"Production and Isolation of pigments from *Monascus purpureus (MTCC 410)* using solid state and submerged fermentation"

A PROJECT

Submitted in partial fulfillment of the requirements for the award of the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

Under the supervision of

Dr. Saurabh Bansal
(Assistant Professor (Sr.Grade) , Biotechnology)

By

Divij chauhan (131571)

To

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN – 173234

HIMACHAL PRADESH, INDIA

May 2017

CONTENTS

CHAPTER 6 References

CERTIFICATE

This is to certify that the work entitled "**Production and isolation of pigments from *Monascus purpureus (MTCC 410)* using solid state and submerged fermentation"** pursued by Mr.Divij chauhan(131571) in partial fulfillment for the award of degree of B.Tech in Biotechnology from Jaypee University of Information and Technology, Waknaghat has been carried out under my supervision. This work has not been submitted to any other university or institute for the award of any degree or appreciation.

Dr. Saurabh Bansal

Assistant Professor

Department of Biotechnology and Bioinformatics

Jaypee University of Information and Technology, Waknaghat

Dist. Solan, HP-17323

Email: saurabh.bansal@juit.ac.in

DECLARATION

I, Divij chauhan do hereby declare that the project report submitted to the Jaypee university of information and technology in partial fulfillment for the award of degree of bachelor of technology in Biotechnology entitled**,"Production and Isolation of pigments from *Monascus purpureus (MTCC 410)* using solid state and submerged fermentation"** is an original piece of research work carried out by us under the guidance and supervision of Dr. Saurabh Bansal.

Divij chauhan

ACKNOWLEDGEMENT

With the grace of God and blessings of our parents it is our utmost privilege to express a word of thanks to those who availed us during this work.

I owe a debt of gratitude to Prof. R. S. Chauhan**,** Professor and Head of Department of Biotechnology and Bioinformatics**.**

With proud privilege and profound sense of gratitude, we acknowledge our indebtedness to our guide Dr. Saurabh Bansal for his keen interest, constructive reproval , unceasing inspiritment and untiring parental guidance. We thank him for giving us project which resonated well with our capabilities and would also prove fruitful in the long run.

I would like to relish Mr. Baleshwer sir, Kamlesh sir and Mamta mam for helping us at every step of the way, providing all material related to the project.

Last but not the least, we would like to convey our heartfelt gratitude to all Ph.D. scholars for helping us.

This experience is greatly appreciated and will surely be subsidiary in the long run.

Divij chauhan(131571)

Date

SUMMARY

Microbial pigments are secondary metabolites, which are engendered or produced by a variety of microorganisms. The present study was conducted to produce and isolate pigments, mainly red ones, from *Monascus purpureus* (MTCC 410).

Monascus purpureus belongs to the family **Monascacese** and to the class **Ascomyceta** whose characteristic feature is the competency to engender secondary metabolites with vigorous yellow, orange and red pigmentation. ***Monascus purpureus*** is a filamentous fungus. Three types of pigments are: Orange Pigment, components are rubropunctain and monascoubrin; Red pigment came from analogues rubropunctaminea and monascorubramine; Yellow pigment consists of monascin and ankaflavin.

In this study, *Monascus purpureus (*MTCC 410) was utilized to produce red, orange and yellow pigments, engendered by solid-state fermentation with the substrate of non-glutinous rice. Later, these pigments were extracted with ethanol and physicochemical analysis of red pigment solution was done.

