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***Monascus* pigment production by solid-state fermentation with corn cob substrate**

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Palanivel Velmurugan and Hyun Hur made equal contributions to this work and should be considered co-first authors.

Abstract

Natural pigments are an important alternative to potentially harmful synthetic dyes. We investigated the feasibility of corn cob powder as a substrate for production of pigments by *Monascus purpureus* KACC 42430 in solid-state fermentation. A pigment yield of 25.42 OD Units/gram of dry fermented substrate was achieved with corn cob powder and optimized process parameters, including 60% (w/w) initial moisture content, incubation at 30°C, inoculation with 4 mL of spores/gram of dry substrate, and an incubation period of 7 days. Pigment yield using corn cobs greatly exceeded those of most other agricultural waste substrates. The pigments were stable at acidic pH, high temperatures, and in salt solutions; all important considerations for industrial applications. Our results indicate the viability of corn cob substrate in combination with *M. purpureus* for industrial applications.

Keywords: Corn cob substrate, *Monascus purpureus*, Natural pigments, Solid-state fermentation, *N*-Acetyl glucosamine

Natural dyes were the main colorants for textiles through the 19th century (1). Since then synthetics have almost completely replaced natural dyes. The wide range of available colors, greater reproducibility, improved quality of dyeing, and economic benefits of synthetic dyes are highly desirable. However, it is well known that some of the synthetic dyes are environmental toxins and negatively impact ecosystems. Recent studies reported that 10–35% of these dyes are lost in wastewater during the dyeing process (2, 3). Due to serious environmental pollution concerns, textile industries are facing extensive problems and several have banned various synthetic coloring agents. Consequently there is a growing demand for eco-friendly, non-toxic colorants for industrial applications.

Natural dyes or pigments are an important alternative to potentially harmful synthetics (3). Numerous studies report that microorganisms of the genus *Monascus* produce red pigments, which are used as coloring agents in food and textiles (4–6). Moreover, in several Asian countries, *Monascus* species are grown on rice grains, and used for coloring some foods (7), increasing the demand for the highly safe pigments. However, the high cost of the current liquid culture-based fermentation technology has limited the industrial use of *Monascus* pigments. Thus, there is a growing need for low cost production of natural pigments or coloring agents.

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 (8). Interestingly, recent studies report that SSF provides a more adequate habitat for fungi, resulting in high pigment production in a relatively low-cost process when agro-industrial wastes are used as substrate. Agro-industrial wastes such as rice bran, wheat bran, coconut oil cake, sesame oil cake, palm kernel cake, groundnut oil cake, cassava powder, spent brewing grain, and jackfruit seed powder have been screened to select the best substrate for pigment production (8). However, to our knowledge no effort has been made to utilize the corn cob for this purpose. The cob is the central core of the maize (*Zea mays* ssp. *mays* L.) seed head or “ear.” When harvesting corn the cob is collected as part of the ear, leaving the corn stover in the field. Corn cobs are an important source of furfural, an aromatic aldehyde used in a wide variety of industrial processes. Although of little nutritional value, corn cobs can be used as fiber in ruminant fodder. For many years corn cobs have also been made into charcoal. The corn cob contains considerable amounts of polysaccharides (such as cellulose and hemicelluloses), which promote fungal growth and thereby increase pigment yield. Corn cobs are a highly economical

substrate for SSF, compared to other substrate previously used for pigment production. The objective of our study was to develop a fermentation process for production of *Monascus* pigments employing SSF using corn cobs. We optimized key parameters to maximize pigment production.

Materials and methods

***Monascus purpureus* culture** — *Monascus purpureus* KACC 42430 was acquired from the Korea Agriculture Culture Collection (KACC). The culture was maintained on potato dextrose agar (Difco, Paris, France), preserved at 4°C, and sub-cultured every three weeks.

Inoculum preparation — Ten milliliters of sterile distilled water was added to fully sporulated (6 to 8 day-old) agar slope culture and the spores were scraped from the agar plates under aseptic conditions. The spore suspension was used as the inoculum.

