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Optimization of Organic Solvents for the Extraction and Purification of Yellow Color Pigment from the Isolate AM2 and its Characterization as a Potent Anti-Oxidant.

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Abstract

Microorganisms are widely used for obtaining various natural products over last few years. Among the various sources of many industrially important products, there has been an increasing trend towards the isolation and purification of pigment producing microorganisms as a replacement of synthetic colorants. Natural pigments and synthetic dyes have been extensively used in various fields such as food production, textile industries, paper production, agricultural practices and researches. In the present study pigment producing microorganisms were isolated from various sources and yellow pigment producing organism was further experimented and studied. Pigment was extracted using different solvents such as 95% v/v methanol, 99.5% Acetone, Acidified ethanol, 75% v/v Methanol:Acetone, 85% v/v Methanol:Acetone, 95% v/v Methanol:Acetone in which 95% v/v Methanol gave maximum extraction (42 mg/100 ml) of the pigment. The hydrogen peroxide scavenging activity obtained was 1 mg/ml of pigment solution with comparison to Ascorbic acid

Key words : Yellow pigment, Extraction, organic solvents, Antioxidants

Introduction

Colors have a strong impact on every creation of life, including the clothes we wear and the food we eat. These colors are divided into two categories 1) natural colors 2) synthetic colors *(*Manikprabhu and lingappa., 2013). Natural colors are pigments, which are produced by living organisms such as Actinomycetes, Fungi, Yeast and bacteria. Synthetic colors have been proved to be toxic and dangerous to mankind (Aberoumond A., 2011). The main source of natural pigments are plants and microorganisms. The most common plant pigments are carotenoids, chlorophylls and betalains. The usage of plant pigments have many limitations due to its non availability throughout the year, its stability and solubility and the large scale pigment production which may lead to the loss of the species. The fungal and bacterial pigments are becoming an alternative source of naturally derived pigments (Arulselvi *et al*.,2013). Pigments have been extensively used in food

production, fish industries, textile industries, paper production, agricultural practices and other technology and also having biological activities as antioxidants (are the molecules which are having capacity to quench the free radical) and anticancer agents (Khanafari *et al.,*2006).

Materials and method

Screening and isolation of pigment producing microorganisms

Soil samples were collected from various areas in and around Navsari city. All the samples were diluted and plated on Nutrient agar medium and incubated at room temperature to obtain isolated colonies. The pigment producing bacterial colonies were selected for further isolation and study.

Morphological and cultural characterization of pigmented isolates

All the pigmented isolates were categorized on the basis of gram reaction and further characterized morphologically by microscopic observation and cultural characteristics exhibited by the isolated colonies on nutrient agar plate after incubation.

Production of pigment into production media

For the production of pigment 10% inoculum of bacterial isolate AM2 was inoculated into sterile production media (peptone 10.0gm, meat extract 3.0 gm, NaCl -5.0 gm, D/W 1000.0 ml, pH 7.4) containing flask and incubated at room temperature on shaker at 120 rpm

Optimization of organic solvent for the pigment extraction

After the production of yellow pigment, the broth was centrifuged at 10,000 rpm for 20 min, the cell pellet were collected and 3 ml of the different organic solvents like 95 % v/v Methanol, 85% v/v Methanol:Acetone, 75% Methanol :Acetone, Acidified Ethanol (Vora *et al.,* 2014), 99.5 % Acetone (Elkenawey *et al*., 2017) were added for the extraction of pigment. The pigmented solvent was kept in water bath at 60°C for 20 min and centrifuged at 10,000 rpm for 5 min. After incubation supernatant was collected into pre weighted crucible and allowed to dry on hot plate at 60°C for further extraction. Purification of the extracted pigment was carried out by using chloroform (Arulselvi P. *et al*., 2013).

