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Screening and Evaluation of Melanogenic Enzyme Tyrosinase Producing Bacterium from Different Crop Soil

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ABSTRACT

The present study is focused on isolation and screening of tyrosinase enzyme produced by bacterial isolates and its application in the production of L-DOPA. 25 bacterial isolates have been screened which were collected from different crop soils. Based on primary screening, out of the 25 isolates the more potential tyrosinase producer was selected and identified by using 16S r-DNA sequencing. The work also dealt with growth study, enzyme activity, L-DOPA production, extraction of melanin pigment and chemical analysis. The pigment showed high absorbance in UV region and decreased when shifted towards the visible region. The melanin pigment was further characterized by FT-IR Spectroscopy.

Keywords: Tyrosinase, Melanin, L-DOPA, Pigment, Crop Soil.

INTRODUCTION

Soil contains a varying range of microflora including tyrosinase producing bacteria and fungi. Tyrosinase (EC 1. 14. 18. 1, monophenol, *O*-diphenol: oxygen oxidoreductase) is a copper-metalloprotein that is ubiquitously dispensed in nature. It is a bifunctional and a monooxygenase enzyme that catalyzes the *O*-hydroxylation and subsequent oxidation of monophenols, *O*-phenols, to quinones (Claus and Decker, 2006). Tyrosinase plays an important role in wound healing and the primary immune response of plant life, sponges and many invertebrates (Danial *et al.*, 2018; Decker and Tuczek 2000). In humans, tyrosinase produces melanin species as a defense against the harmful effects of UV light (Kumar *et al.*, 2011). The enzyme has a significant role in the medical sector, for example, the production of L-DOPA, a favored medication for the treatment of Parkinson's disease (Franciscon *et al.*, 2012). It is also used in the production of hydroxyl tyrosol as an estrogenic intermediate (Zhang *et al.*, 2007), food additives (Decker and Tuczek 2000), and melanin production for therapeutic applications (Pandey *et al.*, 1989). In plants, it is necessary to synthesize phenolic polymers such as tannins, lignin, and flavonoids. In sponges and many invertebrates, they are the major components of wound healing and the primary immune response (Cerenius *et al*., 2004; Muller *et al*., 2004; Van Gelder *et al*., 1997). This also involved in sclerotization of the

cuticle after molting or injury in arthropods. The bacterial origins of tyrosinases are known to be extracellular enzymes, involved in melanin production (Claus and Decker, 2006). In fungi, this enzyme has crucial importance in virulence and survives reproductive organ differentiation, protection of tissue after infection and spore formation (Seo *et al.*, 2003). In bacteria, tyrosinase enzyme is the key enzyme in initiating the melanin biosynthesis pathway and has vital roles in their protection.

Various organisms have been used for the production of tyrosinase enzymes on commercial scale, i. e. bacteria, fungi, and mushrooms, etc. *Streptomyces* species isolated from soil were also used for the *in vitro* production of the extracellular tyrosinase (Rao *et al.*, 2013). Due to multiple sources of tyrosinase, its structural properties are widely distributed in nature along with their distribution in tissues and cells, so no common protein is observed across all species (Mayer, 2006; Jaenickle and Decker, 2003). However, the extracellular bacterial tyrosinases do not have signal sequences, but their secretion is proposed to be assisted by a second protein having a signal sequence (Leu *et al*., 1992; Tsai and Lee, 1998). Compared to plant and fungal tyrosinases, the bacterial tyrosinases also have a shorter sequence, typically encoding amature protein of 30kDa. Common thing in all tyrosinases is their binuclear type 3, consist of two copper atoms in copper centre each connected with six histidine molecules in their active site (Ali *et al.*, 2017). The conversion of phenols to *o*-diphenols by tyrosinase is a potentially attractive catalytic ability (Hearing, 1987). It also plays an important role in the formation of melanin pigment during melanogenesis in melanocytes which are located at the epidermal junction and it is present in these cells that originate from the embryonic neural crest and is responsible for the synthesis of melanin (Kamal *et al.*, 2014). Through oxidation it catalyzes the production of melanin and other pigment from tyrosine (Shepherd *et al.*, 2015). In bacteria it is widely present in *Streptomyces glaucescens* and in fungal species like *Agaricusbisporus* and *Neurosporacrassa* (Chang, 2009).

The first bacterial tyrosinases have been purified from cell extracts of *Streptomycesnigrifaciens* (Nambudiri, 1972) and *Streptomyces glaucescens* (Lerch, 1972). The common features of *streptomyces* species is to synthesis melanin or melanin like pigments (Kuster, 1976). The melanin pigments consist of two types which produced from melanocytes they are eumelanin (black or brown melanin) and pheomelanin (red or yellow melanin (Summers, 2006). The catalization of two different enzymatic reactions occurs in tyrosinase by using molecular oxygen (Decker *et al.*, 2001; Land *et al.*, 2003; Lerch 1995; Solomon *et al*, 1996; Van Gelder *et al.*, 1997). Tyrosine is first oxidized to dipoquinone which in presence either react with cysteine to give a precursor on or reddish brown pheomelanin or cyclase to give a dihydroxyindole precursor of black or brown eumelanin (Mason, 1984). In the earth, most of the living organisms tend to produce melanin which is a universal (but at the same time enigmatic) adaptation (Wood, *et al.*, 1999).

