

Original Research Article

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## Production, Extraction and Partial Purification of Melanin Pigment from Pathogenic *Klebsiella pneumoniae* HM Isolated from Clinical Samples

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### ABSTRACT

Sixty selected isolates showed a positive results for Api20E and CHROMagar as *Klebsiella pneumoniae*. primary screening by using minimal salt media appeared only six isolates have the ability to produce dark brown to black pigment in different levels. In vitro melanization assays showed *K. pneumoniae* HM produces brown colored pigment within 3 days on agar plate containing L-tyrosine. After optimization study, the results showed, the suitable condition for pathogenic *Klebsiella pneumoniae* HM to produce pigment were L-tyrosine foundation, pH 7.2, with temperature 35°C, therefore a pilot study was done in flasks, and the results appeared *Klebsiella pneumoniae* could produces pigment 121.9 mg/l throughout 3 days. Furthermore, the result showed that 2g/l of L- tyrosine was the suitable conc. That led to increasing in the yield amount in addition the production occurred after 24hrs. The sixth pigment producing isolates showed, sensitivity to cephalothin and erythromycin. While about 16.6% from these isolate were resistant to tobramycin, gentamycin and ciprofloxacin, and 66.6% of isolates were resistant to cefotaxime and about 83.3% showed resistant to clarithromycin. The pigment that produced by *Klebsiella pneumoniae* HM was likely to that melanin pigment produced by other bacteria. The physical characteristics of produced pigment was a dark brown pigment in culture medium. The chemical analysis of suspected melanin pigment occurred by solubility's method showed the pigment didn't dissolve in D.W, HCl (1N), ethanol and chloroform but dissolve in NaOH (1N) only. FT-IR spectroscopy was chosen for further characterization of the suspected melanin pigment, melanin exhibited broad absorption band at 3367.48 cm<sup>-1</sup>. The broadening of the band might be due to the hydrogen bonding of OH group with amine group. Another peak at 2929.67cm<sup>-1</sup> which appeared as a minute projection was assigned to N-H stretch. While absorption at 1080.06 cm<sup>-1</sup> may be attributed to aromatic ring CH stretching. Also the FTIR spectrum of extracted melanin result indicated there were amide linkages in the bacterial melanin, throughout a band at 1647.10 cm<sup>-1</sup> revealing the amide present in the molecule.

### Keywords

*Klebsiella pneumoniae*,  
Melanin,  
Bacterial  
melanin,  
L-tyrosine.

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### Introduction

*Klebsiella pneumoniae* an opportunistic pathogen responsible for important nosocomial infections (Maroncle *et al.*,

2002; Malik *et al.*, 2003; Nguyen *et al.*, 2003). Clinically it is the most significant member of the *Klebsiella* genus of

Enterobacteriaceae. These organisms are characterized by non-motile, and characterized by its capsule which are rich in carbohydrate as a complex of polysaccharide (Prescott, 2002). This genus can produce melanin especially the environmental one (Shrishailnath *et al.*, 2010). Melanins are brown to black colored complex natural pigments which are widely distributed throughout all living forms in nature. Melanins have many biological functions which include thermoregulation, photo protection, acting as free radical scavenging, cation chelators, and as antimicrobial agent. In plants melanin play as cell wall strengtheners (Riley, 1997). while in humans it determines the skin color and also plays a important role in protecting the skin against ultra violet light (Huang *et al.*, 2012). Melanins have many application potentials in the agriculture, cosmetics, and pharmaceutical industries. Research has revealed that melanin produced by Streptomyete showed photoprotection and mosquitocidal activity of *Bacillus thuringiensis* sub sp. Israelensis (Liu *et al.*, 1993). In the microorganisms, melanin acts as a protective agent against environmental stresses. For example melanin makes the bacteria resistant to antimicrobials (Lin *et al.*, 2005) and melanins in fungi are involved in fungal pathogenesis (Butler and Day, 1998).

