

**STUDIES ON EVALUATION OF *EPICOCCUM NIGRUM* FOR
PRODUCTION OF POLYKETIDE AND CAROTENOID
PIGMENTS AS POTENTIAL NATURAL FOOD
COLOURANT**

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I here certify that the work, which is being presented in the thesis, entitled “**STUDIES ON EVALUATION OF *EPICOCCUM NIGRUM* FOR PRODUCTION OF POLYKETIDE AND CAROTENOID PIGMENTS AS POTENTIAL NATURAL FOOD COLOURANT.**”, by “**SAWINDER KAUR**” in partial fulfillment of requirement for the award of degree of PhD (Biotechnology) submitted in the School of Biotechnology and Biosciences at **LOVELY PROFESSIONAL UNIVERSITY, PHAGWARA** is an authentic record of my own work carried out under the supervision of **Dr. P.S. PANESAR**, Professor & Head, **DEPARTMENT OF FOOD ENGINEERING AND TECHNOLOGY, S.L.I.E.T., Longowal**, **Dr. Prasad Rasane**, Assistant Professor, **Department of Food Technology and Nutrition, LPU, Phagwara** and **Dr. Sushma Gurumayum**, Assistant Professor, **Department of Basic Engg. and Applied Sciences, Central Institute of Agricultural Engg. and Post Harvest Technology, Sikkim**. The matter presented in this thesis has not been submitted in any other University / Institute for the award of PhD Degree.

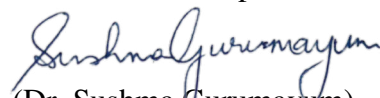
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ABSTRACT

The current study was conducted to evaluate the potential of *Epicoccum nigrum* for pigment production. Among all the media evaluated, Potato dextrose agar (PDA) was observed to be the best media for growth and sporulation while Sabouraud dextrose agar (SDA) was only 29% as efficient as PDA, the best medium. The radial growth rate in case of PDA was 3 ± 0.02 mm/day while in case of SDA it was only 1.09 mm/day. Whilst starch as carbon source was found to increase the radial growth to 5.15 ± 0.02 mm/day, sucrose significantly ($p < 0.05$) influenced the sporulation (224000 ± 1550 spores/ml) of *Epicoccum nigrum*. Among the various nitrogen sources analysed peptone significantly increased ($p < 0.05$) radial growth (6.55 ± 0.02 mm/day) as well as sporulation (220000 ± 2100 spores/ml). The observations also indicated that *E. nigrum* is able to sense and differentiate between light in different wavelength ranges and respond differently in growth and sporulation. The light passing through red colour sheet resulted in better radial growth (8.5 ± 0.02 mm/day) in comparison to unfiltered light (3 ± 0.02 mm/day). Yellow pigment production in terms of hue values was significantly influenced by the presence of dextrose, peptone and darkness. Broken rice and wheat bran were found to be most suitable substrates amongst the all tested for the synthesis of pigment using solid state fermentation. The process parameters i.e. initial moisture content and incubation time for solid state fermentation were optimized to get maximum pigment yield using design expert software. The optimized conditions resulted in pigment yield of 43.1 AU/g at initial moisture content of 41% and incubation time of 11.73 days. Central composite rotatable design (CCRD) was used for deducing optimized conditions for low cost aqueous extraction of yellow pigment and flavanoid. The pigments were extracted under conditions of extraction temperature (40-70°C), mass of fermented rice (0.5-1.5 g) and time (30-90min) using water as extraction media. The optimum conditions generated by the software for aqueous extraction process i.e. extraction temperature of 55.7°C, 0.79 g of fermented matter and extraction time of 56.6 min resulted in yield of 52.7 AU/g yellow pigment and 77.2 AU/g flavanoid.

The box behnken design was found to be appropriate in deducing the optimized conditions for the separation of carotenoid pigment from the fermented substrate. The optimized conditions resulted in carotenoid yield of 432.18 $\mu\text{g/g}$ at extraction temperature of 40.27°C,

20.21 mL/g of solvent to solute ratio and extraction time of 76.22 min from *Epicoccum nigrum* fermented wheat bran.

Preparative thin layer chromatography was used for separating the components. Out of all solvent systems Chloroform (65): Methanol (25): Water (4) resulted in better separation of the pigment with R_f ranging from 0.87-0.91. The separated spot was removed and spectrum was run between the range 350 nm to 600 nm after dissolving the pigment in ethanol. The peak was obtained at 430 nm. The result of LC-DAD-MS shows the major peak has retention time of 11.64 min and λ_{max} of 430 nm suggesting the presence of extended oxypolyene, possibly longer than oxoheptane with proposed molecular formula of $C_{34}H_{44}O_{10}$. Indeed, the molecule showed a similar UV-visible absorption spectrum, as well as ESI-MS molecular ion in positive mode $[M+H]^+$ at m/z 613.32 as the yellow pigment Orevactaene previously isolated from some species of *Epicoccum nigrum* by other researchers.

The yellow pigment obtained by extraction of *E. nigrum* fermented substrate showed a characteristic FTIR spectrum. As per the data obtained the characteristic peaks are visible at 1523.82 cm^{-1} for wheat bran and 1522.85 cm^{-1} for broken rice, it can be assumed that the pigment contain 9-conjugated compound. The presence of strong absorptions for hydroxyls at 3377.47 cm^{-1} (O-H stretching), conjugated carbonyls 1668.48 cm^{-1} (C=O stretch, -C=O, -C=O Conjugated) and additional absorptions for the presence of ester/lactone 1146.72 cm^{-1} confirms the presence of orevactaene.

The stability of yellow pigment was found to be variable at different temperatures and pH conditions. While at $30\text{ }^\circ\text{C}$ only 4% decrease in color intensity was observed after 2 hours, $40\text{ }^\circ\text{C}$ resulted in 8% decrease and at $80\text{ }^\circ\text{C}$ and pH 6.0, 17% of color intensity was lost. These results showed that the yellow pigment produced by *E.nigrum* is heat sensitive and changes in color intensity should be expected in heat processed products. After 180 min at $80\text{ }^\circ\text{C}$ yellow pigments maintained 82 and 76% of the initial color at pH 6 and pH 8 respectively, while pH 4 resulted in 65% decrease in color intensity. Autoclaving resulted in 69% decay and exposure to sunlight for 2 hours showed 1% decay. Thermodynamic parameters for pigment decay at pH 6.0 were represented in terms of enthalpy ΔH , activation energy E_a , free energy ΔG and ΔS . The values observed were 44.52 to 44.93 kJ/mol, 48.48 kJ/mol, 96.60 to 105.18 kJ/mol and -170.50 to -171.85 J/mol/K respectively. All these parameters help in

predicting the quality changes in terms of appearance during thermal processing and optimizing the process.

On the basis of the results obtained, fermented wheat bran and broken rice were better than to unfermented counterpart in most of the antioxidant properties which may be due to increased phenolic content because of fermentation. It was found that methanol was better solvent to extract compounds contributing to DPPH activity. Accordingly, fermented mass as well as the pigment extract might be potential source of antioxidant for use in food products. The pigment extract showed inhibitory effect against strains viz. *Streptococcus pyogenes* MTCC442, *Staphylococcus aureus* MTCC96 and *Candida albicans* MTCC227.

Keywords: *Epicoccum nigrum*, microbial pigment, extraction, flavonoid, carotenoid pigment, FTIR, antioxidant activity, antimicrobial activity

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INTRODUCTION

Color is the most important factor for food acceptance. Typically, color is associated with food we eat such as apple with red, lemon with yellow or carrots with orange. Any unnatural color is associated with spoilage or defect. Thereby, for a consumer it helps in judging the quality of food as well (Aberoumand, 2011). The application of food colors as additives in the processed food is a considerable aspect in deciding the acceptability of processed food for both food industry and buyers. For the manufacturer, adding colors helps in ensuring uniformity and assist in reinforcing the colors that are already present, but have become less intense due to processing treatments. For the consumer, added colors help in refurbishing the original appearance of the foods and make them more attractive and exclusive products (Dufossé, 2006).

The color related regulations vary from country to country. Approximately 43 colorants have been permitted by the European Union to be used as food additives, whereas the United States has allowed about 30 (Mapari et al., 2005). Food Safety and Standards Authority of India (2011) have allowed only 15 colorants including natural and synthetic in its regulations. According to global industry analysts, market of food colors is anticipated to reach \$ 2.97 billion by 2025 (Anon, 2018). Among the different pigments available, the carotenoids alone are anticipated to rise up to \$1.4 billion by 2018 with a growth rate of 2.3% annually (Venil et al., 2014).

Food colors utilized in the processed food industry are produced either synthetically or extracted from natural sources. However, the use of synthetic colors has steadily declined in recent years, owing to side effects such as allergic reactions shown by some of them such as azorubin and tartrazin; toxic effect of tartrazin towards human lymphocytes cells and also their ability to bind with DNA (Duran et al., 2002; Babitha et al., 2006; Yonghui et al., 2013); hyperactivity disorder caused by brilliant blue and tartrazine FD&C Yellow No 5; cancer, DNA damage, neurotoxicity and hyperactive behavior in children caused by allura red (Sabnis, 2010). Therefore, attention towards alternative sources of pigments that are derived from natural sources like plants, animals, insects and micro-organisms, has increased keeping in view the safety concerns about synthetic pigments (Aberoumand 2011). There is need for exploring new sources of natural pigments to substitute synthetic pigments. Conventionally, natural pigments are isolated from plants or insect tissues (Downham and Collins, 2000) which are dependent upon raw material availability and moreover the extracted pigments show a variable profile influenced by extraction conditions and

also being sensitive to heat, light and pH conditions. On the other hand, microorganisms have been gathering more consideration of researchers as a source of natural pigment in comparison to plant and animal sources (Mapari et al., 2005). Pigment synthesis using microbial fermentation process has a number benefits such as relatively simpler extraction, increased product yields, no dearth of substrates, no environmental barrier, and short time duration of production (Mortensen 2006; Poorniammal et al., 2011).

Fungi are metabolically active and capable of producing several metabolites with pharmacological activities, industrial enzymes and pigments (dos Reis Celestino et al., 2014). A number of fungal species found in nature are known to secrete pigments as secondary metabolites, but only a few have been investigated with an aim of synthesis of food colors (Mapari et al., 2006). Much of work has been reported on the fungus, *Monascus purpureus* as it has been traditionally used for years as food color in China, Taiwan and Japan (Prajapati et al., 2014). Besides *Monascus* pigments, filamentous fungi produce several non-carotenoid pigments that exhibit extraordinary ranges of colors (Duran et al., 2002). The microbial pigment diversity is not only related to molecular structures, but also to the variety of hues added to color palette of existing natural colors (Mapari et al., 2006; Mapari et al., 2008). The non-carotenoid pigments produced by the fungi are their secondary metabolites, many of which are having structure similar to polyketides. The enzyme polyketide synthases are partially responsible for the synthesis of polyketide pigments (Shimizu et al., 2005) which exhibit characteristic UV-Vis spectra. Therefore, it is assumed that these polyketides may be considered as a prospective source of new colors that can be promising for food applications. Microorganisms are recognized to secrete different types of polyketide pigments as secondary metabolites, which include carotenoids, fenazine, acilphenol, naphthaquinones, pyrone, sclertiorine and anthraquinone (Poorniammal et al., 2011).

Penicillium spp. has also been reported to produce pigments of polyketide nature similar to those produced by *Monascus* spp. (Ogihara et al., 2001; Jiang et al., 2005; Mapari et al., 2006). However, the production of mycotoxin citrinin along with the pigment in *Monascus* fermented food has limited the use of these genera for natural colorant production (Mapari et al., 2005). Some species of *Aspergillus* sp produce hydroxyanthraquinoid (HAQN) pigments along with several mycotoxins such as secalonic acid, oxaline, citrinin, cyclochlorotine, rugulosin (Goyal et al., 2016). Strains of *Epicoccum nigrum* have been discovered to produce pigment without co-producing mycotoxin (Dufosse et al., 2014).

Epicoccum nigrum (*E. purpurascens*) is an anamorphic ascomycota has universal allocation. It produces dark pigmented conidia and is mostly related to the decomposition of plant tissues (Mims and Richardson, 2005). This fungus is normally found in several plant species usually in inner tissues and exhibit endophytic behavior like other moulds (Schulz and Boyle, 2005; Arnold, 2007). Many studies on *E. nigrum* were focused on the isolation of secondary metabolites with anti-diabetic, antimicrobial and anticancer properties (Shu et al., 1997; Wangun et al., 2007; Guo et al., 2009). Pigments of both polyketide and/or carotenoid origin with color hues in red-orange-yellow spectra have also been reported (Mapari et al., 2008). Yellow pigment with antioxidant potential and water soluble property was also reported by Stricker et al., (1981). Bahrim et al., (2007) investigated the antioxidant effect of flavonoid and a carotenoid complex separated from the *E. nigrum* mycelia upon the cells of the *Saccharomyces cerevisiae* strain. *E. nigrum* pigments improved the cell multiplication and metabolic activity and reduced the intracellular oxidation with significant effects on cell stability and sustainability. This draws the interest towards the potential of fungal sources other than *Monascus*, producing colors similar to commercially available food colors in yellow spectrum.

Owing to the range of pigment hues produced by *E. nigrum* without threat of toxin production, the present study has been designed under following objectives:

- i. Optimization of nutrient composition and media process parameters for fermentation, culture growth and pigment production by *Epicoccum nigrum*.
- ii. Extraction and purification of the pigment.
- iii. Characterization and structure elucidation of the isolated pigment.
- iv. Antimicrobial and antioxidant potential of the pigment.

REVIEW OF LITERATURE

2.1 PIGMENTS AS FOOD ADDITIVES

The appearance of the food is paramount in the process of human choice for selection and eating. The factors contributing to the process of selection and purchase are the influence of the colored appearance of the food or food package and the ambience of the display surroundings. Food coloration improves food presentation and thus became an important goal in the food industry.

Color is perceived by the eye because certain substances selectively reflect different wavelengths of light. The presence of a double bond is an essential requirement for colored materials both synthetic and natural; the number, nature and location of this double bond play a major role in determining a substance's color characteristics (Catherine 1987). The sensation of color is a psychological phenomenon, which is only a part of the overall visual perception of the information detected by the retina of the eye and elucidated by the brain. Human perception of color has proved very effective in determining food quality.

According to regulation by the United States Food and Drug Administration (USFDA), color additives are defined as “any dye, pigment or another substance made by a process of synthetic or similar artifact or extracted, isolated or otherwise derived with or without intermediate or final changes of identity from a vegetative, animal, mineral or other source and that when added or applied to a food drug or cosmetics or to the human body or any part thereof is capable of imparting a color thereto” (Judie 1987).

Today, food products are consumed far from where they are produced. It has been estimated that 75% of the food in developed countries is processed in some form before it reaches the consumer. As a result of processing and transportation of food, degradation of natural pigment and loss of appearance are common phenomena. The challenge in front of the food industry is to manufacture a food with good sensory properties as per consumer's demand and also within the budget (Downham and Collins 2000).

Thus, the use of additives by the food industry is necessary to restore the original food appearance, ensure color uniformity, intensify color normally found in food, protect other components (antioxidants), obtain the best food appearance, preserve the characteristics

associated with food and help as a visual characteristic of food quality. The purpose of food additives must never be to cover up bad processing or manipulation as strategies in food production (Maga and Tu 1995).

2.2 NATURAL PIGMENTS vs. SYNTHETIC PIGMENTS

The food colors can be classified broadly as natural and synthetic colors. Traditionally, natural pigments (Table 2.1) have been extracted from the plants or insect tissues (Downham and Collins, 2000). These include carotenes, chlorophyll, riboflavin, turmeric, curcumin caramel, anthocyanin, betanin etc. However, pigments from natural sources have many disadvantages such as their dependence on the availability of raw materials, batch-to-batch differences of pigment profile, mixtures of varying composition that depends upon the cultivar and climatic conditions, sensitivity to heat, light and pH changes and difficult to illustrate in terms of degree of purity. While synthetic colors (Table 2.2) are classified under those colors which require certification under Food and Drug Administration (FDA) classification (Purba et al., 2015). In India only 8 coal tar dyes are permitted under regulations of FSSAI, 2011 for use in food. These include 3 shades of red viz. Ponceau 4R, Carmoisine, Erthrosine, 2 shades of yellow namely Tartazine and Sunset Yellow FCF, 2 shades of blue Indigo Carmine and Brilliant Blue FCF and one shade of green i.e. Fast Green FCF (FSSAI, 2011). These colors are highly stable under varied processing conditions, but keeping in view the toxic effect of synthetic colors, FSSAI has been constantly updating the regulations and has recommended reducing the maximum limit of food color from 200 to 100 ppm in most of the cases.

Table 2.1: Commonly used approved list of natural colorants

Pigment	Source	Color range	EU code	Application	Reference
Anthocyanins	Black carrot, red cabbage, black grape skin	Scarlet to blue	E163	Beverages, soft drinks, fruit juices and wines	Delgado-Vargas et al., 2000; Mapari et al., 2005; Baines and Seal, 2012
Betanin	Beetroots	Pink to red	E162	Fruit yogurt and ice-cream as well as jams, chewing gum, sauces, and soups	Esatbeyoglu et al., 2015
Carotene	Bixa orellana, paprika, saffron, marigold, tomato, carrot	Yellow to red	E160, E161	butter, margarine, meat products, sauces, marinades, coatings	Mapari et al., 2005; Atodiresei et al., 2012; Baines and Seal, 2012
Chlorophyllian	Nettles or spinach	Green to olive	E140	oils, chewing gum, instant soups, sweets	MacDougall, 2002
Cochineal extract and carmine	Female cochineal insect	Orange to red	E120	Jams, gealtins, baked goods, dairy products, non carbonated drinks	FDA, 2015; MacDougall, 2002
Curcumin	<i>Curcuma longa</i>	Yellow	E100	Mustard, yoghurt, baked goods, dairy products, ice cream	MacDougall, 2002

Table 2.2: List of commonly used/ banned synthetic colorants

Colorant	EU Number	U.S code	Health implications, if any	Reference
Allura Red AC	Banned	F, D & C Red No. 40	Known to cause lymphatic tumors and hyperactivity; Banned	McWatters, 1999; EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009a
Brilliant Blue FCF	E133	F, D & C Blue No. 1	Known to cause chromosomal damage	McWatters, 1999; EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010c
Carmoisine	E122	Ext D & C Red No. 10	Adverse effect on kidney and liver	EFSA Panel on Food Additives and Nutrient Sources added to Food, 2014
Indigo Carmine	E132	F, D & C Blue No. 2	Known to cause brain tumor	McWatters, 1999; EFSA Panel on Food Additives and Nutrient Sources added to Food, 2014
Ponceau 4R	E124	F, D & C Red No. 2	No marked accumulation in any of the tissue during metabolism	EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009f
Quinoline Yellow	E104	D & C yellow No. 1C	Lower dosages may provoke hypersensitive reactions in susceptible individuals	EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009c
Sunset Yellow FCF	E110	F, D & C Yellow No. 6	Known to cause kidney tumors, chromosomal damage and allergy	McWatters, 1999; EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009d
Tartrazine	E102	F, D & C Yellow No. 5	Known to cause thyroid and lymphatic tumors	McWatters, 1999; EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009e

2.3 FUNGAL PIGMENTS

Ascomycetous, basidiomycetous and lichens are a group of fungi that synthesize and secrete intracellular or extracellular diverse classes of pigments as secondary metabolites. These pigments can be broadly categorized chemically as polyketides and carotenoids (Mapari et al., 2010).

The fungus, *M. purpureus* has attracted a great attention of scientists, commercial organizations and general public since it has been traditionally used for centuries as a food colorant in China, Taiwan and Japan. *Monascus* fungi are able to produce various potentially useful metabolites, including both primary metabolites (i.e. Ethyl alcohol, acids, esters and other flavoring compounds) and secondary metabolites (i.e. pigments, lovastatin and antimicrobial agents) (Hsu et al., 2002, Kono and Himeno 1999). The unique characteristics possessed by *Monascus* pigments make them of great interest not only as a natural additive in foods and medicines, but also as novel dyestuffs for the application in biodegradable polymers, textiles and cosmetics (Fabre et al., 1993; De Vrije et al., 1998; Dominguez and Webb 2003). As a natural food colorant, red pigments as well as the yellow pigments have been widely used in food industries, e.g. meat, edible oil, biscuit, bread, cakes and beverages (Srianta *et al.*, 2014). Microbial pigments possess not only the coloring properties, but also numerous clinical properties like antioxidant, anticancer, antiproliferative, immunosuppressive, treatment of diabetes mellitus etc. (Panesar et al., 2015). However, the production of mycotoxin citrinin along with the pigment in *Monascus* fermented food has limited the use of these genera for natural colorant production (Mapari et al., 2005).

Besides *Monascus* pigments, filamentous fungi produce several non-carotenoid pigments (Table 2.3) that exhibit extraordinary ranges of colors (Duran et al., 2002). These are secondary metabolites secreted by the fungi, many of which are structurally polyketides. These polyketide pigments are partially synthesized by enzymes called polyketide synthases (Shimizu et al., 2005) and exhibit characteristic UV-Vis spectra. Therefore, it is assumed that these polyketides may serve a potential source of new colors that can be promising for food use.

Table 2.3: List of promising non carotenoid fungal pigment producers

Fungal source	Color	Reference
<i>Aspergillus sp.</i>	Orange , Red	Joshi et al., 2003; Malik et al., 2012
<i>A.glaucus</i>	Dark red	
<i>Blakeslea trispora</i>	Cream	
<i>Epicoccum nigrum</i>	Yellow-orange	Stricker et al., 1991; Echavarri-Erasun and Johnson, 2004
<i>Heminthosporium catenarium</i>	Red	Malik et al., 2012
<i>Monascus sp.</i>	Yellow-orange	Jůzlová et al., 1996; Malik et al., 2012
<i>Paecilomyces sinclairii</i>	Red	Cho et al., 2002
<i>Penicillium herquei</i>	Yellow	Ishikawa et al.,1991; Robinson et al., 1992
<i>Penicillium oxalicum</i>	Dark red	Sardaryan, 2002; Sardaryan et al., 2004
<i>Penicillium purpurogenum</i>	Yellow-orange, red	Büchi et al., 1965; Francis, 1996

Various species of fungi found in the nature have been reported to produce pigments, but only a few have been explored for the purpose of production of food dyes (Mapari et al., 2006). On the other hand, some pigments of polyketide nature similar to those of *Monascus* spp., by the strains of *Penicillium* spp. had been reported (Ogihara et al., 2001; Jiang et al., 2005; Mapari et al., 2006). On similar terms *Epicoccum sp.* has also been previously reported for the production of pigments of carotenoid and/or polyketide nature as secondary metabolites (Shu et al., 1997; Burge et al., 1976) with color spectra in range of red-orange-yellow; yellow pigment with solubility in

water and having antioxidant potential was also reported by Stricker et al., (1981). The pigment produced varies with the cultivation conditions.

2.3.1 Fermentation conditions

For a profitable production of pigments, the culture medium and the environmental factors must be optimized to maximize productivity. Selection of suitable media composition is essential to develop a successful fermentation process. The medium composition influences both the final biomass yield and growth rate. The carbon and nitrogen sources used are known to influence the pigment production (Lin and Demain , 1991; Subhasree et al., 2011, Miyake et al., 2011).

2.3.1.1 Carbon Source

Utilization of carbon sources for growth and pigment production appears to be strain specific since for many strains of *Monascus* glucose and its oligo and polysaccharides were better than other carbon sources (Lin and Demain 1992; Lin and Demain, 1993). Neither growth nor pigments production by *Monascus pilosus* were observed on the media containing lactose and galactose (Pisareva and Kujumdzieva 2010). Better growth and pigmentation on maltose and fructose of *Monascus* sp. has been reported by Lin and Demain (1991). Also, there is information about growth and pigmentation as well for other species and sugars: on maltose and galactose for *Monascus ruber* IMI 313379 (Omamor et al., 2008), for *Monascus purpureus* LPB 97 on xylose (Babitha et al., 2006), lactose (Rasheva et al., 1998; Babitha et al., 2006), fructose (Tseng et al., 2000) and on maltose for *Monascus purpureus* IMI 314369 (Omamor et al., 2008). In case of jackfruit seed powder no supplementation of carbon source was required as it itself gave the maximum yield for red pigment i.e. 19.5A/g (Babitha et al., 2006). The mycelial growth of *Monascus pilosus* C₁, expressed as dry weight (DW) and the specific pigment production (SPP) was mainly stimulated by glucose as a carbon source. The DW and SPP reached values of 10.89 g DW L⁻¹, and 1.97 OU mg DW⁻¹ and 1.01 OU mg DW⁻¹ for the total red and yellow pigments, respectively. The combination of sodium glutamate with glucose increased effectively the pigments production up to 2.29 OU mg DW⁻¹ and 1.67 OU mg DW⁻¹ respectively, for the total red and yellow pigments (Pisareva and Kujumdzieva 2010). Galactose, sorbose, psicose, and mannitol were found to be good supplements next to glucose, but not xylitol (Nimnoi and Lumyong 2009). Tseng et al., (2000) investigated the effect of different sugars on growth and pigment production by *Monascus purpureus* in broth medium and ground meat. The medium with fructose as carbon source proved to be the most suitable for mycelium growth and pigment production, with maltose

and glucose being the second most productive. Mycelium growth and pigment production were inhibited when sucrose and lactose were used as carbon sources. Pradeep et al., (2013) isolated a new strain of *Fusarium moniliforme* KUMBF1201 from paddy field soil for the production of natural pigment. They reported that glucose (2 %) as the carbon source and yeast extract (2 %) as nitrogen source played a major role in enhanced cell growth and pigment production. Most commonly used complex carbon source is rice suitable for the solid state culture of *Monascus* (Teng and Feldheim, 2001; Fung et al., 2012)

2.3.1.2 Nitrogen Source

Utilization of different nitrogen sources in fermenting media has been known to produce different pH patterns which affect growth and pigment production (Lin and Demain 1992; 1993). A source of nitrogen is required for the growth of most of the microorganisms. In the traditional processes of red yeast rice production, further supplementation with nitrogen source is not required as the rice itself has 5-8% proteins (Carvalho et al., 2003). In case of substrate having low nitrogen content supplementation may help with growth and pigment production.

Organic sources such as monosodium glutamate (MSG) and peptone have been reported to stimulate growth of *Monascus* and red and yellow pigment production (Babitha et al., 2006, Lin and Demain 1992). Supplementation (0.3%) of MSG resulted in maximum pigment production of 72 U/g dry cell mass (Mukherjee and Singh 2011). Decrease in production was observed above this concentration, which may be due to respire-fermentative metabolism. Addition of mono sodium glutamate @0.5% in raw rice were found to yield higher pigment of 0.464 (OD) and 1.314 (OD) U/g *Monascus* fermented rice for both red and yellow pigments respectively (Vidyalakshmi et al., 2009). Babitha et al., (2006) observed that organic nitrogen was unfavorable for pigment production although growth was optimum. In their study jackfruit seed powder without any addition of nitrogen source was not able to produce any water-soluble pigments as analyzed by spectral analysis of water extract.

Inorganic nitrogen sources such as ammonium nitrates also stimulate growth and pigment production. The use of sodium nitrate has been reported to limit the growth and pigment yield (Juzlova et al., 1996). The medium must provide sufficient nitrogen in suitable form to promote both growth and pigment production.

The effect of nitrogen sources on the yield, distribution and light stability of monascus pigments in submerged fermentation was studied by Zhang et al., (2015). As compared to the control, glutamic and 6-furturylaminopurine increased the production of pigments from 4185 to 4303 and 4349 U g⁻¹ respectively. 6-furturylaminopurine addition resulted in an increase in the extracellular pigments from 697 to 1404 U g⁻¹ and also increased the hue of fermentation broth from 0.887 to 1.072. The light stability of extra and intracellular red pigments was improved by adding different nitrogen. The extracellular red pigment containing tryptophan was the most stable in sunlight, followed by phenylalanine, glycine, 6-furturylaminopurine, histidine, and the control, and the worst was glutamate.

Jung et al., (2003) studied the effect of 20 amino acids on the color characteristics of monascus pigment. It was found that the yellow and orange pigments were identical regardless of the amino acid addition but red compounds varied on the basis of the type of amino acid added. Structural analyses confirmed the presence of moieties of the added amino acids in derivative pigments.

2.3.1.3 Trace Elements

Many trace elements such as zinc, iron, manganese and phosphorous have been reported to influence the secondary metabolism of fungi. Higher concentrations can have a negative effect on pigment formation. Dominguez and Webb (2003) reported that supplementation of zinc sulphate at concentrations of 0.1 g L⁻¹ in hard wheat flour increased biomass of *M. purpureus* Went (IMI 210765) and red dye yields up to twofold.

2.3.2 Environmental factors

2.3.2.1 Temperature and pH

The temperature plays a pivotal role in cell metabolism, thus influencing pigment production. It has been reported that at higher temperatures the possibility of transformation in pigments was observed (Carvalho et al., 2003, Carvalho et al., 2005). Commonly, the appropriate pH for growth and pigment production of *Monascus sp.* is 5.5–6.5 (Joshi et al., 2003). Nevertheless, different pH values in the media may affect pigment components. Red pigments produced by *Monascus ruber* were found to be sensitive to acidic pH, while the stability improved under neutral and alkaline conditions (Fabre et al., 1993). On the other hand the absorbance maxima for the pigment produced by *Monascus purpureus* LPB 97 grown on jack fruit seed powder was observed at 469

nm for pH 3.0 which corresponded to orange pigment as confirmed by Thin Layer Chromatography (TLC) of pigment extracts (Babitha et al., 2006). This may be due to the buffering nature of different agro-industrial residues (Pandey et al., 2001). Usually the only initial pH value is adjusted to a suitable value depending on the carbon and nitrogen source used.

Silveira et al., (2011) studied the stability of red pigments under different pH and temperature conditions. The pigment maintained 81, 84, and 85% of the initial color after 180 min at 80°C of treatment at pH 6.0, 7.0, and 8.0, respectively. After sterilization at 121 °C for 15 min, 40, 27, and 20% color degradation of pigment occurred under pH conditions 6.0, 7.0, and 8.0 respectively. In both cases, treatments (80 and 121 °C), there was a tendency of increased pigment stability at higher pH values.

Espinoza-Hernandez et al., (2013) reported that the novel pigment producing strains of *Penicillium* viz. *Penicillium purpurogenum* and *Penicillium pinophilum* showed the highest pigment production at a temperature of 24°C and pH 10, although the pH value in each strain at 24°C made a difference in color between each strain. The *Monascus* sp. showed best pigment production at pH 4.0 (Carvalho et al., 2003). The production potential of water soluble *Monascus* red pigment by *Penicillium* species was studied by Jiang et al., (2005). Potato dextrose broth, malt extract broth and a chemically defined medium containing glutamate as a nitrogen sources were used for production. They concluded that the pigment produced was identified as heat stable, polyketide *Monascus* red pigment. The yield of red pigment obtained from *Penicillium* species grown on malt extract medium was 1107 mg/L.

Fusarium moniliforme KUMBF1201 showed good growth on Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media. It required optimum temperature (28±1°C), pH (5.5) for the growth, pigment production and sporulation (Pradeep et al., 2013).

2.4 PRODUCTION OF PIGMENTS ON AGRO-INDUSTRIAL RESIDUES:

The huge amount of agro-industrial residues generated by various industrial and agricultural activities can be of interest as these can be utilized as low cost raw material to provide the growth of microorganisms (Lopes et al., 2013). The waste generated by different food processing industries have been effectively exploited as substrate for microbial pigment production and discussed as under.

2.4.1 Dairy industry:

Whey is the major waste generated by cheese industry and is rich in lactose and whey proteins. This can be used by several microbes for the growth and secondary metabolite production (Panesar et al. 2015). Marova et al., 2012 used whey as a nutrient source in synthetic media for the production of red pigment using *Rhodotorula glutinis*, *Sporobolomyces roseus* and *Rhodotorula mucilaginosa*. It was reported that all strains effectively utilized whey as a substrate. The highest production yields were observed in *R. glutinis* CCY 20-2-26. The cells showed enrichment of β -carotene by 46 mg/ L. Whey and coconut water were utilized by *R. rubra* MTCC1446 for the enhanced synthesis of yellow-pink pigment (Kaur et al., 2009).

2.4.2 Cereal and legume Industry:

Dominguez and Webb (2003) used hard wheat flour as the sole medium for the growth of *M. purpureus* Went (IMI 210765) for pigment production. They found that the supplementation of zinc sulfate at concentrations of 0.1 g/L increased biomass and red pigment yield up to twofold whilst supplementation with ammonium chloride at 2g/L encouraged the production of yellow and orange dyes.

Corn meal substrate produced maximum pigment (19.4 U/gds) in comparison to soybean meal, peanut meal and coconut residue. The supplementation of corn meal with 8% (w/w) glucose resulted in highest pigment production (129.63 U/gds), followed by coconut residue (63.50 U/gds), peanut meal (52.50 U/gds), and soybean meal (22.50 U/gds) (Nimnoi and Lumyong 2009). The utilization of rice broken by *Monascus ruber* MTCC2326 for pigment production resulted in an overall rise in protein and crude fibre content of *Monascus* fermented rice. A protein content of 17.16 %, crude fiber 6.71 %, fat 1.98 % and ash 1.65 % was found to be present in *Monascus* fermented rice (Vidyalakshmi et al., 2009). Srianta et al., (2016) studied the growth and pigment production of *M. purpureus* on different cereal substrates using solid state fermentation. It was observed that the synthesis of pigments started at the end of logarithmic phase on all substrates tried. Maximum production of pigments was reported on rice followed by Dehulled Sorghum Grain >Whole Sorghum Grain>Corn>Sorghum Bran. In all substrates, the red pigment was produced to the extent of 57-87%, excluding sorghum bran substrate which produced yellow pigment in higher concentrations. Supplementation of media with cellulose waste steam improved the pigment production potential of *Pencillium resticulosum*. Hydrolyzed mung bean waste flour was utilized as nitrogen source for the carotenoid production by *Rhodotrula glutinis* (Tinoi et al., 2005). The carotenoid yield improved by 20% under optimized conditions. Corn cob that accounts

for 30% of the waste of maize is a good source of polysaccharides for example cellulose, hemicellulose and lignin (Zhou et al., 2014). The use of corn cob powder as medium for pigment production by *M. purpureus* KACC42430 yielded 25.42 OD units/g of fermented substrate under optimized conditions of solid state fermentation (Velmurugan et al., 2011).

