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Green Pigment from *Bacillus cereus* M^1_{16} (MTCC 5521): Production Parameters and Antibacterial Activity

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Abstract A bacterial strain, *Bacillus cereus* M^1_{16} (MTCC 5521), isolated and identified in our laboratory produces a green pigment when grown in nutrient broth at stationary condition. Optimum fermentation parameters for maximum pigment production are pH 7.0, temperature 30°C, time of incubation 72 h and inoculum volume 1% from 20 h grown cell suspension. Magnesium ion enhances pigment production whereas calcium and zinc ions inhibit the process. The pigment is better extracted from the fermented broth with chloroform in comparison with diethyl ether, ethyl acetate, and butanol. The extracted crude pigment consists of three fractions as revealed from thin layer chromatogram on silica gel GF254 using ethyl acetate and hexane (1:1) solvent system. The major fraction C_3 shows antibacterial activity against different gram positive bacteria. The proposed structure of C_3 is 9-methyl-1,4,5,8-tetra-azaphenanthrene obtained by elemental analysis, GC-MS, and NMR spectra studies.

Keywords Green pigment · *Bacillus cereus* · Antibacterial activity · Production · Chemical structure

Introduction

Color is the most pleasing attribute of any article and hence it contributes to eye appeal of any marketable commodity. A well-textured food, rich in nutrients and flavor cannot be accepted by general public unless it has the right color. Natural colors are being

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added in food from earlier times, but with increase in demand, synthetic colors like coal-tar dyes were developed and dominated the natural colors [1]. But due to the consumer awareness and concern for healthful and completely balanced food an increasing interest in the food colorants of natural origin has been developed as many of the synthetic colors have been proved to be health hazards and even carcinogenic to humans [1].

At present, fibers are dyed mainly with synthetic dyes. However, natural pigments are still valuable for their natural color tones and people's strong likings towards natural colors. Before the invention of synthetic dyes in the nineteenth century, many of the colors used to dye textiles came from plants. Nature is rich in colors (minerals, plants etc.) and pigment producing microorganisms (fungi, yeast and bacteria). Microbial pigments such as carotenoids, melanins, flavins, and quinines are used in many food industries [2]. The ability to produce pigments varies with the organism and its environment. Cytochrome pigments produced by *Bacillus cereus* T spores was reported way back in 1974 [3]. Phenazine pigments from *Pseudomonas aeruginosa* and their application as an antibacterial agent and food colorants had been investigated by Jayalakshmi et al. in 2008 [4]. Many microorganisms produce pigments during their growth which are substantives as indicated by the permanent staining that is often associated with mildew growth on textiles and plastics [5]. Often microorganisms produce 30% of their dry weight as pigments [5]. An improved method for extraction of β-carotene from *Blakeslea trispora* was developed by Roukas et al. in 2001 [6]. Pigment production often depends on growth and effect of light. The produced pigment may be intracellular or extracellular [7]. Microorganisms would therefore seem to offer great potential for the direct production of novel textile dyes or dye intermediates by controlled fermentation techniques replacing chemical synthesis which has inherent waste disposal problems (e.g., toxic heavy metal compounds). Previously extraction of pigments has been carried out from *Monascus*, *Bacillus*, *Rhodotorula*, *Achromobacter*, *Yarrowia*, *Phaffia*, etc. in trial basis by several scientists and currently five productions are operated in an industrial scale [1, 2, 8].

This manuscript deals with the fermentative production and purification of a green pigment by *B. cereus* M^1_{16} (MTCC 5521), isolated, identified and patented in our department [9].

Materials and Methods

Microorganism

The bacterial strain *B. cereus* M^1_{16} (MTCC 5521) isolated and identified [9] in our laboratory was used in the present study. The organism was maintained by subculturing at a regular interval of time (30 days) on nutrient agar medium (g/L), peptone: 5.0, beef extract: 1.0, yeast extract: 2.0, sodium chloride: 5.0 and agar: 15.0; pH 7.0.

Fermentation and Pigment Production

Composition of both inoculum and growth medium were same as maintenance medium except agar as described above. Inoculum was prepared by transferring one loopfull of cells from slant culture of *B. cereus* M^1_{16} (MTCC 5521) to 50 ml sterile medium in a 250-ml Erlenmeyer flask and incubated at 30°C for 24 h at 120 rpm. Ninety milliliters of growth

medium was inoculated with 1% *v*/*v* inoculum and incubated at 30°C for 72 h under stationary condition. Biomass was separated by centrifugation at 5,000 rpm for 15 min and the clear supernatant was used for the study of pigment production.

