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Full Length Research Paper

Melanin production from marine *Streptomyces*

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Melanin pigments are frequently used in medicine, food and cosmetic preparations. In this study, three strains among 21 *Actinomycetes* sp isolates produced a diffusible dark pigment on starch casein an agar medium which was water soluble. The pigment production was estimated using L-tyrosine as substrate. Among the strains, 3 (F1, F2, F3) were used for further analysis. Optimized culture condition for melanin production were; 1% of starch- best carbon source, 0.2% of soyabean- best nitrogen source, salinity- 15 ppt, temperature- 35°C, pH-7.0 and incubation time- 168 h. Sugarcane waste was found to be the cheaper and best source for melanin production. Melanin also showed activity against potential pathogens. The maximum antimicrobial activity was observed with *Escherichia coli* (20 mm) *and Lactobacilus vulgaris* (20 mm*). F1 strain producing melanin was taken for FT-IR analysis. The FT-IR result confirmed that it was melanin pigment. Therefore, this study proved that sugarcane waste can be used for the production of melanin and it (melanin) has potential anti-bacterial activity.

Key words: Melanin, sugarcane waste, antibacterial, FT-IR.

INTRODUCTION

Actinomycetes are Gram-positive bacteria showing a filamentous growth. They are a group of organisms widespread in nature, and play a significant role in the future of biotechnology, because of their importance as producers of vitamins, enzymes, antitumour agents, immunomodifying agents and mainly antibiotic compounds (Goodfellow et al., 1988; Demain, 1995). According to Sanglier et al. (1993) between 1988 and 1992, more than a hundred new molecules from actinomycetes were discovered. Approximately, 75% of these originated from the *Streptomyces* genus and at least 5,000 documented bioactive compounds are known as being produced by this genus (Anderson and Wellington, 2001). The majority of the antibiotics so far reported are obtained from *Streptomyces*, which are common inhabitants of soil. Numerically, they cover about 80% of total antibiotic products as compared to other genera (Kieser et al., 2000). Oceans account for more than 70% of the earth's surface and the microorganisms growing in marine environments are metabolically and physiologically diverse from terrestrial organisms (Takizawa et al., 1993). *Streptomyces* are prolific sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti–infective and –cancer agents or other pharmaceutically useful compounds (Bibb, 2005). Members of this group have been isolated from different soils, plant materials, waters and marine sediments (Zaitlin et al., 2003; Jensen et al,. 1991). They are able to produce a wide range of molecules with broad spectrum activities, that is, anti-bacterial, -fungal, -tumour, -parasitic and -viral (Atta and Ahmad, 2009; Naeimpoor and Mavituna, 2000).

Actinomycetes from the marine environment have been shown to be highly productive and may even surpass their terrestrial counterparts. New microbial metabolites, particularly antibiotics, are permanently needed due to the increase of resistant pathogens, evolution of novel diseases and toxicity of currently used compounds (Hakvag et al., 2008; Jensen et al., 2005). Approaches to the discovery of new antibiotics are generally based on screening of naturally occurring microorganisms like *Actinomycetes*, (Donadio et al., 2002) in different environmental samples.

The brown-black pigment has been referred to as 'melanin ' and as ' melanin like' because of pigmentation

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Abbreviations: FT-IR, HGA, etc

resulted from the addition of tyrosine to a defined medium upon which he was growing some actinomycetes and Douglas and San Clemente (1956) observed a dark brown pigment in flasks in which mycelial homogenates of *Streptomyces scabies* were mixed with tyrosine, dihydroxyphenylalanine or both. In attempting to correlate pigment formation and antibiotic synthesis by *S. antibioticus*, Sevcik (1957) manometrically determined the presence of phenol oxidase activity, with tyrosine and several other substrates. Characterizing the pigment, Bergey's Manual (1957) refers to the pigment as a 'soluble brown pigment'. Melanins are enigmatic pigments that are produced by a wide variety of microorganisms including several species of bacteria and fungi (Selvakumar et al., 2008). Melanin is a common substance produced by animals, plants and microorganisms. Melanins are pigment of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black (Langfelder et al., 200; Casadevall et al., 2000; Jacobson, 2000). Melanins are among the most stable, soluble and resistant of biochemical materials and they enhance the survival and competitive abilities of organism in certain environments but are not essential for growth and development (Bell and Wheeler 1986). It has been shown to protect micro-organisms against UV-radiation, enzymatic lysis, oxidants and killing by alveolar macrophages (Langfelder et al., 2003; Casadevall et al., 2000; Jacobson 2000). It has also been shown to chelate metal ions, to function as a physiological redox buffer, to provide structural rigidity to cell walls and to help to store water and ions (Langfelder et al., 2003)

Several types of melanins have been described in bacteria, plants, animals, and fungi: eumelanins, phaeomelanins, allomelanins and pyomelanins. Eumelanins are formed from quinines and free radicals. Phaeomelanins are derived from tyrosine and cysteine. Allomelanins are synthesized from nitrogen-free precursors, and pyomelanins are derived from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate and homogentisic acid (HGA) (Kotob et al., 1995). HGA is a metabolite of phenolic metabolism in a wide variety of higher organisms, including mammals, fish, birds, amphibians, and plants. Pyomelanin formation is correlated with HGA production in disparate marine bacterial species: *Vibrio cholerae* and *Shewanella colwelliana* (Kotob et al., 1995).

