Int. J. Drug Res. Tech. 2016, Vol. 6 (2), 79-86 ISSN 2277 - 1506

International Journal of Drug Research and Technology

Available online at http://www.ijdrt.com

Original Research Paper

EXTRACTION AND ISOLATION OF MELANIN FROM ACTINOMYCETES

Shreya Modi, Poonam B Chauhan, Mayur Gahlout and **Hiren Prajapati***

Department of Microbiology, KBS Commerce and Nataraj Professional Science College, Vapi. Dist.Valsad-396195, Gujarat, India

ABSTRACT

Twenty different isolates of actinomycetes collected from different localities in Valsad, were screened for melanin pigment production and isolate S1 found to be highest producer of melanin. At 30˚C melanin pigment was produced and confirmed using L-DOPA (as substrate) optimum carbon and nitrogen for melanin production was found to be 10.4 and 31.3 respectively. Melanin was successfully extracted in a crude form and used for the antimicrobial activity against selected microorganism.

Keywords: Actinomycetes, Melanin pigment, Antibacterial activity, Microorganism.

INTRODUCTION

Actinomycetes are produces dark-brown pigment in the culture media, generally referred to as melanin or melanoid pigments. (Dastager *et al.*, 2006). Melanins are negatively charged compound composed of multi-functional polymers and polyphenolic compounds that are produced by various microorganisms by fermentation oxidation. The term "Melanin" originates from a Greek word "melanos" which means black. It is a pigment which is ubiquitous in nature. Melanin pigment is found in most organisms including human beings, animals as well as microorganisms. The most common form of biological melanin is eumelanin. The other forms are pheomelanin and neuromelanin (Butler and Day, 1998). The biosynthesis pathway of melanin pigment in microorganisms takes place through different pathways, similarly to higher organisms. Melanin pigment can be made using L-tyrosine as precursor through the action of tyrosinase. Tyrosinase is a copper protein that belongs to the group of polyphenol oxidases. Tyrosinase transform the tyrosine into L-DOPA (3, 4-dihydroxyphenyl-Lalanine), which is further converted into dopachrome and autooxidized to indol-5, 6-quinone. Then they are spontaneously polymerized into DOPA-melanin which gives dark brown pigment (Mencher and Heim, 1962; Margalith, 1992).

There are various types of Melanin: Eumelanin (Brown-black), Pheomelanin (Yellow-red), Allomelanins, Neuromelanin. Melanins are frequently used in medicine, pharmacology and cosmetic preparations. Melanin's have the ability to undergo polymerization is interesting in industry for its nanotechnology uses in bio-plastics and biopolymers (Nakato, 2006). The aim of this work is to isolate the melanin producing actinomycetes and investigate the production and purification of melanin pigment by the isolates and to characterize the melanin through its physical and chemical properties.

MATERIALS AND METHOD

Media and Reagents

Tyrosine (CDH Mumbai), Casein hydrolysate (SRL Mumbai), Sodium nitrate (Rankem Mumbai), peptone (HPLC Pvt. Ltd Mumbai), K_2HPO_4 (Rankem Mumbai), Ferric ammonium citrate (Rankem New Delhi), Sodium thiosulphate (Rankem, Thane Mumbai) used were of analytical grade. All these reagents and media were prepared in distilled water.

Screening and Isolation of Melanin Producing Actinomycetes from Soil Sample

Microorganisms were isolated from soil samples collected from the various garden, sugar industry, farms and mangoes farm in Vapi and Valsad region, Gujarat-396001, India.

The medium used for the screening of the melanin producing species is Peptone iron medium (gm/liter), peptone-20, K_2HPo_4-1, Ferric ammonium citrate-0.5, sodium thiosulphate-0.8, Agar-32, pH-7 inoculation with 1 gm of soil sample in 250 ml Erlenmayer flask containing 100 ml sterile peptone iron medium. The inoculated medium was incubated for 5 days at 30 °C in static. After incubation period the medium is serially diluted and plated on tyrosine casein agar plate. The inoculated tyrosine casein agar plate contained (1g of L-tyrosine, 25 g of casein hydrolysate, 10 g of sodium nitrate, 32g agar, pH 7). The plates were incubated at 30 °C for 5-6 days. After incubation period the tyrosine casein agar plates were observed for colonies showing diffusible pigment and were purified by streaking on tyrosine casein agar plate.

