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"LAB SCALE PRODUCTION AND ANTIBACTERIAL POTENTIAL OF MELANIN FROM *STREPTOMYCES BIKINIENSIS*"

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ABSTRACT

Actinomycetes were characterized by production of various pigments on natural or synthetic media. These pigments usually described in terms of various shades of blue, red, rose, yellow, green, brown and black. *Streptomyces* is a major group in actinomycetes as blackish brown (melanin) pigment producer. The melanin production by *Streptomyces species* is the key feature for the classification of the *Streptomyces* group. The present study was focused on production and characterization of melanin producing *Streptomyces*. Soil samples were collected from the Shirala region Dist-Sangli. The total 14 Actinomycete isolates were obtained from soil by performing serial dilution technique and using Glycerol asparagine agar supplemented with Cycloheximide (100µg/ml). Morphology and spore chain arrangement of isolates were studied. Out of 14 isolates 12 were suspected belong to genus *Streptomyces*. The primary screening of 12 isolates for melanin production was carried out on peptone yeast extract iron agar. Two isolates such as Kd8 and Kd14 showed blackish brown diffusible pigment and these isolates were taken for secondary screening by using the tyrosine agar medium. The qualitative tests for pigment melanin were carried out by inoculating in tyrosine broth supplemented with traces of chloroform and incubating at 30^0 C for 48 hrs. Red color was reported in tyrosine broth indicated positive melanin production. On the basis of intensity of red color seen in the tyrosine broth Kd8 was selected for further study. Out of these isolates Kd8 isolate was identified on the basis of morphological, cultural, biochemical and 16S rRNA gene sequencing as *Streptomyces bikiniensis.* 0.8gm/100ml pigment was extracted from peptone yeast extract iron agar. The antibacterial activity of pigment extracted from Kd8 has been investigated. The pigment from isolate kd8 showed inhibitory zone against *Escherichia coli, Staphylococcus aureus, Bacillus species and Salmonella typhi.*

KEY WORDS: ***Streptomyces*, Melanin, pigment, Cycloheximide, antibacterial activity.**

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INTRODUCTION

In 2006, around 7.4 million tons of inorganic, organic and special pigments were marketed worldwide. The global demand on pigments was roughly US$ 20.5 billion in 2009. The worldwide sales are said to increase up to US$ 24.5 billion in 2015, and may reach US$ 27.5 billion in 2018. The toxicity problems caused by those of synthetic pigments to the environment have created a mounting interest towards natural pigments. Among natural pigments, pigments from microbial sources are potentially good alternative to synthetic pigments. Many artificial synthetic colorants, which have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes, have various hazardous effects. To counter the ill effect of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources.[1] Natural pigments can be obtained from two major sources, plants and microorganisms.[2-3] The accessible authorized natural pigments from plants have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility and are often non-available throughout the year. The latter are of great interest owing to the stability of the pigments produced and the availability of cultivation technology.[4-6] The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications.Melanin is seen as a potent pigment which can be used to make an organic photovoltaic cell, useful in preparation of bio plastics, in making UV absorbing optical lenses and also can play an important role in bioremediation.Several species of *Streptomyces* genus produces bioactive molecules like antibiotics, pigments and many extracellular enzymes as glucose isomerase, amylase, cellulases and proteases. *Streptomyces* produces various pigments like yellow, greyish yellow, bluish grey, whitish grey and many other pigments. *Streptomyces species* have ability to produce dark brown pigment known as melanin. It was diffusible, dark pigment which is water soluble. Melanins are mostly used in cosmetics as a component of photo protective creams and sunscreen lotions basically for UV- protection and free radicals scavenging properties.[7] Melanins can be used as UV-protective agent in the bioinsecticide preparation such as in the *Bacillus thuringenesis* (Bt) insecticidal crystals.[8] The melanin producing organism can also be used in bioremediation of radioactive waste such as uranium.[9] As global warming results in the depletion of ozone layer which leads to the increased penetrations of UV radiations from Sun in the Earth's environment. This increased UV radiation causes more skin cancers which mainly includes melanoma (Human Melanocyte Cancer). Melanoma is a malignant tumor of melanocytes which are found predominantly in skin but also in the bowel and the eye. Malignant melanoma of skin accounts for 1, 60,000 new cases annually and in the United States, 59,940 individuals developed melanoma in 2007, leading to an estimated 8,110 deaths. The lifetime risk of developing melanoma is now 1 in 49 for men and 1 in 73 for women.[10] The melanin can be used in vaccine preparation against human melanocyte cancer (melanoma); the lymphocytes of melanoma patients can be restimulated in vitro with autologous tumor cells to generate antitumor cytolytic T lymphocytes (CTL).Such antitumor CTL clones which appear to recognize melanin as an antigen. The melanin antigen may therefore constitute a useful target for the specific immunotherapy of melanoma.[11-12] The anti HIV (Human Immunodeficiency Virus) property of melanin was also reported, as soluble melanin found to be inhibiting replication of HIV in vitro. Thus soluble melanin may represent a new class of pharmacologically active substances which should be further investigated for a potential therapeutic agent in the treatment of AIDS (Acquired Immune Deficiency Syndrome).[13] In 1963, D. E. Weiss *et.al* has reported high electrical conductivity in an iodine-doped and oxidized polypyrrole melanin. A decade later, John McGinness, and *et.al* reported a high conductivity state in a voltage-controlled solid-state threshold switch made with DOPA melanin. Further, this material emitted a flash of light (electroluminescence) when it was