MATERIALS AND METHODS

Sample collection and isolation of Actinomycetes

Marine sediment samples were collected from Vellar Estuary, Tamilnadu of India. Samples were placed in a Petri dish and tightly sealed. The samples were then pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 4 days and subjected to serial dilution (up to 10^{-6}dilution) by adding 1 g of soil sample in 99 ml of 50% sea water. About 1.0 ml of diluted samples were plated on starch casein agar with cycloheximide (25 µg/ml), nalidixic acid (25 µg/ml) to avoid bacterial and fungal contamination by spread plate technique and incubated at 28°C for 7 to10 days.

Characterization

The ability of the isolate to utilize various carbon and nitrogen sources were studied by the method recommended in the International *Streptomyces* project. Carbon sources like dextrose, mannitol, fructose, xylose, sucrose, raffinose, inositol, arabinose, galactose, celibiose, adonitol, melibiose, sorbitol, dulcitol, mannose, lactose and rhamnose were tested on phenol red broth supplemented with 1% carbon source (Nonomura, 1974; Vimal et al., 2009).

Identification of strains

Colony morphology, mycelium pattern, reverse side pigment and carbon utilization were observed. Based on these observations, the strains were identified. (Nonomura, 1974).

Melanin formation

Melanin formation was tested using 10µl of suitable liquid media, dispensed in test tubes and inoculated with one loop full of the spores of the *Streptomyces* and subjected to stationary stage at 37°C for seven days. Melanin pigment was estimated by taking 2 ml of the culture and 1 ml of 0.4% substrate solution (L-tyrosine). The reaction mixture was incubated at 37°C for 30 min and a red coloration was observed and read spectrophotometrically at 480 nm (UV-1601, Shimadzu) (Scribners et al., 1973; Mencher and Heim, 1962).

Optimization of melanin

For optimization of melanin production to find the optimum culture conditions for its production, the strains were cultured at different temperatures (28 to 46°C), carbon sources (1% w/v of starch, glycerol, dextrose, maltose, fructose and glucose), nitrogen sources (1% w/v yeast extract, soyabean meal and peptone), cheaper sources (1% w/v sugar cane waste, rice bran, wheat bran, coconut cake, rice flour) in different pH values (5.0 - 9.5). The impact of salinity on melanin production was evaluated using various salinity (0 to 20 ppt). All the experiments were carried out in 500 ml conical flasks containing 100 ml of production medium (g/l), 3 g of calcium carbonate,1 ml of trace salt element ($FeSO_4.7H_2O$-0.5 g, $ZnSO_4.7H_2O$- 0.5 g, Cu $SO_4.7H_2O$- 0.5 g, $MnCl_2.4H_2O$- 0.5 g, distilled water- 100 ml) in 1000 ml of distilled water (pH 7.5).The culture flasks were maintained in a water bath shaker.

Mass scale production of melanin

Melanin production was performed in a shake flask culture with 1000 ml of production media. The production media contained the following culture conditions; pH 7.0, temperature 35°C, salinity 15 ppt, nitrogen source- 0.2%, soyabean meal and 1.0% of sugarcane waste concentration, for 7 days incubation.

Extraction of melanin pigment

After incubation cultures were centrifuged at 3,000 rpm for 30 min, equal volume of chloroform, ethyl acetate and methanol were

added with cell free supernatant and mixed well. This step was repeated 2 to 3 times. The solvents were then evaporated and powdered while the pigment residues were collected (Sambamurthy and Ellaiah, 1974).

Antibacterial activity of actinomycetes

The purified actinomycetes colonies were screened for antibacterial activity against *Escherichia coli, Lactobacillus vulgaris, Proteus mirabilus, Vibrio cholerae, Stapylococous aereus, Salmonella typhae, Salmonella paratyphae* and *Klesella oxytoca* by well diffusion method. The isolates were grown in a production broth until adequate turbidity was achieved. 100 µl of the actinomycetes broth culture was placed in wells made on Muller Hinton agar plates seeded with the test bacterial pathogen cultures. The plates were incubated at 37°C and observed for inhibition zone after 24 h.

Antibacterial activity of melanin crude pigment extract

Antibacterial activity was tested by well diffusion method. Pathogens like *E. coli, L. vulgaris, P. mirabilus, V. cholerae, S. aereus, S. typhae, S. paratyphae* and *K. oxytoca* were swabbed on a Muller Hinton agar against 100 µl of pigment extract and incubated at 37°C for 24 h.

Fourier-transform infrared (FT-IR) spectrophotometer

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying the types of chemical bonds (functional groups) and therefore, can be used to elucidate some components of an unknown mixture. The molecular characterization was performed using melanin. One milligram of freeze-dried melanin was ground with 100 mg of KBr and pressed with 7,500 kg for 30 s to obtain translucent pellets. Infrared absorption spectra were recorded on a Thermo Niocolet, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively. All measurements consisted of 500 scans, and KBr pellets were used as background reference.

RESULTS

Isolation of melanin producing actinomycetes

The sediment samples were collected from Vellar Estuary, Tamilnadu, India. Actinomycetes were isolated by using starch casein agar. Based on their colony morphology on the master plate, totally 21 different colonies were observed. Three isolates namelyF1, F2 and F3 produced diffusible melanin pigments.

Characterization

Ability to utilize the different carbon sources and their growth at specific pH and temperatures of F1, F2 and F3 strains are recorded in Table 1.