During the process of biosynthezation of melanin, the monophenols or cersolase activity of tyrosinase enzyme can catalyses substrate tyrosine into 3, 4-dihydroxyphenylalanin or DOPA

(o-diphenol) by ortho-hydroxylation reaction. Diphenols and quinones are formed by the combining such subtrates like phenols and diphenols with tyrosine and L-DOPA (Robb, 1984; Whitaker, 1995). L-DOPA which is an amino acid used as drug to treat parkinson's disease (Surwase *et al.*, 2012). Mosanto first developed the asymmetric hydrogenation method for the synthesis of L-Dopa which showed many limitations such as poor conversion rate, low enantioselectivity and high production cost (Min, *et al.*, 2015). The present study dealt with isolation and identification of tyrosinase-producing bacteria from different crop soils, optimization of the substrate concentration for increased production of tyrosinase, growth study, Tyrosinaseactivity, L-DOPA production, Pigmentextraction (Melanin), Chemicalanalysis, UV-visible and FT-IR Spectroscopy of the pigment.

MATERIALS AND METHODS

Isolation of Bacteria Soil samples are collected from the regions around Sankarankovil, Tirunelveli district for the isolation of bacteria producing tyrosinase enzyme. The bacteria were isolated by serial dilution and pour plate method on mineral salt medium composed of $K_2HPO_4$173 g/L, $MgSO_4$. $7H_2O$ 0. 1 g/L, NaCl 4 g/L, $FeSO_4$. $7H_2O$ 0. 3 g/L, $NH_4NO_3$1 g/L, $CaCl_2$. $2H_2O$ 0. 02 g/L, $C_6H_{12}O_6$ 5 g/L, Agar 15 g/L, Peptone 5 g/L and Yeast1. 5 g/L and the plates were incubated at room temperature for 24 h. All tyrosinase producing isolates were maintained on nutrient agar slants and stored at 4º C for further use.

Primary Screening

The primary screening was carried in tyrosine medium for all the isolated bacteria on nutrient agar medium containing NaCl 5 g/L, Beef extract 3 g/L, Peptone 5 g/L, agar 20 g/L supplemented with 1g/L L- tyrosine. All the isolated bacteria were streaked into plates containing tyrosine agar medium and incubated at room temperature for 72 h. The colonies that are grown produce a brown pigment around it. The pigmented colonies are gradually changed its colour to black, which indicates the presence of tyrosinase positive organism. The tyrosinase producing colonies were purified by further repeated quadrant streaking on agar plates containing nutrient agar medium and maintained.

Identification of Isolate

The isolated bacterium was identified by 16SrDNA sequencing. The 16SrDNA sequence was compared with sequences available in public databases, using the BLAST search program on the on the NCBI web site (http://www.ncbi.nlm.nih.gov) to find closely related bacterial 16Sr DNA gene sequences. A phylogenetic tree was constructed by the neighbor -joining method and maximum composite likelihood model.

Growth Study

L- Tyrosinase producing strains were inoculated in flasks containing 50mL nutrient broth and kept at room temperature for 96 h. The culture was harvested at every 6 hour interval for a

period of 96 h. Growth kinetics was obtained by measuring the cell density at 660nm at different intervals.

Tyrosinase Production

The isolated strain was inoculated in Mineral Salt Broth (MSB) with the enhancer (L-Tyrosine) and was incubated at 37°C. At every 6 h of incubation, culture was collected and observations were made. Cell free crude enzyme was prepared by centrifugation at 5000rpm for 10 minutes. To 1mL of supernatant, 0. 5mL of L-DOPA and 1. 5mL of potassium phosphate buffer (pH 6. 5) was added and the absorbance was determined at 475nm using a UV/VIS spectrophotometer.

L-Dopa Production

The L-DOPA assay was determined according to Arnow's method (Arnow, 1937). To 1 ml of supernatant, 1ml of 0. 5NHCl, 1ml of nitrite molybdate reagent and 1ml of 1N NaOH was added and the final volume was adjusted to 5ml with distilled water. The absorbance was measured at 530 nm and the concentration of L- Dopa was determined from Arnow standard curve of L- Dopa.

Screening of Bacteria Capable of Producing Melanin Pigment

The selected strain was grown in 100 ml Erlenmeyer flasks containing 50 ml of minimal medium with or without 0. 1% tyrosine and incubated at room temperature for 96 h. Inoculated culture flasks without L- Tyrosine as well as uninoculated flasks containing L-Tyrosine served as controls. Thereafter, the culture was collected by centrifugation at 8000 xg. For L-Tyrosine dependent pigment production assay, different concentrations of L-tyrosine were supplemented to the above mineral salt media (125, 250, 375, 500, 625, 750, 875, 1000 µg/ml).

