

# Stability Study of the Pigment Extract from a Wild *Pycnoporus sanguineus*

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**Abstract:** The stability of crude pigment of *Pycnoporus sanguineus* strain 28cc under different conditions was assessed. The strain was isolated from the fruit body of the fungus collected in Fenglin National Nature Reserves (China) and had been identified by morphological and rDNA-ITS based approach. The natural pigments were obtained from cultural liquid of submerged culture by adsorption of macro-porous resin (HPD-722) and eluted with 80% ethanol. The natural colorants had a characteristic absorption peak of 416nm (corresponds cinnabarine) used for determination of pigment stability. *P. sanguineus* pigment tolerant to high temperatures and could keep stability during pasteurization temperature. It had orange-red colors in both acidic and alkaline environment, as well as exhibits good stability at pH 4-7. In the alkaline environment, pigments showed increased in absorbance and deepness in color. *P. sanguineus* pigments could tolerate to various metal ions ( $K^+$ ,  $Na^+$ ,  $Cu^{2+}$ ,  $Zn^{2+}$  or  $Al^{3+}$ ) and displayed strong stability to reductant, but oxidant result in a certain degree of fading. The result from this study indicated that *P. sanguineus* pigment displayed good stability under various conditions, which is the basis of their market potential. The obtained characteristics of pigment stability of this strain can be used for development of production of natural pigment.

**Keywords:** *Pycnoporus sanguineus*, Pigment, Stability, Fungi, Identification

## 1. Introduction

With the continuous improvement in our understanding of the molecular, pharmacological and physiological effects of food additives on human health, the security of food additives have attracted much attention. Research have carried out by many scientists, had reported that most artificially synthetic pigments have adverse side effects that may be carcinogenic or even toxic [1]. Due to this reason, the synthetic colorants in food will gradually be limited and eventually phased out. Meanwhile, natural source pigments are becoming more and more attractive, largely due to their natural superiority over artificial pigment, including their nutritional value, pharmacological effects, as well as the higher values in food safety [2-5].

With short production cycles, low space requirements and a

wide range of sources, microorganism-derived natural pigments could show greater capacity to meet the low-cost and high yield requirements of the market [6, 7]. There are dozens ways to successfully produce and apply natural pigments, especially natural pigments extracted from microorganism, which have as few kinds as *Monascus* [8-10]. Thus, the research and exploration of natural pigments is particularly important.

One of the key elements in the successful development of a natural food colorants is its stability, which may be affected by many factors in the environment, including light, temperature, oxidants, pH, metal ions as well as various additives [11, 12].

*Pycnoporus sanguineus* (the family of *Polyporaceae*), is a white-rot saprobic fungus and one of a promising organisms for developing of natural pigments. At least, seven pigments containing the phenoxazone chromophore have been identified amongst the metabolites of *P. sanguineus*, and

produce various shades of red, orange, yellow, and brown color [13].

The fungus exhibits many beneficial pharmacological effects, including promoting tissue regeneration, assisting gas and blood transport, dissolving phlegm, eliminating rheumatism, relieving itching, guiding gas downward, as well as hemostasis and cancer cell inhibition. While many recent publications on *P. sanguineus* have focused on ligninolytic laccase enzymes [12-15], little attention has been paid to pigments produced during its fermentation [16].

The experiment was to isolate the strain of *P. sanguineus* from natural environment and study the stability of natural pigment obtained from its submerged culture under effect of various physical and chemical factors.

## 2. Materials and Methods

### 2.1. Isolation of Wild *P. sanguineus* Strain

The fruiting body of wild *P. sanguineus* was collected from the Fenglin National Nature Reserves of Heilongjiang province. After surface sterilization with 75% ethanol, an incision was made on the collected fruiting bodies of *P. sanguineus* using a scalpel at a sterile bench. The deep-seated, uncontaminated mycelium was then removed aseptically and immediately placed into a plate containing PDA medium (Potato Dextrose Agar containing 200g/L potato, 20g/L glucose and 15~20g/L agar) for culturing. Regular observation on colony growth status and further purification on PDA plates were carried out, and the resulting pure strain of *P. sanguineus* was obtained and stored in the test tube.

