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OPTIMIZATION OF FERMENTATION PARAMETERS TO ACHIEVE MAXIMUM YIELD OF BIOCOLOR FROM FUNGAL STRAINS

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ABSTRACT

Color is the main feature of any food item as it enhances the appeal and acceptability of food. During processing, a substantial amount of color is lost, and makes any food commodity attractive to the consumers; synthetic or natural colors are added. The main food biocolorants are carotenoids, flavanoids, anthocyanidins, chlorophyll, betalain and crocin, which are extracted from several horticultural plants. In addition to food coloring, biocolorants also act as antimicrobials, antioxygens and thereby prevent several diseases and disorders in human beings. Although, biocolorants have several potential benefits, yet tedious extraction procedures, lower color value, higher cost than synthetic dyes, instability during processing etc., hinder their popularity. Although, it is presumed that with the use of modern techniques of biotechnology, these problems in extraction procedures will be reduced. To meet the growing demand, more detailed studies on the production and stability of biocolorants are necessary while ensuring biosafety and proper legislation. Therefore, the present study is one of the approaches that, to isolate a new strain and optimization of fermentation conditions in order to produce appreciable amounts of biocolor.

Keywords: biocolorants, isolation, submerged, fermentation, optimization

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INTRODUCTION

Color becomes the most sensitive part of any commodity not only for its appeal but also it enhances consumer acceptability. In addition, the color of a food substance is important to indicate its freshness and safety that is also indices of good aesthetic and sensorial values. The demand for natural source of such compounds is increasing day by day because of awareness of positive health benefit out of natural compounds. It therefore, necessitates looking into natural

sources of food grade colorants and their use potentials. It is found more justified to use the term biocolorant instead of biopigment. Since the pigments are mostly water insoluble with exceptions of certain pigments of biological origin (PritamChattopadhyay*et al.*2008). Color is the main feature of any food item as it enhances the appeal and acceptability of food. During processing, a substantial amount of color is lost, and makes any food commodity attractive to the consumers; synthetic or natural colors are added. The main food biocolorants are carotenoids, flavanoids, anthocyanidins, chlorophyll, betalain and crocin, which are extracted from several horticultural plants. In addition to food coloring, biocolorants also act as antimicrobials, antioxygens and thereby prevent several diseases and disorders in human beings. Although, biocolorants have several potential benefits, yet tedious extraction procedures, lower color value, higher cost than synthetic dyes, instability during processing etc., hinder their popularity. Although, it is presumed that with the use of modern techniques of biotechnology, these problems in extraction procedures will be reduced, yet to meet the growing demand, more detailed studies on the production and stability of biocolorants are necessary while ensuring biosafety and proper legislation (Rymbai *et al*, 2011). The controversial topic of synthetic dyes in food has been discussed for many years. The scrutiny and negative assessment of synthetic food dyes by the modern consumer have raised a strong interest in natural coloring alternatives. The continuous use of synthetic colors in textile and food industry has resulted in many toxic diseases such as cancer and even some synthetic dyes in textile leads to environmental degradation. So nowadays the best choice to overcome such problem is "biocolor". Since the biocolors are the natural colors which have increased demand, not only for the safety of health and environment but also for their beauty and novelty. Hence, there is worldwide interest in process development for the production of pigments from natural sources. The existing authorized natural food colorants are of either plant or animal origin and have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility, and are often non-availability throughout the year. There are number of microorganisms which have the ability to produce pigments in high yields, among which certain fungi have been reported to produce non-carotenoid pigments but only a few of those have been explored as possible food colorants such as *Monascus, Paecilomyces, Serratia, Cordyceps, Streptomyces* and Yellow-red and blue compounds produced by *Penicillium herquei* and *Penicillium atroventeum*. Today, the major product of the *Monascus* fungus is a natural food coloring often substituted for synthetic food colors because of food safety concerns (Su *et al*. 1973).

MATERIALS AND METHODS

Collection of samples

The survey for isolating some of the untapped wild strains was carried out. In the survey, the areas covered were very diverse, major focused on the areas that were never or very minimally surveyed. So different soil samples in and around Bengaluru district from diversified habitats were collected for the isolation of biocolor producing micro-organisms.