Substrate and solid-state fermentation — Corn cobs were obtained from local agricultural fields around Iksan, South Korea. Cobs were washed thoroughly and dried in sunlight, then ground to 2 mm particle size using a sterile blender. The prepared material was soaked in deionized water at 80°C for 12–48 h to increase porosity and bulk density. Then the material was pressed to remove the water and dried in the shade. Five grams of the dry substrate was placed in a 250 mL Erlenmeyer flask and a nutrient salt solution (2 mL) containing (g/L): KH_2PO_4 , 2; NH_4NO_3 , 5; NaCl, 1; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 (9, 10) was added by adjusting different pH (pH 1–8). Initial moisture content was adjusted to 50% (w/w) with distilled water. Flask contents were mixed thoroughly, autoclaved at 121°C for 20 min, and cooled to room temperature. The flask was inoculated with the *M. purpureus* KACC 42430 spore suspension (6×10^5 spores/mL) and incubated at 30°C and 50% humidity for 7 d. Unless otherwise indicated, these conditions were maintained throughout the experiment. All experiments were conducted in triplicate and means \pm standard deviations are reported.

Biomass estimation — Total fungal biomass was determined by measuring the *N*-acetylglucosamine released by acid hydrolysis of the chitin present in the fungal cell walls (10, 11). In brief, 0.5 g of dry fermented corn cob powder was mixed with 1 mL of concentrated H_2SO_4 . Acetyl acetone reagent (1 mL) was added to the mixture, which was then placed in a boiling water bath for 20 min. After cooling, 6 mL of ethanol was added, followed by 1 mL of Ehrlich reagent (Sigma-Aldrich, Milwaukee, WI, USA) and incubated at 65°C for 10 min. After cooling to room temperature, optical density (OD) was measured at 530 nm against the reagent blank using *N*-acetylglucosamine (Sigma-Aldrich) as the external standard.

Pigment extraction and quantification — After 7 d of incubation, the substrate was dried on aluminum foil at room temperature and ground to a fine powder using a sterile household grinder. Pigment was extracted following the procedure of Babitha et al. (8). In brief, fermented solid substrate was extracted with 5 mL of 90% methanol per gram 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 #1 filter paper.

Following the method of Tseng et al. (12), extracellular pigment was quantified by measuring OD at 412 and 500 nm, representing yellow and red pigment production, respectively and pigment yield was expressed as OD/gdfs. Absorbance was also measured for pigment extract (13–15).

Substrate preparation variables — To study the effect of soaking on pigment production, flasks were placed on a rotary shaker at 100 rpm for 0, 12, 24, and 48 h at 80°C. The following parameters were also evaluated: incubation time (12 to 264 h), 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 salt solution pH after autoclaving). Growth kinetics was estimated from temporal changes in biomass. Pigment production was determined spectrophotometrically, as previously described.

Pigment stability — Pigment stability was determined using the method of Srivastava et al. (16) and Perumal et al. (17). Briefly, glass test tubes containing 10 mL of the fungal pigment were incubated in a water bath at 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C for 10 min. After cooling to room temperature, absorbance was measured using a UV–visible spectrophotometer and percent stability was calculated. Extracts (10 mL) in another set of tubes were adjusted to pH 2, 4, 6, 8, 10, 12 or 14, mixed for 10 min, and absorbance measured. Tubes containing 10 mL of extract also were incubated under stress conditions, including 12 h in a hot air oven at 90°C, 2 h in sunlight, and steaming at 834 kPa for 20 min. Another set of tubes containing 10 mL of the fungal pigment extract were incubated under UV light for 12 h. Test tubes containing 10 mL of extract were amended with 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1% (v/v) salt solution (NaCl and NH_4Cl) and kept in a warm water bath for 1 h to determine stability in textile dyeing. Stability (%E) was calculated:

$$\%E = [(A_0 - A_1)] \times 100/10 \quad (1)$$

where A_0 is pigment absorbance before treatment and A_1 is absorbance after treatment. Absorbance was measured spectrophotometrically at 500 nm of the pigment.

