



Isolation of pigment producing bacteria from rabbit urine

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Abstract

Bacteria produce pigment for various reasons and some bacteria like *Staphylococcus aureus* produces Carotenoid pigment called Staphyloxanthin, which give it a characteristic golden color. Staphyloxanthins a triterpenoid carotenoid, which shows antioxidant properties. This bacterium mostly found in rabbit urine. Normal rabbit urine ranges in color from light yellow to a deep orange-red due to plant pigments or pigments called porphyrins produced by the bladder. Some specimens of rabbit urine yield more than two different isolates, and staphylococci are the most frequently isolated bacteria. Oxalate and uric acid crystals were seen in 6% of the samples, particularly those with significant growths of *Pseudomonas aeruginosa*. Rabbit urine has tremendous application in agriculture it has shown to possess inhibitory activity against bacteria. The chemical element present in rabbit urine are Nitrogen, Phosphorus, Potassium, Calcium, Calcium carbonate, Triple phosphate, Calcium oxalate, Methionine, etc. Rabbit urine is considered an excellent fertilizer because of its chemical composition. It is also an effective organic manure for vegetables and other arable crops. Rabbit urine contains six times most useful elements found in another livestock's waste. The isolation of pigment producing bacteria was done by streak or spread plate method. The Mannitol salt agar medium was used for the isolation procedure. From the isolated culture, various preliminary test, biochemical test and antimicrobial activity were performed. Antioxidant properties can be determined by hydrogen peroxide scavenging activity. High cost of using synthetic medium there is need to develop new low-cost process for the production of pigment as well as for the isolation procedure. The pigments produced by bacteria can be isolated using Ethanol based extraction method. These pigments can be purified and characterized using analytical techniques like TLC. No antibacterial activity of Staphyloxanthin (STX) was detected against the bacteria like *E. coli*, *Salmonella spp.*, *Klebsiella spp.*, *Pseudomonas putida*. Hydrogen peroxide scavenging activity assay was performed to check STX pigment activity.

Keywords: Rabbit urine, *Staphylococcus aureus*, Staphyloxanthin, Antioxidant Test, Tlc

Introduction

Rabbit urine can range in color from pale yellow to rusty red, depending on factor like genetic diets and hydration. Normal rabbit urine is always watery and not thick.

Rabbit can eat variety of plant that contains pigment that can affect the color of their urine. For example, eating carrots and spinach and other veggie contains beta-carotene can cause red urine. when the female rabbit is pregnant it will give clean urine. The red, orange color is believed to be due in part to harmless plant pigment called Porphyrins, it is bladder produce pigment called that can also affect the color of rabbit urine. The foods that have been reported to elicit the greatest change in urine color includes Broccoli, Cabbage, Dandelion, and Carrots.

The continue universal application of synthetic colorants decade that have caused human health vulnerability and environmental pollution. So, it was important to discover novel natural color such as microbial colorants which were safer and better than synthetic colorants [1]. The potential of bacterial pigments for mass generation of varying coloring properties was first possible and is now getting notable importance and attention of both researchers and industries [2]. The bacterial pigment has several pharmacological activities like anti-microbial, anti-cancerous, anti-oxidant property with large of economic potential and there is vast scope for easy and cheap products of natural colorants in all season from bacterial source, compared to plant sources.

Bacterial pigments have many applications in day to day life [3]. Natural strain is slowly replacing synthetic colorants and this change has encouraged the possibilities for the high

production of natural pigment and dye and their sustainable utilization in all prospective field. There is an increases awareness on the harmful influence of synthetic colorants and the resultant boost in the demand for natural colorants.

In some international markets, there are restrictions and limitations in trading and manufacturing of synthetic colorants which move towards alternate natural colorants. There is an increase in demand to color unique products like toys, crayons, textile printings, hand-made papers etc. Using natural colorants.

Rabbit urine is considered as excellent fertilizer because of its chemical composition [4]. Because of its chemical composition (it contains 1.05% Nitrogen, 0.01 %Phosphorous, 0.85% Potassium, and 0.12 %calcium. Crystal CaCO₃, Triple phosphate, and Calcium oxalate, Amino acid: Methionine)

Staphylococcus aureus are found in rabbit urine which is gram positive [5, 6] and produces Staphyloxanthin (Carotenoid) pigment [7]. Staphyloxanthin is a carotenoid pigment that are ubiquitous in nature [8] and show function as Antioxidant [9]. Microbial pigments are now the industrial interest because they often more soluble and stable than those from animal or plant or source and can grow quickly which can lead to high productivity and produce a product throughout the year [10].