Melanin Production and Estimation

Melanin was produced by submerged fermentation; the production was carried out in 250 ml ernlenmeyer flask containing 100 ml of peptone iron medium. (peptone-20, K_2HPo_4-1, Ferric ammonium citrate-0.5, sodium thiosulphate-0.8, Agar-32, pH-7)

Inoculum Preparation

An isolated colony, from the preserved culture plate was transferred in to 50 ml Erlenmeyer flask containing peptone iron broth. The flask was incubated at 30 °C for 48 hrs at static condition. The freshly grown 48 hrs old culture with 1.0 O.D at 600 nm is used as inoculum to inoculate in production medium.

Production Medium

Melanin production was carried out in 250 ml Erlenmayer flask containing 100 ml of the Peptone-iron medium inoculated with 5% inoculum of selected strain and then incubated at 30 ºC for 5-6 days. And the culture suspension was centrifuged at 5000 rpm for 20 min. The cell-free supernatant was used as a source of melanin.

Assay for Melanin Production

Melanin pigment was estimated by taking 2 ml of the supernatant and 1 ml of 0.4% substrate solution (L-dopa). The reaction mixture was incubated at 37°C for 5 min and read spectrophotometrically at 480 nm (UV-1601, Shimadzu) (Scribners *et al.*, 1973; Mencher and heim., 1962).

Optimization of Media Optimization of Melanin Production

Effect of Different Medias

The effect of different medium composition was done using different production media. The fermentation was carried out in 250 ml Erlenmeyer flask with 100 ml of the modified media which is then inoculated with 5% of the inoculums of selected actinomycetes strain and incubated at 30 ºC for 5-6 days. Different media used in this study are given below:

Media I: Starch casein agar media: 10g Starch, 0.3g Casein, 2g KNO_3, 2g NaCl, 2g Potassium, 0.02g $CaCO_3$, 0.01g $FeSO_4$, 32g Agar.

Media II: peptone yeast extract iron media : 5g Peptone, 0.1g Yeast extract, 0.05g Ferric ammonium citrate, 1g K_2HPO_4, 0.08g Sodium thiosulphate, 32g Agar.(pH-6.7)

Media III: Glycerol asparagine agar : 1g L-Asparagine, 1g K_2HPO_4, 10g Glycerol, 1g NaCl, 32g Agar (pH-7.4).

Media IV: Peptone iron media : 20g Peptone, 1g K_2HPO_4, 0.5g Ferric ammonium citrate, 0.8g Sodium thiosulphate, 32g Agar (pH-7).

Effect of Carbon Source

Effect of carbon source on melanin production was studied in the production medium with various simple and complex carbon sources including fructose, maltose, lactose, sucrose, starch and glucose. The flasks were inoculated with 5% of the inoculum and incubated at 30 °C for 5-6 days with static condition. The samples were collected after 5 days .and the culture suspension was centrifuged at 5000 rpm for 20 min. The cell-free supernatant was used as a source of melanin. The samples were collected

after 5 days and sample was used as a source of melanin formation.

Effect of Nitrogen Source

Effect of different nitrogen sources including yeast extract, peptone, beef extract, urea and their combinations, ammonium sulphate, NH_4Cl. A control is represented with peptone containing media.

Effect of pH

Effect of initial pH of the medium on melanin production was studied by adjusting the pH of the production medium in the range of 3 to 9 using 1 N NaOH and 1N HCl after sterilization.

Effect of Temperature

Effect of temperature on melanin production was studied by incubating the production medium with 5% of the inoculum at different temperatures including 20°C, 25°C, 30°C, 35°C and 37°C for 5-6 days under static conditions.