Estimation of Pigment

Concentration of pigment in the supernatant was determined by measuring the absorption at λ max (379 nm) using UV–Visible spectrophotometer (Hitachi, model U 2000) [10].

Extraction of Pigment from the Fermentation Broth

Extraction of the pigment from the fermentation broth was done by solvent extraction method. To each 50 ml of the fermented broth taken in different separating funnels, 25 ml of different solvents were added separately and shaken for several times. Solvent layer containing the pigment was collected and its concentration was estimated.

Fractionation of Crude Pigment

Thin layer chromatography [11] of the crude pigment extracted with chloroform was done using silica gel GF254 as absorbent and solvent system ethyl acetate: hexane (1:1).

Elemental Analysis

Elemental analysis of carbon, hydrogen, and nitrogen of the fraction C_3 was performed in 2400 series II CHN analyzer, Perkin Elmer.

Analysis of Purified Compound by GC-MS and NMR

The purified compound was subjected to GC-MS analysis using Thermo Trace GC Ultra coupled with Thermo Fisher DSQ II mass spectrometers with electron impact ionization at 70 eV to generate mass spectra. 30 m–0.25 mm Thermo TR50 column (polysiloxane column, coated with 50% methyl and 50% phenyl groups) was used for chromatographic separation of metabolites.

NMR spectra of the same compound in $CDCl_3$ was also carried out using Bruker Biospin Avance 400 MHz NMR spectrometer using a 5-mm broad band inverse probe head, equipped with shielded z-gradient accessories.

Test for Antimicrobial Activity

Antimicrobial spectrum of the dried fractions C_1, C_2, and C_3, obtained by TLC of the dried pigment extracted by chloroform from fermented broth was dissolved in DMSO and tested for antimicrobial activity by agar cup plate technique [12] using DMSO as a control against the test organisms viz. *Escherichia coli*, *Bacillus subtilis*, *B. cereus*, *Klebsiella pneumoniae*, *Micrococcus lutea*, *Salmonella typhi*, *P. aeruginosa*, *Staphylococcus aureus*, *Aspergillus niger*, and *Saccharomyces cerevisiae.* The bacteria were grown in nutrient agar medium consisting of (g/L), peptone: 5, NaCl: 5, yeast extract: 2 and beef extract: 1 (pH 7.0) and the yeasts and fungi were grown in Czapek–Dox agar medium [13].

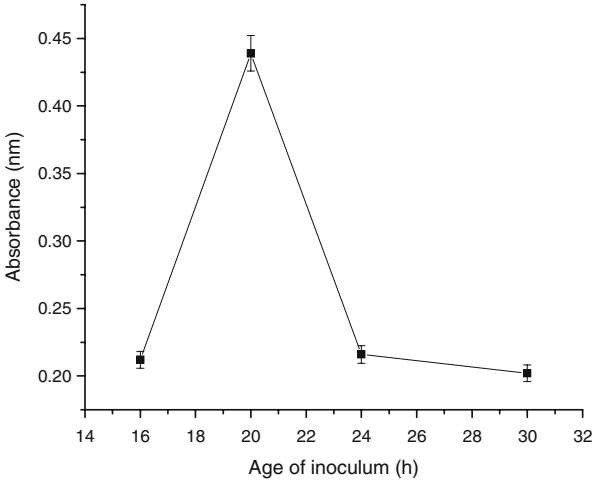

Fig. 1 Effect of age of inoculum on pigment production

Results and Discussion

Age and Inoculum Concentration

In order to optimize the age of inoculum, pigment production was carried out using cell suspension of the organism of different age (viz. 16, 20, 24, and 30 h) as inoculum (1%), other conditions remaining the same (Fig. 1). It was observed that maximum pigment production was achieved with a 20-h-old cell suspension. To study the effect of inoculum volume on pigment production, fermentation medium was inoculated with different volume of 20-h-old cell suspension (0.2–3.0%). It was observed that pigment production increased with increase in the percentage of inoculum up to 1% (Fig. 2) and further increase showed decrease in pigment production.

