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Standardization of Process Parameters for Production of Red pigment from *Penicillium purpurogenum* under Submerged Fermentation

C. Padmapriya[1]*, R. Murugesan[2] and S. Gunasekaran[3]
[1,3] Department of Agricultural Microbiology, [2]Agri Business Development,
Tamil Nadu Agricultural University, Coimbatore - 641003

Microbial pigments are an important alternative to potentially harmful synthetic dyes. An extracellular red pigment producing fungus, *Penicillium purpurogenum* was isolated from soil and its optimal submerged fermentation parameters were evaluated. The optimal process conditions for maximum red pigment production were as follows: Starch (33.41 CVU mL^{-1}), peptone (34.5 CVU mL^{-1}) ammonium nitrate (32.8 CVU mL^{-1}) and incubated under darkness (34.5 CVU mL^{-1}). Addition of the ferrous sulphate gave significant yield (29.4 CVU mL^{-1}) on red pigment production. These results suggest the red pigment from *P. purpurogenum* with potential application in food colouring and textile industries.

Key words: Submerged fermentation, Food colourants, *Penicillium purpurogenum*, Red pigment

There is a growing preference for natural colourants among consumers because of their manifold advantages over synthetic colourants in terms of both health and the environment (Kumar and Sinha, 2004). Among the microbial sources of natural colourants, micro-algae and filamentous fungi are the most explored ones with the former have a limitation of low productivity (Hejazi and Wijffels, 2004). Filamentous fungi, present a readily available potential alternative or additional source to the existing sources of natural colourants owing to their amenability to be produced in higher yields with the available cultivation technology (Teng and Feldheim, 2001; Firn and Jones, 2003). Such a production system would be independent of the availability and external supply of raw materials unlike in case of the production of plants or insects based natural colourants that are currently in use (Mapari *et al*., 2005).

The production of *Monascus* -like pigments from *Penicillium* strains have been reported recently (Mapari *et al* ., 2009). They are homologues of pigments of *Monascus* which have similar chromophore polyketides (Mapari *et al*., 2008). The pigments produced by *Penicillium* (PP-V and PP-R) and *Monascus* (monascorubrine and mona scuscorubramine) are structurally similar (Ogihara and Oishi, 2002).These pigments have a potential use in the food industry because they are not associated with citrinin or any other known mycotoxins production and are non-pathogenic to humans. The genes or the enzymes involved in biosynthesis of the citrinin mycotoxin are not yet characterized in *Penicillium* strains.

*Corresponding author email: agri.padma@gmail.com

Many industries favors the use of submerged liquid fermentation owing to its easier product recovery and purification for secondary metabolite production and the use of standardised conditions interms of culture media choice and typical fermentation parameters (Aldfred *et al.,* 2005). Designing a fermentation medium is a critical and important process as the medium composition can significantly affect the product yield (Panda *et al*., 2007). An optimally balanced culture medium was mandatory for maximal production for the secondary metabolites. Optimization of pigment producing parameters for the isolated strains to explore their industrial possibilities is therefore a necessity. The present study was undertaken with the objective to study the effect of process parameters such as light, carbon, nitrogen and trace elements for red pigment production by *Penicillium purpurogenum* under submerged culturing.

Materials and Methods

Culture and fermentation medium

The fungus used in this study was isolated from soil, collected from Tiger reserve, Parambikulam, India. The red pigment producing fungi was isolated and identified as *Penicillium purpurogenum* and certified through Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi. The ITCC accession number obtained was 8904.12. Stock cultures were maintained on potato dextrose agar (PDA) slants at 4ºC after being incubated at 30ºC for 5-7 days. Yeast phosphate soluble starch (YPSS) medium was used as fermentation medium to assess the pigment production. YPSS medium contained (per L) the following: 15 g soluble starch,

4 g yeast extract, 1 g K_2HPO_4 and 0.5 g $MgSO_4.7H_2O$ in deionised water.

Preparation of inoculum

Pure culture of *P. purpurogenum* from PDA slant was transferred into 250 mL Erlenmeyer flask containing 100 mL of the YPSS medium. After cultivation of 2-3 days, spores were scraped with 5 mL of sterilized water from the mycelial mat under aseptic conditions and the spore suspension was used as the inoculum.

Optimization of culture conditions

The process parameters studied were colour of light, carbon sources, nitrogen sources and trace elements. Growth of fungus and red pigment production was monitored in 7 days interval. All experiments were performed in triplicate and mean ± standard deviation is reported.