DR. SAURABH BANSAL

DIVIJ CHAUHAN

DATE

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

S.R.NO.	GRAPH NO.	TITLE
1	Graph 1	pH stability ($O.D_{500}$)
2	Graph 2	Heat stability ($O.D_{500}$)

ABBREVIATIONS

K_2HPO_4	Dipotassium phosphate
$MgSO_4$ .7H2O	Magnesium sulfate heptahydrate
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
$MnSO_4.H_2O$	Manganese(II) Sulfate Monohydrate
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate
O.D	Optical density

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Pigments are present in all living matter and provide alluring colors to them. They play vital roles in the development of organisms. Pigments can be relegated by their inchoation as natural, synthetic, organic or inorganic. Pigments from natural sources such as plants, animals and microorganisms are known as natural pigments. Out of all the ways used for pigment synthesis, microbial synthesis has emerged as the best possible way for pigment production. Microorganisms have become a substantial source for pigment production due to high growth rate, cheap culture medium, and easy extraction process; all these offer more advantages for microorganisms than for other biological resources [1, 2]. Monascus pigments, which are produced by various species of *Monascus*, have been used as a natural colorant and as traditional food additives in East Asia [3].

Monascus purpureus is a species of mold that is purplish-red in color. It is additionally kenned by the denominations **ang-khak rice mold, corn silage mold, maize silage mold, and rice kernel discoloration**. Monascus is a native organism of China and Thailand can facilely grow in several condition It is being used in food and textile industry, also it has many medicinal uses.

Monascus purpureus species engender an involute cumulation of three categories of pigments, red, orange and yellow each with two component of polyketide; these pigments are the secondary metabolites with a mundane azaphilone skeleton. The orange color incorporates monascorubrine and rubropunactine. Red pigments contain monascorubramine and rubropunactamine that are nitrogen analogues of the orange pigments and the yellow colors incorporates monascin and alkaflavin. Red pigments are of great importance because of their use in textile and food industry. Monascus red pigments are usually produced through fermentation, specifically solid-state fermentation.

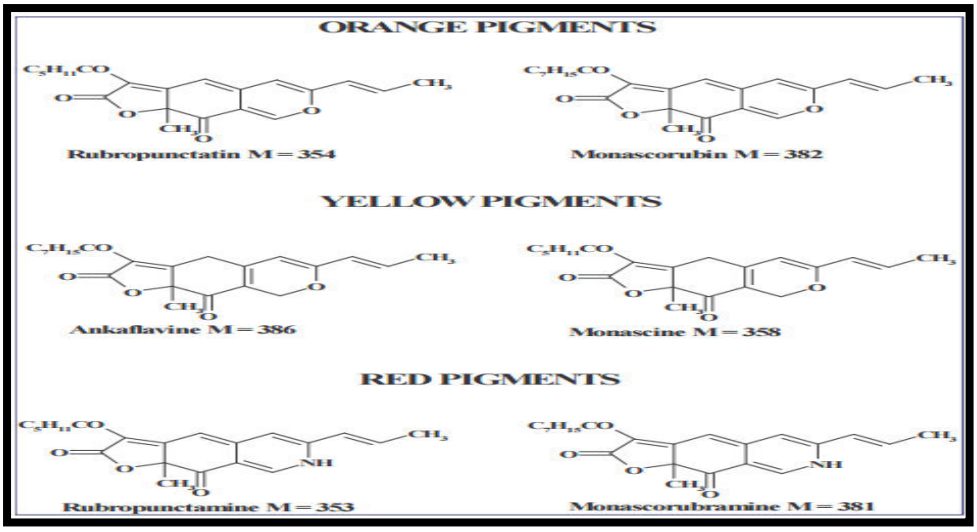


FIG 1: *Monascus purpureus* Pigments

SUBMERGED FERMENTATION

Submerged fermentation is a method of fermentation process in which catalysts and other receptive mixes are submerged in a liquid such as alcohol, oil or a nutrient broth. The procedure is utilized for an assortment of purposes, mostly in industrial manufacturing. Culture control during fermentation is simpler which is appropriate for bacterial development yet less powerful for fungus due to low pigment production.

Mostly, different sources of water-soluble nutrients are used which are uniformly distributed through the fermentation. Usually, innoculum ratio is low and the substrate being used is utilized to its full extent and rapidly which is to be replaced constantly. In this type of fermentation culture control is easy but yield is low. Bacterial and yeast cells are uniformly distributed throughout the liquid and in case of fungus, fungal mycelia cells grow in the form of individual mycelium.

