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In vitro studies of rhodopsin isolation from anoxygenic phototropic bacteria

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Abstract
Phototropic bacterial studies were carried out from lakes in Deulgaon Raja Taluka and Wadali Lake in Amravati which showed presence of *Rhodopseudomonas, Rhodospirillum & Rhodobacter spp*. characterized on the basis of morphological & biochemical properties. Further photosynthetic pigment was isolated from these bacteria and was characterized. These pigment has a wide range of application most significant being artificial retina preparation. Despite tedious cultivation protocol these bacteria were isolated from Lakes in Deulgaon Raja Taluka and Amravati District Lake.

Keywords: *Rhodopseudomonas, Rhodospirillum, Rhodobacter spp,* isolation, biochemical characterization and assay etc

Introduction

Phototrophic bacteria are the group of bacteria which derive energy from sunlight for their growth and the source of carbon is in the form of carbon dioxide or organic carbon. There are two groups of phototrophic bacteria that are oxygenic phototrophic bacteria and oxygenic phototropic bacteria. Oxygenic phototropic bacteria use light as an energy source and they evolve oxygen in photosynthesis process.

Aerobic oxygenic phototrophic bacteria are obligate aerobes which derive energy from sunlight by an oxygenic photosynthesis and anaerobic oxygenic photosynthetic bacteria are gram negative which contain bacteriochlorophyll and use light as an energy source. These are anaerobic and do not evolve oxygen during photosynthesis process. The anoxygenic bacteria grow phototrophically only under anaerobic condition. These organisms are found in anaerobic freshwater or marine environment. They occur beneath the surface of water, as at the bottom of a lake. These bacteria contain various type of insoluble caratenoid pigment. Which absorb light energy and transmit it to the bacteriochlorophyll these bacteriochlorophyll absorb light of long wavelength. The anaerobic anoxygenic phototrophic bacteria can divided into two groups on the basis of their pigmentation that is purple bacteria and green bacteria green photosynthetic bacteria contain bacteriochlorophyll types c or d and minor amounts of bacteriochlorophyll a, these pigments involved in photosynthesis are located in membrane bound vesicle within cell and culture are generally green or brown in colour some of which are green sulphur bacteria. E.g. *Chlorobium tedium* and green non-sulphur bacteria e.g.*Chloroflexus auranticus*

Purple phototrophic bacteria

These bacteria are proteobacteria that are phototrophic that are capable of producing energy through photosynthesis. They are pigmented with bacteriochlorophyll a or b, together with various caratenoid, which give them colour ranging from purple, red, brown and orange. The photosynthetic pigment and structure of photosynthetic apparatus are located within a system of internal membrane that is originated from and being continuous with the cytoplasmic membrane. These membranes consist of small finger like intrusions vesicle, tubules or lamellae parallel to an angle to the cytoplasmic membranes and they carry the photo synthetic apparatus. The reaction centre and light harvesting pigment protein complex surrounding the reaction centre.

Purple bacteria do not produce oxygen because the reducing agent involved in photosynthesis, called purple sulphur bacteria. It is either sulphide or elemental sulphur and the others, called purple non-sulphur bacteria, typically use hydrogen or some other compound in small amount.

The purple sulphur bacteria include the family chromatiaceae. These are a group of anaerobic gamma proteobacteria capable of photosynthesis which found in hot springs or stagnant water. They do not use water as their reducing agent instead they use hydrogen sulphide which oxidized to produce granules of elemental sulphur this in turn oxidized to from sulphuric acid. The colour of culture of purple sulphur bacteria appear orange-brown to purple violet, they are generally found in anoxic zones of lakes and other aquatic habitats where hydrogen sulphide accumulate & also in sulphur springs where geochemically and biologically produced hydrogen sulphide can trigger the formation of bloom of purple sulfur bacteria, these bacteria contain bacteriochlorophyll a or b, and various type of carotenoids which is located in cytoplasmic membrane. These purple sulphur bacteria are classified with chromatiaceae and ectothiorodospiraceae. Which are may be ovoid to rod shaped, coccoid or helical; all genera of purple sulfur bacteria are capable of photolithotrophic growth, by using H_2S or elemental sulphur as election donor for CO_2 fixation.