Melanins are consisting of polymers of phenolic compounds. Depending on the types of melanin present in both prokaryotes and eukaryotes it can be mainly classified into many main types:

**Eumelanins** are black or brown pigment.

**Phomelanins** are yellow to red pigment.

**Allomelanins** are the least studied and most heterogeneous group of melanins (Plonka and Grabacka, 2006).

There is other type of melanin called Neuromelanin (NM) is a dark polymer pigment produced in specific populations of catecholaminergic neurons in the brain. Humans have the largest amount of NM (Fedorow *et al.*, 2005).

Animal melanins may be classified as black eumelanins and yellow-to-brown pheomelanins, whereas melanins from plants, fungi, and bacteria are brown-to-black allomelanins (Nicolaus *et al.*, 1964).

Melanins form a diverse group of pigments synthesized in living organisms in the course of hydroxylation and polymerization of organic compounds. Melanin. It is nearly a ubiquitous pigment, are negatively charged, hydrophobic (Butler and Day, 1998).

These pigments are insoluble in both aqueous and organic solvents, and it is consider difficult to study their structure by conventional biochemical and biophysical techniques (Piattelli *et al.*, 1965). The ability to produce melanin is widespread among microorganisms. From the chemical point of view, the only common feature of microbial melanins is it being a product of oxidative polymerization of various phenolic substances (Przemyslaw and Maja, 2006). Melanins are heterogeneous polymers of dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA) monomers linked by heterogeneous non-hydrolyzable bonds (Crippa, 1989), with only a short-distance ordering (Cheng, 1994).

It was suggested that melanin polymers constitute the building blocks of melanin granules (Zajac, 1994). Therefore, because of the importance of this pigment in microbiota, the present study was a chief to know ability of pathogenic *K. pneumoniae* isolated from clinical samples to produce melanin.

## Materials and Methods

Synthetic melanin and L-tyrosine were procured from Sigma Chemicals Co., St. Louis, USA. Other chemicals and biological materials (analytical reagent grade) were obtained from HiMedia chemicals, Mumbai, India.

### In Vitro Melanization Assay

In this study, 60 clinical samples of *Klebsiella pneumoniae* were collected from different patients, including both sexes with different age, who suffered from infection: (urinary tract infection, wound infection swabs, burn infection swabs, stool, blood and sputum) patients taking care and medications in Al-Yarmouk teaching hospital, Al-Kindi and Central Pediatric hospitals, all in Baghdad /Iraq. These isolates were characterized by using Api 20E Bio-merieux / France and confirmed the identification by CHROMagar.

All isolates were cultured on chemically defined minimal medium containing 1.8% (w/v) agar agar, pH 7.2, with or without L-tyrosine, and incubated for 2-3 days at 35C<sup>0</sup>. Plates were examined daily to monitor the growth of the bacterium and melanin pigment production

### Production of Melanin Pigment

Bacterial isolates capability of highly producing melanin pigment on minimal media agar was peak up and inoculated in test tube contains minimal media broth and incubated 24hrs. we take about 4ml from inoculated culture and inoculate 250 ml Erlenmeyer flask containing minimal media broth consisting from defined components (K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, FeSO<sub>4</sub>·7H<sub>2</sub>O, M ZnSO<sub>4</sub>, MnSO<sub>4</sub>·4H<sub>2</sub>O, NH<sub>4</sub>NO<sub>3</sub>, KH<sub>2</sub>PO<sub>4</sub>, CaCl<sub>2</sub>·2H<sub>2</sub>O, Na<sub>2</sub>MoO<sub>7</sub>·2H<sub>2</sub>O and glucose) with or without L-tyrosine (1 g L<sup>-1</sup>) at pH

7.2. Inoculated culture flasks without L-tyrosine as well as uninoculated flasks containing L-tyrosine served as controls. The medium was autoclaved at 15 psi (121°C) for 20 min; these flasks were inoculated with the bacterium and incubated at 35°C for 72 hr. this method depends on (Shrishailnath *et al.*, 2010).