Monascus has been successfully cultured under submerged culture condition for pigment production. Moreover, studies on pigment synthesis by strains of *M. purpureus* in submerged cultures revealed that the yield is affected by medium composition, pH and agitation. The composition of pigments produced depends upon the nutrient available and the strain being used. This method has many advantages over the solid-state process such as easy control of process parameters, high productivity, large volume processing, reduced fermentation time and cost. However, this method is used only for pigment production in industries, for lab scale purposes solid state is preferred.

SOLID STATE FERMENTATION

Solid state fermentation is a fermentation process occurring in the absence or near absence of free water. It is used in various industries like food, pharmaceutical, cosmetic, fuel and textile. This procedure comprises of depositing a strong culture substrate, for example, rice or wheat grain, on flatbeds in the wake of seeding it with microorganisms, the substrate is then left in a temperature-controlled space for a few days. It is highly specific and produces limited amount of waste.

Solid state fermentation is used mainly in Asian countries for manufacturing process of many food uses. In this type of fermentation oxygen and carbon dioxide play a major role and influence pigment production. It uses culture substrates which have low water levels (reduced water activity), which is particularly appropriate for mould. For biopigment production, the use of agro-industrial byproducts makes the process economically possible. Thus, pigments yield is high at low cost. The most regularly used solid substrates are legume seeds, cereal grains (rice, wheat, barley and corn), wheat bran, as well as lignocellulose materials such as straws, sawdust or wood shavings.

This type of fermentation process has higher volumetric productivity with very lower energy requirements. Low availability of moisture and absence of free water reduces the possibility of contamination, favoring the production of specific compounds. Thus, this type of fermentation is preferred over submerged one.

CHAPTER 2

OBJECTIVE

OBJECTIVE

Monascus purpureus is a filamentous fungus which produces, mainly three pigments orange, yellow and red. It is being used as a colorant and dye in food and textile industry respectively. Also, it has many medicinal advantages. Thus, because of its usage in many industries it is of great importance to everyone, especially to Asian countries.

The main aim of the present study deals with

- Production of pigments using solid and submerged fermentation
- Comparative analysis between solid state and submerged fermentation
- Isolation of pigments
- Evaluation of physiochemical properties of red pigments
- Application analysis as food colorants and dyes

CHAPTER 3

REVIEW OF LITERATURE

3.1 DESCRIPTION OF FUNGUS

Botanical name **:** *Monascus purpureus*

Kingdom **:** Fungi

Class **:** Eurotiomycetidae

Order **:** Eurotiales

Family **:** Elaphomycetaceae

Genus **:** Monascus

Species **:** *M. purpureus*

Common Name **:** Ang-khak rice mold, corn silage mold, maize silage mold

Natural pigments have become of great importance due to negative effects of synthetic ones on humans and environment. Microorganisms produce variety of pigments which can be used as a substitute for synthetic ones. Microorganisms have become a substantial source for pigment production due to high growth rate, cheap culture medium, and easy extraction process; all these offer more advantages for microorganisms than for other biological resources [1, 2]. Monascus pigments, which are produced by various species of Monascus, have been used as a natural colorant and as traditional food additives in East Asia [3].

Pigments of Monascus are combinations of yellow (ankaflavin, monascin), orange (rubropunctatin, monascorubrin) and red (rubropunctamine, monascorubramine) compounds, which possess a range of biological activities [4,5]. The chemical structures of these pigments and metabolites have been characterized as ankalactone, monascolidone, monascopyridine, monasfluore, monascusone, new red pigment, monaspurpurone [6], and monarubrin and rubrupunctin [6]. Various biological activitiesas embryotoxicity, teratogenicity, immunosuppres sive properties, antioxidant properties, and antibiotic and cytotoxic activities of oligoketide *Monascus* metabolites have been evaluated [6].

