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DNA amplification and characterization of pigment producing gene from *Monascus ruber*

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ABSTRACT

The aim of the present work was to investigate the feasibility of jackfruit seed powder as a substrate for the production of pigments by *Monascus ruber* in solid-state fermentation (SSF). Pigment production by the fungus *Monascus* sp. was studied to determine the more pigment in solid culture than in submerged culture. The fungi actively grown in Potato Dextrose Agar (PDA) medium. A pigment yield of 2.85 OD Units/g dry fermented substrate was achieved by employing jackfruit seed powder. Pigment production was carried out 50% initial moisture content, incubation temperature 30 °C, 1×10^6 spores/g dry substrate inoculum and an incubation period of Fourteen days. The pigments were extracted with water and ethanol. The absorbance of these pigments was analyzed in the UV-visible spectrophotometer. The absorbance of these ethanol extract was found to be higher (2.85 OD Units/g). The ethanol extract of each substrate was subjected to TLC and it was determined that extract contains both yellow and red pigments. The genomic DNA was isolated and it was electrophoresed in agarose gel and then it was eluted from the gel for further study. Then the PCR products was electrophoresed along with the DNA marker and it was viewed in the under Gel documentation system. From this we can concluded that this *Monascus ruber* pigments can be used as a natural colorant in the food industry replacing the synthetic ones. Furthermore, the isolation of the specific gene may pave the way for the isolation of novel pigments from the fungus. To the best of our knowledge this is the first report on pigment production using jackfruit seed powder in solid-state fermentation (SSF) and separation by using ethanol extract.

Keywords: Monascaceae, Pigments, TLC, FTIR, DNA, PCR.

INTRODUCTION

The genus *Monascus*, which includes four species: *M. pilosus*, *M. pupureus*, *M. ruber* and *M. floridans*, belongs to the class Ascomycetes and the family Monascaceae. This fungus is a source of various secondary metabolites of

polyketide structure. Fungi are used in many industrial processes, such as the production of enzymes, vitamins, polysaccharides, polyhydric alcohols, pigments, lipids, and glycolipids. Some of these products are produced commercially while others are potentially valuable in biotechnology (Adrio *et al.*, 2003). Since prebiblical times, fungi including both true filamentous fungi and yeasts, have been used to produce products such as beer, wine, bread and cheese. The twentieth century, a golden age of industrial microbiology, yielded a myriad of products made by fermentation processes: solvents, antibiotics, enzymes, vitamins, amino acids, polymers, and many other useful compounds (Demain, 1999). Fungal secondary metabolites are extremely important to our health and nutrition and have tremendous economic impact. (Adrio *et al.*, 2003). In general, a pigment is any material resulting in color in plant or animal cells and is the result of selective absorption. A chemist defined pigments as those compounds with absorption in the UV-A or UV-B regions, and most importantly in the visible region (Bandaranayke, 2006). Pigments are water insoluble substances that are used to color articles like ink, paper, textiles and many more. But certain biopigments like anthocyanin is water-soluble. Moreover, there are some biocolorants, which are not pigment in any sense (structural colors, light emitting luciferin). Hence, biocolourants are coloring agent obtained from biological sources (Chattopadhyay *et al.*, 2008).

The most important characteristic of this fungus is the ability to produce secondary metabolites of polyketide structure which are synthesized by the polymerization of acetyl and propionyl subunits in a similar process to fatty acid synthesis (Juzlova *et al.*, 1996). *Monascus* is known to produce at least six molecular structures of pigment which can be classified into three groups depending on their color. They include yellow pigments Monascin (C21 H26 O5) and ankaflavin (C23 H30 O5), the orange pigments Monascorubin (C23 H26 O5) and rubropunctatin (C21 H22 O5), and finally red pigments monascorubramine (C23 H27 NO4) and rubropunctamine (C21 H23 NO4) (Pattanagul *et al.*, 2007). The color specification of the latter depends on the associated amino acid or protein. Growth of *Monascus ruber* would be directly affected by the composition of starch or type of carbon sources (Lee *et al.*, 2007). Aldohexoses as glucose and dextrose are better carbon sources for the growth of *Monascus ruber*, than sugar alcohols such as Sorbitol and Mannitol while sucrose reduced the growth of the fungus (Babitha *et al.*, 2006).