Identification of actinomycetes

Three strains showed melanin pigmentation. Reverse pigmentation and carbon utilization pattern were observed. Growth at 37°C and pH 7.5 were also observed. Based on these observations, it is found that F1, F2 and F3 are *Streptomyces sp*.

Melanin formation

Red colour formation indicated melanin production by F1, F2 and F3. These strains were observed spectrophotometrically at 480 nm. The optical density values showed the different level of melanin production of F1, F2 and F3. It was plotted against the standard graph of melanin.

Optimization of melanin

Melanin production was optimized at different carbon, nitrogen and cheaper sources, at different salinity, temperatures, pH and incubation times. The best sources and optimized parameters for effective melanin production are the following; starch (carbon source) (Figure 1), Soyabean meal (best nitrogen source) (Figure 2), sugarcane waste (Figure 3), 15 ppt salinity (Figure 4), 35°C (Figure 5), pH 7(Figure 6) and incubation time 168 h (Figure 7).

Mass scale production of melanin

21.13 g/l of crude melanin was extracted by solvent extraction method with 1000 ml of optimized production media at optimized condition. In this process, sugar cane waste was used as a cheap carbon source.

Antibacterial activity of actinomycetes

F1, F2 and F3 showed anti- bacterial activity against all tested pathogenic bacteria.

Antibacterial activity of melanin crude pigment extract

Crude melanin pigment had anti bacterial activity against all tested pathogen and had maximum zone of clearance against *Escherichia coli, Lactobacillus vulgaris* (Table 2 and Figure 8).

Fourier-transform infrared (FT-IR) spectrophotometer

FT-IR results proved that it was a melanin pigment. The chemical properties of the resultant dark pigment were determined including its elemental composition with infrared (IR) spectrum. In our study, IR spectrum of

Table 1. Biochemical characterization of *Streptomyces.*

Hydrolysis test for Isolate	F1	F2	F3
Starch	+	+	-
Casein	+	+	+
Gelatin	+	+	+
Sucrose	-	-	+
Salicin	A	-	-
Terhalose	-	A	A/G
Rhamanose	-	-	A/G
Xylose	-	-	-
Dextrose	A/G	A/G	A/G
Celebiose	-	A	A
Fructose	A/G	A/G	A/G
Galactose	-	-	-
Arabinose	-	-	-
Adonitol	-	-	-
Melibiose	A/G	A/G	A/G
Raffinose	-	A/G	A/G
Sorbitol	-	-	-
Dulcitol	-	-	-
Mannose	A/G	A/G	A/G
Lactose	-	-	A/G
Inositol	A/G	A/G	-
Growth at 37℃ and pH 7.5	+	+	+

A-Acid alone produced, A+G - Acid and Gas produced, - no sugar fermentation.

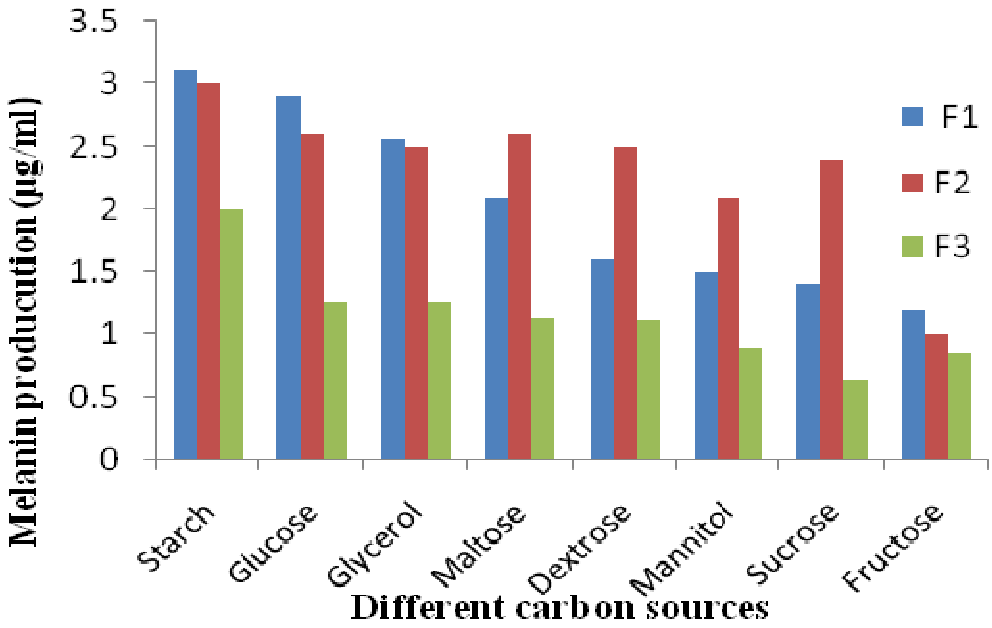

Figure 1. Melanin production with different carbon sources by F1, F2 and F3 strains.

melanin with peaks near 3,381 cm^{-1} was ascribed to –OH bond. The peak at 2,925 cm^{-1} was assigned to C–H stretch bond related to methane group. The peak of 1633 cm^{-1} was amino group NH_2 stretching (Figure 9).