Isolation of fungi for biocolor production

Samples collected from the said location were brought to the laboratory in sterile polythene bags. These samples were aseptically plated on Potato Dextrose Agar (PDA) media by using 0.1 mL of sample (by serial dilution method) was to avoid the overcrowding of the organisms on the plates. The plates were incubated for 48-72 hrs at 28°C. The plates were exhibited isolated colonies of

varied fungi. The isolates obtained were preserved in PDA slants at a temperature of 4°C (Aneja, 1993). Further, the tentatively identified strains were labeled serially as **FS1 to FS20.**

Rapid plate assay for screening of biocolor producer

The fungal isolates obtained from the above steps were further subjected to rapid plate assay for screening of biocolor producing isolates on Sabourauds Dextrose Agar (SDA) media. The plates containing SDA medium were inoculated with the spore suspension of the fungi and incubated at 28°C for a period of one week. The control plate was maintained. The isolate which gave maximum production of biocolor was selected for further studies.

Preparation of inoculum

A spore suspension was prepared from 168 hrs old culture grown on PDA slants by adding 10 mL of sterile distilled water containing 0.1% tween-80 directly over the slants and suspending the spores with a sterile loop. The spore suspensions were standardized to 1x10 spores per mL by addition of sterile distilled water (Lingappa and VivekBabu, 2005). 1 mL spore suspension was used to inoculate 100 mL of experimental medium.

Optimization parameters for enhancement of pigment yield

The process development is the key step in any basic fermentation criteria process development refers to the up-gradation of the factors that influence the metabolic producing capacities of the organism, it involves optimizing the production parameters and suitably enabling the organism into a potent producer of the required metabolite in its best possible quality and concentration the organisms vary in their abilities to produce the required pigment. The extent of pigment production is depend upon a variety of factors, including the makeup of the fermentation medium, particularly composition of the medium and how it was prepared, its physical and environmental conditions. During this optimization process once a particular parameter was optimized the same optimum condition of that specific parameter will be employed in the subsequent studies wherein another parameter is to be optimized. The following parameters were studied to enhance the pigment production.

Optimization of incubation temperature

Five conical flasks containing 100 mL of the SDB media with an optimized pH was inoculated with the spore suspension of fungal culture. The flasks were incubated for a period of 10 days at different temperatures, 20°C, 24°C, 28°C, 32°C, 36°C. Similar conditions were also provided for control strain as to compare the biocolor production.

Optimization of initial pH of the media

Five conical flasks containing 100 mL of the SDB media with initial pH range from 2.0 to 6.5 with a difference of 0.5 were inoculated with fungal spores and incubated at 28°C for a period of 10 days. During the process of fermentation, a standard culture of *Monascus ruber 2326 (*MTCC strain from Chandigarh) was employed as positive control to compare the pigment production abilities of the isolate.

Optimization of Carbon source

The fermentation medium with two different sugar i.e. dextrose and sucrose (30 g/L, 40 g/L and 50 g/L) was tested for optimizing the suitable carbon source. Optimized initial pH and

temperature was adjusted. Similar conditions were also providing for control strain for biocolor yield. The fundamental idea of overall process optimization is to enhance the product yield by taking the optimal physiological conditions.

Pigment extraction

To extract the fungi, the fermented broth was filtered with a filter paper (0.5 μm). After filtration, the paper was rinsed with distilled water several times. The filtered broth was added to 250 mL conical flask along with 20 mL of methanol. The mixture was left to shake for 15 min at 200 rpm to leach the pigments into the solution. 10 mL of culture broth was taken and centrifuged at 6000-10000 rpm for 10 min. The pigment was extracted using ethyl acetate (De Moss and Evans 1959). Supernatant was poured into a separating funnel and 10ml of ethyl acetate was added to it. After formation of two layers, the lower layer was taken and dissolved in 5-10 mL of acidified methanol (1 mL of 1N HCl: 24 mL of methanol). The mixture was then shaken and after releasing its gas the funnel was placed into holder in order to separate the organic and aqueous phases. The organic layer contains the desired pigment. The pigment containing layer was drained in a Petri plate and allowed to evaporate at 60% for overnight. The resulting pigment was obtained as a crude extract.

Analysis of purity of sample

To check the purity of sample, the ethyl acetate and methanolic fractions were spotted on a TLC (Thin Layer Chromatography) plates and developed using n-hexane: ethyl acetate (5:7) solvent system.

Stability test of pigment towards pH changes

The effect of pH on the stability of the pigment extracted from fermented broth was carried out by adjusting the pH at extreme acidic pH (2.0) and extreme alkaline (13.0) using 0.1N HCl and O.1N NaOH respectively.