The purple non – sulphur bacteria are found among the alpha and beta sub-group, including.

Rhodospirillales.		
R*hodospirillaceae*	e. g.	*Rhodospirillum*
Acetobacteraceae	e. g.	*Rhodopila.*
Rhizobials.		
Bradyrhizobiaceae palustris.	e. g.	*Rhodopseudomonas*
Hyphomicrobiaceae	e. g.	*Rhodomicrobium*
Rhodobiaceae	e. g.	*Rhodobium*
Other families		
Rhodobacteraceae	e. g.	*Rhodobacter*
Rhodocyclacae	e. g.	*Rhodocyclas*
Comamonadaceae	e. g.	*Rhodoferax*

The purple non-sulfur photosynthetic bacteria have a wide range of growth modes and are able to grow under photoautotrophic, photoheterotrophic and chemoheterotrophic condition (Imhoff and Truper, 2005) [7]. Switching from one mode to another dependingan condition available, especially: degree of anaerobiosis, availability of carbon source and availability of light. Under anaerobic condition, culture appears orange brown to purple red. This group of microbes are located in water bodies below the layer of oxygenic photosynthetic organism (Oda *et al.,* 2002) [17]. They are also found in habitats such as waste water ponds, sediments, moist soil, and seawater pools (Okubo *et al.,* 2006) [19]. These bacteria exhibit a diversity of shapes i.e. helical, rod-shaped, ovid or spherical. The purple non-sulfur bacteria are photoorganotrophs that is organic substance serve both as carbon source and as electron donor for the reduction of carbon dioxide. Photosynthesis occurs only under anaerobic condition in the presence of light (Blankenship *et.al.,* 1996) [1].

Typically, several discrete layers can be recognized, arranged vertically, where organism distribute themselves according to physiological requirements such as amount of light, Oxygen, nutrient and temperature. The upper layer is green layer which is dominated by cyanobacteria and organoheterotrphic bacteria (Gemerden, 1993) [4]. Below this layer a pink layer is found which contain anoxygenic phototrophic bacteria (Urmeneta *et al.,* 2003) [26]. Which represent purple non-sulphur bacteria? This pink colour is due the pigment present in their membrane purple non-sulphur bacteria. This is reaction centre for energy transfer. This reaction centre contains two basic type of light harvesting complex called LH1 and LH_2. The light energy is used by reaction centre to get excitation state at which low potential electron are transferred to a quinine, then to cytochrome eventually returning to the reaction center. The electron transfer is considered to be cylindrical during, electron transfer proton pumped outside the cell by quinones, creating a proton motive force, the membrane bound ATPase can take advantage of the electrochemical gradient and use it to produce ATP. By generating reducing power, some purple non-sulfur bacteria get their carbon from organic compounds; most photographs are autotrophs that are they get their carbon by converting CO_2 to reduced sugars. The cells store this reducing power in the form of NADPH. The purple non-sulphur bacteria used reduced compound from environment the source of electron to reduce NADP+ to NADPH. As like this pigment work for the survival for the purple non sulphur bacteria which have number of pigment like bacteriochlorophyll a, b, c and bacteriorhodopsin.

The bacteriorhodopsin is a pigment present in some phototrophic bacteria, it is a protein acts as a proton pump that is it captures light energy & uses it to move protons across the membrane out of the cell. It is seven helix trans membrane proteins, usually found in two dimensional crystalline patches known as purple membrane. The repeating element of the hexagonal Lattice is composed of three identical protein chains each rotated by 120 degree relative to the others. Each chain has seven Trans membrane alpha-helix and contains one molecule of retinal buried deep within, the typical structure for retinylidene proteins.

This bacteriorhodopsin is the retinal molecule that changes it's conformation. When absorbing photon, resulting in a conformational change of surrounding protein and the proton pumping action (Hayashi *et al.,* 2003) [6]. It is covalently linked to lysine 216 in the chromophore. After photo isomerization of the retinal molecule Asp 85 becomes a proton accepter of the donor proton from the retinal molecule. This release a proton from a holding site into the extracellular site of the membrane reprotonation of the retinal molecule by Asp 96 restores its original isomerized form. This result in a second proton being released to the extracellular side. Asp 85 release it is proton into the holding site. The bacteriorhodopsin molecule is purple with absorption maxima at 568nm.

