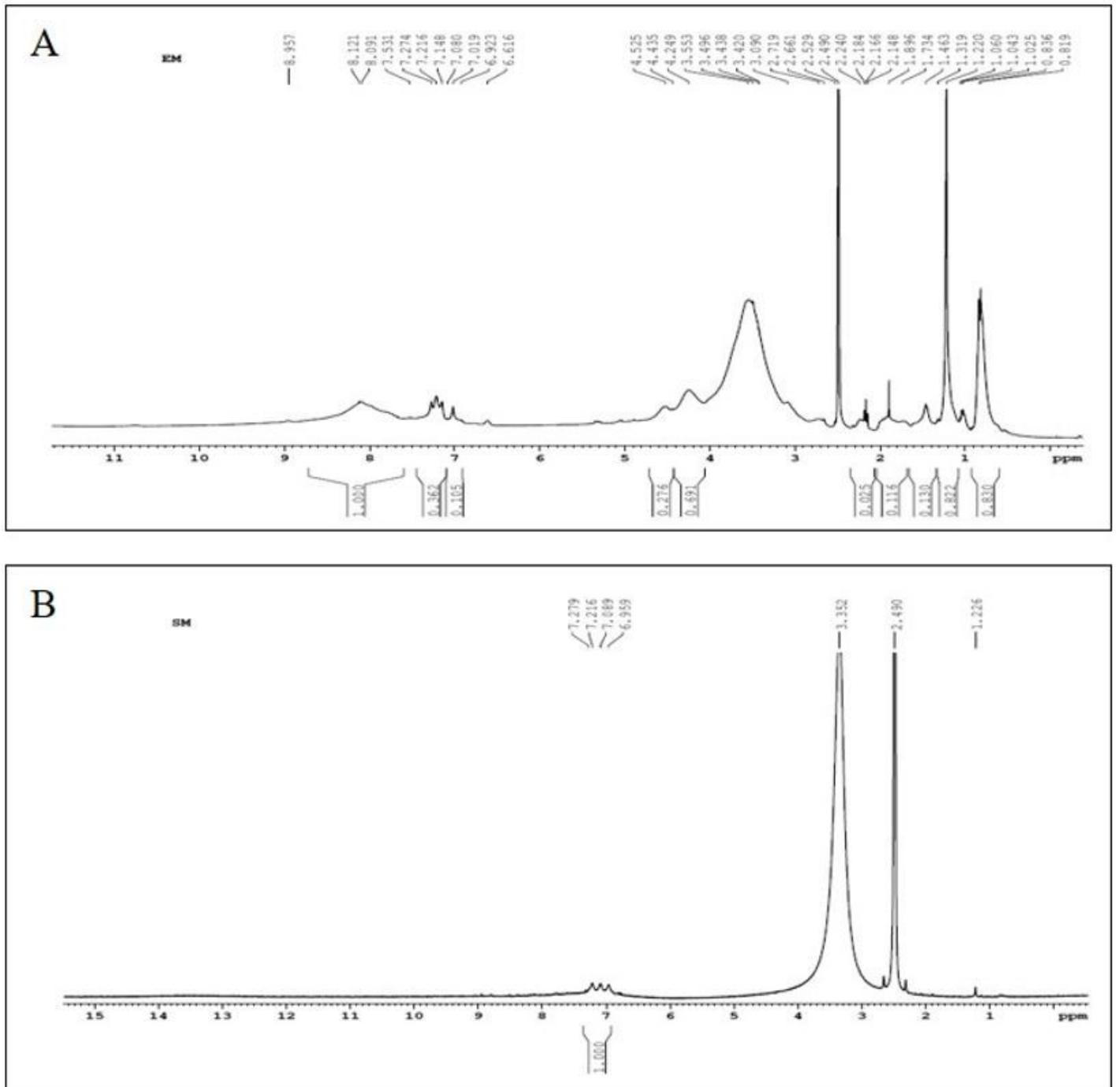


Figure 5

^1H NMR sp.ectral analysis of extracellular melanin from *Prauserella* sp.. (MAPPL 017A) and Synthetic melanin using $\text{D}_2\text{O}/\text{NH}_4\text{OH}$ at pH 10.0 A: Extracellular melanin from *Prauserella* sp.; B: Synthetic melanin