Monascus-fermented products have been used in food, medicine, and industry for more than thousand years in Asian countries, and several excellent agents with a broad range of applications have been found for them. These products can be applied as food or nutritional supplements with multiple therapeutic profits [7-9].

3.2 TYPES OF FERMENTATION

Fermentation is a technique or process of biological conversion of complex substrates into simpler compounds by various microorganisms such as fungi and bacteria. During the metabolic breakdown, apart from main products certain additional compounds or products are synthesized. These products are called secondary products. For the production of pigments, two types of fermentation process including solid state fermentation and submerged fermentation are used.

In nature, solid-state fermentation (SSF) is carried out by mixed or co-cultures of different fungal species. The co-culture of fungi during fermentation may provide help for better biomass and secondary metabolites production. There are several reports of co-culture of fungal species, and mixed cultures have been found to enhance enzyme and organic acid production [10, 11]. 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 [12]. 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 [12].

Monascus pigment fermentations have been performed mainly in solid cultures [13], however production yields have been too low to allow industrial scale production to make it economical. As a means of increasing the pigment yield, much research has focused on submerged cultures [14]. There are reports concerning mutation of strains, changes in nutrients and culture conditions. Submerged fermentation techniques have also been developed including fed-batch cultures, bioreactors [15]. Moreover, studies on red pigment synthesis by various strains of *M. purpureus* in submerged cultures revealed that the yield is affected by medium composition, pH and agitation [16]. Particularly, composition of pigments varies significantly depending on the types of nutrient available, such as nitrogen sources and the strain used [17]. Solid state fermentation is not proper for large-scale industrial pigment production because of low productivity, high labor cost and control concerns [2]. To overcome these limitations of solid state fermentation, several studies have been conducted on submerged fermentation for pigment production from Monascus [18, 19]. Thus, for lab scale purposes soli state fermentation is more feasible and for large-scale industrial pigment introduction submerged fermentation is used.

Substrate being used play a vital role in fermentation process. The outcome of fermentation profoundly differs for every substrate, thus, it is astronomically consequential to cull the right substrate. Fermentation techniques have to be streamlined for every substrate. This is primarily due to the reason that an organism reacts differently to each substrate. The rates of utilization of various nutrients is different in each substrate, thus does productivity. Some of the common substrates used in solid state fermentation are wheat bran, rice and rice straw, hay, fruit and vegetable waste, paper pulp, bagasse, coconut coir, and synthetic media [20]. Some common substrates used in submerged fermentation are soluble sugars, molasses, liquid media, fruit and vegetable juices, and sewage waste water.

CHAPTER 4

MATERIALS AND METHODS

4.1 MICROORGANISMS AND MEDIA

A fungal strain of ***Monascus purpureus*** **(MTCC 410)** was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India, and was used in this study. It was maintained on yeast phosphate soluble starch agar medium preserved at 4°C.

Component of media (g/l)	
Yeast Extract	4.00
Starch	15.00
K_2HPO_4	1.00
$MgSO_4.7H_2O$	1.00
Agar	20.00
Distilled Water	1000.00

Table 1: Yeast phosphate soluble starch agar medium

Seed culture was prepared. It comprise of

Seed Culture Media (g/l)	
Yeast Extract	1.5
Malt Extract	2.5
Glucose	5.0

Table 2: Seed culture media

Two types of fermentation process were carried out. The fermentation media composed of

Fermentation media for solid state (g/l)	
Pre-soaked rice	20.00
Dextrose	74.50
Peptone	5.54
Malt Extract	14.46
NH_4Cl	6.57
$MnSO_4.H_2O$	0.58

Table 3: Fermentation Media For Solid State Fermentation

Fermentation media for submerged (g/l)	
Corn cob	20
Nacl	1
$MgSO_4.7H_{2O}$	1
KH_2PO_4	2
NH_4NO_3	5

Table 4: Fermentation Media For Submerged Fermentation

4.2 CULTIVATION

Selected strain was transferred to yeast soluble starch agar slants from stock culture and incubated at 28 ˚C for 7 days. After 7 days, spores were harvested with sterile distilled water and used for preparation of seed culture. Seed culturing was carried out in flasks containing 50 ml medium inoculated with a full loop of 7 days old culture. Incubate it at 32°C, 160 rpm.