2.4.3 Fruit and vegetable industry:

Incubation of *M. purpureus* LPB97 grown on jackfruit seed as a substrate under dark conditions resulted in better red pigment yield from 14.5 - 22 OD/g of dry substrate. However, pigment production was totally suppressed indirect illumination. Blue and red light also affected culture morphology as well as pigment production (Babitha et al., 2008). The study proposed that phytochrome type of system may be functional in this organism.

Submerged cultivations of *M. purpureus* NRRL 1992 with sugarcane bagasse as carbon source was carried out by Silveira et al., (2013) which could prove to be a low cost substrate. The pigment produced show reasonable stability under high temperatures and/ or acidic pH. First-order kinetics fitted best for thermal degradation of pigments synthesized by *Monascus*.

Concentrated grape must when used as the only carbon source resulted in maximum yield of 5.95mg/l of total carotenoid using strains of *Rhodotrula glutins* (Buzzini and Martini, 2000). Sehrawat et al., (2017) optimized parameters for pigment production using *Monascus purpureus* MTCC 369. Sweet potato peel and pea pod powder when used @ of 7.8 and 3.9% (w/w) respectively, resulted in yield of 21 CVU/g at pH 5.4 for 8 days 9 hours (8.9 days) at 32°C.

2.5 EXTRACTION AND PURIFICATION:

Extraction and purification of colorants is the most important step in the production process. Microbial pigments are produced either as extracellular or intracellular pigments. Although it is easy to collect the extracellular pigments, intracellular pigments required cell disruption and more downstream processing time (Nigam and Luke, 2016). Most commonly organic solvents are used for extraction purposes (Velmurugan et al., 2010). However, their use in large quantities can add to environmental burden, cost and also can result in the creation of degradation products (Dermiki et al., 2009). The main factors affecting selection of extraction procedure are characteristics of the molecule of interest, extraction yield, reproducibility, cost, extraction time and solvent requirement (Wang and Weller, 2006). Conventional methods include maceration, solvent extraction, percolation and pressurized liquid extraction, but these require large amount of

solvents and may result in degradation of extracted molecules. The supercritical carbon di oxide has been used successfully for the extraction of various bioactive molecules from diverse substrates (Jaren-Galan et al., 1999). Pasquet et al., 2011 used microwave assisted extraction using different solvent in order to increase the selective solubilization of polar and non- polar pigments. Zhu et al., 2017 evaluated the performance of ultrasound assisted extraction for pigment recovery from seaweed extract. They reported that maximum carotenoid yield was obtained at 50°C and ultrasonic power from 100-300W facilitated the extraction of pigments.

Monascus pigments include both hydrophilic and hydrophobic constituents as per their solubility in water (Qian and Wu 2010). Extraction of pigments were normally carried out using different concentrations of ethanol (Johns and Stuart 1991; Babitha et al., 2006; Babitha et al.,2007; Vidyalakshmi et al., 2009; Lai et al., 2011), the hydrophobic components were separated by organic solvents such as n-hexane, benzene, methanol, ethanol, etc., and the hydrophilic components were separated by water (Sato et al., 1997; Sweeny et al., 1981; Lin and Iizuka 1982).The use of different IL–citrate buffer aqueous two-phase systems was studied for the purification of a red colorant from the broth of *P. purpurogenum* DPUA 1275 by Ventura et al., (2013).

2.6 STABILITY EVALUATIONS:

Stability testing is essential while evaluating the marketable/business potential of secondary metabolites (Sant’Anna et al., 2010). During heat treatments, number of desirable and undesirable changes might occur, causing the destabilization of the constituents. Therefore, information of pigment behavior under different processing and storage conditions is of importance from a technical point of view targeting food applications. Mathematical modelling epitomize a potent and crisp method to display the physical behavior of molecules in mathematical terms consisting of equations that provide an outcome based on data input (van Boekel 2008). Thus, extrapolation of biomolecule behavior is possible by the application of appropriate models and by undertaking thermodynamic studies. Several kinetic models have been anticipated to model the heat degradation of food constituents.

2.6.1 Thermal stability

A pigment produced by *Monascus purpureus* CMU001 on agricultural residue was not stable at high temperature and extensive contact with UV light. The strength of red pigment decreased

30.57 and 5.41% after autoclaving and pasteurization, respectively (Nimnoi and Lumyong 2011). Bahrim et al., (2007) investigated the antioxidant action of crude pigment extracts from the *E. nigrum* mycelia upon the cells of the *Saccharomyces cerevisiae* strain. Results showed that *E. nigrum* pigments increased the cell count and metabolic activity and decreased the intracellular oxidation with progressive effects on cell stability and sustainability. The stability of red color produced by *P. purpurogenum* GH2 under the conditions used for food and beverage pasteurization was best represented by a first order model at pH 6 up to 80°C, and at 80°C for pH 4–8, with a z-value of $48.7 \pm 0.9^\circ\text{C}$ and a D value at (80°C and pH 6) of 981 ± 5 min (Morales-Oyervides et al., 2015). Velmurugan et al., (2011) investigated the relative stability of red *Monascus* pigment under different thermal conditions. It was found that the pigment was stable upto 95.9% under sterilization conditions, but changed to brown color when subjected to dry heat at 60°C for 12 hours.

2.6.2 pH stability

The red *Monascus* pigment retained its original color at pH 5, 6 and 7. The pigment was lost at more acidic conditions and at pH above 7 the color changed to brown shade suggesting its application in neutral or near neutral pH foods. The change of color may be credited to changes below or above the dissociation constant of pigment molecules (Velmurugan et al., 2011). Furthermore, the stability of pigment from *Monascus* has been broadly reported by many researchers such as Carvalho et al. (2005) who stated that the loss of color intensity at high temperature and low pH might be due to crude behavior of pigment extract, whose decomposition may affect degradation manner. On the contrary, Lin and Demain (1992) stated that the pigments are stable over a broader range of pH.

2.6.3 Photostability

The photostability of extra and intracellular *Monascus* red pigments was enhanced with the addition of different nitrogen. While, the extracellular red pigment having tryptophan was the most stable to sunlight, followed by phenylalanine, glycine, 6-furturylaminopurine, histidine, and the control, and the least was glutamate (Zhang et al., 2013). *Monascus* pigment produced using corn cob as substrate retained its color on sunlight exposure for 2 hours and UV light for 12 hours. The retention was up to 99.2% (Velmurugan et al., 2011).

MATERIALS AND METHOD

3.1 MICROORGANISM AND ITS MAINTENANCE

Epicoccum nigrum strain used in the experiments has been isolated, identified and deposited at National Fungal Culture Collection of India situated at Agharkar Research Institute (ARI), Pune with accession number (NFCCI-3667). The microorganism was maintained in potato dextrose agar plates and slants and was sub cultured once every month.

3.2 FERMENTATIONS

3.2.1 Agar Plate Cultures

3.2.1.1 Agar medium

In agar plate study, five solid media viz. Potato Dextrose Agar (PDA), Malt extract Agar (MEA), Czapek's Dox Agar (CDA), Sabouraud's Dextrose Agar (SDA) and Yeast Extract Agar (YEA) were used to observe their effect on growth and production of pigment. The selection of media was based on compositions commonly used for pigment production from fungal species.

The diameter of the fungal colony was measured through its center with a metric ruler. Measurement was made after regular intervals of 24 h. A linear regression line of a colony radius (Carvalho et al., 2005) against time was used to obtain the growth rate (mm day^{-1}). Observations were made for sporulation (spores/mL), dry cell weight (g).

Effect of different carbon (fructose, dextrose, starch, sucrose, sorbitol and ethanol) and nitrogen sources (Ammonium chloride, ammonium sulfate, peptone, sodium nitrate and yeast extract) was studied by using CDA as the base media.

To investigate the wavelength dependence of parameters under study, agar plate study was carried out under variable light conditions, utilizing the blue, yellow, green and red illumination. In all the treatments, the plates were analyzed for radial growth, sporulation and dry cell weight after 10 days of incubation

3.2.1.2 Color

The digital imaging method was used for color determination on agar plate cultures (Yam and Papadakis, 2004). The result was deduced as follows: L* designates lightness from 0 (black) to 100 (white). A positive value of a* represents red while a negative value of a* indicates green.

Likewise positive and negative values of b^* indicate yellow and blue, respectively. Chroma values represent the saturation or purity of color. Hue angle values indicate the degree of redness, yellowness, greenness and blueness maximum being at 0, 90, 180, and 270 respectively (Mapari et al., 2008).

3.2.1.3 Cultivation Method

The culture was maintained on Potato Dextrose Agar (PDA) at 4°C and sub cultured aseptically every fortnight. Mycelia of *Epicoccum nigrum* was cultivated by growing on PDA plates for 7 days. The mycelium was inoculated in triplicate at the center of the agar plates. The inoculated plates were then incubated for 7 days at 28±2°C.

3.2.2 Solid-State Fermentation

3.2.2.1 Fermentation conditions:

For inoculum, mycelia were scraped off from 7 days-old actively growing cultures in sterile distilled water.

3.2.2.2 Selection of substrate

Various substrates *viz.* broken rice, wheat bran, corn bran and sugarcane bagasse were used as substrate for SSF. The substrates were dried at 60°C till constant weight. The dried substrate was passed through a mixer and sieved using an appropriate mesh sieve to get uniform particle size of 0.25mm and stored in a dry place till further use. Ten grams of dried substrate was filled in 250 ml Erlenmeyer flask and mixed with a known quantity of distilled water to adjust the moisture at 70%. The flasks were then autoclaved at 121°C for 15 min, cooled, inoculated with 10% of the spore suspension under sterilized conditions. For uniform spreading of the spores the contents were mixed with sterile rod. After inoculation, the flasks were incubated at 25±2°C for 12 days. Every day, the flasks having inoculated substrate were shaken manually to loosen it up. After the desired period the pigment was extracted and yield calculated.

4.5.1.2 Influence of initial moisture content

For optimization of initial moisture content, 10 g of selected substrate was taken in Erlenmeyer flask and de-ionized water was added to attain the initial moisture content of approximately 30, 40, 50, 60 and 70% (g of water in 100g of wet substrate) in different flasks. The substrate is then left undisturbed to equilibrate at 30°C for 1 hr. The flasks were then autoclaved at 121°C for 15

min, cooled, inoculated with 1ml of the spore suspension under sterilized conditions. After adding inoculum, the flasks were incubated at $25\pm 2^{\circ}\text{C}$ for 12 days.

3.2.2.4 Influence of Incubation time

For optimization of incubation time, 10 g of selected substrate was taken in Erlenmeyer flask and de-ionized water was added to achieve selected initial moisture content. The substrate is then let undisturbed to equilibrate at 30°C for 1 hr. The flasks were then autoclaved at 121°C for 15 min, cooled, inoculated with 10% of the spore suspension. The flasks were incubated at 25°C for 12 days.

After fermentation the flasks were autoclaved at 121°C for 15 min and the contents were then dried at 50°C for 24 hours. The dried substrate was ground in pestle and mortar and used for extraction and estimation of pigment.

3.2.2.5 Influence of carbon source on pigment production

To check the influence of carbon source on pigment production various carbon sources *viz.* dextrose, galactose, maltose, sucrose, lactose and xylose were used in concentration of 4% w/w and 8%w/w of substrate (Babitha et al, 2006).

3.2.2.6 Influence of nitrogen source on pigment production

Different sources of nitrogen such as ammonium chloride, yeast extract, peptone, mono sodium glutamate (MSG), sodium glutamate, oil cake and urea were used with concentration 1% w/w of substrate (Vidyalakshmi *et al.*, 2009).

3.2.2.7 Experimental design

Response Surface Methodology (RSM), an assemblage of statistical and mathematical techniques, was used for planning the studies to optimize the process for the desired level of responses (Myers, 1971). Sarkar et al. (1998, 2004), Sharma (2002) reported the usefulness of the methodology for analyzing and optimizing the responses of multivariate system. In response surface experiments, attempts are made to identify the response of a system as a function of the explanatory variables. The developed empirical model is used to study and optimize the system parameters for desired level of responses.

A central composite rotatable design with four factorial points, four star points ($\alpha = \pm 1.414$) and five replicates at the center point, all in duplicates, resulting in 13 number of experiments (Table 3.1), was employed to optimize the system parameters (Montgomery, 2017).

The range and the levels of the experimental variables used in the coded and uncoded form in the center, the factorial and star points of design are presented in Table 3.2. The level of parameters was carefully selected based on the literature available. The coding of the levels was done using the following equations:

$$X_1 \text{ (Initial moisture content, \%)} = (x_1-60)/10 \quad \text{-----} \quad (3.1)$$

$$X_2 \text{ (Incubation Time, Days)} = (x_2-12)/3 \quad \text{-----} \quad (3.2)$$

The experimental design in coded (x) and actual level (X) of variables is given in Table 3.3. The dependent variable (y) was associated with the coded variables (x_i , $i = 1$ and 2) by a second order polynomial equation (Eq. 3.3) as given below

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 + \varepsilon \quad \text{-----} \quad (3.3)$$

The coefficients of the polynomial were denoted by b_0 (constant), b_1 , b_2 (linear effects); b_{12} , (interaction effect); b_{11} , b_{22} (quadratic effects); and ε (random error). The statistical investigation of the collected data and three-dimensional graph plotting was done using Design Expert software version 7.0.

Table 3.1 Experimental design in coded form of response surface analysis

Coded Variable				Number of Experiments
X_1	X_2	Combinations	Replications	
± 1	± 1	4	1	4
± 1.414	0	2	1	2
0	± 1.414	2	1	2
0	0	1	5	5

^aCode '0' is for centre point of the parameter range investigated ' ± 1 ' for factorial points, and ' ± 1.41 ' for star points; X_1 , Initial moisture content, X_2 , Incubation time (days)

Table 3.2. Coded and uncoded parameter levels

Experimental Variables	Code	Coded level				
		-1.414	-1	0	1	1.414
Initial moisture content, %	X_1	45.86	50	60	70	74.14
Incubation time (days)	X_2	7.76	9	12	15	16.24

Table 3.3: The central composite experimental design (coded and actual levels of two variables) employed for the pigment production process

S.No.	Initial Moisture content (%)-coded	Incubation Time(days)-coded	Initial Moisture content (%)	Incubation Time(days)
1.	1.414	0	74.14	12
2.	0	0	60.0	12
3.	0	0	60	12
4	1	-1	70	9
5	0	1.414	60	16.24
6.	1	1	70	15
7.	-1	1	50	15
8.	0	-1.414	60	7.76
9.	0	0	60	12
10	0	0	60	12
11	-1	-1	50	9
12	0	0	60	12
13.	-1.414	0	45.86	12

3.3 EXTRACTION AND ESTIMATION OF PIGMENT

3.3.1 Extraction of pigment produced by solid state fermentation

2 g of dried fermented substrate was taken in conical flasks and mixed with different solvents in ratio of 1:10 for extraction of pigment. The various solvents used were hexane, benzene, acetone, methanol, ethanol and distilled water. Extraction was carried out by keeping the flask on a rotary shaker at 200 rpm for 60 min. The contents were then filtered through what man's filter no. 1. The extracted pigment was analyzed by spectroscopy (Shimadzu, Japan). Extraction of pigment was carried out by two methods:

3.3.1.1 Single solvent extraction:

2 g of dried fermented substrate was extracted with individual solvent (hexane, benzene, petroleum ether, acetone, methanol, ethanol and distilled water). After adding each solvent flask were kept on a rotary shaker for one hour at 200 rpm.

Extracted sample was analyzed with the help of UV Spectrophotometer. The spectrum scanning was done from 300nm to 600nm to find out dominating wavelength and pigment yield calculated (Venkatachalam et al., 2018).

3.3.1.2 Continuous Solvent Extraction –

For continuous extraction solvents on the basis of increasing polarity were used in the order hexane, benzene, petroleum ether, acetone, methanol, ethanol and distilled water. After adding each solvent flask were kept on rotary shaker for one hour at 200 rpm.

Extracted sample was analyzed with the help of UV Spectrophotometer. The spectrum scanning was done from 300nm to 600nm to find out dominating wavelength.

Pigment yield was calculated by using following equation (3.4)

$$\text{Pigment yield (ODunits/gm)} = \frac{\text{OD} \times \text{dilution factor} \times \text{total volume of pigment}}{\text{dry weight of moldy substrate}} \text{ ----- (3.4)}$$

3.3.2 Estimation of dry weight of moldy substrate

2 g of wet substrate after fermentation was taken in a pre-weighed dish along with 5 ml of ethanol. The dish was kept in hot air oven set at 105°C. The contents were dried till constant weight is achieved. After drying the dish was shifted to a desiccator for cooling. When the temperature of the petri dish reached room temperature, the dish with the dry moldy substrate was weighed.

3.3.3 Optimization of aqueous extraction of water soluble pigments produced by *Epicoccum nigrum*

Response surface modeling is a powerful statistical tool and technique efficiently used in investigating multiple independent parameters and their interactive effects (Jakobsen and Bertelsen, 2000; Hsu and Chung, 2001) and developing mathematical models that perfectly anticipates the overall process (Maran and Manikandan, 2012). The focal advantage of RSM is less number of experiments required to get enough data for statistically adequate results (Kaur et al., 2009). Several biochemical and food biotechnological processes have been successfully

optimized using response surface methodology (Gomes et al., 2013). The extraction and purification of microbial pigment are the most important step for its application purposes.

For the extraction process the experiments were conducted as per the experimental design (Table 3.4) generated by Design expert software version 7.0. The required amount of mass of fermented matter was put into a 250 ml Erlenmeyer flask and the volume was made 50 ml using distilled water as solvent under study. The flask was capped to avoid evaporation during the experiments. The flasks were incubated at different temperatures for a designated time as per the design. After incubation the contents were strained through Whatmann paper no. 1 and the filtrate was used for determination of pigment yield.

3.3.3.1 Experimental Design

Response surface methodology (RSM) was used in the designing of experiments because it highlights the analysis and modeling of the experiment wherein the dependent variable is affected by numerous factors and the aim is to optimize the conditions (Montgomery 2017). A three-factor five-level central composite rotatable design (CCRD) was used (Myers 1976). The process parameters were the extraction temperature (X_1), mass of fermented matter (solute) (X_2) and time (X_3). The parameters and their levels were finalized on the basis of initial experiments. The levels used under study are: extraction temperature (X_1 ; 29.8-80.2°C), mass of solute (X_2 ; 0.2-1.8 g) and time (X_3 ; 9.5-110.5 min). The three independent variables were coded as -1.682 -1, 0, 1 and +1.682. The experimental design in coded (x) and actual level (X) of variables is given in Table 3.4. The dependent variable (y) was associated to the coded variables (x_i , $i = 1, 2$ and 3) by a second order polynomial equation (Eq. 3.5) as given below

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad \text{----- (3.5)}$$

The coefficients of the polynomial were denoted by b_0 (constant), b_1, b_2, b_3 (linear effects); b_{12}, b_{13}, b_{23} (interaction effects); b_{11}, b_{22}, b_{33} (quadratic effects); and ε (random error). The statistical investigation of the collected data and three-dimensional plotting was done using Design Expert software version 7.0.

Table 3.4: The central composite experimental design employed in the extraction process

Independent variable(Coded Factor)			Independent variable(Actual Factor)		
A:Extraction temp (°C)	B:Mass of fermented rice (g)	C:Time (min)	A:Extraction temp (°C)	B:Mass of fermented rice (g)	C:Time (min)
-1	-1	1	40	0.5	90
1	-1	1	70	0.5	90
0	0	1.682	55	1	110.5
0	0	0	55	1	60
0	0	0	55	1	60
0	0	0	55	1	60
-1	1	-1	40	1.5	30
0	0	-1.682	55	1	9.5
0	0	0	55	1	60
-1	-1	-1	40	0.5	30
0	0	0	55	1	60
1	-1	-1	70	0.5	30
1	1	1	70	1.5	90
0	0	0	55	1	60
1.682	0	0	80.2	1	60
-1.682	0	0	29.8	1	60
1	1	-1	70	1.5	30
0	1.682	0	55	1.8	60
0	-1.682	0	55	0.2	60
-1	1	1	40	1.5	90

3.3.3.2 Extraction of carotenoids

For the extraction process the experiments were conducted as per the Box-Behnken experimental design (Table 4.23). The dried fermented mass was soaked in HCl solution (3 mol/L) at 28°C, shaking the contents at 100 rpm for period of 30 min, and afterwards centrifuged for 20 min at 10,000 rpm. The supernatant was discarded, and acetone was then added to the one gram of sample as per design. Carotenoids extraction was undertaken at time and temperature conditions of design under the shaking condition of 100 rpm. The supernatant obtained after extraction by centrifugation (10,000 rpm, 20 min) was used for estimation (Gu et al., 2008).

Carotenoid Estimation

The optical density of supernatant was determined by UV-vis spectrophotometer (Shimadzu, Japan) at 445 nm. The total carotenoids yield (mg/g dried mass) was evaluated using formula (4.3) given by Deming et al., 2006 as follows:

$$\text{Carotenoid yield } \left(\frac{\mu\text{g}}{\text{g}} \text{ of dried mass} \right) = \frac{1000 A D V}{0.16 W} \quad (4.3)$$

Where, A represents optical density of the extract at 445 nm, D denotes dilution factor, V is the volume of solvent, 0.16 is the extinction coefficient of carotenoids, and W is weight of the dried mass of fermented matter.

3.4 ISOLATION AND PURIFICATION OF THE PIGMENT:

3.4.1 Column Chromatography:

A crude pigment extract obtained from fermented substrate was used for pigment purification. The filtrate obtained was concentrated by using a rotary evaporator till dryness. The extract was loaded into a chromatographic column packed with silica gel (60-120 mesh) suspended in methanol. The adsorption column was eluted with analytical grade methanol. The impurities and substrate residue extracted along with the pigment gets adhered to the gel and the pigment is eluted down with methanol. After packing of column concentrated extracts were used for

separation. To purify extract different solvent system were used : Hexane (100%), Hexane: Ethyl Acetate in proportion of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, Ethyl Acetate (100%), Ethyl Acetate: Methanol in proportion of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and Methanol (100%) respectively. The solvent fractions eluted from the column were collected and spectrum was analyzed using UV-Vis spectrophotometer (SHIMADZU UV-1800, Japan). Collected fractions were further used for TLC analysis (Parmar and Singh, 2018).

3.4.2 Thin Layer Chromatography:

The fraction obtained from the column were concentrated and applied to preparative TLC (20cm×20 cm, silica gel G). The running solvents tried were: Acetone (30): Hexane (70); Ethyl Acetate (100): Formic Acid (11): Acetic Acid (11): Water (26); Chloroform (65): Methanol (25): Water (4); Chloroform (95): Methanol (5) to find out the best solvent mixture given higher R_f value (Parmar and Singh, 2018). The solvent mixture was kept in a chromatographic chamber for 15 min for equilibration. The sample was spotted on silica gel plates with the help of capillary tube and air dried. The TLC plates were placed in chamber dipped in solvent system and allowed to run. TLC plate was carefully removed and air dried. Retention factor (R_f) value was calculated according to the following equation (3.6)

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \text{-----} (3.6)$$

The best separated target bands were scraped off, dissolved in ethanol, and separated from silica through filtration (Whatman No. 1 paper). The sample was concentrated to desiccation at 45°C under reduced pressure in a rotary evaporator.

3.4.3 High Performance Liquid Chromatography:

To further check the purity of the pigment the purified product was analyzed on a C18 column using an analytical HPLC at 430 and 450 nm. The LC system was operated using a water/acetonitrile gradient system starting from 15% (v/v) acetonitrile, which was increased linearly to 100% in 20 min with a holding time of 5 min (Mapari et al., 2008).

3.5 CHARACTERIZATION AND STRUCTURE ELUCIDATION OF THE PIGMENT

3.5.1 Chemical characterization:

Chemical characterization of the extracted pigment was performed through UV–Vis spectra, FTIR and LC-DAD-MS and NMR analyses.

3.5.1.1 FTIR analysis:

For structure determination the extracted pigment was subjected to FT-IR analysis (Shimadzu 8400S FT-IR spectrophotometer, equipped with KBr beam splitter) using approximately ratio of 1:9 mg sample to KBr. FT-IR spectrophotometer was operated at a spectral range of 4000–400 cm^{-1} with a maximum resolution of -0.85 cm^{-1} . The spectra were interpreted using the guidelines of Stuart (2004).

High-resolution LC-DAD-MS:

The analysis was performed on Waters, Micromass Q-TOF micro system equipped with a photodiode array detector and separation Module of Waters Alliance 2795. The Xbridge Waters C_{18} LC column with $250 \times 4.6 \text{ } 5 \mu\text{m}$ i.d. was used. The LC system was coupled with a Z spray electrospray positive ionization source (ESI+). The injection volume was kept 15 micro liters with flow rate of 0.7 ml/min.

Samples were analyzed in positive ESI (ESI+) using a water- CH_3CN gradient system starting from 15% CH_3CN , which was increased linearly to 100% in 20 min holding this for 5 min or starting from 5% CH_3CN keeping this for 2 min and increasing to 100% in 18 min keeping this for 5 min. The water was buffered with 10 mM ammonium formate and 20 mM formic acid (HCOOH) and the CH_3CN with 20 mM HCOOH (Nielsen, 2005).

The desolvation temperature was $250 \text{ }^\circ\text{C}$; source block temperature was $100 \text{ }^\circ\text{C}$; and the desolvation flow was ca.550 lts/hr and cone gas flow was 25lts/hr. The capillary was held at -2780 V , and data were collected as centroid data from m/z 100-900. The potential difference between the skimmers was 30V.

The compounds were identified by comparing LCMS spectra with library and literature by their m/z value, and wavelength absorbance pattern.

NMR analysis

^1H NMR spectra were recorded on a Bruker Avance 500 DRX spectrometer (Bruker, Karlsruhe, Germany) at 300 MHz. Coupling constants (J) in Hz. Abbreviations: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, brs = broad.

^{13}C NMR spectra including DEPT were recorded on a Bruker Avance 500 DRX spectrometer (Bruker, Karlsruhe, Germany) at 75 MHz. Chemical shifts were measured relative to tetramethylsilane as standard.

2D-NMR spectra

^1H - ^1H COSY (^1H , ^1H -correlated spectroscopy), HSQC (Heteronuclear Multiple Quantum Coherence)

3.5.2 Evaluation of Pigment Stability:

The thermal stability test was performed at different temperatures keeping the pH constant at 6.0. The pigment extracts were incubated in a water bath at 30, 40, 60 or 80 °C from 0 to 360 min. For pH stability studies, temperature was kept constant at 80 °C and pigment extracts with variable pH values (4.0–8.0) were incubated from 0 to 180 min. For checking the effect of sterilization on pigment stability autoclave treatment (121 °C for 15 min) was given. The buffer solutions used were 0.2 M sodium citrate–phosphate (pH 4.0, 5.0, and 6.0) and 0.2 mol/L sodium phosphate (pH 7.0 and 8.0). In all the experiments, pigment yield was measured and expressed as percentage of relative absorbance (Silveria et al., 2011).

3.5.2.1 Statistical analysis for kinetic modeling of thermal degradation:

The data obtained of residual color for thermal degradation through time was fitted to first order kinetic models. Analysis was performed using statistical criteria in which coefficient of determination (r^2), chi-square (χ^2), and standard error of means (S.E.M.) were evaluated. These criteria have been used efficaciously to compare the kinetics of thermal inactivation models of several bioactive compounds (Corradini and Peleg 2004; Sant'Anna et al., 2010).

First Order Kinetic model: Pigment degradation during the thermal treatment of pigment extracts was considered to follow first order kinetics (Chandran et al., 2014; Ludikhuyze et al., 1999) indicating a logarithmic order of degradation which is expressed as

$$\frac{A}{A_0} = \exp(-kt) \quad (3.7)$$

The dependence of the degradation rate constant (k) on temperature was quantified by the Arrhenius equation,

$$k = A_0 \exp\left(-\frac{E_a}{RT}\right) \quad (3.8)$$

In the equations, A/A_0 represents the residual absorbance (426 nm) at time t (min), k (min^{-1}) is the inactivation rate constant at a given temperature, A_0 is the Arrhenius constant, E_a the activation energy, and R the universal gas constant ($8.31 \text{ J mol}^{-1} \text{ K}^{-1}$). The experimental data were statistically evaluated on the basis of coefficient of determination (r^2), chi-square (χ^2), and standard error of means (S.E.M.). These criteria have been used successfully to compare the kinetics of thermal inactivation models of several bioactive compounds (Corradini and Peleg 2004; Shalini et al., 2008; Sant'Anna et al., 2010)

Chi Square (χ^2) –

$$\chi^2 = \frac{\sum(a_{\text{measured}} - a_{\text{predicted}})^2}{n-p} \dots\dots\dots (3.9)$$

Standard Error Mean

$$SEM = \frac{\sum(a_{\text{measured}} - a_{\text{predicted}})^2}{\sqrt{n}} \dots\dots\dots (3.10)$$

Root Square Mean Error

$$RMSE = \sqrt{\frac{\sum(a_{\text{measured}} - a_{\text{predicted}})^2}{n}} \dots\dots\dots (3.11)$$

Where p is the number of parameters and n is the number of observations.

3.6 ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF PIGMENT:

3.6.1 Antimicrobial Activity

Both bacterial and fungal species were used for antimicrobial assay. The strains were procured from The Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. Four bacterial strains and 4 fungal strains were taken to examine the antimicrobial activity of the pigment. For bacterial culture nutrient agar medium and for fungal culture potato dextrose agar medium was used. The screening for the antimicrobial activities was performed based on the radial diffusion technique (Kalemba and Kunicka 2003). Assay plates were prepared using Muller-Hinton agar. Muller Hinton agar, sterilized in a flask and cooled to 45 to 50°C, was distributed to sterilize petri dishes. After the agar has solidified, 24 h, old microbial cultures were serially diluted up to 10^{-9} and 0.1 ml of culture was spread on the medium plate. Wells of diameter 6mm were made on the plates using sterile well borer and pigment extract was dispensed within them for a study of

antimicrobial activity. The plates were incubated at 37°C and size of inhibition zones around the hole was measured after 24 h of incubation. For pigment extract methanolic extract was concentrated to dryness and by using DMSO concentration of extract was made 5mg/ml.

The test organisms under study were

1. *Escherichia coli* MTCC443
2. *Pseudomonas aeruginosa* MTCC424
3. *Staphylococcus aureus* MTCC96
4. *Streptococcus Pyogenes* MTCC442
5. *Candida albicans* MTCC227
6. *Aspergillus Niger* MTCC282
7. *Trichoderma harzianum* MTCC792
8. *Fusarium moniliforme* MTCC156

3.6.2 Antioxidant properties

For making the methanolic and ethanolic pigment extracts 10 g of substrate was extracted with 100 ml of respective solvent for 24 h at 25°C under shaking conditions. The contents were strained after extraction through Whatman No. 1 paper. The residues left after extraction was re-extracted with additional 100 ml of solvent. The collected extracts were then rotary evaporated at 40°C to dryness. The dried extract was collected and used for antioxidant properties by dissolving in distilled water to a concentration of 50 mg/ml and further diluted to 0.01, 0.1, 1, 5, 10 and 20 mg/ml for further use (Lee at al., 2009).

3.6.2.1 Total phenolic content (TPC)

Total phenolic content of pigment extract was measured spectrophotometrically (Shimadzu, Japan) at 765 nm by using Folin Ciocalteu's reagent. The reaction mixture was prepared by mixing 0.5 ml of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO₃. The samples were kept in a water bath at 45°C for 45 min. The absorbance was measured using spectrophotometer at 765 nm. The concentration was noted (mg/ml) using a calibration curve of Gallic acid. The results were represented as Gallic acid equivalents (GAE) (mg GAE/g of sample) (Stankovic, 2011).

3.6.2.2 Total Flavonoid content (TFC)

Total flavonoid content was determined spectrophotometrically by Aluminum chloride method (Chang et al., 2002). In the pigment extract (0.5 ml) 1.5 ml of methanol, 0.1 ml 1 M potassium acetate, 0.1 ml of 10% aluminum chloride and 2.8 ml of distilled water was added. The contents were kept at room temperature for 30 min and absorbance of the mixture was observed at 415 nm. The calibration curve was prepared using quercetin standard. The results were represented in terms of quercetin equivalent/g of sample

3.6.2.2 DPPH Radical Scavenging Activity

The antioxidant activity of the pigment extracts was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay (Lee et al., 2003). Briefly, the stock reagent solution was prepared by dissolving 24 mg of DPPH in 100 ml methanol. The working solution was obtained by mixing 10 ml of stock solution with 45 ml methanol to obtain an absorbance value of 1.1 ± 0.02 at 517 nm, using a spectrophotometer. The different concentrations of pigment extracts were allowed to react with 3 ml of DPPH solution. The mixture was shaken briskly and kept at room temperature in dark conditions for 30 min. The mixture was measured spectrophotometrically at 517 nm. A control sample with no added extract will also be analyzed and the results will be expressed as radical scavenging activity (% RSA).

$$\% \text{radical scavenging activity} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100 \quad \text{-----} \quad (3.12)$$

Where, A = absorbance at 517 nm.

3.6.2.3 FRAP (Ferric Reducing/Antioxidant Power) Assay

The FRAP assay was performed as previously described by Benzie and Strain 1996. Briefly the working FRAP reagent produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10:1:1 ratio prior to use and heated to 37°C in water bath for 10 min. pigment extracts of various concentrations (1–5 mg/ml) was allowed to react with 2.7 ml of the FRAP reagent. The final volume of the reaction mixture was made up to 3 ml with distilled water. The reaction mixture was kept in dark for 30 min. The reading of the colored product (ferrous tripyridyltriazine complex) was then taken at 593 nm.