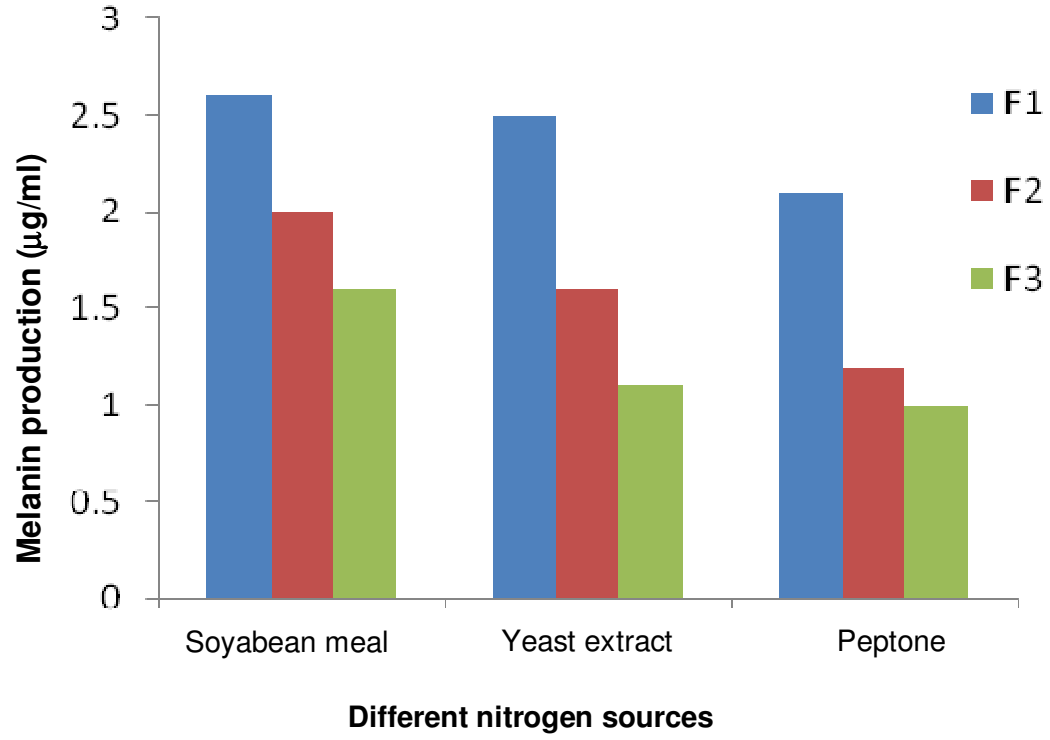

Figure 2. Melanin production with different nitrogen sources by F1, F2, and F3 strains.

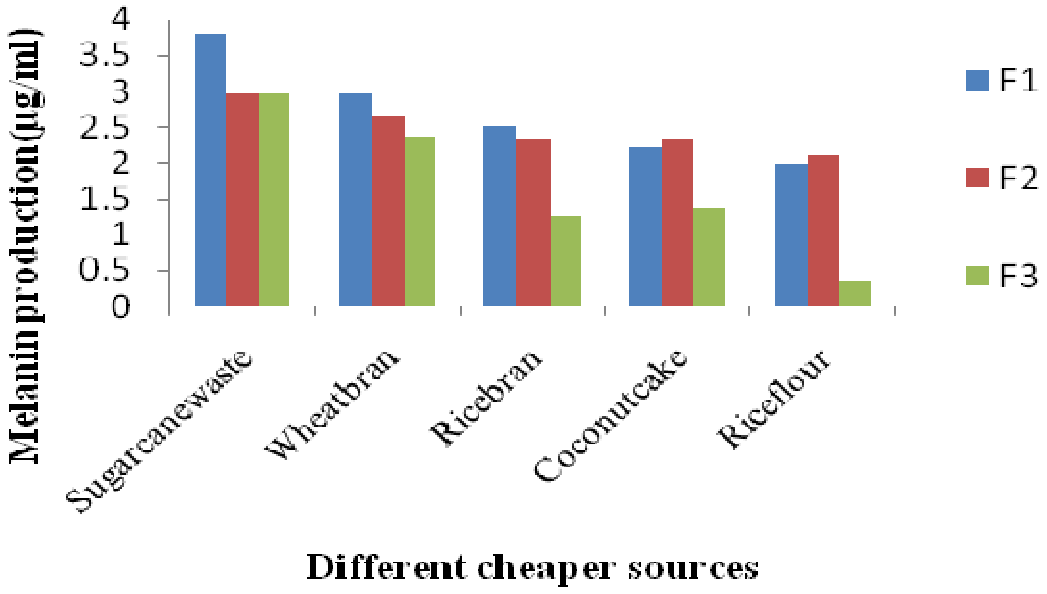

Figure 3. Melanin production with different cheaper carbon sources by F1, F2 and F3 strains.

DISCUSSION

Actinomycete strains were isolated from marine sediments, collected from Vellar Estuary, Tamilnadu, India. A total of 21 different actinomycete colonies were recorded among which 3 streptomyces produced melanin pigment. A diverse actinomycete community in marine sediments have been extensively investigated (Kathiresan et al., 2005), and also diverse actinomycete community in the Chesapeake Bay has been observed (Takizawa et al., 1993).