In this experiment sediment sample were collected from nearby Wadali and University lake for isolation of phototropic bacteria by inoculation of serially diluted sediment sample into basal salt medium that is succinate agar medium. And incubating it at 30^0C for 4-7 days. After incubation reddish bloom observed in broth and red colour colony on succinate agar plate. Then it characterized morphologically, culturally & biochemically, further four Rhodopseudomonas spp., four Rhodobacter spp. & two Rhodospirillum spp. were found. And then subjected to rhodopsin production from it.

Material and Methods

Sample collection

Sample were collected from lakes in Deulgaon Raja Taluka, Total ten sediment sample were collected & the site of sample collection was choose on the basis of amount sunlight fall on that site.

Methods

1. Enrichment of Anoxygenic phototropic bacteria

The sediment sample was collected then it is diluted by using serial dilution method up to10^{-5} and this dilution is inoculated into the media that is Basal salt medium which give only growth of phototropic bacteria in the presence of light & anaerobic condition, for maintaining anaerobic condition the anaerobic jar is applicable. After inoculation the inoculated broth is kept in anaerobic jar in the presence of light. For 4-7 days, after 4-7 days the growth is seen in the form of reddish bloom, the total ten sample are inoculated in different ten tube after appropriate dilution.

2. Screening of rhodopsin producing bacteria

Screening was done by sub culturing. The culture was subculture on plates and slant, then the subsequent sub culturing was done for seven times and then the plate are observed for the red colonies when this red colonies was observed, it was transferred to slant, Likewise this ten isolates of rhodopsin producing bacteria were isolated then this isolates are subjected for the production of rhodopsin pigment. The media are firstly inoculated with the separate isolate in separate media and then incubated in the anaerobic jar for 4-7 day. When the reddish growth was observed the broth culture was subjected to the exaction.

3. Identification of bacteria

Identification of Isolates was based on Morphological, cultural and biochemical characteristics. Morphological characteristics**:** The morphological Characters were examined under microscope by performing gram staining & motility. Biochemical Characteristics**:** The Biochemical characters were examined by conducting the following test.

1. Catalase test
2. Indol
3. Methyl Red
4. Voges Prosker's
5. Citrate Test
6. Sugar Test

a. Arabinose
b. Dextrose
c. Fructose
d. Lactose
e. Mannitol
f. Xylulose
g. Sucrose
h. Rhamnose

Production of rhodopsin

The Production of rhodopsin was carried out by inoculation of red colonies present on slant in to the broth medium and then incubated in anaerobic jar for 4-7 day. After 4-7 days. The reddish bloom was observed which indicated the rhodopsin production.

Extraction of rhodopsin

The centrifuge tube was washed and autoclaved, after that the sample was taken into centrifuge tube aseptically. Then centrifuge it at 8000 rpm for 15 min. the cell pellet was sediment at bottom. So, discard the supernatant and add sodium lauryl sulphate into the centrifuge tube containing cell pellet, sodium Lauryl sulphate was used for disruption of cell because, rhodopsin pigment was present in cell membrane. After addition of sodium lauryl sulphate centrifuge it at 8000 rpm for 10 min. After centrifugation supernatant was discarded and disrupted cell pellet containing rhodopsin which was dissolved in ethanol. Centrifuge it at 8000 rpm for10 min. Then the cell pellet sediment at bottom of tube and pigment in ethanol these supernatant was taken for spectrophotometer and the absorption maxima was get on 530 nm.