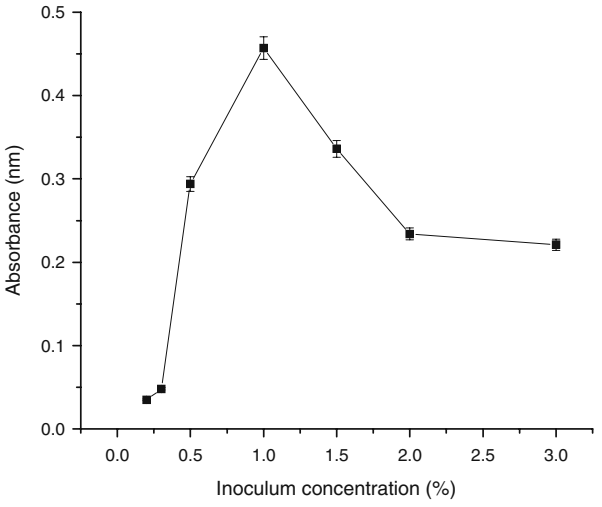

Fig. 2 Influence of inoculum concentration on pigment production

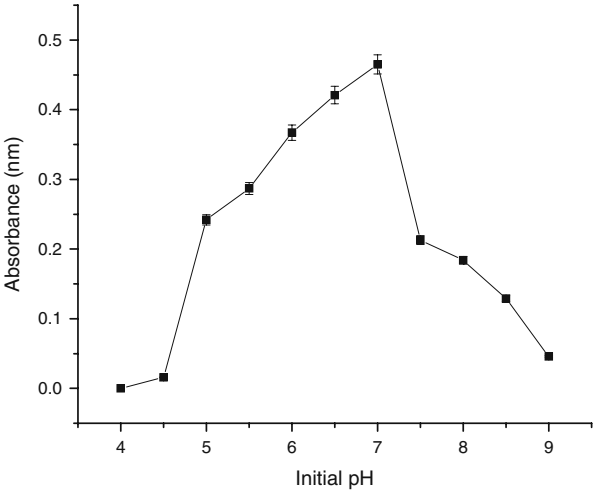

Fig. 3 Effect of initial pH of the medium on pigment production

Influence of pH and Temperature

To study the effect of pH on pigment production, 50 ml of the fermentation medium having pH values adjusted to 5.0–9.0 taken in a 250-ml Erlenmeyer flask was inoculated with 20-h-old cell suspension (1% *v*/*v*) and incubated at 30°C for 72 h at stationary condition. Production of pigment increased with increase in initial pH from 5.0 to 7.0 and then decreased sharply (Fig. 3). Effect of temperature on pigment production was studied over a temperature range of 20–45°C at pH 7.0, other conditions remaining the same. The results showed that 30°C was the optimum temperature for maximum pigment production (Fig. 4).

Period of Fermentation and Shaking Speed

Effect of incubation time on pigment production under above mentioned optimized conditions showed (Fig. 5) that the production was maximum after 72 h of fermentation.

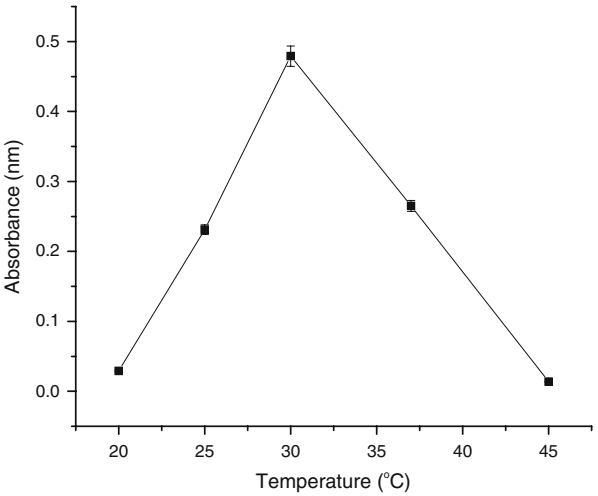

Fig. 4 Effect of temperature on pigment production

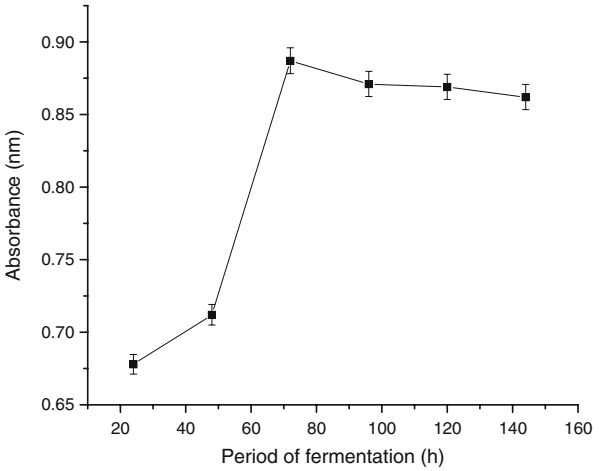

Fig. 5 Time course for pigment production

The fermentation was carried out as usual at stationary and also under shaking condition (60 to 120 rpm). Maximum production of pigment was observed at stationary condition (Fig. 6). This probably indicates that oxygen is inhibitory to the synthesis of this pigment.