RESULTS AND DISCUSSION

Isolation of fungi for biocolor production

The isolation pattern of fungi is presented in table1. In the present study, 20 isolates were isolated and named serially from FS1 to FS20. Amongst the samples used for the isolation of fungi, soil from rotten vegetables yielded maximum number of isolates (12), whereas rotten fruits, garden soil and agricultural soil yielded 5, 2, 1 isolates respectively.

SL.NO	SOURCES	NO. OF FUNGAL ISOLATES
01	Garden Soil	02
02	Agricultural Soil	01
03	Rotten Vegetables	12

04	Rotten Fruits	05

Table 1: Isolation pattern of fungal isolates for biocolor production

Rapid plate assay for screening of biocolor producer

The results from plate assay are presented in Figure 1. The results revealed that all isolates showed different range of zone of diameter. Therefore, for the convenience, the grouping of strains of fungi has been done on the basis of zone of diameter they exhibited. It is proposed that the strain exhibiting zone of diameter above 0.9 mm are referred as good or high bicolor producers, those strains with zone of diameter 0.6 to 0.9 mm and those having below 0.6mm zone of diameter may be referred to as moderate and poor biocolor producers respectively. As per this grouping the isolate FS10 exhibited higher zone of diameter and considered as potential strain for biocolor production among isolates obtained from the different samples. As such, strain FS2, FS9, FS15 can be treated as moderate biocolor producers and remaining isolates treated as poor biocolor producers.

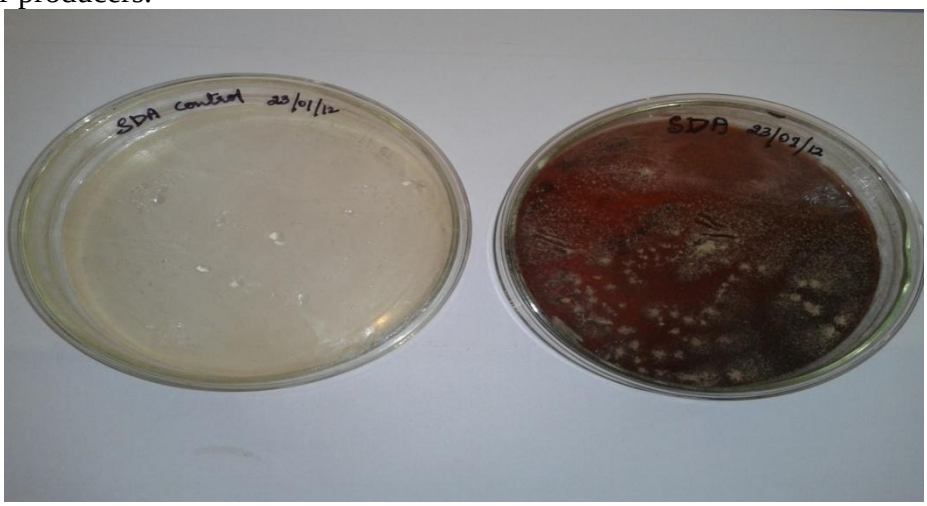


Figure 1: Rapid Plate Assay for Biocolor Production

Identification of potential biocolour producing isolate FS10

On the basis of its morphology (Figure 2) and microscopic features the strain was identified as *Penicillium sp.*