Antibacterial Activity of Melanin

The antibacterial activity of melanin was carried out by agar well diffusion technique against 24 h old cultures of Gram-positive bacteria. (*Staphylococcus aureus*, *Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli, salmonella typhi A, Salmonella typhi B*). Wells with 1cm diameter were made on sterile Muller-Hinton agar plates. Bacterial cultures were swabbed on the surface of the agar and melanin pigment was added into the wells. The plates were incubated at 30°C for 24 h. Antibacterial activity of the melanin pigment was determined by measuring the growth inhibition zone around the well.

FTIR of Melanin Extract

The melanin extract of RV 1 was characterized by FTIR spectroscopy (Fourier transform infrared spectroscopy, Perkin Elmer Spectra GX). The FTIR analysis was carried out in the mid IR region of 400–4,000 cm^{-1}. The control and melanin extract samples were mixed with spectroscopically pure KBr in the ratio of 5:95 to form a uniform pellets, which was then fixed in sample holder, and the analysis was carried out.

RESULT AND DISCUSSION

Isolation and Screening of Actinomycetes Producing Melanin from Soil Sample

Various sample from farm, KBS Garden, sugarcane farms and mangoes farm was used to isolate melanin producing actinomycetes. A total of 20 actinomycetes strain were obtained, showing pigmented colonies in tyrosine-casein agar plate. This 20 isolates were selected as melanin producing actinomycetes and purified by sub-culturing on tyrosine-casein agar plates.

The screening in liquid medium shows isolate no. S1 showed maximum melanin production of 10.6 mg/ml after 5 days of incubation at 30°C under static condition. Isolate M1 (9.6 mg/ml), F1 (8.2 mg/ml) and S2 (10.5 mg/ml) showed comparable melanin activity after 5 days incubation at 30°C. However, isolate no M3 showed negligible amount of melanin activity after 5 days of incubation at 30 °C.

Optimization of Melanin Production

The present work involves optimization of different parameters governing melanin production. The effects of various carbon sources, nitrogen sources, incubation temperature, etc. on melanin production were examined by one factor at a time method.

Effect of Different Medium Composition on Melanin Production

The result in figure 1 shows the effect of different media on melanin production by isolate S1. It reveals that isolate S1 shows maximum melanin production in medium 4 (7.25 mg/ml) whereas medium 1 give lowest melanin production (2.93 mg/ml).

Effect of Different Carbon Sources on Growth and Melanin Production

To determine melanin production supplementation of various carbon sources is required (Narang and Satyanarayana, 2001). These sources consist of glucose, fructose, maltose, lactose, sucrose and starch (Figure-2) This indicates that starch is the most efficient source for maximum production of melanin. Similar results obtained by Venkatesan *et al.* (2008) who reported that starch was the most effective carbon source for the production of

melanin, followed by glycerol and fructose. In contrast with the results obtained by Hewedy and Ashour (2009), showed that *Kluyveromyces marxianus* and *Streptomyces chibaensis* produces the pigment with all carbon sources tested.

Effect of Nitrogen Sources on Growth and Melanin Production

Nitrogen is the secondary energy sources and plays an important role in the growth and production of melanin. In this study the effect of supplementary organic nitrogen sources (Peptone, Urea) and inorganic nitrogen sources ($(NH_4)_2SO_4$, NH_4Cl) on the production of melanin by S1 strain using peptone iron broth was determined. The maximum activity was shown in yeast- extract containing medium (11.6 mg/ml).Similar results obtained by Venkatesan *et al.*, 2008, who isolated six strains of Streptomyces.

Effect of Incubation Temperature on Growth and Melanin Production

Effect of Temperature significantly influenced the growth, development and, in general, metabolic activities of an actinomycetes. Different incubation temperature used were 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C. Maximum melanin production was achieved at incubation temperature of 30°C.