Standard curve for FRAP

	1	2	3	4	5	6	7	8
H ₂ O	1000	985	970	940	880	820	760	700
1mM Std (μl)	0	15	30	60	120	180	240	300
FRAP (ml)	2	2	2	2	2	2	2	2
Std Conc (μM)	0	5	10	20	40	60	80	100

FRAP value calculated as

FRAP value of sample (μM)

$$= \frac{\text{Absorbance of sample} \times \text{FRAP value of standard } (\mu\text{M})}{\text{Absorbance of standard}}$$

------(3.13)

3.7 Statistical analyses

The statistical importance of the evaluated data was analyzed by one-way analysis of variance. Differences between the mean values were tested using the least significant difference multiple range test. Values were considered significant when $P < 0.05$, except when otherwise indicated. Each treatment was conducted in triplicate, and all experiments were repeated at least twice.

RESULT AND DISCUSSION

Experiments were conducted to study the potential of *Epicoccum nigrum* for production of polyketide and carotenoid pigments and their extraction and characterization. The results obtained have been discussed under different subheadings

4.1 Agar plate study

The agar plate study was aimed to categorize medium components for growth parameters and pigment production with a focus on yellow to orange pigment. Five different media compositions were used to analyze the growth, sporulation and the pigment production by *Epicoccum nigrum*. Growth was depicted by measurements of the colony diameter at different time intervals to estimate the colony radial growth rate (K_r) and spore count using Neubauer chamber. The pigment coloration, intensity was recorded in terms of L^* , a^* , b^* , hue angle and chroma values.

Nutritional prerequisites are critical for successful development of fungus in lab as well as at industrial level. Manipulation in the nutritional components may help in improving the yield of targeted secondary metabolite (Walker and White 2005). Basal medium selection is the most important step in media optimization.

4.1.1 Influence of different medium

The effect of different composition on growth parameters (radial growth and biomass), sporulation and pigmentation ability in terms of L^* , a^* , b^* , hue angle and chroma value of *E. nigrum* after 7 days of fermentation period have been given in Table 4.1. All culture media favored the growth of the test fungi to various degrees. There was a significant difference in the growth over different media at $p < 0.05$ (Table 4.1). A speedy growth was observed on Potato dextrose agar (PDA) in comparison to other media. On this medium, the fungal colony acquired a diameter of 43.2 mm on the 7th day (Fig. 4.2). The colony radial growth rate was 0.125 mm/hr. Growth rate decreased in Malt extract agar (MEA), Yeast extract agar (YEA) and Czapeck dox agar (CDA). With respect to radial growth, the least suitable media were SDA as it was only 29% as effective as PDA, the best medium. Similar behavior was visible for sporulation that varied significantly for different media under study. The highest sporulation was induced by PDA ($2.85 \times 10^5 \pm 2 \times 10^3$ spores/mL) followed by MEA, YEA, and CDA and lowest by SDA ($1.67 \times 10^5 \pm 5 \times 10^2$ spores/mL) as shown in Table 4.1. PDA is one of the most commonly used culture media

for most of the fungi owing to its simple composition and its ability to support vegetative growth. Saha et al., (2008) also reported PDA to be the best culture media for mycelia growth.

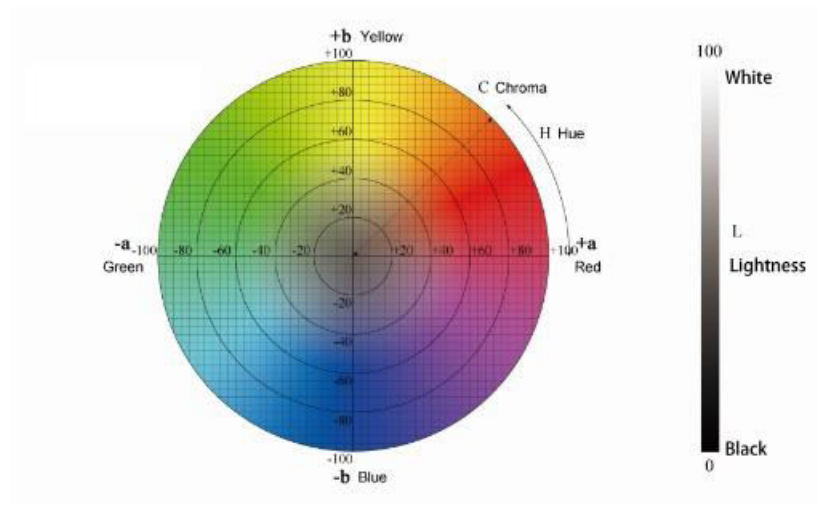


Fig. 4.1 CIE LAB color Coordinates

Pigment production using different media formulations on agar plates were quantified using a computer vision system in terms of L^* , a^* , b^* , hue angle and chroma value. The measurements are based on light reflected from the surface (Mapari et al., 2006). The variation in lightness L^* , a^* , b^* , hue and chroma values on different media are shown in Table 4.1. The various media produced different patterns of variation. At the end of the incubation period, the color in medium CDA showed the maximum value of a^* i.e. 62.06 ± 0.08 (Table 4.1). The hue angle ranges from 44.90 ± 0.01 to 79.94 ± 0.05 . This indicated that the color produced to be red-orange-yellow on the basis of the CIELAB color system (Fig. 4.1) as explained in Chapter 3. This infers that the pigment produced is the same, but synthesized in higher or lower amount on varying media formulations (Mapari et al., 2008). Similar variations in hue angle were shown on *E. nigrum* liquid pigment extracts obtained from different media (Mapari et al., 2006).

Table 4.1 Influence of different media on the growth, sporulation and pigmentation ability of *E. nigrum*

Evaluation	PDA	CDA	SDA	MEA	YEA
Radial growth rate (mm/day)	3±0.02 ^a	2.2±0.01 ^b	1.09±0.01 ^c	2.95±0.01 ^d	2.76±0.03 ^e
Sporulation (spores/mL)	2.85×10 ⁵ ±2×10 ^{3a}	2.24×10 ⁵ ±1.4×10 ^{3b}	1.67×10 ⁵ ±5×10 ^{2c}	2.5×10 ⁵ ±2×10 ^{3d}	2.37×10 ⁵ ±2×10 ^{3e}
Dry cell weight (g)	0.08±0.005 ^a	0.03±0.003 ^b	0.025±0.002 ^b	0.06±0.005 ^c	0.04±0.004 ^d
L	60.54±0.06 ^a	52.49±0.13 ^b	59.36±0.08 ^c	59.65±0.08 ^d	83.05±0.08 ^e
a*	29.42±0.03 ^a	62.06±0.08 ^b	48.31±0.01 ^c	27.13±0.08 ^d	6.01±0.01 ^e
b*	56.81±0.02 ^a	61.82±0.18 ^b	62.05±0.07 ^b	56.74±0.03 ^a	34.05±0.07 ^c
Hue	62.60±0.01 ^a	44.90±0.01 ^b	52.05±0.08 ^c	64.41±0.13 ^d	79.94±0.05 ^e
Chroma	64.05±0.07 ^a	87.71±0.02 ^b	78.66±0.02 ^c	62.80±0.12 ^d	34.82±0.25 ^e

Data presented as means± SD of triplicate readings. Mean values within each row with different superscripts are significantly different (p < 0.05)

The increased growth rate or sporulation did not necessarily result in higher pigment production in the media under study. In general, the pigments are the secondary metabolites produced by fungi and their production usually occurs in stationary phase (Calvo et al., 2002). However, the metabolic versatility results in multiple responses under variable nutritional and environmental conditions. The amount of biomass did not certainly result in higher pigment production (da Costa Souza et al., 2016). Fig. 4.2 depicts the change in colony diameter over time on different defined medium.

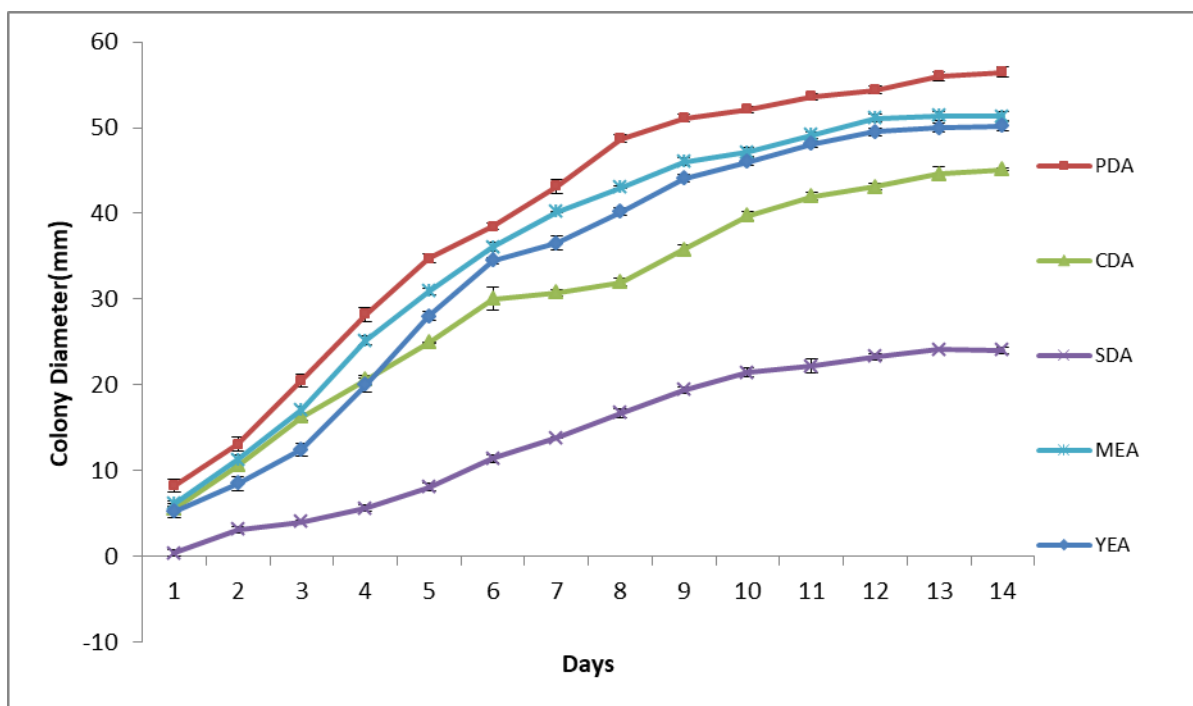


Fig. 4.2. Colony diameter of *Epicoccum nigrum* on different agar media viz. Potato dextrose (PDA), Czapeck dox (CDA), Sabouraud dextrose (SDA), Malt extract (MEA) and Yeast extract (YEA)

4.1.2 Influence of different carbon sources

In order to study the influence of different carbon sources on growth, sporulation and the pigment production CDA was used as base medium and replacing the carbon source with the carbon sources under study. Carbon sources significantly affected the parameters under study viz. radial growth, sporulation, dry cell weight and color parameters. The results of carbon sources are depicted in Table 4.2 and Fig. 4.3. Different carbon sources affected the growth parameters differently, while starch induced maximum radial growth (5.15 ± 0.02 mm/day), highest sporulation (224000 ± 1550 spores/mL) was induced by sucrose and dry cell weight (0.81 ± 0.02 g) was maximum when fructose was used. The requirement for the type and quantity of carbon differs among fungal species and the growth characteristics might vary within the strains (Gao et al., 2007). Shah et al., (2005) also reported that there is no correlation between radial growth and conidial production in case of the fungus *Metarhizium anisopliae*. Present study too showed that the carbon inducing maximum sporulation was different from the one that induced the best colony growth. Our results are in accordance with findings of Rajput and Shahzad (2015) who reported that sucrose is the best carbon source for conidial production of *T. polysporum*. Abdullah et al.,

(2005) also observed that mycelia yield of *T. harzianum* appreciably increased when sucrose was used as a carbon source in the culture media. Monga (2001) claimed that sucrose and glucose were the significant inducer for conidial production of *T. harzianum* and *T. koningii*. However, Jayaswal et al., (2003) reported that the maximum growth and sporulation of *T. viride* was found on supplemented medium containing peptone, sucrose and trehalose as compared to action of these carbon sources individually.

Different carbon sources affected the color production differently. The hue angle varies from 58.72 ± 0.59 (sucrose) to 84.79 ± 0.11 (sorbitol) (Table 4.2) indicating that it represents light orange to yellow color. The chroma value for sorbitol as carbon source was highest (41.24 ± 0.05) amongst all other carbon sources representing the brightest color. The nutrients can control the expression of genes of interest and trigger the metabolic pathways vital for pigment production (Pradeep et al., 2013). Thus, depending upon species some sources of carbon can be more effectively assimilated and promote higher yield of desired secondary metabolite (dos Reis Celestino et al., 2014).

Table 4.2 Influence of carbon sources on growth, sporulation and pigment production

	Dextrose	Fructose	Sorbitol	Starch	Sucrose
Radial growth (mm/day)	4.75 ± 0.02^a	4.3 ± 0.015^b	5.1 ± 0.015^c	5.15 ± 0.02^d	4.6 ± 0.03^e
Spore Count (spores/mL)	114000 ± 1557^a	182000 ± 1635^b	160000 ± 1885^c	136000 ± 1420^d	224000 ± 1550^e
Dry Cell Weight (g)	0.54 ± 0.01^a	0.81 ± 0.02^b	0.69 ± 0.01^c	0.15 ± 0.05^d	0.18 ± 0.06^d
L*	75.42 ± 0.16^a	64.62 ± 1.7^b	59.79 ± 0.12^c	63.33 ± 0.19^b	63.35 ± 1.59^b
a*	6.68 ± 0.14^a	10.20 ± 0.03^b	3.74 ± 0.07^c	7.80 ± 0.16^d	11.65 ± 0.23^e
b*	24.29 ± 0.17^a	23.91 ± 0.10^b	41.07 ± 0.05^c	19.36 ± 0.22^d	19.16 ± 0.08^d
Hue	74.61 ± 0.41^a	66.88 ± 0.16^b	84.79 ± 0.11^c	68.06 ± 0.64^d	58.72 ± 0.59^e
Chroma	25.19 ± 0.13^a	26.04 ± 0.09^b	41.24 ± 0.05^c	20.88 ± 0.15^d	22.42 ± 0.05^e

Data presented as means \pm SD of triplicate readings. Mean values within each row with different superscripts are significantly different ($p < 0.05$)

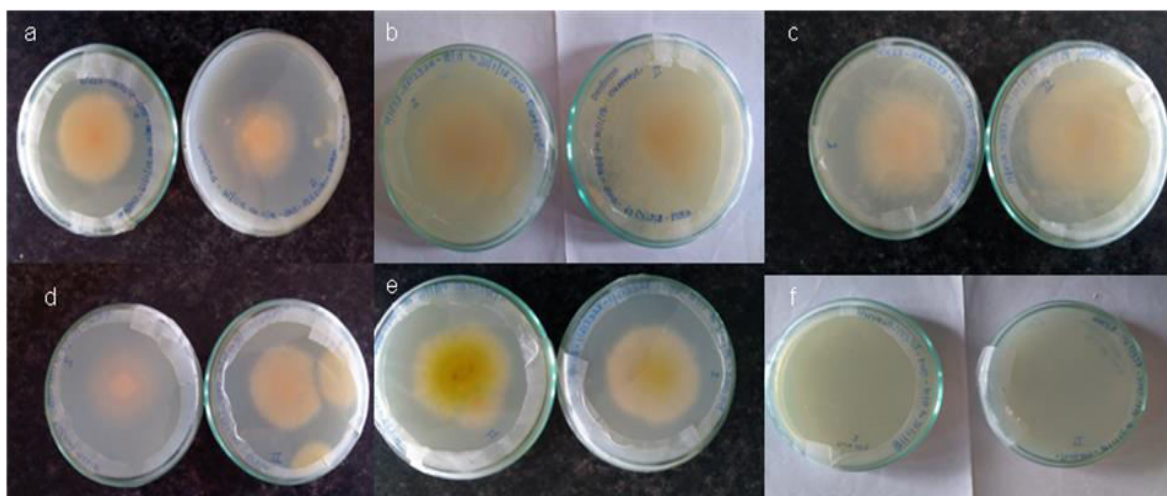


Fig. 4.3 Influence of different carbon sources on growth parameters: a) fructose, b) dextrose, c) starch, d) sucrose, e) sorbitol, f) ethanol

4.1.3 Influence of different nitrogen sources

The influence of nitrogen sources was examined on Czapeck Dox agar medium by replacing nitrogen source with ammonium chloride, ammonium sulfate, peptone, sodium nitrate and yeast extract. It was observed that peptone and yeast extract influenced the radial growth ($6.55 \pm 0.02 \text{ mm/day}$ and $6.5 \pm 0.03 \text{ mm/day}$) and yellow color production positively at $p < 0.05$, hue angle being 76.03 and 70.15 respectively as reported in Table 4.3 and Fig. 4.4. Sodium nitrate, ammonium chloride and ammonium sulfate showed moderate to little effect on radial growth as well as on sporulation, while peptone and yeast extract induced sporulation. Quality as well as quantity of the different nitrogen source used in the media to significantly influence not only the growth and differentiation, but also the fungal secondary metabolite production (Niehaus et al., 2014). The ability of the strain to utilize nitrogen sources decides the reproduction capability and degree of growth of fungus. The consumption of nitrogen during the growth process may result in a pH change in the culture medium and thereby affecting the pigment composition. Conversely, the pH value itself, is considered as an important, dictatorial factor that affects the pigment composition (Shi et al., 2015). Ammonium sulfate and sodium nitrate also repressed sporulation of *Penicillium camemberti* in solid medium, while potassium nitrate induced the sporulation

(Krasniewski et al., 2006). Similar findings have also been reported for *Monascus* (Shi et al., 2015) and *Aspergillus* (Skromne et al., 1995).

Table 4.3 Influence of nitrogen sources on growth, sporulation and pigment production

	Ammonium Chloride	Ammonium sulfate	Peptone	Sodium Nitrate	Yeast Extract
Radial growth (mm/day)	3.8±0.02 ^a	3.6±0.021 ^b	6.55±0.02 ^c	4.8±0.02 ^d	6.5±0.03 ^c
Spore Count (spores/mL)	158000±1720 ^a	154000±1335 ^b	220000±2100 ^c	160000±1600 ^a	206000±2050 ^d
Dry Cell Weight (g)	0.7±0.01 ^a	0.55±0.02 ^b	0.33±0.02 ^c	0.74±0.01 ^d	0.46±0.02 ^c
L*	54.23±0.23 ^a	56.62±0.12 ^b	57.20±0.09 ^c	65.42±0.15 ^d	42.64±0.05 ^e
a*	19.30±0.16 ^a	19.35±0.08 ^a	14.15±0.08 ^b	9.28±0.03 ^c	16.46±0.09 ^d
b*	22.46±0.17 ^a	23.89±0.12 ^b	56.91±0.06 ^c	22.81±0.04 ^d	45.60±0.01 ^e
Hue	49.32±0.45 ^a	50.97±0.13 ^b	76.03±0.08 ^c	67.85±0.10 ^d	70.15±0.10 ^e
Chroma	29.61±0.02 ^a	30.74±0.14 ^b	58.64±0.05 ^c	24.63±0.03 ^d	48.48±0.03 ^e

Data presented as means± SD of triplicate readings. Mean values within each row with different superscripts are significantly different ($P < 0.05$)

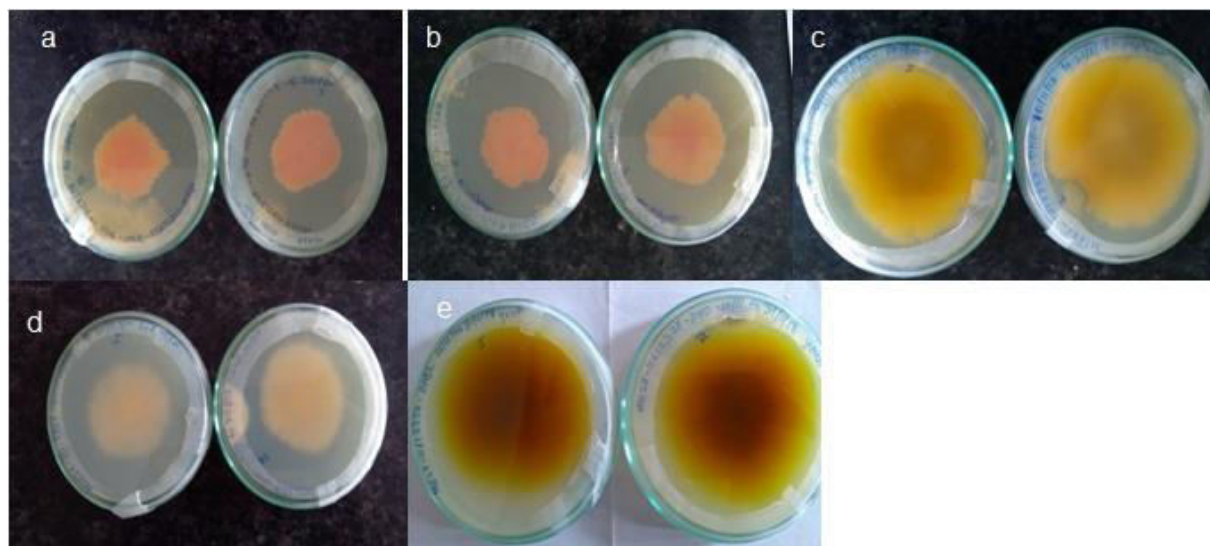


Fig. 4.4 Influence of different nitrogen sources on growth parameters: a) Ammonium chloride, b) ammonium sulfate, c) peptone, d) sodium nitrate, e) yeast extract

A change in the visible color was observed with the change in the nitrogen source which was supported by the hue data. It was observed that peptone and yeast extract affected (Fig. 4.4) yellow color production positively ($p \leq 0.05$), hue angle being 76.03 and 70.15 respectively. Addition of ammonium salts resulted into a pigment with a tinge of red color while peptone and yeast extract favor yellow color production. The corresponding hue angle for ammonium chloride and ammonium sulfate was 49.32 ± 0.45 and 50.97 ± 0.13 respectively. Nitrogen sources are considered to be very important for regulating the gene expression of interest and stimulating the metabolic pathways responsible for pigment synthesis (Chatterjee et al., 2009). In the current work, peptone and yeast extract favored the pigment production the most in comparison to rest of the nitrogen sources. Peptone is one of the most commonly used nitrogen sources in various culture media. It provides many essential nutrients such as peptides and amino acids and number of fungi have the capability to consume it during metabolism resulting in increased pigment secretion (Celestino et al., 2014; Quereshi et al., 2010). Organic nitrogen compounds not only provide nitrogen, but also carbon and sulfur to the culture media which are used by fungi as an energy source. On the other hand, yeast extract also supplies, vitamins and coenzymes required for the growth of fungus. Pigment production is favored by medium containing organic nitrogen sources (Pradeep et al., 2013). In the present study yeast extract demonstrated results similar to peptone i.e. increased pigment production.

4.1.4 Influence of light

Light is considered to be one of the regulators of growth, reproduction and metabolite production, all of which are significant expressions for the existence and spreading of the fungal classes (Babitha et al., 2008).

When looking at the development of the fungus, sporulation preferred normal illumination, while under complete darkness; there was an increase in the growth and dry cell weight. To investigate the wavelength dependence of parameters under study, agar plate study was carried out under variable light conditions, utilizing the blue, yellow, green and red illumination. In all the treatments, the plates were analyzed for radial growth, sporulation and dry cell weight after 10 days of incubation. It was observed that radial growth rate was the maximum under red light (8.5 ± 0.02 mm/day) followed by green light, yellow, blue and dark (5.8 ± 0.02 mm/day) (Table 4.4). Under the influence of red light, the colonies showed prolific growth (Fig. 4.5) while when blue

light was passed, contraction in the colony size was noticed. Similar conclusions were reported by Babitha et al., (2008) for *Monascus purpureus*. Sporulation was stimulated significantly on light exposure as shown in Table 4.4. Natural light stimulated the sporulation significantly ($2.85 \times 10^5 \pm 2000$ spores/mL) followed by red, green, yellow, blue and complete darkness ($1.68 \times 10^5 \pm 2200$ spores/mL). These findings indicate that *E. nigrum* differentiates between light in different wavelength ranges and respond differently in growth and sporulation. Kilpatrick and Chilvers (1981) also deduced that *Epicoccum* species seem to be variable in its requirement and response to light in terms of natural light and complete darkness. In case of *E.nigrum*, no reports are available to influence of diverse wavelength of light on growth, sporulation and pigment production.

The effect of light on the synthesis of biopigments has been reported in the recent past. Miyake et al., (2008) stated that while red light improved red pigment synthesis, blue light repressed it in case of *Monascus* cultures. Babitha et al., (2008) observed that the red light only slightly affected biopigment production by *Monascus purpureus*, whereas green and blue light considerably repressed the biopigment synthesis. In the present study the effect of light under similar conditions was examined on pigment production in terms of L* a* b* values, hue angle and chroma values. The hue angles of the petri plates under exposure to different wavelengths ranged from 54.34-64.63 being dark orange to yellow. This shows that the color compounds are similar, but are secreted in variable amounts on exposure to light of a specific wavelength. The chroma value denotes the intensity of a gloss. In a color wheel, values near to the center having same L* value show dull or gray colors, whereas values near to the circumference represents bright colors. As per the observations, natural light, darkness, yellow and red light produce bright yellow colors while blue and green light produced dull colors in comparison (Table 4.4)

Table 4.4 Influence of light of different wavelength on growth, sporulation and pigment production

	Red (780-600nm)	Yellow (595-577nm)	Blue (492-455nm)	Green (577-492nm)	Dark	Control
Radial Growth (mm/day)	8.5±0.02 ^a	7.0±0.05 ^b	6.1±0.03 ^c	7.2±0.02 ^d	5.8±0.02 ^e	3±0.02 ^f
Spore Count (spores/mL)	252000±1883 ^a	210000±1550 ^b	206000±2100 ^c	226000±1575 ^d	168000±2200 ^e	285000±2000 ^f
Dry Cell Weight (g)	0.47±0.02 ^a	0.42±0.01 ^a	0.5±0.02 ^b	0.55±0.01 ^b	0.13±0.02 ^c	0.08±0.005 ^d
L*	54.25±0.16 ^a	56.83±0.07 ^b	47.14±0.13 ^c	43.79±0.14 ^d	56.90±0.15 ^b	60.54±0.06 ^f
a*	39.84±0.17 ^a	19.12±0.15 ^b	31.61±0.17 ^c	25.59±0.13 ^d	27.52±0.17 ^c	29.42±0.03 ^f
b*	59.16±0.14 ^a	41.91±0.14 ^b	44.07±0.17 ^c	41.05±0.1 ^d	60.78±0.2 ^e	56.81±0.02 ^f
Hue	56.04±0.18 ^a	65.45±0.11 ^b	54.34±0.06 ^c	58.05±0.07 ^d	65.63±0.05 ^b	62.60±0.01 ^e
Chroma	71.32±0.03 ^a	46.07±0.18 ^b	54.24±0.24 ^c	48.37±0.16 ^d	66.77±0.28 ^e	64.05±0.07 ^f

Data presented as means± SD of triplicate readings. Mean values within each row with different superscripts are significantly different ($P < 0.05$)

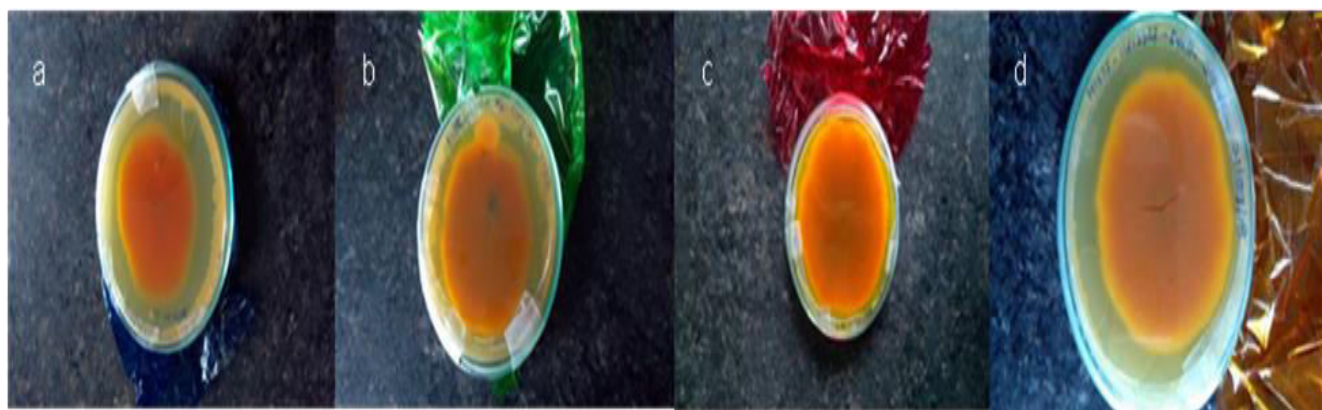


Fig. 4.5 Influence of light on growth, sporulation and pigment production by *E. nigrum*

4.5 Solid-State Fermentation

4.5.1 Fermentation conditions:

For inoculum, mycelium was scraped off from 7 days-old growing culture in sterile distilled water.

4.5.1.1 Selection of substrate

Various substrates viz. broken rice, wheat bran, and corn bran and sugarcane bagasse were used as substrate for SSF. The results are shown in Table 4.5

Table 4.5 Influence of different substrates on pigment production by *E. nigrum*

Wavelength (nm)	Pigment Yield, AU/g of dry matter			
	Broken Rice	Wheat bran	Corn bran	Sugarcane Bagasse
350	84.00±0.25 ^{aA}	0.03±0.18 ^{bB}	2.98±0.21 ^{aC}	84±0.15 ^{aA}
370	79.59±0.30 ^{bA}	0.65±0.05 ^{bB}	8.19±0.12 ^{bC}	NA
390	55.90±0.22 ^{cA}	54.26±0.11 ^{cA}	4.36±0.12 ^{bB}	NA
410	36.52±0.15 ^{dA}	60.79±0.10 ^{dB}	3.21±0.15 ^{aC}	20.37±0.12 ^{bD}
430	24.38±0.25 ^{eA}	55.64±0.20 ^{eB}	2.52±0.11 ^{dC}	NA
450	18.18±0.18 ^{fA}	43.24±0.10 ^{fB}	1.95±0.05 ^{eC}	6.97±0.05 ^{dD}
470	13.50±0.22 ^{gA}	26.54±0.06 ^{gB}	1.45±0.05 ^{fC}	NA
490	9.81±0.35 ^{hA}	15.85±0.13 ^{hB}	0.65±0.10 ^{gC}	NA
510	7.12±0.20 ^{iA}	9.41±0.10 ^{iB}	0.29±0.15 ^{hC}	3.86±0.12 ^{dD}
530	5.52±0.25 ^{jA}	5.35±0.15 ^{jA}	0.12±0.10 ^{iB}	NA
550	4.38±0.10 ^{kA}	2.99±0.18 ^{kB}	0.04±0.005 ^{jC}	2.60±0.18 ^{eB}

Data presented as means± SD of triplicate readings. Mean values within each column with different small superscripts are significantly different ($P < 0.05$). The different capital superscripts in rows are significantly different ($P < 0.05$)

In order to select the best substrate for microbial pigment production by *E. nigrum* four substrates were screened viz. broken rice, wheat bran, corn bran and sugar cane bagasse. The amount of pigment produced with different substrates varied to a great extent. As per the graph (Fig. 4.5) different substrate supported pigments with different dominating wavelength. While broken rice and sugar cane bagasse showed maximum absorbance at 350-370nm, wheat bran supported the pigment with a dominating wavelength around 410-430nm. This may be due to differences in the composition of the substrate. Coarse corn bran was unable to support growth and pigmentation. This may be due to poor digestion of the substrate by the organism, so was rejected further in this study. Broken rice and wheat bran were selected as the substrates for further studies.

4.5.1.2 Influence of initial moisture content

The moisture content of the substrate is one of the critical factors influencing the growth of microorganism and product formation (Singhania et al., 2009). Moisture content can affect water activity of the system, but high moisture content can adversely affect the mass transfer of oxygen and carbon dioxide due to water logging of substrates. Usually, the optimal moisture content falls in the range of 40-70% (Raimbault, 1998). The result shown in Table (4.6) indicates that production of pigment was highly dependent on the initial moisture content of rice substrate.

After fermentation, rice with initial moisture content of 50% seemed dry with poor growth of *Epicoccum*, whereas rice with 60% moisture content showed better growth and pigment production. With high initial substrate moisture of 70 and 80%, the rice grains became too sticky thereby hampering the growth of fungus and pigment production and its excretion into the substrate. For the pigments, the maximum values at 60% initial moisture content were 84 ± 0.13 AU/g dry matter at 350-370 nm and 40.9 ± 0.10 AU/g at 410 nm.