The orange pigments, monascorubin and rubropunctatin are synthesized in the cytosol from acetyl coenzyme A by the multienzyme complex of polyketide syntheses I. These compounds possess unique structures that are responsible for their high affinity to compounds with primary amino groups (so called aminophiles). Reactions with amino acids yield the water soluble pigments monascorubramine and rubropunctatin. *Monascus ruber* pigments can be reduced oxidized and react with other products especially amino acids to form various derivative products sometimes called the Complexed pigments. The free pigments are insoluble in water. However their complexation with proteins and peptides in the culture medium makes them soluble in aqueous media. Glutamylrubropunctatin and Glutamylmonascorubrine are complex pigments of *Monascus ruber* (Juzlova *et al.*, 1996),

They have low water solubility, sensitive to heat and unstable in pH range of 2-10. Stability of pigments can be affected by Acidity, Temperature, Light, Oxygen, Water activity and Time. The carbon source, nitrogen source and pH have been shown to influence pigment production by *Monascus* sp., (Su, 1978). The color can be influenced by the culture conditions, in particular, by the pH value, phosphorus and nitrogen sources in the substrate. The pigments can easily react with amino group containing compounds in the medium such as Proteins, amino acids and Nucleic acids to form water soluble pigments.

Monascus compounds have application as pharmaceuticals or food additives (Kraiak *et al.*, 2000). In the former case, monocolin K was found to inhibit cholesterol synthesis thus reducing hypolipidemia, and lovastatin was found to reduce serum cholesterol and triglyceride (Panda *et al.*, 2009). The red pigment has been of increasing interest to the food industry because products are extracellular and water soluble making them easy to use. Applications include the increased red coloring meat, fish, and ketchup (Hamano and Kilikian, 2006). It can also be used in traditional foods to replace nitrate or nitrite for quality improvement. Colorants can be added to fruit flavored yoghurt for enhancing the color of the fruit (Juzlova *et al.*, 1996).

Many of the studies involving *Monascus* have dealt with the general culture conditions to improve pigment production. *Monascus* is probably a xerophilic fungus, which grows in a wide variety of substrates (Babitha *et al.*, 2006). Some natural substrates that have already been tested, besides rice and other cereals, are

cassava starch, wheat bran, wheat meal, bread meal, corn meal and diary milk. Various agro industrial residues such as rice bran, wheat bran, cassava, etc., have been exploited for pigment production (Babitha *et al.*, 2006).

With the advances in biotechnology, molecular genetics have been employed for rapid identification of different kinds of fungi (Lee *et al.*, 2006). Molecular technology can greatly enhance detection sensitivity, as well as simplify and expedite the identification of fungi. Several methods, including random amplified polymorphic DNA (RAPD), arbitrary primed PCR (AR-PCR), restriction fragment length polymorphism (RFLP), PCR-PFLP and DNA sequencing, have recently been used for the authentication of biological materials. In this present study deals analyses the production of secondary metabolites or pigments from the fungi namely *Monascus ruber* with optimized substrates, and also analyses the experimental techniques used to characterize the mass multiplication of pigments production, analyses the role of pigments in the various industries applications for future needful. Secondary metabolites were analyzed by using FTIR.

MATERIALS AND METHODS

Collection and maintenance of *Monascus ruber*

The materials used for the production of the *Monascus ruber* pigments, the methodology adopted to produce the *Monascus ruber* pigments, and characterize their pigments. The culture *Monascus ruber* MTCC 2326, obtained from Microbial Type Culture Centre, Chandigarh, India and mass multiplied in the laboratory of CLRI, Chennai. The fungus *Monascus ruber* was maintained on Potato Dextrose Agar (PDA) slants and incubated at 30°C-32°C for seven days, preserved at 4°C and sub cultured once every four weeks. The culture medium for cultivation of *Monascus ruber* Potato Dextrose Agar (PDA) was obtained from Hi-media laboratories, Mumbai, India. The spores were scraped off under aseptic conditions to produce spore suspension which to be used as the inoculums.