### Pigment Extraction

The method of pigment extraction from this *K. pneumoniae* HM strain grown medium was followed as described by Nicolaus (1964). The 3-days-grown cell suspension was disrupted by using a Vibracell ultrasonicator in an ice bath for 3-min periods; each period of disruption was of 30-s cycles followed by a 1-min off cycle during which the medium and oscillator probe were cooled in ice. The disrupted broth was acidified with 1 N Hcl to pH 2 and allowed to stand for one week at room temperature. Then this suspension was boiled for 1 h to prevent the formation of melanoidins and then centrifuged at 10,000 ×g for 10 min by Fava *et al.*, (1993). Dark brown pigment pellet was formed and washed three times with Hcl(0.1 N) and then with water. The pellet was soaked with ethanol absolute and the mixture incubated in a boiling water bath for 10 min and then kept at room temperature for 1 day. The pellet was washed with ethanol absolute two times and then dried in air. The extracted pigment pellets were stored at -20C<sup>0</sup> for farther use in subsequent analyses.

### Partial purification of the pigment

The purification of the melanin pigment was carried out as the standard protocols described by Harki *et al.*, (1997), the purification was done by column method using silica gel column material of 60-120 mesh size, the silica gel was loaded into a chromatographic column after activation by

heating at 90 C for 30 mins. and suspended by PBS buffer pH 7 and allowed to settle, the 3ml of the sample was loaded to the top of the column. The pigment was eluted by phosphate buffer pH 7.0 and with flow rate 30ml/hr.

### **Precipitation the partial purified pigment**

According to Tomomi (2014) method, the fractions that contain the pigment were recorded the highest absorbance. These fractions were treated with absolute ethanol by adding 4ml to each fraction and left it for one day, then centrifuged at 10000 gx, the precipitant was dried at room temperature and stored at -20C.

### **Analysis of pigment by FT-IR**

The partial purified pigment was ground with IR grade KBr (1:10) and pressed into disks under high pressure using a pellet maker. The FT-IR spectrum was recorded at 4,000-400 cm<sup>-1</sup> using a FT/IR-4100; Shimadzu-Japan).

### **Results and Discussion**

All 60 selected isolates showed positive results for Api20E and CHROMagar as *Klebsiella pneumoniae* Figure 1.

### **Selection Pigment Production isolates**

primary screening of 60 isolates of *K. pneumoniae* by using minimal salt media appeared only six isolates have the ability to produce dark brown to black pigment, but in different levels depending on time of incubation figure 2, on the other hand, important note need be take care, the producing *K. pneumoniae* HM may loss its ability of production in next culturing, but in several culturing retained its ability. These phenomena's was related to the lack of

melanin synthesis, throughout using in the construction of combinatorial libraries of cyclic peptides and proteins as Scott *et al.*, (1999) mentioned.

The colonies capable of producing brown pigment were picked up and coded as *Klebsiella pneumoniae* HM. In vitro melanization assays were performed to determine whether *K. Pneumoniae* HM produces pigment from L-tyrosine. Cells were spread onto agar plates with / without L-tyrosine. The isolates turned to brown colored within 3 days on agar plate containing L-tyrosine (Fig. 2), but not pigment production observed with that lacking L-tyrosine. After optimization study, the results showed, the suitable condition for pathogenic *Klebsiella pneumoniae* HM to produce pigment were L-tyrosine foundation, pH 7.2, with temperature 35 C, therefore a pilot study for ability of isolates to produce pigment was done in flasks, and the results appeared *Klebsiella pneumoniae* HM could produces pigment 121.9 mg/l throughout 3 days, and the pigment production was graduated during the time of incubation Fig 3.

Furthermore, the result showed that 2g/l of L- tyrosine was the suitable one, because it was proportional with the amount of pigment production and with the incubation time. Increasing the concentration of this amino acid to 2 g/l led to elevation in the yield amount and the production occurred after 24hrs, while decreasing the concentration of L-tyrosine less than 1g/l, the time taken for pigment production was increased to 4-7 days, and the amount of pigment was decline.

