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Research Paper

OPTIMIZATION OF PIGMENT PRODUCTION BY *Kocuria flava* MAJOD ISOLATED FROM GARDEN SOIL

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Abstract

In recent years, the utilization of natural pigments in food products, dyes, cosmetics & pharmaceutical manufacturing processes has increased dramatically. In order to meet the increasing demand for natural sources, pigment-producing microorganisms can be exploited at the industrial level. The current study was carried out with an objective to isolate and characterize pigment producing organisms from rhizosphere soil samples collected from different areas in Mumbai. In our study, nineteen pigment-producing bacteria were isolated on sterile nutrient agar plates, and extraction of these pigments from overnight grown cell-mass was done using methanol. The quantitative analysis of pigment production was carried out with the help of UV-Visible Spectrophotometer (at λ_{max}445nm). Among the test isolates, *Kocuria flava* majod which was identified using 16S rRNA gene sequencing analysis was found to be the most promising. The optimization studies revealed that maximum pigment production by *K. flava* majod can be achieved in nutrient broth (pH7) medium supplement with sucrose (0.8%) and urea (1%) when it is grown at 30˚C for 48h in the presence of light under shaker conditions (120rpm). In addition, a 1% inoculum size adjusted to the optical density of 0.7 measured spectrophotometrically at 540nm was found to be optimum for pigment production. Partial characterization of the extracted pigment using paper chromatography and Fourier-Transform Infrared Spectroscopy analysis revealed it to be a carotenoid. The pigment also showed good antioxidant activity evaluated by 2,2-diphenyl-1-picrylhydrazyl assay, and antibacterial activity against various laboratory cultures.

Key words: *Pigment, Fourier-Transform Infrared Spectroscopy, 16S rRNA, Kocuria flava majod*.

INTRODUCTION

Colors provide an attractive outlook for any product, which in-turn attracts consumers increasing its demand at an industrial level. These colors may be synthetic dyes or organic pigments utilized in various food, textile or pharmaceutical industries. Of lately, the increasing awareness about the harmful effects of synthetic colors and dyes on health and environment has shifted the preferences of manufacturers and consumers towards organic sources for pigment production [1].

In general, pigments may be produced organically by living organisms such as plants and microorganisms, or they can be inorganic in nature either found freely in the environment or reproduced by chemical synthesis in laboratories [2]. Although, organic pigments were known, and used as colorants since prehistoric times; the synthetic dyes eventually captured the modern market due to its ease of production, lower capital investment and superior coloring properties. Moreover, they do not impart flavor to food products and very small amounts are required for coloring process [3]. Considering these advantages, negligible studies were carried out in the past regarding the safety of synthetic colors and dyes on health. As a result, more and more profit-seeking industries entered the market, over the years affecting the natural environment. It is only in the past few decades that the toxic nature of these dyes and colorants are studied in detail and the pollution caused by them, in addition to its harmful effects like carcinogenic or mutagenic properties, are reported.

The increasing awareness and health consciousness among common people have instigated manufacturers to present them with safer yet attractive alternatives in terms of colorants. The primary source of pigments used until recently was various parts of plants viz., leaves, fruits, vegetables and flowers. However, they suffer from many drawbacks such as un-availability throughout the year, pigment un-stability and solubility. Moreover, the commercial use of plants for pigment production may lead to loss of valuable species among them. It is for this reason that, microorganisms such as fungi and bacteria are studied as an alternate source of naturally derived pigments [3]. Micro-organisms can easily surpass the drawbacks presented by plant pigments since they can grow rapidly, independent of weather conditions, in low-cost nutrient medium to produce controllable and predictable yield. Moreover, microbial pigments are generally primary or secondary metabolites having medicinal or nutritional properties that can act as sources of vitamin, minerals, anti-oxidants etc [4]. Examples of bacterial pigments include light harvesting cell organelle chlorophyll, flavonoids, betalain, melanin and carotenoids [5, 6]. Among these, carotenoids represent the largest and most diverse class of natural colored compounds with over 700 structures identified in plants, algae, fungi and bacteria [6].

Today, microbial carotenoids are exploited as the major source for pigment production owing to its availability. It also confers several biological properties which are advantageous to humans. They act as provitamin A as well as a chemoprotective agent

in biological systems [7]. In addition, they also show anti-oxidant and antimicrobial properties [3, 8].

Considering the scope of microbial-derived pigments, the current study was carried out with an aim to isolate pigment-producing bacteria from soil, optimize the physicochemical parameters required to scale-up its production and identify the type of pigment produced. Various applications of the pigment produced viz., the anti-oxidant, anti-bacterial and dyeing properties were also studied.

MATERIALS AND METHODS

Chemicals and media used in our study

The nutrient medium used for screening, isolation and optimization studies were purchased from Hi-media Laboratories. The chemicals and organic solvents used in our study were of highest purity and analytical grade, and purchased from Difco laboratories Ltd.

Screening and isolation of pigment-producing bacteria

Twenty rhizosphere soil samples were collected from various locations in Mumbai city for screening of pigment-producing organisms. One gram of these samples was suspended in 10mL of sterile phosphate buffered saline, vortexed, and the sediments were allowed to settle for 10mins. The supernatant was then serially diluted ten-fold using sterile phosphate buffered saline, and 0.1mL aliquots from 10^{-1}, 10^{-2} and 10^{-3} tubes were plated on sterile Nutrient Agar (NA) medium supplemented with 1% glycerol. These plates were incubated at 30°C for 24-48h and observed for the appearance of isolated and pigmented colonies. The pigmented colonies were further isolated on NA plates to ensure its purity, and colony characteristics of the pure isolates were studied. It was maintained on NA slants containing 1% glycerol at 4°C until further use.

Extraction of pigments and determination of its absorption spectra

A 1% culture suspension of pigment-producing isolates, adjusted spectrophotometrically to an optical density of 0.8 at 540nm, was inoculated in a sterile Nutrient Broth (NB) supplemented with 1% glycerol and incubated at 30°C for 3days. The cells were harvested by centrifugation at 5,500rpm for 20mins. The obtained pellet was washed with sterile saline and centrifuged at 5000rpm for 15mins. The pigments were extracted from the cell-mass by mixing it with 5mL ethanol and vortexing it for 1-2mins. It was again centrifuged at 5000rpm for 15mins. The pellet thus obtained was incubated in a water bath at 60°C for 15mins until all visible pigments were extracted. The resulting supernatant was analyzed by UV spectrophotometer to determine the absorption spectrum of the extracted pigment which was measured in the UV-Visible region between the wavelength 200-800nm [9, 10].

Identification of potential pigment-producing bacteria

Identification of the most promising isolate was done on the basis of morphological, cultural and biochemical tests using Vitek 2C and the strains were confirmed by 16S rRNA gene sequencing analysis which was carried out by Sai Biosystems Pvt Ltd, Nagpur, Mumbai.

Optimization of various physicochemical parameters for pigment production

For carrying out optimization studies, a 1% culture suspension, adjusted spectrophotometrically to an optical density of 0.8 at 540nm, of test isolate was inoculated in 50mL nutrient medium and incubated at 30°C for 48h under shaker conditions. The cells were harvested by centrifugation at 5,500rpm for 20mins and the cell mass was determined. The pigments were extracted using organic solvents and analyzed spectrophotometrically at 445nm (λmax for carotenoids). The optimization studies were carried out by varying one parameter at a time by keeping the others constant. These varying parameters included nutrient media for testing nutritional requirement of the test isolate (NB, Luria Bertani, M9 mineral medium, King's B and Polypeptone yeast extract glucose broth), organic solvents for pigment extraction (methanol, acetone, ethanol, ethyl acetate, chloroform, methyl acetate, acetic acid; and 1:1 mixture of methanol: acetic acid, methanol: ethanol and ethanol: acetic acid), aeration (i.e., static or shaker conditions), incubation time (24h, 48h, 72h, 96h, 120h), pH (4-10), temperature (4°C, 30°C, 37°C, 45°C and 55°C), inoculum size (0.5%, 1%, 2%, 3%, 4% and 5%), culture density (0.2-0.9 $O.D_{540nm}$) and effect of light and dark conditions on its growth [7, 11-17].