Substrate Selection

The best suitable substrates were selected for maximum yield of pigment production. Selected substrates are as follows: jackfruit seed powder, grape seed powder, soybean powder, wheat bran, rice bran, rice, bread powder, combination of rice bran and wheat bran were obtained dried at 60°C for twelve hours and then grounded. Ten gram of each substrate was added in 250mL conical flask and a salt solution (4 mL) containing: KH_4PO_4 ; NH_4NO_4 ; NaCl_2 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added. The contents were mixed thoroughly and then autoclaved at 121°C for 15 min. On cooling, they were inoculated with the spore suspension containing 1×10^6 spores/ml of *Monascus ruber* and incubated at 30°C for fourteen days.

Preparation of *Monascus* Red Pigment

The fungal spores were grown on the substrates. After incubation for about two weeks these substrates were filtered and taken for further studies. These cultures were taken with known volume of water and shaken well. This extract was then filtered and collected. The same fungal cultures were treated with solvent (Ethanol) and kept in the shaker for 12 hrs. This extract was again filtered and collected in another beaker.

Absorption Spectra of Red Pigment

The analysis of pigment production was done by measuring absorbance maxima of pigment extract by spectral analysis using a double beam spectrophotometer at 500nm (Shimadzu, UV1601). Water extract and extract of pigment was determined by Spectrophotometer.

Thin Layer Chromatography (TLC) Analysis

Crude extract was analyzed by thin layer chromatography (TLC). Concentrated ethanol were applied to Silica Gel 60 plates (Merck, Darmstadt, Germany) and developed with a solution of chloroform/methanol/water (volume ratio of 90:25:4) to compare the R_f values of the pigments.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantitative some components of an unknown mixture. It can be applied to the analysis of solids, liquids, and gasses. Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride (salt). Salt is transparent to infrared light. The drop the pigment extracts

which forms a thin film between the plates this film, which is also transparent in the IR, and is used for pigment functional group identification.

Polymerase Chain Reaction (PCR)

The total genomic DNA was isolated from the fungus *Monascus ruber* and Separation of DNA is achieved by electrophoresis in agarose. The presence of DNA fragments in the gel can be determined directly by viewing in the UV transilluminator after staining with ethidium bromide which intercalates between the stacked bases of the DNA molecules and fluorescence bright orange when irradiated with UV light. The isolated DNA eluted from the agarose gel by using gel elution method. The eluted purified DNA subjected to PCR amplification. Each PCR reaction was carried out 25 μ l containing template DNA, dNTPs, MgCl₂, Taq DNA polymerase (Sigma Aldrich chemicals, Bangalore, India), primer and PCR buffer. Amplification was performed in Thermo cycler. Programmed for an initial denaturation at 94°C for 1 minute, 50°C for 1 minute and 68°C for 2 minutes, 72°C for 5 minutes for 35 cycles and a final auto extension step. PCR products were resolved in 0.8% agarose gel, visualized by using UV transilluminator.

RESULTS

Growth of the fungi *Monascus ruber*

The fungi *Monascus ruber* was grown in Potato Dextrose agar (PDA) and it was incubated at 30°C. The growth of the fungi was observed within three to five days, which it has slight pigmentation. The growth and pigmentation of the fungi occurs, thereby the spores of the fungi can be readily taken as an inoculums.

Production of Pigments in various substrates

Various substrates are produced pigments from *Monascus ruber*. The substrates used are as follows, jackfruit seed powder, grape seed powder, soybean powder, wheat bran, rice bran, rice, bread powder and combination of rice bran and wheat bran.