Optimization of Volume of Medium

Fermentation experiment was conducted under optimum conditions using different volume of medium (40–125 ml) in a 250-ml Erlenmeyer flask. From the results (Fig. 7), it was observed that 90 ml medium in a 250-ml Erlenmeyer flask was found to be optimum showing the highest amount of pigment production. In the range of 50–100 ml liquid volume, the pigment production did not differ appreciably.

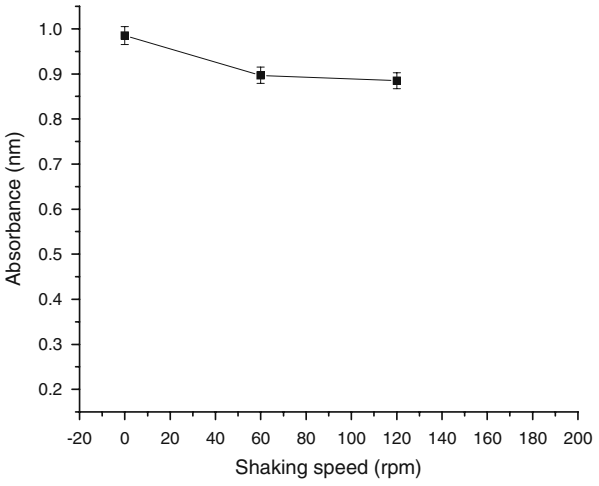

Fig. 6 Effect of shaking speed on pigment production

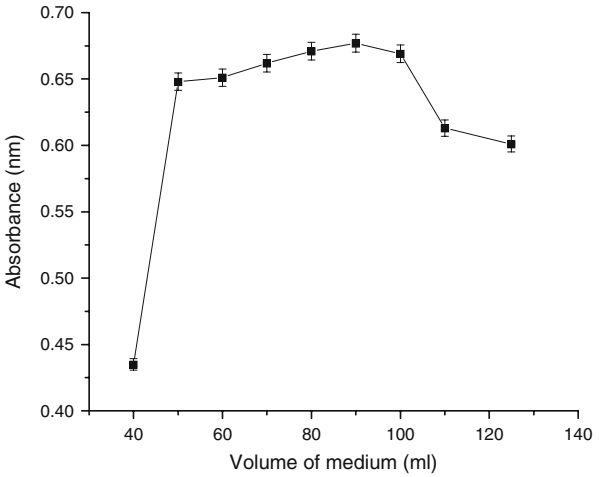

Fig. 7 Effect of volume of medium on pigment production

Effect of Medium Composition

To investigate the effect of medium composition six different media including the fermentation medium as described in Table 1 have been screened for pigment production. Fermentation was conducted with optimum conditions as described above. Medium-5 has been found to be the best for the pigment production in comparison to others (Fig. 8). In the second phase, concentration of the beef extract, peptone, and sodium chloride in the selected medium was varied to optimize their amount. Concentration of beef extract was varied (0.1–1%) keeping the concentrations of other ingredients constant. It was found that concentration of beef extract in the medium has profound influence on the pigment production and 0.9% was found to be optimum (Fig. 9). Concentration of peptone was varied (0.1–0.9%) while beef extract and sodium chloride concentration were kept at 0.9% and 0.5%, respectively. Peptone has been found to have a significant influence on pigment production, optimum being 0.4% (Fig. 9). Similarly, effect of sodium chloride (0.4–1%) on pigment production was studied in presence of 0.9% beef extract and 0.4% peptone. Like peptone, sodium chloride also has marked influence on pigment production and the optimum was found to be 0.7% (Fig. 9). Thus, it may be concluded that fermentation medium containing beef extract, peptone, and