Table 4.6 Influence of initial moisture content on pigment production by *E. nigrum*

Wavelength (nm)	Pigment Yield, AU/g of dry matter				
	40%	50%	60%	70%	80%
350	71.92±0.18 ^{aA}	75.6±0.20 ^{aB}	84±0.31 ^{aC}	84±0.22 ^{aC}	73.5±0.12 ^{aA}
370	73.29±0.21 ^{aA}	79.59±0.11 ^{bB}	84±0.11 ^{aC}	79.59±0.05 ^{bB}	67.2±0.10 ^{bD}
390	49.58±0.15 ^{bA}	55.86±0.05 ^{cB}	57.56±0.15 ^{bC}	55.90±0.11 ^{cB}	50.82±0.05 ^{cA}
410	31.71±0.11 ^{cA}	36.54±0.21 ^{dB}	40.91±0.10 ^{cC}	36.52±0.11 ^{dB}	34.23±0.10 ^{dD}
430	20.74±0.10 ^{dA}	24.71±0.12 ^{eB}	28.05±0.18 ^{dC}	24.38±0.12 ^{eB}	24.06±0.15 ^{eB}
450	15.03±0.28 ^{eA}	18.58±0.10 ^{fB}	21.25±0.20 ^{eC}	18.18±0.20 ^{fB}	16.84±0.11 ^{fA}
470	10.75±0.15 ^{fA}	13.92±0.15 ^{gB}	15.77±0.02 ^{fC}	13.50±0.06 ^{gB}	13.65±0.12 ^{gB}
490	7.53±0.12 ^{gA}	10.37±0.20 ^{hB}	11.48±0.05 ^{gC}	9.81±0.17 ^{hD}	9.62±0.07 ^{hD}
510	5.29±0.18 ^{hA}	7.89±0.22 ^{iB}	8.48±0.17 ^{hC}	7.12±0.08 ^{iB}	6.72±0.18 ^{iD}
530	3.91±0.08 ^{iA}	6.38±0.25 ^{jB}	6.74±0.11 ^{iB}	5.52±0.12 ^{jC}	5.04±0.20 ^{jC}
550	2.94±0.05 ^{jA}	5.25±0.12 ^{kB}	5.54±0.10 ^{jB}	4.38±0.08 ^{kC}	3.84±0.15 ^{kD}

Data presented as means± SD of triplicate readings. Mean values within each column with different small superscripts are significantly different ($P < 0.05$). The different capital superscripts in rows are significantly different ($P < 0.05$).

4.5.1.3 Effect of Incubation time

Table 4.7 summarizes the influence of incubation time on pigment production. As per the result obtained the maximum pigment production occurred after 12 days and at certain wavelengths it may occur in 9 days depending upon the type of pigment. As it is clear from the Table (4.7) the pigment production started on the 3rd day and become maximum (84.00±0.15 AU/g) by the 12th day at 350 nm. For yellow pigment (410-430 nm) maximum pigment yield was obtained between 9th and 12th day, i.e. 43.47±0.02 to 58.59±0.02 AU/g of dry matter.

Table 4.7 Influence of incubation time on pigment production by *E. nigrum*

Wavelength (nm)	Pigment Yield, AU/g of dry matter				
	3	6	9	12	15
350	14.72±0.18 ^a	76.54±0.12 ^b	80.22±0.11 ^c	84.00±0.15 ^d	84.00±0.13 ^d
370	13.29±0.15 ^a	56.49±0.10 ^b	73.29±0.04 ^c	79.59±0.10 ^d	73.92±0.10 ^c
390	10.73±0.08 ^a	59.64±0.02 ^b	57.54±0.12 ^b	55.90±0.12 ^c	49.35±0.09 ^d
410	8.19±0.12 ^a	48.09±0.13 ^b	58.59±0.02 ^c	36.52±0.11 ^d	31.71±0.02 ^e
430	6.26±0.10 ^a	28.90±0.11 ^b	33.81±0.05 ^c	43.47±0.02 ^d	24.78±0.06 ^e
450	5.23±0.05 ^a	31.29±0.08 ^b	22.36±0.07 ^c	33.18±0.13 ^d	24.78±0.12 ^e
470	4.41±0.11 ^a	20.37±0.14 ^b	15.90±0.15 ^c	13.50±0.08 ^d	12.85±0.14 ^e
490	3.78±0.10 ^a	12.62±0.02 ^b	10.94±0.13 ^c	9.81±0.05 ^d	8.61±0.10 ^e
510	3.28±0.13 ^a	7.83±0.05 ^b	7.50±0.15 ^b	7.12±0.11 ^b	5.88±0.09 ^c
530	3.00±0.15 ^a	5.44±0.11 ^b	5.50±0.10 ^b	5.50±0.13 ^b	5.25±0.11 ^b
550	2.77±0.05 ^a	4.09±0.12 ^b	4.14±0.10 ^b	4.39±0.17 ^b	4.20±0.18 ^b

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

4.5.1.4 Optimization of the fermentation conditions for pigment production

The study of the combined effect of initial moisture content and incubation time on pigment yield was undertaken using experimental design generated by Design Expert (Stat-Ease, Inc., Version 7.0). The experimental results are reported in the Table (4.8).

Final equation in terms of coded factors

$$\text{Pigment yield} = 42.99 + 1.71A - 1.20B - 2.69AB - 10.13A^2 - 7.98B^2 \text{ -----(4.1)}$$

Where A is initial moisture content (%) and B is incubation time (days)

Table 4.8 Effect of initial moisture content (IMC) and incubation days on dependant variable i.e. Pigment yield

S.No.	IMC (%) -coded	Incubation Time (days)-coded	IMC (%)	Incubation Time (days)	Pigment Yield (AU/g)
1.	1.41	0	74.14	12	24.38
2.	0	0	60.0	12	43.47
3.	0	0	60	12	42
4	1	-1	70	9	30.81
5	0	1.41	60	16.24	24.78
6.	1	1	70	15	23.56
7.	-1	1	50	15	24.7
8.	0	-1.41	60	7.76	28.89
9.	0	0	60	12	43.5
10	0	0	60	12	42.8
11	-1	-1	50	9	21.2
12	0	0	60	12	43.2
13.	-1.41	0	45.86	12	20.7

Table 4.9 Analysis of variance of fitted models

Source	Sum of squares	Df	Mean Square	F value	P value (Prob>F)	
Model	1092.01	5	218.40	412.71	<0.0001	Significant
A-IMC	23.37	1	23.37	44.17	0.0003	
B-incubation time	11.43	1	11.43	21.60	0.0023	
AB	28.29	1	28.89	54.59	0.0002	
A ²	714.14	1	714.14	1349.50	<0.0001	
B ²	443.49	1	443.49	838.07	<0.0001	
Residual	3.70	7	0.53			
Lack of fit	2.15	3	0.72	1.85	0.2784	NS
Std. Dev.		0.73		R ²	0.9966	
Mean		31.85		Adj-R ²	0.9942	
C.V%		2.28		Pred. R ²	0.9838	
PRESS		17.74		Adeq Precision	45.894	

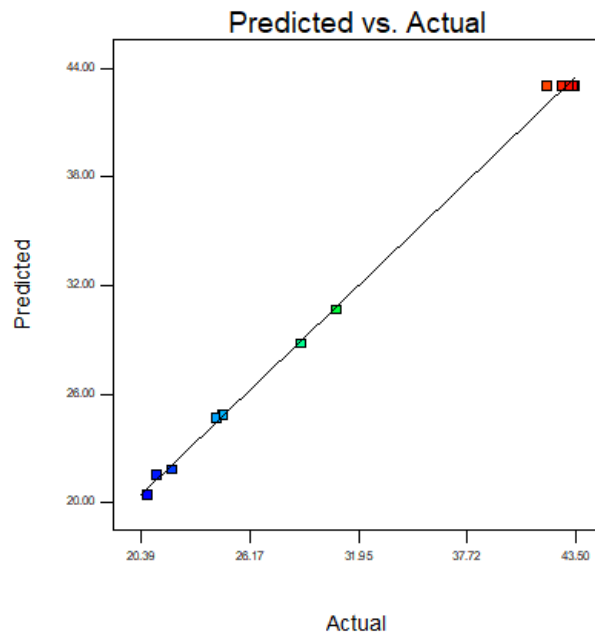


Fig. 4.6: Observed and predicted values of pigment yield

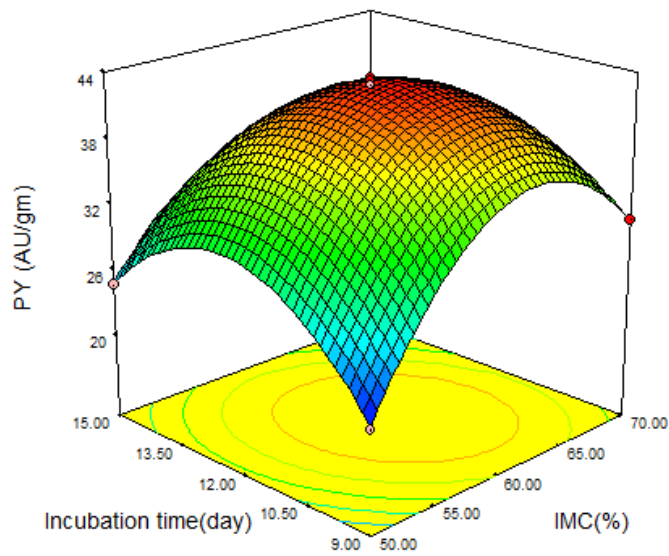


Fig. 4.7 A 3-D plot showing the effect of Incubation time and initial moisture content on pigment yield

Effect of variables on pigment yield

The pigment yield ranged from 20.7 AU/g to 43.5AU/g as depicted in Table 4.8. The empirical relation between response and variable is given by the equation, initial moisture content showed a significant ($p < 0.001$) positive effect at the linear level showing an increase in response with increase in initial moisture content, but after a particular amount of initial moisture content the yield starts decreasing, probably because of the accumulation of substrate particles, decrease of volume and thereby reducing the aeration, growth and metabolite production (Faseleh et al. 2012). Under low moisture conditions, the available oxygen is sufficient, but the water content is not enough to support metabolic activity and heat dissipation (Aparna and Reddy, 2012). Moisture content showed a negative effect at quadratic level, which represents higher response. The second variable i.e. incubation time showed a significant ($p < 0.01$) negative effect on pigment yield at linear as well as quadratic level. The interactive effect of the two variables was significant and negative ($p < 0.001$). Significant negative interaction suggests that the level of one of the variables can be increased while that of the other will decrease for constant value of response.

Adequacy of model was also tested by the graph representing observed versus predicted values (Fig.4.6). If the model is a perfect fit, all the points will be uniformly distributed around the line passing near the origin and at an approximate angle of 45° .

A plot between observed and predicted values of pigment yield is shown in Fig. 4.6 It is clear from the figure that the points are uniformly distributed over the experimental range and meet other requirement for a good fit.

The process conditions were optimized using the software. The criteria applied for numerical optimization was: the process variables were kept within range and response was kept maximum. The conditions optimized using design expert software are: 61% initial moisture content and incubation time of 11.73 days will result in pigment yield of 43.1 AU/g with overall desirability value of 0.98.

4.6 Effect of carbon supplementation on water and acetone extractable pigments

The results showing the effect of different carbon source supplementation in broken rice on water extractable pigments are reported in Table 4.10. It was observed that out of all carbon sources tried 4% dextrose was able to improve the pigment yield by 63% at 410 nm followed by maltose, sucrose, lactose and galactose. Utilization of carbon sources for pigment production appears to be

strain as well as fungal source specific. Similar results were reported for some strains of *Monascus* where glucose and its oligosaccharide and polysaccharides were better than other carbon sources for pigment production (Lin and Demain, 1992). Commonly used carbon sources as growth substrates for production of secondary metabolites by fermentation process is corn starch, glucose, molasses and sucrose. The suitable carbon source is important for pigment synthesis during non-growth phase. These not only provide energy for growth and pigment production, but also provide carbon for the synthesis of cell structures, metabolites and organic molecules (Said, 2010). The yield is normally limited due to the negative effect exercised by the carbon source because of carbon catabolite regulation which is widely observed in microbes and helps in sequential and organized utilization of carbon sources when more than one is present. In the presence of multiple sources of carbon the cell catabolize the one which rapidly supplies energy and carbon for growth. Therefore the synthesis of enzyme utilizing other substrates is suppressed until the primary substrate is exhausted (Sanchez and Demain, 2002).

At 8% (Table 4.11), there was an inhibition in pigment production in all the cases. This could be mainly because of high concentration of glucose, which results in the initiation of Crabtree effect or respiro-fermentative metabolism (Lee et al., 2011) along with high production of ethanol (Hajjaj et al., 2015). Also, higher concentration of carbon might decrease pigment production due to osmotic pressure exerted by high concentration of carbon source (Said, 2010). Keeping in view the pigment yield, broken rice can be supplemented with 4% dextrose to improve the water extractable pigment yield.

Table 4.10: Effect of supplementation of carbon sources (4%) on water extractable pigments

Wavelength nm	Control	Dextrose	Galactose	Lactose	Maltose	Sucrose	xylose
350	47.88±0.15 ^a	72.54±0.10 ^b	31.82±0.15 ^c	37.16±0.10 ^d	41.64±0.11 ^e	6.60±0.12 ^f	39.44±0.10 ^g
370	36.96±0.11 ^a	58.56±0.05 ^b	25.88±0.11 ^c	29.83±0.13 ^d	33.35±0.12 ^e	41.85±0.13 ^f	31.84±0.02 ^g
410	14.07±0.07 ^a	22.96±0.11 ^b	11.78±0.13 ^c	15.17±0.14 ^d	17.71±0.05 ^e	15.31±0.10 ^d	15.04±0.08 ^d
430	12.60±0.12 ^a	20.12±0.15 ^b	10.25±0.10 ^c	13.49±0.06 ^d	15.41±0.03 ^e	13.98±0.03 ^d	13.23±0.13 ^d
450	10.92±0.10 ^a	15.70±0.13 ^b	7.71±0.09 ^c	10.72±0.11 ^a	11.49±0.15 ^d	12.10±0.05 ^d	10.20±0.15 ^a

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

Table 4.11: Effect of supplementation of carbon sources (8%) on water extractable pigments

Wavelength nm	Control	Dextrose	Galactose	Lactose	Maltose	Sucrose	Xylose
350	47.88±0.02 ^a	5.19±0.12 ^b	5.37±0.03 ^b	51.23±0.05 ^c	4.96±0.04 ^b	29.58±0.10 ^d	31.82±0.02 ^e
370	36.96±0.04 ^a	31.69±0.01 ^b	35.51±0.02 ^a	38.26±0.05 ^c	29.19±0.02 ^d	23.66±0.02 ^e	25.30±0.07 ^f
410	14.07±0.05 ^a	8.88±0.03 ^b	8.96±0.05 ^b	19.18±0.04 ^c	7.85±0.05 ^d	13.95±0.10 ^a	12.69±0.01 ^e
430	12.6±0.06 ^a	8.07±0.05 ^b	8.12±0.07 ^b	16.33±0.02 ^c	7.19±0.03 ^d	12.15±0.12 ^a	11.25±0.03 ^e
450	10.92±0.05 ^a	7.00±0.02 ^b	7.16±0.03 ^b	11.88±0.10 ^a	6.37±0.02 ^c	8.98±0.02 ^d	8.78±0.05 ^d

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

The acetone extractable pigment was inhibited by supplementation of carbon sources (Fig. 4.10) which might be due to hindrance created in the pigment synthesis pathway. The higher glucose concentration above the optimum level showed reduced biomass and pigment production due to Crabtree effects (Lee et al., 2001). Chen and Johns, (1994) also reported the similar effect of decreased pigment production by *Monascus* at high glucose concentration (50 g/L). The results are shown in Table 4.12 and 4.13.

Table 4.12: Effect of supplementation of carbon sources (4%) on acetone extractable pigments

Wavelength	Control	Dextrose	Galactose	Lactose	Sucrose	xylose
350	84.00±0.02 ^a	7.12±0.02 ^b	4.53±0.02 ^c	7.62±0.02 ^b	8.79±0.03 ^d	9.27±0.02 ^e
370	84.00±0.02 ^a	6.84±0.01 ^b	4.10±0.10 ^c	6.96±0.01 ^b	8.47±0.07 ^d	8.52±0.05 ^d
410	31.50±0.05 ^a	9.99±0.05 ^b	4.56±0.02 ^c	7.77±0.04 ^d	8.10±0.01 ^e	8.05±0.12 ^e
430	27.93±0.03 ^a	8.96±0.01 ^b	4.23±0.03 ^c	7.17±0.03 ^d	7.56±0.02 ^d	7.39±0.10 ^d
450	23.31±0.07 ^a	8.01±0.03 ^b	3.80±0.05 ^c	6.63±0.05 ^d	6.80±0.05 ^d	6.78±0.01 ^d

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

Table 4.13: Effect of supplementation of carbon sources (8%) on acetone extractable pigments

Wavelength	Control	Dextrose	Galactose	Lactose	Sucrose	xylose
350	84.00±0.02 ^a	6.07±0.05 ^b	8.42±0.07 ^c	6.64±0.01 ^b	13.31±0.02 ^d	6.58±0.02 ^b
370	84.00±0.02 ^a	5.53±0.04 ^b	7.86±0.05 ^c	6.22±0.05 ^d	11.26±0.05 ^e	6.08±0.10 ^d
410	31.50±0.05 ^a	7.01±0.12 ^b	8.09±0.08 ^c	5.32±0.05 ^d	11.12±0.03 ^e	5.45±0.03 ^c
430	27.93±0.03 ^a	6.38±0.07 ^b	7.69±0.10 ^c	5.08±0.12 ^d	9.92±0.01 ^e	5.12±0.04 ^d
450	23.31±0.06 ^a	5.73±0.08 ^b	7.29±0.12 ^c	4.79±0.14 ^d	8.73±0.05 ^e	4.64±0.05 ^f

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

4.7 Effect of Nitrogen supplementation on water and acetone extractable pigment

Utilization of different nitrogen sources has been known to show a different pH pattern in fermentation thereby affecting the pigment production. In our study the effect of nitrogen supplementation resulted in improved yield of water extractable pigment (Table 4.14). While the pigment yield at 410 nm (yellow color) improved from 14.07 AU/g to 68.92 AU/g when sodium nitrate was added, followed by sodium glutamate (32.19 AU/g) and mono sodium glutamate (MSG) (31.23 AU/g). Babitha et al., (2006) also reported MSG to be most suitable for pigment production. Flavanoid production (350-370 nm) was also significantly improved by sodium nitrate, sodium glutamate and MSG addition (Fig. 4.8).

Supplementation of nitrogen inhibited the acetone extractable pigments (Table 4.15). This suggested that the broken rice alone produces more amount of acetone extractable pigments and supplementation results in a decrease in pigment yield. Pigments being secondary metabolites are produced via enzymatic pathways that happen via individual proteins, free or complexed, or through polypeptides carrying out a various enzymatic steps, e.g., polyketide synthases and peptide synthetases (Demain, 1998). The metabolite production is influenced by nitrogen regulation. The control of enzyme production is generally exercised by the intracellular nitrogen pool. Many secondary metabolic pathways are negatively affected by nitrogen sources favorable for growth, e.g., ammonium salts. As a result, a slowly assimilated amino acid is often used as the nitrogen source to encourage high production of secondary metabolites. Information concerning the mechanism(s) underlying the negative effect(s) of ammonium and certain amino acids on

industrial processes is scarce (Demain, 1996). The effect of different nitrogen sources are shown in Fig. 4.8.

Table 4.14: Effect of supplementation of nitrogen sources on water extractable pigments

Wavelength	Control	Yeast	Urea	Sodium nitrate	Sodium glutamate	MSG	Ammonium Chloride	Peptone	Oil cake
350	47.88±0.03 ^a	51.23±0.06 ^b	44.54±0.05 ^c	84.00±0.10 ^d	84.00±0.12 ^d	10.69±0.04 ^e	51.23±0.05 ^b	50.96±0.05 ^b	27.57±0.03 ^f
370	36.96±0.02 ^a	38.26±0.04 ^b	42.08±0.02 ^c	78.67±0.03 ^d	78.65±0.02 ^d	84.00±0.02 ^e	38.26±0.04 ^b	41.74±0.02 ^c	22.17±0.02 ^f
410	14.07±0.04 ^a	19.18±0.03 ^b	25.84±0.03 ^c	68.92±0.06 ^d	32.19±0.01 ^e	31.23±0.05 ^e	19.18±0.05 ^b	11.91±0.04 ^f	13.05±0.05 ^g
430	12.6±0.03 ^a	16.33±0.02 ^b	23.04±0.10 ^c	48.85±0.02 ^d	28.24±0.03 ^e	27.01±0.12 ^f	16.33±0.03 ^b	10.59±0.03 ^g	11.22±0.03 ^h
450	10.92±0.05 ^a	11.88±0.01 ^b	19.25±0.03 ^c	33.21±0.05 ^d	21.15±0.02 ^e	22.14±0.02 ^f	11.88±0.03 ^b	8.86±0.04 ^g	8.01±0.10 ^g

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

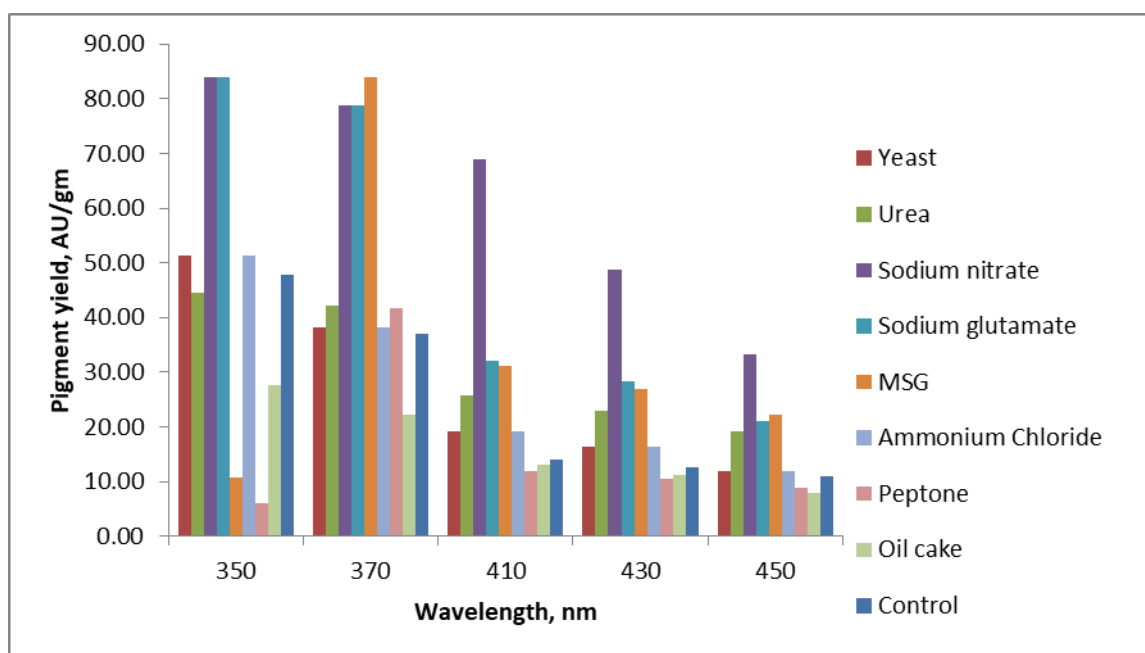


Fig 4.8. Effect of supplementation of nitrogen sources on water extractable pigments

Table 4.15: Effect of supplementation of nitrogen sources on acetone extractable pigments

Wavelength	Control	Yeast	Urea	Sodium nitrate	Sodium glutamate	MSG	Ammonium Chloride	Peptone	Oil cake
350	84.00±0.25 ^a	4.48±0.50 ^b	3.53±0.15 ^c	7.37±0.18 ^d	7.81±0.18 ^d	7.33±0.14 ^d	2.25±0.25 ^e	4.26±0.13 ^b	2.87±0.12 ^c
370	84.00±0.25 ^a	4.37±0.55 ^b	3.10±0.20 ^c	7.43±0.15 ^d	7.79±0.15 ^d	6.52±0.12 ^e	2.08±0.20 ^f	3.95±0.12 ^c	2.64±0.20 ^f
410	31.50±0.15 ^a	3.18±0.15 ^b	2.11±0.12 ^c	6.73±0.12 ^d	5.41±0.20 ^d	5.39±0.10 ^d	1.70±0.11 ^c	3.98±0.17 ^b	3.16±0.10 ^b
430	27.93±0.07 ^a	2.95±0.11 ^b	2.00±0.11 ^b	6.08±0.13 ^c	4.91±0.15 ^d	4.86±0.02 ^d	1.65±0.15 ^b	3.63±0.15 ^e	2.83±0.13 ^b
450	23.31±0.15 ^a	2.55±0.05 ^b	1.83±0.05 ^c	5.29±0.10 ^d	4.29±0.07 ^e	4.29±0.05 ^e	1.51±0.13 ^c	3.26±0.03 ^f	2.38±0.14 ^b

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in rows are significantly different ($P < 0.05$)

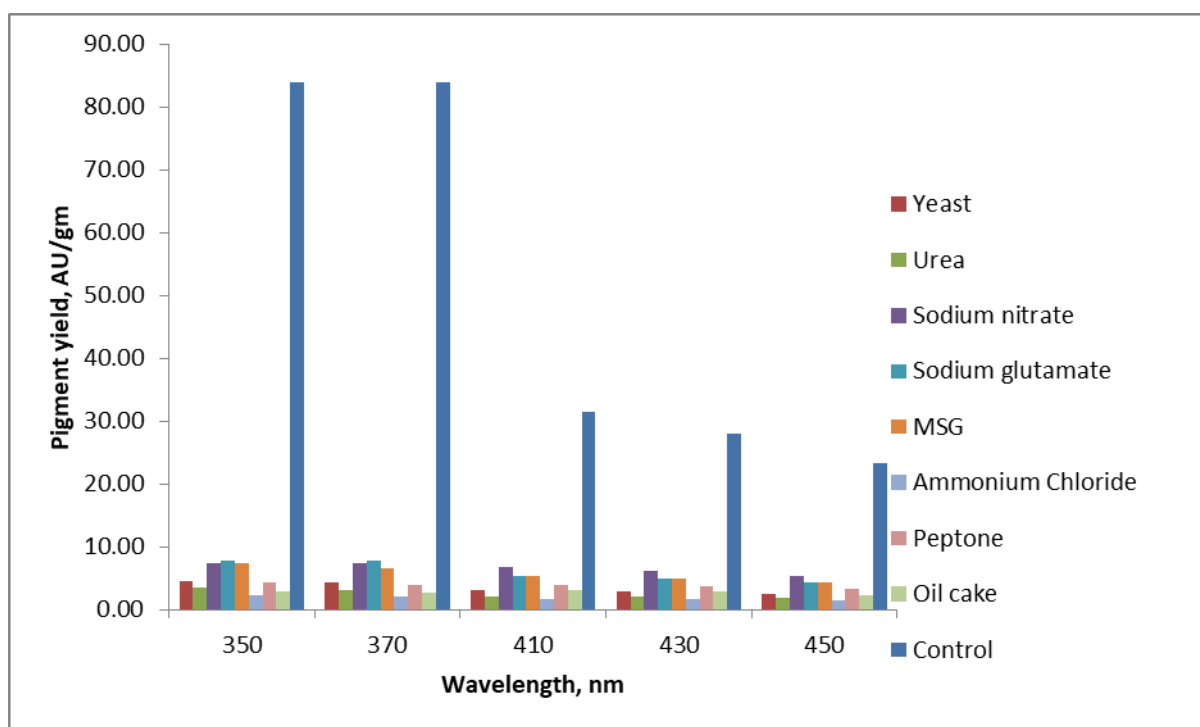


Fig 4.9. Effect of supplementation of nitrogen sources on acetone extractable pigments

4.8 EXTRACTION AND ESTIMATION OF PIGMENT

Although solid state fermentation is considered to be the best method for secondary metabolite production, final recovery of pigment depends on the extraction method used as the pigments are

substrate bound. In the present study, pigment extraction based on the polarity of the pigment resulted in high recovery of pigments.

4.8.1 Single solvent and continuous solvent extraction:

In this method of extraction, the pigment was extracted as ethanol, methanol, acetone, benzene, hexane, petroleum ether and water soluble. In case of wheat bran as substrate, maximum pigment yield of 35.2 AU units/ g dry mass was observed at λ_{\max} at 406 nm with methanol followed by distilled water 20.74 AU units/ g dry mass (λ_{\max} at 425nm), acetone with 20.6 AU units/ g dry mass (λ_{\max} at 415nm) and ethanol 19.26 AU units/ g dry mass (λ_{\max} at 428 nm) as depicted in Table 4.16. The rest of the solvents extracted very less amount of pigment in the range of 410-430 nm which is the range for yellow-orange pigment as shown in figure (4.11). Flavonoids (350-370 nm) are extracted the maximum by acetone to the order of 20.81 AU units / g dry mass. It was observed that out of the all solvents evaluated methanol, ethanol, acetone and distilled water were the best solvents and can be used for the extraction of polar and non-polar compounds secreted by the fungus.

In case of broken rice as substrate, maximum pigment yield of 24.58 AU units/ g dry mass was observed at λ_{\max} at 415 nm with methanol followed by ethanol, with 8.27 AU units/ g dry mass (λ_{\max} at 426nm) and acetone 7.53 AU units/ g dry mass (λ_{\max} at 431nm) Rest of the solvents extracted very less amount of pigment in the range of 410-430 nm which is the range for yellow-orange pigment. Distilled water extracted maximum of flavonoids (350-370 nm) to the order of 27.0 AU units / g dry mass followed by acetone.

The results showed that the type and extent of pigment production is dependent on the substrate and its composition. Single solvent was not able to extract all the pigments as the both polar and non-polar solvent soluble pigments were secreted by *E. nigrum* (Fig. 4.10).

Table 4.16: Extraction of pigment from fermented wheat bran with single solvent at a time

Sr. No.	Substrate	Solvents	Wavelength (nm)	Absorbance	Pigment Yield (AU/g)
1	Wheat Bran	Ethanol	428.00	2.853	19.26
2		Methanol	406.00	3.911	35.20
3		Acetone	415.50	3.751	20.63
4			346.00	3.783	20.80
5		Hexane	465.50	0.025	0.15
6			430.50	0.013	0.08
7			378.50	0.009	0.05
10		Benzene	493.00	0.110	0.96
11			465.50	0.127	1.11
12			437.50	0.118	1.03
13		Petroleum Ether	478.50	0.114	0.97
14			451.50	0.126	1.07
16		Distilled Water	425.00	3.950	20.74

Table 4.17: Extraction of pigment from fermented broken rice with single solvent at a time

Sr. No.	Substrate	Solvents	Wavelength (nm)	Absorbance	Pigment Yield (AU/g)
1	Broken Rice	Ethanol	426.00	1.003	8.27
2		Methanol	415.50	2.731	24.58
3			337.50	3.972	35.75
4		Acetone	431.40	1.205	7.53
5			346.20	0.738	4.61
7		Hexane	476.00	0.089	0.67
8			451.50	0.106	0.79
9			349.00	0.019	0.14
10		Benzene	464.00	0.159	1.27
11			440.00	0.142	1.14
12		Petroleum Ether	475.00	0.078	0.70
13			452.00	0.094	0.86
14		Distilled Water	348.50	4.000	27.00
15			426.00	3.901	20.71

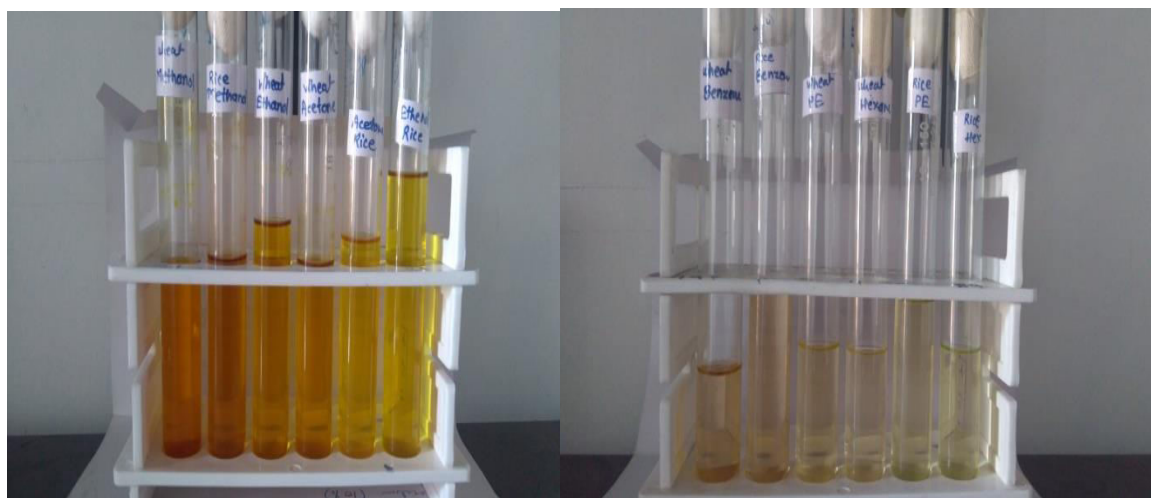


Fig 4.10. Extraction of pigment from with single solvents

Since the single solvent method does not result in extraction of complete pigment from fermented substrate, pigments were extracted based on polarity using different solvents starting from less polar to highly polar (Fig. 4.11). This helped to extract maximum of the pigment from the fermented substrate.

Table 4.18: Extraction of pigment from fermented wheat bran based on polarity of solvent

Sr. No.	Substrate	Solvents	Wavelength (nm)	Absorbance	Pigment Yield (AU/g)
1	Wheat Bran	Hexane	485.50	0.049	0.28
2			455.50	0.051	0.29
3			429.00	0.043	0.27
4			373.50	0.044	0.25
5			348.00	0.061	0.35
6		Benzene	352.00	0.063	0.56
7		Petroleum Ether	341.50	0.029	0.23
9		Acetone	432.50	2.093	14.13
10		Methanol	425.00	1.284	11.56
11		Ethanol	428.00	1.026	9.23
12		Distilled Water	433.00	4.000	32.00



Fig 4.11. Extraction of pigment based on polarity of solvent

Table 4.19: Extraction of pigment from fermented broken rice based on polarity of solvent

Sr. No.	Substrate	Solvents	Wavelength (nm)	Absorbance	Pigment Yield (AU/g)
1	Broken Rice	Hexane	451.50	0.069	0.52
2			347.00	0.011	0.08
3		Benzene	434.50	0.054	0.40
4			410.00	0.051	0.38
5			328.50	0.084	0.63
6		Petroleum Ether	333.50	0.006	0.05
7		Acetone	431.50	1.349	9.11
8			336.00	0.996	6.72
9		Methanol	444.00	3.456	30.24
10		Ethanol	423.50	0.220	2.14
11		Distilled Water	435.00	4.000	34.00

It was observed in continuous extraction based on the polarity of the solvents that the solvents acetone, methanol, ethanol and distilled water are extracting the maximum of the pigments.