Optimization of nutrient sources

In addition to above mentioned physicochemical parameters, the effect of various carbon sources (1% maltose, mannitol, glucose, sucrose, sorbitol, xylose, galactose, glycerol and fructose), organic and inorganic nitrogen sources (potassium nitrate, tri-ammonium citrate, sodium nitrate, beef extract, yeast extract, tryptone, urea, ammonium chloride, ammonium nitrate, ammonium oxalate and ammonium dihydrogen phosphate) and C/N ratios (1:1, 2:1,3:1, 4:1 and 5:1) on pigment production by the test isolate was also studied [8,18].

Characterization of the pigments

The extracted pigments were characterized by paper chromatography and Fourier Transform Infrared (FTIR) spectroscopy technique.

Paper chromatography

The pigment extracted in methanol was concentrated by evaporating the solvent in an incubator at 55°C. Two mL of this extract was then mixed with 5mL petroleum ether and partitioned with an equal volume of 90% methanol, followed by vigorous shaking in

a separating funnel. A 50µL sample was collected from the epiphase and hypophase thus created after partitioning of the above mixture and used for carrying out paper chromatography. The mobile phase used i.e., methanol: benzene: ethyl acetate (5: 70: 25) was allowed to run until it reached 3/4th of the paper and the chromatogram was analyzed visually for banding patterns [19].

The relative Rf values were calculated as:

$$Rf = \frac{d(sample)}{d(solvent)}$$

Where d(sample) is the distance traveled by the sample and d(solvent) is the distance traveled by the solvent system.

Fourier-transform infrared spectroscopy (FTIR)

The structural analysis of the extracted pigment was done using FTIR analysis, where an infra-red spectrum helps in identifying the presence and position of various functional groups of the pigments. The analysis was carried out at P.S. Ramanathan Advanced Instrumentation Centre, Matunga, Mumbai.

Applications of the extracted pigment

Anti-bacterial activity of the pigment extracted from test isolate

The antimicrobial potential of the extracted pigment was tested against ten laboratory cultures viz., *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pyogens, Salmonella typhi, Proteus vulgaris, Escherichia coli, Staphylococcus aureus* 6538p, *Vibrio cholera, Salmonella paratyphi A* and *Salmonella paratyphi B* using agar well diffusion method. A molten Mueller and Hinton agar butt (20mL) was bulk seeded with 0.1mL culture (0.1O.D$_{540nm}$) and poured in sterile petri-plates. Wells of 6mm diameter was punched after solidification of the medium using a sterile cork borer and 50µL of the extract was added to them. The plates were incubated at 37°C for 24h and zones of inhibition were measured [11].

Anti-oxidant activity of the pigment extracted from test isolate

The antioxidant activity of the isolated pigment was determined using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay. Different concentrations (1-5mg/mL) of the extracted pigment were made using methanol, and 1mL of each dilution was mixed with 3mL freshly prepared methanol solution of DPPH (40µg/mL). This mixture was incubated for 30min in the dark at room temperature (30°C). The extracted pigment solution prepared in 3mL methanol without DPPH was used as blank and a methanol solution of DPPH (devoid of pigment) served as control. The ascorbic acid solution was used as a standard in our study [20].

The absorbance of the solutions was recorded using a UV-VIS spectrophotometer at 445nm and percent (%) inhibition of DPPH radicals was calculated by using the formula:

I (% inhibition) = [(Control – Test)/ Control)] × 100.

Dyeing ability of the pigment extracted from test isolate

In order to determine the dyeing ability of the extracted pigment, a cotton fabric and polyester cloth were cut in 5×5inch dimensions. The cloth pieces were then dipped in the extracted pigment solution for 10min and allowed to air dry in petri plates [4]. It was observed for the intensity of color visible on the fabric after dyeing.

RESULTS AND DISCUSSION

Screening, isolation and identification of pigment-producing bacteria

In our study, 19 organisms were isolated from the 20 rhizosphere soil samples collected from Mumbai. Among these isolates, the one showing the highest intensity of extracted pigment, in the wavelength region of 400-600nm as analyzed by the spectrophotometer, was selected for further studies. The spectral scan of the pigment produced by the most promising organism is represented in Fig.1. It produced dark yellow pigment and showed a λ_{max} at 445nm indicating that the pigment produced may be a carotenoid. The culture was identified as *Kocuria* sp., by cultural, morphological and biochemical analysis carried out using Vitek 2C, and 16s rRNA analysis further confirmed the species as *Kocuria flava* majod. The identified isolate was deposited as *Kocuria flava* majod at National Centre for Biotechnology Information (NCBI) with an accession number LC377273.

Optimization of various physicochemical parameters for pigment production

The results for the optimization of physicochemical parameters for pigment production are represented in Fig. 2-10. The optimized culture condition for pigment production by *K. flava* majod was achieved when it was grown in NB (pH7) medium supplement with sucrose (0.8%) and urea (1%) and incubated at 30˚C for 48h in the presence of light under shaker conditions (120rpm) using 1% inoculum adjusted spectrophotometrically to an optical density of 0.7 at 540nm.

Optimization of media for pigment production

In our study, the maximum pigment production was observed in NB growth medium (Fig. 2), and hence it was used for further optimization parameters. The NB medium contains peptone and meat extract which serves as an organic carbon and nitrogen source respectively, hence providing the organism with the essential growth factors and vitamins which are necessary for their propagation. In a similar study, Micrococcus sp.,

isolated from soil environment was reported to show maximum pigment production in Trypticase Soy Broth medium [21]. In another study, *Streptomyces flavofuscus* ARITM02 was found to produce pigment in starch casein broth, and failed to produce pigments in other test nutrient medium [22].

Screening of organic solvent for maximum pigment extraction

The result for screening of organic solvent for maximum pigment extraction is represented in Fig. 3. It was observed in acetic acid. It has been reported earlier, that the choice of solvent is one of the crucial steps in the process of extraction since it has the ability to separate analytes of interest from a mixture of compounds based on the affinity with the solvent [23]. In similar studies, different solvents like chloroform, acetic acid, ethanol, petroleum ether and ethyl lactate were screened for extraction of pigments from bacterial species [16, 23, 24]. In addition to extraction properties of solvents, a study has also reported the effective use of chemicals such as ethanol and acetic acid as an inducer and/or precursor of carotenoid (astaxanthin) synthesis in *Phaffia rhodozyma* strain. In their study, the addition of 10g/L ethanol or 5g/L acetic acid resulted in a relatively high concentration of astaxanthin i.e., 45.62 mg/L and 43.87 mg/L respectively [16].

Effect of aeration on pigment production

The Fig. 4 represents the effect of aeration on pigment production by *K. flava* majod. In our study, maximum pigment production was obtained at shaker condition, indicating that aeration is an important factor for the same. The *K. flava* majod is an obligatory aerobic organism and is dependent on oxygen for energy and biosynthesis. Presumably, our observed results are a representation of general characteristics of *K. flava* majod. Similar to our findings, maximum pigment (Prodigiosin) production in *Serratia marcescens* was observed under shaker conditions at 27°C in 48h [17]. In contrast, a study has also reported decreased production of pigment in *Bacillus cereus* M^{1}_{16} (MTCC 5521) with an increase in the rate of aeration. In addition, they also reported a reduction in pigment production with an increase in agitator speed during the process of fermentation. Hence, confirming the inhibition of pigment production in presence of oxygen [25].