Table 1 Composition of different media used for pigment production

Medium	Composition
Medium 1	$NH_4H_2PO_4$—0.1%, glucose—0.5%, NaCl—0.5%, $MgSO_4$ $7H_2O$—0.02%, K_2HPO_4—0.1%, $CaCO_3$—0.01%, pH—7
Medium 2	Peptone—1%, yeast extract—2%, Na_2HPO_4—0.7%, pH—7
Medium 3	Beef extract—0.1%, yeast extract—0.2%, NaCl—0.5%, peptone—0.5%, pH—7
Medium 4	Beef extract—0.3%, peptone—0.5%, pH—7
Medium 5	Beef extract—0.3%, peptone—0.5%, NaCl—0.5%, pH—7
Medium 6	Beef extract—0.3%, peptone—0.5%, glucose—0.2%, pH—7

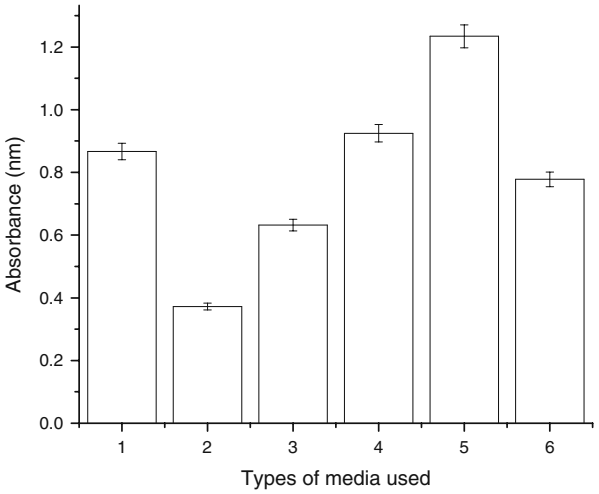

Fig. 8 Effect of different media on pigment production

sodium chloride in the concentration of 0.9%, 0.4%, and 0.7%, respectively, will be optimum for pigment production using *B. cereus* M^{1}_{16} (MTCC 5521).

Influence of Minerals and Phosphate on Pigment Production

To investigate the effect of minerals (viz. Na^{+1}, K^{+1}, Ca^{+2}, Fe^{+3}, Mg^{+2}, Zn^{+2}) and PO_4^{-3} on pigment production these were incorporated into the medium (range 0–1%) and fermentation was carried out under optimum conditions. In a typical experiment, the salt of the trace element under test was first omitted (if present in the basal medium) and then added to the medium in graded doses in separate flasks to determine its optimal concentration. In each subsequent experiment, the basal medium was so altered to include an optimal amount of a mineral (Fig. 10).

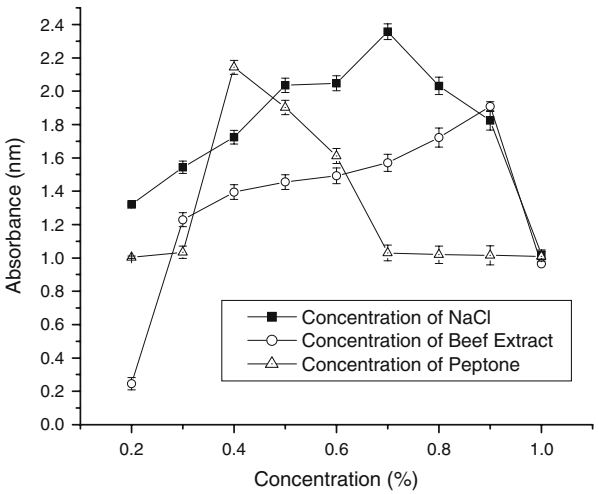

Fig. 9 Effect of concentration of beef extract, peptone and NaCl on pigment production