FIG 2 Seed Culture Media

4.3 FERMENTATION

Seed culture media was transferred to fermentation media and was fermented. For soild state fermentation for 14-16 days at 30 ˚C and for and for submerged fermentation it was fermented for 7-14 days in complete darkness

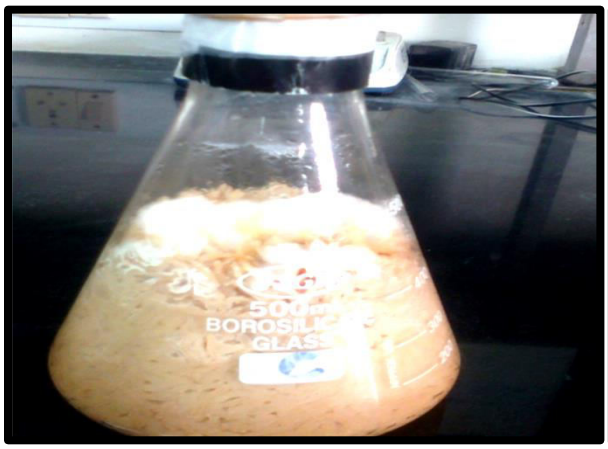


Fig 3: Solid State Fermentation Media

Fig 4: Submerged Fermentation Media

4.4 PIGMENT EXTRACTION

After an incubation period , mycelium was harvested followed by supernatant filtration using a sterilized muslin cloth. Later, two volumes of 95% (v/v) ethanol was added to exhausted culture broth according to the following procedure [21]:Firstly, 60% of the required solvent volume was diluted, a resulting mixture was kept on a rotary shaker for incubation at 180 rpm, 30°C for 30 min. It was followed by a centrifugation of ethanolic mixture for 15 min at 4000 rpm. The pellet was dispersed in the remaining volume of ethanol and centrifuged again at 4000 rpm for 5 min, the supernatants were then collected and filtered. With help of the rotary evaporator the powder form of the pigments was obtained.

Fig 5 : Extraction of pigment

4.5 PHYSIOCHEMICAL ANALYSIS

The physiochemical analysis of red pigment solution was done,mainly pH stability and heat stability.

pH Stability

Red pigment solution was made, with pH adjusted to several values between 3 to 8 with with 0.1N NaOH or dil. HCl.The color intensity was read as absorbance at 500 nm ,against water as blank.

Heat Stability

For determining the heat stability the red pigment solution was incubated for several temperatures viz. 60,70,80,90 for 15 mins.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 PIGMENT PRODUCTION

After 4th fermentation process the pigments were produced. A thin layer of pigments was observed in both submerged fermentation and solid state fermentation. However, contamination was observed in submerged one and no contamination in solid state fermentation.

Fig 6: Pigments Production

5.2 ISOLATION OF PIGMENTS

A thin layer of pigment was observed in flask that underwent solid state fermentation. Yellow and red pigments were produced. Two pigments; yellow and red were extracted from the solid state fermentation (fig. 7 and fig. 8)

