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REGULAR ARTICLE

OPTIMIZATION OF FERMENTATION CONDITIONS FOR RED PIGMENT PRODUCTION FROM *PHOMA HERBARUM* (FGCC#54) UNDER SUBMERGED CULTIVATION

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SUMMARY

An extracellular pigment-producing coelomycetous phytopathogenic fungi belonging to the genus *Phoma* was isolated from phyllosphere of *Parthenium hysterophorus* L. The cell free culture filtrate contained the red pigment. The optimal culture conditions for red pigment production were as follows: Sucrose (742 units), Potassium Nitrate (784 units), pH (736 units), temperature (749 units).

Key words: *Phoma herbarum* (FGCC#54), pigment, Cell Free Culture Filtrate (CFCF), Biomass

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1. Introduction

Pigments have been reviewed for their use in dyes and biological activities by several workers (Iturriaga et al. 2005; Johnson and White 1969). Nature is rich in colors (minerals, plants, microalgae etc) and pigment-producing microorganisms (fungi, yeasts, bacteria) are quite common. Among the molecules produced by microorganisms are carotenoids, melanins, flavins, quinones and more specifically monascins, violacein or indigo. There are many fungi which produce anthraquinones as a secondary metabolite. Fungal anthraquinones as polyketide-derived secondary metabolite occur widely in many genera of fungi. Anthraquinones are a group of aromatic organic compounds encompassing several hundreds of compounds, differing in the nature and positions of the substituents. This class of compounds has shown a wide variety of biological activities (Chan et al. 2005). Fungal anthraquinones as polyketide-derived secondary metabolite occur widely in many genera of fungi (Rai 2002). From pathogenic fungi alone are known novel alkaloids, terpenoids, peptides, macrolides, phenolics and numerous other classes of compounds (Strobel et al. 1991). *Phoma* are among the most prolific producers of secondary metabolites. *Phoma exigua* Desm. produces pigments (Rai 2002) Anthraquinones have also been isolated from *Phoma foveata* (Bick and Rhee 1966), while other fungi too have been reported to exhibit biological activity (Martinkova et al. 2008). The production of pigments by *Phoma* spp. and their biotechnological potential are exhaustively studied by several scientists (Rajak and Rai 1983, Rai and Rajak 1991, Rai and Rajak, 1993, Sonar 2002; Kshirsagar 2004, Deshmukh 2006).

2. Material and Methods

Recovery of the organisms

Tissues from the diseased portion of the weed were cut down into about 1 mm pieces with the help of sterilized blade and forceps and under aseptic conditions transferred to Petri-dishes containing pre sterilized PDA medium. The Petri-dishes were incubated at 28±1^{0}C in BOD incubator (Yorco) and examined regularly. As soon as growth appeared they were transferred to PDA slants.

Fermentation medium

Richard's Broth was used as fermentation medium to assess the pigment

production. (KNO_3=10 gm; KH_2PO_4=5 gm; $MgSO_4$. 7 H_2O=2.5 gm; Sucrose=35 gm; $FeCl_3$=100µg; Distilled Water =1000 ml)

Preparation of cell free culture filtrate

500 ml Erlenmeyer flasks containing 250 ml of pre-sterilized fermentation medium i.e. Richard's Broth was seeded with 5mm discs separated by sterilized cork borer from 10 days old vigorously growing culture on PDA medium at 28±1°C in a BOD incubator (Remi, India). Inoculated flasks were incubated at in a BOD incubator for 7, 14, 21 and 28 days.

Extraction of cell free culture filtrate

Under aseptic conditions, the metabolized broth was filtered through a pre-weighed Whatman filter paper no. 1 and was centrifuged at 4000 x g for 15-20 min. The pellet was thrown and the supernatant was re-filtered *in vacuo* by microfiltration using sterile microfilters, 0.45 µm, Minisart (Sartorius, Gottingen, Germany) to obtain crude culture filtrate (Walker and Templeton 1978).