Effect of pH on Growth and Melanin Production

The different pH taken were 3.0 to 9 and maximum melanin activities was observed at pH 7. A further increase in pH reduced the melanin. Neutral pH was preferable for melanin production by the actinomycetes isolate in starch nitrate medium. Similar results have been shown by Ulukus, 1984; Ali *et al.*, 2011.

Antibacterial Activity of Melanin

The antibacterial activity of melanin was monitored against Gram-positive (*Staphylococcus aureus, Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli, Salmonella typhi A, Salmonella typhi B*).Results shown in Table 1 indicate that melanin has antibacterial activity against all the tested microbial strains. The lowest zone of inhibition was exhibited by *S. typhi* (16 mm) and highest zone of inhibition was observed in *S. aureus* (20 mm).

FTIR Analysis of Melanin

The FTIR spectrum of the extracted melanin shows a peak around 3431 cm^{-1}, correspond to the OH group, Peak observed around 1636 cm^{-1} observed due to bending of secondary NH group, Peak at 1541 cm^{-1} may occurs due to nitro like compounds. The peak centered at 1063 cm^{-1} is the indication of CH in-plane of aliphatic structure. Similar, results has been shown by Agnes *et al.*, (2015) and Mohamed *et al.*, (2013).

CONCLUSION

In present study melanin is produced by specific actinomycete isolate S1. The optimum temperature and pH for melanin production was found to be 30°C and 7 respectively. The optimum carbon and nitrogen source for melanin production was found to be starch and nitrogen respectively.

ACKNOWLEDGEMENT

I would like to thank to my principle and all the faculty members of K.B.S. Commerce & Nataraj Professional Science College for providing all the laboratory facilities and for their support provided at all the steps of this study.

Table 1: Antibacterial activity of Melanin

Test organism	Zone of inhibition	
	Melanin Extract	Tetracycline
B.cereus	18 mm	15 mm
S.aureus	20 mm	16 mm
E.coli	17 mm	15 mm
S.typhi A	16 mm	13 mm
S.thphi B	18 mm	13 mm