4.8.2 Optimization of aqueous extraction of water soluble pigments produced by *Epicoccum nigrum*

All the experiments were carried out as per the experimental design (Table 3.4) to find out the optimized conditions and investigate the effect of independent variables on the water soluble pigment extraction from *Epicoccum nigrum* fermented broken rice. The predicted results were obtained by the model fitting technique in Design Expert software version 7.0 and the values compared well with the experimental values. The observed results are shown in Table 4.20. Regression equations were generated by fitting the data to various models.

Table 4.20: Effect of extraction temperature, mass of fermented rice and time on response variables

Independent variable			Response	
Extraction temp (°C)	Mass of fermented rice (g)	Time (min)	Yellow Pigment (AU/g)	Flavonoid (AU/g)
40	0.5	90	39.6	39.56
70	0.5	90	27.78	31.2
55	1	110.5	24.52	13.52
55	1	60	52.01	77.02
55	1	60	51.89	77.25
55	1	60	52.5	75.28
40	1.5	30	22.07	28.64
55	1	9.5	25.53	23.56
55	1	60	51.5	76.58
40	0.5	30	31.51	42.16
55	1	60	51.9	75.68
70	0.5	30	25.13	50.6
70	1.5	90	15.93	29.81
55	1	60	52	76.23
80.2	1	60	16.31	36.96
29.8	1	60	26.6	32.24
70	1.5	30	24.21	46.54
55	1.8	60	26.6	61.91
55	0.2	60	45.48	70.16
40	1.5	90	20.79	31.1

Table 4.21: ANOVA and regression coefficients of second order polynomial model for the response variables

Variables	DF	Estimated coefficients		F value	
		Yellow pigment	Flavonoid	Yellow pigment	Flavonoid
Model	9	+51.97	+76.34	1318.20**	611.65**
X₁	1	-2.80	+1.80	358.21**	27.26*
X₂	1	-5.33	-3.02	1298.26**	76.67**
X₃	1	-0.038	-3.89	0.066 ^{NS}	126.97**
X₁ X₂	1	+1.94	+2.07	100.28**	20.96*
X₁ X₃	1	-1.56	-4.50	64.76**	99.37**
X₂ X₃	1	-2.54	+0.97	172.46**	4.58 ^{NS}
X₁²	1	-10.82	-14.77	5646.69**	1929.06**
X₂²	1	-5.66	-3.65	1546.56**	118.12**
X₃²	1	-9.56	-20.45	4405.93**	3697.56**
Lack of fit	5			4.79 ^{NS}	4.58 ^{NS}
R²		0.999	0.998		
Adj. R²		0.998	0.996		
CV%		1.60	2.56		

* Significant at 5% significance level; ** Significant at 1% significance level

4.8.2.1 Second order polynomial equations and statistical analysis

Based on the CCRD experimental design, the empirical relationship between the independent variables and investigational results obtained were indicated by a second-order polynomial equation with linear, interaction and quadratic terms. The equations generated by software in coded factors are presented below

$$\text{Yellow pigment } \left(\frac{AU}{g}\right) = 51.97 - 2.80x_1 - 5.33x_2 - 0.038x_3 + 1.94x_1x_2 - 1.56x_1x_3 - 2.54x_2x_3 - 10.82x_1^2 - 5.66x_2^2 - 9.56x_3^2$$

(4.1)

$$\text{Flavanoid } \left(\frac{\text{AU}}{\text{g}}\right) = 76.34 + 1.80x_1 - 3.02x_2 + 3.89x_3 + 2.07x_1x_2 - 4.50x_1x_3 + 0.97x_2x_3 - 14.77x_1^2 - 3.65x_2^2 - 20.45x_3^2 \quad (4.2)$$

Regression analysis and analysis of variance (ANOVA) were used to test the capability and fitness of the models and F-test was conducted to evaluate the significance of each independent variable. The analysis of variance results in Table 4.21 show F-value for yellow pigment and flavonoid as 1318.20 and 611.65 respectively, suggesting that the model is significant at $p < 0.01$. Coefficient of determination (R^2) and adjusted- R^2 were calculated to verify the adequacy and fitness of the model. The values of R^2 were found to be 0.999 and 0.998 for yellow pigment and flavonoid, respectively, which means that 99% of experimental data is well fitted. The high value of adjusted- R^2 (0.998 for yellow pigment and 0.996 for flavonoid) supports a high association between the experimental and anticipated values. The low coefficient of variation value (1.60 and 2.56) represents a high degree of accuracy and consistency of experiments performed (Liyana-Pathirana and Shahidi, 2005).

4.8.2.2 Effect of variables on yellow pigment extraction

Yellow pigment from fermented broken rice was separated by the aqueous extraction process. The process was accomplished as per the experimental design at different extraction temperatures, times and mass of fermented matter in order to find out the best extraction conditions. The empirical relationship between variables and responses is given by equation 4.1. Three dimensional response plots were used to describe the effect of the independent variables on the pigment extraction. These plots demonstrated the significance of two variables at a time on the extraction process while the third variable was maintained as constant. The surface plots are illustrated in Fig. 4.12-4.14.

Under the influence of the process parameters under study the yield of yellow pigment ranged from 15.93 to 52.5 AU/g (Table 4.20)

Extraction temperature and mass of fermented matter significantly affected ($p < 0.01$) the yellow pigment extraction in linear as well as the quadratic manner while time affected the pigment yields only in quadratic term. All the process parameters showed a negative effect in linear as well as quadratic level. Temperature and mass exhibited a positive interactive effect. The temperature might result in softening of tissue, thereby increasing the solubility of pigment, thus giving higher extraction rate (Kushwaha et al., 2017). Increase in temperature increased the pigment yield in the initial stages, but above 55°C there was a significant decrease in yield. This might be due to heat sensitive nature of yellow pigment pigment. The response surface plot (Fig. 4.12) displays the

interaction effect of the process parameters (extraction temperature and mass of fermented rice) on the yellow pigment extraction. Both parameters showed a strong and positive effect on the extraction effectiveness and the total amount of pigment extracted was increased with an increase in both the temperature and the mass of the sample used in the extraction process.

The yellow pigment yield improves with increasing mass and time due to the fact that the water can enter into the large amount of mass. But above 60 min there is a decrease in yield which might be because of decomposition or oxidation of pigment under the influence of temperature, light and oxygen (Maran and Manikandan, 2012). There is no study reported till date on extraction process of yellow pigment of *E.nigrum*.

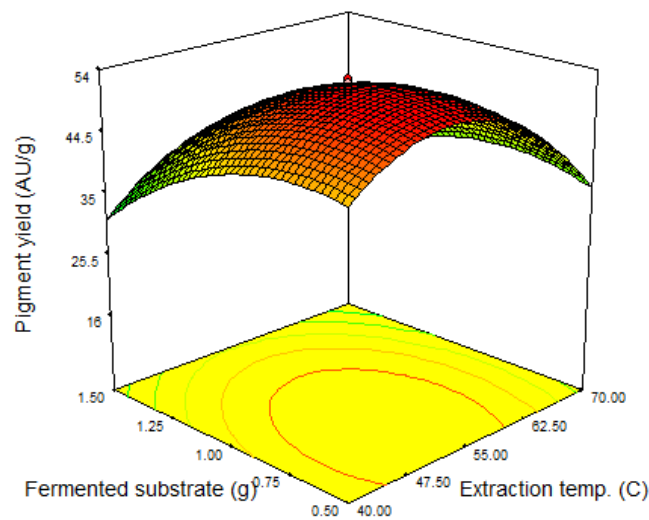


Fig. 4.12. Response plot depicting effect of mass of fermented matter and extraction temperature on yellow pigment extraction

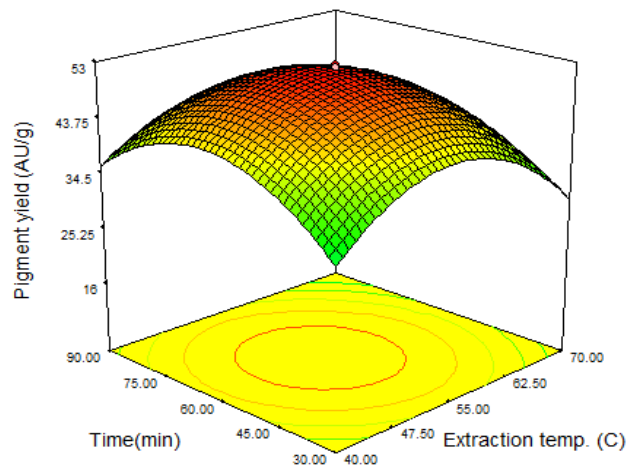


Fig. 4.13. Response plot depicting effect of extraction time and extraction temperature on yellow pigment extraction

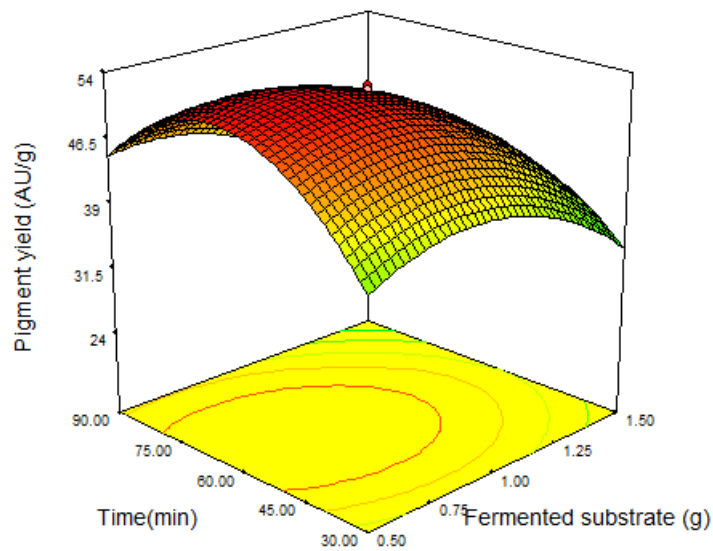


Fig 4.14. Response plots depicting the effect of extraction time and mass of fermented matter on pigment extraction

4.8.2.3 Effect of variables on Flavanoid extraction

The yield of flavonoid ranged from 13.52-77.25 AU/g (Table 4.20). The empirical relationship between variables and responses is given by equation 4. Extraction temperature showed a

significant ($p < 0.01$) positive effect at linear and negative effect on quadratic level. While, mass of fermented matter negatively affected ($p < 0.01$) the flavonoid yield, the extraction of flavonoids from fermented rice using water was based on the principle of solid–liquid extraction. Highest extraction was noticed when mass was 1g that might be due to diffusion of solvent in the matrix and releasing the water soluble components in the solvent phase (Candrawinata et al., 2014). On further increasing the mass of broken rice there was a decrease in yield which might be due to insufficient solvent phase for extraction purposes (Maran et al., 2013). The effect of temperature and mass on flavonoid yield was positively significant at $p < 0.01$ (Fig 4.15). The increase in temperature results in better penetration and thereby affecting the extraction rate of flavonoids. But higher temperatures might affect the stability of flavonoids as it is dependent on the structure of distinct flavonoids (Sharma et al., 2015). Heating is considered responsible for a number of degradative changes in fresh vegetables such as oxidation, thermal degradation, and leaching of bioactive compounds (Kalt, 2005). The positive and negative effects of heat depend upon the morphology and nutritional properties of vegetables (Turkmen et al., 2005). Some researchers reported the damage of phenolic compounds at higher temperatures, thus causing a loss in the yield (Yılmaz et al., 2015; Sun et al., 2011).

Mass of the fermented rice exhibited a significant negative effect at linear and quadratic level ($p < 0.01$) but a positive interactive effect with temperature and treatment time. The increased time may result in better diffusion and solubilization of the water soluble component as elucidated by Fick's second law of diffusion that a final equilibrium may reach in a certain period of time (Fig 4.16). Moreover, an extended extraction time increases the chances of oxidation and decomposition of flavonoids (Tan et al., 2013). Fig. 4.17 depicts the effect of the mass of fermented matter and extraction time on flavonoids.

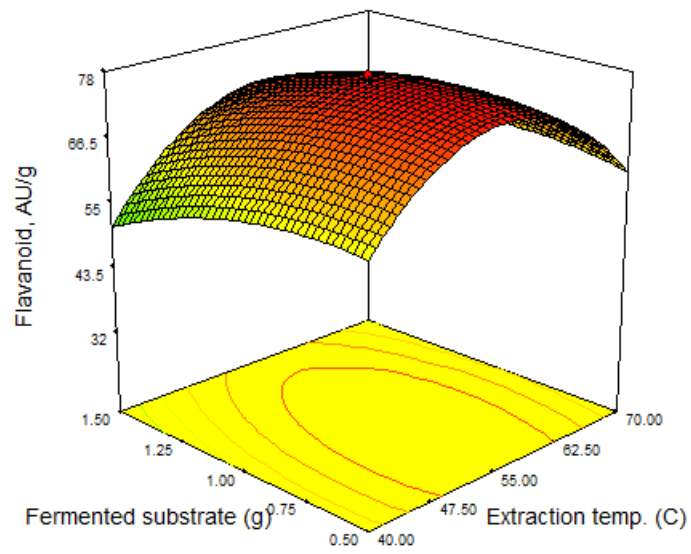


Fig 4.15. Response plot depicting effect of mass of fermented matter and extraction temperature on flavonoid extraction

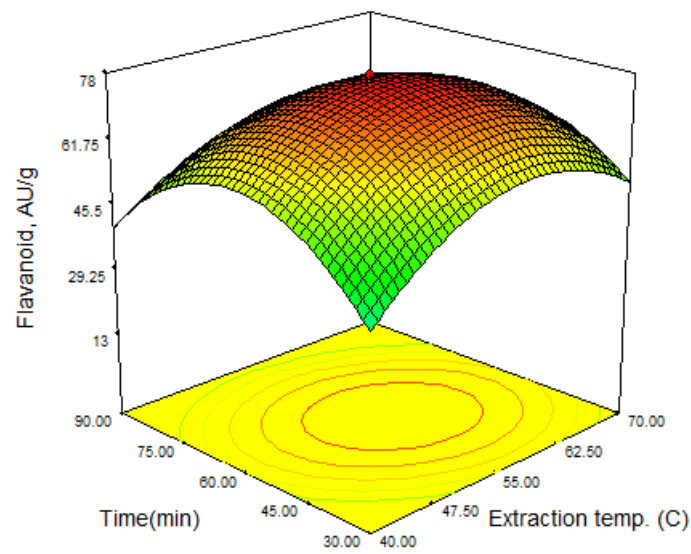


Fig. 4.16. Response plot depicting effect of extraction time and extraction temperature on flavonoid extraction

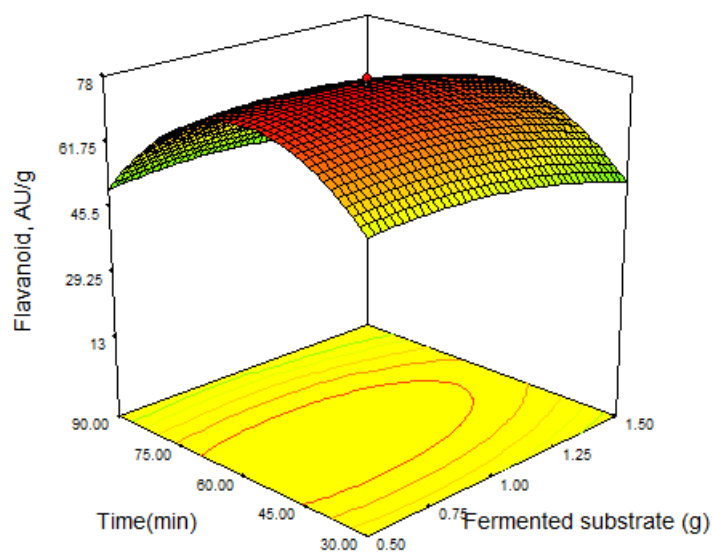


Fig 4.17. Response plots depicting the effect of extraction time and mass of fermented matter on flavanoid extraction

4.8.2.4 Selection of optimum conditions

Design expert software version 7.0 was used to optimize the conditions for the extraction process to get the maximum yield of yellow pigment and flavonoids. The criteria applied to the numerical optimization was: the independent variables were kept within range and responses were kept at the maximum. By applying the methodology of the desired function, the optimized level of different variables was obtained. It indicated that an extraction temperature of 55.7°C, 0.79 g of fermented matter and extraction time of 56.6 min will yield the yellow pigment and flavonoid pigments in the order of 52.78 AU/g and 77.22 AU/g respectively with overall desirability value of 1.000. The overlay plot (Fig 4.18) for graphical optimization with conditions yellow pigment: 50AU/g (lower value) and flavonoid: 75AU/g (lower value) shows the optimum zone for maximum yield.

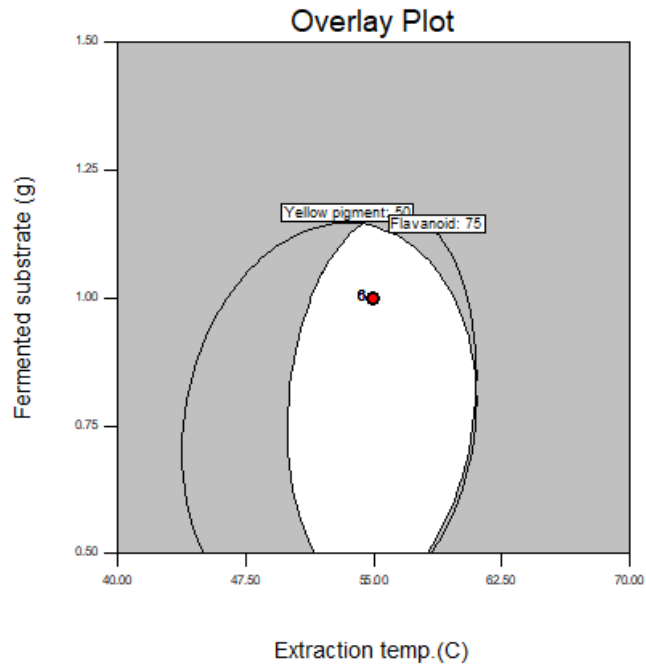


Fig 4.18. Overlay plot between extraction temperature and mass of fermented rice at constant time 60 min

4.8.2.5 Verification of optimized conditions

The fitness of the model was investigated by setting up the experiment at the optimum conditions generated by the software, i.e. extraction temperature of 55.7°C, 0.79 g of fermented matter and extraction time of 56.6 min. The experimental values obtained are shown in Table 4.22 which are within 95% of the anticipated value. This shows the suitability of the quadratic model.

Table 4.22: Experimental and predicted values at optimum conditions

Response	Experimental value	Predicted Value
Yellow pigment (AU/g)	51.6 ^a	52.78 ^a
Flavonoid (AU/g)	76.8 ^b	77.23 ^b

Mean values with different superscripts in rows are significantly different ($P < 0.05$)

4.8.3 Optimization of extraction of acetone soluble pigments produced by *Epicoccum nigrum*

In this work, the influence of extraction time, temperature and solvent-solid ratio on the carotenoids yield from the substrate fermented with *E. nigrum* was investigated using Box-Behnken experimental design.

4.8.3.1 Box-Behnken Experimental Design and statistical analysis

Box-Behnken designs (BBD) are a class of second-order rotatable or almost rotatable designs based on three-level incomplete factorial designs. The number of experiments (N) required for the development of design is calculated as $N = 2k(k - 1) + C_0$, (where C_0 is the number of central points and k is number of factors) (Ferreira et al., 2007). The process parameters chosen for extraction process were chosen on the basis of lab trials. The levels used under study are: extraction temperature (30-50°C), solvent to solute ratio (10-30 ml/g) and extraction time (30-120 min). The carotenoid yield was correlated to process variables by second order polynomial equation (Eq. 4.4) as given below

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (4.4)$$

Where y is the carotenoid yield, b_0 represents model intercept, x_1 , x_2 and x_3 are process variables under study and b_{ij} represents regression coefficients.

The experiments were carried out as per the Box Behnken design depicted in Table 4.23 so as to find out the optimized conditions and investigate the influence of extraction parameters on carotenoid yield from *E. nigrum* fermented wheat bran. The predicted results obtained from Design Expert software version 7.0 by model fitting techniques related well with the experimental values. The empirical relation between independent and dependent variables were expressed with linear, interaction and quadratic terms in the form of second order polynomial equation.

Table 4.23: Effect of Extraction time, temperature and solvent: solid ratio on carotenoid yield

Extraction time, min	Temperature,(°C)	Solvent: solid	Carotenoid yield, (µg/g)
30	30	20	241.50
75	40	20	427.50
75	30	10	214.56
30	50	20	393.00
75	40	20	435.75
30	40	30	230.44
75	40	20	439.88
75	50	10	239.56
120	30	20	381.00
120	40	10	231.13
75	50	30	235.13
120	50	20	251.63
30	40	10	330.44
75	30	30	228.56
75	40	20	425.25
120	40	30	350.50
75	40	20	431.75

The software generated equation in coded factors is given below:

$$\begin{aligned}
 \text{carotenoid yield } \left(\frac{\mu\text{g}}{\text{g}}\right) = & 432.03 + 2.36x_1 + 6.71x_2 + 3.62x_3 - 70.22x_1x_2 + 54.84x_1x_3 - \\
 & 4.61x_2x_3 - 29.54x_1^2 - 85.71x_2^2 - 116.86x_3^2
 \end{aligned}
 \tag{4.5}$$

Analysis and variance and regression analysis were used to test the fitness of model and F-test was done to estimate the importance of each independent variable. The results shown in Table (4.23) depict the F value of carotenoid yield as 449.91 suggesting the model is significant at $p < 0.001$.

Table 4.24 Analysis of variance of fitted models

Source	Sum of Squares	df	Mean Square	F-Value	P value (Prob > F)	
Model	132526.33	9	14725.15	449.91	< 0.0001	significant
X₁ : Extraction time	44.53	1	44.53	1.36	0.2816	
X₂ :Temperature	360.30	1	360.30	11.01	0.0128	
X₃ :Solvent: solid	104.67	1	104.67	3.20	0.1069	
X₁ X₂	19722.69	1	19722.69	602.60	< 0.0001	
X₁ X₃	12031.40	1	12031.40	367.61	< 0.0001	
X₂ X₃	84.99	1	84.99	2.60	0.1110	
X₁²	3673.14	1	3673.14	112.23	< 0.0001	
X₂²	30929.81	1	30929.81	945.02	< 0.0001	
X₃²	57503.92	1	57503.92	1756.97	< 0.0001	
Residual	229.10	7	32.73			
Lack of Fit	87.15	3	29.05	0.82	0.5473	not significant
Pure Error	141.95	4	35.49			
Cor Total	132755.44	16				

Coefficient of determination and adjusted R² values, 0.998 and 0.996, respectively verified the adequacy of the model fitness and also represented a high level of association between experimental and predicted results (Liyana-Pathirana and Shahidi, 2005).

4.8.3.2 Influence of variables on carotenoid yield

Carotenoids were extracted from the *E. nigrum* fermented wheat bran using acetone as solvent as per the experimental design at different extraction time, temperature and solvent-solute ratio so as to optimize the extraction conditions. The empirical equation generated is given by equation 4.5. Three dimensional plots depict the effect of different variables on carotenoid extraction keeping one variable constant. The response plots are shown in Fig. 4.19-4.21

Under the effect of parameters under study the yield ranged from 214.56 to 439.88 µg/g (Table 4.23)

Extraction temperature and solvent to solid ratio significantly affected the carotenoid extraction in linear at $p < 0.01$ and $p < 0.1$ respectively as well as in a quadratic manner ($p < 0.001$) while extraction time affected the pigment yield only in quadratic term. At the linear level the effect was positive with temperature dominating over the solvent-solid ratio, which means that increase in these variables increased the pigment yield. The temperature might result in increased solubility of pigment due to tenderization of tissue, thus giving improved extraction rate (Kushwaha et al., 2017). It could be observed that above 40°C the carotenoid yield decreased. This might be due to that above this temperature the carotenoid might get degraded or rearrangement of the structure could occur (Gu et al., 2008). For solvent-solute ratio, maximum extraction occurs at 20 mL/g below or above which the carotenoid yield drastically decreased when the temperature is 40°C . A significant positive interactive effect ($p < 0.1$) was observed.

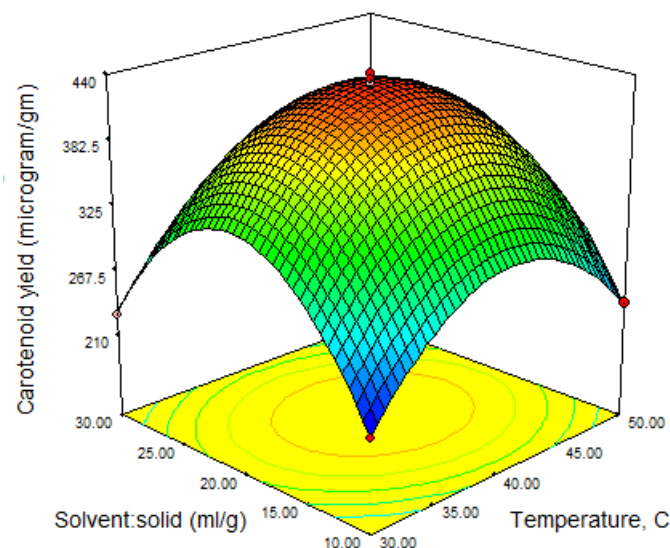


Fig 4.19. Response plots depicting the effect of solvent:solid ratio and extraction temperature on carotenoid extraction

The influence of extraction temperature and time is shown in Fig 4.20. Variation in temperature and time showed that maximum yield resulted when extraction time is 75 min at 40°C . The interactive effect is significantly negative ($p < 0.0001$). The increase in extraction time resulted in decrease which might be due to oxidation of carotenoids because of longer exposure to oxygen, light and other environmental conditions (Gu et al., 2008; Gang and Zora, 2001)

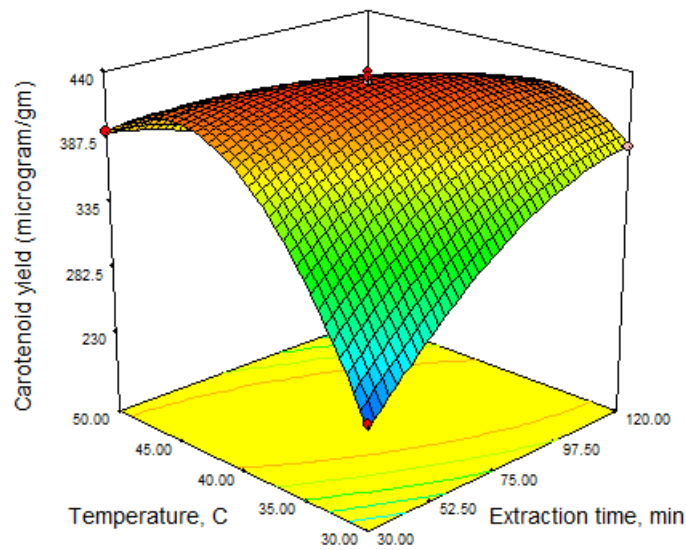


Fig. 4.20. Response plots depicting the effect of extraction temperature and time on carotenoid extraction

The carotenoid yield improves with increasing solvent: solute ratio and extraction time because of the reason that the solvent can diffuse into the large amount of substrate . The interactive effect is significantly positive at $p < 0.0001$. But above 75 min there is a decrease in yield which might be because of decomposition or oxidation of pigment under the influence of temperature, light and oxygen (Maran and Manikandan, 2012).

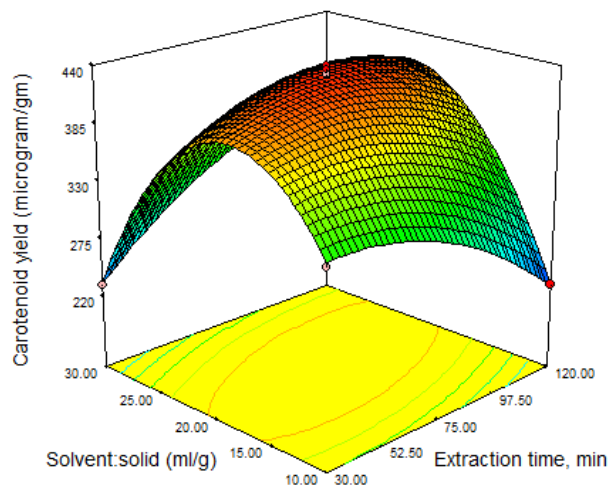


Fig. 4.21. Response plots depicting the effect of solvent: solid ratio and time on carotenoid extraction

4.8.3.3 Selection of optimum conditions

Design expert software version 7.0 was used to optimize the process conditions for the extraction to get the maximum yield of carotenoid. The criteria applied to the numerical optimization was: the process variables were kept within range and the response was kept at the maximum. The optimized level of different variables was obtained by using the procedure of the desired function. It showed that an extraction temperature of 40.27°C, 20.21 mL/g of solvent to solute ratio and extraction time of 76.22 min will yield the carotenoid pigments in the order of 432.18 µg/g with overall desirability value of 0.96. The overlay plot (Fig 4.22) for graphical optimization with conditions carotenoid yield: 220 µg /g (lower value) and 430 µg /g (upper value) shows the optimum zone for maximum yield.

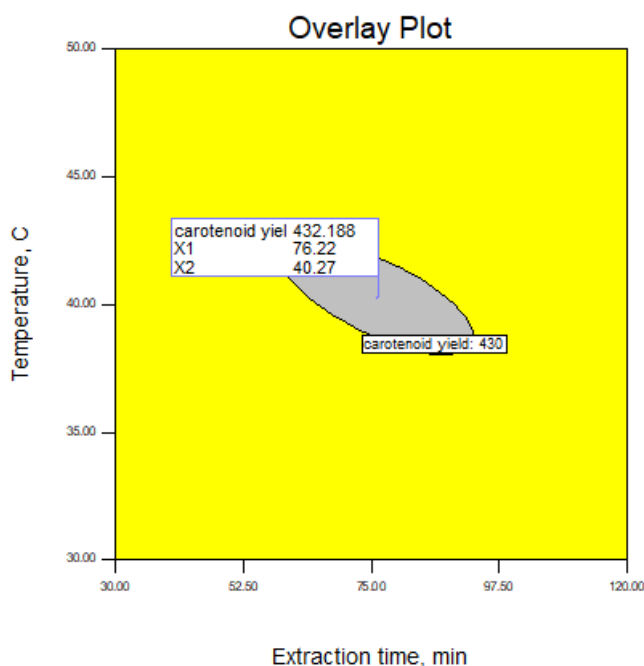


Fig. 4.23. Overlay plot between extraction temperature and time at constant solvent: solid ratio of 20.2 mL/g

4.8.3.5 Verification of optimized conditions

The model fitness was verified by setting up the experiment at the optimum conditions generated by the software, i.e. extraction temperature of 40.27°C, 20.21 mL/g of solvent to solute ratio and extraction time of 76.22 min. The experimental value obtained was 430.85 µg/g which is within 95% of the predicted results which shows the suitability of the model. Under the optimal conditions generated by Design expert software by numerical optimization approach, the

experimental data of responses were in close conformity with the anticipated pigment yield. This optimization can provide the basis for scaling up for industrial extraction process with optimum utilization of energy, time, raw material and solvent.

4.9 Isolation and Purification of Pigments

Ethanolic and methanolic extracts were purified by column chromatography and thin layer chromatography (Fig. 4.23).

Table 4.25 Absorbance values of fractions collected with different solvents

Solvent	Peak Wavelengths, nm	Absorbance
Hexane: Ethyl acetate (8:2)	424	0.928
	343	0.888
Hexane: Ethyl acetate (7:3)	587	0.135
	442	0.179
Methanol 100%	422	0.188
Hexane: Ethyl acetate (6:4)	367	0.067

Table 4.26: R_f values of the separated spots under the influence of different solvent systems

Sr. No	Solvent system	Solvent front	Sample		R _f Value	
			MR	MW	MR	MW
1	Acetone (30): Hexane (70)	14	0.7	1.5	0.05	0.11
2	Ethyl Acetate (100): Formic Acid (11): Acetic Acid (11): Water (26)	11	2.5	3.5	0.23	0.32
			7.5	7.8	0.68	0.71
3	Chloroform (65): Methanol (25): Water (4)	11.5	10	10.5	0.87	0.91
4	Chloroform (95): Methanol (5)	11	2	7	0.18	0.64

In column chromatography as both hydrophilic and hydrophobic solvent systems were used for purification, the clear separation of fractions was not achieved, reason behind that might be polarity of pigments quite close. In the present set of solvent systems good separation occurred

in Methanol (100%), Hexane: Ethyl acetate with proportion 8:2 and 7:3. As Table 4.25 shows peak wavelengths obtained after purification by using the column.

As column chromatography was not completely successful in separating the pigments, further purification was attempted using preparative thin layer chromatography. The purified yellow fraction obtained from column chromatography was used for TLC. The crude pigment extract was applied on to the TLC plate using a 10 μ L pipette tip. The solvent was allowed to evaporate. The spotted TLC plates were then placed in developing chamber containing the solvent system. The different solvent systems evaluated were Acetone (30): Hexane (70); Ethyl Acetate (100): Formic Acid (11): Acetic Acid (11): Water (26); Chloroform (65): Methanol (25): Water (4) and Chloroform (95): Methanol (5). Out of all solvent systems Chloroform (65): Methanol (25): Water (4) resulted in better separation of the pigment with R_f ranging from 0.87-0.91. After purification with TLC, silica gel part where the spots were observed that parts collected separately and again dissolved in solvent. Using UV-Vis Spectrophotometer spectrum was run between the range 350 nm to 600 nm (Fig.4.24). The peak was obtained at 426 nm and the same has been reported for orevactaene pigment secreted by *E. nigrum* reported by Mapari et al., (2008).