### 2.2. rDNA-ITS Based Strain Identification

The purified strain was cultured in PDA liquid medium on a shaking platform at 28 °C for 7 days. DNA was subsequently extracted using the CTAB method [17]. 1μL fungal DNA was amplified for the ITS-rDNA sequence using the “universal” fungal primers ITS1/ITS4 (forward primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3', reverse primer ITS4: 5'-TCCTCCCTTATTGATATGC-3') in a final volume of 25μL, including 17.5μL ddH<sub>2</sub>O, 2.5μL 10×Ex Taq Buffer, 1.5μL dNTP, 0.5μL Ex Taq enzyme, and 1μL for each ITS1 and ITS4 primers. The amplification protocol was set at 7 min initial denaturation at 94.0 °C and then 35 cycles of 1min denaturation at 94.0°C, 0.5min annealing at 56.5°C, 1 min extension at 72.0°C, 7 min termination of extension at 72.0°C. The PCR products were separated by electrophoresis on a 1% agarose gel and isolated bands were recovered by Agarose Gel DNA Purification Kit (TaKaRa, USA). The purified PCR products were ligated to the pMD18-T vector, and transformed into JM109 competent cells. Transformed cells were screened using ampicillin-containing plates and isolated colonies were cultured in 1.5mL LB for amplification. Bacterium PCR was carried for validity. The recombinant clones were sequenced in Sangon (Sangon Biotech, Shanghai), and sequences were analyzed using BLAST (Basic Local Alignment Search Tool) to

search for similarity with the sequences deposited in Gene Bank, NCBI (<http://blast.ncbi.nlm.nih.gov/>).

### 2.3. Preparation of *P. sanguineus* Pigments

Scaled-up cultures of the purified strain 28cc were grown at 28°C in shake flasks (140 turn/min) containing PDA liquid medium. After 15 days, the fermentation broth was filtrated by four layers of gauzes and the mycelium was discarded. The orange crude pigment was then recovered by putting the filtrate through macro-porous resin (HPD-722) for adsorption for 12h at room temperature, and eluted with 80% ethanol.

### 2.4. Characteristic Absorption Wavelength of *P. sanguineus* Pigments

The crude pigment extract was subjected to spectral scanning through a UV-vis spectrophotometer from 300 nm to 800 nm to obtain its characteristic absorption peak, then used for subsequent determination of pigment stability. The absorbance at the wavelength with a characteristic absorption peak was used to determine the pigment concentration, i.e., higher absorbance value indicates higher concentration. In addition, the color value (CV) was determined at the characteristic absorption wavelength to evaluate the effects of macro-porous resin treatments. The crude pigment extract after macro-porous resin adsorption was rotary evaporated. 0.1g of which were weighed (with accuracy of 0.0001) and dissolved to 100mL, absorbance at the characteristic wavelength of  $\lambda_{\max}$  was determined and CV was calculated according to formula:

$$CV = E_{1\text{cm}}^{1\%} \lambda_{\max} = \frac{A \times n}{m} \times \frac{1}{100}$$

CV - the color value, A - absorb light readings, n - the dilution factor and m - express quality.

### 2.5. Determination of *P. sanguineus* Pigment Stability

#### 2.5.1. Influence of pH on the Pigment Stability

Using HCl and NaOH, the pH value of the pigment solution was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 respectively. After incubating for 1 h at a room temperature (RT, 20°C), the absorbance at characteristic wavelength pick of the solution was determined.

#### 2.5.2. Effect of Metal Ions on the Pigment Stability

0.25 mol/L of K<sup>+</sup>, Na<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> or Al<sup>3+</sup> was added to the crude pigment solution, which was buffered to a pH=7 using HCl and/or NaOH. The solution was held for 1 h at RT and subjected to absorbance determination at characteristic wavelength.

#### 2.5.3. Influence of Temperature on the Pigment Stability

Regulating the pH value to 7, the pigment solution was incubated at RT, or in 40°C, 60°C, 80°C, and 100°C water-baths for 1 h. After cooling to RT, the absorbance at characteristic wavelength was determined.

### 2.5.4. Influence of Oxidant/Reductant on the Pigment Stability

The pH value of the pigment solution was regulated to 7, and in term of oxidant, H<sub>2</sub>O<sub>2</sub> of 0.3%, 0.6%, 1.5% and 3% (v/v) was added, while Na<sub>2</sub>SO<sub>3</sub> of 0.02 g/L, 0.04 g/L and 0.06 g/L were used to test the effect of reductant. After holding for 1 h at RT, the absorbance at characteristic wavelength of the solution was determined.

