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Full Length Research Paper

Pigment production from a mangrove *Penicillium*

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A mangrove *Penicillium* producing red pigment was cultured in an optimized medium that was designed by the authors previously and used in this study. The purpose of this study was to identify the pigment and also to study the effect of bio elements on pigment production. Pigment from the medium was efficiently extracted using chloroform, ethyl acetate and n-butanol. Most of the red pigment was extracted into ethyl acetate and further purified by preparative thin layer chromatography. From ^{1}H and ^{13}C NMR data supported by electronic imaging mass spectrometry, structure of the compound was elucidated as 2-(4-acetyl phenyl) acetic acid. The yield of pigment produced was studied with respect to various salts and bio elements. Salts at high concentrations (sodium chloride, ammonium sulfate, and sodium nitrite) had a drastic effect on pigment yield because most of the pigment remained adhered to the mycelium instead of diffusing into the medium. Also, when bio elements were supplemented to the medium; calcium, iron, and zinc enhanced pigment yield whereas; potassium, magnesium, copper and manganese did not have significant impact on pigment production. Lead had a drastic negative effect on the pigment yield. Therefore, this study proves that salts and bio elements play a major role in the production of various metabolites from mangrove fungi.

Key words: *Penicillium*, 2-(4-acetyl phenyl) acetic acid, bio elements, salts, soluble pigment.

INTRODUCTION

Fungi have been the source of many important anti-bacterial agents including penicillin's and cephalosporins, both of which have been used heavily for the past 50 to 60 years. Overall, many metabolites are being produced by *Aspergillus* and *Penicillium* species which are salt tolerant, fast growing species and are easily obtained from many substrates (Bugni and Ireland, 2004; Isaka et al., 2000; Park et al., 1999; Udagawa et al., 2000). Marine fungi are well known sources for novel biologically active secondary metabolites (Bugni and Ireland, 2004) and have been found to produce different metabolites compared to terrestrial organisms (Spery et al., 1998). Pigments from natural sources are one group of industrially significant metabolites, because of the negative impacts artificial synthetic colorants have on human health. Natural pigments have widely been used

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in foodstuff, cosmetics, and pharmaceutical manufacturing processes (Francis, 1987; Kim et al., 1995). Microbial pigments can become highly significant when the production yield is high and the pigments are highly stable (Cho et al., 2002).

The ocean environment is a rich source for novel metabolite producing microbes, but only during last two decades the studies on marine metabolites have increased. Some previous studies showed that the marine *Penicillium* produced pigments (PP-V and PP-R) and these are similar in structure to the pigments produced from *Monascus* (monascorubrine and monascuscorubramine) (Ogihara and Oishi, 2002); pigments from *Monascus* are being used in the food industry since long time. Mohan and Vijay-Raj (2009) also described pigment production and radical scavenging activity from a *Penicillium* sp NIOM-2 isolated from marine sediment in India. Three other halophilic fungal strains *Hortaea werneckii*, *Phaeotheca triangularis*, and *Trimmatostroma salinum* isolated from the salterns in the eastern coast of the Adriatic Sea, produced melanin pigments at saturated concentrations of sodium chloride (Tina et al., 2006). Details of several other studies on marine fungi producing significant metabolites are seen but literature on industrial application of potential marine fungal pigments is very minimal and this drives our interest to study the pigment potential of a mangrove *Penicillium*.

The purpose of this research was to isolate fungal pigments from mangrove sediments because mangroves exist under conditions of high salinity, extreme tides, high temperature, and various other stress conditions (Kathiresan and Bingham, 2001). Therefore microbes growing under these conditions will have the potential to produce different significant metabolites to cope with these stresses.