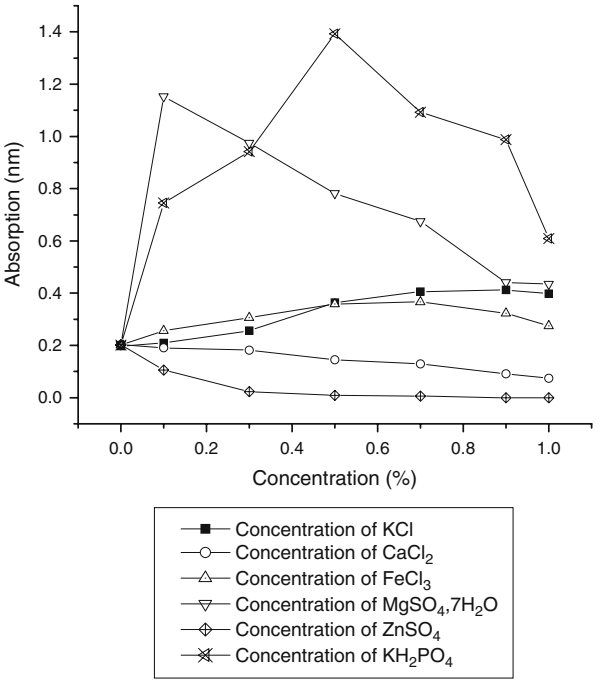

Fig. 10 Effect of minerals and phosphate on pigment production

It appeared from the results that pigment production is influenced by the concentration of KH_2PO_4 (0.5%), $MgSO_4,7H_2O$ (0.1%), and NaCl (0.7%). $FeCl_3$ and KCl have no significant effect on pigment production whereas $ZnSO_4,7H_2O$ and $CaCl_2,2H_2O$ have some inhibitory effect on pigment production.