In this study, Table 1 shows the hydrolysis and

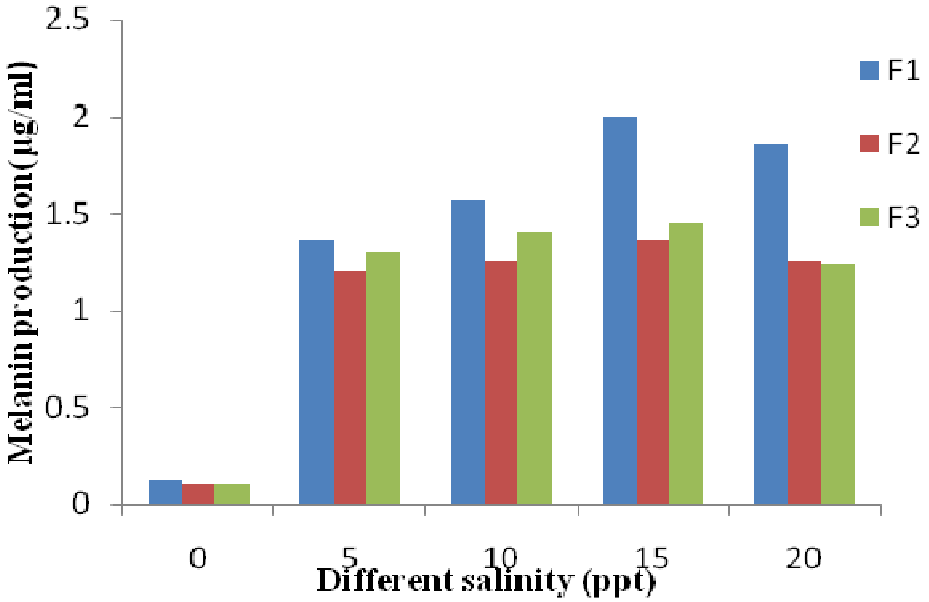

Figure 4. Melanin production with different salt concentration (ppt) by F1,F2 and F3 strains.

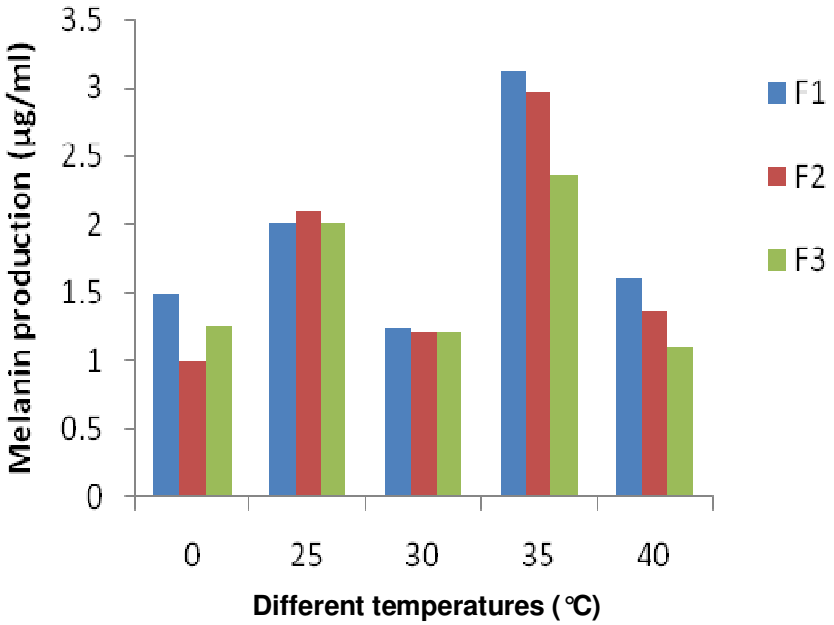

Figure 5. Melanin production at different temperatures (°C) by F1, F2, F3 strains.

utilization of different sugars by streptomyces. Actinomycetes have hydrolytic properties of starch, lipid, gelatin and urea (Gomathynayagam et al., 2001). Previous studies suggested that actinomycetes are considered to contribute to the breakdown and recycling of organic compounds and also revealed that different carbon sources are used for the production of melanin by streptomyces (Sunanda et al., 2009). In our stuyd, we observed that all the three isolates could degrade almost all available complex substances. Reports on the degradation and enzymatic activity of streptomyces also revealed a much similar result (Laidi et al., 2006; Ibrhaim, 2006).

Optimum melanin production was observed in 1.0% of starch concentration (Figure 1), 0.2% soyabean meal concentration (Figure 2), 1.0% of cheaper source (Figure 3), 15% of salinity (Figure 4), 35°C temperature (Figure 5), pH 7.0 (Figure 6) and incubation time of 192 h (Figure