Research on mangrove pigments is very less; it has been recorded that microorganisms from mangroves open up new areas for potential biotechnological exploitation (Gopal and Chauhan, 2006). During this study period, we isolated nearly 100 mangrove fungi from Godavari mangroves of India. Most of the fungi were pigment producers and a red pigment-producing *Penicillium* was selected to study the pigment, its optimization conditions and the effect of metals and salts on pigment yield. In this paper, pigment extraction method, pigment structure and the impact of different bio elements and salts on pigment yield were explained. The main objective of this study was to identify which bio elements have a positive effect on pigment production and also to study if salts effect pigment production even when the fungus was from a marine origin.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The mangrove fungus DLR-7 isolated from Godavari Delta, Andhra Pradesh, India was identified as *Penicillium* sp. according to Alexopolus and Mims (1979). Stock cultures of (Karuna et al., 2009) this *Penicillium* were maintained on potato dextrose agar slants prepared with 50% aged seawater; the cultures were revived every month and stored at 4°C until used in the experiment. Inoculum for these studies was prepared by growing the fungus initially at 25°C on potato dextrose agar (PDA) plates for seven days. Plates having uniform growth and sporulation were selected and a 0.7 cm^2 plug from the outer zone of the colony was punched with a sterile cutter. The plugs were transferred to 100 ml of culture medium in 250 ml flasks and incubated under static conditions at 25°C until maximum pigment was produced (Gunasekaran and Poorniamaal, 2008).

Culture conditions

An optimized basal culture medium was designed for red pigment production using potato extract prepared in the laboratory. Two hundred grams of potatoes were cleaned, sliced and cooked for 30 min with 500 ml of distilled water; the cooked potato slices were mashed and the liquid was filtered through a muslin cloth (Aneja, 2003). Then, 500 ml of seawater was added to the medium and autoclaved. Optimization of culture medium was carried out using various carbon and nitrogen sources and altering different physical parameters (Lathadevi et al., 2014). Results from the optimization experiments concluded that xylose (2% w/v) and glycine (1% w/v) when supplemented to potato extract and pH adjusted to 3.0, produced a high yield of pigment. Therefore, this medium was further used to extract and identify the pigment. The medium was also supplemented with different salts and bio elements to study if they have any effect on the concentration of the pigment produced.

Extraction of pigment

Extraction of pigment from the liquid culture media was carried out by different solvents viz. non polar to polar (Padmavathi and Prabhudessai, 2013). Chloroform, ethyl acetate, and n-butanol were used for the extraction process; all the solvents used were obtained from Qualigens Fine Chemicals Pvt. Ltd., (Mumbai, India). Five liters of culture medium were prepared and about 500 mL were dispersed in ten 1 L conical flasks. The media were autoclaved and about 0.7 cm^2 plugs from the outer zone of the *Penicillium* culture plates were transferred to the media. The inoculated flasks were incubated under static conditions at 25°C (Gunasekaran and Poorniamaal, 2008) for 12 days and the cultures were then harvested. The culture medium was then passed through filter paper (No. 1; Whatman, India Liasion Office, Mumbai, India). A three stage multi-contact/counter current extraction method was used to extract the pigment. Three 500 ml separating flasks were used, 200 ml of the filtered broth was added to the flasks, and 100 ml of distilled solvent was added to the first flask, shaken well and allowed to stand until the aqueous and organic layers separated. Organic layer was transferred to the second flask treated as in the previous step and repeated with the third flask as shown in Figure 1.

Finally, solvent with the pigment was transferred to a clean conical flask and extraction was repeated until no more pigment diffused into the solvent. The entire culture broth was extracted in the same way with chloroform, ethyl acetate, and n-butanol. Finally, the solvents were stripped off with the help of a rotary vacuum evaporator and the amount of pigment was weighed and purified by chromatography. Absorbance of the extracts was measured using a UV-visible spectrophotometer (Model 117, Systronics, India).