switched. Melanin also shows negative resistance, a classic property of electronically-active conductive polymers.[14-15] Melanin voltage-controlled switch, an active organic polymer electronic device was engineered in 1974 by Smithsonian Chip collection. These early discoveries were lost until the recent emergence of such melanins in device applications, in particular, electroluminescent displays. This pigment had many applications in different sectors such as printing, dyeing, pharmaceuticals and food industries. Shirala region of Maharashtra comes under heavy rain fall. Blackish brown (melanin) pigment had studied throughout the world by different groups of scientists but no one had reported melanin producing *Streptomyces* from Shirala region. Natural dyes can provide the much needed alternative to the complex world of the chemical dyes. So this work was undertaken.

MATERIALS AND METHODS

MATERIALS

1. Soil sample- Soil samples were collected from the villages around Shirala Dist. - Sangli, M.S. India and used in this study for isolation of actinomycetes.

2. Glycerol asparagine broth, Glycerol asparagine agar and Cycloheximide.

3. Tyrosine agar, Peptone yeast extract iron agar, Tyrosine broth and Chloroform.

METHODS

1. Isolation of Actinomycetes

The soil samples were collected from the villages around Shirala Dist.-Sangli, M.S. India and enrichment of soil sample was carried out in Glycerol asparagine broth supplemented with Cycloheximide (100µg/ml). 10-fold serial dilution of the sample was prepared up to 10^{-6} and 0.1ml aliquots of each dilution was inoculated into Glycerol asparagine agar (L-asparagine- 0.1g, K_2HPO_4-0.1g, glycerol- 1g, trace salt solution-0.1ml, agar- 2.5g, distilled water-100ml pH-7.4). To avoid the growth of fungal contaminant medium were supplemented with Cycloheximide (100µg/ml). Plates were incubated at room temperature (28°C) and monitored periodically over 5 to 7 days. Pure isolates were transferred on the slants of same medium and preserved at 4±2°C for further study.

2. Identification of Streptomyces

Morphological characteristics were studied with cover slip culture technique. Cultural characteristics were recorded on Glycerol asparagine agar medium. Biochemical characters were recorded on the basis of sugar utilization potential, enzymatic activities and growth under inhibitory substances. On the basis of spore mass color, the substrate mycelium color, the shape of the spore chain, morphological and cultural characters Actinomycetes suspected to be *Streptomyces* were sorted.