Dye, pigments and colorants are isolated from natural source and are all widely used in everyday life. Such as used of pigment in food production, agricultural practices, paper production and textile industries. It was noted that

carotenoid function as antioxidant and suggested STX can protect *S. aureus* against oxidative stress [9, 11, 12]

Color have been used to add or enhance aesthetic values of compound that used. Color play an important role in human lifestyle and human culture. The color produced by microorganism is known as Bio-colors. The pH of urine was basic in nature and hence it is integrated with the use of chemical fertilizers to increases soil fertility, reducing soil acidity and increases crop yields since it is foliar feed [13].

The technique used for collection of rabbit urine sample (Albino Rabbit) was "Clean catch technique" (Barry, Smith and Turck 1975). Wet mount examination was done by using light microscope, the uric acid crystals, RBC and Pus cells was observed. The isolated bacteria are *Staphylococcus aureus* and was determined by using Bergey's manual of determinative bacteriology. The Staphyloxanthin pigment was extracted using ethanol extraction method and was purified partially by organic solvent and TLC [14]. The pigment is used in color paint, nail paint, textile industries, in pharmaceutical industries, as a dye. Hydrogen peroxide scavenging activity assay was performing to check the staphyloxanthin activity. *S. aureus* overnight in broth media with increasing iron concentration and enhanced their susceptibility to kill H₂O₂ [15]

Materials and methods

Sample collection

"Clean catch technique" used for collection of rabbit urine sample (Albino Rabbit) [16]. The sample collected from group of Rabbit with the help of sterile syringe in clean or sterile test tube.

Wet-mount examination of rabbit urine sample

10ml of rabbit urine sample was centrifuged at 2000 rev/min for 10 min and the supernatant was discarded. The pellet was examined for parasites, pus cells and RBCs Crystals using a microscope with a 40X objective and a 10X eyepiece [16]. The pH of urine sample was check by using 'Multistix' reagent strips (Ames Co., Miles Laboratories Ltd, Stoke Poges, UK) [16, 17, 18].

Isolation of microorganism

Serial dilution of rabbit urine sample upto 10⁵ was perform and by selecting appropriate media such as mannitol salt agar [19] least concentrated sample was spread on sterile MSA agar plate and incubate at 37°C for 24 to 72hrs [20, 21].

Bacterial characterization and identification

The colonies appeared on mannitol salt agar were selected for further tests. confirmation of *S. aureus* was determined according to Bergey's manual of determinative bacteriology [22]. These characteristics include; colonial morphology, size of colony, color and the effect on the media such as pigments appear on mannitol salt agar and ability to ferment mannitol [23]. For the identification of bacteria biochemical tests were performed [14, 24].

Detecting the ability of *S. aureus* isolates to produce staphyloxanthin

In order to identify the ability of *S. aureus* to produce the Staphyloxanthin, 10 ml of Brain-Heart Infusion Broth (BHIB) was inoculated with 100µl of *S. aureus* and incubated at 37°C for 18 hours [25]. Bacterial growth was determined by measuring the absorbance at 620 nm. A

volume of 100µl of the inoculum from isolate was streaked on culture media including: Mannitol salt agar or Trypticase soya medium incubated at 37°C for 2 days and then incubated at 20°C for 2days [14]. Yellowish colored pigment was observed which show the positive result [14, 26].

Determination of optimal conditions for staphyloxanthin production

To determine the optimum conditions, the effect of some factors on pigment production were studied [14]

Optimum temperature: A volume of 100µl of bacterial inoculum (10³cell/ml) was cultured at different temperatures (20°C, 28°C, 37°C) for 24 to 48hrs.

Optimum pH: A volume of 100µl of bacterial inoculum (10³cell/ml) was cultured at different pH values (4,5, 6, 7, 8,) using NaOH and HCL.

Optimum incubation time: A volume of 100µl of bacterial inoculum (10³cell/ml) was cultured and incubated at 37°C for different times (18, 24, 48, 72 hrs.)