Effect of incubation period on pigment production

The effect of incubation period on pigment production by *K. flava* majod is presented in Fig. 5. The optimum pigment production was observed within 48h under shaker conditions, after which it was found to decline. Similar to our study, optimum carotenoid production by *Exiguobacterium aurantiacum* FH was observed in 48h, beyond which, a decline in both growth and pigment production was observed [19]. In another study, the non-pathogenic strain of pigment-producing *Pseudomonas fluorescens* was isolated from soil and studied for the production of natural dye. It also showed maximum pigment production in 48h [26].

Effect of pH and temperature on pigment production

The Fig. 6 and 7 represents the effect of pH and temperature on pigment production by *K. flava* majod. The optimum pigment production was observed at pH 7 and a temperature of 30°C. Similar to our findings, studies have reported maximum pigment production at 30ºC and pH7.0 in *Aspergillus carbonicus* as well as a fungal pigment producing strain isolated from spoiled mango viz., *Talaromyces verruculosus* [27, 28]. Another study reported optimum growth at 37°C and pH8.0 for *Micrococcus roseus*, and 35°C, pH7.0 for *Micrococcus luteus* [21]. *Streptomyces flavofuscus* ARITM02 isolated from rhizophere soil also showed optimum pigment production at 35°C, pH7.5 [22]. In a similar study, Streptomyces species were isolated from soil and the cultures were screened for actinorhodin production. The culture showed a narrow range of incubation temperature and pH for relatively good growth and pigment production; where maximum growth, as well as pigment intensity, was observed at 30°C and pH7.6-8.0 [29].

Effect of inoculum size and culture density on pigment production

The optimum inoculum size and culture density for maximum pigment production in *K. flava* majod were determined to be 1% culture suspension adjusted spectrophotometrically to an optical density of 0.7 at 540nm (Fig. 8 and 9 respectively). However, the maximum growth was obtained when NB was inoculated with a 7% inoculum size. In another study, *Monascus purpureus* showed optimum pigment production in ethanol and glucose medium when inoculated with a spore suspension of 10^5 and 10^7spores/mL respectively [30]. A high inoculum size significantly increases the biomass within a short span of time by utilizing all the essential nutrients in the medium, at the same time compromising pigment production. Our findings thus indicate that the production of pigments in bacteria is dependent on extracellular nutritional and/or environmental conditions.

Effect of light on pigment production

In order to elucidate the effect of light on pigment, *K. flava* majod was incubated in light and dark under optimized culture conditions evaluated in our study. The growth, as well as pigment production, was found to be optimum in presence of light (Fig.10). A study carried out to examine the pigments produced by *E. aurantiacum* FH suggested that the color stability of carotenoids is a major issue in bacterial pigments. In their study, a good stability in the produced carotenoids was obtained after exposing it to a temperature of 100°C for 5h, in which case only 7.04% color loss was observed. However, residual color equivalent to 83.24 and 86.27% were recorded after 24h exposure to pH 5 and 9 respectively [19].

Optimization of nutrient sources

The results for the optimization of nutrient sources for pigment production are represented in Fig. 11-15. Optimization of media components has been predicted to play a significant role in enhancing pigment production, and different nutrient sources have different impacts on the same. In our study, the optimum pigment production was observed when NB medium was supplemented with 0.8% sucrose and 1% urea in the ratio of 1:2. Also, the pigment activity was found to decrease with increased concentration of nitrogen sources. This is because *K. flava* majod possess an inducible gene to produce pigments and in presence of readily available nitrogen sources, it prefers to bypass the investment of ATP required to regulate its production. A similar study reported 2% glucose and 2% yeast extract as an optimum carbon and nitrogen source respectively for pigment production in *Fusarium moniliforme* KUMBF1201 [31]. Another study also reported a significant effect of carbon and nitrogen sources on pigment production in UV resistant *Micrococcus roseus* and *Micrococcus luteus* isolated from soil. Optimum production of pigment was observed when the culture medium was supplemented with sucrose and KNO_3 [21]. Similar to our findings, 1% urea was reported to be the optimum source for pigment production in Pseudomonas MCCB 103 and marine bacterium, Rhodospirullum sp. [18].

In our study, the cell growth required relatively high concentrations of carbon and nitrogen sources for carotenoid production, while carotenoid biosynthesis required much lower concentrations of sucrose and urea. A possible explanation for the low optimal nutrient concentrations required may be the synthesis of carotenoids as secondary metabolites, which are produced when the cells are under stress (such as nutrient limitation) and the cell growth (primary metabolism) is suppressed [32].

Characterization of the pigments

Paper chromatography

For separation of various components, carotenoid extracted from *K. flava* majod was mixed with equal volumes of petroleum ether and 90% methanol. It formed two layers viz., upper petroleum ether layer called epiphase and lower methanol layer called hypophase. Structures of carotenes and xanthophylls form the basis of their separation upon partitioning. As xanthophylls do not contain any hydroxyl group it remains in the hypophase while the carotenoid contains two hydroxyl groups are found in epiphase. Also, the carotenoids migrate with the solvent front, while monohydroxylated compounds migrate to an intermediate distance and dihydroxylated compounds remain close to the baseline of the chromatography sheet [33].

In our study, the epiphasic and hypophasic layers were therefore analyzed by paper chromatography, which confirmed the presence of carotenoid. The results revealed a single spot in epiphase with an Rf value of 0.70 (Fig. 16). This value is close to the

findings of Godinho et. al. (2008), who reported the presence of dark yellow spot (Rf value of 0.77) for carotenoids obtained from *Microbacterium arborescens*-AGSB [34].

Fourier-transform infrared spectroscopy (FTIR)

The yellow pigmented bacterial strain of *K. flava* majod gave sharp peaks at 1925.04, 2256.81, 2361.94, 2135.29, 4249.36, and 4333.27 cm^{-1} as depicted in the spectrum scan (Fig. 17). Other peaks were also observed at 1658.85, 1758.19, 2414.02, 2541.32, 2743.86, 4249.39 and 4398.85 cm^{-1}. In the present study, the bands at approximately 1658.85, 1758.19 and 2256.81 cm^{-1} appears due to the bending vibration of methylene –CH2 (scissoring) which is also seen in beta-carotene standard fingerprint peak at 1925.04 cm^{-1} and can be assigned to lycopene pigments, while peak between 1650-4450 cm^{-1} in *S. suarezii* KK6, *K. turfanensis* KK7 and *K. rosea* KK12 are attributed to the β-ionone ring of beta-carotene due to the C-H, (–CH3) symmetrical bending and also observed in standard beta-carotene fingerprint region [6].

Application of the extracted pigments

Anti-bacterial activity of the pigment extracted from test isolate

Our current study was extended to evaluate the antibacterial effect of the carotenoids produced by *K. flava* majod. The pigment extracted in methanol demonstrated bactericidal activity against *Staphylococcus aureus*, *Staphylococcus aureus* 6538p and *Vibrio cholerae*, with zones of inhibition of 14, 15 and 12mm respectively. Present results are consistent with a previous study that showed the inhibitory activity of pigment produced by Micrococcus species on gram-positive bacteria [21]. Also, the pigment produced by *Rhodotorula glutinis* showed effective antibacterial activity against both gram-negative and gram-positive bacteria, with *Bacillus cereus* and *Salmonella enteritidis* showing the lowest and highest sensitivity to this pigment, respectively. In addition, the highest MIC and MBC values were observed against *S. enteritidis* and *E. coli*, respectively [35].

Anti-oxidant activity of the pigment extracted from K. flava majod

The ability of carotenoids to utilize singlet oxygen molecules or reactive oxygen species is well known. In our study, the anti-oxidant assay of carotenoids produced by *K. flava* majod showed the IC50 value to be 3.2 (mg/mL) as represented in Table 1. In another study, the carotenoid pigments isolated from *M. roseus* and *M. luteus* showed significant UV protective nature and antioxidant IC50 value of 3.5-4.5 mg/mL [21]. The carotenoids extracted from *Halococcus morrhuae*, *Halobacterium salinarium* and *Thermus filiformis* were reported to be 0.85µg/mL, 0.84µg/mL and 2.41µg/mL respectively. The difference in IC values was related to the presence of acyclic carotenoids with both large numbers of conjugated double bonds and of hydroxyl groups in the major carotenoid of the halophilic microorganisms [36].