Chromatographic analysis

Thin layer chromatography (TLC, Sigma-Aldrich, Hyderabad, India),

Take the culture medium after 12 days of incubation

Filter the medium and remove the fungal mass

Extraction and concentration of the pigment

Take about 200 ml of filtered medium in three 500 ml separating flasks

Add 100ml of distilled chloroform to the first flask

Transfer the organic layer from the first flask

Transfer the organic layer from the second flask

Completely extract the entire culture medium with chloroform

Strip off chloroform and weigh the pigment

Add 100ml of distilled ethyl acetateto the chloroform pretreated medium

Transfer the organic layer from the first flask

Transfer the organic layer from the second flask

Completely extract the entire medium with ethyl acetate

Strip off ethyl acetate and weigh the pigment

Add 100ml of distilled n- butanol to the ethyl acetate pretreaed medium

Transfer the organic layer from the first flask

Transfer the organic layer from the second flask

Completely extract the entire medium with n-butanol

Strip off n-butanol and weigh the pigment

Figure 1. Extraction of pigment from the culture medium: This figure explains the process how pigment is being extracted from the culture medium.

was performed to study the number of pigment components present in each crude pigment fraction obtained from solvent extraction. The ethyl acetate fraction had more pigment but fewer pigment components, which were further purified by preparative TLC using chloroform: methanol (1:1). The pigment was later identified by nuclear magnetic resonance spectroscopy (NMR, Jeol 400 MHZ, USA).

Effect of salts on pigment production

The culture medium was prepared as described in the earlier section and about 50 ml of the medium was dispersed into 100 ml conical flasks. These flasks were then treated with different concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 and 10% w/v), sodium nitrite, and ammonium sulfate separately and adjusted to pH 3.0. The media were then autoclaved and inoculated with the *Penicillium* cultures as described earlier. This study was performed to determine if salts in the culture medium affect the concentration of pigment produced. All the experiments with salts were performed in triplicates and compared to a control (media not treated with salts).

Effect of bio elements on pigment production

This experiment was designed to study the effect of bio elements on pigment production. The culture medium was supplemented with

Table 1. Characteristics of the pigment fractions after solvent extraction.

Fraction	Color	λmax (nm)	Weight (mg/L)
CF	Light Yellow	450 470 510	70
EaF	Deep Red	450 470 510	600
BF	Red	510	250

CF, Chloroform fraction; EaF, Ethyl acetate fraction; BF, Butanol fraction.

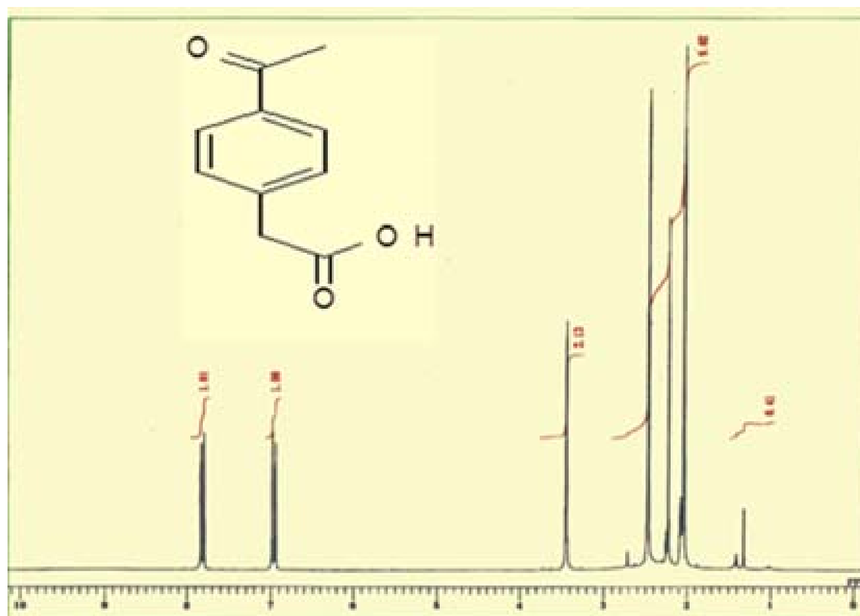

Figure 2. ^{1}HNMR spectrum of the compound [2-(4-acetyl phenyl) acetic acid] in $CDCl_3$.