Pigment yield

About 1 mL of the culture broth was dissolved with 5 mL of 90 % (v/v) methanol. The solvent and sample were kept on a rotary shaker at 200 rpm for 1 h, allowed to stand for 15 min and filtered through Whatman filter paper (47 mm). The clear supernatant was collected and after dilution, absorbance of red pigment was measured at 500 nm using Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The absorbance values were converted in to pigment units using by the following formula (Johns and Stuart, 1991):

$$\text{Pigment yield (colour value units } mL^{-1}) = \frac{\text{OD} \times \text{Dilution} \times \text{Total volume of solvent}}{\text{Amount of sample (mL)}}$$

Biomass yield

The mycelium was separated from the fermented broth and washed twice with distilled water over a preweighed Whatman No.1 filter paper. The filter paper with fresh mycelium was dried in hot air oven at 80°C for 4 h and weighed (Olsson and Nielsen, 1997).

Result and Discussion

Effect of light on red pigment production

In the fungal kingdom, light plays a key role as a regulator for growth, pigment production, asexual and sexual reproduction, all of which are important aspects for the survival and dissemination of the fungal species (Babitha *et al.,* 2008). At the present study the lower pigment production (19.63 CVU mL^{-1}) was observed in the white unscreened light. Incubation in total darkness resulted in increased red pigment production (34.5 CVU mL^{-1}) (Table 1). A low yield of all the shades of pigments was observed in green wavelength, whereas the yellow wavelength inhibited the production of pigments. The darkness, red and blue wavelength observation finds significance as it is against the postulated photo -protective role of biopigments. The results are in agreement with Velmurugan *et al.* (2009) who reported the effect of light on extra and intracellular pigment production. These observations indicate that existence of photoreceptors responsive to dark and light in fungi. The lower pigment production was observed under green and yellow light, it is possible that an enzymatic pathway, which may be induced by nutrient exhaustion, degrades the pigment. Enzyme degradation of secondary metabolites is a common phenomenon in fungi (Johns *et al.,* 1982). Physiological and morphological response of the fungi towards different wavelength of light suggested that a phytochrome type of system might be operative in this organism.

Table 1. Effect of different light on growth and pigment production of fungal isolate

Light	Pigment yield (CVU mL^{-1})	Biomass yield (g 100mL $broth^{-1}$)
Red (780–622 nm)	30.02 (± 0.34)	2.20 (± 0.02)
Yellow (597–577nm)	2.65 (± 0.03)	0.52 (± 0.01)
Green (577–492 nm)	11.48 (± 0.13)	0.56 (± 0.05)
Blue (492–455 nm)	28.33 (± 0.32)	1.92 (± 0.02)
White light	19.63 (± 0.22)	1.75 (± 0.02)
Darkness (no light)	34.5 (± 0.39)	2.42 (± 0.02)
SEd	0.38	0.02
CD (p = 0.05)	0.84	0.06

CVU – Colour value units; Values are mean (±SD) of three replicates

Effect of carbon and nitrogen sources on red pigment production

During the microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but also as important energy source.

Among the carbon sources evaluated in this study, starch gave a maximum yield (33.41 CVU mL^{-1}) of red pigment (Table 2). Gunasekaran and Poorniammal (2008) also reported soluble starch to be the best carbon source for red pigment production by *Penicillium* sp.

Table 2. Effect of carbon sources on growth and pigment production of fungal isolate

Carbon source	Pigment yield (CVU mL^{-1})	Biomass yield (g 100mL $broth^{-1}$)
Maltose	19.26 (± 0.22)	2.26 (± 0.02)
Fructose	26.47 (± 0.30)	3.10 (± 0.03)
Xylose	16.39 (± 0.18)	1.96 (± 0.02)
Sucrose	22.54 (± 0.26)	1.76 (± 0.02)
Glucose	20.55 (± 0.23)	1.44 (± 0.01)
Lactose	12.48 (± 0.14)	1.96 (± 0.02)
Galactose	15.27 (± 0.17)	3.03 (± 0.03)
Methanol	0.00 (± 0.00)	0.00 (± 0.00)
Glycerol	1.83 (± 0.02)	1.73 (± 0.01)
Starch	33.41 (± 0.38)	2.25 (± 0.02)
SEd	0.31	0.03
CD (p = 0.05)	0.66	0.07