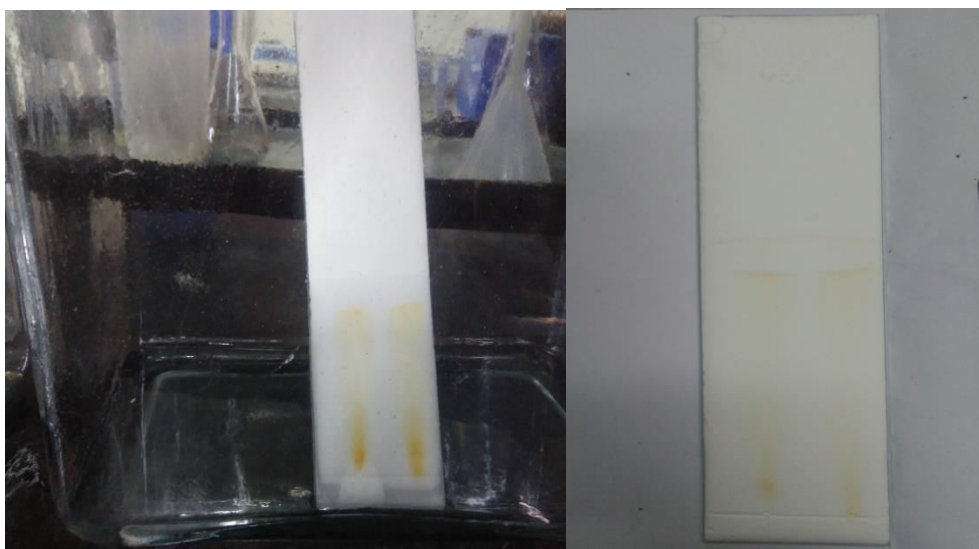


Fig. 4.23. Thin layer chromatography of extracted pigment

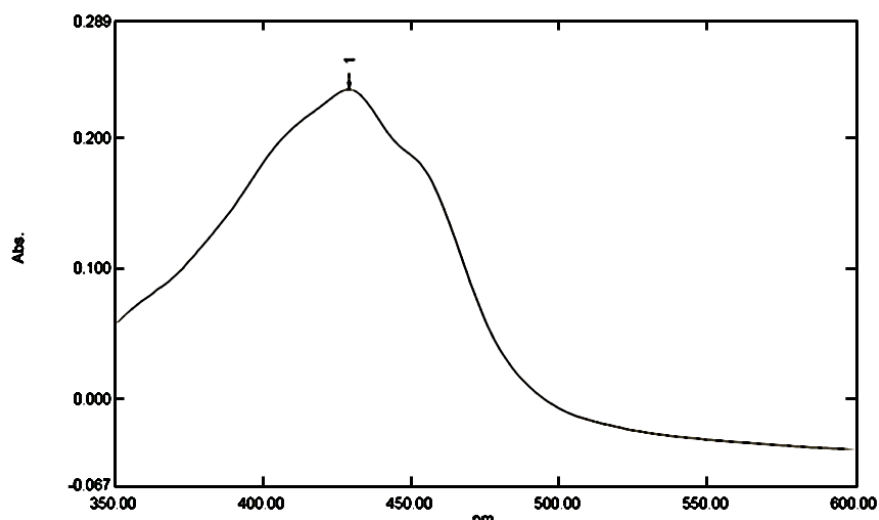


Fig. 4.24. Peak obtained of the yellow pigment purified by TLC

5.0 Characterization and structure elucidation of pigment

Structure Elucidation

Spectroscopy techniques are nowadays commonly used for determination of structure of molecules. Structure elucidation is important for understanding the physicochemical properties of the molecule. Several spectroscopic tools are used for structure determination such as NMR, mass spectrometry, and IR spectroscopy (Singh et al., 2014).

5.1 Mass spectroscopy analysis

The eluted sample was analyzed by LC-DAD-MS, absorption spectrum, measured from 400-700nm, and the mass spectra was determined and compared with spectral library and literature database for identification (Fig. 4.25-4.27). The result shows the major peak has a retention time of 11.64 min and λ_{max} of 430 nm suggesting the presence of extended oxypolyene, possibly longer than oxoheptane (Shu et al., 1997) with proposed molecular formula of $\text{C}_{34}\text{H}_{44}\text{O}_{10}$ (Fog. 4.26). Indeed, the molecule showed a similar UV-visible absorption spectrum, as well as ESI-MS molecular ion in positive mode $[\text{M}+\text{H}]^+$ at m/z 613.32 as the yellow pigment Orevactaene (Fig. 4.28-4.29) previously isolated from some species of *Epicoccum nigrum* by other researchers (da Costa Souza et al., 2016; Mapari et al., 2006; Shu et al., 1997).

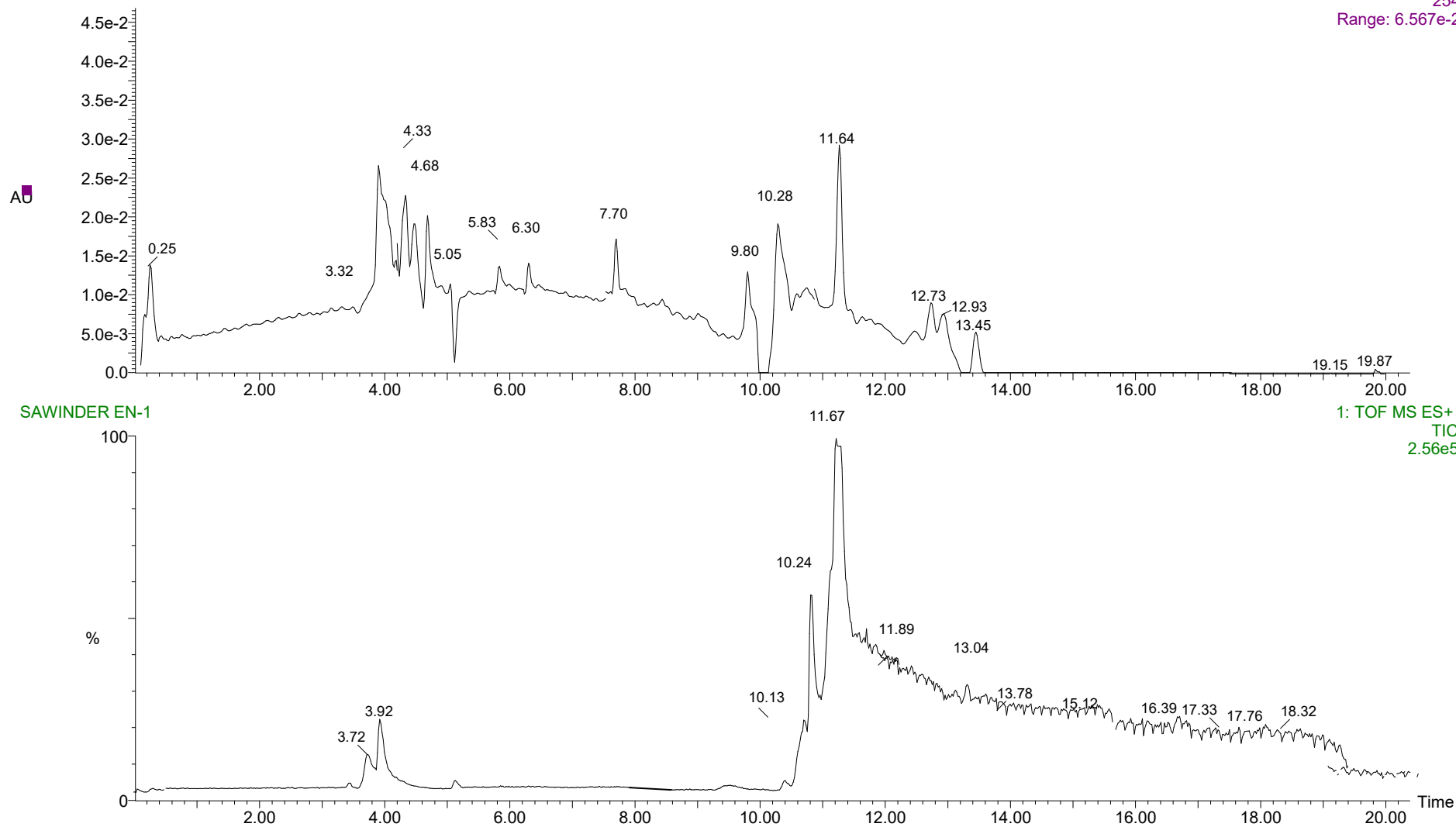


Fig 4.25. Chromatogram of the yellow pigment extracts from *Epicoccum nigrum* and Total ion chromatogram (m/z 100-1000) from positive ion electrospray.

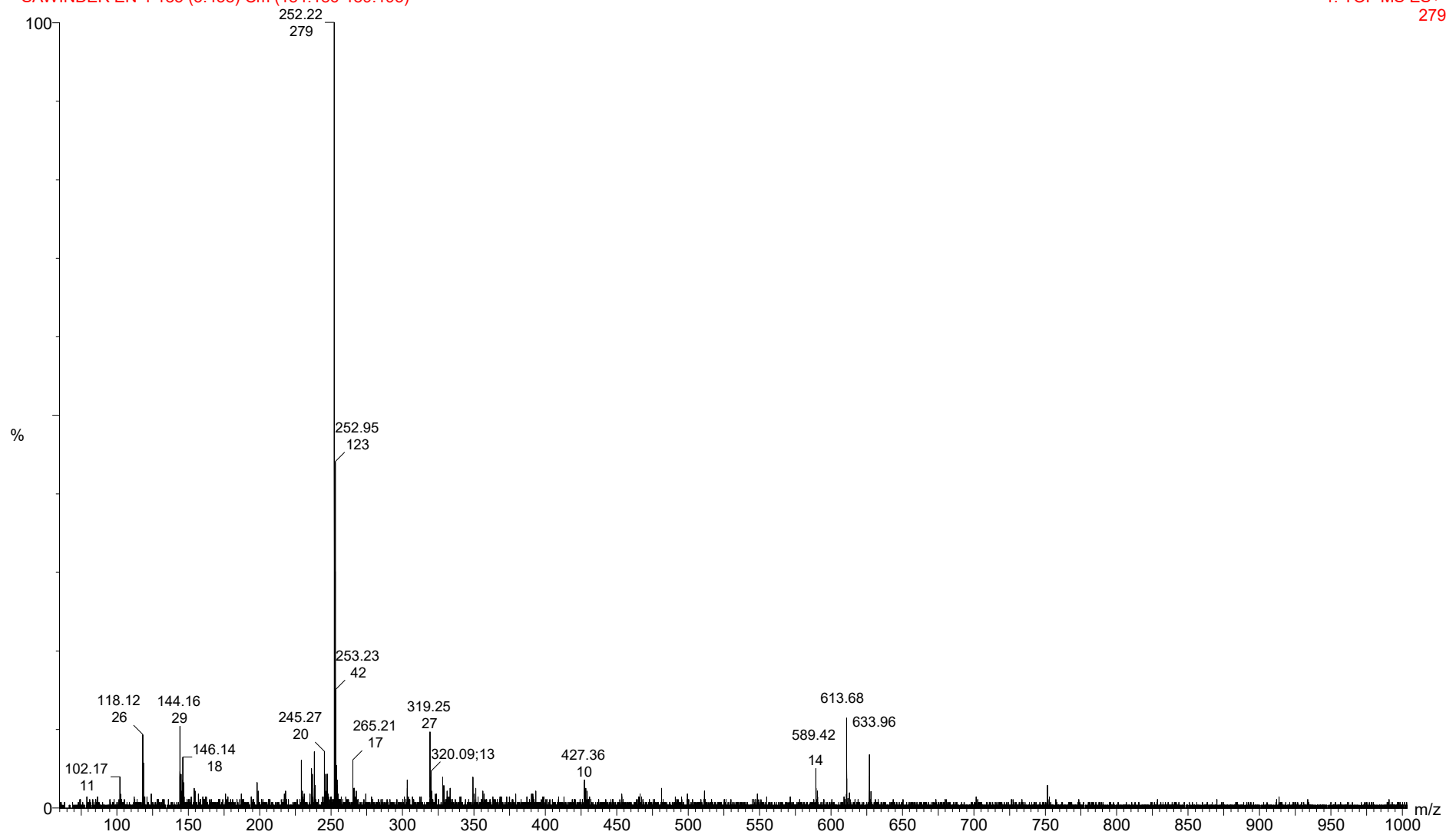


Fig. 4.26. Mass spectrum of yellow pigment from ESI

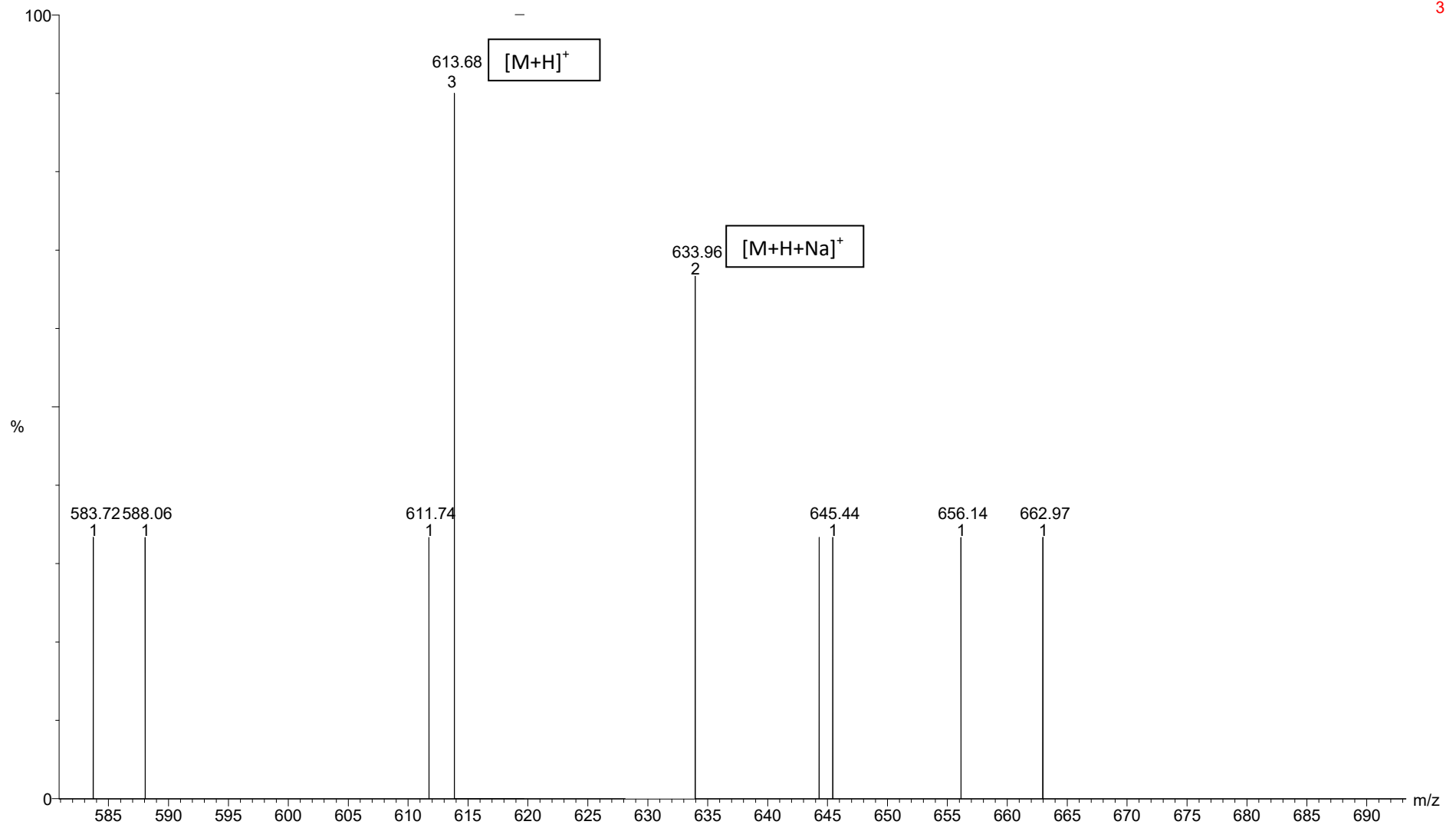
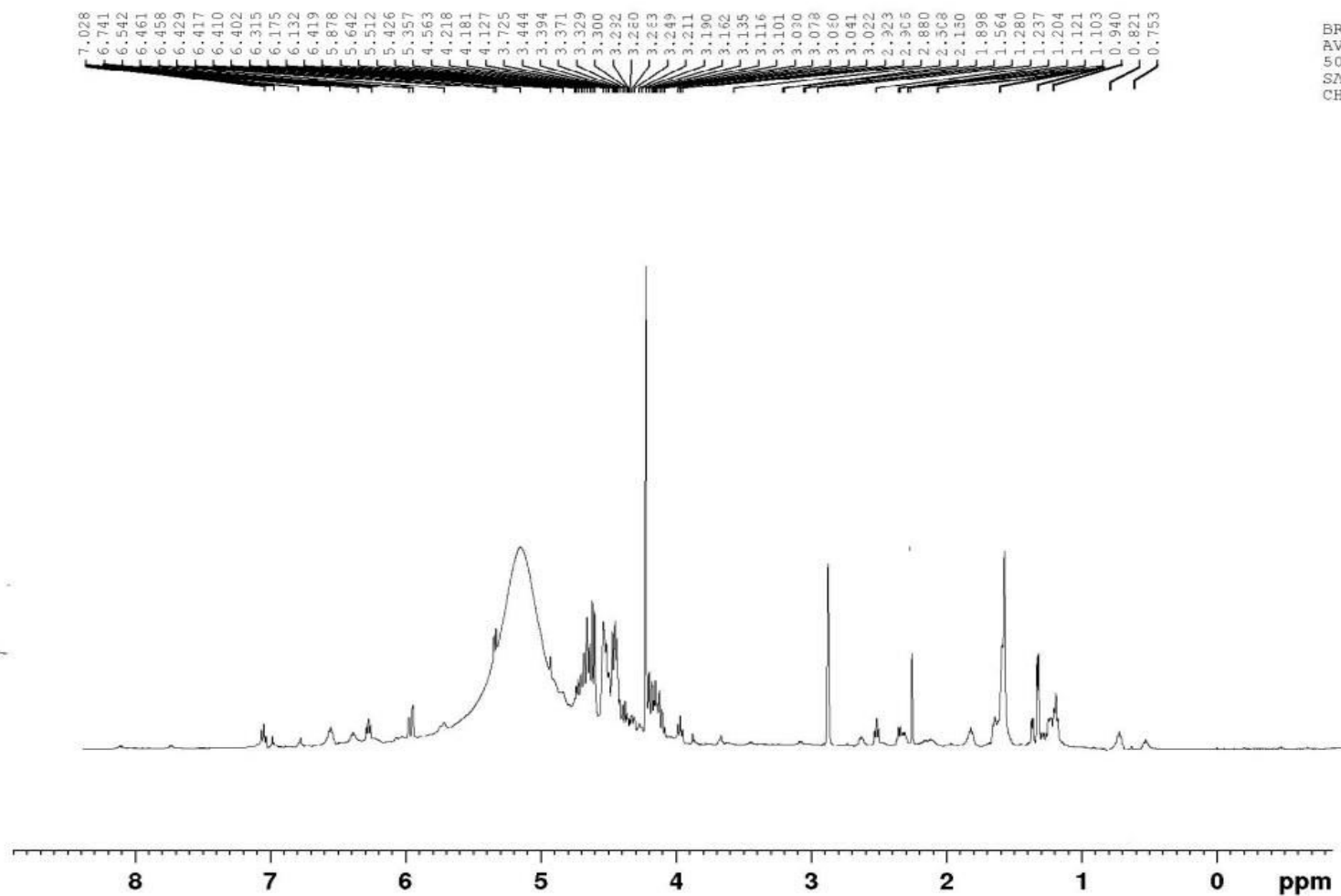


Fig. 4.27. Mass spectrum of yellow pigment from ESI+

NMR spectroscopy

Fig. 4.29 depicts the ^1H spectrum of yellow pigment produced by *E. nigrum*

^1H NMR (DMSO- d_6 , 300 MHz) δ 4.21 (d, 1H, $J = 9.5$ Hz H-3), 4.12 (dd, 1H, $J = 9.5, 9.3$ Hz, H-4), 3.24 (dd, 1H $J = 9.3, 2.8$ Hz, H-5), 6.17 (s, 1H, H-7), 6.31 (d, 1H, $J = 15.2$ Hz, H-9), 7.02 (dd, 1H, $J = 15.2$ Hz, 11.4, H-10), 6.41 (dd, 1-H, overlapped, H11), 6,74 (dd, 1H overlapped, H-12), 6.40 (dd, 1H overlapped, H-13), 6.54 (dd, 1H, overlapped, H-14), 6.41 (dd, overlapped, H-15), 6..42 (dd, 1H, overlapped, H-16), 6.46 (dd, overlapped, H-17), 6.45 (dd, 1H, overlapped, H-18), 6.13 (s, 1H, H-20), 5.51 (d, 1H, 10.3, H-22), 2.88 (m, H-23), 1.12,1.28 (m, 2H, H-24), 1.23 (m, 1H), 1.10, 1.20 (m, 2H, H-26), 0.82 (t, 3H, $J = 7.6$ Hz, H-27), 0.75 (d, 3H, $J = 7.2$ Hz, H-28), 0.94 (d, $J = 7.1$ Hz, 3H, H-29), 8.06 (brs, 1H COOH), 1.89, (s, 3H, H-31), 3.72 (dd, 1H, $J = 6.2, 2.8$ Hz, H-32), 3.37 (m, 1H, overlapped), 3.39, 3.44 (m, 2H, H-34).



BRUKER
AVANCE NEO
500 MHz NMR SPECTROMETER
SAIF, PANJAB UNIVERSITY,
CHANDIGARH

Current Data Parameters
 NAME May19-2019
 EXPNO 190
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20150122
 Time 17.11 h
 INSTRUM Avance Neo 500
 PROBHD 1H19470_0933 (4
 PULPROG zgpg30
 TD 65536
 SOLVENT dmsd
 NS 0
 DS 0
 SWH 14705.883 Hz
 FIDRES 0.149789 Hz
 AQ 2.2202240 sec
 RG 12.4984
 TM 34.000 msec
 DE 6.79 usec
 TE 301.5 K
 D1 1.0000000 sec
 TDR 1
 SFO1 500.1320885 MHz
 NUC1 1H
 P0 3.33 usec
 P1 10.00 usec
 PL1 22.02300072 W

F2 - Processing parameters
 SI 65536
 SF 500.1320885 MHz
 WDW EM
 SSR 0
 LB 0.20 Hz
 GB 0
 PC 1.00

Fig. 4.28. ^1H spectrum of yellow pigment produced by *E. nigrum*

Fig. 4.29 depicts the ^{13}C spectrum of yellow pigment produced by *E. nigrum*

^{13}C NMR (DMSO- d_6 , 75 MHz): δ 167.5 (s, C-1), 101.1 (s, C-2), 74.2 (d, C-3), 67.4 (d, C-4), 75.1 (d, C-5), 162.1 (s, C-6), 101.2 (d, C-7), 157.3 (s, C-8), 122.4 (d, C-9), 134.3 (d, C-10), 131.4 (d, C-11), 138.4 (d, C-12), 128.7 (d, C-13), 136.8 (d, C-14), 133.3 (d, C-15), 136.1 (d, C-16), 132.1 (d, C-17), 139.5 (d, C-18), 134.2 (s, C-19), 130.3 (d, C-20), 131.1 (s, C-21), 146.4 (d, C-22), 32.1 (d, C-23), 43.8 (t, C-24), 32.6 (d, C-25), 29.4 (t, C-26), 11.2 (q, C-27), 19.1 (q, C-28), 21.2 (q, C-29), 169.4 (s, C-30), 13.1 (q, C-31), 69.1 (d, C-32), 79.1 (d, C-33), 60.6 (t, C-34).

The analysis of ^{13}C and DEPT 135 revealed the presence of 34 carbons, including two carboxyl carbons, 18 sp^2 carbons (1 of which is oxygenated), 14 sp^3 carbons (5 oxymethines, and 1 oxymethylene) and two carboxyl carbons. The presence of carboxyl carbons was confirmed by the ^{13}C NMR data, which showed two signals at δ 162.1 and 169.4. The ^1H - ^1H COSY revealed the alignment H-9/H-10/H-11/H-12/H-13/H-14/H-15/H-16/H-17/H-18, H-22/H-23/H-24/H-25/H-26/H-27 and H-3/H-4/H-5/H-32/H-33/H-34

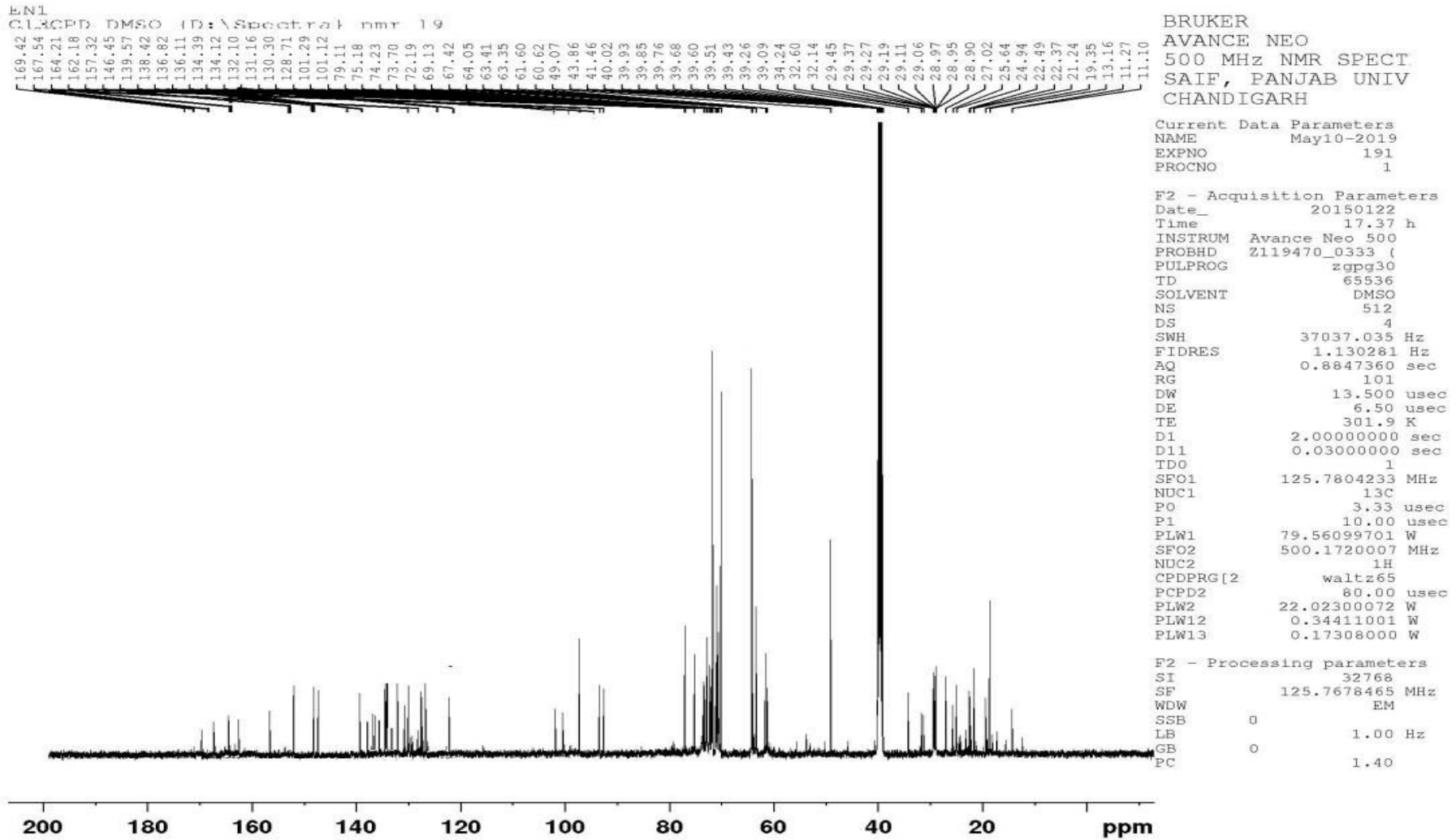
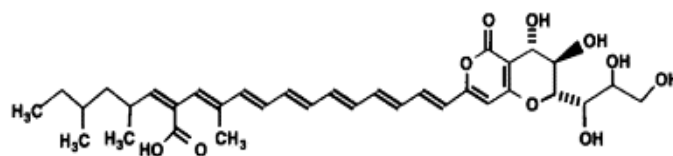


Fig.4.29. ^{13}C spectrum of yellow pigment produced by *E. nigrum*



Orevactaene

The search for this structure in the database revealed the known orevactaene, which has been reported to be a binding inhibitor of HIV-1 Rev protein to the Rev response element (RRE) with an IC_{50} value of $3.6 \mu\text{M}$ from *Epicoccum nigrum* WC47880 (Shu et al., 1997).

Among all, IR is one of the most simple to perform, less time consuming and requires less sample quantity. Several significant compounds and biological molecules have been characterized by IR spectroscopy such as polysaccharides (Jiao et al., 2011), terpenes (Manzoo et al., 2004) and flavanones (Briggs and Colebrook, 1962).

Carotenoids symbolize the major and most varied class of natural pigments. More than 700 structures have been described from plants and microbial sources (Fons et al., 2011). Carotenoids belong to the class of tetraterpenoids with 40 carbon atoms. Structurally, carotenoids can be subclassed into carotenes and xanthophyll (Singh et al., 2014). The variation in color, ranging from pale yellow through bright orange to deep red, is directly related to their structure (Rao and Rao, 2007).

The yellow pigment obtained by extraction of *E. nigrum* fermented substrate showed a characteristic FTIR spectrum (Fig. 4.30- 4.31). The IR spectrum shows band at 1026.16 (trans distributed $-\text{C}=\text{C}-$, $\text{C}-\text{O}$ stretching), 1146.72 ($\text{C}-\text{O}$ stretching, Aliphatic $\text{C}-\text{O}$ stretching, $\text{C}-\text{O}$ stretching, trans distributed $-\text{C}=\text{C}-$, $\text{C}=\text{C}$, $\text{C}-\text{C}$, $\text{C}-\text{CH}_3$), 1244.13 ($\text{C}-\text{O}$ stretching, Aromatic $\text{C}-\text{O}$ stretching, Aliphatic $\text{C}-\text{O}$ stretching, $\text{C}-\text{O}$ stretching, $\text{C}-\text{O}$ acetate), 1441.84 ($-\text{CH}_2$), 1480.42 ($-\text{CH}_2$), 1522.85 ($-\text{CH}_2$, $-\text{C}=\text{C}-$, $\text{C}-\text{C}$, $\text{C}-\text{CH}_3$), 1668.48 ($\text{C}=\text{O}$ stretch, $-\text{C}=\text{O}$, $-\text{C}=\text{O}$ Conjugated), 1731.17 ($\text{C}=\text{O}$ Acetate), 2927.08 ($\text{O}-\text{H}$ stretching, $-\text{C}-\text{H}$ stretching) and 3377.47 ($\text{O}-\text{H}$ stretching) for fermented broken rice. Wheat bran show characteristic peaks at 1040.63, 1151.54, 1243.16, 1440.87, 1472.7, 1523.82, 1593.25, 1667.52, 1732.13, 2856.67, 2927.08 and 3339.86. The slight difference in peaks can be because of the difference in composition of the substrates. Typically, carotenoid $\text{C}=\text{C}$ stretching modes are around 1520 cm^{-1} e.g. spheroidene, β -carotene (Maxime and

van Grondelle, 2012). The carotenoids show specific peaks at 1515-1522 cm^{-1} , 1156-1157 cm^{-1} and 1011-1020 cm^{-1} . The characteristic peak of crocetin have been reported at 1536, 1165 and 1020 cm^{-1} while β - carotene, which is a 9-conjugated C=C, carotenoid show strong bands at 1524, 1156 and 1007 cm^{-1} , an acyclic 11-conjugated carotene lycopene shows strong bands at 1510, 1156 and 1004 cm^{-1} (Schulz et al., 2005). The slightest deviation from the characteristic peaks in samples under study may occur due to oxidation problems. The shift of peak on first wave number has been correlated with number of conjugated bonds, i.e. 1536 cm^{-1} (7) \rightarrow 1524 cm^{-1} (9) \rightarrow 1510 cm^{-1} (11) (Schulz et al., 2005). As per the data obtained (Table 4.27) the peaks are visible at 1523.82 cm^{-1} for wheat bran and 1522.85 cm^{-1} for broken rice, it can be assumed that the pigment contain 9-conjugated compound.

The presence of strong absorptions for hydroxyls at 3377.47 cm^{-1} (O–H stretching), conjugated carbonyls 1668.48 cm^{-1} (C=O stretch, -C=O, -C=O Conjugated) and additional absorptions for the presence of ester/lactone 1146.72 cm^{-1} confirms the presence of oreovactaene (Shu et al., 1991) in FBR and same for FWB with absorptions shown at 1151.54 cm^{-1} , 1667.52 cm^{-1} and 3377.47 cm^{-1} .