Thin Layer Chromatography

The qualitative analyses of the pigments produced by the substrates were analyzed by thin layer chromatography, which was shown in the **Figure 1**. The R_f values of the pigments were interpreted in the **Table 1**, which shows that Rice, Rice Bran, bread powder and combination of wheat bran and Rice bran have higher R_f values than the other substrates. The table one shows that rice, rice bran, bread powder and combination of wheat bran and rice bran have higher R_f values 3.20, 0.73, 0.90, 0.90 for ethanolic extract, and 2.065, 2.60, 0.67, 0.70 for water extract respectively. These indicate the respective R_f values of yellow, orange and red for the pigment extracts. R_f values of substrates were essentially the same. These results indicated that the same yellow, orange and red components were produced during fermentation, regardless of the initial pH of the substrate. Contaminants were not identified on TLC plates, since the spots obtained were in agreement with the results of spectral analysis. This could be attributed to the buffering activity of the substrates.

Spectrophotometric Analysis

From the spectral analysis, the absorbance of the water extracts and ethanol extracts of the pigments from the substrates were analyzed. The ethanolic extracts have higher absorbance than the water extracts of the substrates. The higher absorbance was absorbed in the substrates such as rice, bread powder and rice bran has been shown. The optical density values obtained were shown in the **Table 2**. The optical density of the ethanolic extract of rice, bread powder, rice bran, combination of rice bran and wheat bran were 3.20, 3.60, 3.20, 3.80 respectively, which shows higher absorbance, whereas the optical density of the water extracts of the above substrates were 2.60, 2.85, 2.60, 2.50 respectively which have lower absorbance with the supplementation of the additional source, these substrates gives the maximum yield of red pigment as well as yellow pigment.

FTIR analysis

The infrared (IR) spectrum of the *Monascus* red pigment was shown in **Figure 2**. The main absorbance peak include 3424.95, 2915.12, 2361.62, 1654.35, 1458.06 and 1045.16 cm⁻¹. The peak at 3424.95 cm⁻¹ shows a broad peak so it may be OH group. The peak at 2915.12 is CH₂ group. The peaks at 2361.62 and 1654.35 cm⁻¹ indicated that there

might be NH group present. The peaks at 1458.06 cm^{-1} and 1045.16 cm^{-1} indicate that there the presence of C=O groups.

Amplification of the DNA of *Monascus ruber*

The DNA was isolated from the fungi and it was electrophoresed in agarose gel. From the gel, the DNA was eluted and it was subjected to polymerase chain reaction under optimum condition. The PCR product was obtained and it was electrophoresed along with one-kilo base pair (1KB) marker and it was shown in the **Figure 3**. The identified gene fragments observed in the lane 1 was at the 260bp also the gene amplified at 519bp when compared to ladder. The lane 3 which acts a negative control.

DISCUSSION

Growth of the Fungi

They have low water solubility, sensitive to heat and Unstable in pH range of 2-10. Stability of pigments can be affected by Acidity, Temperature, Light, Oxygen, Water activity and Time. The carbon source, nitrogen source and pH have been shown to influence pigment production by *Monascus sp.* (Su, 1978). The color can be influenced by the culture conditions, in particular, by the pH value, phosphorus and nitrogen sources in the substrate. The productivity were reduced too much lower levels in media with glucose, sucrose or starch as a carbon source. Sources of carbon and nitrogen are also increases the pigment production by *Monascus sp.*, reported by Pastrana, 1995). Carel and Shepherd, 1977 explained the effect of different nitrogen sources on pigment production and sporulation of *Monascus sp.*, in submerged shaken culture.

Substrate Selection and Effect of Carbon Supplementation

Cultivation of *Monascus ruber* in solid media has a long tradition in Asian countries to produce a red colorant used as a food ingredient. The fungus produced highest pigment production on rice, bread powder, rice bran substrates and lowest yield was observed from the other substrates such as jackfruit seed powder, grape seed powder, and soybean powder and wheat bran. The advantage of using rice as a substrate is that fermentation period was shorter than compared with that of Chairote *et al.* (2007) who used three week for fermentation of sticky rice (Korker 6 & Sanpatory 1). In this experiment, the substrates were supplemented with glucose; pigment production was increased six fold after one-week incubation. Monosaccharides are readily metabolized sugar alcohols less so and consequently pigment production is reduced (Babitha *et al.*, 2006). Rice with addition of glucose might be better for pigment production when compared with sticky rice since it induced a threefold higher pigment production and shorter fermentation period. Although, in fermented RD6, the addition of soybean milk gave a darkened color, fermentation without soybean milk gave the highest yield of momacolin K and compactin (Chairote *et al.*, 2008). Our results agreed with those of Lin *et al.* (1992) who concluded that the utilization of carbon source for growth and pigment production depended on strain specification. Glucose and its oligopolysaccharide were better than other carbon sources for both growth and pigment production. The high amount of pigment is produced by using *Monascus sp.*, cultivated over rice. In this present study, rice and rice bran as a growth substrate for *Monascus sp.*, which produce high amount of pigment.