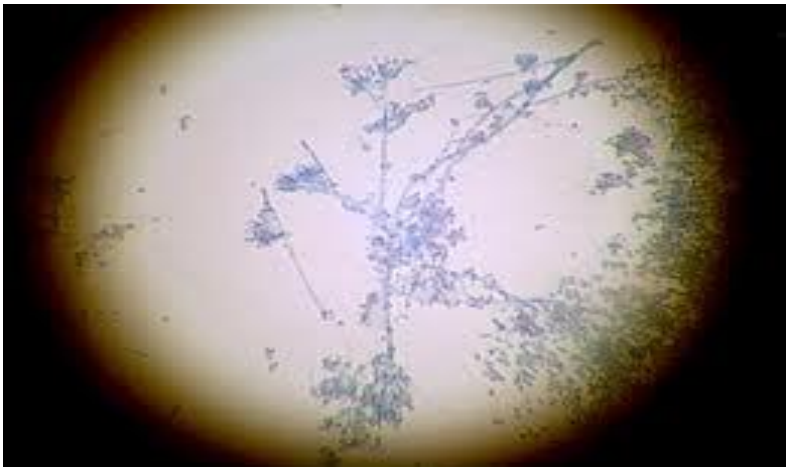
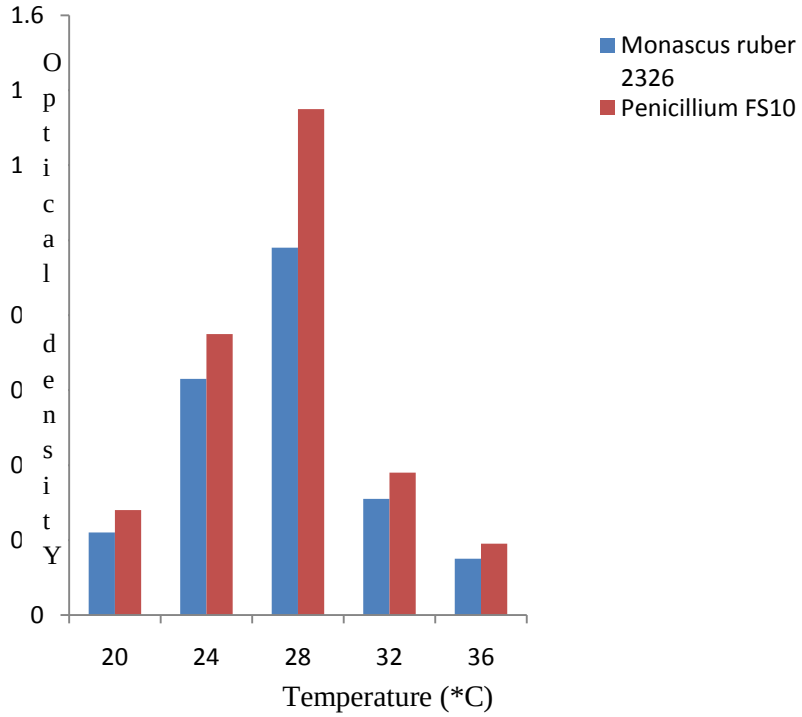


Figure 2 Microscopic appearance of *Penicillium FS10*

Effect of initial temperature on biocolor production

The effect of different initial temperature levels on the production of biocolor by *Penicillium FS10*and *Monascusruber MTCC 2326* on Sabourauds Dextrose Broth (SDB) are presented in Graph 1. The perusal of data indicated that the Biocolor production increased significantly with increase in temperature from 20°C - 36°C for *Penicillium FS10* and *Monascusruber MTCC 2326*. The decrease in biocolor production was observed above 28°C in all the days of fermentation period on Sabourauds Dextrose Broth (SDB). The maximum biocolor production of 0.1Å and 0.08Å was observed at 36°C respectively for *Penicillium FS10* and *Monascusruber MTCC 2326* after 168hrs of fermentation period.



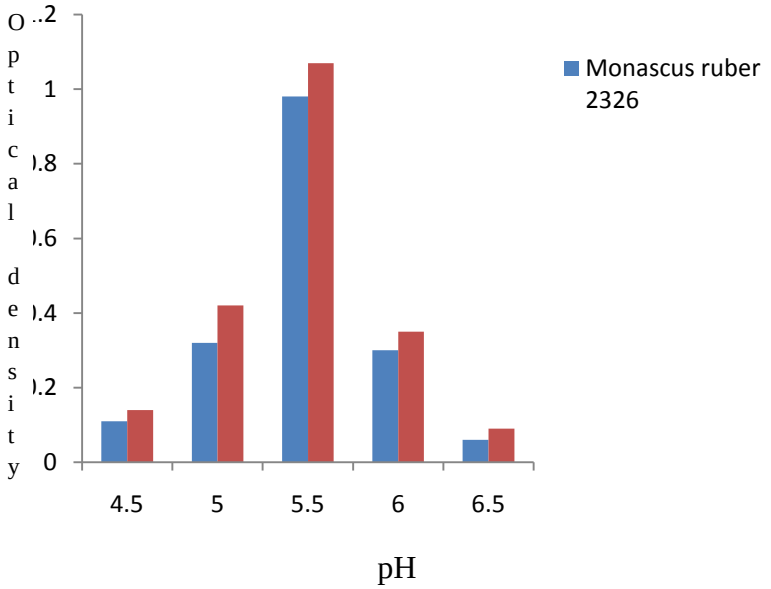
Graph 1: Effect of initial temperature on pigment production by *monascus ruber2326* and *Penicillium FS10*