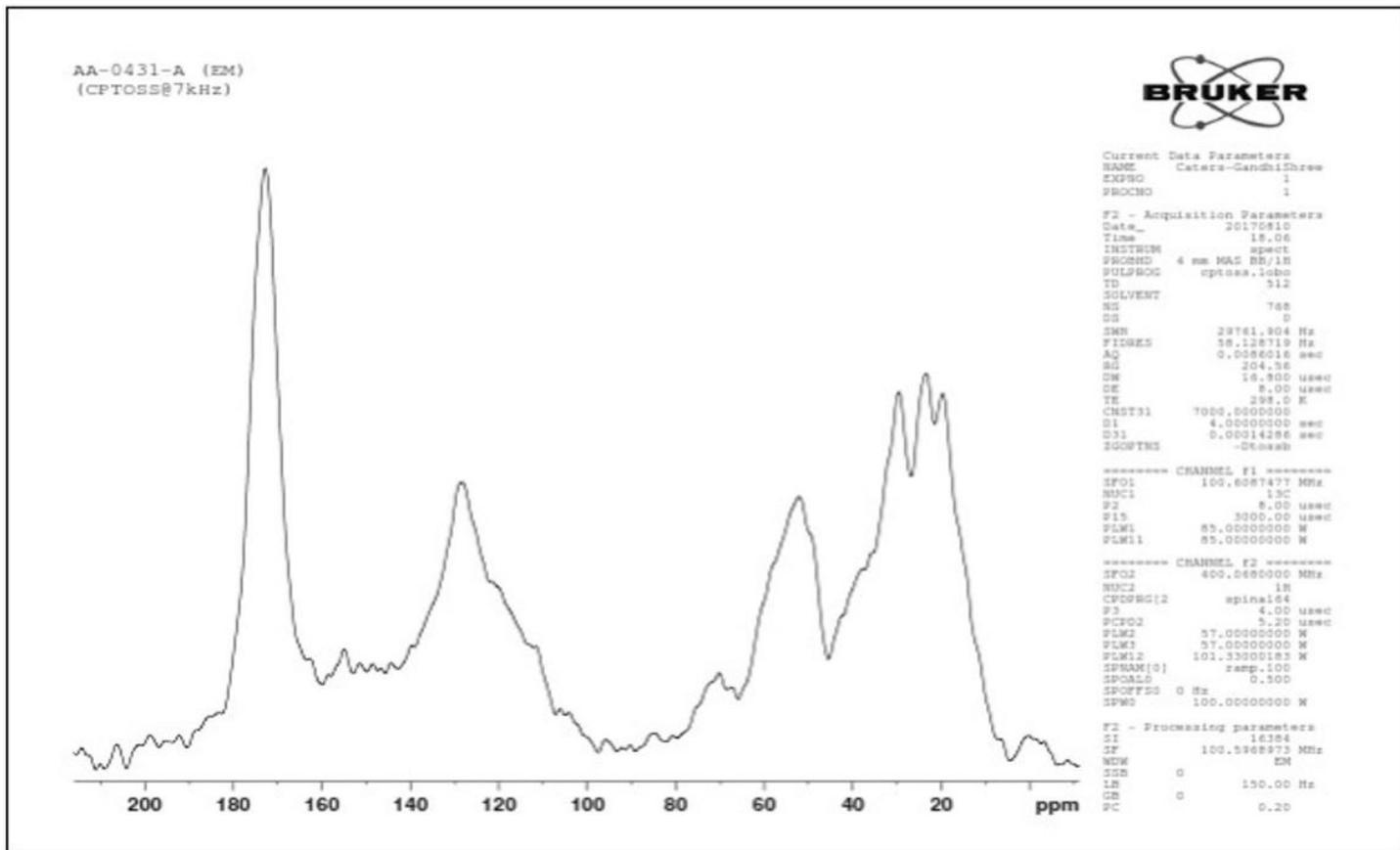


Figure 6

¹³C CP/MAS sp.ectral analysis of extracellular melanin from *Prauserella* sp.. (MAPPL 017A)