## 3. Results

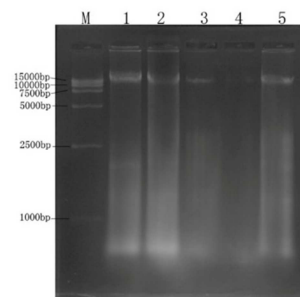
### 3.1. The Isolation and Identification of *P. sanguineus* Strain

The fruiting bodies of wild *P. sanguineus* were sessile and coated with hydrophobic suberin. Its pileus was 3-5 cm in diameter and approximately 3-6 mm thick, with no fine fur on its surface. The tubule showed the same dark red color with small pipe orifice and circle (Figure 1). It is consistent with the description of *P. sanguineus* [18].

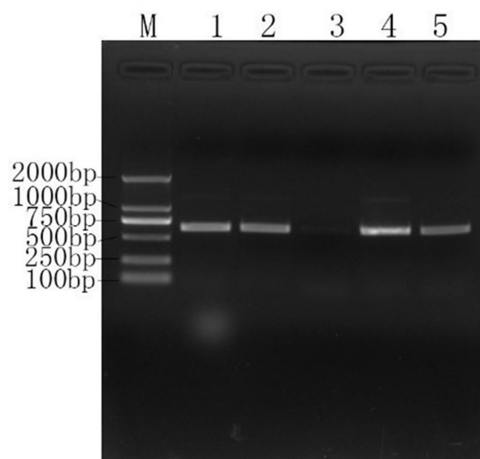


**Figure 1.** A schematic representation of Sporocarp of a wild *P. sanguineus* collected from Fenglin National Nature Reserves.

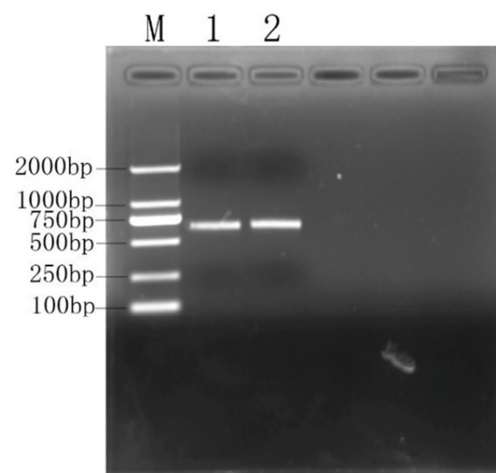
For rDNA-ITS based strain identification the mycelium genome was extracted by CATB method and separated. Genomic products greater than 15kb could be observed (Figure 2). PCR products on an agarose gel showed strong band intensity, between 500 and 750bp, at approximately 650bp (Figure 3). The recovered PCR products were inserted into the pMD18-T vector and transformed into competent cell JM109. After appropriate culture and selection, PCR detection on bacteria cells to confirm the inserted clone, was conducted and displayed intense clear and bright single band as showed (Figure 4). This suggested that approximate 650bp ITS-rDNA had been successfully inserted into the pMD18-T vector, and the transformed JM109 was sent to Sangon (Sangon Biotech, Shanghai) for sequencing. The returned sequence was queried against GenBank (nr database) using blast, and 99% similarity with *Pycnoporus sanguineus* was reported. Combined with observed morphological characteristics, the strain 28cc is *P. sanguineus*. The sequence had been deposited in GeneBank with accession number of KC920825.



**Figure 2.** The image of Gel electrophoresis of the purified strain genome DNA extracted by CTAB analysis.



**Figure 3.** The image of Gel electrophoresis of PCR products.

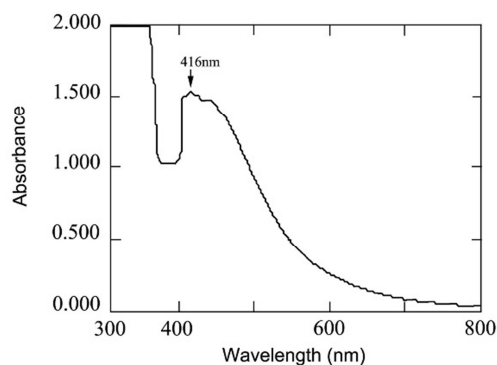


**Figure 4.** The image of Gel electrophoresis of bacterium PCR.

### 3.2. Characteristic Absorption Wavelength and Composition of Pigments

The isolated crude pigments from submerged culture of *P. sanguineus* exhibited an orange-red color. The spectrum of the natural pigments displayed a wide range of absorption wavelengths (Figure 5). Within this spectrum, the strongest characteristic absorption wavelength was found to be at 416 nm (Figure 5). This peak corresponds to cinnabarine. Cinnabarine is a major pigment of *P. sanguineus* which can