Temperature (°C)	Optical Density(650nm)	
	Monascusruber 2326	***Penicillium FS10***
20	0.22	0.28
24	0.63	0.75
28	0.98	1.35
32	0.31	0.38
36	0.15	0.19

Table 2: Effect of Temperatureon Biocolor production

Effect of initial pH on biocolor production

The effects of different initial pH on biocolor production by for *Penicillium FS10* and *Monascus ruber MTCC 2326* are presented Graph 2. The effect of initial pH on the biocolor production revealed that the yield of biocolor increased with the increase in the initial pH of the media up to 5.5 units for *Penicillium FS10* and *Monascus ruber MTCC 2326* respectively, these increasing peaks were observed up to 168hrs of fermentation period and there after the yield decreased as pH levels and fermentation period increased for both the organisms. The maximum biocolor 1.07Å and 0.98Å was obtained at pH 5.5 at 72 hrs of fermentation period for both *Penicillium FS10* and *Monascus ruber MTCC 2326* respectively. The least biocolour production of 0.09Å and 0.06Å was observed at pH 6.5 respectively for *Penicillium FS10* and *Monascus ruber MTCC 2326* after 72 hrs of fermentation period.



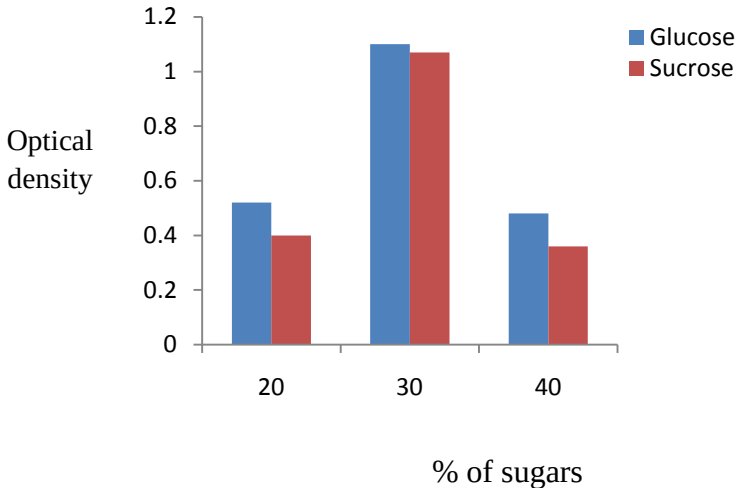
Graph 2.Effect of initial ph on pigment production by *Monascus ruber2326* and *Penicillium FS10*

pH	*Optical Density (650nm)*	
	Monascusruber 2326	***Penicillium FS10***
4.5	0.11	0.14
5	0.32	0.42
5.5	0.98	1.07
6	0.3	0.35
6.5	0.06	0.09

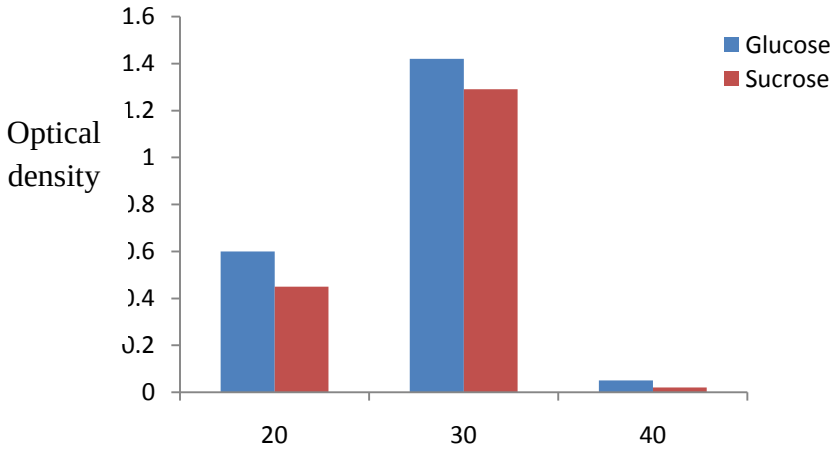
Table 3. Effect of pH on Biocolor production