Pigment Extraction

The bacteria that grown in minimal slat medium supplemented with tyrosine broth produced black colour pigment. This cell suspension was disrupted by using a sonicator (ULTRASONIC PROBE SONICATOR 20Khz/ 120w) in an ice bath at a normal power of 70W to disrupt the cell for 3 min period, each period of disruption was of 15 s followed by 1 min off for which the medium and oscillator probe to be cooled in ice. The disrupted cell was then acidified with 1N HCl to pH 2. The acidified cells are kept under room temperature for a period of 1 week. Then this suspension was boiled for 1hr to prevent formation of melanoidins and then centrifuged at 8000 rpm for 10 min. The formed black pigment pellet was washed three times with 15ml of 0. 1N HCl and then with distilled water. To this pellet, 10ml of ethanol was added and the mixture then incubated in a boiling water bath for 10 min and then kept at room temperature for 1 day. The pellet was washed 2 times with ethanol and

then air dried. The pellets of the extracted pigment were pooled for use in subsequent analysis.

Chemical Analysis of the Pigment

The chemical analysis of melanin pigment was carried out by the modified method of Fava *et al*., 1993. The solubilities of the blackpigment in distilled water, 1 N HCl, 1 N NaOH, ethanol, acetone, chloroform, benzene, and phenol were checked. Reactions with oxidizing agent viz., 30% hydrogen peroxide (H_2O_2) was also determined.

UV Visible Spectroscopic analysis of the Extracted Pigment

The partially purified melanin was prepared using 0. 1N NaOH and the alkaline solution was scanned from 180 to 900nm wavelengths. A 0. 1N NaOH was used as blank (Wenlin *et al*, 2007).

FTIR Spectroscopic Studies

The purified melanin pigment was subjected for FTIR Spectroscopic analysis. The FT-IR spectrum was recorded at 4000 to 400cm^{-1}using a Perkin Elmer spectrophotometer.

RESULTS AND DISCUSSION

Soil is a source of indigenous bacteria therefore, in this study, for the isolation of tyrosinase producing bacterial strains we have collected soil samples from different agricultural soils.

Isolation of Bacteria

Based on various morphological characteristics, a total of 25 isolates were obtained from different soil environments by serial dilution and pour plate method. These 25 isolates were allowed for primary screening. (PLATE 1)

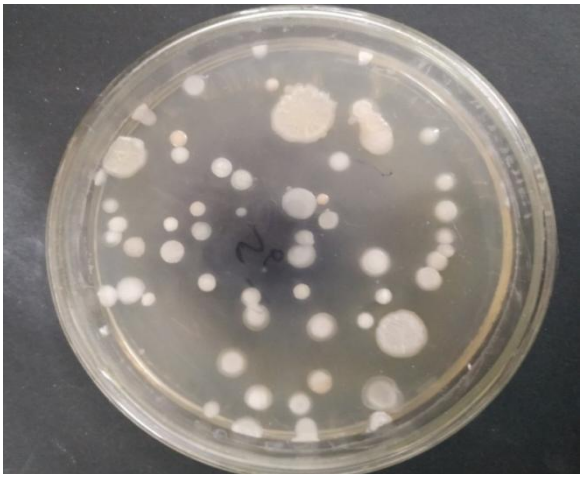

Plate 1.Shows Isolation of Bacteria by Serial Dilution and Pour Plate Method

Tyrosinase Producing Bacteria

Out of 25 isolates, the strain S6 showed more melanin pigment due to presence of tyrosine compared to the other strains. This isolate produced blackish brown pigmentation on tyrosinase agar medium, which indicates melanin production (PLATE 2).