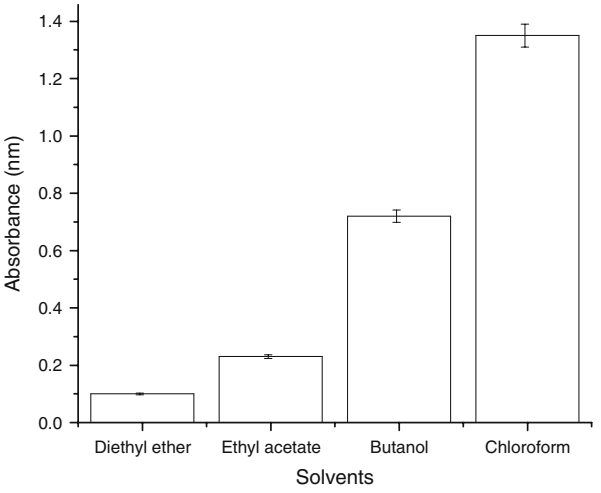

Fig. 11 Extraction capacity of pigment by different solvents

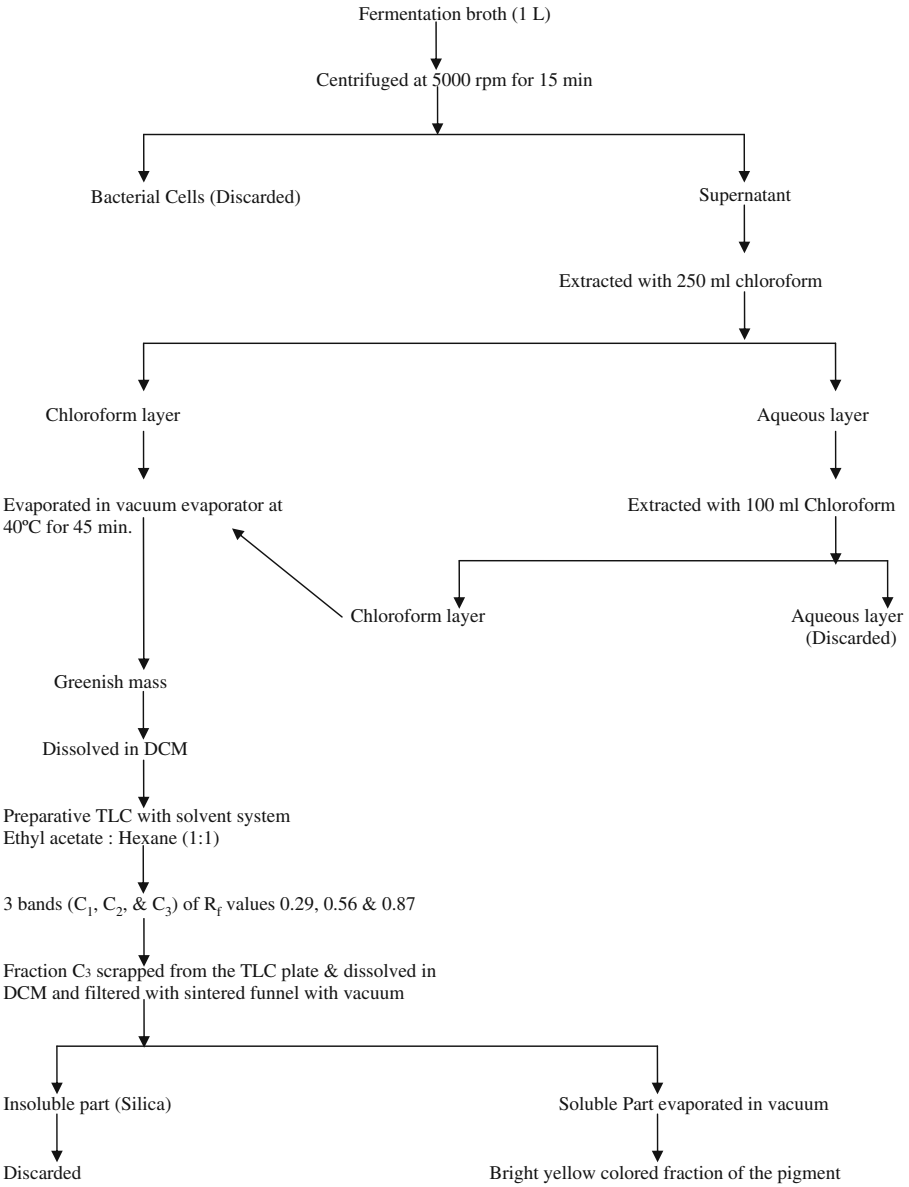

Fig. 12 The flow diagram of isolation of the pigment from fermentation broth

Extraction of Pigment

Different solvents such as diethyl ether, ethyl acetate, butanol, and chloroform were used to extract the pigment from the broth. Chloroform (1:1) has been found to be the most suitable solvent to extract the pigment (Fig. 11). The detail procedure for extraction and fractionation of the pigment are shown in the flow-sheet (Fig. 12).