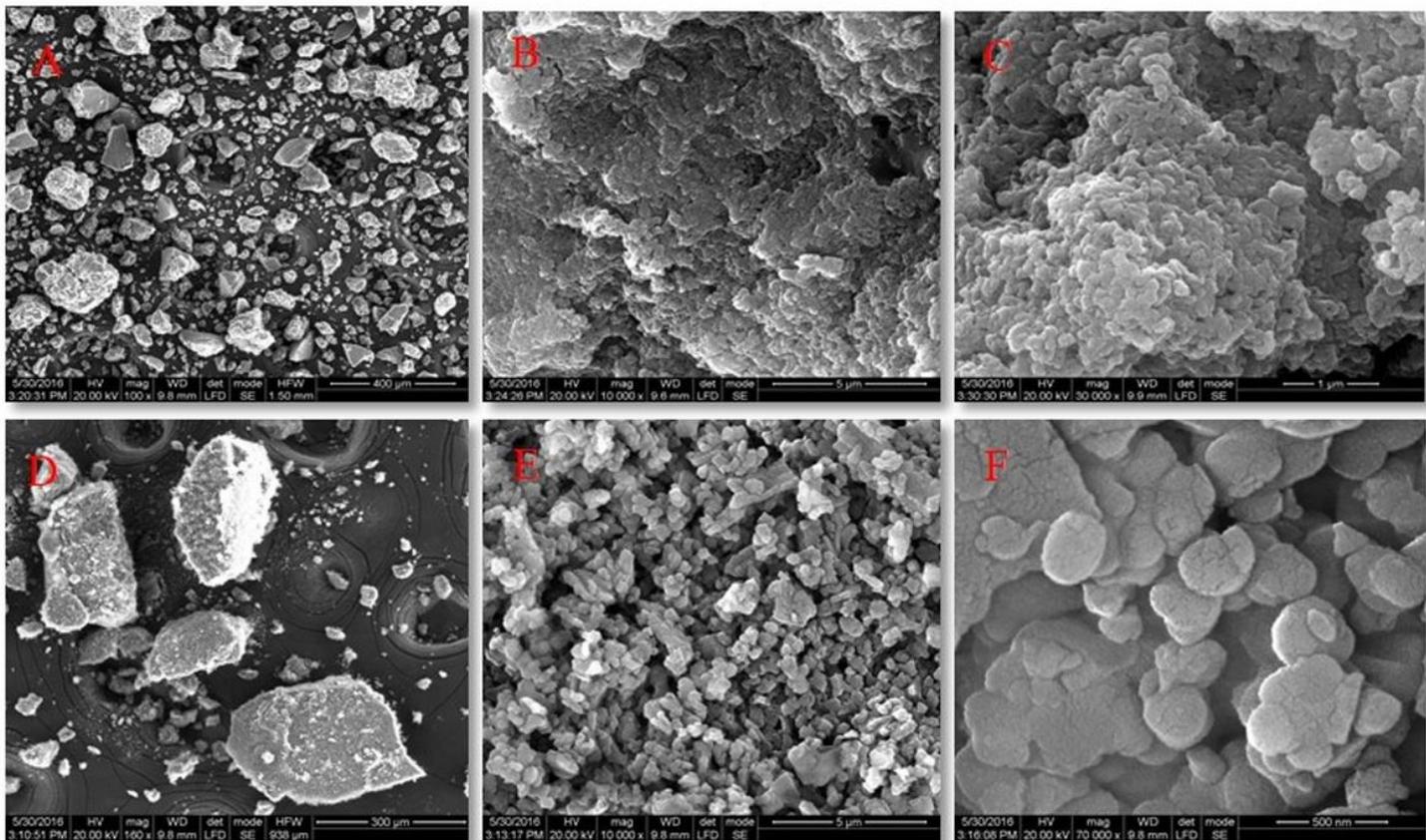


Figure 7

SEM micrographs of extracellular melanin (A-C) from *Prauserella* sp.. (MAPPL 017A) and Synthetic melanin (D-F)