Fig 7: Dark Yellow and Red Pigments

Fig 8: Red Pigments

5.3 PHYSIOCHEMICAL ANALYSIS

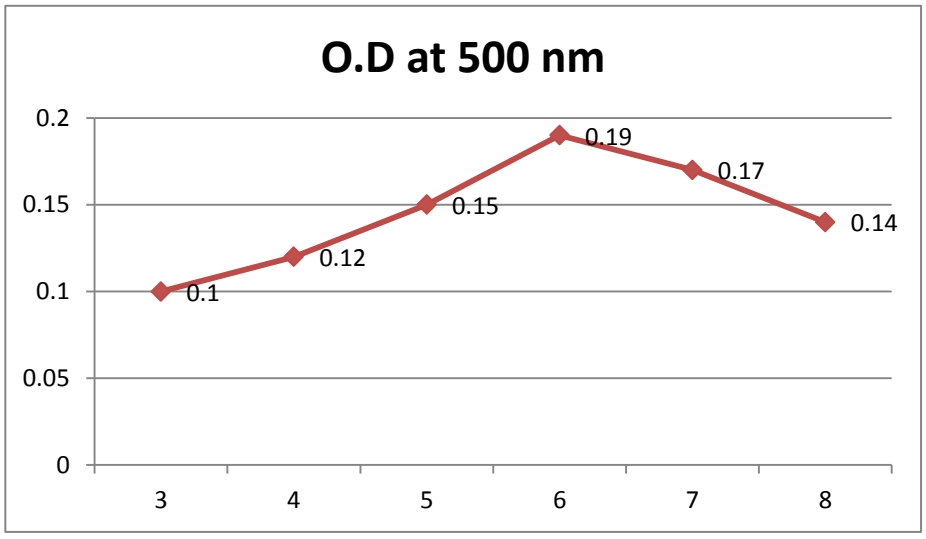
Physiochemical analysis of the red pigment solution was done; pH stability and Heat stability.

pH Stability

Monascus red pigments were tested at various pH ranging from 3-8. Optical density was taken at wavelength 500 nm. Monascus red pigments were stable at pH 5-7, degradation of red colour was observed at pH greater than 8.

pH Stability (O.D at 500nm)	
pH	**O.D**
3	0.1
4	0.12
5	0.15
6	0.2
7	0.18
8	0.14

Table no. 5 pH stability ($O.D_{500}$)



Graph No 1: pH stability

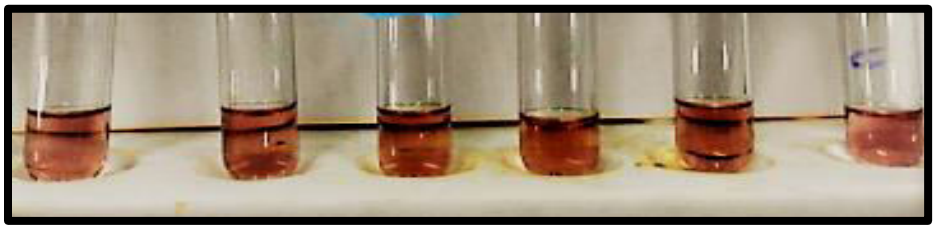


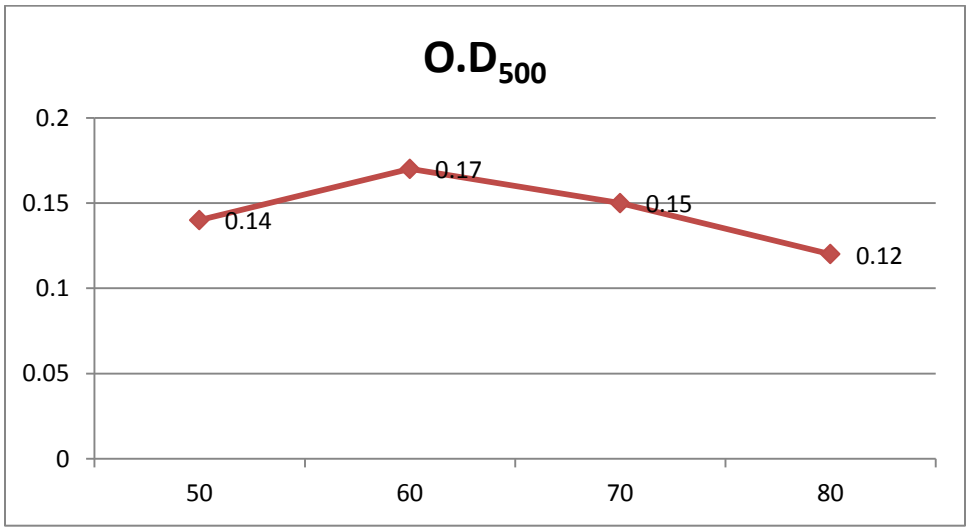
Fig 9 : pH stability (pH range 3-8)