On the other hand, the increasing in the amino acid concentration more than 2g/l led to appear crystal particles in the media that affect negatively in the capability of *K.*

*pneumoniae* HM to produces pigment, and the extraction method become more complicated.

### **Antimicrobial sensitivity of melanin producing *K. pneumoniae* HM**

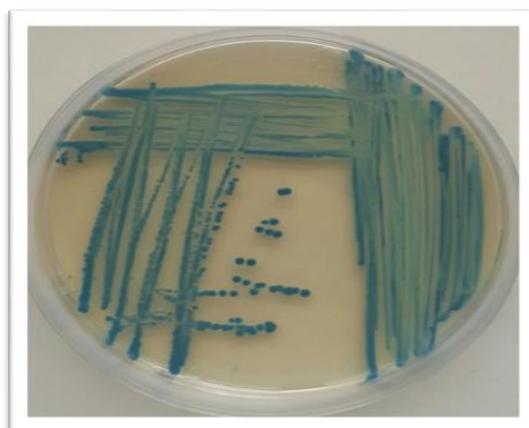
The sixth pigment producing isolates were tested against 7 antimicrobials and shows the isolates were sensitive to cephalothin and erythromycin in percent 100%. While about 16.6% from melanin producing isolate were resistant to tobramycin, gentamycin and ciprofloxacin, and 66.6% of isolates were resistant to cefotaxime and about 83.3% showed resistant to clarithromycin table1. This result did not act as a characteristic phenomenon's for these isolates and mostly are not significantly different from other *K. pneumoniae* isolates.

### **Characterization of Melanin Produced by *Klebsiella pneumoniae* HM isolate**

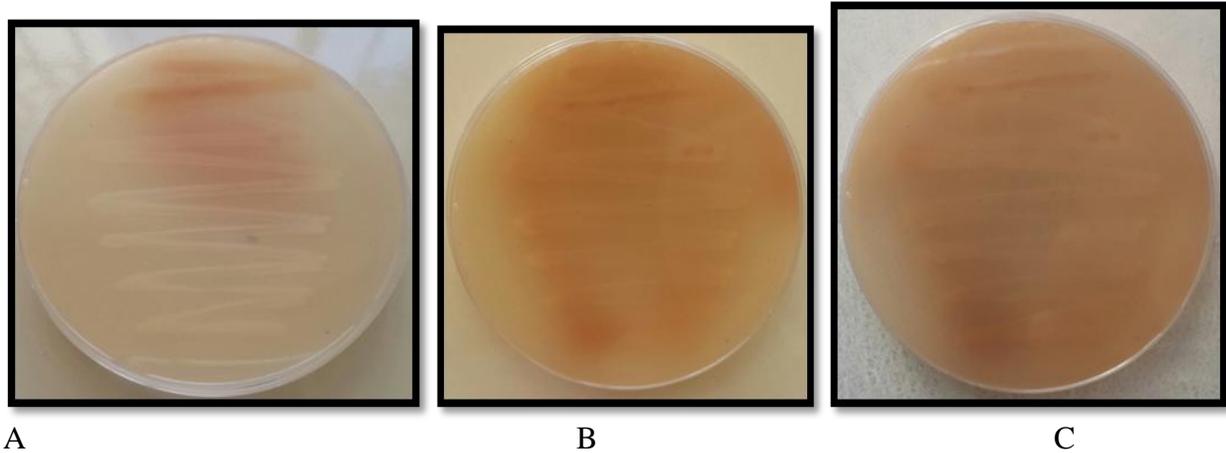
The observed pigment that produced by *Klebsiella pneumoniae* HM shown a true melanin, as revealed by a number of physical and chemical tests. These tests