CVU – Colour value units; Values are mean (±SD) of three replicates

A stimulatory effect of nitrogen source on pigment formation has been reported by Hamdi *et al.* (1998). It is well known that utilization of different nitrogen sources in fermentation had effects on

microorganism growth and pigment production (Lin and Demain, 1991). In the present study, out of 10 nitrogen sources tested for growth and pigment production, inorganic nitrogen source ammonium nitrate (32.8 CVU mL^{-1}) and organic nitrogen source peptone (34.5 CVU mL^{-1}) were preferred by *P. purpurogenum* (Table 3). According to Chen and Johns (1993) ammonium and peptone served as good nitrogen sources that yielded better growth and pigmentation of *Monascus purpureus.* Organic sources of nitrogen were rapidly absorbed without much biochemical transformation. The effect of organic nitrogen is complicated, because it may serve as a carbon source and can promote the protein -bound dissolution of red pigment into the culture broth. The higher polarity of the red pigments is known to enhance their binding to water-soluble nitrogen containing organic compounds (*i.e.* peptone), which has been proposed as the solubilisation mechanism (Broder and Koehler, 1980). Neveen (2011) also reported that peptone had a positive effect on growth and pigment production of *P. purpurogenum*. The highest significant for extra and intracellular pigment (90% and 77%, respectively) was shown with peptone in *P. purpurogenum*.

Table 3. Effect of nitrogen sources on growth and pigment production of fungal isolate

Nitrogen source	Pigment yield (CVU mL^{-1})	Biomass yield (g 100mL $broth^{-1}$)
Peptone	34.5(± 0.36)	2.80 (± 0.03)
Beef extract	11.8 (± 0.13)	1.53 (± 0.01)
Soya peptone	13.6 (± 0.15)	1.92 (± 0.02)
Malt extract	18.2 (± 0.21)	2.17 (± 0.02)
Yeast extract	25.7 (± 0.28)	3.22 (± 0.03)
Potassium nitrate	0.0 (± 0.00)	0.76 (± 0.01)
Sodium nitrate	15.2 (± 0.17)	1.95 (± 0.22)
Ammonium nitrate	32.8 (± 0.37)	1.83 (± 0.02)
Ammonium chloride	23.7 (± 0.28)	1.44 (± 0.01)
Ammonium sulphate	28.5 (± 0.35)	0.82 (± 0.01)
SEd	0.34	0.04
CD (p = 0.05)	0.77	0.09

CVU – Colour value units; Values are mean (±SD) of three replicates

Table 4. Effect of trace elements on growth and pigment production of fungal isolate

Trace element source	Pigment yield (CVU ml^{-1})	Biomass yield (g 100ml $broth^{-1}$)
Copper sulphate	0.0 (± 0.00)	0.0 (± 0.00)
Ferrous sulphate	29.4 (± 0.33)	1.5 (± 0.07)
Zinc sulphate	12.5 (± 0.14)	1.8 (± 0.02)
Magnesium sulphate	15.3 (± 0.17)	0.7 (± 0.01)
Manganese sulphate	17.2(± 0.23)	2.1 (± 0.02)
SEd	0.29	0.02
CD (p = 0.05)	0.66	0.05

CVU – Colour value units; Values are mean (±SD) of three replicates

Effect of trace metals on red pigment production

In order to investigate the effect of trace elements on red pigment production and mycelial growth, *P. purpurogenum* was cultivated in YPSS medium. Each trace element was added to the culture medium at a concentration of 0.05 per cent. The maximum red pigment production of 29.4 CVU mL^{-1} was achieved in ferrous sulphate supplemented medium followed by manganese sulphate (Table 4). These results are consistent with Lee *et al*. (2001), who reported Fe^{2+} has the strongest stimulatory effect on red pigment production. Some stimulatory effect has also been attributed to Mn^{2+}, because trace metals have important effects on secondary metabolism (Weinberg, 1989). The other bioelement like copper ions appeared to be detrimental on both red pigment production and mycelial growth in *P. purpurogenum*.

Conclusion

From the results of the present study it can be concluded that, a red pigment could be isolated from *P. purpurogenum* in submerged fermentation. The optimal carbon, nitrogen and mineral sources such as starch, peptone, ammonium nitrate and ferrous sulphate in YPSS medium were standardised for an improved growth and red pigment production of *P. purpurogenum*. This type of optimization study will be helpful to biotechnological approach for strain improvement and for the benefit of food and textile industries.

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