Biomass estimation in liquid media

For fungal growth, cultures were incubated at 28±1^{o}C. At the end of the incubation period, the contents of each 15 ml Erlenmeyer flask were filtered through a pre-dried and pre-weighed Whatman No.1 filter paper. The filter papers were dried at 90±1^{o}C in hot air oven (Remi, India) for 24-36 hr, then cooled down in a vacuum dessicator to obtain a constant dry weight (Chung and Tzeng 2004). Each experiment was run in triplicates and data was statistically proven**.**

Extracellular red pigment

Red pigment production was indirectly evaluated by measuring the absorbance of the culture filtrate at 500nm in Spectrophotometer (Shimadzu). One unit of pigment is defined as equivalent to 0.01 O.D at 500 nm light absorption (Gunasekaran and Poorniammal 2008)

Optimization of culture condition

The physiochemical parameters studied were pH, temperature, carbon, nitrogen, C:N ratio and inoculum age Experiments were conducted in shake flasks, fungal growth and pigment production were monitored in 7 days interval and all experiments were performed in triplicates.

Statistics- Each experiment was performed atleast three times. The data are given in figures as Mean ±SE and the bars denote ±S.E. values. Data were analyzed by Analysis of Variance (ANOVA) Genstat, Hyderabad, India with a significant level of ($P \leq 0.05$).

3. Results

Effect of pH and temperature on red pigment production

The pH of the culture medium has been reported to play a key role in pigment synthesis. *Phoma* sp. FGCC#54 was cultivated at different initial pH levels (3.0-12.0). Results (Table-1) depict that biomass and pigment production were significantly affected with increase in pH levels. Lower pH values support greater pigment production in comparison with higher values. Similar biomass production results are consistent with those obtained for pigment production. The highest biomass and pigment production was recorded when initial pH of culture medium was set at pH 4.0. *Phoma* sp. FGCC#54 was cultivated under various incubation temperatures (0-40^{o}C) for both mycelial growth and pigment production. Consequently the optimum temperature suitable for both these parameters was found to be 28±1^{o}C (Table-2).

Table 1: Effect of different pH on the production of pigment and biomass by *Phoma* sp. FGCC#54

S. No.	pH	Pigment Production (500 nm)	F. pH	Biomass (gm/L)
1	3.0	695	7.00	7.86±0.01
2	4.0	736	7.69	12.6±0.04
3	5.0	700	6.67	11.46±0.02
4	6.0	680	6.86	10.73±0.02
5	7.0	660	7.20	8.93±0.06
6	8.0	240	7.74	7.73±0.01
7	9.0	100	7.81	5.66±0.04
8	10.0	0	8.25	3.73±0.03
9	11.0	0	8.60	2.40±0.02
10	12.0	0	9.56	0.93±0.03
	SEm±			0.05
	$LSD_{5\%}$			0.14

Values are means of triplicates
SEm = Standard error of Mean
LSD5%= Least Significant Difference at P≤0.05

Table 2: Effect of different temperatures on pigment production by *Phoma* sp. FGCC#54

S. No.	Incubation Temperature (°C)	Pigment Production (500 nm)	F. pH	Biomass (gm/L)
1	0	0	3.84	0.00±0.00
2	5	0	3.84	0.06±0.00
3	10	0	4.00	1.20±0.03
4	15	0	5.10	4.06±0.03
5	20	80	6.54	8.00±0.04
6	25	650	6.77	9.66±0.03
7	28	749	6.91	13.00±0.03
8	30	720	7.00	12.00±0.02
9	35	336	7.20	10.53±0.04
10	40	220	7.34	7.73±0.03
	SEm±			0.04
	$LSD_{5\%}$			0.11

Values are means of triplicates
SEm = Standard error of Mean
LSD5%= Least Significant Difference at P≤0.05