Pigment Analysis

Monascus pigments are the group of azaphilones that is the metabolite synthesized from the polyketide chromophores and beta-keto acids by esterification; also red pigment and citrinin are common synthesis pathways in this fungus controlled by pksCT gene of &7,838bp with a single intron (Chairote *et al.*, 2008). In this study, pigment extracts produced by *Monascus ruber* were separated by thin layer chromatography. Supplementation with the carbon source might affect the pattern of the pigment production. The results showed that the R_f value for the yellow pigment was same for all extracts, but in contrast the red pigment was present in more than one spot in each of that extracts. These results were different from those of Babitha *et al.* (2006) who showed that lowest R_f value. This result indicated that, during fermentation yellow and red pigments were produced and the supplementation of the glucose affected the pigment production. The various types of red pigment production indicated by R_f value might be dependent on the effect of the substrate type. To produce pigment, it can be easily react with amino group containing compounds in the substrate such as proteins, amino acids or nucleic acids, according to Dufossee *et al.* (2005). The colour specification of *Monascus* red pigments greatly depends on the amino acids or proteins which the pigment was associated and they also found that the synthesis of the new red pigment might start with the restraint

of the esterification course between beta-keto acid and polyketide chromophores. Crude extract of *Monascus ruber* was analyzed by thin layer chromatography (TLC). Concentrated ethanol extracts were applied to Silica Gel 60 plates (Merck, Darmstadt, Germany) and developed with a solution of chloroform/methanol/water (volume ratio of 90:25:4) to compare the R_f values of the pigments (Babitha *et al.*, 2006).

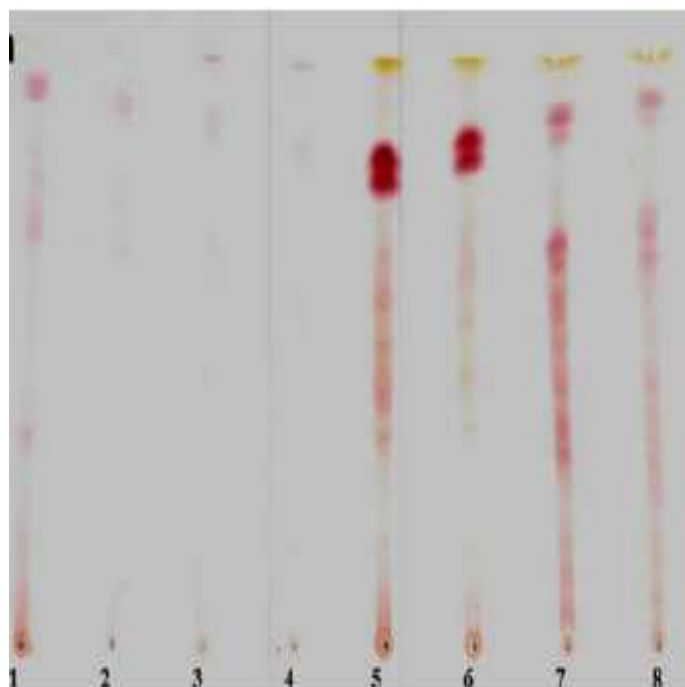


Fig 1. TLC analysis of pigment extracted from fermented substrate. 1. Jackfruit seed powder, 2. Grape seed powder, 3. soybean powder, 4. Wheat bran, 5. Rice bran, 6. Rice, 7. bread powder, and 8. combination of rice bran and wheat bran