Results and discussion

General observations — The applicability of corn cob waste as a substrate for pigment production was evaluated using *M. purpureus* KACC 42430. Soaking the corn cob powder at 80°C for 48 h resulted in greater pigment production (Figure 1). Fermentation yielded a highest red pigment concentration (25.42 OD Units/gdfs) using corn cob compared to other agro industrial wastes such as coconut oil cake (0.118 OD Units/gdfs), groundnut oil cake (0.150 OD Units/gdfs), sesame oil cake (0.375 OD Units/gdfs), tamarind seed powder (1.146 OD Units/gdfs), cassava flour (1.458 OD Units/gdfs), wheat bran (3.525 OD Units/gdfs), spent brewing grain (4.356 OD Units/gdfs), palm kernel cake (7.650 OD Units/gdfs), and jackfruit seed powder (12.113 OD Units/gdfs) (6). Spectral analysis indicated maximum absorbance at 490 nm (data not shown), confirming red pigment production by *M. purpureus* KACC 42430. Our results are consistent with studies reporting greater pigment production by *M. purpureus* LPB 97 using jackfruit seed powder as substrate (9) and by *M. purpureus* CMU001 using inexpensive agricultural products and residues (10). Corn cobs contain 32.3–45.6% cellulose and 39.8% hemicelluloses (primarily composed of pentosan and 6.7–13.9% lignin). Cellulose is a polysaccharide of glucose units that serve as the main structural component of cob cell walls. Hemicellulose is a less complex polysaccharide that can more easily be broken down to simple monosaccharides. The extracellular hydrolytic enzymes of *M. purpureus* degrade the complex polysaccharides (cellulose and hemicellulose) into simple molecules and thereby increase the bioavailability of the sugars. The increased bioavailability of sugars directly enhances the growth rate of fungi as well as pigment production.

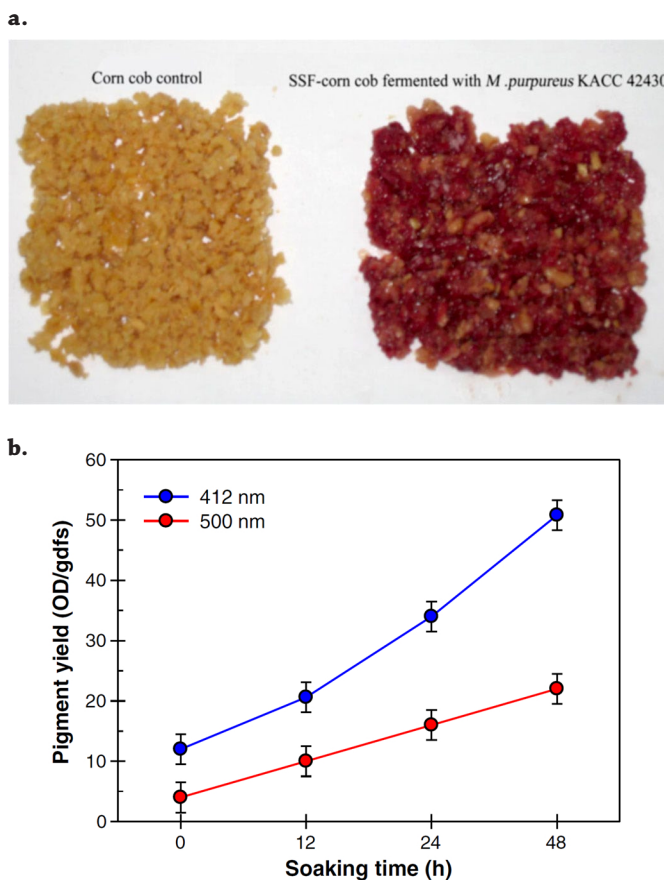


Figure 1. (a) Solid state fermentation (7 days incubation at 30°C with 60% (w/w) moisture content) of non-fermented and fermented corn cob. (b) Pigment yield (412 and 500 nm) in solid state fermentation by *M. purpureus* KACC 42430 in response to substrate soaking time.