Dyeing ability of the pigment extracted from test isolate

The pigment extracted from *K. flava* majod was assessed for its dyeing ability, and it was observed that only the cotton fabric showed notable coloration, whereas polyester fabrics retained very light color (Fig. 18). In another study, the highest concentration of pigment in media was obtained by boiling the mycelia of *Talaromyces verruculosus* and using ferrous sulfate as a mordant [28]. It is suggested that natural dyes/pigments perform better in presence of a mordant which helps in binding the colored molecules to the fabric [37].

CONCLUSION

The isolation and optimization of carotenoid pigment produced by *K. flava* majod were successfully carried out in our study, and it showed effective production of pigments at neutral pH and room temperature indicating its possible exploitation at the industrial level. Although the dyeing ability for polyester fabric material was found to be weak in our study, it can be further improved by use of mordant, and the pigment can be readily used for dyeing cotton fabrics.

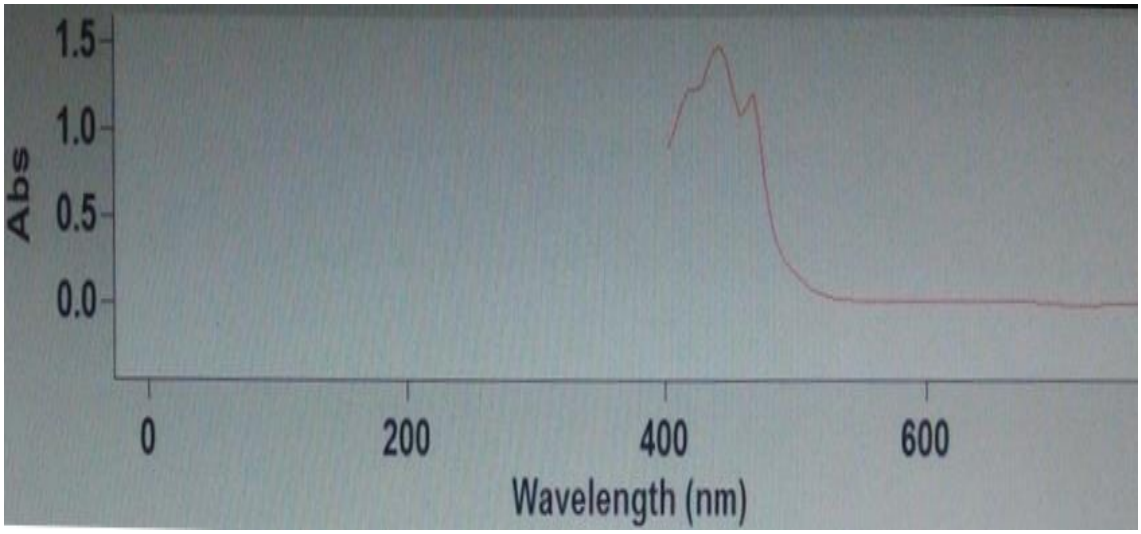

Fig. 1: Spectrum scan (λmax 445nm) of the pigment produced by *Kocuria flava* majod