Effect of carbon and nitrogen sources on red pigment production

In order to determine a suitable carbon source for the red pigment production, *Phoma herbarum* (FGCC#54) was cultivated in the basal medium containing various carbon sources. Of the ten carbon sources examined the disaccharides Sucrose, Maltose, Lactose were relatively more favorable to the mycelial growth. Maximum production of pigment was observed in sucrose as the C-source. Maximum biomass production was reported in sucrose (Table 3). In this study, the inorganic nitrogen sources- KNO_3 and $NaNO_3$ had a positive effect on mycelial biomass and pigment production whereas the organic nitrogen sources- peptone and yeast extract proved to be good supporters for biomass and pigment production. Other inorganic nitrogen sources- ammonium oxalate, ammonium tartarate, ammonium chloride, urea and basal media without

nitrogen source strongly inhibited the red pigment synthesis. Of all the nitrogen sources tested, KNO_3 and $NaNO_3$ gave the highest pigment yield followed by Peptone and yeast extract. (Table-4).

Table 3: Effect of carbon sources on pigment and biomass production by *Phoma* sp. FGCC#54

S. No.	Carbon sources	Pigment Production (500 nm)	F. pH	Biomass (gm/L)
1	Mannose	200	6.96	6.2±0.04
2	Fructose	314	6.44	7.4±0.03
3	Glucose	328	6.88	8.3±0.02
4	Sucrose	742	6.93	12.7±0.04
5	Maltose	620	6.76	10.7±0.02
6	Lactose	615	6.80	10.4±0.04
7	Sorbitol	0	6.18	1.86±0.03
8	Citric acid	0	1.93	1.53±0.03
9	Starch	510	6.71	9.86±0.03
10	Dextrin	556	6.75	10.26±0.04
11	No Carbon	0	4.75	1.66±0.04
	SEm±			0.05
	$LSD_{5\%}$			0.14

Values are means of triplicates
SEm = Standard error of Mean
LSD5%= Least Significant Difference at P≤0.05

Table 4: Effect of nitrogen sources on pigment and biomass production by *Phoma* sp. FGCC#54

S. No.	Nitrogen Sources	Pigment Production (500 nm)	F. pH	Biomass (gm/L)
1	Potassium Nitrate	784	6.96	12.46±0.03
2	Sodium Nitrate	762	6.20	11.33±0.04
3	Ammonium Nitrate	0	1.97	5.33±0.03
4	Ammonium Sulphate	0	1.30	2.66±0.02
5	Ammonium Oxalate	0	4.81	2.86±0.03
6	Ammonum Tartarate	0	1.30	4.33±0.03
7	Ammonium Chloride	0	0.67	3.60±0.03
8	Urea	0	6.03	2.00±0.04
9	Peptone	600	7.44	9.80±0.04
10	Yeast Extract	688	6.54	8.33±0.03
11	No Nitrogen	0	3.33	3.33±0.02
	SEm±			0.05
	$LSD_{5\%}$			0.14

Values are means of triplicates
SEm = Standard error of Mean
LSD5%= Least Significant Difference at P≤0.05

Effect of C/N ratio on red pigment production

The effect of C/N ratio on pigment production was investigated using Richards Medium. A shown in Table 5, mycelial growth and pigment production were maximal at a C/N ratio of 4C:6N. It is noteworthy that further change of C/N

ratios higher than 4C:8N or lower than 4C:6N) resulted in a decrease in red pigment production.

Effect of inoculum age on red pigment production

In order to determine the effect of inoculum age on pigment production, *Phoma herbarum*FGCC#54 was cultivated in the optimal medium with different inoculum ages from 6-12 day old culture at 28±1°C. The optimal inoculum age resulted in a decrease in mycelial growth (Table 6).