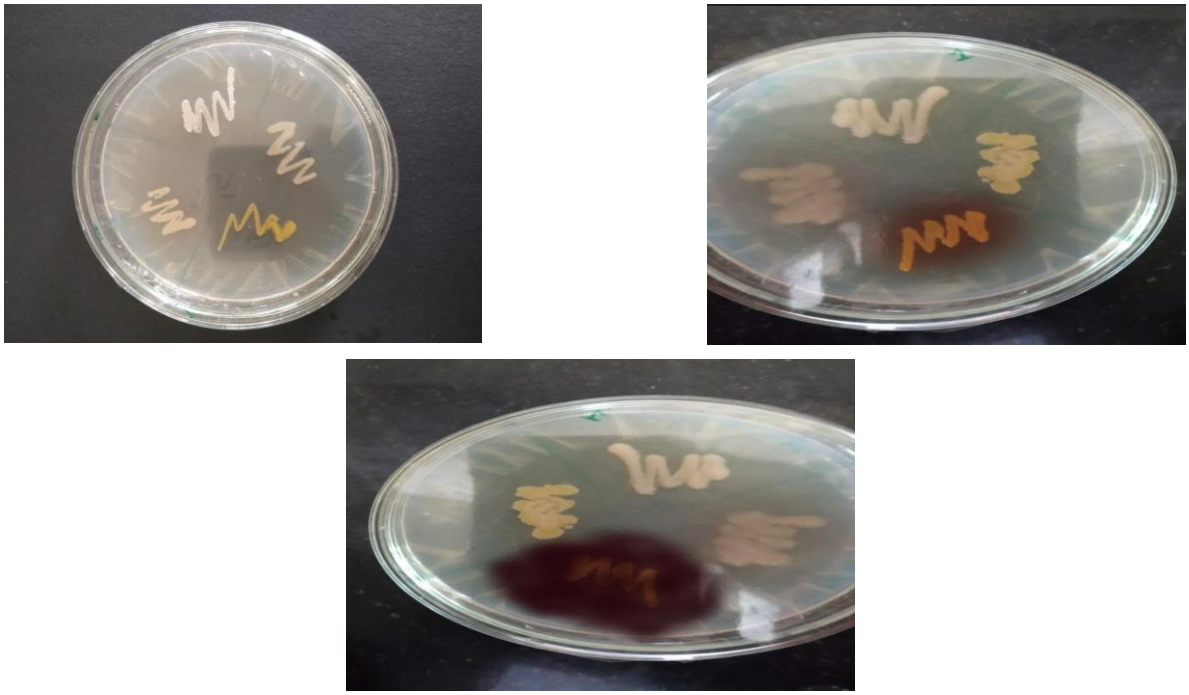

Plate 2.Shows Primary Screening in Tyrosine Medium

Similarly, (Sharma *et al*.,**)** also isolated 21 isolates of Actinomycetes and used in tyrosine agar medium for primary screening of tyrosinase enzyme and also reported that, the formation of brown colour was mainly due to melanin (Raval *et al*., 2012). The tyrosinase producing strain S6 shows more melanin pigment due to presence of tyrosine compared to the other strains. After primary screening, these isolates were further checked for extracellular tyrosinase activity. Among 25isolates the strain S6 showed maximum tyrosinase activity.

Purification of Bacteria

Based on primary screening the most potent strain was selected and purified by quadrant streaking method. Individual colonies were obtained and selected for further studies (PLATE 3).

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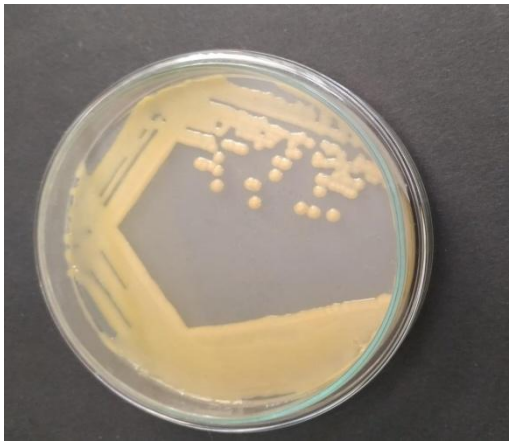

Plate 3.Shows Purification of Culture by Quadrant Streaking Method

Identification

16S rDNA Sequencing

The isolatedstrain was then identified through 16S rDNA sequencing. Strain S6 Showed 99% homology with *Chryseobacterium cucumeris*. All the 16S rDNA sequences of related strains have been retrieved from the NCBI database. Genome accession numbers are shown in parenthesis: 0. 005 denotes the genetic distance. The phylogenetic relationship of the strain S6 is shown in (FIG. 1)

Phylogenetic analysis

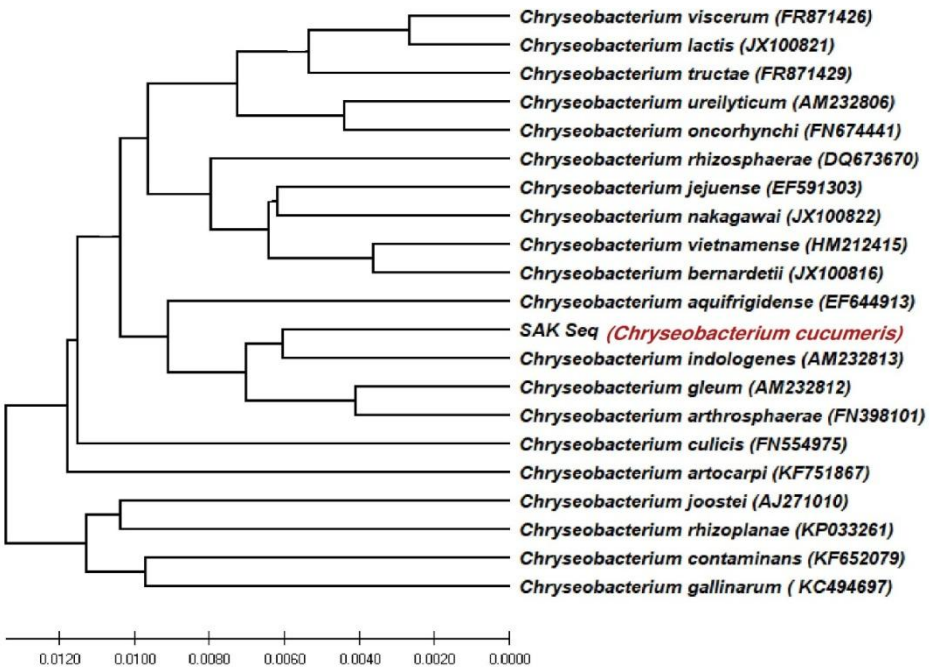

Figure 1.Phylogenetic Tree Showing the Position of Isolate S6 with Reference to Related Strains

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Growth Study and Tyrosinase Production

The growth of the isolated strain *Chryseobacterium cucumeris* was studied for every 24 hr over a period of 168hr of incubation at the absorbance of 660nm. It showed the maximum growth at72hr of incubation, after which the growth declined (FIG 2).