Substrate pH — Substrate pH is one of the most important factors determining microbial growth and metabolic activity in SSF. *M. purpureus* biomass and pigment yield were determined at different initial substrate pH levels (Figure 2). Fungal growth was completely inhibited at pH 1 and 2. However, at pH 3.0 and 4.0, the λ_{\max} shifted to 480 and 390 nm, respectively. These observations are consistent with Babitha et al. (8), who reported a similar λ_{\max} shift for *Monascus* pigments at pH 3. Yongsmith et al. (18) reported that a lower substrate pH promotes synthesis of yellow pigments, whereas a higher pH results in red pigments. In our study, yield differed between pH 5 and 6; yellow pigment (26.42 OD Units/gdfs at 412 nm) production was maximal at pH 6 and red pigment (24.18 OD Units/gdfs at 500 nm) was maximal at pH 5. 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. (8), who reported maximum pigment production by *M. purpureus* at pH 4.5 to 7.5, while using jack fruit seed as substrate in solid state fermentation. *N*-acetyl glucosamine concentration was greatest at pH 5 (270 mg/gdfs) followed by pH 6, indicating maximum growth and pigment production.

Temperature — Temperature is one of the most critical factors in SSF based on fungi cultures. Because of the mesophilic nature of *M. purpureus*, maximum growth and pigment production was obtained at 30°C (Figure 3). Results are in agreement with Domsch et al. (19) and Babitha et al. (8), who reported an optimum temperature of 30°C to 37°C for *Monascus* sp. An interesting observation is that the maximum absorbance shifted to 390 nm (which corresponds to yellow pigments) when the temperature was above 30°C. Babitha et al. (8) and Carvalho et al. (20) similarly reported shifts in absorption maxima at different incubation temperatures. Maximum pigment production occurred at 30°C (41.14 OD Units yellow pigment/gdfs at 412 nm and 25.52 OD Units red pigment/gdfs at 500 nm); production decreased drastically at higher temperatures. At 30°C, maximum *N*-acetyl glucosamine concentration was 160 mg/gdfs.

Inoculum size — Numerous studies have shown the large influence of inoculum size on product yield in SSF and our observation was similar. Inoculating with 4 mL of spores/gram of initial dried substrate (gds) maximized yellow pigment yield (28.12 OD Units/gdfs at 412 nm), followed by red pigment (22.14 OD Units/gdfs at 500 nm), with a biomass of 220 mg/gdfs (Figure 4). These results are in agreement with previous studies (8, 21, 22). Too little inoculum resulted in insufficient biomass and smaller amounts of product, whereas too much inoculum produced excessive biomass and depleted the nutrients required for pigment formation (8).

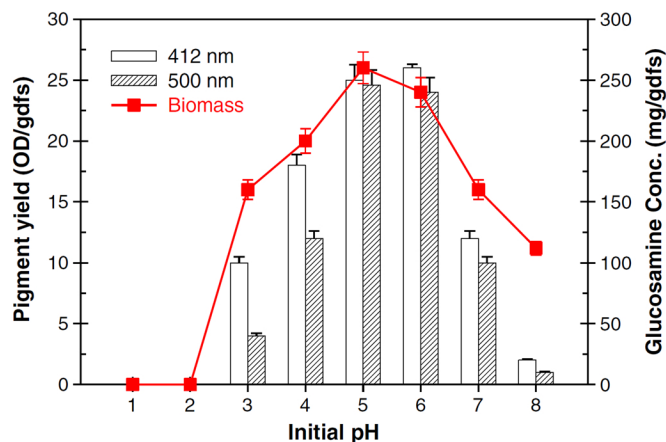


Figure 2. Growth (glucosamine concentration) and pigment yield (optical density 412 and 500 nm) of *M. purpureus* in solid state fermentation at different initial substrate pH, incubation temperature, inoculum size, incubation period and initial moisture content.

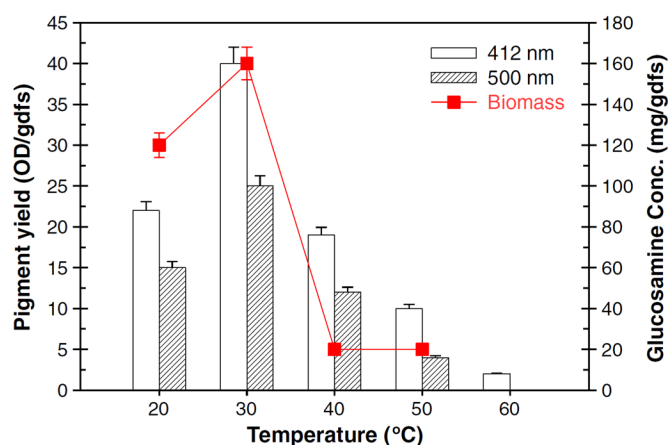


Figure 3. Growth (glucosamine concentration) and pigment yield (optical density 412 and 500 nm) of *M. purpureus* in solid state fermentation at different incubation temperatures, inoculum size, incubation period and initial moisture content.