Heat Stability

The heat stability of pigment was checked at various temperatures ranging from 50˚C to 80˚C. They were incubated for at different temperatures for 15 minutes. O.D was taken at 500 nm. At 60˚C pigments were found stable, colour intensity decreased above 70˚C.

Heat Stability ($O.D_{500}$)	
Temperature	**O.D**
50	**0.14**
60	**0.17**
70	**0.14**
80	**0.13**

Table 6: Heat Stability ($O.D_{500}$)



Temperature range

Graph No 2 : Heat stability ($O.D_{500}$)

5.4 Discussion

Bio-pigments are of great importance to humans because of their vast benefits and non-toxicity. Over the years, microorganisms have emerged as the important source for pigment production. Fungi, out of all microorganisms display a wide range of fascinating colours.

Monascus purpureus produces mainly 3 pigments: yellow, orange and red. Pigments were produced from *Monascus purpureus* by two process solid state and submerged fermentation. Contamination was observed in submerged one, thus, pigments were isolated from the flask that underwent solid state fermentation. Mainly two pigments yellow and red were isolated.

Further, after isolation physiochemical analysis of the red pigment solution was done. pH stability and heat stability of the pigments was checked. Monascus red pigment was stable at pH 6-7, showing maximum absorbance at pH 6. Persistent exposure to acidic and basic conditions results in loss of colour. pH 3 & 8 showed complete loss of colour. Thus, pigments were found to be stable at pH 6-7.

Heat stability was done, red pigment solution was incubated for temperature ranging from 50˚C to 80˚C for 15 minutes and absorbance was read at 500 nm. From thermal profile analysis, it was observed that red pigments are stable at temperature 60˚C for 15 mins. However, above 70˚C the colour intensity decreased. At 80˚C there was complete loss of colour, maybe due to breakdown of pigment molecules in solution.

CHAPTER 6

CONCLUSION

6.1 CONCLUSION

Microorganisms produce pigments, which have become important to us. These pigments are used in various industries such as food and textile. Fungi produce more fascinating pigments than any other microorganism. Many fungi such as *Monascus* species, is known for producing vast range of pigments.

In this study, *Monascus purpureus* strain (MTCC 410) was manipulated to produce pigments, by two fermentation process: solid state fermentation and submerged fermentation. Red and yellow pigments were produced and isolated.

The physiochemical analysis of red pigment solution was done, mainly pH stability and Heat stability. Monascus red pigments were tested at various pH ranging from 3-8. Optical density was taken at wavelength 500 nm. Monascus red pigments were stable at pH 5-7, degradation of red colour was observed at pH greater than 8. The heat stability of pigment was checked at various temperatures ranging from 50˚C to 80˚C.They were incubated for at different temperatures for 15 minutes. O.D was taken at 500 nm. At 60˚C pigments were stable, colour intensity decreased above 70˚C.

Thus, Stability of the pigment at high temperature and low pH can easily be known by physiochemical analysis.

REFERENCES

REFRENCES

[1] Unagul P, Wongsa P, Kittakoop P, Intamas S, Srikiti-Kulchai P, Tanticharoen M. Production of red pigments by the insect pathogenic fungus *Cordycepsunilateralis BCC 1869*. Ind Microbiol Biotechnol. 2005; **32**: 135-140.