Pigment characterization by TLC (Butanariu, M., 2016)

The purified pigment was analyzed by thin layer chromatography with silica gel. The solvent system consisted of Benzene:Ethyl acetate (2:1;v/v). The chromatography chamber with the solvent was kept for 20 min for the equilibration. The sample was spotted on the silica gel sheet using a capillary tube and air dried. The TLC sheet was then dipped in the solvent system. After 45 min TLC sheet was carefully removed and the retention factor (R_f) value was calculated according to the following equation from chromatogram.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by solvent}}$$

Hydrogen peroxide scavenging activity

Hydroxyl radical activity was measured by the salisylic acid method. Powdered pigment was dissolved in methanol for making of 1.0 mg/ml solution, and aliquots were prepared viz.

0.1ml, 0.2ml, 0.4 ml, 0.6ml, 0.8ml and 1.0 ml, the same method was used for Ascorbic acid with the same aliquot. The final volume was made up to 1.0 ml by distilled water. The following addition of 1.0 ml of 9 mMol/L salisylic acid, 1.0 ml of 9 mMol/L Ferrous sulphate ($FeSO_4$), 1.0 ml of 9 mMol/L H_2O_2 was carried out. The reaction was activated by adding H_2O_2 and the reaction mixture was incubated for 60 min at 37°C in a water bath. After incubation, the absorbance of the mixture was measured at 510 nm using UV-Vis spectrophotometer. The control tube did not contain pigment solution, the self tube did not contain H_2O_2 and the blank tube did not contain pigment solution and salisylic acid, Ascorbic acid was used as positive control compound (Geng,M. *et al*., 2012).

Hydroxyl radical scavenging activity was calculated as per the following equation.

$$Sa\% = 1 \left[\frac{-Asample - Aself - Ablank}{Acontrol} \right] X \quad 100$$

Where Sa% = scavenging activity of tested sample (%), A_{sample} = Absorbance of tested sample, A_{blank} = Absorbance of the blank, A_{self} = Absorbance of selves, $A_{control}$ = Absorbance of the control.

Results and Discussion

Among the isolates obtained from the various samples, 10 isolates showed characteristic pigments of varied coloration. Out of the 10 pigmented isolates, AM2 showed a characteristic yellow pigment which was selected for further study. The isolate AM2 was categorized as gram positive cocci shaped organisms occurring singly and in clusters with a characteristic colonial characteristics of medium sized, circular shaped with smooth surface and entire margin, the colonies showed a convex elevation

Optimization of solvent extraction

By using 6 different organic solvents, maximum extraction of yellow pigment (42mg/100ml) was observed in 95% v/v Methanol as compared to the other solvents i.e 75% v/v methanol:Acetone, 85% v/v Methanol:Acetone, 95% v/v Methanol:Acetone, Acidified Ethanol, and 99.5% Acetone (Table1).

Sr. No.	Organic solvents	Extraction of pigment
1	95 % v/v Methanol	42 mg
2	95% v/v Methanol : Acetone	22 mg
3	85 % v/v Methanol : Acetone	16 mg
4	75 % v/v Methanol : Acetone	NIL
5	Acidified Ethanol	NIL
6	99.5% v/v Acetone.	NIL

Table 1: Quantification of extracted pigments with different organic solvent

The highest amount of pigment was extracted in methanol solvent at concentration of 95% v/v while no extraction was observed in ethanol as well as acetone (Fig 1).

Fig 1: Pigment extraction in different solvent system

Characterization of extracted pigment

Thin layer chromatography was performed of the extracted pigment (Fig 2a) using solvent system consisted of Benzene:Ethyl acetate (2:1 v/v). The calculated Rf value of the bacterial pigment was observed to be 0.66 (Fig 2b) which is similar to standard carotenoid Rf value (Vora J.U.,*et al*,2014) indicative of carotenoid nature of the pigment.