Molecular Identification of Streptomyces

One of the *Streptomyces* having potential to produce maximum tyrosinase was identified by using 16s rRNA sequencing. Genomic DNA was isolated using the Insta Gene™ Matrix Genomic DNA isolation kit. Using below 16S rRNA universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler. Name of the primer used for forward sequencing was 27F with sequence details AGAGTTTGATCMTGGCTCAG having a number of Bases 20. Name of the primer used for reverse sequencing was 1492R with sequence details TACGGYTACCTTGTTACGACTT having number of bases 22. 16S rRNA gene fragment was amplified using universal primers as above mentioned. Single-pass sequencing was performed on each template used16s rRNA universal primers. 518F primer with the sequence CCAGCAGCCGCGGTAATACG having number of base pairs 20 was used for forward sequencing.800R primer with the sequence TACCAGGGTATCTAATCC having number of base pairs 18 was used for forward sequencing. The fluorescent labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The phylogeny analysis of sequence with the closely related sequence of BLAST results was performed followed by multiple sequence alignment.

3. Primary screening

Peptone yeast extract iron agar

The isolates were streaked on Peptone yeast extract iron agar (pH 6.7) containing Peptic digest of animal tissue-1.5%, Protease peptone-0.5%, Yeast extract-0.01%, Ferric ammonium citrate-0.005%, K_2HPO_4-0.1%,

Sodium thiosulphate-0.008% Agar-2%. Plates were incubated at 30^0C for 48hrs to observe brown pigmented colonies that gradually changed its color to black were considered as an indication of melanin positive organism.

4. Secondary screening methods

Tyrosine agar

The isolates showing positive results in primary screening were streaked on tyrosine agar (pH 7) containing Asparagine-0.1%, L-tyrosine-0.5%, K_2HPO_4-0.05%, $MgSO_4.7H_2O$-0.05%, NaCl-0.05%, $FeSO_4.7H_2O$-0.000001%, $CuCl_2.2H_2O$-0.0000027% , $CoCl_2.6H_2O$-0.000004%, Sodium molybdate.$2H_2O$-0.0000025%, Zinc chloride-0.000002%, Boric chloride-0.000285%, manganese chloride $4H_2O$- 0.00018%, Sodium tartarate-0.000177% and agar-2%. All the plates were incubated at 30°C for 48 hrs the occurrence of brown pigmented colonies that gradually changed its color to black was considered as indication of melanin formation.[14]

Tyrosine broth

The isolates from secondary screening were inoculated into 50 mL of 0.1% tyrosine broth with few drops of Chloroform in 100mL Erlenmeyer flask and incubated at 30^0C for 48hrs. The deep red color shows the positive results.[14]

5. Production of melanin

Melanin production was carried out as per method of Manivasagan following with slight modification.[16] Composition of the production medium used was Peptone yeast extract iron broth (pH 6.7) containing Peptic digest of animal tissue-1.5%, Protease peptone-0.5%, Yeast extract-0.01%, Ferric ammonium citrate-0.005%, K_2HPO_4-0.1%, Sodium thiosulphate-0.008%.All the experiments were carried out in 500 ml Erlenmeyer flasks containing 100ml of Tyrosine broth medium. Sterile medium was inoculated with 5% of inoculum incubated at 30^0C for 4 days.

6. Extraction of melanin

Melanin extraction was carried out as per method of Mohanasrinivasan.[17]After the incubation, pH of the fermented broth was adjusted to 12 using 2N NaOH and centrifuged at 4000 rpm for 15 minutes. Supernatant was collected and pH of the supernatant was again adjusted to 2 using 2N HCl. Recentrifugation was done at 4000 rpm for 15 minutes. The obtained pellet was collected and suspended in methanol. The pigment was extracted from methanol by using a rotary vacuum evaporator and the amaranth colored pellet obtained was dried and stored at 5°C.

