Madras Agric. J., 101 (10-12): 406-410, December 2014
https://doi.org/10.29321/MAJ.10.001225

Production of Red Pigment from *Penicillium purpurogenum* by SSF with Cassava Processing Waste

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The fungal pigments are a good alternative to currently used synthetic colourants and / or natural colourants derived from plant materials. The aim of the present study was to investigate the feasibility of cassava processing waste as a substrate for production of pigments by *Penicillium Purpurogenum* in solid state fermentation (SSF). A pigment yield of 40.13 Colour Value Units (CVU) / g of dry fermented substrate was achieved by using cassava processing waste with optimized process parameters such as 50 % initial moisture content, inoculation with 4.0 ml of spores (25×10^5 spores) / gram of dry substrate and an incubation period of 15 days with 1 % peptone at 30°C,. Thus, utilization of cassava processing waste for red pigment production in this study could provide the most effective use of natural resources and lead to technology development for further cost reduction.

Key words: Solid state fermentation, Cassava processing waste, *Penicillium purpurogenum*, Red pigment

Pigments, which are either natural or synthetic, play an important role in food, textile and pharmaceutical industries as colourants. Synthetic red pigments such as azorubin or tartrazin cause allergic reactions (Fabre *et al*., 1993) and Citrus red having carcinogenic and tetragenic effects (Marlin *et al*., 1987). The scrutiny and negative perceptions of synthetic food pigments by the modern consumer have given rise to a strong interest in natural colouring alternatives for human safety and environmental conservation (Dufosse, 2006).

Natural pigments can be obtained from two major sources, plants and microorganisms. The accessible authorized natural pigments from plants have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility and often non-availability throughout the year. The later are of great interest owing to the stability of the pigments produced and the availability of cultivation technology (Parekh *et al*., 2000). *Monascus* species are well known to produce pigments like monascorubrine, rubropunctatine (Juzlova *et al.,* 1996 and Pattanagul *et al*., 2007) and more recently monascusones from a *Monascus* mutant have been identified (Ratana and Toshima, 1987). On the other hand, *Monascus*-derived pigments contain citrinin and the production of mycotoxin limits the use of *Monascus* as a producer of food colourants (Liu *et al*., 2005). Therefore, it is interest to search for alternative pigment producing organisms. The production of *Monascus* -like red pigments from *Penicillium* strains have been reported recently. These pigments have a potential use in the food industry because they are not associated with citrinin production (Mapari *et al*., 2008). However, the high cost of the current liquid culture-based fermentation technology has limited the industrial use of red pigment from *Penicillium purpurogenum*. There is a growing need for low cost production of natural pigments or colouring agents (Pandey, 2003).

Solid state fermentation (SSF) has emerged as an effective alternative for liquid culture- based fermentation technology. The substrates used in SSF supply the basic nutrients to the microorganisms and serve as an anchor for the cells (Babitha *et al*., 2007). Interestingly, recent studies report that SSF provides a more adequate habitat for fungi, resulting in high pigment production in a relative low-cost production process when agro-industrial wastes are used as substrate (Velmurugan *et al*., 2011).

The starch manufacturing industries from cassava, which also produce the solid wastes, from the peelings and the screening of starch slurry before sedimentation, called as cassava processing waste (CPW). Annual production of CPW in India is 380 kilo tonnes and processing of one tonne of cassava roots gives 151 kg of peelings and 51 kg of CPW. Cassava processing waste is a highly economical substrate for SSF and it contains complex of starch, cellulose, hemicelluloses, pectin, fiber and protein which promote the fungal growth and thereby increase the pigment yield (Sonali and Lal, 2008). However, to our knowledge no effort has been made to utilize the cassava processing waste for pigment production. The objective of this study was to develop a fermentation process for production of red pigment from *P. purpurogenum* employing SSF using CPW.

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Materials and Methods

Culture

The fungus used in this study was isolated from soil and maintained at Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore during selection and screening of pigment producing microorganisms. The red pigment producing fungus was 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. The strain was maintained by routine weekly transfer, under aseptic conditions to potato dextrose agar (PDA) slants and stored at 4ºC after being incubated at 30ºC for 5-7 days.

Cassava sago waste

Dried CPW was obtained from sago factory, Salem. CPW was characterized for protein (Lowry *et al*., 1951), starch (Hedge and Hotreiter, 1962), Total soluble solids (TSS), cellulose (Updegroff, 1969), crude fiber (Prosky *et al*., 1988), ash (Reuter *et al*., 1986) and moisture content using standard methods.