Table 1. R_f values of ethanol extracted pigments and water extracted pigments from various substrates

| Sl.No | Substrate | R_f value | |
|-------|------------------------|-------------|-------|
| | | Ethanol | Water |
| 1. | Jack fruit seed powder | 0.65 | 0.50 |
| 2. | Grape Seed powder | 0.75 | 0.60 |
| 3. | Soybean Powder | 0.42 | 0.20 |
| 4. | Wheat bran | 2.70 | 2.11 |
| 5. | Rice bran | 3.20 | 2.65 |
| 6. | Rice | 3.20 | 2.60 |
| 7. | Bread powder | 0.73 | 0.67 |
| 8. | Rice bran + Wheat bran | 0.90 | 0.70 |

Table 2. Optical density observation of *Monascus ruber* grown in different medium at 500nm by UV –visible spectrophotometer

| S.No | Substrate | Ethanol + Pigment | Water + Pigment |
|------|------------------------|-------------------|-----------------|
| 1. | Jackfruit Seed powder | 2.85 | 2.00 |
| 2. | Grape Seed powder | 2.08 | 1.64 |
| 3. | Soybean powder | 2.50 | 1.85 |
| 4. | Wheat bran | 2.70 | 2.11 |
| 5. | Rice bran | 3.20 | 2.65 |
| 6. | Rice | 3.20 | 2.60 |
| 7. | Bread Powder | 3.60 | 2.85 |
| 8. | Rice bran + Wheat bran | 3.80 | 2.50 |