Table 4.27 FTIR peak data of pigment obtained from fermented wheat bran and broken rice

FWB		FBR	
Peak	Intensity	Peak	Intensity
534.3	85.61	409.89	66.802
		434.96	64.766
		675.11	59.999
714.65	86.86	721.4	59.784
1040.63	74.643	1026.16	50.806
1151.54	86.67	1146.72	57.651
1243.16	87.768	1244.13	58.701
		1324.18	59.098
1403.26	87.599		
1440.87	88.624	1441.84	60.153
1472.7	92.771	1480.42	63.326
1523.82	98.627	1522.85	65.984
1593.25	86.565		
1667.52	90.433	1668.48	62.004
1716.7	98.057		
1732.12	97.921	1731.17	64.134
		1763.96	64.111
1813.15	101.719	1814.11	62.511
		1858.48	62.539
		1883.55	62.039
2856.67	91.127		
2927.08	84.205	2927.08	58.706
3339.86	86.322	3377.47	54.55

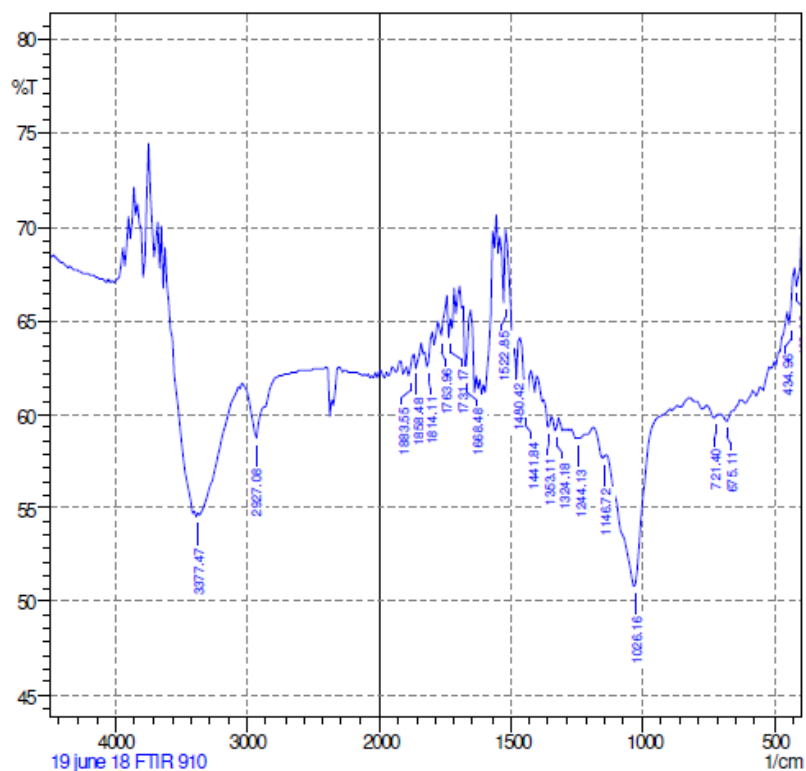


Fig. 4.30. FTIR spectrum of pigment extracted from broken rice

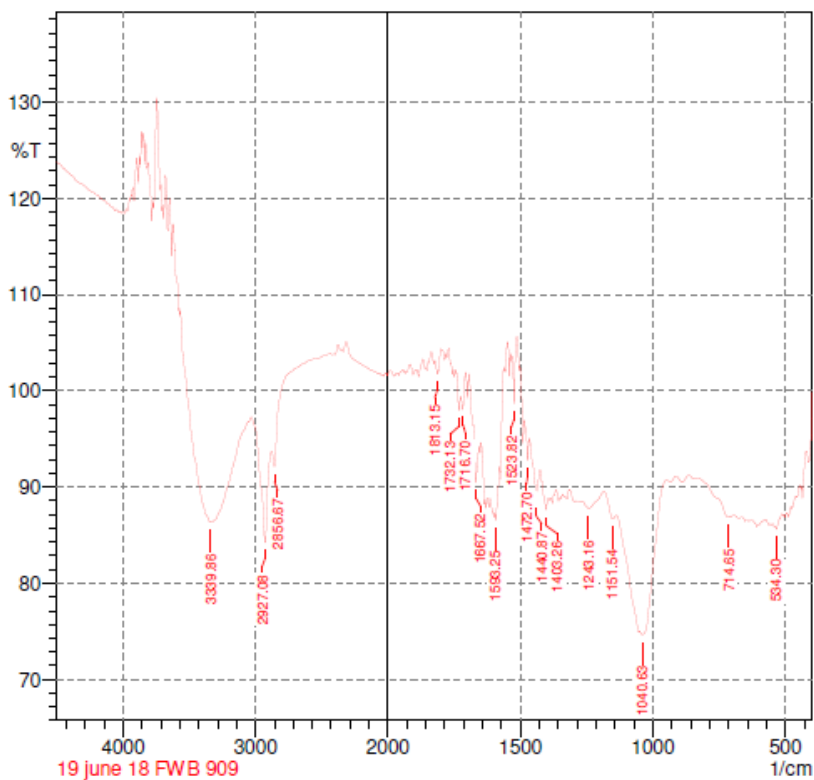


Fig. 4.31. FTIR spectrum of pigment extracted from wheat bran

4.5.2 Evaluation of pigment stability

For thermal and pH stability studies, pigment extracts were kept at variable temperature and pH conditions.

4.5.2.1 Mathematical modeling of pigment degradation kinetics

The characterization and evaluation of natural pigments are mainly based on absorption spectra obtained spectroscopically (Delgado-Vargas et al., 2000). Most of the degradation reactions are temperature dependent and can result in the change in the intensity of color within due course of time. The change in the spectral and visual properties is due to the structural changes brought in by the thermal treatment (Giménez et al., 2015). Knowledge of thermal stability of a colorant is important to design productive strategies to prevent change in visual appearance. The influence of temperature and pH on the stability of water soluble yellow pigment was investigated. Figure (4.32 and 4.33) shows the residual absorbance (A/A_0) of the pigment extract as a function of the time at the four temperatures (30, 40, 60 and 80 °C) investigated. It can be noticed that, the rate of color degradation, increased as the temperature increased (Table 4.32 and 4.33)

Table 4.28: Effect of temperature and time on residual absorbance obtained from broken rice

t (min)	10 °C	30 °C	40 °C	60 °C	80 °C
0	0.99±0.01 ^a	0.97±0.005 ^a	0.92±0.005 ^a	0.79±0.004 ^a	0.58±0.003 ^a
30	0.99±0.01 ^a	0.97±0.007 ^a	0.88±0.003 ^a	0.74±0.002 ^a	0.51±0.002 ^a
60	0.99±0.01 ^a	0.95±0.002 ^a	0.87±0.005 ^a	0.70±0.003 ^{ab}	0.49±0.005 ^b
90	0.99±0.01 ^a	0.94±0.001 ^a	0.86±0.004 ^b	0.69±0.005 ^b	0.48±0.006 ^b
120	0.99±0.01 ^a	0.92±0.002 ^a	0.85±0.005 ^b	0.65±0.005 ^b	0.45±0.003 ^b
150	0.99±0.01 ^a	0.90±0.005 ^b	0.83±0.002 ^b	0.48±0.002 ^c	0.42±0.001 ^b
180	0.99±0.01 ^a	0.89±0.002 ^b	0.78±0.005 ^c	0.43±0.005 ^c	0.35±0.005 ^c
210	0.99±0.01 ^a	0.87±0.005 ^b	0.67±0.004 ^d	0.35±0.005 ^d	0.32±0.005 ^c
240	0.99±0.01 ^a	0.85±0.003 ^b	0.63±0.002 ^d	0.34±0.001 ^d	0.19±0.003 ^d

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in column are significantly different ($P < 0.05$)

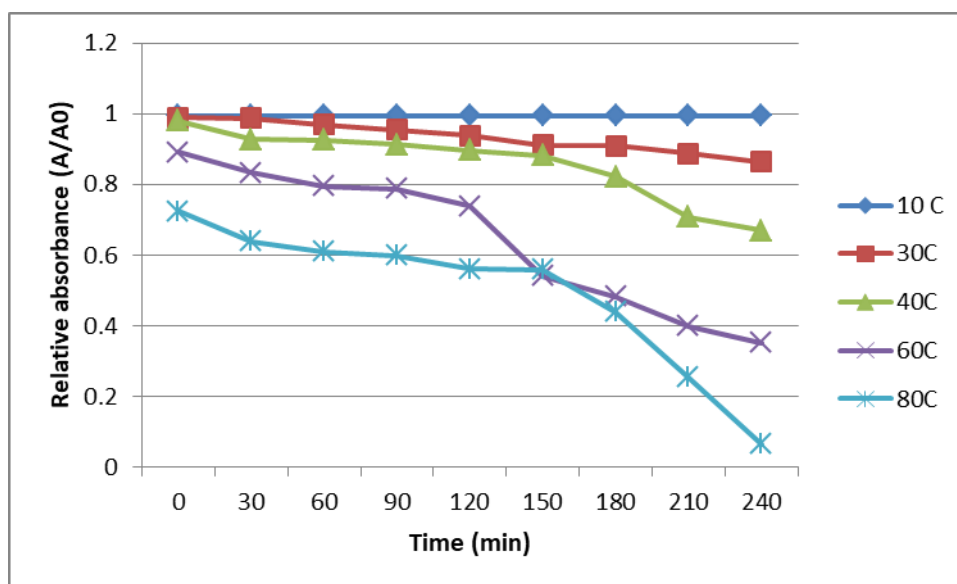


Fig.4.32 Effect of temperature and time on the residual absorbance of orevactaene obtained from broken rice

Table 4.29: Effect of temperature and time on residual absorbance for pigment produced in FWB

t (min)	10°C	30°C	40°C	60°C	80°C
0	0.99±0.01 ^a	0.77±0.01 ^a	0.95±0.005 ^a	0.52±0.01 ^a	0.61±0.01 ^a
30	0.99±0.01 ^a	0.69±0.01 ^b	0.58±0.02 ^b	0.50±0.02 ^a	0.59±0.02 ^a
60	0.99±0.01 ^a	0.48±0.03 ^c	0.54±0.01 ^b	0.47±0.01 ^a	0.58±0.01 ^a
90	0.99±0.01 ^a	0.38±0.02 ^d	0.42±0.03 ^c	0.32±0.02 ^b	0.54±0.005 ^b
120	0.99±0.01 ^a	0.28±0.02 ^e	0.30±0.02 ^d	0.29±0.01 ^c	0.53±0.01 ^b
150	0.99±0.01 ^a	0.22±0.03 ^e	0.29±0.01 ^d	0.25±0.03 ^c	0.25±0.02 ^c
180	0.99±0.01 ^a	0.17±0.01 ^f	0.18±0.03 ^e	0.08±0.01 ^d	0.21±0.01 ^d
210	0.99±0.01 ^a	0.10±0.05 ^g	0.12±0.01 ^f	0.02±0.005 ^e	0.17±0.03 ^e
240	0.99±0.01 ^a	0.03±0.02 ^h	0.05±0.02 ^g	0.01±0.01 ^f	0.14±0.02 ^f

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in column are significantly different ($P < 0.05$)

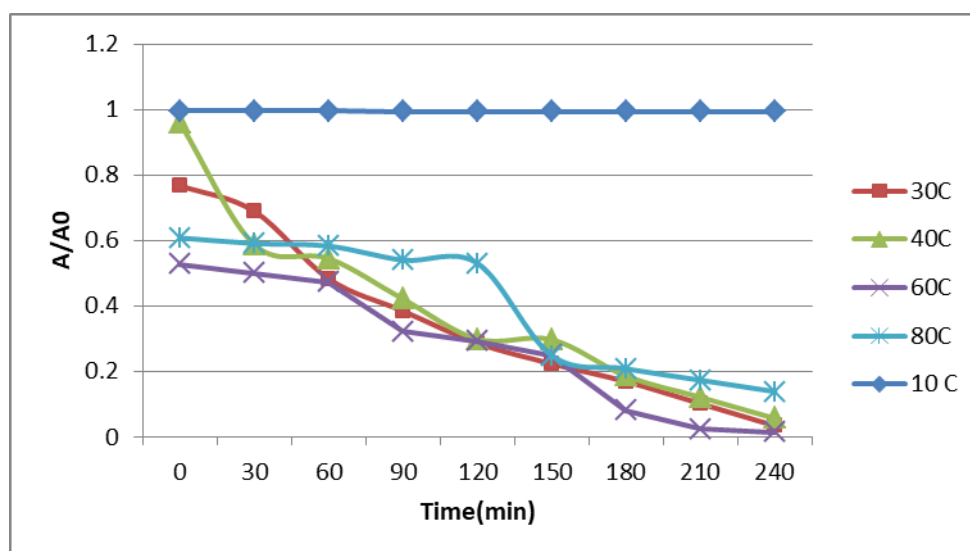


Fig. 4.33. Effect of temperature and time on the residual absorbance of orevactaene obtained from fermented wheat bran.

Kinetic models are commonly used to optimize the industrial process, save time and minimize the effect of treatment on product quality (Patras et al., 2010). The kinetic study of color degradation during thermal processing helps to quantify color under varied time, temperature combination after having the idea of order of reaction.

Information of kinetic parameters such as rate constant (k), half-life period ($t_{1/2}$) and activation energy values (E_a) is important for predicting the fate of pigment during processing and storage. The first order kinetic equation has been frequently employed to predict and explain the thermal degradation of natural as well as microbial pigments, such as anthocyanin (Kechinski et al., 2010) and monascus pigment (Silveira et al., 2008). The kinetic parameters for pigment derived from *Epicoccum* fermented wheat bran and broken rice are summarized in Table (4.28 and 4.29).

Table 4.30: Thermal degradation of yellow pigment at different temperatures (FBR)

Temperature (°C)	FBR			
	$k(\text{min}^{-1})$	$t_{1/2}(\text{h})$	D value (h)	z value (°C)
10	0.00004±0.00001	288.75±1.2	962.5±10.5	37.42±2.5
30	0.00014±0.00001	82.5±1.5	275±13.5	
40	0.00024±0.00003	41.25±1.2	137.5±16.07	
60	0.00072±0.0001	14.26±2.0	47.53±10.05	
80	0.002±0.0005	5.25±2.25	17.5±1.5	

Table 4.31: Thermal degradation of yellow pigment at different temperatures (FWB)

FWB				
Temperature (°C)	k (min ⁻¹)	t _{1/2} (h)	D value (h)	z value (°C)
10	0.00004±0.00001	288.75±1.2	962.5±10.5	
30	0.0009±0.00001	82.5±1.5	275±12.5	
40	0.0014±0.0003	48.12±1.1	160.41±15.00	
60	0.0020±0.0002	16.04±2.5	53.47±10.03	38.24±2.5
80	0.0072±0.0005	5.77±2.15	19.25±1.2	

The decimal reduction time (D value) is defined as the time required for a one log reduction of the initial absorbance at a given temperature. It is calculated by plotting the residual absorbance on a log scale against time. The z value is the temperature needed to reduce the D value by one log cycle and obtained by plotting D values on a log scale against the temperatures. These values help to describe first order reactions. D values for thermal degradation of orevactaene pigment secreted by *Epicoccum nigrum* using wheat bran and broken rice as substrate ranged from 962.5 to 17.5 h in the temperature range of 10-80 °C and z value was found to be 37-38 °C. Half- life period ranged from 288.75 to 5.25 h at 10-80 °C. Effect of conventional heating on *P.purpurogenum* GH2 pigment showed a D value of 43.54 to 6.4 h and t_{1/2} ranged 13.1 to 1.94 h in the temperature range of 60-90 °C (Aguilar-Machado et al., 2017). *Monascus* pigment D values ranged from 357 to 34 h in the temperature range of 30–80 °C, and z value was calculated to be 50 °C. Half-life times varied from 107.5 to 10 h at 30–80 °C (Silveira et al., 2013). Chandran et al., (2014) studied the degradation kinetics of beetroot color using different cooking methods, t_{1/2} for beet-root puree color degradation ranged from 365 to 21 min for temperature range from 50-120 °C (Chandran et al., 2014)

First order equation shown better results for experimental data, such as showing higher R² values ranges from 0.811 to 0.940, lower χ^2 , SEM and RMSE in the range of 4.90 to 6.07, 8.17 to 10.11 and 1.65 to 3.35 respectively in case on fermented broken rice. In case of fermented wheat bran, it shows higher R² values ranges from 0.870 to 0.924, lower χ^2 , SEM and RMSE in the range of 2.83 to 6.74, 4.72 to 11.24 and 1.25 to 2.41 respectively.

Choosing equation which has less measurable parameter is recommendable for the predictive modeling reason behind that it is stable to use easy model. (Schokker and Boekel, 1997). It shows

that first order equation is better to explain the temperature degradation of pigments produced by *E. nigrum*. This equation is frequently used to explain the thermal destruction of various natural pigments like anthocyanin (Reyes and Cisneros-Zevallos 2007; Harbourne et al., 2008; Kechinski et al., 2010)

The degradation rate constants (k) were used to calculate activation energy using Arrhenius equation (Eq 3.8). A plot between Ln k on a log scale and reciprocal of temperature is used to find the activation energy. The k-values obtained gave a good fit to Arrhenius equation (Fig. 4.34 and 4.35); thus k_0 and E_a were calculated. E_a represents the energy hurdle that molecules need to overcome in order to undergo reaction and is considered as important criteria for analyzing pigment's thermal stability (Liu et al., 2008).

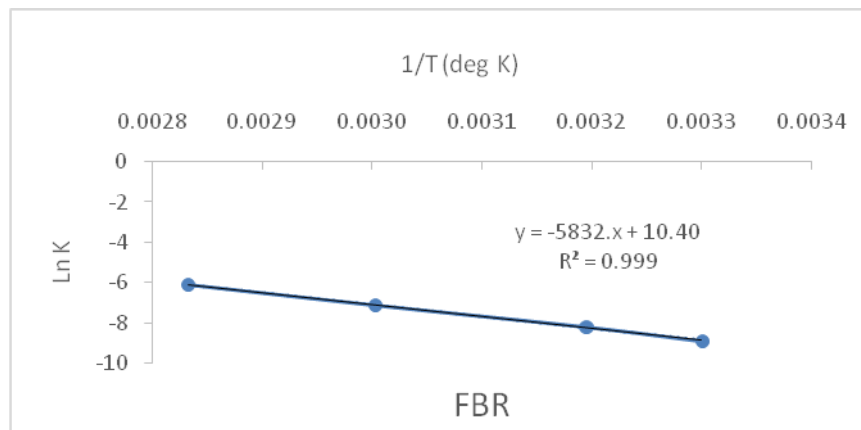


Fig. 4.34. Temperature dependence of reaction rate on degradation of pigment obtained from broken rice

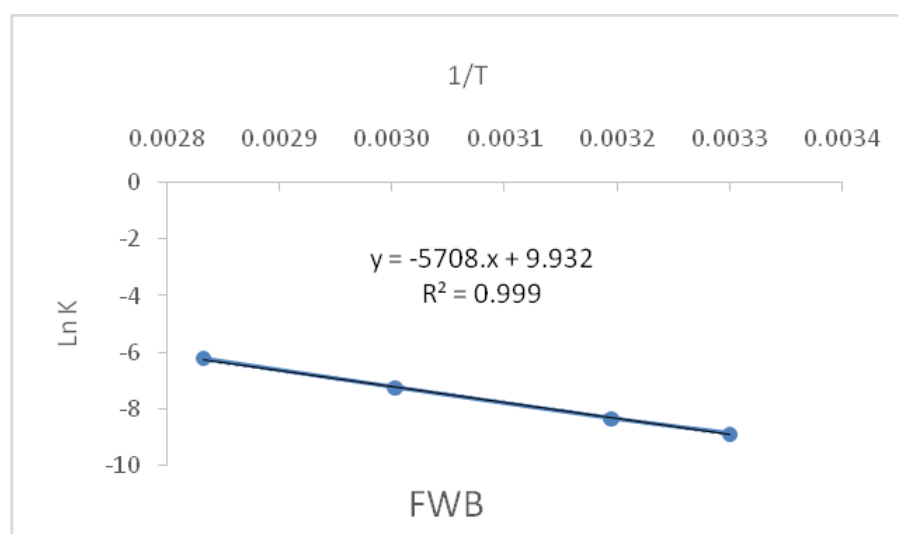


Fig 4.35. Temperature dependence of reaction rate on degradation of pigment obtained from wheat bran

E_a obtained was 48.48 kJ/mol for pigments extracted from fermented broken rice and for pigments extracted from fermented wheat bran it was 47.45 kJ/mol. In case of *Monascus* pigment degradation E_a was observed 40.8 kJ/mol (Silveira et al., 2013). While other yellow food colorants like lutein, riboflavin, curcumin, β -carotene, gardenia yellow, Opuntia betaxanthin had an activation energy of 3.2, 36.4, 23.7, 6.5, 31.2, 43.7 kJ/mol respectively (Giménez et al., 2015). For beet root pigments, E_a values were 94.01 and 97.16 kJ mol⁻¹, respectively (Liu et al., 2008).

The obtained value for k_0 for *E. nigrum* pigment was 9.932 min⁻¹ for FWB and 10.40 min⁻¹ for FBR. First order model and Arrhenius equation can be combined as follows:

For FWB

$$\frac{A}{A_0} = \exp \left[-9.932 \exp \left(\frac{-5708}{8.3147T} \right) t \right] \text{-----(4.6)}$$

For FBR

$$\frac{A}{A_0} = \exp \left[-10.40 \exp \left(\frac{-5832}{8.3147T} \right) t \right] \text{-----(4.7)}$$

Where, t is the time in minutes and T is temperature in Kelvin. These combined equations give an idea of the effect of time temperature combination on pigment stability in terms of residual pigment which is an important issue for the food industry. Orevactaene discoloration observed at 30, 40, 60 and 80°C was variable. While at 30 °C only a 12% decrease in color intensity was observed after 4 hours, 40°C resulted in 31% decrease and at 80°C 56% of color was vanished. The results showed that the orevactene pigment produced by *E.nigrum* is heat sensitive and changes in color intensity should be expected in heat processed products. Similar results are reported for *Monascus* pigments by Wong and Koehler (1983); Fabre et al., (1993) and Carvalho et al., (2005). However, further studies can be conducted to see the interactive effect of food components on pigment stability.

Stability of *Epicoccum* pigments at different pH values were studied at 80°C. At acidic pH, i.e., pH 4 pigment precipitation was observed which may restrict its use in acidic foods. After 180 min yellow pigments maintained 93% and 83% of the initial color at pH 6 and pH 8. At high temperature with increased pH, it resulted that there is an increase in stability of pigments. Autoclaving treatment was also performed for *Epicoccum* pigments. Pigments produced on wheat

bran shown less degradation in color, i.e. 45% (Fig. 4.36) as compared to pigments produced on broken rice i.e. 69% (Fig. 4.37). Faber et al., (1993) stated sensitivity of Monascus pigments towards low pH. Natural pigment commonly show such level of degradation which can be compensated by proper pigment dosage.

Table 4.30: Effect of pH and time on residual absorbance of orevactaene from wheat bran

t	pH4	pH6	pH8
0	0.77±0.01 ^a	0.65±0.01 ^a	0.70±0.01 ^a
30	0.66±0.02 ^b	0.58±0.02 ^a	0.69±0.02 ^a
60	0.55±0.01 ^c	0.58±0.005 ^a	0.69±0.01 ^a
90	0.44±0.03 ^d	0.57±0.01 ^a	0.68±0.01 ^a
120	0.32±0.02 ^e	0.52±0.007 ^a	0.68±0.01 ^a
150	0.22±0.01 ^f	0.51±0.01 ^a	0.67±0.02 ^a
180	0.11±0.005 ^g	0.50±0.01 ^a	0.67±0.03 ^a

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in column are significantly different ($P < 0.05$)

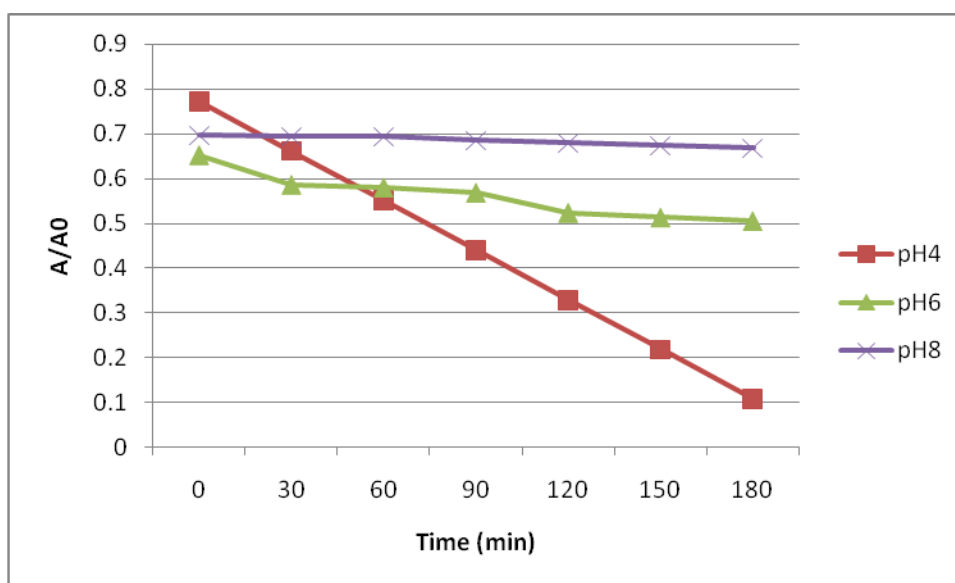


Fig. 4.36: Effect of pH and time on the residual absorbance of yellow pigment obtained from fermented wheat bran.

Table 4.31: Effect of pH and time on the residual absorbance of yellow pigment obtained from fermented broken rice.

t	pH4	pH8	pH6
0	0.74±0.01 ^a	0.78±0.01 ^a	0.70±0.01 ^a
30	0.59±0.01 ^b	0.77±0.02 ^a	0.68±0.01 ^a
60	0.51±0.02 ^c	0.75±0.01 ^a	0.66±0.02 ^{ab}
90	0.45±0.01 ^d	0.74±0.03 ^a	0.61±0.01 ^b
120	0.42±0.02 ^e	0.69±0.02 ^b	0.56±0.01 ^c
150	0.27±0.01 ^d	0.67±0.03 ^b	0.56±0.03 ^c
180	0.07±0.03 ^e	0.66±0.03 ^b	0.55±0.02 ^c

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in column are significantly different ($P < 0.05$)

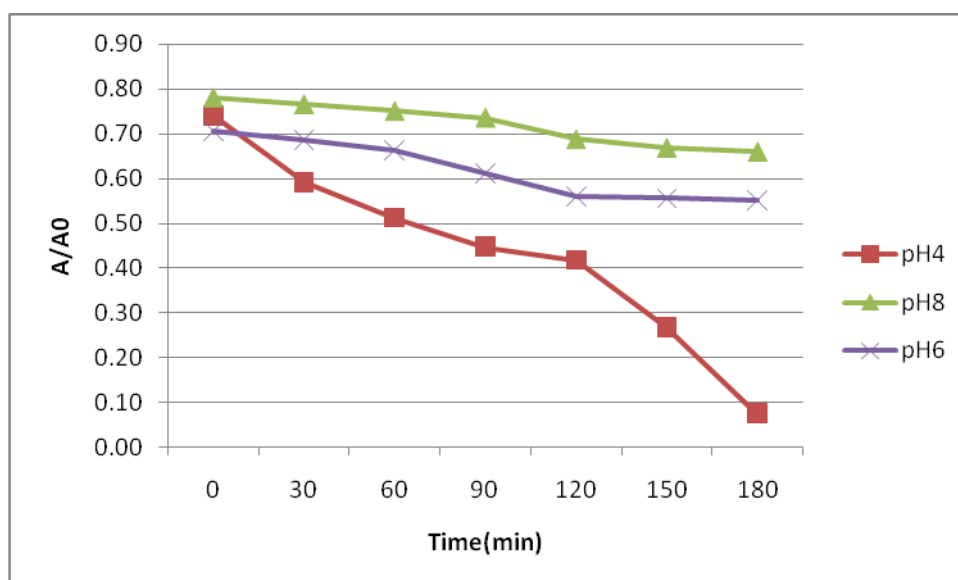


Fig.4.38. Effect of pH and time on the residual absorbance of yellow pigment obtained from fermented broken rice

Stability of pigments during sunlight exposure was also checked. Pigments were exposed to sunlight for 2 hours during time 12 pm to 2 pm. While *Epicoccum* pigments produced on wheat bran resulted only 1% degradation of color and pigments produced on broken rice shown 13% color degradation. In this study the degradation of color observed, which is commonly found in natural pigments (Carvalho et al., 2005).

Thermodynamic equation parameters were derived by using mathematical expressions based on absolute reaction rate theory (Rasane et al., 2018)

$$k = \frac{K_B T}{h} e^{\frac{\Delta S^*}{R}} e^{\frac{-\Delta H^*}{RT}} \text{-----(4.8)}$$

Where, K_B = Boltzmann's constant, 1.38×10^{-23} J/K

h = Plank's constant, 6.63×10^{-34} Js

ΔS^* = entropy of activation, J/mol.K

ΔH^* = enthalpy of activation, J/mol

The enthalpy was calculated from activation energy (E_a) using the following equation (4.7):

$$\Delta H^* = E_a - RT \text{ (4.7)}$$

The free energy of activation (ΔG^* , KJ/mol) was calculated using equation (4.8):

$$\Delta G^* = \Delta H^* - T\Delta S^* \text{ (4.8)}$$

Thermodynamic parameter estimation might provide information related to the kinetics of temperature degradation of pigments and modification in stability of structure. Table 4.30 and 4.31 shows calculated values of ΔH^* , ΔG^* and ΔS^* . For orevacatene pigment the enthalpy of activation (ΔH^*) ranged from 44.52 to 45.97 KJ mol⁻¹, entropy of activation (ΔS^*) varied from -166.72 to -171.85 J/mol.K and free energy of activation (ΔG^*) ranged from 96.60 to 105.18 kJ/mol for the temperature range of 30-80°C.

Values of ΔH^* for thermal degradation represent a measure of the number of non-covalent bonds broken and ΔS^* measure the disorderliness of the system. The decreasing value of ΔH^* with increase in temperature indicate conformation modifications have occurred (Bhatti et al., 2006) From thermodynamic point of view ΔG values indicates that degradation reaction conditions are not supportive at higher temperatures (Basu et al., 2008). It may be because of negative entropic contribution in the degradation process. Negative ΔS values indicate that system shows a low level of disorderliness (Bhatti et al., 2006).

Table 4.32 Thermodynamic parameter value for *E. nigrum* yellow pigments decay at pH 6.0

FBR				
Temperature(°K)	Ea (kJmol ⁻¹)	ΔH(kJmol ⁻¹)	ΔG (kJmol ⁻¹)	ΔS (Jmol ⁻¹ K ⁻¹)
303	48.48±1.64	44.93±1.51	96.60±3.5	-170.50±7.5
313		44.85±1.55	98.47±1.5	-171.29±8.1
333		44.69±1.47	101.89±1.9	-171.78±8.5
353		44.52±1.55	105.18±2.2	-171.85±8.2

Table 4.33 Thermodynamic parameter value for *E. nigrum* yellow pigments decay at pH 6.0

FWB				
Temperature(°C)	Ea (kJmol ⁻¹)	ΔH(kJmol ⁻¹)	ΔG (kJmol ⁻¹)	ΔS (Jmol ⁻¹ K ⁻¹)
303	47.45±1.58	45.97±1.38	96.60±3.5	-167.10±7.7
313		45.87±1.35	98.07±1.2	-166.72±8.0
333		45.72±1.33	101.56±2.0	-167.71±8.1
353		45.55±1.35	104.90±2.5	-168.13±8.2

4.6 Antimicrobial and Antioxidant activity of Pigments

4.6.1 Total Phenol and Flavonoid content

Phenolics are the secondary metabolites with various medicinal and functional properties. These compounds help in neutralizing reactive oxygen species or free radicals by donating an electron or hydrogen atom in aqueous solutions (Nile and Park 2015). In the present study, the phenolic content was measured by the Folin Ciocalteu method (Table 4.36) and flavonoids by Aluminum chloride method. It was observed that the fermentation with *Epicoccum nigrum* has resulted in an

increase in phenolic and flavonoid compounds and yield was further dependent upon the solvent used for extraction. There was a significant difference at $p < 0.05$ in the phenolic and flavonoid compounds production in unfermented and fermented substrates. There was significant positive 2 fold increase ($p < 0.05$) in phenolic content and 1.8 fold in flavonoid content in broken rice substrate after fermentation with *Epicoccum nigrum* when extracted with ethanol. On the other hand, methanolic extraction resulted in 1.6 fold increase in both phenolic and flavonoid compounds as shown in Table 4.36.

Table 4.34: Total phenol content and flavonoid content in different pigment extracts

	Total Phenol content (mg GAE/ml)	Total Flavonoid content (mg QE/ml)
Ethanolic broken rice (EBR)	6.69±0.37 ^a	3.29±0.13 ^a
Ethanolic fermented broken rice (EFBR)	12.27±0.32 ^b	6.01±0.63 ^b
Ethanolic wheat bran (EWB)	7.68±0.38 ^A	5.40±0.16 ^A
Ethanolic fermented wheat bran (EFWB)	11.86±0.46 ^B	6.82±0.23 ^B
Methanolic broken rice (MBR)	8.43±0.34 ¹	3.42±0.11 ¹
Methanolic fermented broken rice (MFBR)	13.27±0.80 ²	5.81±0.44 ²
Methanolic wheat bran (MWB)	9.42±0.38 ³	5.91±0.19 ²
Methanolic fermented wheat bran (MFWB)	13.81±0.16 ²	6.59±0.23 ³

Data presented as means± SD of triplicate readings. Mean values within each the different superscripts in column are significantly different ($P < 0.05$)

McCue and Shetty (2005) reported that bioprocessing of soy milk by kefir cultures could increase phenolic contents and, thus, enhance the scavenging abilities on DPPH radicals. It seems that wheat bran and broken rice might contain more phenolic and flavonoid components formed during fermentation. The increase in phenolic and flavonoid contents during the fermentation process might result from the various processes of glycosylation, deglycosylation, methylation, sulfate conjugation by filamentous fungus (Nguyen et al., 2014). All of the fungi secrete many different types of enzymes during the fermentation process, e.g., hydrolases, cellulose- or xylan-degrading enzymes, and esterase, that can soften the kernel structure, break down the cereal cell walls, and release esterified and insoluble-bound nutrient substances (Yokoyama and Hayakawa, 2002; Koseki et al., 2010). Similar results have been reported by Babitha et al., 2007 and Suraiya et al., 2017 where the phenolic and flavonoid content increased with the fermentation time. Lee et al., 2008 have shown that the total phenolic and anthocyanin contents and antioxidative activity of black bean enhance remarkably after fermentation by filamentous fungi.