Extraction and purification of staphyloxanthin pigment

the pigment of *Staphylococcus aureus* was extracted by using ethanol extraction method. Enriched broth was transferred in centrifuge tubes and centrifuged at 6000 rpm for upto 10 min [27]. Supernatant discarded and pellets were used for further process. 1 ml of 1 N NaOH solution was added in the pellet and this mixture were separated in another clean test tube. The mixture was boiled in a hot water bath for 60 min. Equal volume of ethanol was added in the tube. Centrifuged at 3000 rpm for 20 min gives a turbidity free color solution (supernatant). It was mixed well with equal volume of Petroleum ether and vigorously the tube was shaken on the vortex. The tube was kept for 10 min. Layer was separated, Then the upper layer was discarded [3, 28] and the lower layer was added in the evaporating dish. After the evaporation the extract was stored at 4°C [29, 30]. The absorbance of pigment was determined by UV vis-spectrophotometer at 450nm [31].

Thin layer chromatography

The extracted pigment was analyzed by TLC with Silica gel [29]. The solvent system consists of Benzene: Methanol: Acetic acid (87:11:2) [14]. The spot of extracted pigment was place on the silica gel TLC sheet. The TLC sheet was then dipped in the solvent system wait to run solvent system up to 3/4 on the TLC sheet. After that, the strip was kept in the hot air oven for 5 min to become dry [32]. The strip was observed under UV light.

The Rf value was calculated according to following equation:

Distance of spot sample movement
Distance of spot solvent movement

Rf =

After development of the spot-on TLC, the plate was recovered by scraping the appropriate portion of silica gel into a tube and eluting with methanol. To determine the quantity of staphyloxanthin pigment, following equation was used [14, 31, 33, 34].

V(A-0.0051)
0.175W

Total pigment unit/cell =

Whereas

A: Is the absorbance value of the diluted Staphyloxanthin extraction at 450nm.

V: Is the final volume of the extract Staphyloxanthin.

W(g): Is the weight of the dried powder of staphyloxanthin.

0.175: Is the extraction coefficient of carotenoids

Antibacterial activity of Staphyloxanthin pigment

The activity of Staphyloxanthin was tested by the well-diffusion method. The Mueller-Hinton Agar (MHA) plates and Nutrient agar plate were inoculated with 10³cell/ml of indicator isolates (Pseudomonas aeruginosa, Pseudomonasputida, Staphylococcus spp., Escherichia coli, Klebsiella spp., Salmonella spp., Shigella spp.,) (obtained from department of microbiology, Kopargaon / university of SPPU). By preparing the media; bacterial suspension was spread on the plate and the wells were prepared in the same plate which filled with 100 µl of staphyloxanthin solutions (final concentration 0.2 g/ml by diluted 0.4 gm staphyloxanthin partial purified powder with 2 ml distilled water). The plates were incubated at 37°C for 24-48 hr.

Antioxidant property of Staphyloxanthin pigment

Chemical

The chemical used in this study are phosphate buffer, Hydrogen peroxide (H₂O₂), sodium hydroxide (NaOH) and potassium dihydrogen phosphate(K₂HPO₄)

Other: Distilled water, sample of Staphyloxanthin pigment.

Preparation of phosphate buffer (pH7.4)

In 1000mL of distilled water 6.8gm of (K₂HPO₄) (potassium dihydrogen phosphate) and 1.5gm of NaOH (sodium hydroxide) was dissolved.

Preparation of 40mM Hydrogen Peroxide

In 50ml of phosphate buffer 4.4 ml of H₂O₂ (30%) was dissolved; to final 40mM concentration. Fresh Hydrogen Peroxide was prepared before the experiment.

Hydrogen peroxide scavenging activity assay

The hydrogen peroxide scavenging assay was determined according to Ruch *et al* method [35]. 10mg of Staphyloxanthin pigment were weighed on a balance and diluted in 1000 micro litter of distilled water. In clean sterile test tubes, a 100 µL of sample (Staphyloxanthin) added into 400 µL of phosphate buffer then 600 µL of 40mM hydrogen peroxide solution added and vortexed them for 5 min. Incubated for 10min at room temperature. The blank test tube contains only phosphate buffer without hydrogen peroxide was consider as a blank for control. Measure the absorbance of Sample and blank(control) at 230nm [36, 37, 38, 39].

The abilities to scavenge the hydrogen peroxide were calculated using the formula [36]

Ao-A1
Ao

% scavenged(H₂O₂) = ×100

where,

Ao: Absorbance of control

A1: Absorbance of sample

Result And discussion

Wet-mount examination of rabbit urine sample

By performing wet mount examination of rabbit urine sample under the light microscope the RBCs, Uric acid Crystals, pus cells were observed [16].