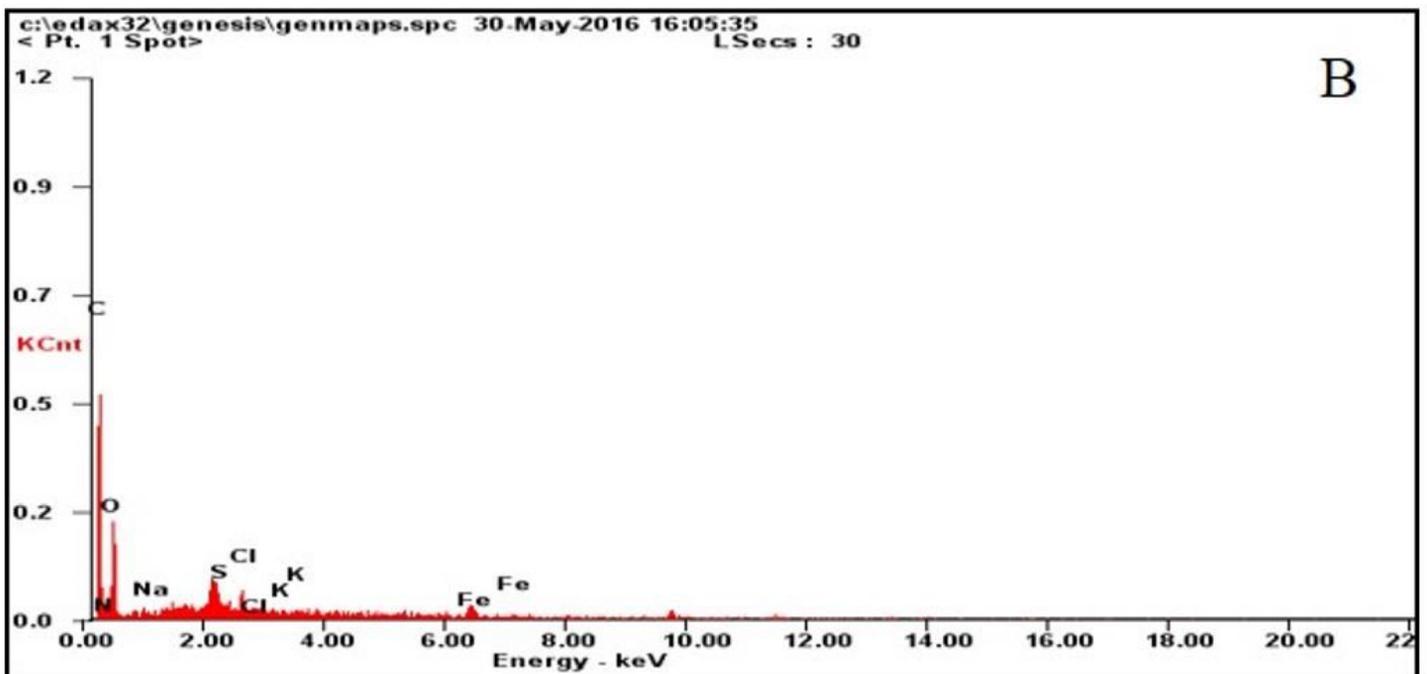
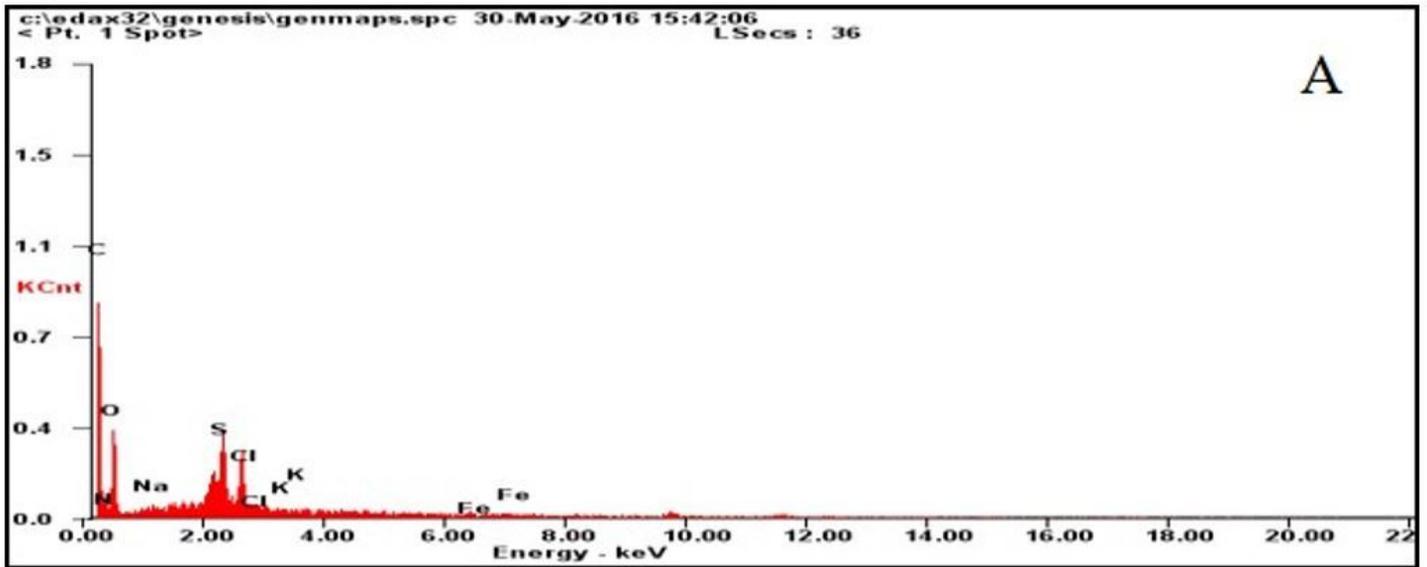