Table 5: Effect of C: N ratio on pigment production by *Phoma* sp. FGCC#54

C	0C	1C		2C		4C		6C		8C		10C	
N		F. pH	Dry wt Gm/l	F. pH	Dry wt Gm/l	F. pH	Dry wt Gm/l	F. pH	Dry wt Gm/l	F. pH	Dry wt Gm/l	F. pH	Dry wt Gm/l
0N		5.24	0.46±0.05	5.48	0.53±0.05	5.33	2.53±0.05	6.14	5.2±0.04	6.17	0.80±0.04	6.20	0.46±0.05
1N		6.20	0.47±0.04	6.15	0.46±0.04	6.68	3.20±0.04	6.29	4.6±0.05	6.34	0.93±0.05	5.84	0.40±0.03
2N		6.38	0.40±0.03	6.43	0.40±0.04	6.65	4.60±0.04	6.20	2.13±0.05	6.25	0.95±0.04	6.14	0.33±0.04
4N		6.21	0.40±0.04	6.36	0.38±0.03	6.54	7.20±0.03	6.25	1.26±0.04	6.30	1.06±0.05	6.17	0.46±0.03
6N		6.42	0.46±0.04	6.59	0.33±0.03	6.75	10.15±0.04	6.34	0.66±0.05	6.36	0.75±0.04	6.20	0.40±0.04
8N		6.00	0.48±0.04	6.29	0.53±0.04	7.52	9.13±0.04	6.30	0.93±0.04	6.33	0.66±0.05	6.06	0.48±0.05
10N		6.21	0.26±0.04	6.34	0.55±0.04	7.34	6.66±0.05	6.22	0.60±0.05	6.24	0.80±0.04	6.05	0.33±0.04
SEm±	0.04		0.06		0.05		0.03		0.10		0.08		0.05
$LSD_{5\%}$	0.13		0.28		0.19		0.15		0.24		0.17		0.23

Values are means of triplicates
SEm = Standard error of Mean
LSD5%= Least Significant Difference at P≤0.05

Table 6: Effect of nitrogen sources on pigment and biomass production by *Phoma* sp. FGCC#54

S. No.	Inoculum age (days)	Pigment Production (500 nm)	F. pH	Biomass (gm/L)
1	6	0	3.84	0.00
2	7	60	4.56	1.82
3	8	140	5.87	5.73
4	9	380	6.24	7.80
5	10	760	6.88	12.40
6	11	740	7.19	10.60
7	12	690	7.38	9.75
	SEm±			0.03
	$LSD_{5\%}$			0.11

Values are means of triplicates
SEm = Standard error of Mean
LSD5%= Least Significant Difference at P≤0.05

4. Discussion

In contrast to maximum pigment production and biomass highest biomass and pigment production recorded at initial pH of culture medium pH 4.0, in other microorganisms it was found to be the best at pH 9.0 (Unagul et al. 2005; Gunasekaran and Poorniammal 2008). In contrast to the optimal temperature presently obtained for both mycelial growth and pigment production, Gunasekaran and Poorniammal (2008) found to be 30°C. Fungi usually require long periods for submerged culture, exposing them to contamination risk, this optimal temperature is regarded as favourable for *Phoma herbarum* (FGCC#54.) This is quite similar to anthraquinone production by *Penicillium oxalicum* (Sardaryan et al. 2004). It has been reported that various types of peptone supported greater pigment production in many kinds of pigment producing fungi (Cho et al. 2002). Gunasekaran and Poorniammal, 2006 reported a C/N ratio of 1:1 to be the best for biomass and pigment production by *Penicillium* sp. According to Cho et al. (2002) carotenoid content of pink pigment decreased as C/N ratio increased. Amongst several fungal physiological properties, the inoculum age usually plays an important role in fungal development (Glazebrook et al. 1992; Bae et al. 2000).

Optimization of culture conditions for pigment production by *Phoma herbarum*FGCC#54 an improvement in red pigment production was thus achieved under optimal culture conditions by using submerged fermentation. The optimization of physico-chemical properties of pigment produced by *Phoma herbarum* FGCC#54 and its phytotoxicity to target weeds demonstrates the feasibility of commercial production of this pigment as a potential herbicide after further investigations.

Properties of *Phoma herbarum* FGCC#54 red pigment

Parameter	Properties
Colour content (OD units)	784
Water solubility	Soluble
Hue (at 0.1%)	Dark Red
Hygroscopy	Little

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