indicated that the pigment is likely to that melanin pigment, produced by other bacteria such as *Aeromonas media* (Lewis *et al.*, 1998) and *Escherichia coli* (Huang *et al.*, 2009) and fungi *Cryptococcus neoformans* (Gibello *et al.*, 1997) and *Pleurotus cystidiosus* (Selvakumar *et al.*, 2008). The physical characteristics of produced pigment from *Klebsiella pneumoniae* HM was a dark brown pigment in culture medium as a dead-end product. On the other hand, the chemical analysis of suspected melanin pigment occurred by solubility's method by using several solvents such as distilled deionized water, 1 N HCl, 1 N NaOH, ethanol and chloroform were checked. The results showed the suspected melanin pigment not dissolve in D.W, HCl (1N), ethanol and chloroform but dissolve in NaOH (1N) only, these results were agreed with Shrishailnath, (2010) who mentioned that bacterial extracted melanin was dissolved only in alkaline solution such as NaOH (1N), and aggregated by acidic solution, this result was proven the extracted melanin form pathogenic *K. pneumoniae* HM in this study was as melanin pigment extracted by other bacteria.

**Fig.1** *K. pneumoniae* incubated at 37°C for 24 hrs on CHROMagar



**Fig.2** Screening of bacterial strain for pigment production, where HM cells were grown with L-tyrosine and incubated for 3 days. A-Pigment production after 24hr. B- pigment production after 48hr. C- pigment production after 72hr.



**Fig.3** 1- The dark brown-colored flask were selected for melanin production by *Klebsiella pneumoniae* HM cells grown with L-tyrosine 2- Without L-tyrosine, the cells were unable to produce pigment. 3- The uninoculated flask shows the autooxidation of L-tyrosine is not likely a cause.



**Fig.4** The partial purification process of pigment by using silica gel column mesh (90-120) with flow rate ml/min. and at the absorbance at 380nm.