form approximately 50% of the seven its pigments [16]. The purification of crude pigment by treatment of cultural liquid by the macro-porous resin shows in the increase CV value of the pigment solution from 2,59 to 19,06 (in 6,36 times) that is good value for the market.

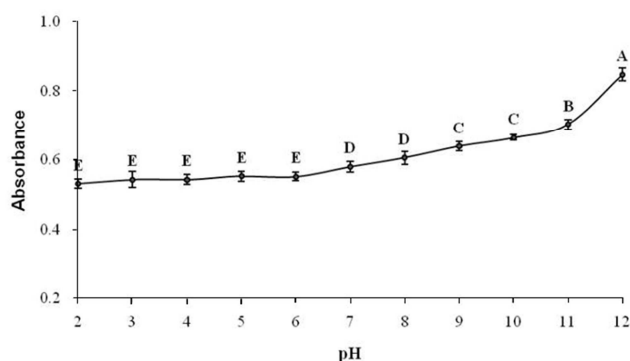


**Figure 5.** The absorption spectrum of the crude pigment extracts from a UV-vis spectrophotometer.

### 3.3. Pigment Stability

#### 3.3.1. The Stability of *P. sanguineus* Pigment at Different pH

The absorbance of the crude pigment extract increased with increasing pH (Figure 6), with a corresponding deepening in color. In an acidic environment (pH=2-7), changes in absorbance varied slightly (0.528-0.587 optical density (OD)). In an alkaline environment (pH=7-12) with elevation values of pH, the absorbance displayed a increase from 0.587 to 0.850 OD. Thus, exposure to strong alkaline resulted in a stronger effect on pigment absorbance. This suggests that pigments from *P. sanguineus* remained stable under acidic environments and relatively weak in alkaline conditions, the pigment became unstable under strong alkaline circumstances.

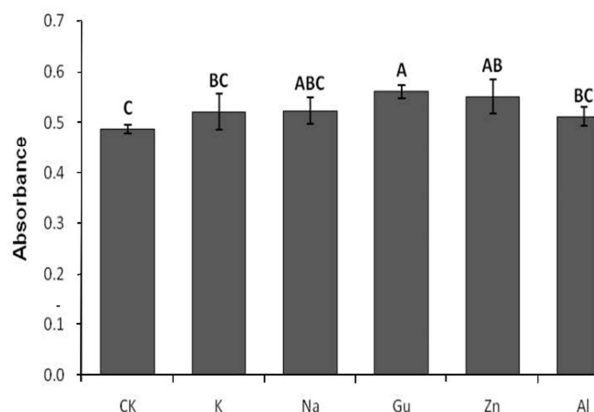


**Figure 6.** Absorbance of pigment as a function of pH values. Each absorbance value is an average of three replications and the error bar shows a standard deviation. Different letters denote significant differences ( $P \leq 0.05$ ) among different treatments.

#### 3.3.2. $\text{Cu}^{2+}$ and $\text{Zn}^{2+}$ Augment Absorbance of *P. sanguineus* Pigments

$\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Al}^{3+}$  did not significantly affect the absorbance wavelength of the crude pigments, implying that *P. sanguineus* pigments had a good tolerance to  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Al}^{3+}$

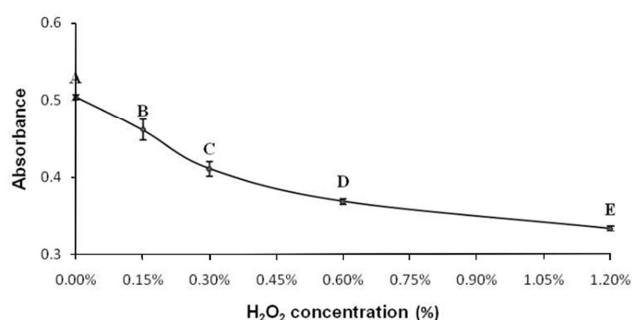
at low concentrations (Figure 7). Nevertheless, with the addition of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , the absorbance of *P. sanguineus* pigment was significantly enhanced, suggesting that  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  may have a hyperchromic effect on *P. sanguineus* pigment.