7. Antimicrobial Potentiality

The antimicrobial activities were measured as described by Abd El-Nasser.[18] The crude pigment extracted from *Streptomyces* was studied for antimicrobial activities against Gram positive organisms such as *Staphylococcus aureus* and *Bacillus species* and Gram negative organisms such as *Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris* bacteria. The Muller- Hinton medium was used for testing antibacterial activity. Composition of Muller- Hinton medium was (Infusion from beef -3%, Acid hydrolysate of casein-1.75%, Starch-0.5%, Agar-2%, Distilled water-100% and pH-7.2-7.4).

RESULTS AND DISCUSSION

Isolation of melanin producing Streptomyces

A total 14 actinomycetes were isolated from soil samples. The isolates were tentatively identified, out of 14 isolates 10 were belonged to genus *Streptomyces.* Rest of the four isolates two were *Nocardia* one become *Streptoverticillium* and one *Madhuromycetes*. The isolates were screened for melanin pigment production. On tyrosine agar (Fig. 1) and peptone yeast extract iron agar medium (Fig. 2) two isolates showed brown colored pigmentation. Pigmentation around the colonies gave positive indication for the melanin production. All isolates were inoculated in tyrosine broth supplemented with a few drops of chloroform for confirmation test of melanin. The color of the inoculated tyrosine broth changed from light pink to brown and ultimately to deep red with further incubation (Fig. 3). The color intensity of isolate Kd8 was much higher than Kd14.On this ground Kd8 was selected for further studies.

Figure.1
"Pigmentation on Tyrosine agar of isolates Kd14 and Kd8"

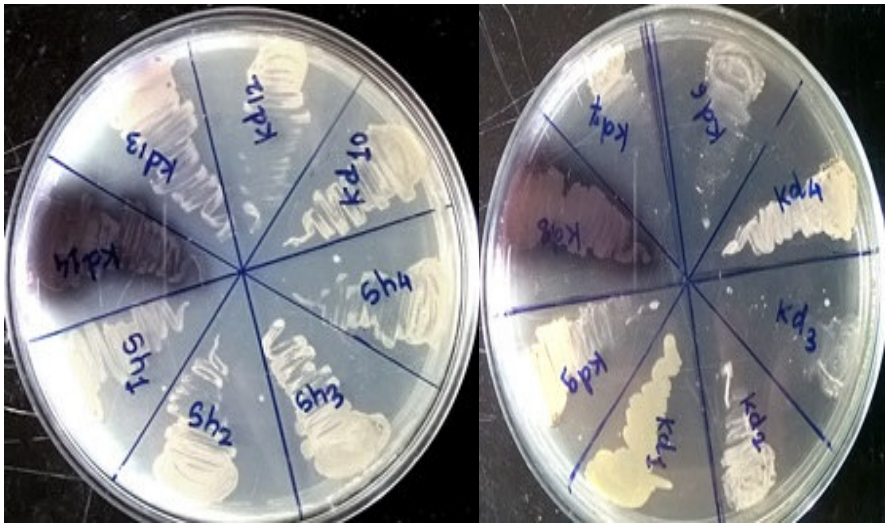


Figure.2
"Pigmentation on PYIA of isolates Kd14 and Kd8"