Figure 8

EDAX analysis of Extracellular melanin pigments from *Prauserella* sp.. (MAPPL 017A) A: Extracellular melanin; B: Synthetic melanin

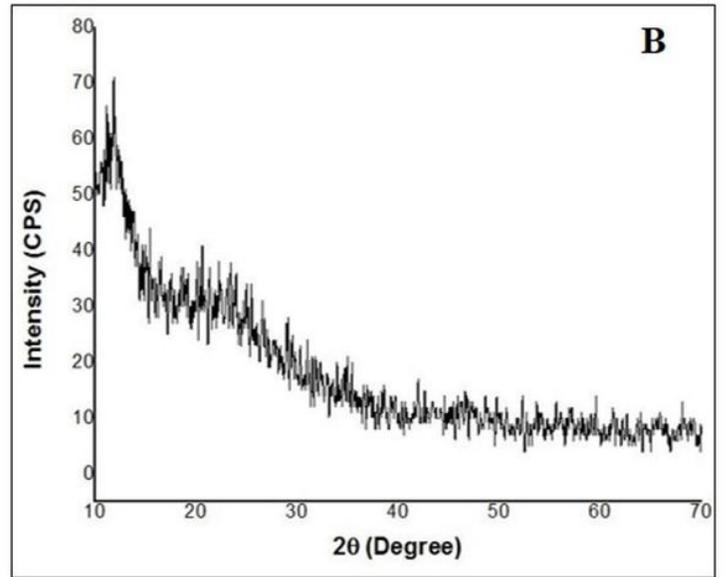
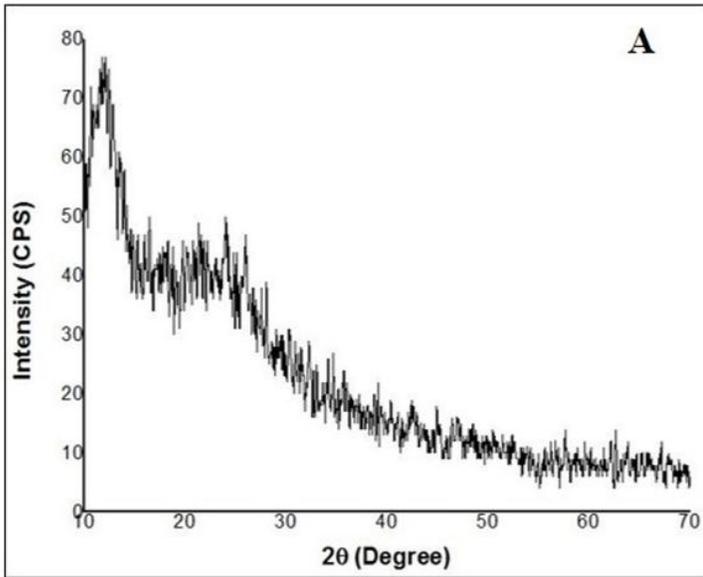


Figure 9

XRD pattern of the melanin pigments from *Prauserella* sp.. (MAPPL 017A) A. Extracellular melanin; B. Synthetic melanin

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

22 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)