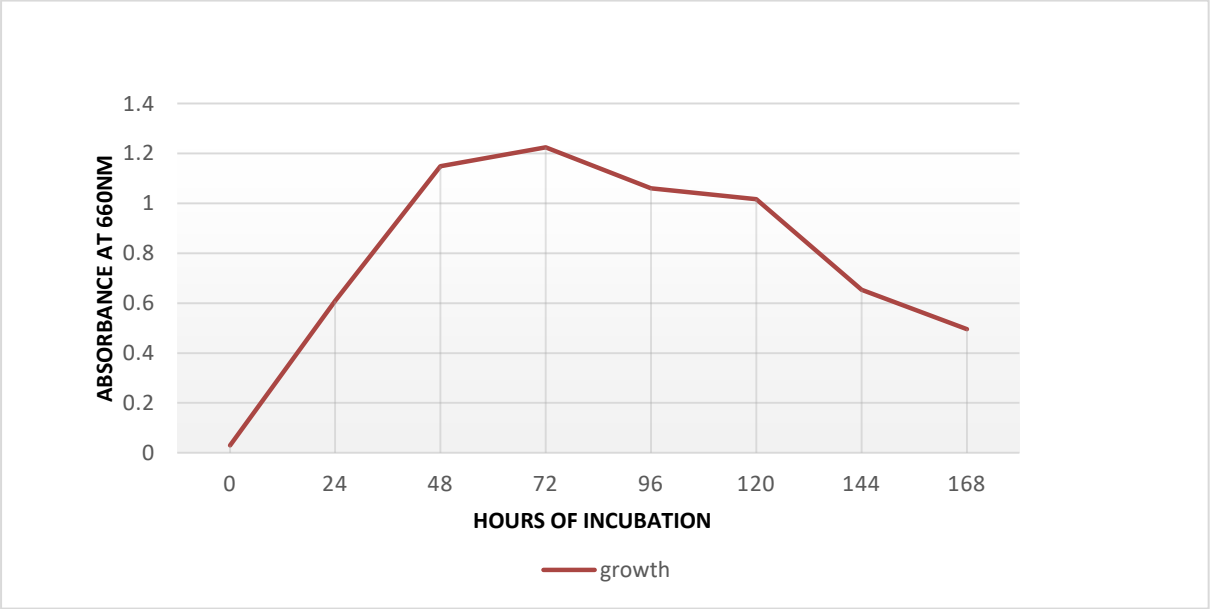

Figure 2.Shows Growth Curve

Tyrosinase assay was carried out to check the activity of tyrosinase enzyme at an absorbance of 475 nm. The production of tyrosinase was maximum 0. 7396mg/ml at 120hrs of incubation. The tyrosinase production was seen in the declining phase of the growth (FIGURE 3).

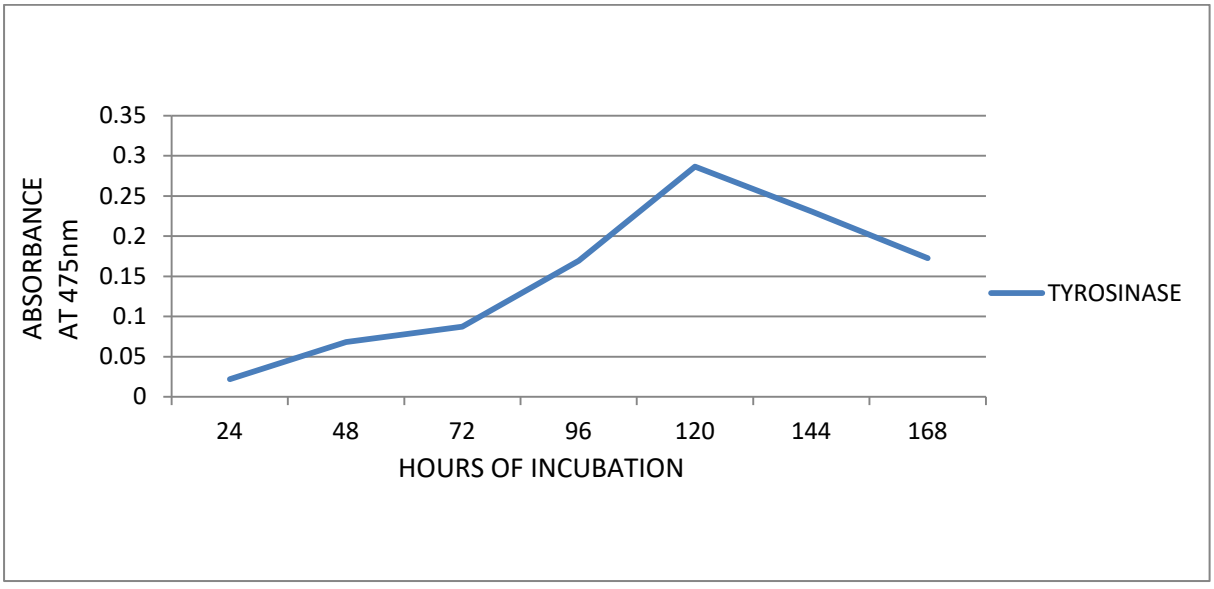

Figure 3.Shows Production of Tyrosinase at Every 24hrs of Incubation

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Tyrosinse Assay

The enzyme activity was conducted at different concentrations of tyrosinase ranging from 0. 1 to 1 (mg/ml) using and Arnow's method at an absorbance of 475 nm for every 24 hr. The present study showed a maximum production of 0. 528 mg/ml of enzyme activity for 0. 6 mg/ mlconcentration at 120hrs (FIGURE 4)