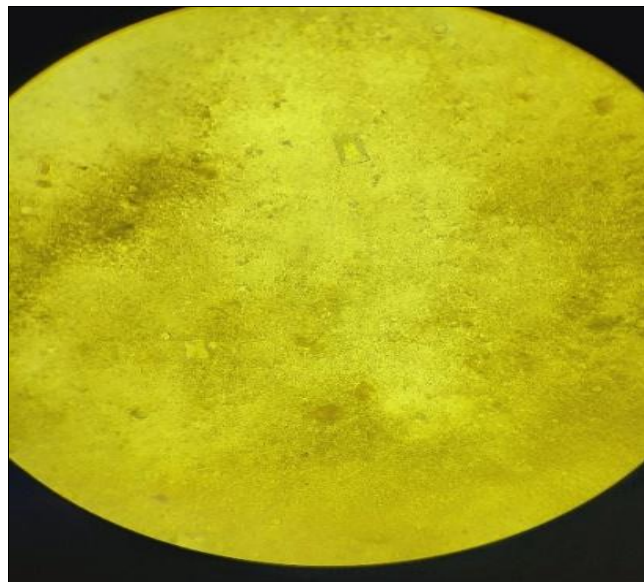


Fig 1: Wet mount examination of rabbit urine: RBC + Pus cells + Uric acid crystal

Isolation of microorganism

After spreading the least concentrated colonies on MSA agar plate [19] and incubation of 37oC for 24 -72hrs yellowish golden color pigmented colonies was observed [40].



Fig 2: Microbe isolated on MSA plate showing: yellowish golden colored Staphyloxanthin pigmented colonies and Mannitol Fermentation

Bacterial characterization

Table 1: Morphological and Biochemical characterization

Media	Mannitol salt agar
Size	2mm
Shape	Circular
Color	Yellowish golden color
Margin	Entire
Consistency	Sticky
Elevation	Convex
Opacity	Opaque
Gram character	Grampositivecocci
Catalase	Positive
Oxidase	Negative
Indole	negative
Methyl red	positive
Voges proskauer	positive
Citrate utilization	positive
Mannitol fermentation	positive

Determination of optimal conditions for staphyloxanthin production

- By growing the *S. aureus* bacterial strain on MSA agar under the different temperature 18°C, 28°C and 37°C it was observed that the favorable temperature for the bacteria is 37°C. [21,41]

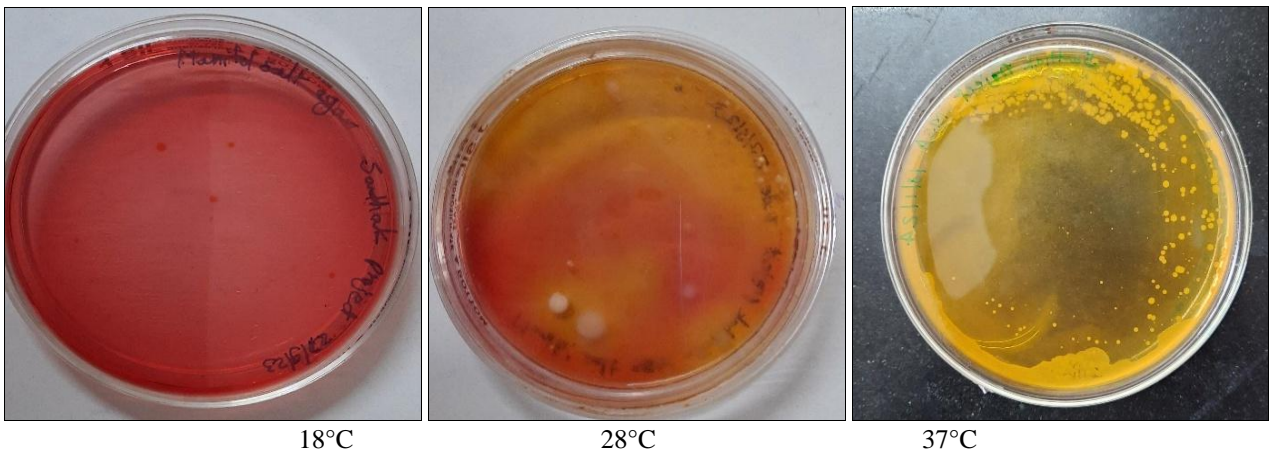


Fig 3: *S. aureus* grow on different temperature at 18°C ,28°C, 37°C respectively

- It was observed that pH7 show the high turbidity [42] and maximum pigmentation [3] and show maximum absorbance at 450nm [14].