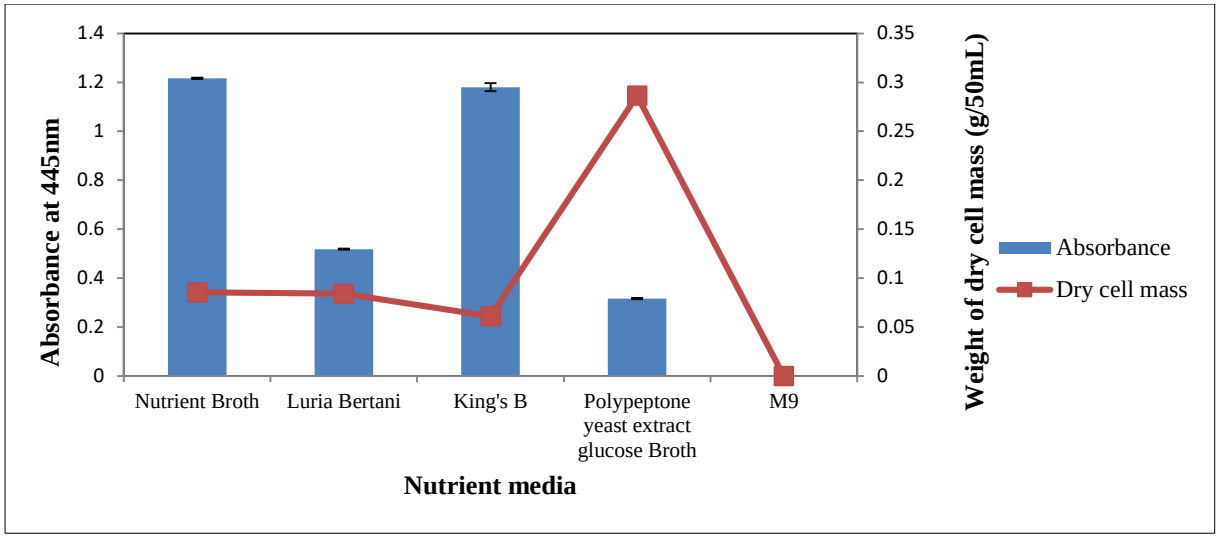

Fig. 2: Optimization of media for pigment production by *K. flava* majod

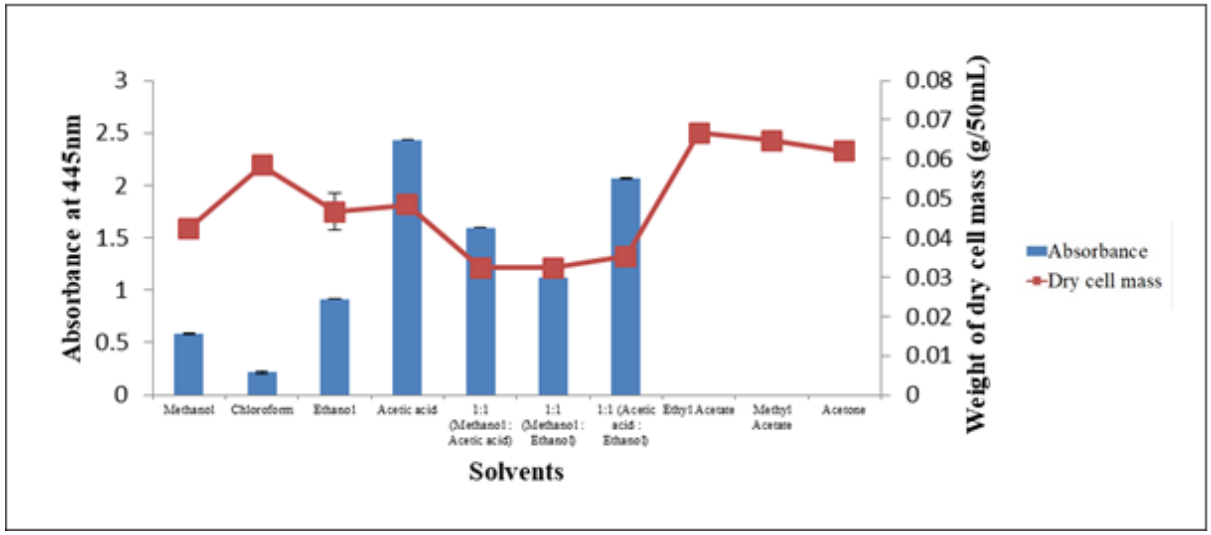

Fig. 3: Screening of organic solvent for maximum pigment extraction

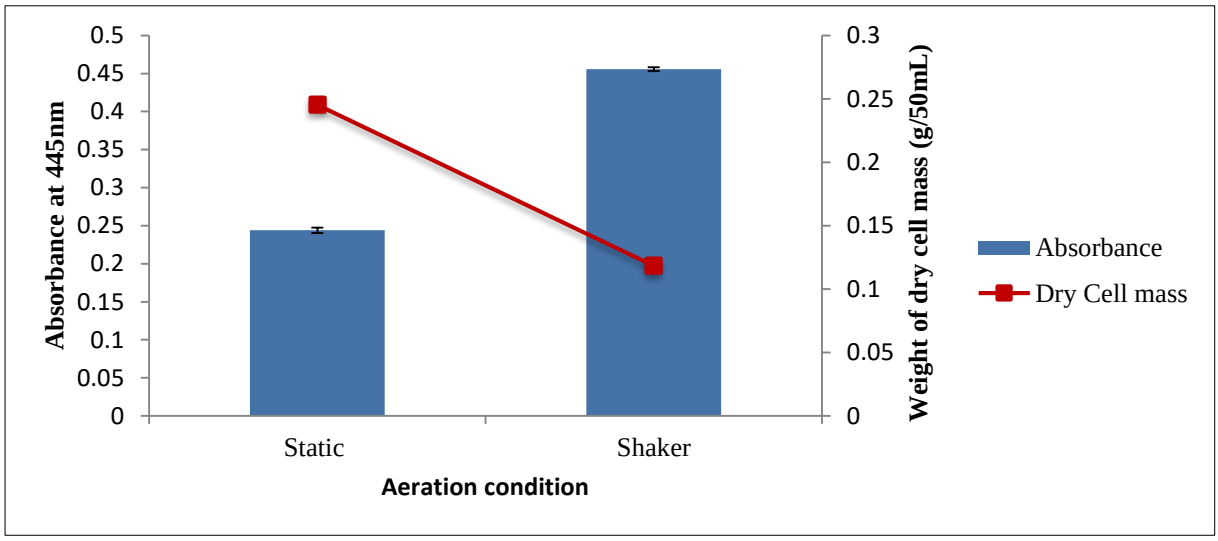

Fig. 4: Effect of aeration on pigment production by *K. flava* majod

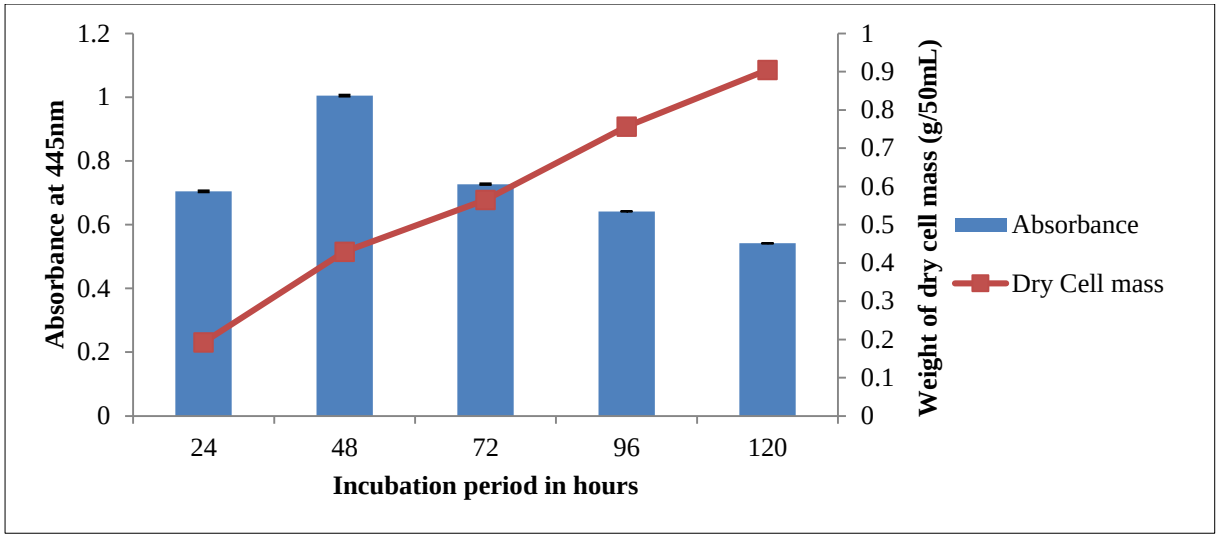

Fig. 5: Effect of incubation period on pigment production by *K. flava* majod

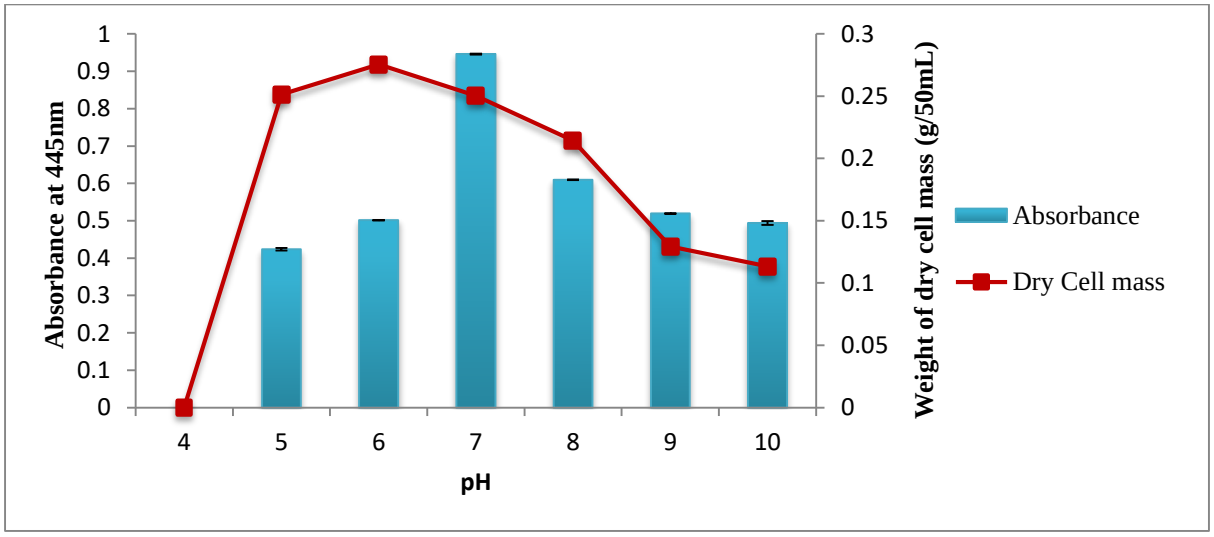