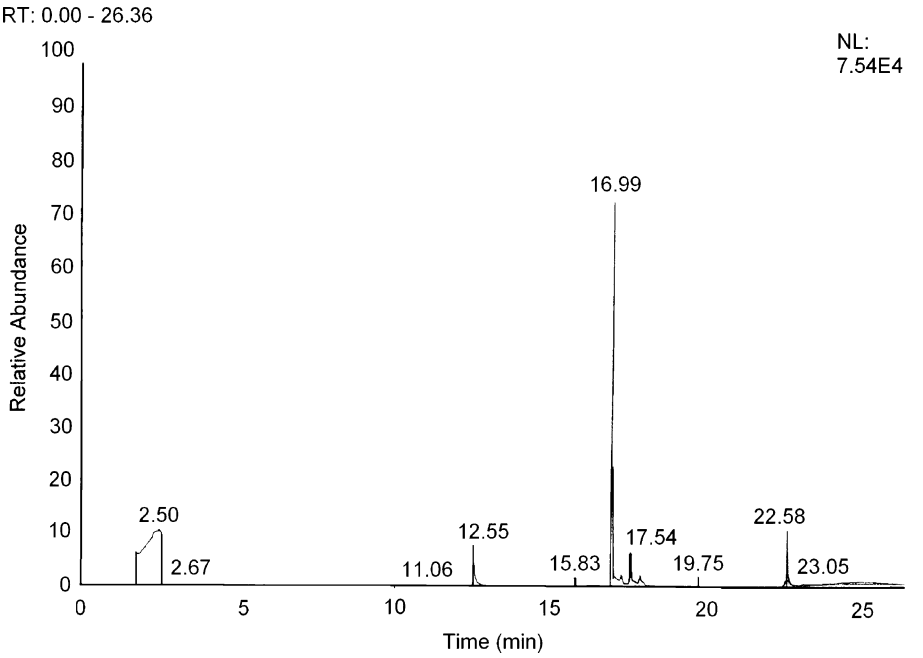

Fig. 13 GC spectra of fraction C_3 of the pigment

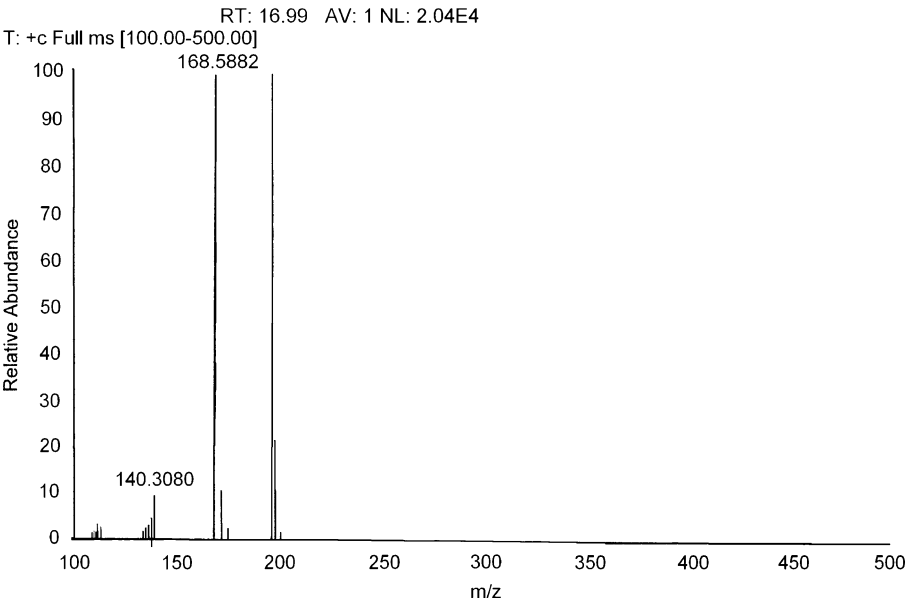

Fig. 14 MS spectra of fraction C_3 of the pigment

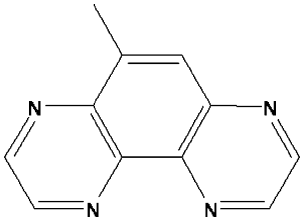

Fig. 15 Proposed structure of the pigment fraction C_3 (9-methyl-1,4,5,8-tetra-azaphenanthrene)

Fractionation of Crude Pigment

Thin layer chromatography of the crude pigment was done on silica gel GF254 as adsorbent using the solvent system ethyl acetate:hexane (1:1). The crude pigment was clearly separated in three distinct spots C_1, C_2, and C_3 with R_f values 0.29, 0.56, and 0.87, respectively. The spots were scraped off, dissolved in dichloromethane, washed with water, and dried over anhydrous sodium sulfate under vacuum to obtain the purified pigment.

Elemental Analysis of the Fraction C_3

Elemental analysis of the fraction C_3, purified by thin layer chromatography shows the following composition:C=79.02%, H=11.28%, N=1.17%, O (by difference)=8.53%

Analysis of the Purified Compound by GC-MS and NMR

Major peak appeared at 16.99 min (Fig. 13). The corresponding mass fragmentation data showed peaks at *m*/*z* 196 (97%), 169 (100%), 142 (32%), 115 (18%); (Fig. 14). Matching the mass fragmentation data with existing library (NIST/Wiley) indicates the probable compound is 9-methyl-1,4,5,8-tetra-azaphenanthrene (Fig. 15).

The ^{1}H NMR signals of the same compound in $CDCl_3$ solvent appeared at δ 1.4 (3H, s), δ 7.7 (1H, s), δ 8.3 (4H, d) and corresponding ^{13}C signals appeared at δ 29.7, δ 120, δ 129.19, δ 129.69, δ 130.47, δ 130.77, δ 131.84, δ 141.20, δ 143.86, δ 144.16 (2C). The

Table 2 Antimicrobial property of the fraction C_3

Bacteria	Zone of inhibition		
	C_1	C_2	C_3
Escherichia coli	−	−	−
Bacillus subtilis	−	−	+++ (8−10 mm)[a]
Bacillus cereus	−	−	++ (5 mm)[a]
Klebsiella pneumoniae	−	−	+ (2 mm)[a]
Micrococcus lutea	−	−	+ (2 mm)[a]
Salmonella typhi	−	−	−
Pseudomonas aeruginosa	−	−	−
Staphylococcus aureus	−	−	+ (2 mm)[a]
Aspergillus niger	−	−	−
Saccharomyces cerevisiae	−	−	−

[a] Excluding the diameter of the well which is 10 mm

NMR data clearly support the structure of the proposed compound by GC-MS analysis. Elemental analysis also supports the proposed structure.

Antimicrobial Activity

Dried pigment fractions (C_1, C_2 and C_3) obtained by fractionation of the crude pigment was dissolved in DMSO (2 μg/ml) and tested for antimicrobial activity by agar cup plate technique [12] against the test organisms using DMSO as a control.

It was observed (Table 2) that only fraction C_3 shows antibacterial activity against *B. subtilis, B. cereus, K. pneumoniae*, *M. lutea* and *S. aureus*. However, it shows no inhibitory effect on *E. coli, S. typhi*, *P. aeruginosa*, *A. niger*, and *S. cerevisiae*.

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