Solid state fermentation

Five g of the dry CPW was placed in 250 ml Erlenmeyer flask and a nutrient solution (3 ml) containing (g/L): KH_2PO_4 (2), NH_4NO_3 (5), NaCl (1) and $MgSO_4 .7H_2O$ (1) was added by adjusting different pH (2 to 9). Initial moisture content was adjusted to 50 % (w/w) with distilled water. Flasks contents were mixed thoroughly, autoclaved at 121°C for 20 min and cooled to room temperature. The flask was inoculated with 2 ml of spore suspension containing $6\times10_5$ spores ml^{-1} of *P. purpurogenum* and incubated at 30°C for 15 days. All experiments were conducted in triplicate and mean ± standard deviation is reported.

Pigment extraction and estimation

After 15 days of incubation, the substrate was dried on aluminium foil at room temperature and ground to fine powder. The fermented solid substrate was extracted with 5 ml of 90 per cent methanol per g of dry fermented substrate (gdfs). The mixture was placed on a rotary shaker at 200 rpm for 1 h, allowed to stand for 15 min and filtered through Whatman No. 1 filter paper (Babitha *et al.*, 2007). The extracted pigment was quantified by measuring OD at 500 nm and pigment yield was expressed as colour value units per g of dry fermented substrate (CVU $gdfs^{-1}$) (Tseng *et al*., 2000).

Biomass estimation

Total fungal biomass was determined by measuring the N -acetyl glucosamine released by acid hydrolysis of the chitin present in the fungal cell wall (Sakurai *et al* ., 1977). About 0.5 g of dry fermented substrate was mixed with 1 ml of conc. H_2SO_4. Acetyl acetone reagent (1 ml) was added to the mixture and incubated in a boiling water bath for 20 min. After cooling, 6 ml of ethanol was added, followed by 1 ml of Ehrlich reagent and incubated at 65ºC for 10 min. After cooling, the optical density was measured at 530 nm against the reagent blank using N-acetyl glucosamine as the standard.

Process optimization studies

To study the effect of incubation period on pigment production, flasks were incubated for varying periods (3, 5, 7, 10, 15, 20, 25 and 30 days). Growth kinetics was estimated by estimating the biomass at various incubation periods and by relating the pigment producing capability of the fungus. The following parameters were also evaluated: moisture content of the substrate (adjusted to 40%, 50% and 60% w/w with distilled water), temperature (20°C, 30°C, 40°C, 50°C and 60°C; for fungal growth and pigment production), inoculum size, and substrate pH (by adjusting the pH of salt solution after autoclaving). Pigment production was determined spectrophotometrically, as previously described.

Result and Discussion

Physico - chemical properties of CPW

The physicochemical properties of CPW used for red pigment production are presented in Table 1. The major component starch was approximately 0.59 mg g^{-1}. The second highest component was cellulose at 0.24 mg g^{-1}. It also contains 0.12 mg g^{-1} crude fiber, 0.01 mg g^{-1} protein and 0.02 mg g^{-1} ash. The extracellular hydrolytic enzymes of *P. purpurogenum* degrade the complex polysaccharides into simple molecules and thereby increase the bioavailability of sugars (Dhale and Vijay raj, 2009). The increased bioavailability of sugars directly enhances the growth rate of fungi as well as pigment production.

Effect of initial pH of the substrate on pigment production

Substrate pH is one of the important factors determining microbial growth and metabolic activity in SSF. *P. purpurogenum* biomass and pigment yield were determined at different initial substrate pH levels (Table 2). Fungal growth was completely inhibited at pH 2 and 3.Yongsmith *et al*. (2000) reported that a lower substrate pH promotes synthesis of yellow pigments, whereas a higher pH results in red pigments. In this study, red pigment production was maximal at pH 6 (28.44 CVU $gdfs^{-1}$). Pigment production was reduced at pH 7 and 8, and was completely inhibited with further increases in pH. These results are consistent with Babitha *et al*. (2007) who reported maximum pigment production by *Monasus purpureus* at pH 4.5 to 7.5. N-acetyl glucosamine concentration was greatest

Table 1. Chemical composition of CPW

Constituents	mg g^{-1}
Carbohydrates	0.77 (± 0.90)
Protein	0.01 (± 0.02)
Starch	0.59 (± 0.69)
TSS	0.45 (± 0.52)
Crude fibre	0.12 (± 0.14)
Cellulose	0.24 (± 0.27)
Ash	0.02 (± 0.02)
Moisture	0.11 (± 0.12)

at pH 6 (21.56 g $gdfs^{-1}$ followed by pH 7, indicating maximum growth and pigment production. Velmurugan *et al*. (2011) found that the best results for the production of red pigments were obtained at pH 6 while using corn cob as a substrate in SSF. Mendez *et al*. (2011) reported that highest level of red pigment production from *P. purpurogenum* GH2 was obtained at pH 5.