**Figure 7.** The effect of metal ions on pigment absorbance. The columns represent the average of three replications and bars show the standard deviation. Different letters denote significant differences ( $P \leq 0.05$ ) among treatments.

#### 3.3.3. Sensitivity of *P. sanguineus* Pigments to High Temperatures

Heat stability is critical to minimizing color degradation. *P. sanguineus* pigment solutions exposed to temperatures between 20°C and 60°C demonstrated no significant change in absorbance (Figure 8). At the temperatures range between 60°C and 100°C, the absorbance decreased dramatically with increases in temperature, which suggests that exposure to high temperatures for extended periods of time may lead to the degradation of *P. sanguineus* pigments. Therefore, high temperatures should be avoided during the application process of *P. sanguineus* pigment.

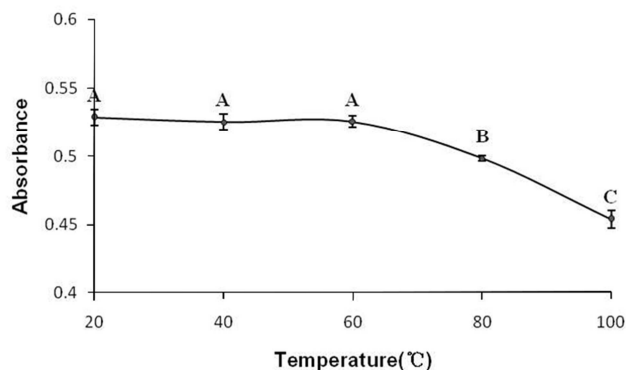


**Figure 8.** Absorbance of pigment at different temperatures (Values are averages of three replications and bars show a standard deviation. Different letters denote significant differences ( $P \leq 0.05$ ) among different treatments

#### 3.3.4. Effect of $\text{H}_2\text{O}_2$ and $\text{Na}_2\text{SO}_3$ on the Stability of *P. sanguineus* Pigment

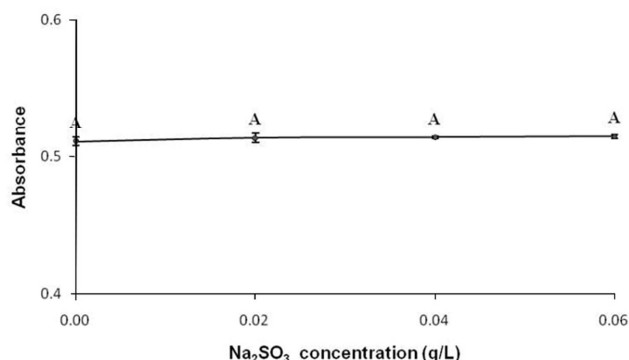
All concentrations of  $\text{H}_2\text{O}_2$  had significant effects on *P. sanguineus* pigment due to its oxidation, resulting in the decrease of absorbance (Figure 9). With the increase in  $\text{H}_2\text{O}_2$  concentration, the extent to which oxidation on *P. sanguineus*

pigment gradually increased, and when  $H_2O_2$  concentration was up to 1.2%, the absorbance of *P. sanguineus* pigments decreased by 33.7%. Therefore, the application of *P. sanguineus* pigments should avoid being exposed to oxidant such as  $H_2O_2$  due to its poor tolerance to oxidizing.



**Figure 9.** Absorbance of pigment at different concentrations of  $H_2O_2$ . Values are average of three replications and bars show a standard deviation. Different letters denote significant differences ( $P \leq 0.05$ ) among different treatments.

The tested concentrations of  $Na_2SO_3$  have no obvious effect on *P. sanguineus* pigments (Figure 10), suggesting their good tolerance to reductant such as  $Na_2SO_3$ , which implied that they could meet the reducing stability requirements in the fields of food, cosmetics, etc.



**Figure 10.** Absorbance of pigment at different concentrations of  $Na_2SO_3$ . Values are average of three replications and bars show a standard deviation. Different letters denote significant differences ( $P \leq 0.05$ ) among different treatments.