Incubation time — The amount of pigment produced varied with incubation time (Figure 5). Maximum yellow and red pigment production was obtained at 168 h (33.42 OD Units/gdfs at 412 nm and 15.28 OD Units/gdfs at 500 nm, respectively), with a biomass of 199 mg/gdfs at 144 and 168 h. Production decreased from 192 to 264 h, likely due to the decline phase of the fungus.

Moisture content — Substrate moisture content plays a key role in fungal growth, enzyme activity, and metabolite production in SSF (18, 23–25). The effect of substrate moisture content on red pigment production and biomass was presented in Figure 6. Yellow and red pigment production was maximal at 60% moisture content (18.92 OD Units/gdfs at 412 nm and 14.26 OD Units/gdfs at 500 nm, respectively). This can be attributed to effective utilization of sugars in the substrate. Results are consistent with Yongsmith et al. (18), who reported maximum glucoamylase activity and pigment production by *Monascus* sp. KB9 at 60% moisture. Pigment yield decreased above or below 60% moisture. The lower yield at high moisture content is due to agglomeration of substrate, reducing oxygen supply for *M. purpureus*. The decrease in pigment production at low moisture content is a result of low nutrient availability due to reduced nutrient salt dissolution, as well as less efficient heat exchange and oxygen transfer (8, 26). Results are similar to those of Johns and Stuart (15) and Babitha et al. (8), who reported reduced pigmentation at substrate moisture content below 40%.

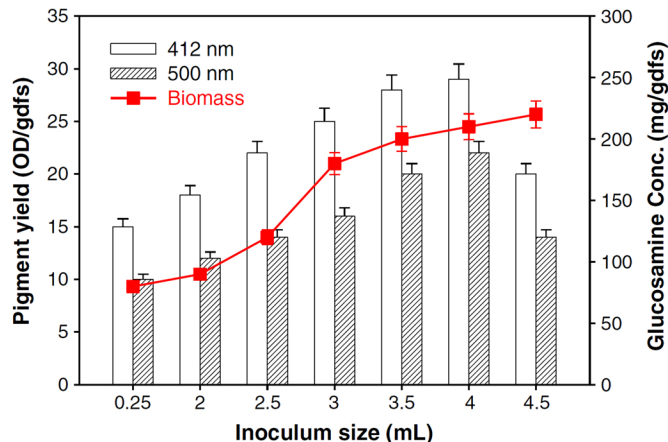


Figure 4. Growth (glucosamine concentration) and pigment yield (optical density 412 and 500 nm) of *M. purpureus* in solid state fermentation in response to inoculum size, incubation period and initial moisture content.

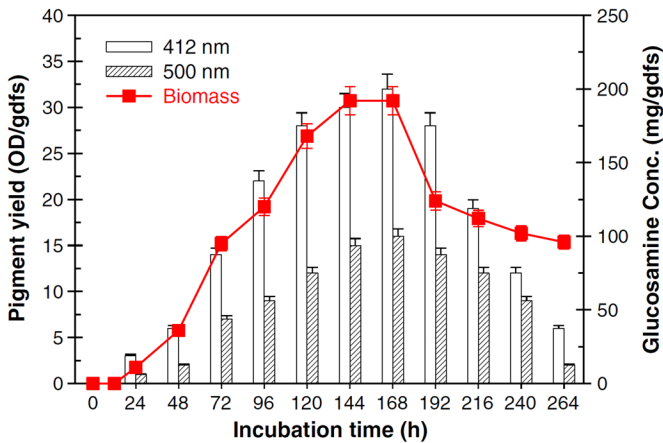


Figure 5. Growth (glucosamine concentration) and pigment yield (optical density 412 and 500 nm) of *M. purpureus* in solid state fermentation in response to incubation period and initial moisture content.