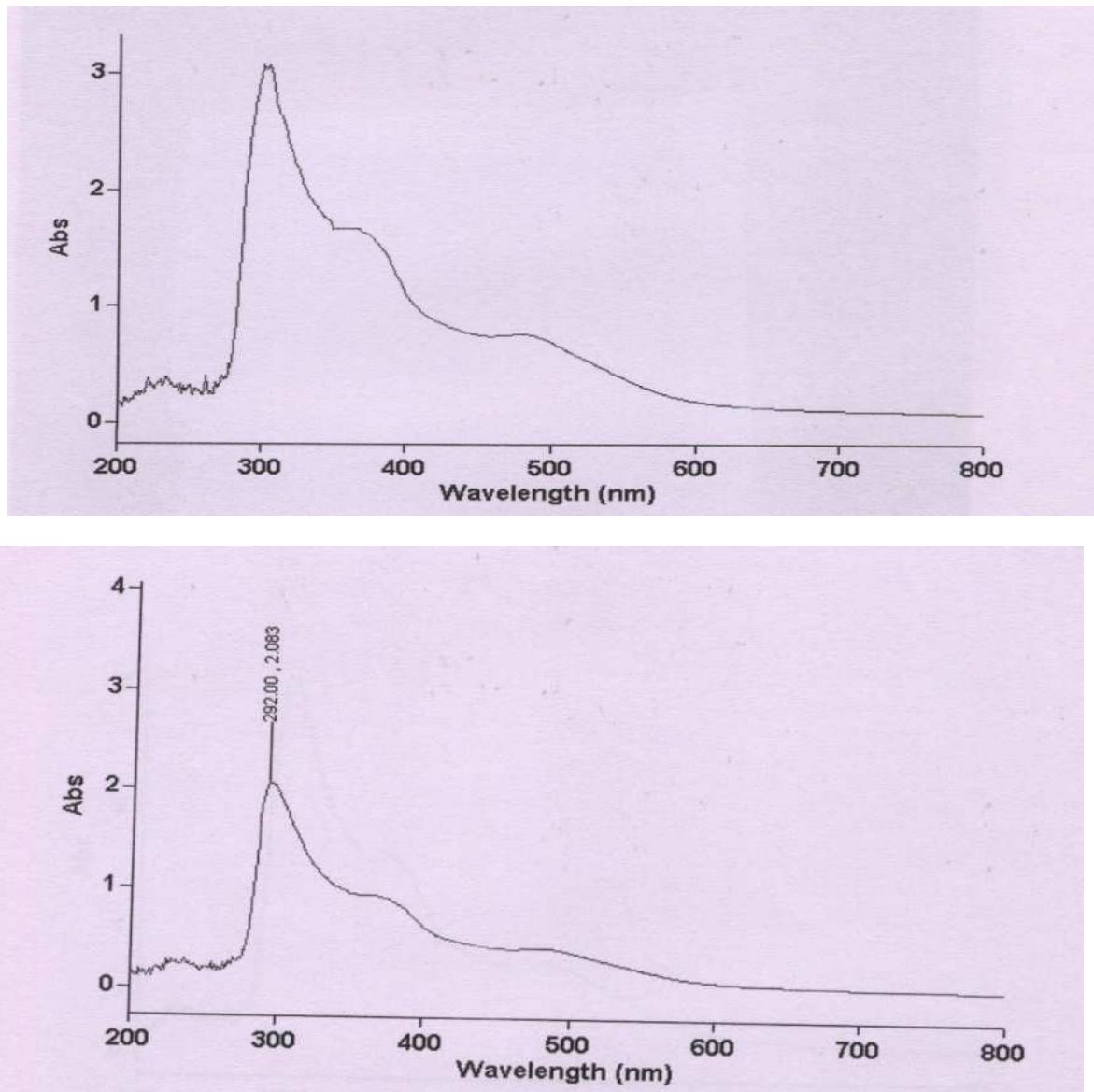


Fig 2. (a, b) Absorbance peak of the Pigment extract in the Fourier Transform Infra Red Spectroscopy

Amplification of DNA

Molecular technology can greatly enhance detection sensitivity, as well as simplify and expedite the identification of fungi. Several methods, including random amplified polymorphic DNA (RAPD), arbitrary primed PCR (AR-PCR), restriction fragment length polymorphism (RFLP), PCR-RFLP and DNA sequencing, have recently been used for the authentication of biological materials (Lotufo *et al.*, 1994; Lakra *et al.*, 2007; Taylor and Ford, 2007). Although they have been proven to be efficient in taxonomic identification and in distinguishing genuine crude drugs from their substitutes or adulterants in previous reports, the application of these methods is limited by the high cost of the fine quality template DNA that is required in these experiments. The quality and concentration of the template DNA, the ratio of template to primer, and slight fluctuations of reacting components or cycling parameters heavily affect the reproducibility of RAPD analysis. In regards to the PCR-RFLP method, the length of PCR products also confines its utilization, as the number of restriction enzyme sites is limited in DNA segments between two primers. Although sequence analysis of PCR products is quite precise and stable, the relatively high expense of DNA sequencing and the sensitivity of contamination in the PCR reaction using universal primers obstruct its wide

acceptance in quality control of medicinal materials (Wang *et al.*, 1997). This study presents an efficient method for identifying the pigment-producing gene in the *Monascus ruber*. Based on the DNA sequence, the primers exactly match a specific DNA sequence. Therefore, a high stringency PCR reaction with the primers for *Monascus ruber* has a positive signal, which amplifies the DNA. Furthermore, the process of identification by Polymerase chain reaction (PCR) is much simple and convenient to use.

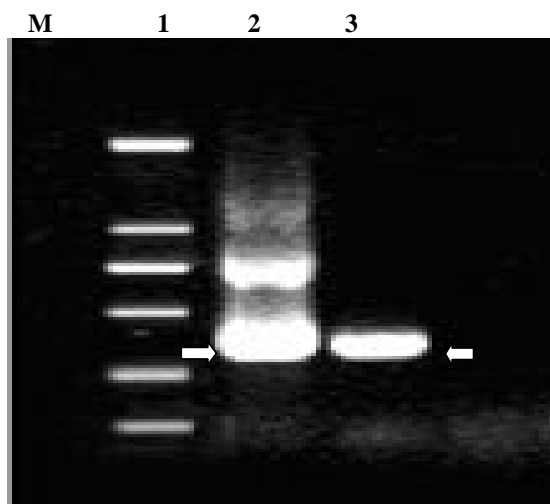


Fig 3. PCR amplification of the *Monascus ruber*, M-DNA marker, Lane 1&2-Positive control; Lane 3-Negative control.

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