Fig 4: Staphyloxanthin production from *S. aureus* grown in different pH values

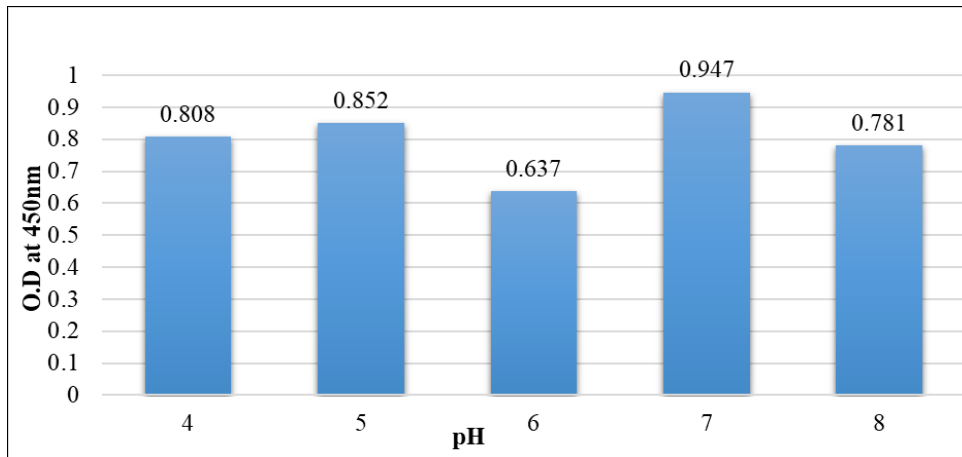


Fig 5: Staphyloxanthin production from *S. aureus* grown in different pH value

- By incubating the bacterial culture at different time interval, it was observed that optimum incubation time of 48-72hrs are the best for pigment production. [14]

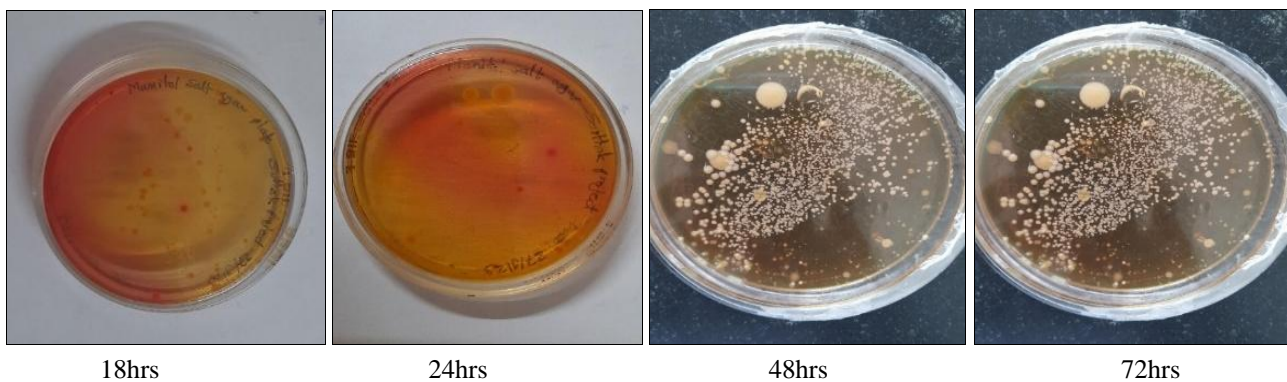


Fig 6: Optimization of time period on MSA plate

Extraction and purification of Staphyloxanthin pigment



Fig 7: Extracted pigment

The pigments extracted were yellowish orange type golden in color. The OD of the pigment was measured and further move for antimicrobial activity. The optimum incubation time period for pigment production was found that 72hrs, because the pigment is secondary metabolite [14, 43, 44] which required time to produce. The pigments produced in this

study have shown different OD. The peak optical density was at 450nm and the pigment which showed maximum optical density was yellowish orange type golden color staphyloxanthin pigment [45]. The best result of extraction of Staphyloxanthin pigment was with ethanol and the amounts is 0.2 g/ml. The RF value was 0.375, which was nearly similar to the results [14, 46]. The results show that the lower production of pigment was obtained when 250 ml medium was used (8.0118 unit/cell). while, the production increases when the volume of the media was 50 ml/flask. When the volume of culture media was 25 ml the ratio of surface area to the volume of media was high which provided high ratio of oxygen that make bacterial growth higher than the pigment production because the pigment is a secondary metabolite and is not necessary for bacterial growth [47, 48].