Result & Discussion

Screening and Assay of anaerobic phototrophic bacteria

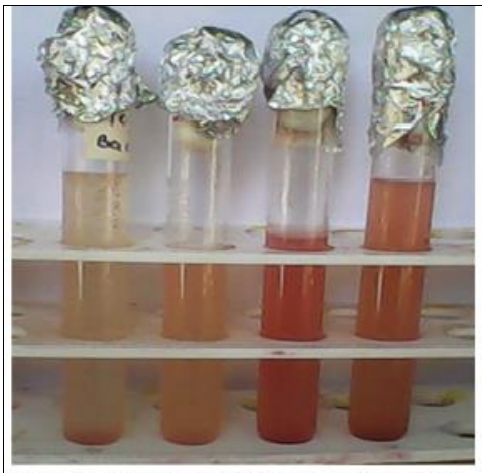

Fig.1 Characteristic red coloured growth of PNSP

Fig2. Culture flask before incubation after inoculation

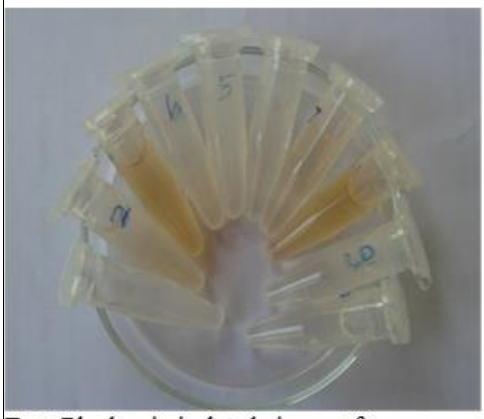

Fig3. Rhodopsin isolated pigment from screened

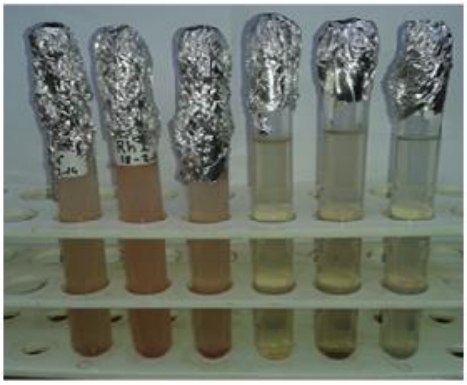

Fig.4 for confirmation 3 tubes incubated at light (left Side) and 3 tubes incubated in dark (Right side)

Table 1: Morphological & biochemical characteristic of phototropic bacteria.

Culture designation	Cultural characteristics							Morphological properties				Biochemical characteristics															Identified Bacterial species
	Colour	Size	Margin	Shape	Elevation	Opacity	Consistency	Gram reaction	Shape	Endospore	Motility	Catalase	Oxidase	Indol	MR	VP	Citrate	Arabinose	Glucose	Fructose	Lactose	Mannitol	Rhamnose	Xylulose	Sucrose	Salicilin	
RH1	Pink	1 mm	Regular	Circular	Convex	Opaque	Soft	-	Short rod	-	M	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Rhodospirillum spp.*
RH2	Red	0.5 mm	Regular	Circular	Convex	Opaque	Soft	-	Short rod	-	M	-	-	-	-	-	+	-	-	-	-	+	+	-		-	*Rhodopseudomonas spp.*
RH3	Pink	0.5 mm	Irregular	Circular	Raised	Opaque	Soft	-	Long rod	-	M	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	*Rhodobacter spp.*
RH4	Red	1mm	Regular	Circular	Raised	Opaque	Soft	-	Long rod	-	M	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	*Rhodobacter spp.*
RH5	Red	1 mm	Regular	Circular	Convex	Opaque	Hard	-	Short rod	-	M	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	*Rhodopseudomonas spp.*
RH6	Pink	1mm	Regular	Circular	Convex	Opaque	Soft	-	Long rods	-	M	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	*Rhodobacter spp.*
RH7	Red	.01 mm	Regular	Circular	Raised	Opaque	Soft		Long rod	-	M	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-	*Rhodobacter spp.*
RH8	Red	0.1 mm	Regular	Circular	Convex	Opaque	Soft	-	Short rod	-	M	-	-	-	-	-	+	-	-	+	-	+	+	-	-	-	*Rhodopseudomonas spp.*
RH9	Pink	0.5mm	Irregular	Circular	Convex	Opaque	Hard	-	Short rod	-	M	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	*Rhodospirillum spp.*
RH10	Red	0.1mm	Regular	Circular	Convex	Opaque	Soft		Short rod	-	M	-	-	-	-	-	+	-	+	+	-	-	+	-	-	-	*Rhodopseudomonas spp.*

Short forms: M=Motile, - = Negative, + = Positive.