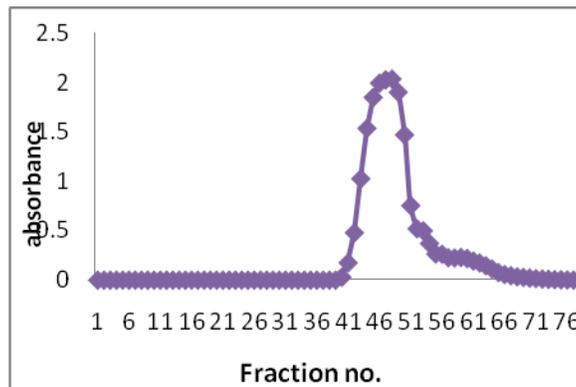


Fig.5a&b FTIR analysis of standard melanin; FTIR analysis of crude melanin

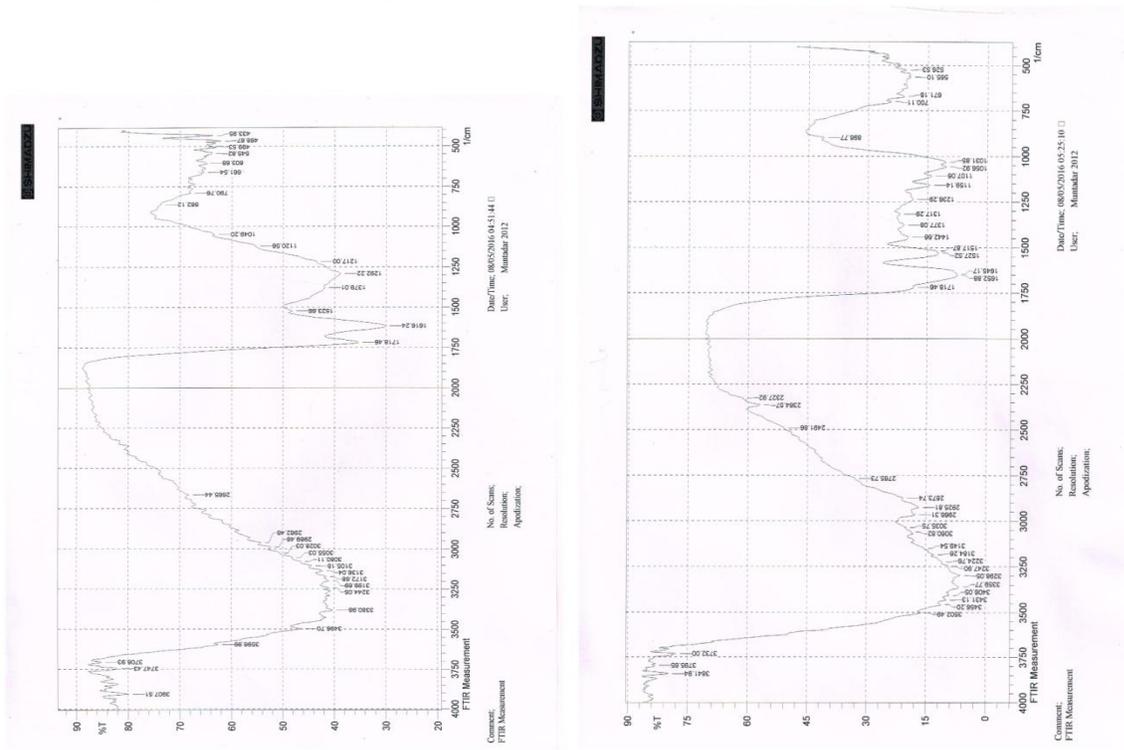
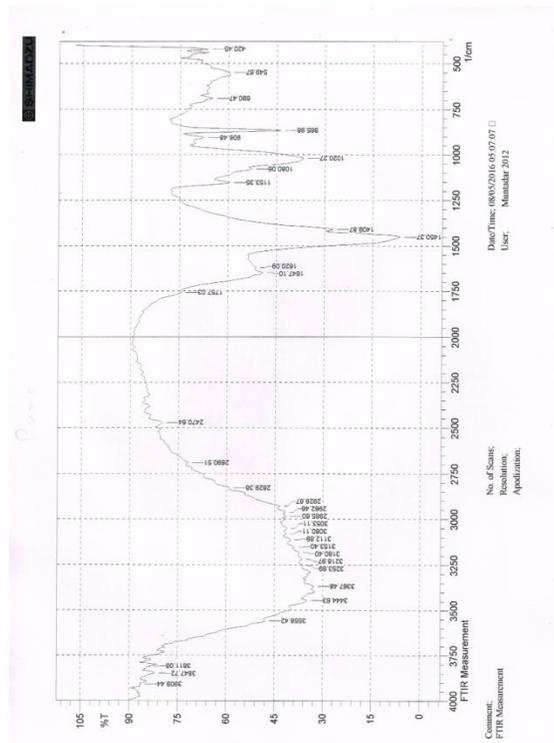


Fig.5c FTIR analysis of pure melanin



Melanin was partially purified by silica gel column method Fig 4. The purified pigment was become ready to use for farther analysis

### FTIR analysis

FT-IR spectroscopy was chosen for further characterization of the suspected melanin pigment, since it is regarded as the most informative, well-resolved, and non-destructive method, providing information on functional groups and detailed structural analysis of melanin Shrishailnath, (2010). The IR spectrum of bacterial melanin isolated from *Lysobacter oligotrophicus* (tomomi *et al.*, 2014) with peaks to 3366  $\text{cm}^{-1}$  is described to -OH and -NH bonds in the melanin. In the present study, it exhibited broad absorption band at 3367.48  $\text{cm}^{-1}$ . The broadening of the band might be due to the hydrogen bonding of OH group with amine group.

Another peak at 2924.99  $\text{cm}^{-1}$  which appeared as a minute projection was assigned to N-H stretch, While in present study the peak is read at 2929.67  $\text{cm}^{-1}$  this result was closely related with that indicated by Banerjee *et al.*, (2014).

