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Full Length Research Paper

Seperation, identification and analysis of pigment (melanin) production in *Streptomyces*

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Nine strains among 180 *Streptomyces* isolates produce a diffusible dark brown pigment on both peptone-yeast extract agar and synthetic tyrosine-agar. They also show the positive reaction to L-tyrosine or L-dopa substrates. The pigment has been referred to be as merely as dark brown water-soluble pigment, as melanoid or melanin. The different carbon and nitrogen sources which influence the pigment production in the *Streptomyces* isolates were also investigated, and the carotenoid content in the pigment was analyzed. The melanin formation in the *Streptomyces* species is the key feature for the classification of the Stretomyces group.

Key words: Pigment, melanin, *Streptomyeces*, taxonomy.

INTRODUCTION

Actinomycetes also synthesizes and excrete dark pigments, melanin or melanoid, which are considered to be a useful criterion for taxonomical studies (Zonova, 1965; Arai and Mikami, 1972). Melanin compounds are irregular, dark brown polymers that are produced by various microorganisms by the fermentative oxidation, and have the radioprotective and antioxidant properties that can effectively protect the living organisms from ultraviolet radiation (Vinarov et al., 2002). Melanins are frequently used in medicine, pharmacology, and cosmetics preparations.

Biosynthesis of melanin with tyrosinase transform the tyrosine into L-DOPA (3, 4-dihydroxy phenyl-L- alanine), which is further converted into dopachrome and auto-oxidized to indol-5, 6-quinone. The later it is polymerized spontaneously into DOPA-melanin which gives dark brown pigment until the further examination (Mencher and Heim, 1962).

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MATERIALS AND METHOD

Isolation

Soil samples from different locations of the Gulbarga region of India were collected and screened for pigmented microorganisms (Grant et al, 1990)especially *Streptomyces* by applying standard serial dilution plate technique, using Starch casein nitrate agar and Glycerol asparagine agar (Cochrane, 1961). The plates were incubated at 28ºC for 3 to 4 weeks. After incubation, typically pigmented, dry, powdery colonies were selected from mixed plate culture and maintained on fresh medium to get pure cultures. The pure cultures were stored at 4ºC until further examination.

Characterization

Culture characterizations were determined according to the International *Streptomyces* project (ISP) (Shirling and Gottlieb, 1970). The general criteria used for Streptomyces identifications (Table 1) are morphology, aerial mycelium, substrate mycelium, spore morphology, production of diffusible pigments, production of melanin pigment, and utilization of various carbon and nitrogen sources (Armen et al, 2004:Gottlieb, 1961: Simon et al, 1999).

Melanin formation

Melanin formation was tested on peptone-yeast extract iron agar

Table 1. The physiology and biochemical characterization of *Streptomyces* isolates.

Isolates	DAS69	DAS123	DAS131	DAS135	DAS178	DAS143	DAS147	DAS165	DAS178
Pigmentation:									
Solid Medium	Dark Brown	Red	Red	Brown	Red	Red	Yellow	Yellow	Brown
Water Soluble	Brown	NP	Brown	Red	NP	Pink	Yellow	NP	Red
Reduction of nitrate	+	+	+	+	+	+	+	+	+
Catalase	+	--	+	--	+	+	+	+	+
Hydrolysis:									
Starch	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+
Utilization of:									
Mannitol	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+
Phenyl Alanine	+	+	+	+	+	+	+	+	+
Rhamonose	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+
Growth at									
Temperature-45^{O}C	+	+	+	+	+	+	+	+	+
PH - 12	+	+	+	+	+	+	+	+	+

and synthetic tyrosine agar. 10 µl of suitable liquid media were dispensed in test tubes and inoculated with one loop full of the spores of the *Streptomyces* and subjected to stationary stage at 27ºC for seven days. Melanin pigment was estimated by taking 2 ml of the culture and 1 ml of 0.4% substrate solution (L-tyrosine or L-dopa). The reaction mixture was incubated at 37ºC for 30 min for L-tyrosine and 5 min for L-Dopa and red coloration resulting from dopachrome formation was observed and read spectrophotometrically at 480 nm (UV-1601, Shimadzu). When there was no coloration within these periods, the reaction mixture was further incubated for as long as 2 h. After incubation melanin was then formed within 30 min (Scribners el al., 1973)

Effect of carbon sources on melanin formation

The basal medium of the following composition was used with 1% glycerol, starch, dextrin, lactose, sucrose, fructose or glucose as the sole carbon source; 2.0 g of NaNO3, 1.0 g of K2HPO4, 0.5 g of MgSO4.7H2O, 0.5 g of KCl, 0.01 g of FeSO4.7H2O in 1000 ml of distilled water (pH 7.2).

Effect of nitrogen sources on melanin formation

The effect of nitrogen sources (L-asparagine, L-arginine, L-citrulline, L-histidine, glycine, L-lysine, L-proline or L-tyrosine) on the pigment production (melanin) in *Streptomyces* was studied with same basal medium using 1 % of glycerol as the carbon source. All carbon and nitrogen sources were prepared in 10% solution, sterilized with bacteriological filters, and added to the basal medium to give final concentration of 1%.

RESULTS AND DISCUSSION

The effect of carbon sources on the pigment (melanin) production by the *Streptomyces* spp. is recorded in Table 2. Starch is the most effective carbon source for the production of melanin, followed by glycerol and fructose. The comparative efficiency of various nitrogen sources for the production of melanin by the *Streptomyces* is reported in Table 3. The amino acids like citrullin, argine, lysine and proline were found to be the most effective nitrogen sources. The comparative estimation of mycelial mass with the pigmentation was showed in Table 4 and Figure 1. Strain DAS 139 is most effective in producing the melanin pigment with the less mycelial growth (Figure 2).