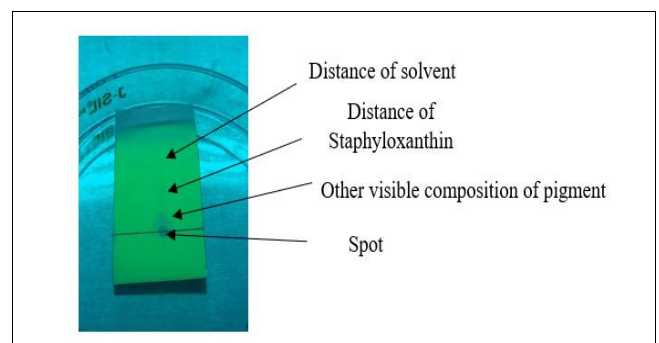


Fig 8: Partial purification of Staphyloxanthin from *S. aureus* by TLC

Antibacterial activity of Staphyloxanthin pigment and Hydrogen peroxide scavenging activity

The antibacterial activity of the STX pigment was examined against several bacteria such as *E. coli*, *Salmonella spp.*, *Klebsiella spp.*, *Pseudomonas putida*. The results revealed that staphyloxanthin has no antibacterial activity against these bacteria that used in this study. Other study^[49] reported the STX pigment extraction did not show antibacterial activities against several gram negative and gram-positive bacteria at concentration 0.25 mg/ml. Hydrogen peroxide scavenging activity of STX pigment was 32.65%^[36]

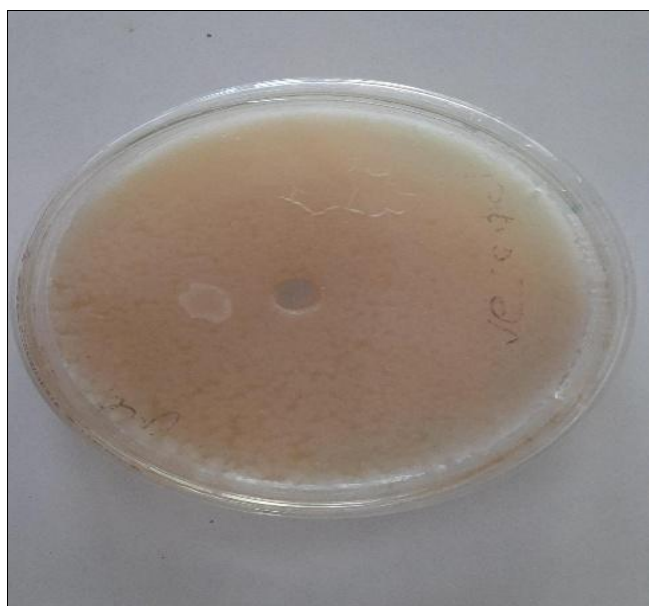


Fig 9: Antibacterial activity of Staphyloxanthin pigment showing no zone of inhibition

Conclusion

From the results of the study, it can be concluded that ethanol extraction method was suitable for isolating carotenoid pigment from rabbit urine. According to Bergey's manual the yellowish orange golden colored carotenoid pigment producing isolate was tentatively identified as may be *Staphylococcus aureus*. The shape of the isolate was cocci shaped, Gram positive bacteria and shows the mannitol fermentation. The isolate was catalase positive and oxidase negative. The effect of pH on pigment production observed that at pH 7 organism able to synthesize maximum pigment. The effect of temperature on pigment production was observed at 18°C, 28°C and 37°C which showed that at 37°C showed maximum pigment production. Extracted Yellowish orange golden colored carotenoid pigment has maximum Absorbance at 450 nm. The Rf value of pigment was found to be 0.375. After taking optical density of control and sample at 230 nm by calculating the result of Hydrogen peroxide scavenging activity of pigment was 32.65% Which is nearly similar to result. Therefore, from characterization pigment extracted it was Staphyloxanthin.

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