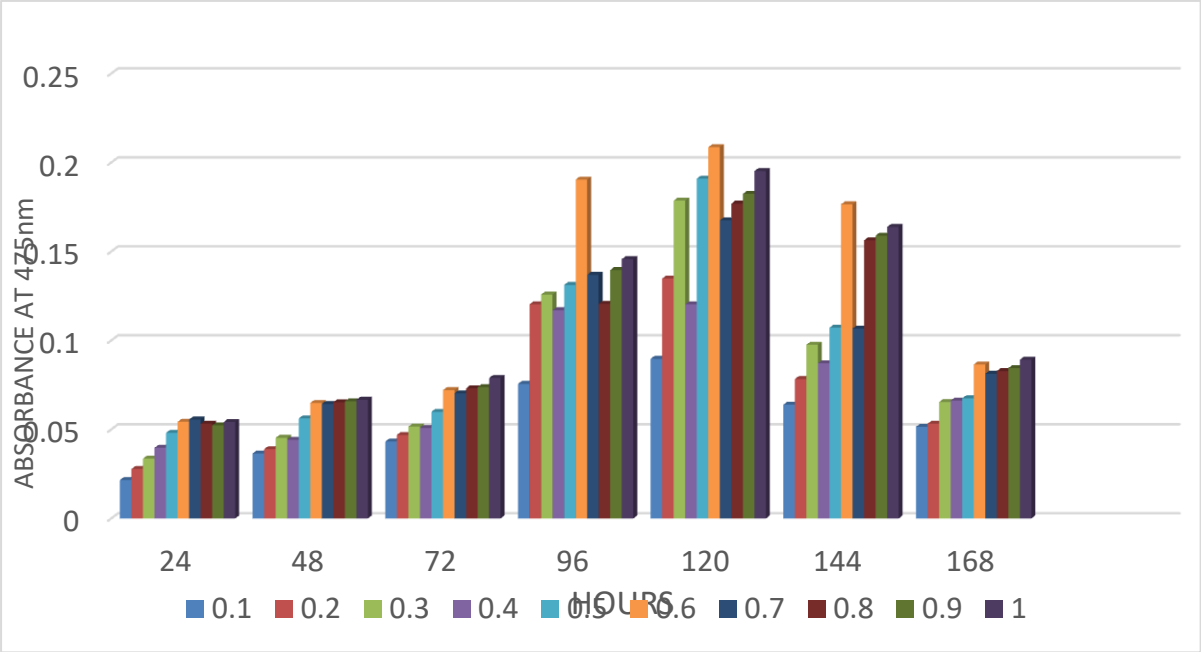

Figure 4.Shows Enzyme Assay at Different Substrate Concentration

L-DOPA Assay

The L-DOPA assay was done at an absorbance of 530 nm for every 24 hrs of incubation. In the present study the L-DOPA production was seen maximum (0. 558 mg/ml) at 144 hrs of incubation. L DOPA is a compound of clinical importance, which is produced only after the production of tyrosinase. This indicates that the production of L-DOPA is a result of tyrosinase activity (FIGURE 5).

Tyrosinase (1. 14. 18. 1) is a copper containing enzyme and has two catalytic activities. It catalyzes orthohydroxylation of monophenols to diphenols by cresolase activity. It also successively oxidizes diphenols to quinone by catecholase activity (Min and Yoo, 2009). Accordingly, based on tyrosinase activity L-Dopa can be produced by o- hydroxylation of L-Tyrosine by cresolase activity (Min and Yoo, 2010) therefore in the present investigation bacterial isolates which exhibited maximum tyrosinase activity were further assayed for L-Dopa production. Previously, Surwase, *et al.*, for the first time reported that the bacterial strain has the ability to utilize L-Tyrosine for producing L-Dopa. They isolated *Bacillus* sp. JPJ & *Brevundimonas*sp. SGJ having the ability to convert L-Tyrosine to L-Dopa from soil samples which can be more favorable for industrial fermentation than plant, fungi and yeast.