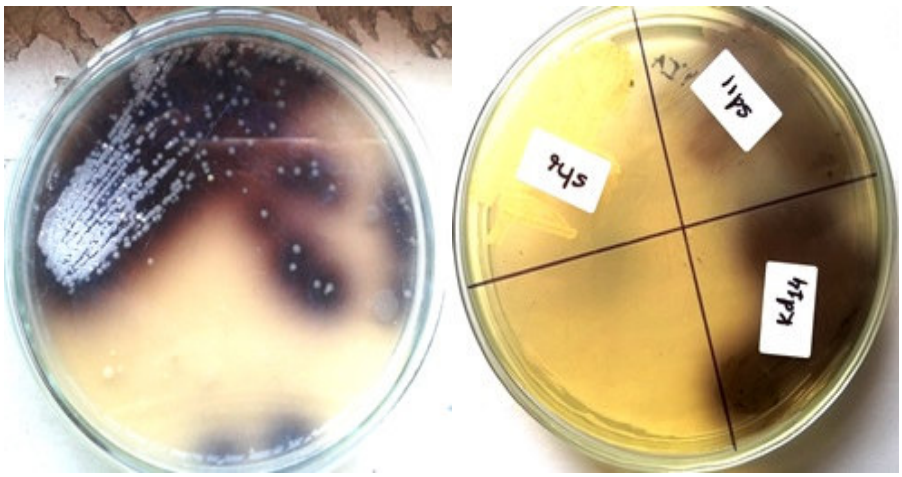
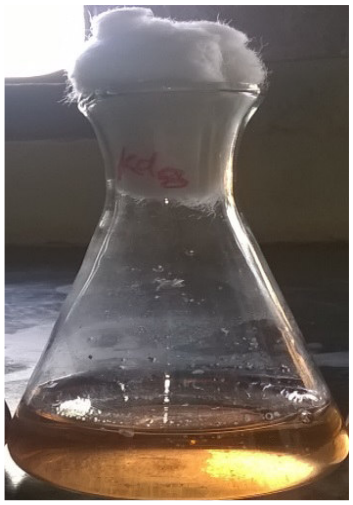


Figure.3
"Growth in tyrosine broth"



Identification of Streptomyces

Isolate Kd8 was identified and found as *Streptomyces* on the basis of morphological, cultural, biochemical identification. Morphological characters were studied by using cover slip culture technique (Aerial and substrate mycelia with spore chain arrangement showed in Fig. 4and 5). Biochemical characters of melanin producing isolate Kd8 was reported (Table.1).

Figure.4
"Spore chain morphology under Light Microscope (kd8)"

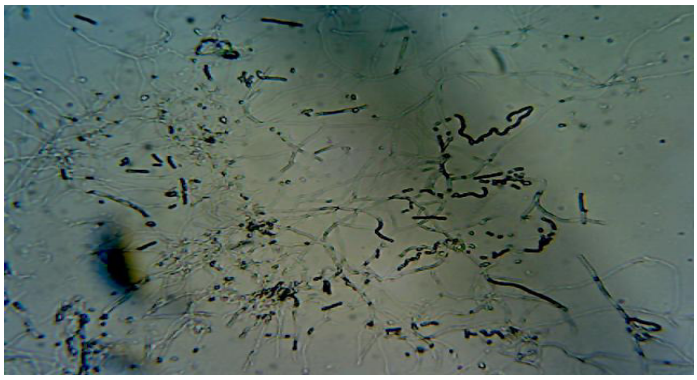


Figure.5
"Morphological feature of isolate Kd8 under SEM"

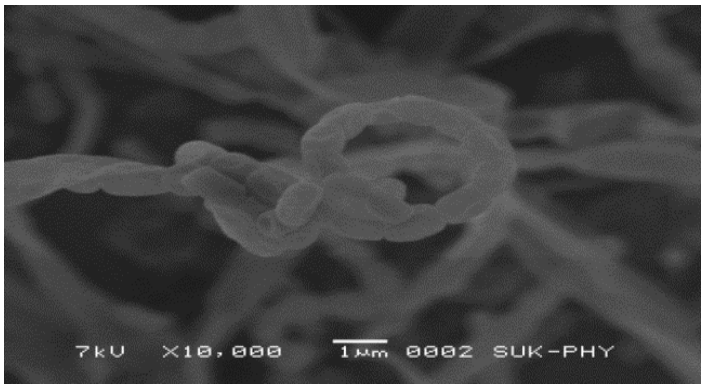


Figure.6
"Production of melanin in PYIA broth"

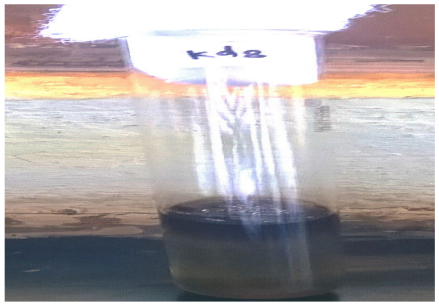


Figure.7
"Extraction of melanin from PYIA broth"