Fig. 6: Effect of pH on pigment production by *K. flava* majod

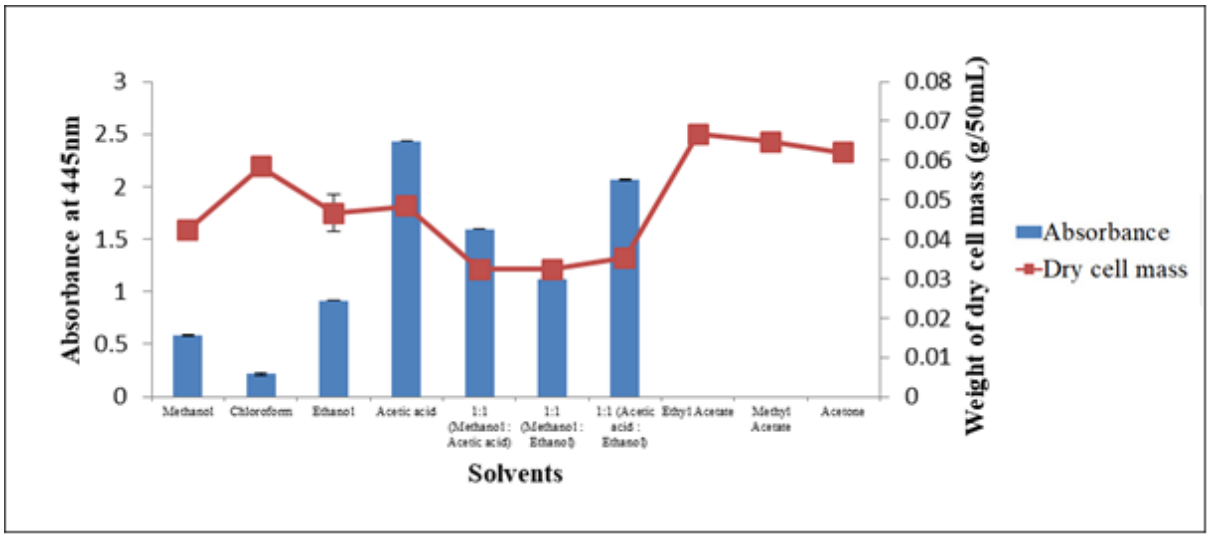

Fig. 7: Effect of temperature on pigment production by *K. flava* majod

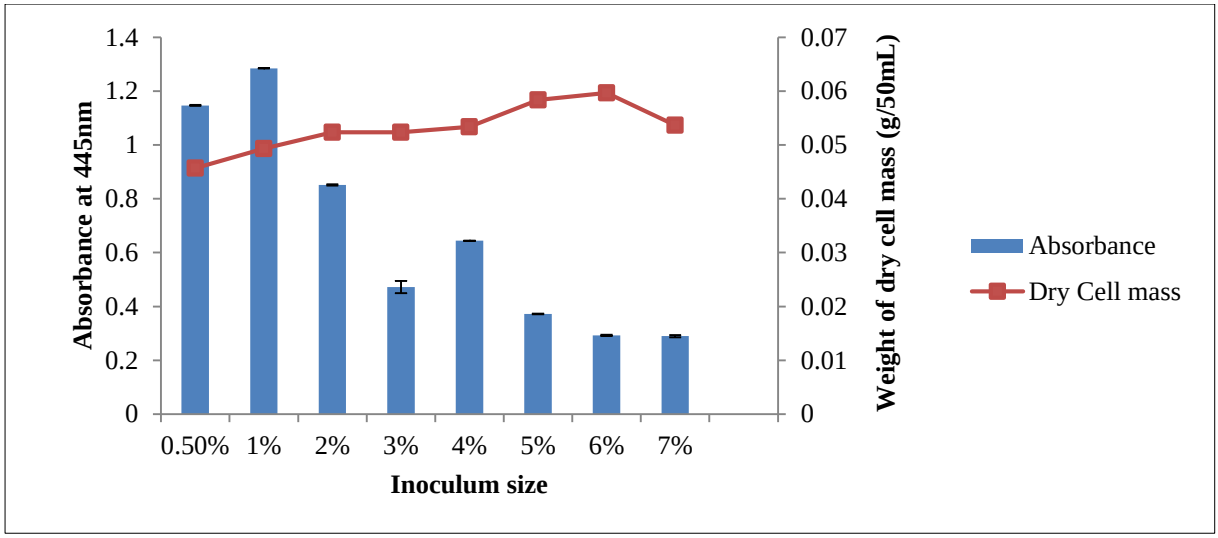

Fig. 8: Effect of inoculum size on pigment production by *K. flava* majod

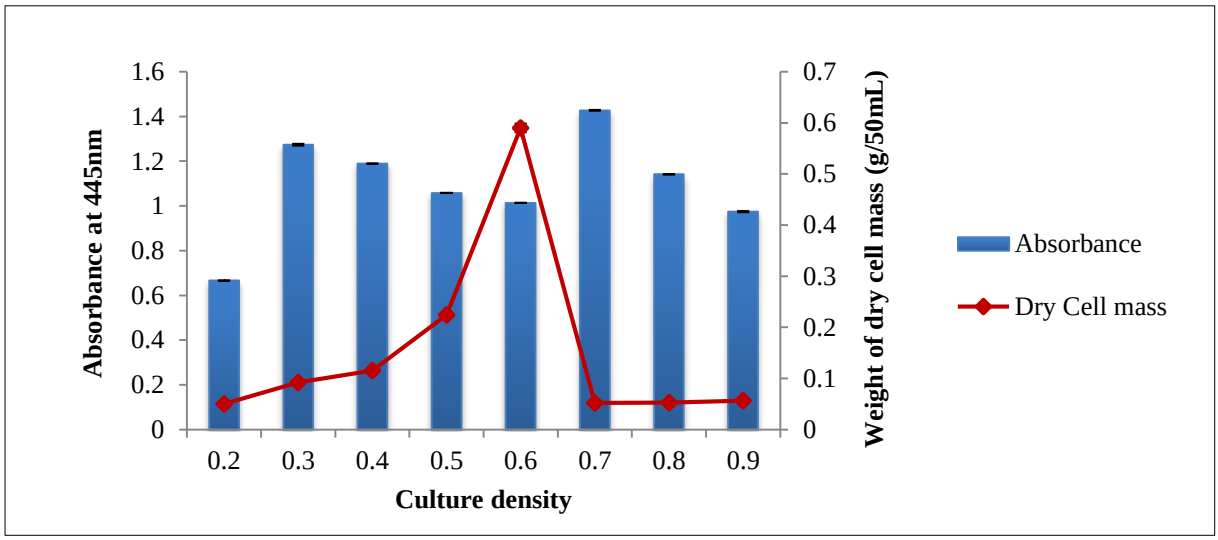

Fig. 9: Effect of culture density on pigment production by *K. flava* majod

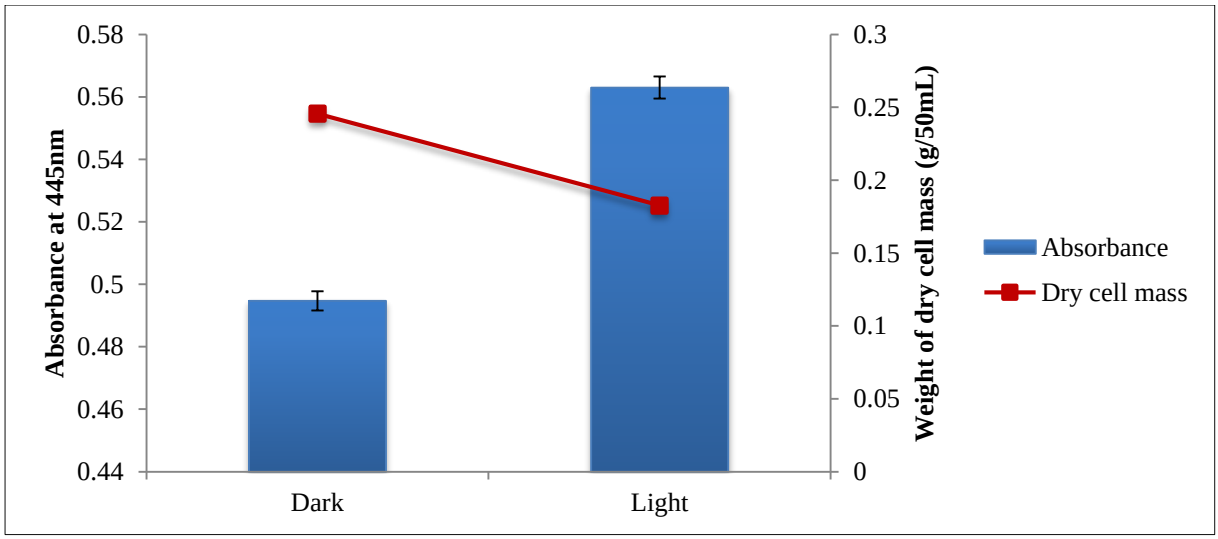

Fig. 10: Effect of light and dark condition on pigment production by *K. flava* majod