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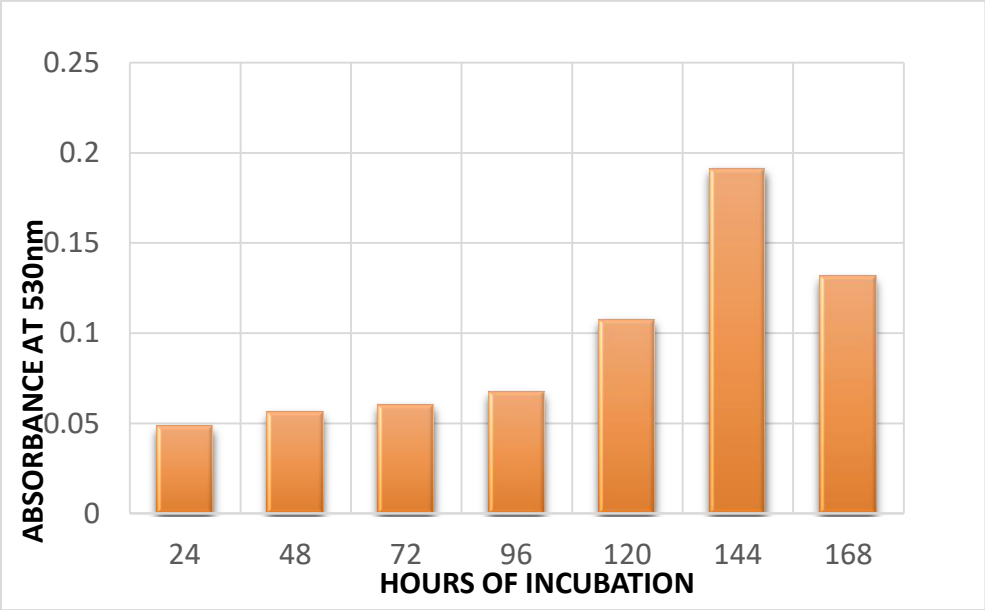

Figure 5.Shows Production of L-Dopa

In Vitro Melanization

In vitro melanization assay was performed to determine whether the pigment is produced from L-tyrosine. In the present study it was found that the tyrosinase producing bacterium *Chryseobacterium cucumber is* does not produce melanin pigment inliquid medium lacking L-tyrosine. Pigment production w as not observed in control flasks, indicating that autooxidation of chemical constituents in the medium does not likely cause any pigmentation (PLATE 4).

Plate 4.Shows Tyrosine Broth Culture

Increase in the growth of the bacterium increased the utilization of L-tyrosine and the pigment production. The maximum growth of the bacterium was observed at 3 days of incubation along with the maximum amount of melanin (1mg/l) production and the concentration of pigment was dependent on the L-Tyrosine concentration (500µg). The maximum utilization of Ltyrosine was observed after 5days of incubation. It was also found that melanin production was initiated after 18 dayswhen the culture entered into decline phase, which showed that the maximum L-tyrosine was utilized before its polymerization into melanin.

Pigment Extraction and Chemical Analysis

The black pigment was extracted by vibrating the cell using sonicator followed by acidification. Then they were separated and washed thoroughly. After that the pigment was used for various studies. The chemical analysis of the melanin pigment is summarized in TABLE 1.

Table 1.Chemical Properties of Melanin Pigment

Serial No.	Test	Result
1	Water	Insoluble
2	Solubility in organic solvents	
	a) Ethanol	Insoluble
	b) Chloroform	Insoluble
	c) Acetone	Insoluble
	d) Benzene	Insoluble
	e) Phenol	Soluble
3	1M KOH/NaOH	Soluble
4	Color	Blackish brown
5	Precipitation in 3 N HCl	Precipitated readily
6	Reaction with oxidizing agent (H_2O_2)	Decolorized (black to colorless)
7	UV visible absorption	Linear relationship between log absorbance and wavelength between 400 and 600 nm

It was observed that the pigment produced by *Chryseobacterium cucumeris*was shown to be a true melanin, as revealed by a number of physical and chemical tests. These tests indicate that the pigment is likely to be a melanin pigment, as observed for the melanin isolated from other bacteria such as *Aeromonas media* (Lewis *et al*., 1998, Vernon *et al*., 1992) and *Escherichia coli* (Christine *et al*, 2008; Huang *et al*.,2009) and fungi *Cryptococcus neoformans* (Gibello *et al*., 1997) and *Pleurotuscystidiosus* (Selvakumar *et al*., 2008). The *Chryseobacterium cucumber is* produced a dark brown pigment in culture medium as a dead-end product. It is

reported that microbes predominantly produce melanin pigment via tyrosinases, laccases, catecholases, and the polyketide synthase pathway (Carlos *et al*., 2008).

UV-Visible Spectroscopy Analysis

The UV-Visible spectroscopy analysis was done at an absorbance of (180 to 900nm) for the extracted melanin pigment (FIGURE 6). It shows maximum absorbance in the UV region 200-350 nm,with the peak at 257. 6 but decreased towards the visible region. This phenomenon is due to the presence of the complex conjugated structure in melanin. The increase in wavelength, decreases the absorbance of melanin pigment.

This propertyof melanin is confirmed by comparing with the previous descriptions of melanin pigment and measurements of synthetic melanin (Rosas *et al*., 2000, Schaefer *et al*., 1953, Sajjan *et al*., 2010, Vernon *et al*., 1992). An increase in wavelength decreases the absorbance of melanin pigment progressively. Hence, the slopes of linear plots are often used to identify melanin pigments. There was a linear relationship between log absorbance and wavelength from 400 to 600 nm, which is one of the most important criteria for the characterization of melanin, Schaefer *et al*., 1953showed that the log of optical density of a melanin solution, when plotted against wavelength, produces a linear curve with negative slopes. Such characteristic straight lines with negative slopes have been obtained for melanin produced by some fungi (Ravishankar *et al*., 1995, Selvakumar *et al*., 2008).