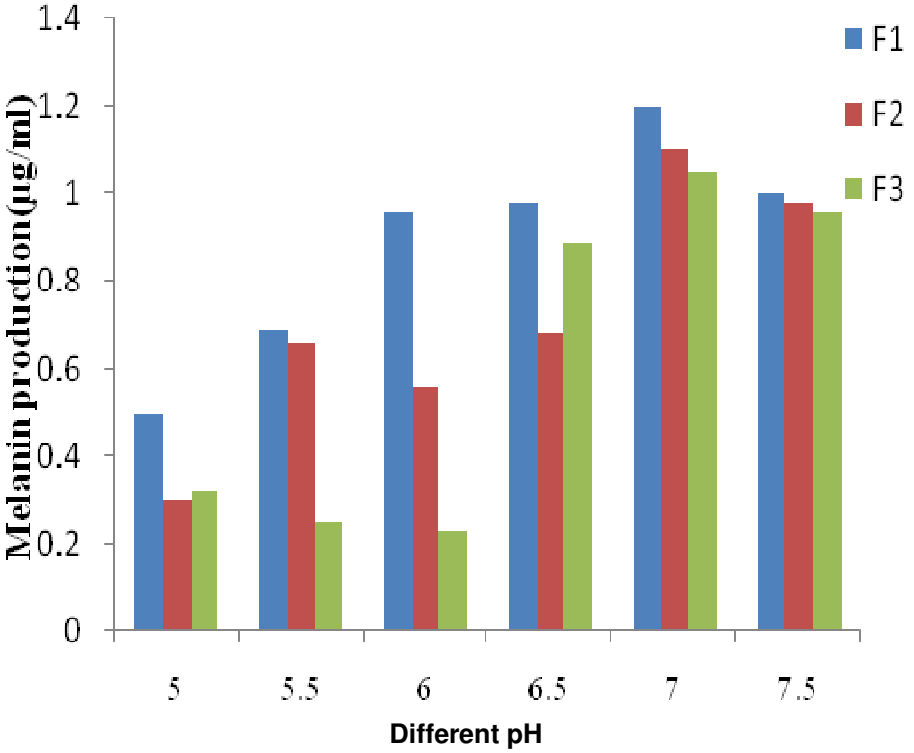

Figure 6. Melanin production at different pH level by F1, F2 and F3 strains.

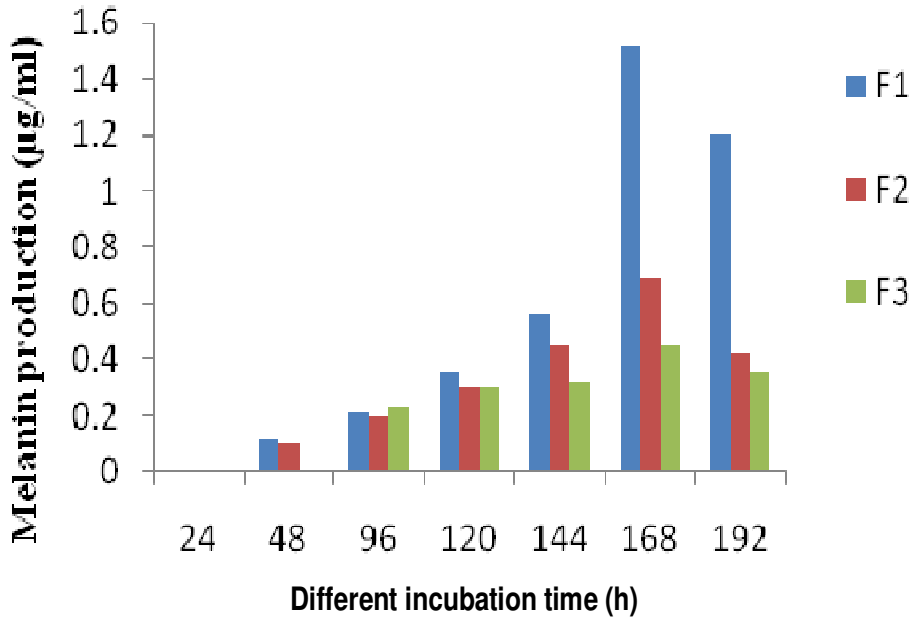

Figure 7. Time course of melanin production at different incubation periods (h) by F1, F2, F3 strains.

7). Dastager et al. (2006) found that starch was the most effective carbon source for the production of melanin, followed by glycerol and fructose. Zapata (1953) also suggested that the best carbon sources for pigment production were D-mannose, glycerol, raffinose, and D-xylose. Mannitol gave scant pigmentation, L-arabinose gave almost negligible, and inulin none. These results agree with those of Cochrane and Conn (1961).

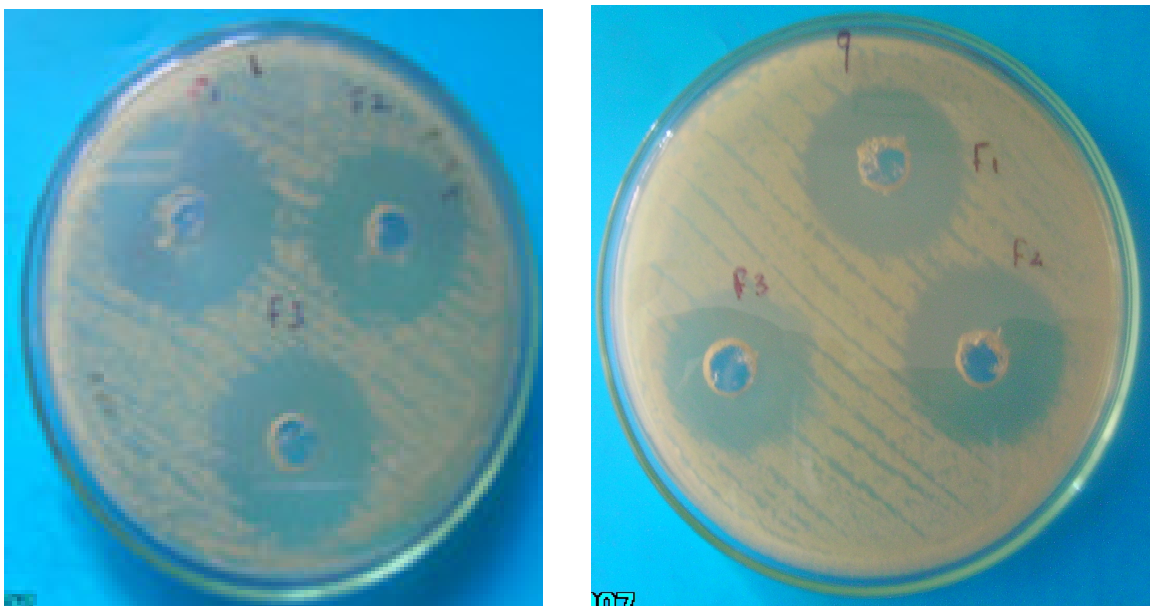

Figure 8. Antibacterial activity of melanin pigment (F1, F2 and F3) against (a) *E. coli* and (b) *L. vulgaris*.