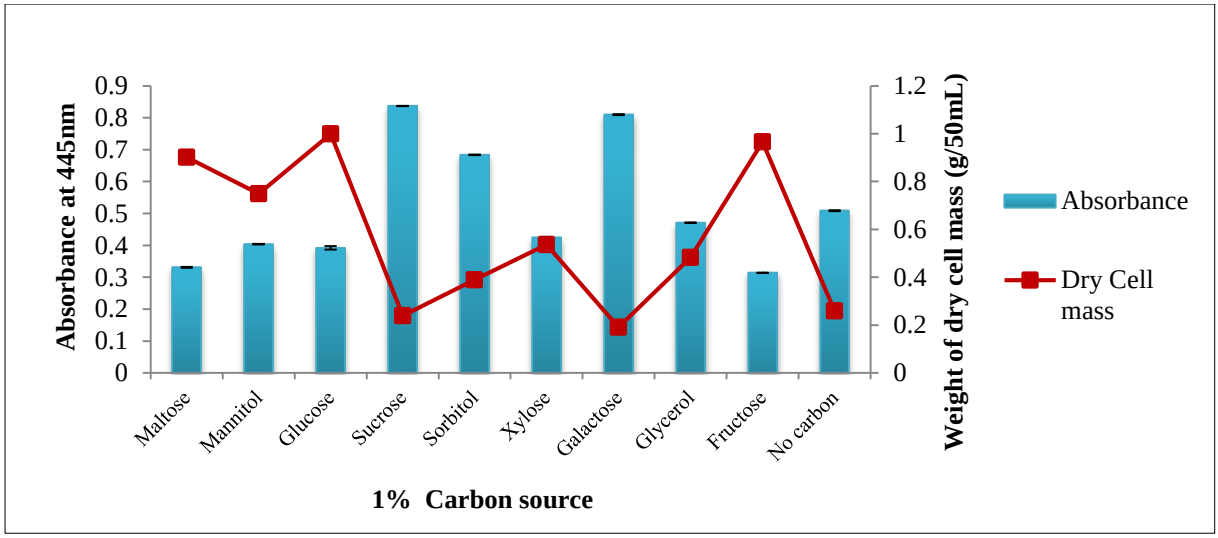

Fig. 11: Optimization of carbon sources for pigment production by *K. flava* majod

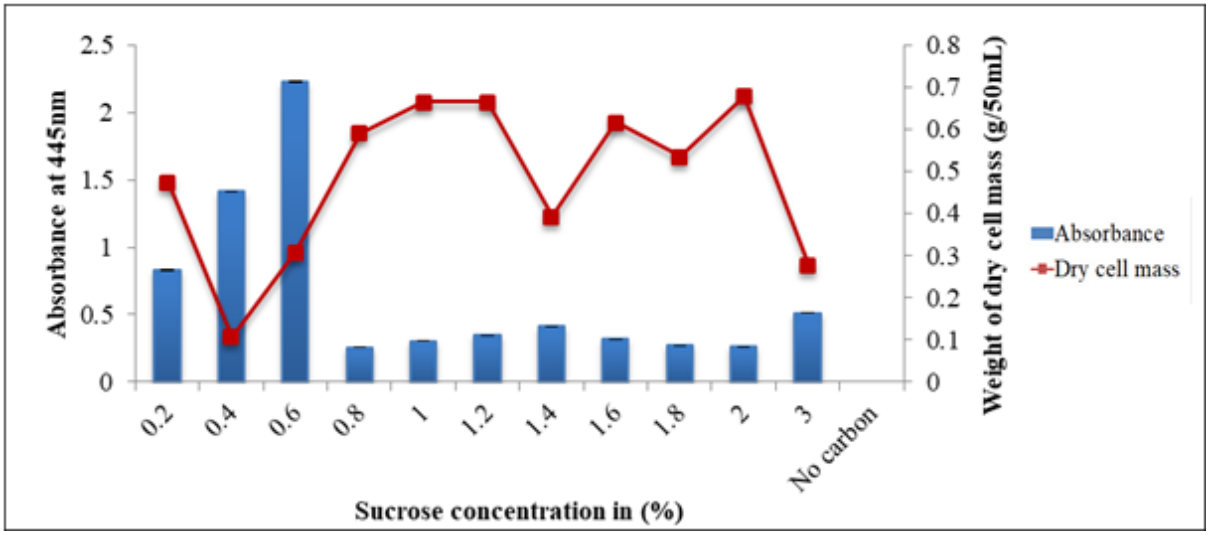

Fig. 12: Optimization of Sucrose concentration for pigment production by *K. flava* majod

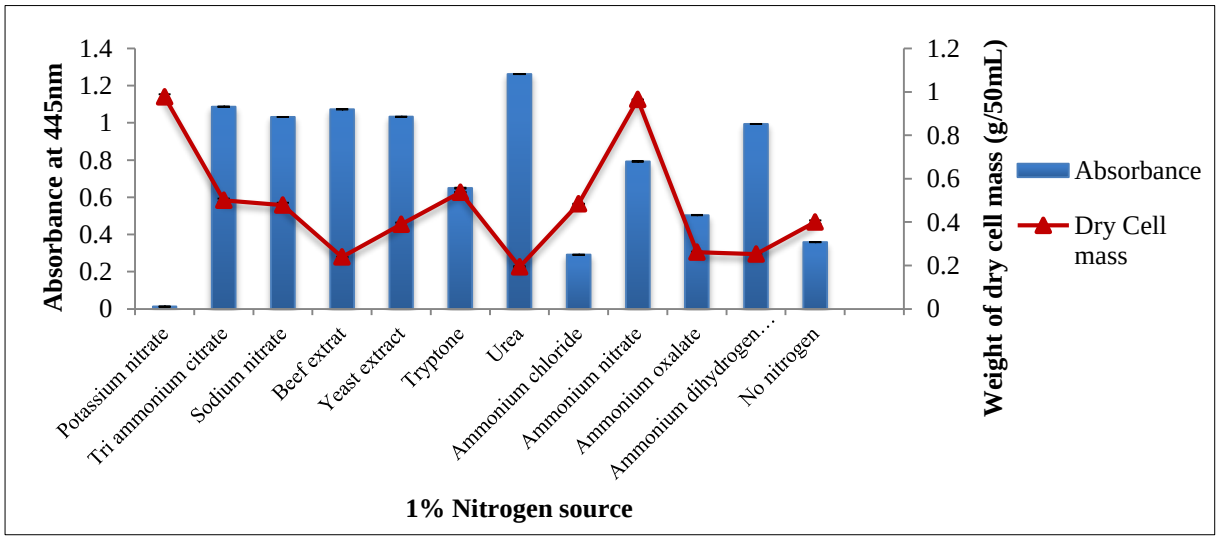

Fig. 13: Optimization of Nitrogen sources for pigment production by *K. flava* majod

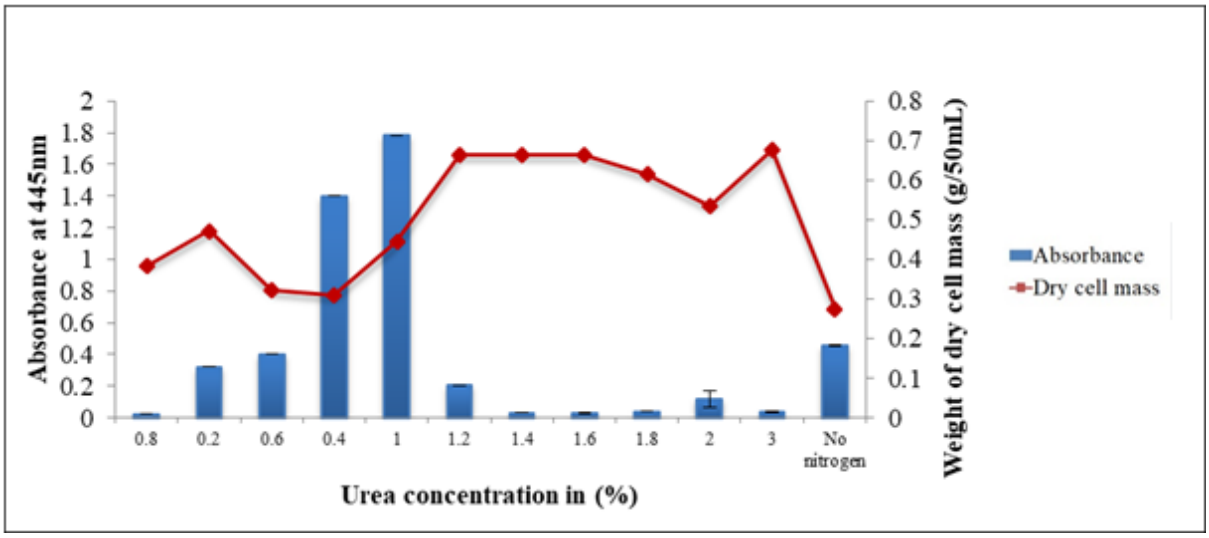

Fig. 14: Optimization of Urea concentration for pigment production by *K. flava* majod