different concentrations of calcium sulfate ($CaSO_4 \cdot 2H_2O$), copper sulfate ($CuSO_4 \cdot 5H_2O$), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), potassium chloride (KCl), lead nitrate ($PbNO_3$), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), manganese sulfate ($MnSO_4 \cdot H_2O$), and zinc sulfate ($ZnSO_4$). These trace metal concentrations were used at concentrations typically found in sea water. Stock solutions ranging from 10^5 to 10^6 ppb were prepared for calcium, magnesium and potassium. From the stock, different working concentrations (10^5 ppb to 15×10^5 ppb) were prepared and added to the culture media. For metals such as iron, copper, manganese, lead and zinc, a pre-stock solution was prepared and the required volumes were taken to obtain the respective concentrations of 0.05 ppb to 1 ppb. 50 ml of media was prepared with pH 3.0, sterilized and inoculated with the test organism as described above and incubated at 25°C for 12 days. All experiment treatments were performed in triplicates and compared to control media (media without trace elements).

Statistical analysis

Data analysis for treatments with salts and trace elements was performed to study their effect on pigment production. Multiple comparative analyses were performed using R version 2.15.3 for Statistical Computing (http://www.r-project.org/). Linear regression function "lm ()" in R was used to determine significant differences between the treatments using {lm (formula = y ~ x)}. Treatments statistically significant ($p < 0.05$) are considered to be positively affecting pigment production. Regression coefficients and p values of significant treatment comparisons were depicted in the table.

RESULTS AND DISCUSSION

The absorption maxima (λmax) and weight of the pigment fractions derived by solvent extraction are presented in Table 1. The chloroform fraction was colorless whereas, the ethyl acetate and n-butanol fractions were deep red. Chloroform and ethyl acetate extracts had fewer components and were fairly pure, but the butanol fraction had a large number of compounds. As the primary interest of this research was to investigate the red pigment, the ethyl acetate fraction was selected and further purified to elucidate the structure of the red pigment. Preparative TLC of the ethyl acetate fraction yielded a crystalline compound with a melting point of 156°C. It had a strong blue fluorescence on the TLC plate under UV; however, it appeared as a green arrow headed spot after spraying with 5% sulfuric acid in methanol. From ^{1}H, ^{13}C nuclear magnetic resonance (NMR) data supported by electronic impact mass spectroscopy (EIMS) , the structure of the compound was elucidated as [2-(4-acetyl phenyl) acetic acid] as presented in Figure 2. However, during the chromatographic separation, the compound turned out to be colorless.

The ^{1}H NMR spectrum of the compound gave signals

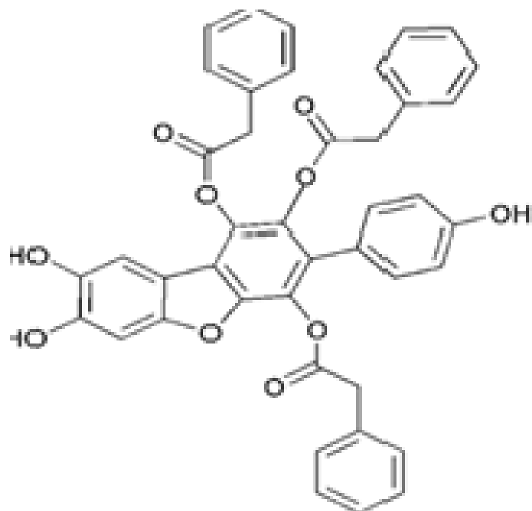

Figure 3. Structure of Ganbajunin-B: This is the assumed structure of the parent molecule from which the pigment component is derived.

at δ 7.82 (2H, d, *J*=7.5Hz), 6.92 (2H, d, *J* = 7.5Hz), 3.45 (2H, s), and 2.2 (3H, s) as shown in Figure 2. High resolution proton magnetic resonance (PMR) showed a pair of doubles in the aromatic region. From the foregoing spectral data, structure of the compound was established as 2-(4-acetylphenyl) acetic acid. ^{13}C NMR supported the structure assigned: δ 22.4, 41.6, 128.7(2C), 129.7 (2C), 135.5, 135.9, 178.4 and 196.2. Further, the structure was confirmed by EIMS, which displayed molecular ion at *m/z* 178. From the literature the parent molecule of this compound was emphasized to be Ganbajunin B that has a red color (Figure 3).