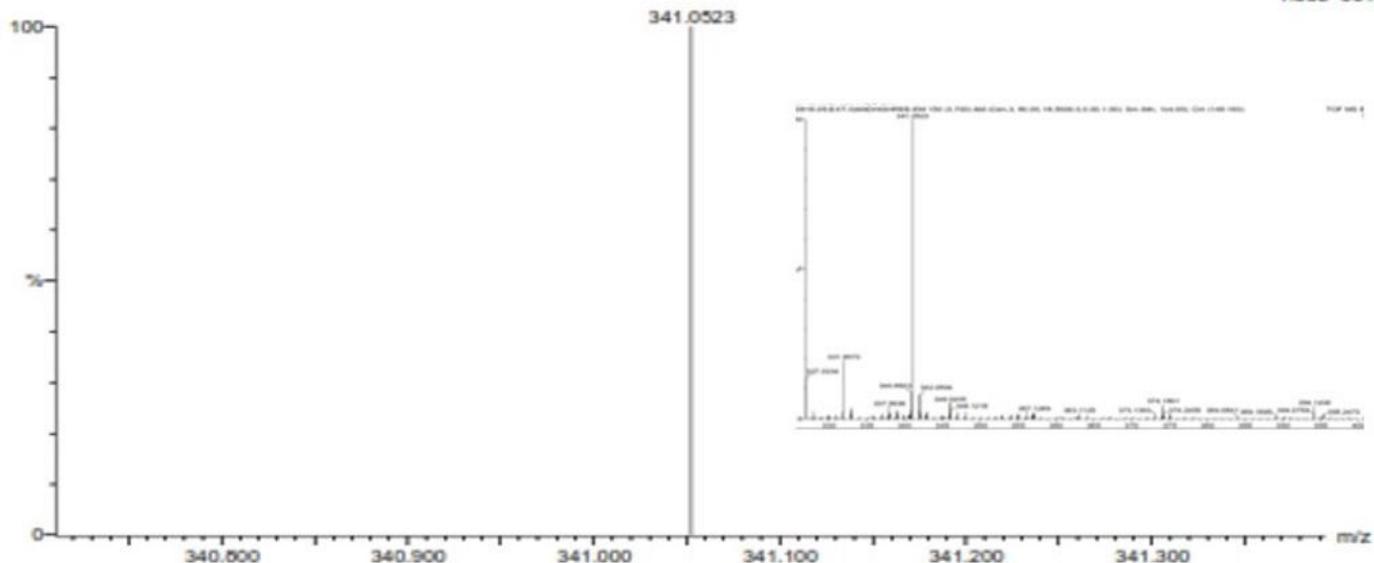
Elements Used:

C: 0-18 H: 0-10 N: 0-2 O: 0-4 Na: 0-1

EXT-GANDHISHREE-EM

060916-05-EXT-GANDHISHREE-EM 150 (3.700) AM (Cen,3, 80.00, Ht,5000.0,0.00,1.00); Sm (Mn, 1x4.00); Cm (148:163)

TOF MS ES+
1.09e+001



Minimum: -1.5
Maximum: 5.0 10.0 50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
341.0523	341.0538	-1.5	-4.4	14.5	n/a	C18 H10 N2 O4 Na

Figure 10

ESI-MS analysis of extracellular melanin from *Prauserella* sp.. (MAPPL 017A)