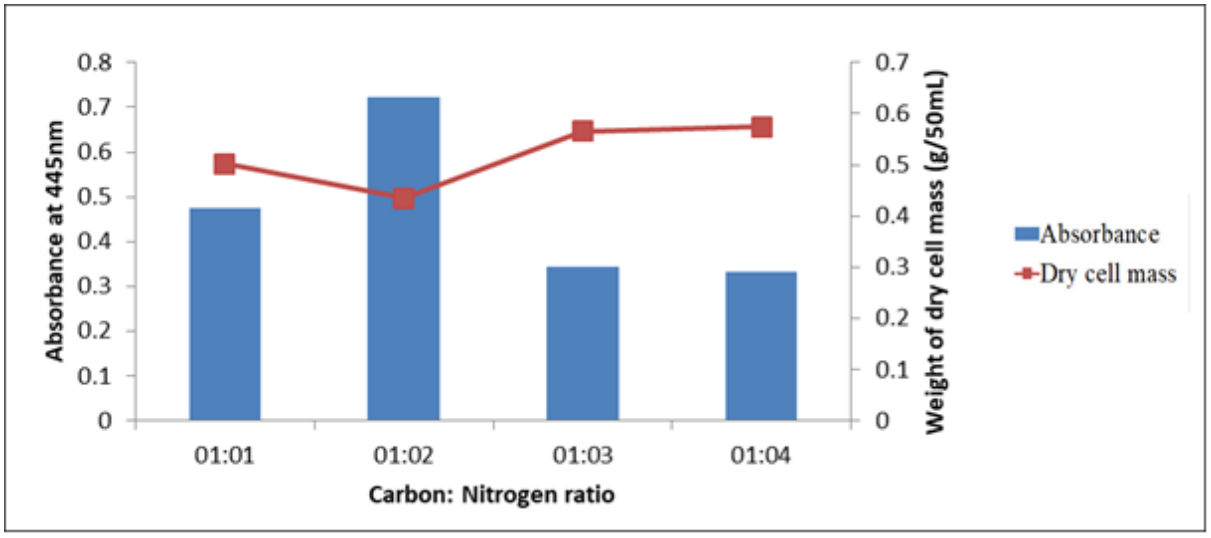

Fig. 15: Optimization of Carbon and nitrogen ratio for pigment production by *K. flava* majod

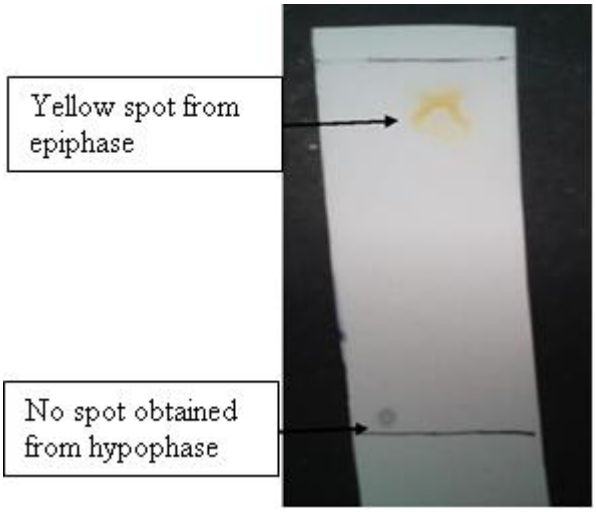

Fig. 16: Paper chromatography of pigment produced by *K. flava* majod

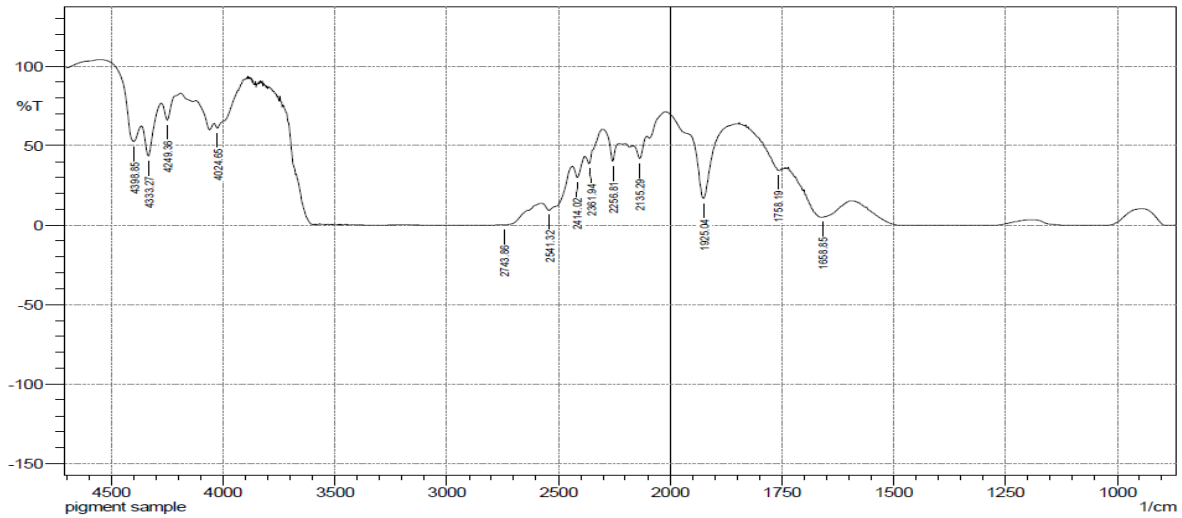

Fig. 17: FTIR spectral scan of pigment produced by *K. flava* majod

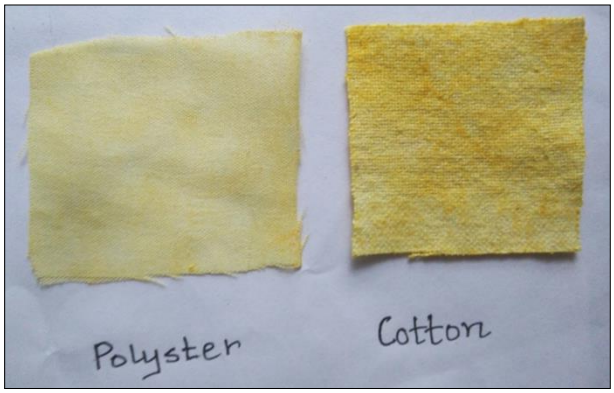

Fig. 18: Dyeing ability of the pigment extracted from *K. flava* majod

Table 1: DPPH assay for determining the antioxidant activity of the pigment extracted from *K. flava* majod

Carotenoid concentration (mg/mL)	% DPPH (radical scavenging activity)
2	18.11111
4	21.22222
6	25.22222
8	24.77778
10	23.11111
20	22.88889
30	25.55556
40	30.33333
50	31.77778
Control	0
Standard (Ascorbic Acid)	80
IC50 Value	3.2 (mg/mL)

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