Table 2. Effect of initial pH of substrate on red pigment yield by *P.purpurogenum*

pH	Pigment yield (CVU $gdfs^{-1}$)	Biomass yield (g $gdfs^{-1}$)
2	0.0	0.0
3	0.0	0.0
4	9.55 (± 0.11)	17.23 (± 0.19)
5	12.88 (± 0.14)	19.57 (± 0.22)
6	25.44 (± 0.32)	21.56 (± 0.24)
7	22.12 (± 0.26)	20.89 (± 0.24)
8	12.87 (± 0.14)	11.16 (± 0.12)
9	2.07 (± 0.02)	4.39 (± 0.05)
SEd	0.24	0.23
CD (p = 0.05)	0.51	0.50

CVU – Colour value units; gdfs - gram of dry fermented substrate; Values are mean (±SD) of three replicates

Effect of temperature on pigment production

Temperature is an important factor as it influences metabolic activities and microbial growth. The results of influence of different temperature on red pigment and biomass production are presented in Table 3. From the results it was evident that maximum pigment production was occurred at 30ºC

Table 3. Effect of temperature on growth and red pigment production by *P.purpurogenum*

Temperature (ºC)	Pigment yield (CVU $gdfs^{-1}$)	Biomass yield (g $gdfs^{-1}$)
20	16.72 (± 0.19)	12.56 (± 0.14)
30	26.12 (± 0.36)	22.31 (± 0.25)
40	12.47(± 0.14)	7.32 (±0.08)
50	5.98 (± 0.06)	5.46 (± 0.06)
60	0.0(± 0.00)	0.0 (± 0.00)
SEd	0.27	0.19
CD (p = 0.05)	0.62	0.44

CVU – Colour value units; gdfs - gram of dry fermented substrate; Values are mean (±SD) of three replicates

(31.52 CVU $gdfs^{-1}$), which clearly indicated the mesophilic nature of the fungus. Pigment production was decreased drastically at higher temperatures from 40 to 60ºC. At 30ºC, maximum N-acetyl glucosamine concentration was 22.31 g $gdfs^{-1}$. Results are in agreement with Babitha *et al*. (2007) who reported red pigment production was highest at 30ºC, and decreased at temperatures higher than 40ºC accompanied by an increase in the production of yellow pigments.

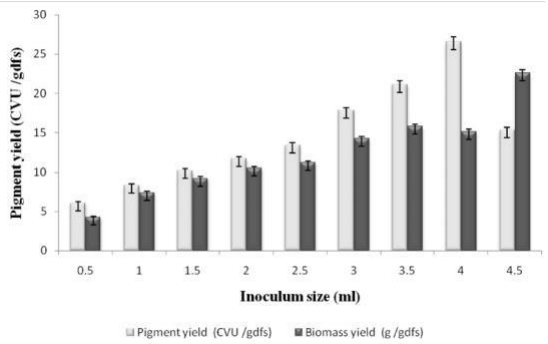

Fig 1. Effect of inoculum size on growth and red pigment production by *P.purpurogenum*

Effect of inoculum size on pigment production

The effect of inoculum size on red pigment production using CPW was studied with different inoculums sizes ranging from 0.5 to 4.5 ml of spores suspension (Fig 1). Pigment production significantly increased (24.13 CVU $gdfs^{-1}$) up to an inoculum size of 3.5 ml of spores per gram of initial dried substrate (spores gds^{-1}). Inoculating with 4 ml of spores gds^{-1} maximized the red pigment production (31.18 CVU $gdfs^{-1}$) with a biomass of 15.10 g $gdfs^{-1}$. Higher inoculum of 4.5 ml of spores gds^{-1} produced too much biomass (22.56 g $gdfs^{-1}$) with less pigment yield (15.36 CVU $gdfs^{-1}$). A low inoculum density of 0.5 ml spores gds^{-1} resulted in insufficient biomass causing reduced product formation, whereas higher inoculum of 4.5 ml spores $gdfs^{-1}$ produced too much biomass and depleted the substrate of nutrients required for pigment production (Pandey *et al*., 2000).