The ability to act as antioxidant depends mainly on the chemical structures of phenolic compounds, their orientation and number of hydroxyl groups attached to the aromatic ring (Sánchez- Moreno et al., 1998).

4.6.2 Antioxidant activity

The secondary metabolites produced by fungal species possess multi-functional properties. Hence it is important to study its biological activities. In the present study antioxidant activities of the pigment secreted by *Epicoccum nigrum* was assessed using different methods such as DPPH, ABTS, FRAP and reducing power.

4.6.2.1 Radical Scavenging activity

The antioxidant activity of the pigment extract was evaluated by inhibiting free radical DPPH, expressed in terms of the ability to scavenge the free radical which is an extensively used method to assess the antioxidant capacity in a short time interval (Rufino et al., 2009; Sánchez-Moreno et al., 1998). The concentration of antioxidant required to decrease the initial concentration of DPPH by 50% (EC_{50}) is the most frequently used factor to measure the antioxidant properties of a substance (Rufino et al., 2009); the lower the EC_{50} value, the higher its antioxidant power.

DPPH radical is widely used to assess the antioxidant potential of sample owing to its high stability donation capacity (Ferreira et al., 2006). At 1 mg/mL with regard to ethanolic and

methanolic pigment extracts, scavenging abilities on DPPH radicals were in the descending order: BHT > MFBR > MFWB > EFBR > EFWB. At 20 mg/ml, scavenging abilities of BHT on DPPH radicals was 86.32%, while the methanolic extracts, MFBR and MFWB exhibited scavenging abilities of 78.74 and 77.46% respectively (Fig. 4.38). On the other hand ethanolic extracts EFBR and EFWB showed a moderate scavenging ability of 53.22 and 49.5%. It was found that methanol was better solvent to extract the compounds contributing to DPPH activity. The pigment complex consists of carotenoids and flavonoids and thereby contributing to the antioxidant activities.

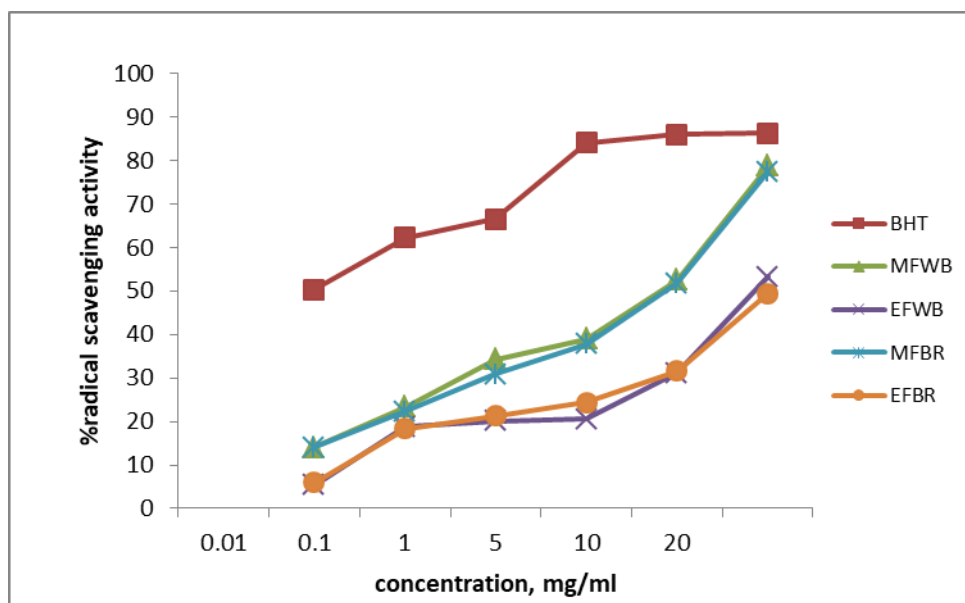


Fig. 4.38 Scavenging ability of methanolic and ethanolic pigment extracts on 1,1-diphenyl-2-picryl hydrazyl radicals.

EC₅₀ value was 5.31 and 5.42 mg/ml for MFBR and MFWB respectively against 1.56 mg/ml of BHT as given in Table (4.37). The DPPH activity of MFBR and MFWB used in present study are comparable to *Monascus* fermented rice (IC₅₀ of 4.38 mg/ml) (Tseng et al., 2006; Yang et al., 2006) and ethanolic and methanolic extracts of *Monascus* fermented soybeans with DPPH activity of 2.85-4.97 mg/mL and 1.80-2.70 mg/ml respectively (Lee et al., 2009). Some foods such as red onion (1.80 mg/mL), guava (2520 μmolTE/100g), red grapes (1350 μmolTE/100g) and garlic (6.40 mg/ml) (Prakash et al., 2001; Thaipong et al., 2006; Qusti et al., 2010) have IC₅₀ value similar to our results.

In ABTS method, the scavenging ability increased with increase in concentration. At 1 mg/mL MFWB scavenged 78.53% of ABTS whereas scavenging abilities of EFBR, MFBR and EFWB was 38.6, 72.53 and 41.6 %, whereas, the scavenging abilities of BHT was in the range of 81.2-

98.7% in the concentration range of 1-20mg/mL (Fig. 4.39). At 20 mg/mL the results were in order of standard antioxidant BHT > MFBR > FBR > MFWB > EFWB, which represents a higher variation than in DPPH assay. EC₅₀ value was 4.5 and 4.85 mg/mL for MFBR and MFWB respectively against 0.07 mg/mL of BHT as given in Table (4.32). The results are higher than monascal polished rice (0.59mg/mL) (Yang et al., 2006).

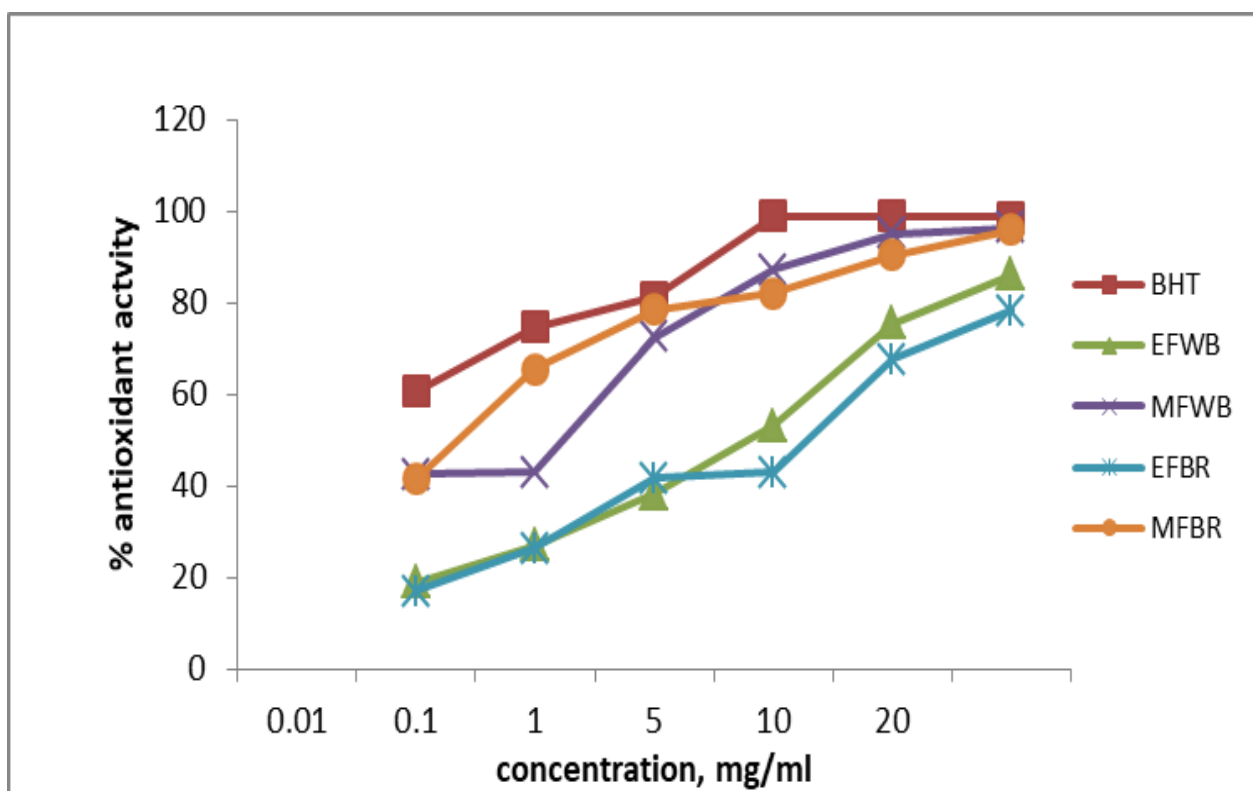


Fig.4.39. Scavenging ability of pigment extracts on ABTS radical

Table 4.35 EC₅₀ values of ethanolic and methanolic pigment extracts from fermented wheat bran and broken rice

	EC ₅₀ value* (mg extract/ml)	
	FWB	FBR
Ethanolic		
ABTS radical scavenging activity	4.85±0.02 ^{aA}	4.5±0.02 ^{aA}
DPPH radical scavenging activity	7.83±0.05 ^{aB}	7.68±0.08 ^{aB}
FRAP	0.61±0.01 ^{aC}	0.8±0.05 ^{aC}
Methanolic		
ABTS radical scavenging activity	1.94±0.02 ^{aA}	2.68±0.12 ^{bA}
DPPH radical scavenging activity	5.43±0.01 ^{aB}	5.31±0.01 ^{aB}
FRAP	0.21±0.02 ^{aC}	0.52±0.03 ^{bC}

*EC₅₀ value (effective concentration), the effective concentration at which the antioxidant activity was 50%; the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and ABTS radicals were scavenged by 50%; and ferrous ions were chelated by 50%, respectively. EC₅₀ value was obtained by interpolation from linear regression analysis. Each value is expressed as mean ± standard error ($n = 3$). Means with different small letters within a row are significantly different ($P < 0.05$). Means with different capital letters within a column at a specific antioxidant attribute are significantly different ($P < 0.05$).

4.6.2.2 FRAP

Results of ferric reducing capacities of different extracts are shown in table. The trend is similar with DPPH and ABTS results. The FRAP values were expressed as $\mu\text{M Fe}^{2+}/\text{ml}$ of sample. In this study the ferric reducing potential was in the order of EFWB > BHT > EFBR > MEWB > MFBR (Fig. 4.40).

Although BHT was good in antioxidant activities and used as food additives in microgram level, the *Epicoccum* fermented rice and wheat bran could be used in higher amounts as a food ingredient and might serve as possible protective agents to reduce oxidative damage. On the basis of the results obtained, fermented wheat bran and broken rice were superior to unfermented counterpart in most antioxidant properties due to the results of increased phenolic content.

Accordingly, fermented mass as well as the pigment extract might be potent antioxidants for use in food products.

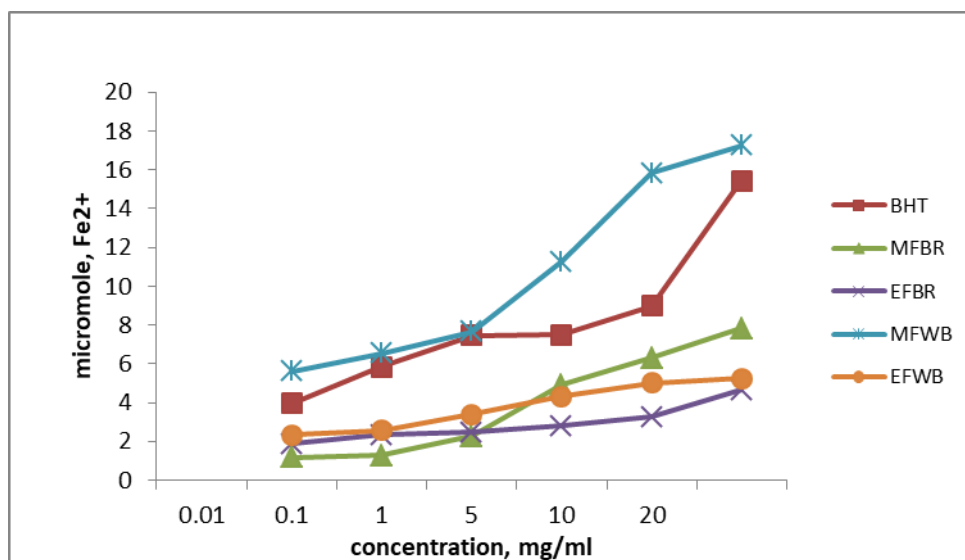


Fig. 4.40. Ferric reducing power of methanolic and ethanolic pigment extracts

Antimicrobial activity:

Entophytic fungi are known to protect plants against several biotic stresses, in part via production of secondary metabolites with biological activities (de Lima Favaro et al., 2012). Yellow pigment produced by *Epicoccum nigrum* was submitted for antimicrobial activity against eight different microbial strains. The qualitative antimicrobial activities of pigments against strains by the diffusion method are shown in Table (4.38). The pigment extract showed an inhibitory effect against only three strains viz. *Streptococcus pyogenes* MTCC442, *Staphylococcus aureus* MTCC96 (Fig. 4.42-4.43) and *Candida albicans* MTCC227 (Fig. 4.44) (Table 4.39). The pigments that showed antimicrobial activity against test microorganisms (Table 4.40) were used to determine effect in different concentration. In all experiments a control test was carried out with DMSO in the absence of pigments, to verify the inhibitory effect on the growth of the target microorganisms. In all cases, the DMSO did not inhibit the microorganism growth.

Table 4.36: Qualitative antimicrobial activity of pigment extract against test organism

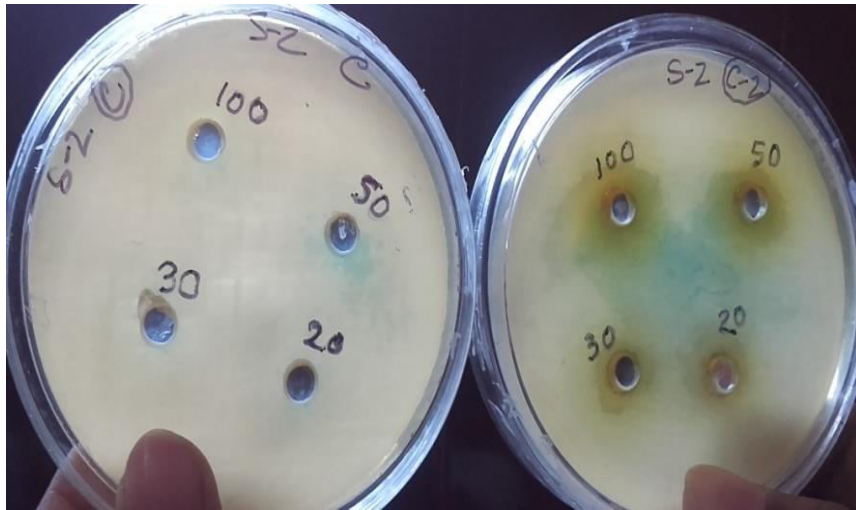
S.No.	Test Organism	Inhibition
1	<i>Escherichia coli</i> MTCC443	(-)
2	<i>Pseudomonas aeruginosa</i> MTCC424	(-)
3	<i>Staphylococcus aureus</i> MTCC96	(+)
4	<i>Streptococcus Pyogenes</i> MTCC442	(+)
5	<i>Candida albicans</i> MTCC227	(+)
6	<i>Aspergillus Niger</i> MTCC282	(-)
7	<i>Trichoderma harzianum</i> MTCC792	(-)
8	<i>Fusarium moniliforme</i> MTCC156	(-)

Table 4.37 Zone of inhibition (mm) shown by pigment extract against test organisms

S.No.	Test Organism	Volume of extract (μ l)	Zone of inhibition, mm
1	<i>Escherichia coli</i> MTCC443 [Gram negative]	100 μ l	0
2	<i>Pseudomonas aeruginosa</i> MTCC424 [Gram negative]	100 μ l	0
3	<i>Staphylococcus aureus</i> MTCC96 [Gram positive]	100 μ l	10.5 \pm 0.3
4	<i>Streptococcus pyogenes</i> MTCC442 [Gram positive]	100 μ l	11.5 \pm 0.7
5	<i>Candida albicans</i> MTCC227 [Yeast]	100 μ l	6.0 \pm 0.5
6	<i>Aspergillus niger</i> MTCC282 [Fungus]	100 μ l	0
7	<i>Trichoderma harzianum</i> MTCC792 [Fungus]	100 μ l	0
8	<i>Fusarium moniliforme</i> MTCC156 [Fungus]	100 μ l	0

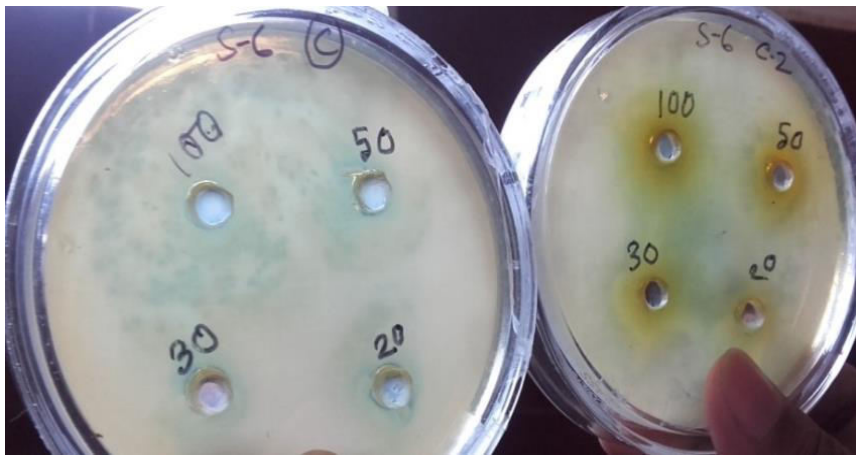
Table 4.38 Zone of inhibition (mm) shown against selected organisms after 24 and 72 hour incubation

Test organisms	Zone of Inhibition (mm)				
	Volume of extract(μ l)	24 hr.		72 hr.	
		C1	C2	C1	C2
<i>Streptococcus pyogenes</i> MTCC442	100	11	12	35	26
	50	10	11	12	19
	30	9	10	24	14
	20	8	9	20	13
<i>Staphylococcus aureus</i> MTCC96	100	10	11	21	30
	50	9	9	19	25
	30	7	8	16	23
	20	5	7	13	17
<i>Candida albicans</i> MTCC227	100	6	8	10	9.25
	50	5	7	8	7
	30	3	2.25	5	6
	20	0	0	0	0



A

B



C

D

Fig. 4.41: Antibacterial activity of crude pigment extract against *Streptococcus pyogenes* MTCC442 (A-zero hour; B -24 hour) and *Staphylococcus aureus* MTCC96 after 24 hrs. incubation (C- zero hour; D-24 hour)

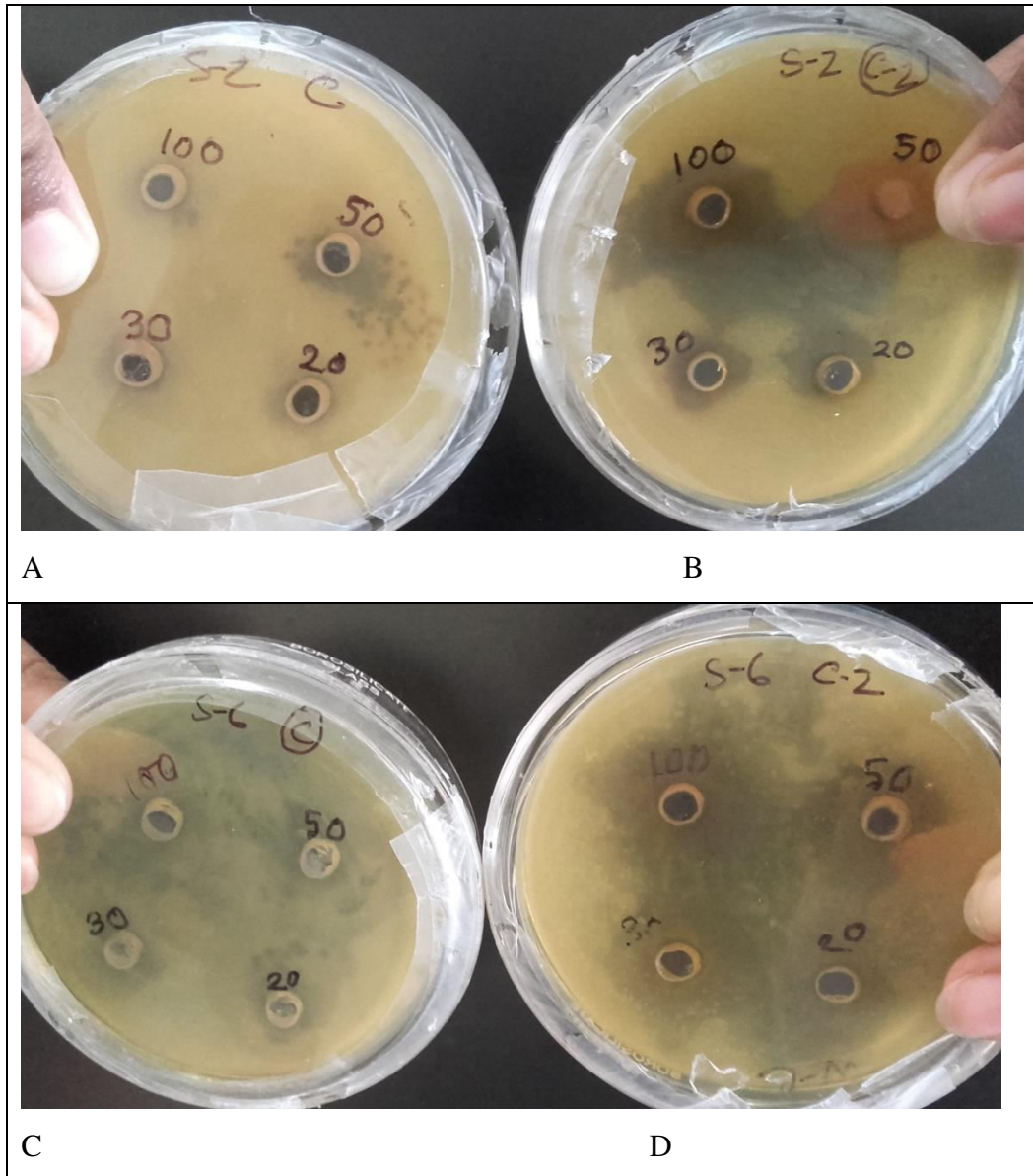


Fig.4.42: Antibacterial activity of crude pigment extract against *Streptococcus pyogenes* MTCC442 (A-zero hour; B -72 hour) and *Staphylococcus aureus* MTCC96 after 24 hrs. incubation(C- zero hour; D-72 hour)

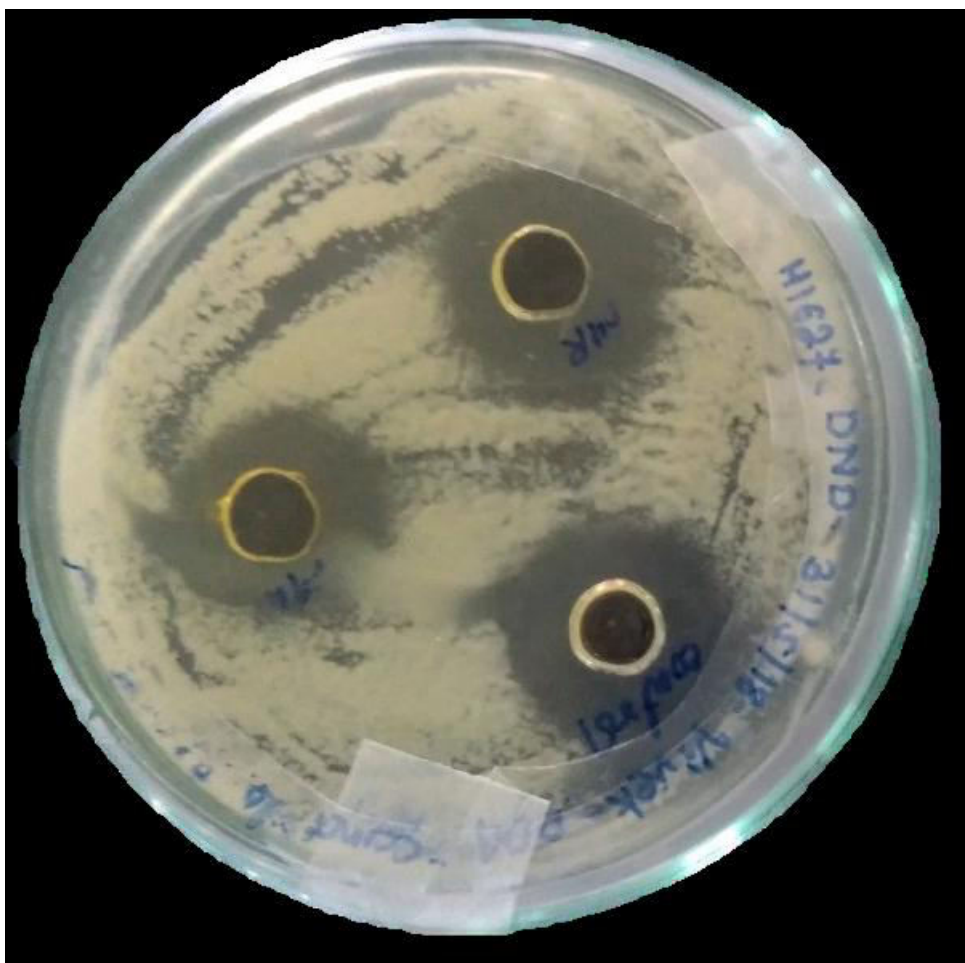


Fig 4.43. Antimicrobial activity of pigment extract against *Candida albicans* MTCC227

The mean diameter of the inhibition zone was more against *Streptococcus pyogenes* MTCC442 which shows *S. pyogenes* MTCC442 was more sensitive to yellow pigment with strongest zone of 11.5 ± 0.7 mm after 24 hr incubation when 100 μ l of 5mg/ml of pigment extract was used which increased to 30.5 ± 6.36 mm after 72 hr incubation (Table 4.40). *E. nigrum* is known to produce a number of antifungal compounds depending on the media on which it grows (Brown et al., 1987). While some have been already characterized: epicorazine A, epicorazine B (Deffieux, Filleau & Baute, 1978), epirodins A and B (Ikawa et al., 1978) and flavipin (Fatima et al., 2016). Zhang et al., (2007) showed the isolation of epicoccin A compound showing modest antimicrobial activity.

Among the test organisms used in the antimicrobial study, two Gram positive bacteria were most susceptible to the pigment extract. The reason may be the presence of lipopolysaccharide in the cell wall of Gram negative bacteria. The LPS of cell wall can prevent the influx of active compounds to cytoplasmic membrane of these bacteria. The difference in susceptibility pattern of test organisms viz., bacteria, yeast and molds to pigment can be attributed to several factors such

as inactivation by enzymes, changes in cellular permeability towards the antimicrobial agent, active efflux, overproduction of target enzymes, bypass of drug action and intercellular cooperation (Manila and Murugesan, 2014; McKeegan et al, 2002).

The antimicrobial effect is considered to occur through various means such as reducing the transportation of nutrients, oxygen and metabolites, interfering with cell membrane permeability and use of iron, reaction with enzymes in the spore and vegetative cells (Xu, 2011; Kim et al., 2006a). Organisms in their active exponential growth phase provide ready access to their cellular targets to an antimicrobial agent (Naidu, 2000).

Kim et al. (2006a) observed that cell surfaces of *E. coli* KCCM 11234 submitted to antimicrobial activity was coated with Monascus pigments when analyzed by electron microscopy. Additional adsorption occurred with the hydrophobic L-tyrosine and L-phenylalanine derivatives indicating that some hydrophobic interaction between the pigment and the pigment-coated cells were induced. The pigment adsorption degree and antimicrobial activity seem to depend on the hydrophobicity of the pigment. Kim et al. (2006b) studied the oxygen uptake rates and specific oxygen uptake rates (qO_2) of *E. coli* cells treated with an L-phenylalanine Monascus pigment derivative. The ratio $qO_2/qO_{2 \max}$ decreased proportionally with the increase in the concentration of both the control red and the L-phenylalanine pigment derivatives limiting the transfer of oxygen and nutrients into the cells.

SUMMARY AND CONCLUSION

The present study was conducted to explore the potential of *Epicoccum nigrum* for the production of polyketide and carotenoid production. So, the objectives of the proposed research work were: a) Optimization of nutrient composition and media process parameters for fermentation, culture growth and pigment production by *Epicoccum nigrum* b) Extraction and purification of the pigment c) Characterization and structure elucidation of the isolated pigment d) Antimicrobial and antioxidant potential of the pigment.

It was observed in the study that growth parameters viz. radial growth, sporulation and dry cell weight, and pigment production using *Epicoccum nigrum* cultivated on solid medium were greatly influenced by the medium composition. Among the various media evaluated PDA was found to be the best with respect to growth parameters, especially sporulation, thus can be used as basal media for cultivation of *Epicoccum nigrum*. While starch as carbon source increased the radial growth, sucrose significantly influenced the sporulation of *Epicoccum nigrum*. Among the various nitrogen sources peptone significantly increased radial growth as well as sporulation. The light passing through a red color sheet resulted in better radial growth in comparison to unfiltered light. Yellow pigment production was significantly influenced by the presence of dextrose, peptone and darkness. It was concluded that depending upon the requirement the nutritional components may be varied to get the desired secondary metabolites.

Broken rice and wheat bran were found to be a most suitable substrate amongst the all tested for the production of pigment through solid state fermentation.

Pigment production conditions were optimized with respect to initial moisture content and incubation days. The conditions optimized using design expert software are: 61% initial moisture content and incubation time of 11.73 days will result in pigment yield of 43.1 AU/g with overall desirability value of 0.98.

Central composite rotatable design under response surface methodology was used for deducing optimized conditions for low cost aqueous extraction of oreovactaene and flavonoid. The pigments were extracted under conditions of extraction temperature (40-70°C), mass of fermented rice (0.5-1.5 g) and time (30-90min) using water as extraction media. The optimum conditions generated

by the software for aqueous extraction process i.e. extraction temperature of 55.7°C, 0.79 g of fermented matter and extraction time of 56.6 min resulted in pigment yield of 52.7AU/g oreovactaene and 77.2 AU/g flavanoid.

The box behnken design was found to be appropriate in finding out the optimized conditions for the extraction of carotenoid from the fermented substrate. The extraction temperature of 40.27°C, 20.21ml/g of solvent to solute ratio and extraction time of 76.22 min for extraction of carotenoid pigments (432.18µg/g) from *Epicoccum nigrum* fermented wheat bran were optimized.

The extracted crude pigment was partially purified using column chromatography. In column chromatography as we have used both polar and non-polar solvent system for purification the clear separation of fractions was not achieved, reason behind that might be polarity of pigments quite close. Further preparative thin layer chromatography was used for separating the components. Out of all solvent systems Chloroform (65): Methanol (25): Water (4) resulted in better separation of the pigment with R_f ranging from 0.87-0.91. The separated spot was removed and spectrum was run between the range 350 nm to 600 nm after dissolving the pigment in ethanol. The peak was obtained at 426nm and as per literature the compound is oreovactaene. The yellow pigment obtained by extraction of *E. nigrum* fermented substrate showed a characteristic FTIR spectrum. As per the data obtained the characteristic peaks are visible at 1523.82 cm^{-1} for wheat bran and 1522.85 cm^{-1} for broken rice, it can be assumed that the pigment contain 9-conjugated compound.

Yellow pigment stability was found to be variable at different temperatures under study. While at 30 °C only 4% decrease in color intensity was observed after 2 hours, 40°C resulted in 8%, decrease and at 80°C and pH 6.0, 17% of color intensity was lost. These results showed that the oreovactaene pigment produced by *E.nigrum* is heat sensitive and changes in color intensity should be expected in heat processed products. After 180 min at 80°C yellow pigments maintained 82 and 76% of the initial color at pH 6 and pH 8, while pH 4 resulted in a 65% decrease in color intensity at 80°C Autoclaving resulted in 69% decay and exposure of pigment to sunlight for 2 hours showed 1% decay. The half-life period for pigment at different temperatures varied from 82.5-5.25 h. The decimal reduction time decreased from 275 h to 17.5 h with increase in temperature. Thermodynamic parameters for pigment decay at pH 6.0 were represented in terms of enthalpy ΔH , activation energy E_a , free energy ΔG and ΔS . The values observed were 44.52 to 44.93 kJ/mol, 48.48 kJ/mol, 96.60 to 105.18 kJ/mol and -170.50 to -171.85 J/mol/K respectively.

All these parameters help in predicting the quality changes in terms of appearance during thermal processing and optimizing the process.

At 20 mg/ml, scavenging abilities of BHT on DPPH radicals was 86.32% while the methanolic extracts, MFBR and MFWB exhibited scavenging abilities of 78.74 and 77.46% respectively. On the other hand ethanolic extracts EFBR and EFWB showed a moderate scavenging ability of 53.22 and 49.5%. It was found that methanol was better solvent to extract the compounds contributing to DPPH activity. On the basis of the results obtained, fermented wheat bran and broken rice were superior to unfermented counterpart in most antioxidant properties due to the results of increased phenolic content. Accordingly, fermented mass as well as the pigment extract might be potential antioxidants for use in food products.

The pigment extract showed inhibitory effect against only three strains viz. *Streptococcus pyogenes* MTCC442, *Staphylococcus aureus* MTCC96 and *Candida albicans* MTCC227.

It can be concluded from the present study that *E. nigrum* can be successfully used for the production of yellow pigment using agro industrial waste. The crude yellow pigment exhibits antioxidant and antimicrobial properties. The pigment can be extracted with water and has stability at pH near to neutrality and would be suitable for low temperature processed foods.

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