[2] Parekh S, Vinci VA, Strobel RJ. Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol. 2000; **54**: 287-301.

[3] Carels M, Shepherd D. The effect of different nitrogen sources on pigment production and sporulation of Monascus sp. in submerged shaken culture. Can J Microbiol 1977;23:1360–72.

[4] Patakova P. *Monascus* secondary metabolites: Production and biological activity. Ind Microbiol Biotechnol. 2012; **40**: 169-181.

[5] Feng Y, Shao Y, Chen F. *Monascus* Pigments. Appl Microbiol Biotechnol. 2012; **96**: 1421-1440.

[6] LoretMO,Morel S. Isolation and structural characterization oftwonewmetabolites from Monascus. J Agric Food Chem 2010;58:1800–3.

[7] Shi YC, Pan TM. Beneficial effects of *Monascuspurpureus* NTU 568-fermented products: A review. Appl Microbiol Biotechnol. 2011; **90**: 1207-1217.

[8] Ajdari Z, AbdGhani M, Khan Ayob M, Bayat S, Mokhtar M, Abbasiliasi S, Khoramnia A, Sulaiman Rahman H, Mehrbod P, Ajdari D, Ariff AB. Hypocholesterolemic Activity of *Monascus* Fermented Product in the Absence of Monacolins with Partial Purification for Functional Food Applications. Sci World J. 2014; 1-12.

[9] Ajdari Z, Ebrahimpour A, Abdul Manan M, Hamid M, Mohamad R, Ariff AB. Nutritional Requirements for the Improvement of Growth and Sporulation of Several Strains of *Monascuspurpureus* on Solid State Cultivation. Biomed Biotechnol. 2011; 1-9.

[10] Banerjee, R.; Mukherjee, G.; Patra, K.C. (2005). Microbial transformation of tannin–rich substrate to gallic acid through co culture method. *Bioresource Technol.* 96(8), 949-953.

[11] Plackett, R.L.; Burman, J.P. (1946). The design of optimum multi factorial experiments. *Biometrika* 33(4), 305-325.

[12] Babitha, S., Soccol, C. R., and Pandey, A.: Solid-state fermentation for the production of Monascus pigments from jackfruit seed, Bioresour. Technol., 98, 1554–1560 (2007).

[13] Johns MR, Stuart DM. Production of pigments by Monascus purpureus in solid culture. J Ind Microbiol 1991;8:23–8.
[14] Lin CF. Isolation and cultural condition of Monascus sp. for the production of pigment in a submerged culture. J Ferment Technol 1973;51:407–14.

[15] Kim HJ, Kim JH, Oh HJ, Shin CS. Morphology control of Monascus cells and scaleup of pigment fermentation. Process Biochem 2002;38:649–55.

[16] Hamdi M, Blanc PJ, Goma G. Effect of aeration conditions on the production of red pigments by Monascus purpureus growth on prickly pear juice. Process Biochem 1996;31:543–7.

[17] Miyake T, Isato K, Nobuyuki N, Sammoto H. Analysis of pigment compositions in various Monascus cultures. Food Sci Technol Res 2008;14:194–7.

[18] Yang S, Zhang H, Li Y, Qian J, Wang W. The ultrasonic effect on biological characteristics of *Monascus* sp. Enzyme Microb Technol. 2005; **37**: 139-144.

[19] Vendruscolo F, Tosin I, Josegiachini A, Schmidell W, Luizninov J. Antimicrobial activity of *monascus* pigments production in submerged fermentation. Food Process Preserv. 2013; 1-6.

[20] Pandey, A., Selvakumar, P., Soccol, C.R., Singh - Nee Nigam, and Poonam (1999) Solid state fermentation for the production of industrial enzymes. Current Science, 77 (1): 149-162.

[21]- Velmurugan P, Kamala-Kannan S, Balachandar V, Lakshmanaperumalsamy P, Chae JC, Oh BT. Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. Carbohydr Polym. 2010; **79**: 262-268.