Table 2. Antibacterial activity of melanin.

Pathogen	F1(mm)	F2(mm)	F3(mm)
Escherichia coli	20	19	18
Lactobacillus vulgaris	20	20	19
Proteus mirabilus	19	15	15
Vibrio cholera	19	19	17
Stapylococous aereus	19	18	18
Salmonella typhae	18	17	12
Salmonella paratyphae	17	16	11
Klebsella oxytoca	17	10	9

When starch was used as carbon source, 8.55 g/l melanin production was observed. Carreira et al. (2001) showed that glucose was the best carbon source for yeast while Dastager et al. (2006) indicated that starch was the effective carbon source for *Streptomyces* spp. followed by glycerol and fructose. Chaskes et al. (2008) had a different opinion: In the case of *Cryptococcus gattii*, the carbon source was fructose. In this study, soyabean meal was observed as the best source for pigment production compared to yeast extract and peptone. Kathiresan et al. (2005) reported that maximum anti-fungal compound production in soyabean meal was the same with yeast extract. Hewedy et al. (2009) suggested that it was feasible to combine yeast extract and peptone into a single medium to increase production of pigment from both strains. As a result, a good increase in growth and pigmentation at 6 g/l concentration for each was obtained. In contrast, a weight of 4 to 1 for D-tryptophan/ D-proline gave excellent growth and strong dark pigmentation for *C. gattii* (Chaskes et al., 2008).

In order to economize melanin production, cheaper carbon sources like sugarcane waste, wheat bran, rice bran, coconut cake, rice flour were used. Among the substrates used, melanin production was the highest in sugarcane waste (Figure 3). The maximum yield was 21.13 g/l when compared to other substrates. Mohamed Rizk et al. (2007) found that molasses was the best raw material for streptomyces growth and antibiotic pro-duction followed by wheat bran, corn corbs, rice bran, saw dust. In our result, sugarcane waste is the best source for pigment production, being cheaper followed by wheat bran, ricebran, coconut cake and rice flour. Selvameenal et al. (2009) observed that the quantity of crude pigment extracted was found to be 420 mg/10 g by using wheat bran as substrate.