Absorption at 1080.06  $\text{cm}^{-1}$  may be attributed to aromatic ring CH stretching; this result is similar to that approved by Arun *et al.*, (2015).

The FTIR spectrum of melanin from fungus *Gliocephatrichum simplex* (Jalmi *et al.*, 2012), showed there was a band at 1658  $\text{cm}^{-1}$  revealing the amide 1 and amide 2 present in the molecule. Closely band was appeared at 1647.10  $\text{cm}^{-1}$  in the present study, this result indicated there was amide linkages also in the bacterial melanin. There was important note attached the result of FTIR in this study, the mild differences between bacterial extracted melanin in this study with

other microorganisms might be due to laboratory working conditions, in addition may be the usable bacteria was pathogenic one Figure 5A, B, C.

### References

- Arun, G., Eyini, M. and Gunasekaran, P. 2015. Characterization and biological activities of extracellular melanin produced by *Schizophyllum commune*. *Indian J. Experimental Biol.*, Vol 53, pp. 380-387.
- Banerjee, A., supaker, S. and Banerjee, R. 2014. melanin from the nitrogen – fixing bacterium *Azotobacter chroococcum*: aspectroscopic characterization, *PloS*, e84574.
- Butler, M.J. and Day, A.W. 1998. Fungal melanins: a review, *Can. J. Microbiol.*, 44(12): 1115-1136.
- Cheng, J., Moss, S. C., Eisner, M., and Zschack, P. 1994. X-ray characterization of melanins - I. *Pigment Cell Res.*, 7: 255-262.
- Crippa, P.R., Horak, V., Prota, G., Svoronos, P. and Wolfram, L. 1989. Chemistry of Melanins: The Alkaloids. Vol. 36, pp. 234-323. Academic Press, New York.
- Fava, F., Gioia, D.D. and Merchetti, L.1993. Characterization of a pigment produced by *Pseudomonas fluorescens* during 3-chlorobenzoate co-metabolism. *Chemosphere*, 27: 825-835.
- Fedorow, H., Tribl, F., Halliday, G., Gerlach, M., Riederer, P., Double, K.L. (2005). Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson's disease. *Prog Neurobiol.*, 75(2): 109–124.
- Gibello, A., Suárez, M., Luis, A.J. and Martín, M. 1997. Molecular cloning and analysis of the genes encoding the