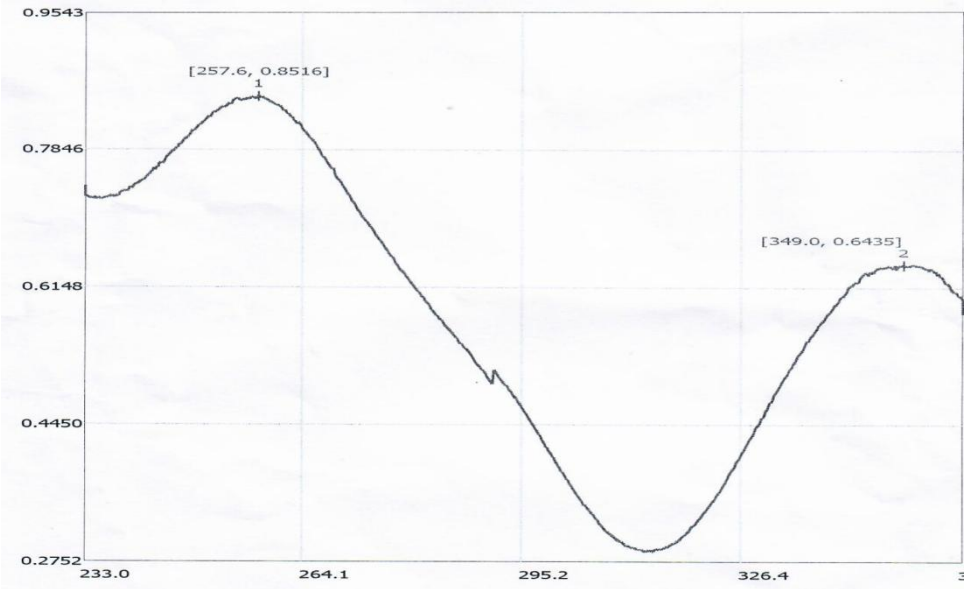

Figure 6.Uv-Visible Spectroscopy

FT- IR Spectroscopy Analysis

FT-IR spectroscopy was chosen for further characterization of the pigment, since it is regarded as the most informative, well-resolved, and non-destructive method, providing information on various functional groups and detailed structural analysis of melanin (Pierce

et al., 1995). FIG. 7 **s**hows the result of FTIR Spectroscopy with various peak values. The IR spectrum of the melanin pigment showed a broad absorption at 3,397. 3 cm^{-1}, which revealed the presence of the -OH group. The broadening of the band might be due to the hydrogen bonding of the-OH group with the -NH group. The peak occurred at 2,930 cm-1, which indicates as -CH. Absorption at 1,627. 73 cm-1 was attributed to aromatic ring C=C stretching. These characteristic properties of the IR spectrum of this pigment were similar to earlier reports (Hoti *et al*., 1993, Selvakumar *et al*., 2008).

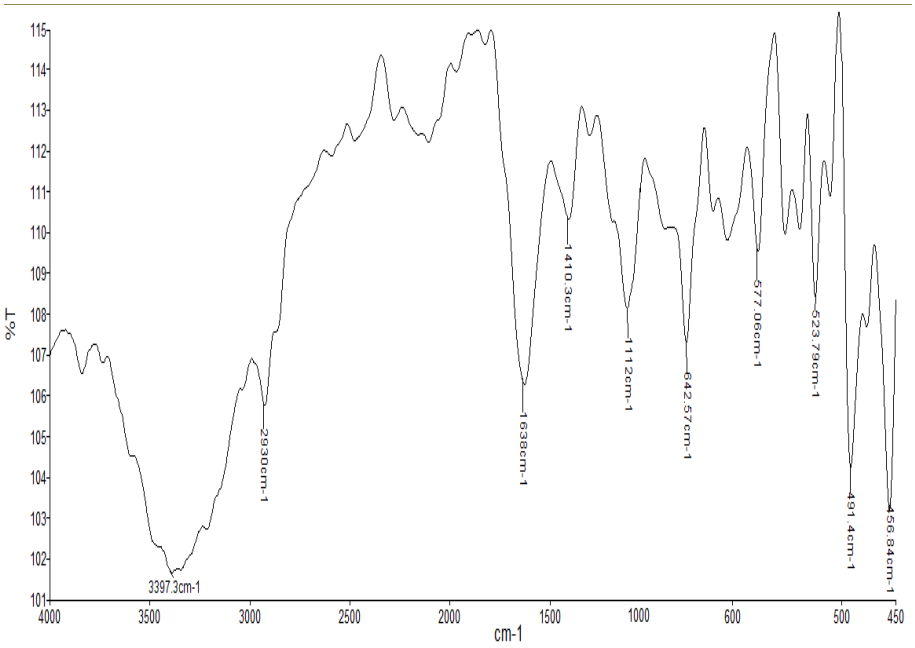

Figure 7.FT-IR Spectroscopy Analysis of the Melanin Pigment Extracted from *Chryseobacterium Cucumeris*

CONCLUSION

The present work was carried out to isolate and identify a potent tyrosinase producing strain from soil. The most tyrosinase producing isolate was identified by 16S rDNA sequencing as *Chryseobacterium cucumeris* and was designated as SAK. The isolated strain showed 99% similarity with *Chryseobacterium cucumeris* using BLAST analysis.

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