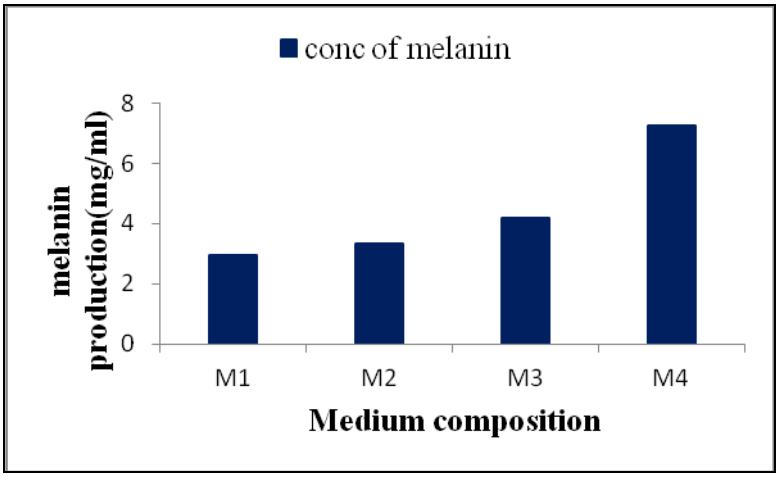

Figure 1: Effect of different medium on melanin production

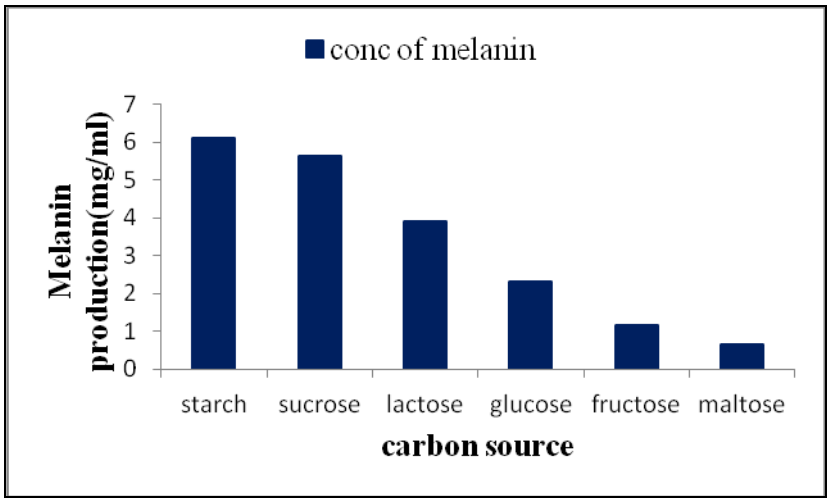

Figure 2: Effect of carbon source on melanin production

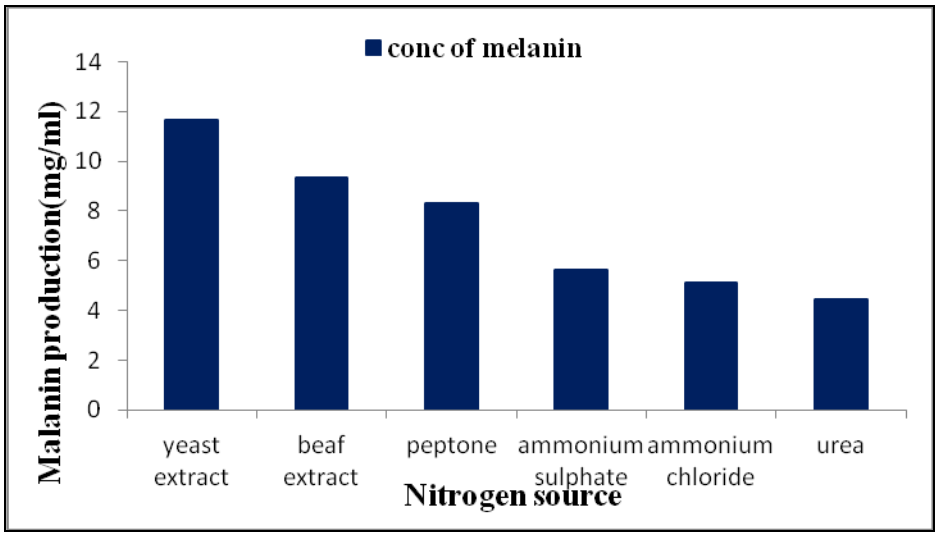

Figure 3: Effect of nitrogen source on melanin production