Figure.8
"Antibacterial activity of melanin against Bacillus Species"

Figure.9
"Antibacterial activity of melanin against Escherichia coli"

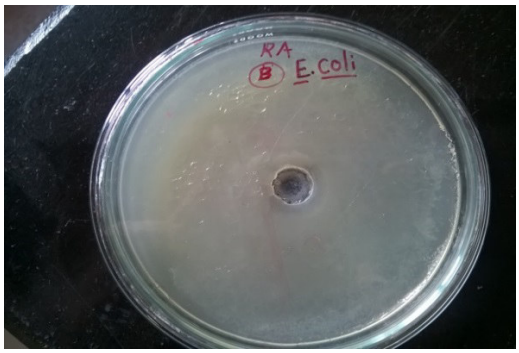
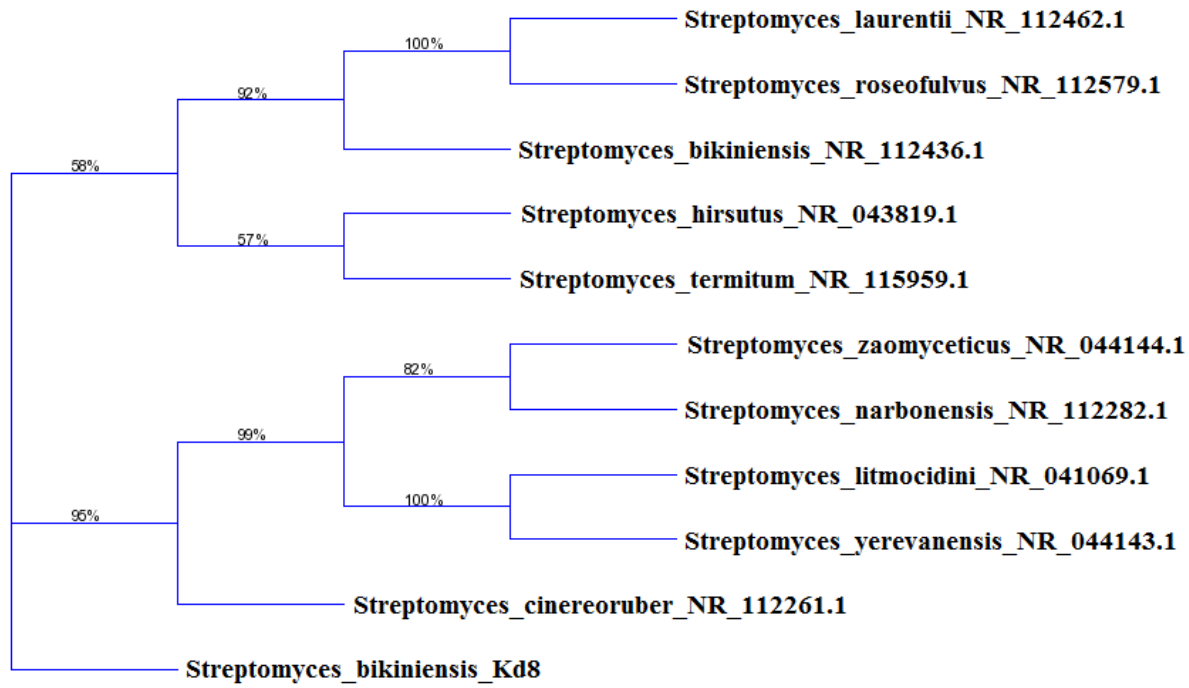