- 4 hydroxyphenylacetate hydroxylase from *Klebsiella pneumoniae*. *Arch. Microbiol.*, 167: 160-166.
- Harki, E., Talou, T. and Dargent, R. 1997. Purification, characterization and analysis of melanin extracted from *Tuber melanosporum* vitt., *Food Chem.*, 58: 69-73.
- Huang, H.C. and Chang, T.M. 2012. Antioxidative properties and inhibitory effect of *Bifidobacterium adolescentis* on melanogenesis, *World J. Microbiol. Biotechnol.*, 28(9): 2903-12.
- Huang, Y., Lai, X., He, X., Cao, L., Zeng, Z., Zhang, J. and Zhou, S. 2009. Characterization of a deep-sea sediment metagenomic clone that produces water-soluble melanin in *Escherichia coli*. *Mar. Biotechnol.*, 11: 124-131.
- Jalmi, P., Bodke, P., Wahidullah, S., Raghukumar, S. 2012. The fungus *Gliocephalotrichum simplex* as source of abundant, extracellular melanin for biotechnology applications. *World J. Microbiol. Biotechnol.*, 28: 505- 512.
- Jalmi, P., Bodke, P., Wahidullah, S., Raghukumar, S. 2012. The fungus *Gliocephalotrichum simplex* as source of abundant, extracellular melanin for biotechnology applications. *World Journal of Microbiology and Biotechnology* 28: 505- 512.
- Lewis, F.G. and Antony, M.G. 1998. Melanin and novel melanin precursors from *Aeromonas media*. *FEMS Microbiol. Lett.*, 169: 261-268.
- Lin, W. P., Lai, H. L., Liu, Y. L., Chiung, Y. M., Shiau, C. Y. and Han, J. M. 2005. Effect of melanin produced by a recombinant *Escherichia coli* on antibacterial activity of antimicrobials, *J. Microbiol. Immunol. Infect.*, 38(5): 320.
- Liu, Y.T., Sui, M.J., Ji, D.D., Wu, I.H., Chou, C. and Chen, C.C. 1993. Protection from UV irradiation by melanin of mosquitocidal activity of *Bt. var. israeliensis*. *J. Invertebr. Pathol.*, 62: 131-136.
- Malik, A., Hassani, S.E., Shahid, M., Khan, H.M., and Ahmad, A.J. 2003. Nosocomial *Klebsiella* infection in neonates in a tertiary care hospital: Protein profile by SDS-page and *kel* typing as epidemiological markers. *Indian J. Med. Microbiol.*, 21(2): 82-86.
- Maroncle, N., Balestrino, D., Rich, C., and Forestier, C. 2002. Identification of *Klebsiella pneumoniae* genes involved in intestinal colonization and adhesion using signature-tagged mutagenesis. *Infect. Immun.*, 70(8): 4729–4734.
- Nguyen, Thi, P.L., Yassibanda, S., Aidara, A., Le Bouguenec, C., and Germani, Y. 2003. Enteropathogenic *Klebsiella pneumoniae* HIV-infected adults, Africa. *Emerg. Infect. Dis.*, 9(1): 135–137.
- Nicolaus, R.A., Piattelli, M. and Fattorusso, E. 1964. The structure of melanins and melanogenesis - IV: On some natural melanins. *Tetrahedron*, 20: 1163-1172.
- Piattelli, M., Fattorusso, E., Nicolaus, R. A. and Magno, S. 1965. The structure of melanins and melanogenesis. V. *Ustilago melanin*. *Tetrahedron*, 21: 3229-3236.
- Plonka, P.M. and Grabacka, M. 2006. Melanin synthesis in microorganisms — biotechnological and medical aspects, *Acta. Biochimica Polonica.*, 53: 429–443.
- Prescott, L. 2002. *Microbiology* 5 th. Ed. MacGraw- Hill Companies, U.S.A.
- Przemyslaw, M.P. and G. Maja. 2006. Melanin synthesis in microorganisms:

- Biotechnological and medical aspects. *Act a Biochem. Pol.*, 53: 429-443.
- Riley, P. 1997. Melanin, *Int. J. Biochem. Cell. Biol.*, 29(11): 1235–9.
- Scott, C.P., Abel-Santos, E., Wall, M., Wahnnon, D.C. and Benkovic, S.J. 1999. Production of cyclic peptides and proteins in vivo. *Proc. Natl. Acad. Sci. USA*, 96: 13638-13643.
- Selvakumar, P., Rajasekar, S., Periasamy, K. and Raaman, N. 2008. Isolation and characterization of melanin pigment from *Pleurotus cystidios* (telomorph of *Antromyocopsismacrocapa*). *World J. Microbiol. Biotechnol.*, 24: 2125-2131.
- Shrishailnath, S., Kulkarni, G., Yaligara, V., Kyoung, L., Karegoudar, T. 2010. Purification and physiochemical characterization of melanin pigment from *Klebsiella* sp. GSK. *J. Microbiol. Biotechnol.*, 20: 1513–1520.
- Tomomi, K., Wakao, F., Tomoe S. and Tadayuki, I. 2014. Characterization of water-soluble dark-brown pigment from Antarctic bacterium, *Lysobacter oligotrophicus*. *J. Biosci. Bioengi.*, vol xx no. xx,1-4,
- Zajac, G., Gallas, J.M., Cheng, J., Eisner, M., Moss, S.C. and Alvarado-Swaisgood, E. 1994. The fundamental unit of synthetic melanin: A verification by tunneling microscopy of X-ray scattering results. *Biochem. Biophys. Acta*, 1199: 271-278.

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