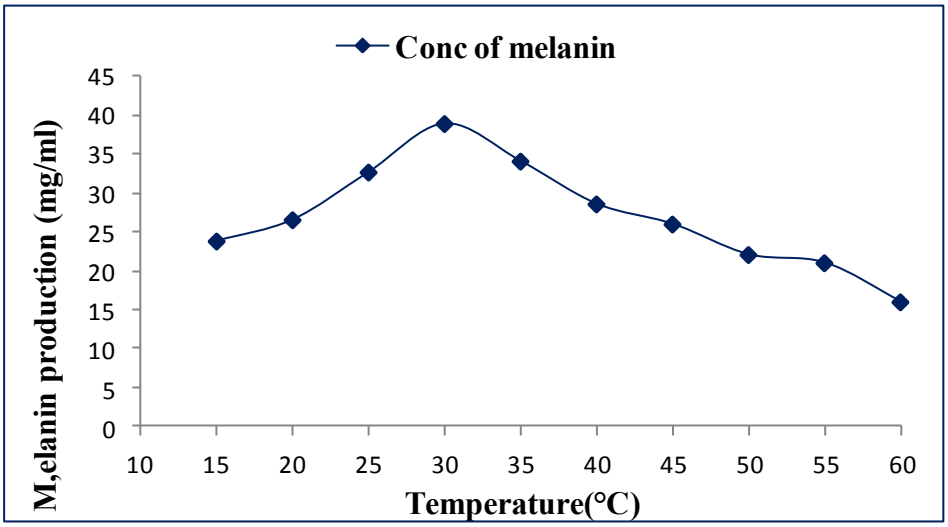

Figure 4: Effect of temperature on melanin production

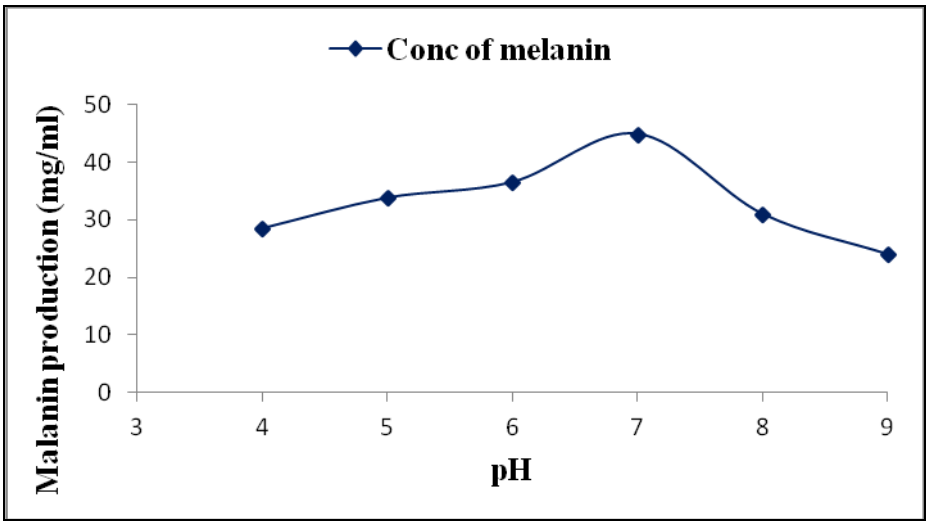

Figure 5: Effect of pH on melanin production

Figure 6: Antimicrobial activity of; A: Melanin and B: Negative control C: Positive control