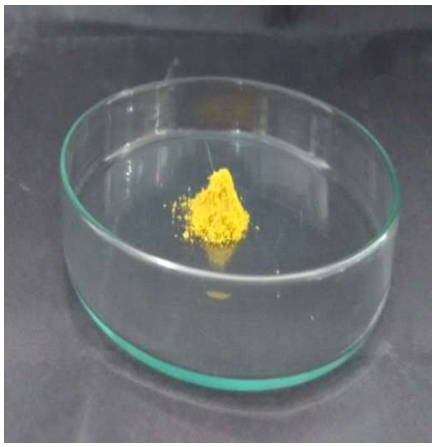


Fig 2a Purified yellow coloured pigment

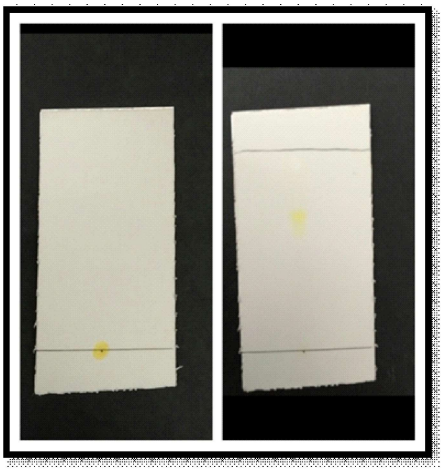
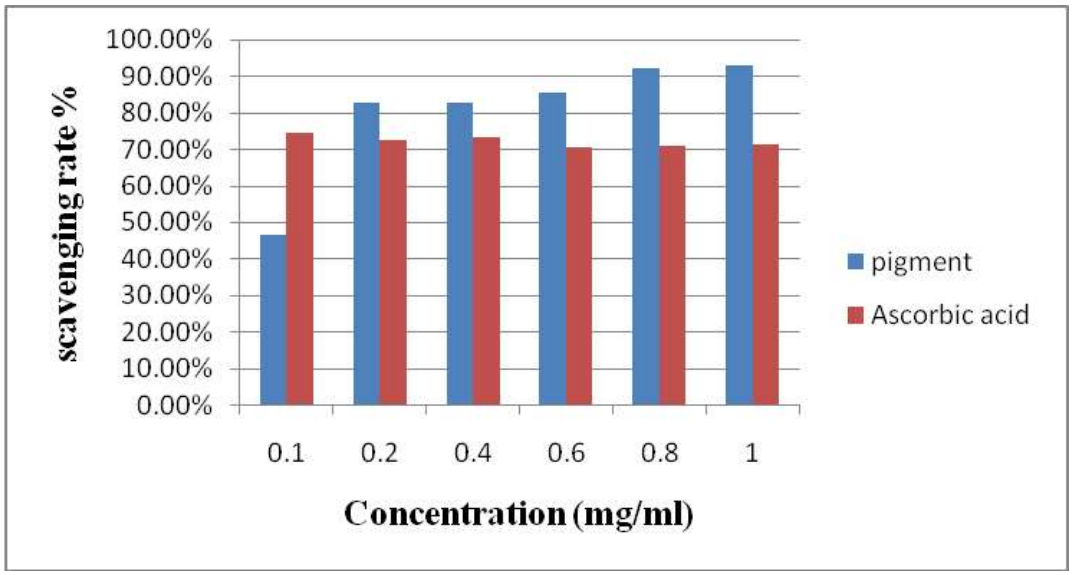


Fig 2b TLC of extracted pigment

Anti-scavenging activity

Hydroxyl radical scavenging activity was measured by salicylic acid method. Hydroxyl radicals the most active in the reactive oxygen species , and it cause damage to the organism. Hydroxyl radicals can react with the variety of molecules such as proteins, polypeptides, to cause bimolecular oxidative damages and cell necrosis or mutations.



Bacterial AM2 pigment had a scavenging activity toward hydroxyl radicals in a dose dependant manner. The scavenging activity of pigment was obviously lower than that of the ascorbic acid when concentration was below 0.2, But the scavenging activity was relatively higher than that of the ascorbic acid when the concentration was over 0.2 which indicates the pigment to have a potent anti-oxidant activity.

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