Effect of incubation period on pigment production

The results of effect of incubation period on red pigment and biomass production are presented in Fig 2. The amount of pigment production varied with incubation time. Maximum red pigment production

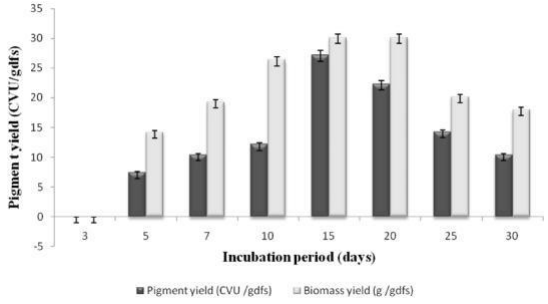

Fig 2. Red pigment production at different incubation period by *P.purpurogenum*

was obtained after 15 days of incubation (40.13 CVU $gdfs^{-1}$) with a biomass of 30.12 g $gdfs^{-1}$ at 15^{th} and 20^{th} days of incubation. Pigment production was decreased from 20^{th} to 30^{th} day, likely due to the decline or death phase of the fungus. The depletion of essential nutrients present in the substrate and the accumulation of inhibitory products such as acids decreased the fungal growth and red pigment production after 30 days of incubation.

Effect of initial moisture on pigment production

For SSF, moisture is a key parameter to control the growth of microorganism and metabolite production (Pandey, 2003). The effect of initial moisture content of the substrate on red pigment production and biomass was presented in Fig 3.

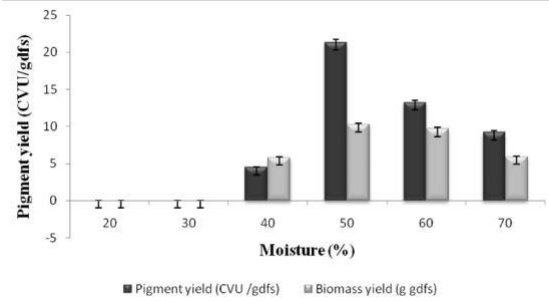

Fig 3. Effect of initial moisture on red pigment production by *P.purpurogenum*

Maximum red pigment production was observed at 50 per cent moisture content (21.33 CVU $gdfs^{-1}$) followed by 60 per cent moisture content (13.26 CVU $gdfs^{-1}$). This can be attributed to effective utilization of sugars in the substrate. Pigment yield was reduced above or below 50 per cent moisture content of CPW. No pigment and biomass production was observed at substrate moisture content below 40 per cent. It may be concluded that 50 per cent moisture content of the substrate was optimum for red pigment production. This result was similar to

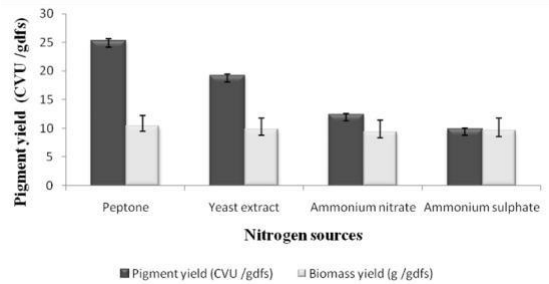

Fig 4. Effect of nitrogen sources on red pigment production by *P.purpurogenum*

the findings of Johns and Stuart (1991) who reported that initial substrate moisture content less than 40 % gave less pigmentation, but that of 50-56 % could give the highest pigmentation. Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer process and decrease in initial moisture level results in reduced solubility minimizes heat exchange, oxygen transfer and low availability of nutrients to the culture (Carrizales and Rodriguez, 1981).

Effect of nitrogen sources on pigment production

In order to investigate the effect of nitrogen sources on red pigment production and mycelia growth, *P. purpurogenum* was grown in CPW by solid state fermentation. Each nitrogen sources were added to the substrate at a concentration of 1 %. Maximum pigment yield was observed with CPW supplemented with 1 % peptone (25.17 CVU $gdfs^{-1}$) followed by 1 % yeast extract (19.11 CVU $gdfs^{-1}$) and for 1 % ammonium nitrate (12.37 CVU $gdfs^{-1}$) followed by ammonium sulphate (9.82 CVU $gdfs^{-1}$) (Fig 4). The pigment yield was higher in organic nitrogen sources than inorganic nitrogen sources. The study reveals that the addition of nitrogen source improves the metabolic activity of the organism to produce increased quantity of the pigment. The results are similar to those of Vidyalakshmi *et al.* (2009).

Conclusion

The results of our study indicate the feasibility and applicability of cassava processing waste a sago industry by-product, for production of red pigment from *P. purpurogenum* by SSF. The highest yield of red pigment (40.13 CVU $gdfs^{-1}$) indicates that cassava processing waste is an effective substrate for SSF. Cassava processing waste is economical and environmentally safe to end users. To our knowledge this is the first report on red pigment production using powdered cassava processing waste in SSF.

Acknowledgement

The authors kindly acknowledge the funding granted by Indian council of Agricultural Research (ICAR) under the scheme “Application of microorganisms in Agriculture and allied sciences (AMAAS)”.

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Received: August 29, 2013; Revised: May 2, 2014; Accepted: July 8, 2014