Effect of salts on pigment production

When the fungal cultures were exposed to different salts (as we listed them in the method section) under optimized culture conditions, the soluble red pigment that was supposed to diffuse into the medium, was essentially absent, especially when sodium nitrite and ammonium sulfate were present in the medium. These electrolytes might have altered the pH and prevented diffusion of the pigment, since in the control without salts, the pigment was soluble. In media containing sodium chloride at various concentrations, there was a soluble red pigment but the concentration of pigment was less than that of the control. When the concentration of sodium chloride increased to more than 2% w/v, the concentration of pigment produced was less. Pigment was produced at a significant concentration in medium containing sodium chloride at 1.5% w/v as shown in Table 2. Secondary metabolite composition and production varies with salt concentration. Halo tolerant marine fungal species have evolved unique metabolic mechanisms in response to salt concentrations. In the marine environment, these fungi must have osmoregulatory mechanisms since the biosynthesis of solutes for osmoregulation is energetically costly. Fungi may exhibit decreased secondary metabolite production/slower rates of metabolite production in the presence of high salt concentrations. These findings suggest that marine-derived fungal metabolite production is modulated by the salt concentration of sea water (Bugni et al., 2003). When the sodium chloride concentration increased from 8 to 10% w/v, pigment solubility decreased where most of the pigment was present in the mycelia. High salt conditions also affected the fungal spores because brown spores were observed instead of red.

Effect of trace elements on pigment production

Several studies reported that trace elements are important factors affecting pigment production in several microorganisms (Kim et al., 1998; An et al., 2001). Calcium at 10^5 ppb increased the pigment yield when compared to the control. But increased calcium concentrations, such as $6x10^5$ ppb, inhibited sporulation and decreased pigment production. Potassium at $2×10^5$ ppb produced less pigment than the control. This may be because potassium plays an important role in ionic balance (osmoregulation), enzyme activity and cell physiology (Kavanagh, 2011). Therefore potassium may be used for fungal growth but does not enhance pigment production. The treatments with magnesium, copper, and manganese also had less pigment than the control groups. Zinc and iron stimulated pigment production when compared to the control. Lead at concentrations of 50 ppb inhibited pigment production and at lower concentrations did not have a positive effect on the pigment yield when compared to the control. Studies by Cuero and Ouellet (2005) demonstrated that metal ions such as zinc, copper, and iron have a stimulatory effect on fungal growth and gene expression.

Iron is thought to be necessary for both primary and secondary metabolism. In particular, iron is a constituent of the active centres of the cytochrome P-450 of various mono oxygenases involved in the biosynthesis of clavine alkaloids (Boonyapranai et al., 2008). Metal ions such as zinc, iron,and copper have a regulatory effect at the cellular and molecular levels, on fungal growth and metabolite synthesis. However, the effect depends on the type and concentration of the metal ions, as well as on the presence of ligands in the substrate and/or host (Weinberg, 1977; Hughes and Poole, 1989; Cuero, 2001). Studies by Cuero and Ouellet (2004), demonstrated a stimulatory effect of the metal ions zinc, copper, and iron on gene expression, growth and metabolite production by phyto pathogenic fungi.

In this study trace elements such as calcium, zinc, iron,

Table 2. Comparative regression analysis of the treatments and control on pigment production.