35°C is the optimum for melanin production. Hewedy et

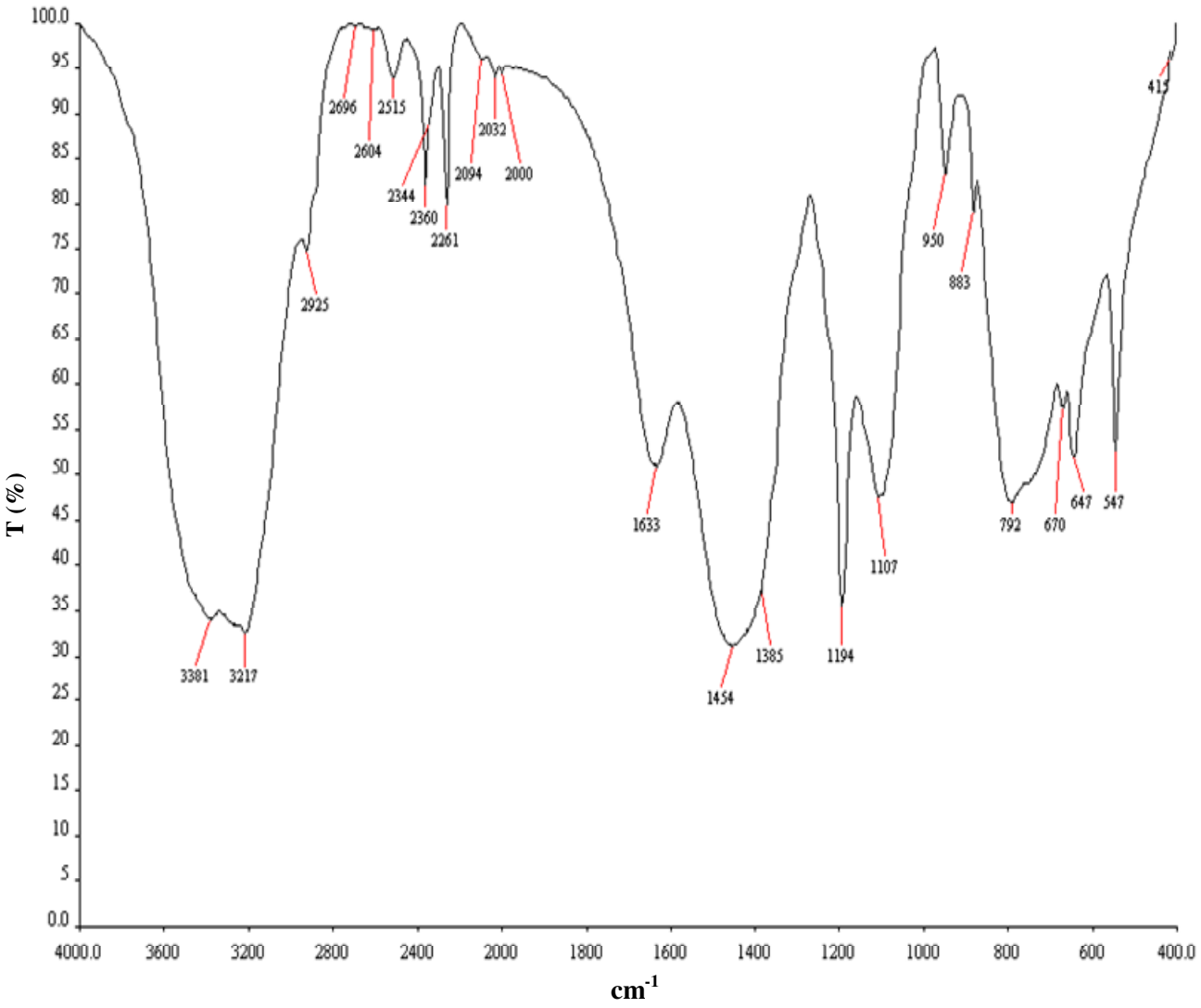

Figure 9. FT-IR analysis of melanin.

al. (2009) found that pigment production was done for 14 days at 32°C, which was the optimum temperature for growth and production of pigment. Optimum pH 7 was the best for melanin production but Carreira et al. (2001) found that the best pH for *Yarrowia lipolytica* was approximately 6.5. Coyne and Harthi (1992) reported that the initial pH ranged from 5.8 to 6.5 in *V. cholerae* 569B; the favourable pH for *C. gattii* ranged from 5.35 to 7.35 for pigment production. Chaskes et al. (2008) and Sanchez-marroquin' and Zapata (1953) found that the optimum pH lies between 7.3 and 7.7. This factor is paramount because deviations of less than a unit with reference to the optimum point showed great differences in the relative pigment intensity. A pH lower than 5.4 or higher than 9.9 allowed neither growth nor pigment production. F1, F2 and F3 strains exhibited potential antibacterial activities. This study confirmed antibacterial activity of streptomyces isolated from sediments, collected at Vellar Estuary Tamilnadu, India. From the ten thousand known microbial metabolites, about 150 to160 (0.2 to 0.3) compounds were practically proved as successful leading compounds. The value of actinomycetes to society in terms of providing useful drugs and to the pharmaceutical industry for revenue generation is immense. Actinomycetes products such as antibiotic- like streptomycin and novobiocin firmly cemented these chemically prolific bacteria in the center stage of natural products in drug discovery research. The crude melanin pigment showed significant antibacterial activity against 8 pathogenic organisms (Table 2). From the results, it is evident that melanin extract showed strong anti-bacterial activity against both *E. coli* and *L. vulgaris* strains at a concentration of 1µl of pigment extract and the range of the zone of inhibition was between 17 to 20 mm. The extract also showed strong activity against *S. aeureus*, *P. mirabilis*, *V. cholerae*, *S.*

typhi, *S. paratyphi* and *K. oxytoca*. Sultan et al. (2002) found that the active metabolite from the *Actinomycetes* sp. showed significant antibacterial activity against 14 pathogenic organisms. In our study, the maximum antibacterial activity was observed with E. *coli*, and *L. vulgaris* followed by *S. aeureus*, *P. mirabilis*, *V. cholerae*, *S. typhi*, *S. paratyphi* and *K. oxytoca*. Selvameenal et al. (2009) found that purified pigment has 20 mm zone of clearance against *E. coli*. In our study, 20 mm zone of clearance was observed in the crude pigment, having activity against *E. coli*. Mohan Remya et al. (2008) found that the antimicrobial efficacy of the isolates was tested with the extract against the pathogens. The maximum activity was observed with *E. coli* (22 mm). Mustafa Oskay et al. (2004) found that the most antibacterial activity was on the phytopathogen bacteria.

One of the main tests for identifying melanin is IR spectrum. The chemical properties of the resulting dark pigment were determined including its elemental composition with infrared (IR) spectrum (Harki et al., 1997). In our study, IR spectrum of melanin with peaks near 3,381 cm^{-1} was ascribed to the –OH bond. The peak at 2,925 cm^{-1} was assigned to the C–H stretch bond related to methane group. The peak at 1,633 cm^{-1} was the amino group with NH_2 stretching. Hewedy et al. (2009) found that melanin exhibited bright spectral absorption lines of 2925 to 2938 cm^{-1} peak related to the hydroxy group (OH), peak at 3344 to 3436 cm^{-1} relates to the amino second group (NH) and peaks at 1243 to 1305 cm^{-1} relates to the anhydride group (C-O) in synthetic melanin and all extracted microbial pigment. Also, there were 2925 to 2938 cm^{-1} peaks relating to the methane group (CH), 1628 to 1651 cm^{-1} peaks relating to the amino group (NH) in the pigment extracted from *K. marxianus*, *S. chibaensis* and synthetic melanin. The synthetic and the present natural microbial melanins had the same chemical properties for each organism. The mixed culture differed in functional groups (C-N). This may be a strain-related phenomenon, or C/N ratio might have resulted from impurities which were difficult to remove from melanin. Based on this finding, we suspect that the resulted pigment may be eumelanin not allomelanin, which lacks nitrogen. Plonka Grabacka's (2006) study also came to the same conclusion.

Conclusion

There should be more researches to find new and low cost sources for antibacterial compound production. This study points out that the cheapest source for maximum production of melanin is sugarcane waste compared to other cheaper sources, and that the melanin pigment has antibacterial activity.

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