Influence of carbon source on biocolor production

The results on the studies pertaining to the production of biocolor by *Penicillium FS10* and *Monascus 2326* on Sabourauds Dextrose Broth (SDB) supplemented with different concentrations of carbon sources like glucose and sucrose are presented in Graphs 3A and 3.B respectively. The process economizations for biocolor production with carbon sources supplemented to the media were carried out with concentration of 20%, 30% and 40%. The results revealed that all the carbon sources employed under the present study have enhanced the biocolor production for the tune of 1.42 Å, 1.29 Å and 1.10 Å, 1.07 Å at 30% glucose and sucrose at 168 hrs of fermentation for strains *Penicillium FS10* and *Monascus ruber MTCC 2326* respectively. In case of monosaccharide (glucose) the maximum biocolor production of 1.42 Å and minimum biocolor production of 0.05 Å was observed, whereas disaccharides like sucrose yielded maximum biocolor production of 1.29 Å and minimum biocolor of 0.02 Å.



Graph 3A. Optimization of carbon source for pigment production by *Monascusruber 2326* on 7th day of fermentation



Graph 3.B optimization of carbon source for pigment production by *Penicillium FS10* on 7th day of fermentation

Concentration (µg)	Carbon Source	
	Glucose	Sucrose
20	0.60	0.45
30	1.42	1.29
40	0.05	0.02

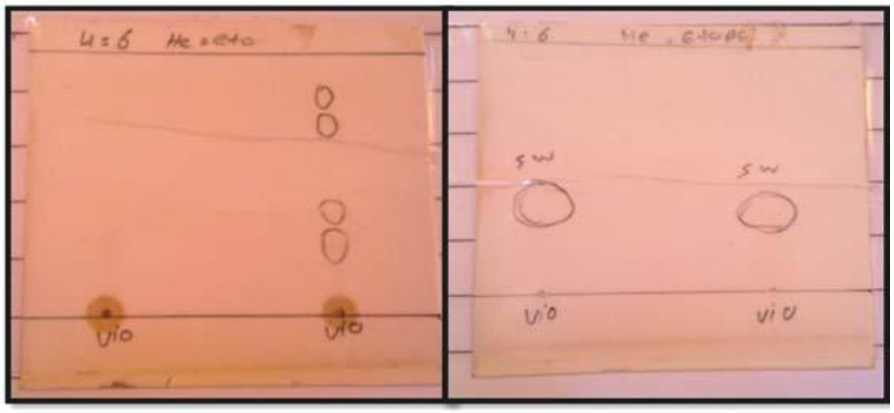
Table 4: Influence of Carbon source on *Penicillium FS10*

Concentration (µg)	Carbon Source	
	Glucose	Sucrose
20	0.52	0.4
30	1.10	1.07
40	0.48	0.36

Table 5.Influence of Carbon Source on *Monascus ruber MTCC 2326*

PURITY OF SAMPLE

TLC test showed presence of impurities in pigments with methanol, where sample collected using ethyl acetate seemed to be pure (Figure 3). This fact can be explained by assuming that present impurities in the sample extracted using methanol are highly polar; it is also known that ethyl acetate is a semi polar solvent. Therefore, by using ethyl acetate in extraction stage just poorly-water soluble pigment will migrate to the organic phase of solvent and impurities will remain inside the supernatant.



1 **2**

Figure 3.TLC result of Pigment extracted with 1) methanol and 2) ethyl acetate

CONCLUSION

Fungi from the genus *Penicillium sp.* are a promising source for natural color additive and reducing blood cholesterol. However, before effectively applying *Penicillium* to foods or dietary supplement, it is important to select and control the fermentation condition to obtain large amounts of required substances such as pigment but with little or no citrinin. Despite this toxicity

problem, *Penicillium* pigments may be quickly produced in large scale throughout the year in industrial facilities, so that it might become an industrially important pigment. The key is to find strain which produce pigment with as little citrinin as possible. In the present study, fungi as source of biocolor production are studied. Mainly due to the enhanced yield and exceptional stability of biocolor, it has a broader perceptive in varied commercial applications. Here, it aimed to isolate and select biocolor producing potent fungi, using standard screening techniques. *Penicillium FS10*, the locally isolated strain was subjected to submerged fermentation for biocolor production. The studies related to process development involving optimization of different fermentation conditions (physical and nutritional) towards enhancement of biocolor production were carried out. The present work describes the effect of incubation temperature and initial pH and also aimed to optimize the carbon source for the increased yield of biocolor production under liquid surface fermentation by *Penicillium FS10.* The results obtained are compared with those of the standard strain, *Monascus ruber 2326.*

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