The pigmentation of *Streptomyces* is distinct enough to allow ready delineation in most *Streptomyces* cultures when combined with other fundamental features, such as color of the surface aerial mycelium after sporulation, sporophore morphology and spore surface. Sometimes it is quite difficult to determine, whether the diffusible pigments produced are melanoid (dark brown) or merely a brown substance, especially when complex organic

Table 2. Effect of Carbon source on Melanin formation by *Streptomyces.*

Carbon Source	Isolates (OD at 480nm)								
	DAS69	DAS123	DAS131	DAS135	DAS178	DAS143	DAS147	DAS165	DAS139
Glycerol	0.165	0.147	0.158	0.141	0.149	0.176	0.163	0.226	0.251
Starch	0.172	0.153	0.162	0.153	0.154	0.192	0.172	0.251	0.281
Dextrin	0.092	0.110	0.097	0.104	0.118	0.121	0.091	0.181	0.110
Lactose	0.052	0.047	0.054	0.047	0.059	0.069	0.059	0.124	0.128
Sucrose	0.128	0.123	0.147	0.131	0.146	0.112	0.121	0.141	0.161
Fructose	0.167	0.152	0.158	0.149	0.152	0.171	0.162	0.201	0.148
Glucose	0.056	0.049	0.053	0.050	0.060	0.069	0.063	0.111	0.147

Table 3. Effect of Nitrogen source on Melanin formation of *Streptomyces*

Nitrogen Source	Isolates (OD at 480nm)								
	DAS69	DAS123	DAS131	DAS135	DAS178	DAS143	DAS147	DAS165	DAS139
L-Aspergine	0.141	0.138	0.113	0.123	0.142	0.109	0.128	0.143	0.137
L-Argine	0.487	0.471	0.462	0.455	0.432	0.481	0.492	0.511	0.531
L-Citrulline	0.671	0.632	0.651	0.649	0.638	0.642	0.647	0.654	0.661
L-Histidine	0.081	0.079	0.071	0.083	0.070	0.068	0.073	0.080	0.085
Glysine	0.071	0.069	0.061	0.073	0.068	0.059	0.063	0.069	0.073
L-Lysine	0.281	0.274	0.289	0.282	0.278	0.271	0.288	0.273	0.291
L-Proline	0.241	0.249	0.239	0.235	0.242	0.247	0.231	0.229	0.247
L-Tyrosine	0.088	0.083	0.089	0.073	0.077	0.081	0.085	0.083	0.071

Table 4. Comparison of Pigment production with the mass (mg) of the *Streptomyces* isolates

Isolates	Growth in gm	Pigmentation OD at 480 nm
DAS69	1.16	0.165
DAS123	0.88	0.121
DAS131	0.55	0.158
DAS135	0.93	0.124
DAS178	0.88	0.149
DAS143	1.03	0.176
DAS147	2.65	0.226
DAS165	1.16	0.173
DAS139	0.60	0.251

media is employed. The present study reveals that the method of testing melanin production by L-tyrosine or L-dopa as a substrate may be the good criterion for the identification and classification of *Streptomyces*.

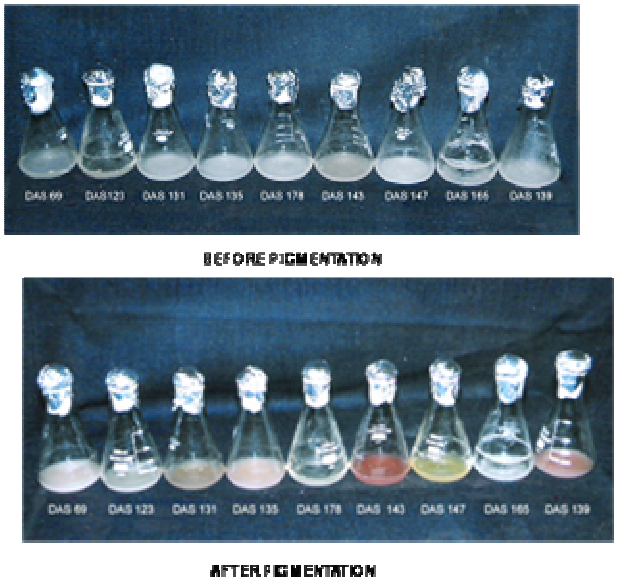

Figure 1. Pigments of the potential actinomycetes.

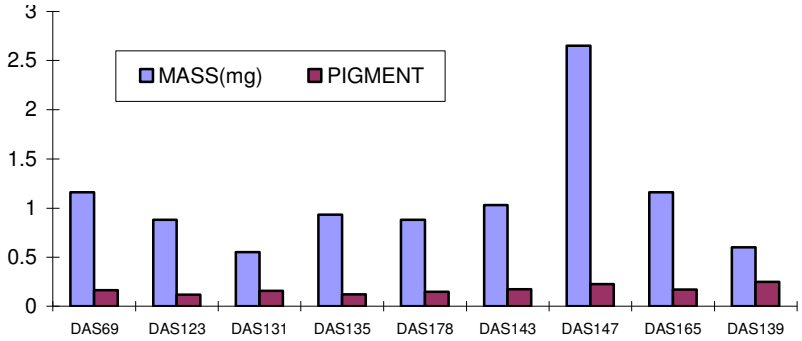

Figure 2. Schematic representation of Pigmentation versus growth of mass culture in mg.

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