Pigment stability — The extracted *Monascus* pigments were subjected to various physical and chemical treatments and results are presented in Table 1. The pigment is soluble in water and color was red at 500 nm. The pigment powder is essentially non-hygroscopic in nature and the Munsell hue test indicated a red color. The original color was retained at pH 5, 6 and 7. Under more acidic conditions (pH 1–4) the pigment lost its color and precipitated at the bottom of the tubes; the color changed at alkaline pH (pH 8–14). A brown shade was observed at pH 8, orange at pH 9, yellow at pH 10, and pale red at pH 14. The color change can be attributed to protonation/dissociation below/above the molecular dissociation constant of the pigment molecules. The presence/absence of color for a specific pigment is a function of pH due to ionization of aromatic —OH groups and tautomerism of —O(—) with =O. Changes in the relative proportions of dissociated/undissociated molecules (with respective colors) would produce the resulting coloration, such as orange at pH 9, yellow at pH 10, and red at 14.

Thermostability of the pigment was determined at 40°C to 100°C (Table 1). The constancy of absorbance at 500 nm indicates thermostability. Color was also retained when the pigments were subjected to steaming and sunlight exposures. The pigment was stable in solution containing up to 0.5% (w/v) sodium chloride and ammonium chloride. However, pigment color changed to brown during 12 h of exposure to hot air.

Table 1. Red pigment properties and stability after various treatments.

Parameter	Properties	Observed stability	Relative stability (%)
Water solubility	Soluble	—	—
Color (λ_{max})	500 nm	—	—
Hygroscopy	Little	—	—
Hue	Dark red	—	—
pH 2–14 (10 min)	—	pH 4, 5, 6, 7	99.0
Dry heat (60°C for 12 h)	—	—	86.2
121°C (20 min)	—	—	95.9
UV light (12 h)	—	—	99.2
Sun light (2 h)	—	—	99.4
Sodium chloride (0.1–1% w/v, pH 7.0, 1 h)	—	0.1, 0.2, 0.3, 0.4, 0.5, 0.6%	96.1
Aluminum chloride (0.1–1% w/v, pH 7.0, 1 h)	—	0.1, 0.2, 0.5%	90.2

—, Not applicable

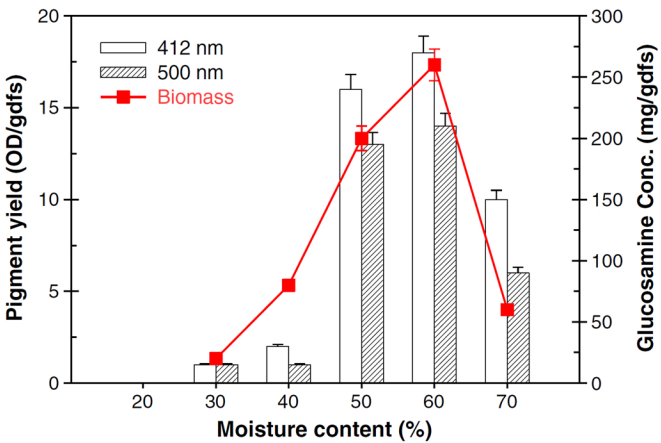


Figure 6. Growth (glucosamine concentration) and pigment yield (optical density 412 and 500 nm) of *M. purpureus* in solid state fermentation in response to initial moisture content.

The results of our study indicate the feasibility and applicability of the corn cob, an agricultural byproduct, for SSF production of pigments from *M. purpureus* KACC 42430. The highest yield of the pigments (25.42 OD Units/gdfs) indicates that corn cob powder is an effective substrate for SSF. As mentioned above pulverized corn cob enhance the enzymatic digestibility of *M. purpureus*, resulted in promoting greater pigment production. This enhanced pigment production compared to previously reported substrates. The corn cob is economical and environmentally safe to end users. To our knowledge this is the first report on pigment production using corn cob powder in SSF. Our future work will focus on the chemical nature of the pigments (isochromene derivatives, specifically azaphilone compounds) and the toxin citrinin, a byproduct of the fermentation process.

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