Table.1
Characterization of isolate kd8

Sr.No.	Isolate	Characteristic			Result
1	Kd8	**Morphological characters**	Spore chain morphology Straight to flexuous		+
			Pigmentation of substrate mycelium (colony reverse) Yellowish brown.		+
			Diffusible pigments(Melanoid pigments)		+
		Pigmentation characters	Pigmentation on PYIA (Blackish brown)		+
			Pigmentation on Tyrosine agar (Blackish brown)		+
			Tyrosine broth (Red color)		+
		Carbon utilization potential	Glucose		+
			Sucrose		+
			Mannitol		+
			Xylose		+
			Arabinose		+
			Lactose		-
			Trehalose		+
			Fructose		+
		Nitrogen utilization potential	L-phenylalanine		+
			L-Cysteine		-
			L-Histidine		+
			DL-Valine		+
		Enzyme activity	Catalase		+
			Oxidase		+
			Lecithinase		-
			Lipase		+
			Protease		+
			Nitrate reductase		+
			H_2S production		+
		Degradation activity	Gelatin		+
			Starch		+
			L-Tyrosine		+
			Urea		+
		Growth at temperatures	4^0C		-
			10^0C		-
			37^0C		+
			50^0C		-
		Growth in presence of inhibitory compounds	Crystal violet (0.0001%)		-
			Phenol (0.1%)		+
			Sodium azide	0.001%	+
				0.002%	-
			Sodium chloride	4%	+
				7%	+
				10%	-
				13%	-

**** Where + = positive -= negative***

Figure.10
"Phylogenetic tree of Streptomyces bikiniensis (Kd8)"



Extraction of melanin

Extraction of melanin from *Streptomyces* was carried out by using Mohanasrinivasan method. The results of melanin produced from *Streptomyces* are shown in table.2.The amount of melanin produced by *Streptomyces* in Peptone yeast extract iron broth was 0.8gm/100 ml.

Table 2
Extraction of melanin

Production medium for Melanin	Weights	Weights in gm/100ml
Peptone yeast extract iron broth/100ml	Initial weight (Empty Petriplate)	39.22
	Total weight (Petriplate+ Pigment extracted)	40.02
	Difference (Weight of pigment)	0.8

Antibacterial activity of melanin from Streptomyces by using agar diffusion method

Antibacterial activity of crude extract melanin from *Streptomyces* was carried out by using agar diffusion method Table 3. The crude melanin produced from the *Streptomyces* showed antibacterial activity against all tested pathogen except *Proteus species*. Melanin showed highest zone diameter of inhibition against *Bacillus species* while least inhibitory zone diameter against *Escherichia coli*.

Table 3
Antibacterial activity of melanin

Sr.No.	Pathogen	Zone diameter (mm)
1	*Bacillus species*	29
2	*Staphylococcus aureus*	25
3	*Salmonella typhi*	23
4	*Escherichia coli*	22
5	*Proteus vulgaris*	*R

Where= Resistant

Mohanasrinivasan *et.al.,* [17] isolated pigment producing actinomycetes from a rhizosphere soil of ornamental plants and identified as *Streptomyces coelicolor MSIS1* (FR856603).The pigment was produced in shake flask as well as in bioreactor. The

results were evident that there was threefold increased in the pigment production in bioreactor compared with shake flask. The extracted pigment was characterized based on TLC, HPLC and FT-IR. The HPLC data showed that the compound may be one of actinorhodinic acid but on the other side FT-IR data infers that there were no presence of aromatic ring but prominent aliphatic stretch has been found. Vasanthabharathi *et.al.,*[19] studied three strains among 21 *Actinomycetes sp i*solates produced a diffusible dark pigment on starch casein an agar medium which was water soluble. 21.13 g/lit of crude melanin was extracted by a solvent extraction method with 1000ml of optimized production media at optimized condition. Therefore, this study was proved that sugarcane waste can be used for the production of melanin and it (melanin) has potential anti-bacterial activity. Jing *et.al.,*[20] studied high-level production of melanin by a novel isolate of *Streptomyces kathirae*. Forty-five bacterial strains that produced diffusive pigments were isolated from 40 soil samples. 13.7g/lit pigment production was from a *Streptomyces kathirae* strain designated SC-1.

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