Substrate	Comparison	Estimate of difference	Standard Error	t value	P-Value
Sodium chloride (%)	1.5*2.0	3.03	0.10	-29.99	1.72×10^{-15}
	1.5*Control	-10.33	0.10	102.24	$< 2\times10^{-16}$
Calcium (ppb)	10^5*5×10^4	12.22	0.11	-103.27	$< 2\times10^{-16}$
	10^5*Control	4.87	0.11	-41.15	2.74×10^{-14}
Potassium (ppb)	2×10^5*10^5	4.03	0.11	-33.96	2.7×10^{-13}
	$2\ \times10^5$*Control	-8.51	0.11	71.72	$< 2\times10^{-16}$
Magnesium (ppb)	15×10^5*10^6	4.12	0.10	-37.55	1.87×10^{-15}
	15×10^5*Control	-16.82	0.10	153.30	$< 2\times10^{-16}$
Iron (ppb)	0.5*1	13.7	0.12	-108.45	$< 2\times10^{-16}$
	0.5*Control	0.61	0.12	-4.82	0.0004
Copper (ppb)	0.5*1	0.36	0.11	-3.02	0.0106
	0.5*Control	-19.24	0.11	161.39	$< 22\times10^{-16}$
Manganese (ppb)	0.1*2	7.7	0.11	-64.95	$< 2\times10^{-16}$
	1*Control	-13.24	0.11	111.68	$< 2\times10^{-16}$
Lead (ppb)	0.01*0.05	-10.59	0.14	-73.46	1.31×10^{-12}
	0.01*Control	-8.29	0.14	57.50	$9.29\ \times10^{-12}$
Zinc (ppb)	100*50	0.09	0.13	-0.65	0.524
	100*Control	4.27	0.13	-31.14	$7.56\ \times10^{-13}$

*: indicates comparison; +: P > 0.05 is not significant for the treatment comparisons. Estimate of difference is the difference of sample means and control means.

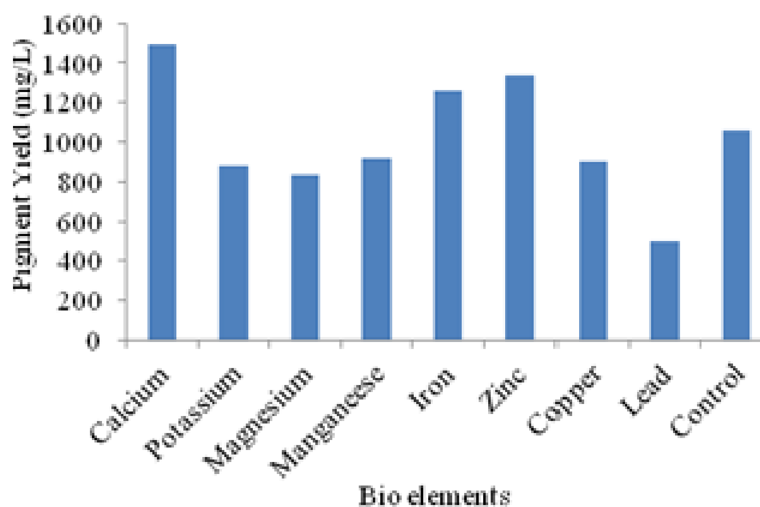

Figure 4. Effect of bio elements on pigment yield: This graph explains how different bio elements affect pigment yield.

and manganese positively influenced pigment yield when compared to the control (without trace elements). Similar findings were observed by Boonyapranai et al. (2008) (Figure 4) on a soil fungus, *Fusarium verticilloides*. Of all

the trace elements studied, calcium produced maximum pigment (1,490 mg/L) while the control yielded 1,065 mg/L of pigment. Bio elements such as zinc and calcium effect fungal growth and gene expression (Cho et al., 2002).

Limited literature is available on pigments from mangrove fungi. This is the first study providing the investigations on the effect of trace elements on mangrove fungal pigments. Observations recorded will significantly contribute for further work in this area. Pigments from terrestrial fungi like *Monascus* are being industrially used in the food industry, but there are no studies on the industrial use of pigments from mangrove fungi. The pigment component 2-(4-acetylphenyl) acetic acid isolated during this study is similar to Ganbajunin B, which is a brownish red pigment isolated from a mushroom *Thelephora vialis.* This compound has strong antioxidant properties and this mushroom is a favorite food in China due to its strong flavor and taste.

The pigment component isolated from mangrove fungus in this study has significant characters such as the compound isolated from the mushroom (*T. vialis)* and further detailed studies such as toxicity tests are required. These studies will be helpful to explore the industrial uses of mangrove fungal pigments.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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