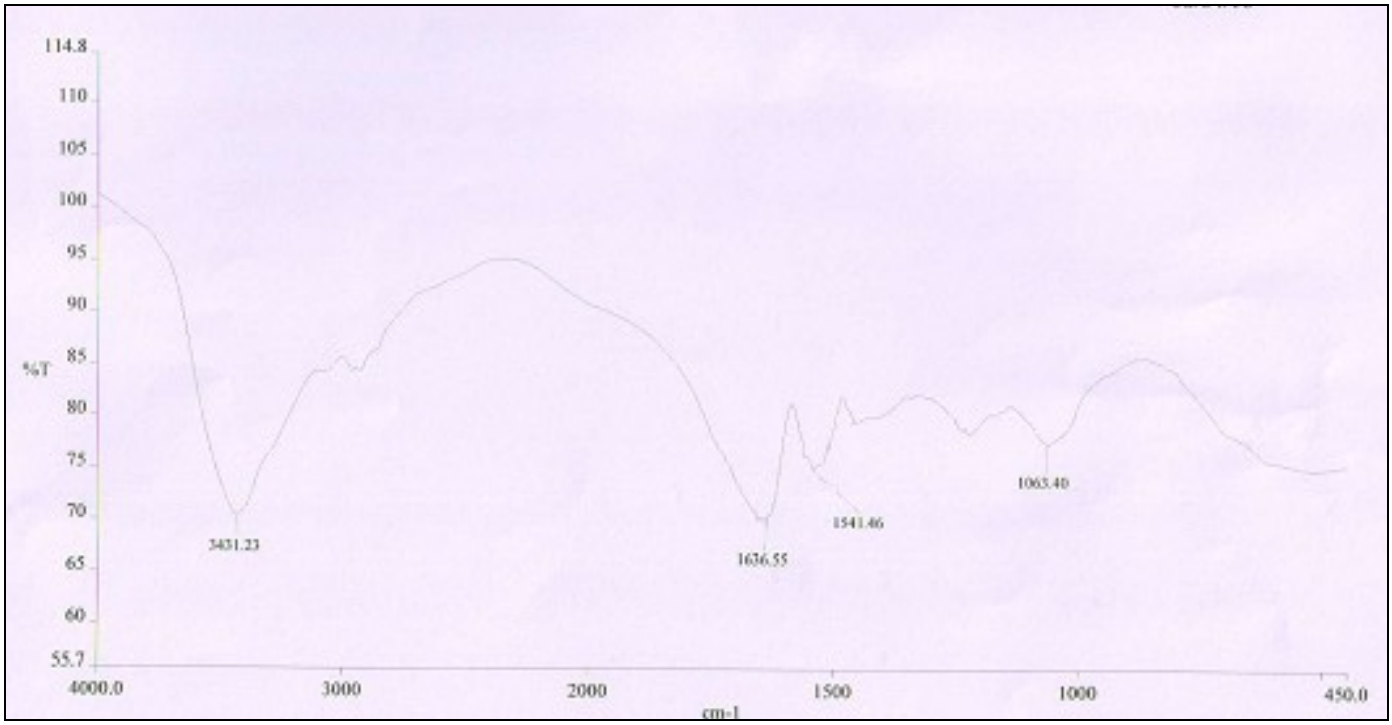

Figure 7: FTIR spectrum, transmittance (T%) of the extracted melanin

REFERENCES

1. Butler, MJ and Day (1998), "AW Fungal melanins: a review", ***Can. J. Microbiol***, 44, 1115-1136.
2. Dastager, SG; Wen-Jun, Li; Dayanand, A; Shu-Kun, Tang; Xin-Peg, Tian Xiao-Yang Zhi; Li-Hua, Xu and Cheng-Lin, Jiang (2006), "Separation, Identification and analysis of pigment (Melanin) production in Streptomyces", ***African Journal of Biotechnology,*** Volume 5,1131-1134.
3. Denizci, AA (1996), "A study on the detection and production of antibacterial antibiotics from Actinomycetes which isolated from the soils of Aegean and Eastern Black Sea regions of Turkey, ***PhD Thesis***, Ege University, Institute of Science, Izmir"
4. Mencher, JR and Heim, AH (1962),"Melanin biosynthesis by *Streptomyces* lavendulae", ***J Gen Microbial***, 28, 665-670
5. Nesbitt, BF; Kelly, JO; Sargeant, K and Sheridan, A (1962), "*Aspergillus flavus* and of melanin pigment from Streptomyces species D5 isolated from Desert soil, Rajasthan, India", ***Drug Invention Today***, 3 (3),12-13
6. Hua, SST; Grosjean, OK and Baker, JL (1999), "Inhibition of aflatoxin biosynthesis by phenolic compounds", ***Letters in Applied Microbiology***, 29, 289-291.
7. Venkatesan, S; Ganesan, R and Muthuchelian, K (1980), "Isolation, Identification and violaceoniger", ***Egyptian Journal of Botany***, 23 (3), 187-190
8. Agnes, Mbonyiryivuze; Bonex, Mwakikunga; Simon, MokhotjwaDhlamini and Malik, Maaza1 (2015), "***Physics and Materials Chemistry***", Vol. 3, No. 2, 25-29
9. Mohamed, Tawfiek; Shaaban, Sabha; Mahmoud, Mabrouk; El-Sabbagh and Asmaa, Alam (2013), "***Life Science Journal***", 10(1)

Correspondence Author:

Hiren Prajapati*

Department of Microbiology, KBS Commerce and Nataraj Professional Science College, Vapi. Dist.Valsad-396195, Gujarat, India

Cite This Article: Shreya, Modi; Poonam, B Chauhan; Mayur, Gahlout and Hiren, Prajapati (2016), "Extraction and isolation of melanin from actinomycetes", ***International Journal of Drug Research and Technology***, Vol. 6 (2), 79-86.

INTERNATIONAL JOURNAL OF DRUG RESEARCH AND TECHNOLOGY