Screening of phototropic bacteria was done by inoculation of serially diluted sediment sample into the succinate broth medium at 30^0c for 4-7 days in anaerobic jar, after incubation period reddish growth was observed in broth (Fig.1 and 2), then these broth culture was used for isolation of phototropic bacteria on succinate agar plate, which result in red colonies on succinate agar plate. The confirmation of phototropic bacteria was done by incubating inoculated culture in dark & light, which show reddish growth in only that culture flask which kept in presence of light. (Fig.4)

Extraction of rhodopsin (Fig.3) from phototropic bacteria was done by centrifugation. Firstly the cells were separated and treated with alcohol and then sodium lauryl sulphate (detergent) which leads to lysis of cell and then absorbance observed at 530nm by spectrophotometric method.

From the above morphological, cultural & biochemical properties of isolated phototropic bacteria (Table.1). All isolates shows characters like, all are gram negative motile rods, and some utilizes sugar like glucose, fructose, Mannitol. It was concluded as the given sediment sample contain *Rhodopseudomonas spp.*, *Rhodobacter spp.*& *Rhodospirillum spp.* Prominently, which was then further used for production rhodopsin.

The present study suggest that rhodopsin production from phototropic bacteria, ten isolates was isolated from sediment sample of Wadali lake and University lake, out of ten, four *Rhodopseudomonas spp*., four *Rhodobacter spp*., & two *Rhodospirillum spp*. Which show different absorption maxima depends on its rhodopsin production. The highest absorption shown by RH10, RH8, RH3, RH5 & RH6 i.e.0.214, 0.206, 0.200, 0.189 & 0.184. And lowest absorption shown by RH1 & RH9 i.e.0.100, 0.122.And medium absorption shown by RH2, RH4, & RH7 i.e.0.164, 0.142, & 0.140. Means the prominent rhodopsin producers was *Rhodopseudomonas* spp. & *Rhodobacter* spp. comparatively, than *Rhodospirillum* spp (Table.2).

Table 2: Spectrophotometric detection of rhodopsin pigment at 530 nm

Bacteria code	Identified bacteria	Absorbance at530 nm
RH1	*Rhodospirillum spp.*	0.100
RH2	*Rhodopseudomonas spp.*	0.164
RH3	*Rhodobacter spp.*	0.200
RH4	*Rhodobacter spp.*	0.140
RH5	*Rhodopseudomonas spp.*	0.189
RH6	*Rhodobacter spp.*	0.184
RH7	*Rhodobacter spp.*	0.142
RH8	*Rhodopseudomonas spp.*	0.206
RH9	*Rhodospirillum spp.*	0.122
RH10	*Rhodopseudomonas spp.*	0.214

In present study, total ten isolates were obtained from sediment sample of Lakes in Deulgaon Raja taluka. Morphological, Cultural and Biochemical characteristics were carried out. Further four *Rhodopseudomonas spp*., four *Rhodobacter spp*. & *Rhodospirillum spp*. were obtained. This isolate were subjected to the production of rhodopsin. Hence, rhodopsin production was observed in red coloration of culture flask, this rhodopsin production was low at initial days and high at seventh day, the rhodopsin production limited with addition of carbon source. This rhodopsin production was observed by taking absorbance at 530nm on spectrophotometer. The absorbance was directly based on the rhodopsin production. Thus, highest rhodopsin production was observed in *Rhodopseudomonas spp. Rhodobacter spp*. and lowest in *Rhodospirillum spp*. Thus, *Rhodopseudomonas spp*. & *Rhodobacter spp*. was good rhodopsin producers. Similar, work carried out by collecting sample from Cabo Rajo saltern, microbial mats, after incubation, it shows reddish bloom containing rhodopsin pigment which were analyzed by UV-spectroscopy which show absorbance peak from 300-1100nm (Feliciano *et al*.,2010) [3]. This rhodopsin has wide pharmaceutical and technological application.

Conclusion

Phototropic bacterial studies were carried out from University lake and Wadali lake which showed presence of *Rhodopseudomonas, Rhodospirillum & Rhodobacter spp*. characterized on the basis of morphological & biochemical properties. Further photosynthetic pigment was isolated from these bacteria and was characterized. These pigment has a wide range of application most significant being artificial retina preparation. Despite tedious cultivation protocol these bacteria were isolated from Lakes in Deulgaon Raja Taluka.

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