## 4. Discussion

The investigation on *P. sanguineus* pigments stability under different conditions provided a significant base to its development and application [19, 20]. Usually, under conditions of high temperature or strong acid/alkaline, or in the presence of metal ions, natural pigments display poor stability compared to artificially synthesized colorants, which restricts the application of natural pigments in various fields. Some natural pigments, such as betaxanthin, are susceptible to high temperature ( $>40^\circ C$ ) and degraded or faded during cooking or pasteurization processing [21]. *P. sanguineus*

pigment has a certain degree of tolerance to high temperatures and can keep stability during pasteurization. The long time cooking can lead to its degradation. Often natural pigments display different colors at different pH values, for example, pigment generated by fermenting *Streptomyces coelicolor* is red when put in acid environment and blue under alkaline condition [22]. *P. sanguineus* pigment shows orange-red colors in both acidic and alkaline environments and exhibits good stability at former condition, therefore, it can stably exist in a vast majority of foods or cosmetics (pH 4-7). In alkaline environment, *P. sanguineus* pigments showed increase in absorbance and deepness in color, suggesting that more consideration should be given when applying or using these pigments. In addition, natural pigments are susceptible to the effects of metal ions, catalytic action of which can lead to their decomposed discoloration or transformation into insoluble salts. This case would not occur in *P. sanguineus* pigments because of its good tolerance to various metal ions, so with catalytic decomposition by metal ions, derived from ironware during the production, transport and storage of the pigment could be circumvented. Furthermore, adding oxidant or reductants should be considered as one of the important factors that affect the stability of pigment during production. *P. sanguineus* pigment displayed strong stability to reductants, favored its application in all kinds of fields, nevertheless, adding oxidant could cause a certain degree of fading. So, adding antioxidant such as tocopherol (Vitamin E) and octyl palmitate during the application of *P. sanguineus* pigment could assure its stability [23].

The pharmacological effects of *P. sanguineus* pigments are equally interesting and may provide an additional consideration in their use. For example, the antibiotic pigment cinnabarine has been shown to exert anti-microbial activity against bacteria from both humans and food [24, 25], as well as protective anti-viral effects when administered to mice in vivo [25]. The 2-amino-phenoxazin-3-one type pigments from *P. sanguineus* may have anti-inflammatory effects through modulation of immune subsets and protect against cancer [26, 27]. Although the exact effects remain to be determined, these active properties will simultaneously make the *P. sanguineus* pigments an attractive candidate as a dye potentially exclude them from certain applications.

Several natural dyes that provide either a red-orange or red color have been identified and are currently used in the pharmaceutical, cosmetic, and food industries; these include annatto (derived from the seed of the achiote), as well as cochineal and carmine (derived from cochineal insects). However, these compounds can induce an adverse hypersensitivity response and have been linked to various cases of anaphylaxis, urticaria, and angioedema in sensitive individuals [28]. Other work has proposed robust applications for natural red pigments derived from *Monascus ruber* (*M. ruber*) in the food industry [29, 30]. A recent study investigating red and orange pigments, produced by *M. ruber* in submerged fermentation, which have revealed properties that are complementary to those identified by us in the *P. sanguineus* pigments, including greater stability at alkaline pH [31]. Thus,

purified pigments from *P. sanguineus* may potentially serve as an ideal alternative to other natural and synthetic pigments for red-orange coloration under certain conditions.

## 5. Conclusion

*P. sanguineus* pigment displayed good stability under various conditions, which is the basis of their market potential. The obtained characteristics of pigment stability of this strain can be used for development of production of natural pigment.

The production and purification of the stable pigments following fermentation of fungi has several advantages over the chemical production of synthetic dyes in that it is environmentally friendly, it is performed at low temperatures (28°C), and does not involve expensive chemicals or strong acids/alkali. To date, the only pigment that are produced economically through fermentation are beta-carotene and riboflavin. The process of optimizing fermentation conditions to increase the yield of *P. sanguineus* pigments will be helpful in promoting an economical, large-scale production program and may contribute to a better understanding of this natural material.

Finally, stringent refinement methods must be established for quality control purposes to guarantee the safety and purity of the extracted *P. sanguineus* pigments. Further studies should also be conducted to establish and/or rule out possible adverse effects in humans.

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