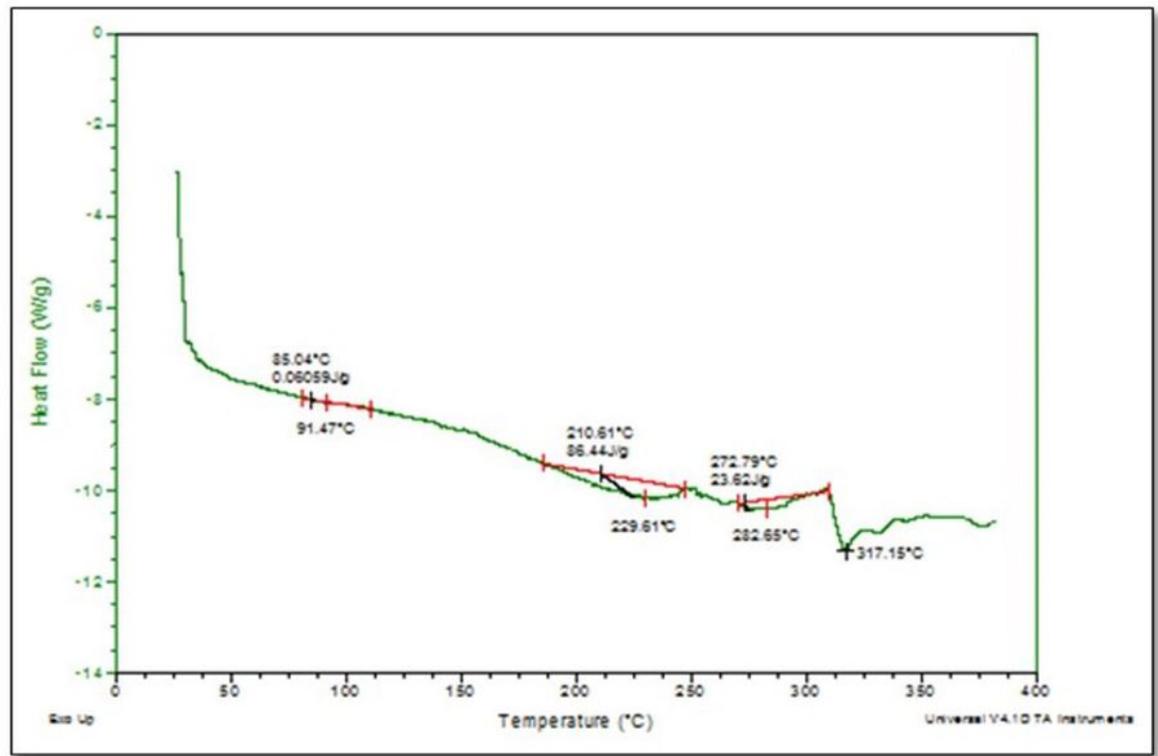
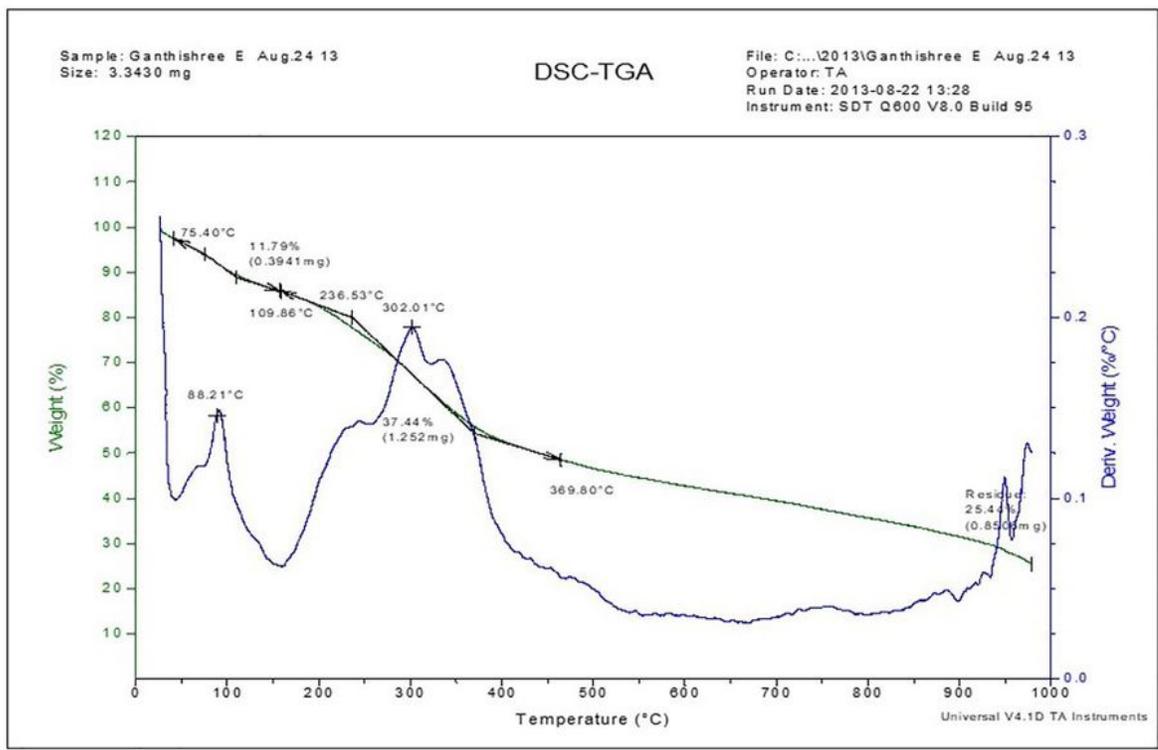


Figure 11

A. Thermal gravimetric analysis (TGA) of extracellular melanin from *Präuserella* sp.. (MAPPL 017A). B. Differential scanning calorimetric (DSC) analysis of extracellular melanin from *Präuserella* sp.. (MAPPL 017A).