CHAPTER 3

LOCALISATION OF CAROTENOID PIGMENTS
IN PSYCHROTROPHIC MICROCOCCUS ROSEUS
INTRODUCTION

A good proportion of psychrotrophic bacteria isolated from soils and water samples from Antarctica were observed to be pigmented (Courington and Goodwin, 1955; Shivaji and Ray, 1995) and the predominant pigmented bacterial strains belonged to the genera *Flavobacterium* (Holmes et al., 1984), *Sphingobacterium* (Shivaji et al., 1992), *Micrococcus* and *Planococcus* (Miller and Leschine, 1984; Shivaji et al., 1988), *Arthrobacter* (Shivaji et al., 1989), *Corynebacterium* (Madden et al., 1979), *Janthinobacterium* (Shivaji et al., 1991) etc. Despite this obvious predominance of pigmented bacteria, very few studies have been directed towards characterisation of pigments with respect to the chemical structure, biosynthesis, localisation and physiological function. In fact, such studies even on the pigments of mesophilic bacteria are limiting. In the genus Micrococcus, seven out of a total of nine species are pigmented and the pigments have been identified as carotenoids. Further, the nature of the pigments also varied between the species thus making them phenotypically either red (*M. agilis, M. roseus*), red/pink (*M. radiodurans*), yellow (*M. luteus, M. varians*) etc. (Kocur, 1986).

In this chapter *in vivo* localisation of the carotenoid pigments in *M. roseus* was ascertained by photoacoustic spectroscopy (PAS). Such *in vivo* studies are important especially because carotenoids are very unstable and are known to change isomerically during the extraction process. PAS is a simple and convenient technique which has been used effectively to obtain absorption spectra of optically opaque and light scattering biological samples as varied as skin, malarial parasite, algal cells, lobster-shell etc. (Mackenthun et al., 1979; Carpentier et al., 1983; Anjo and Moore, 1984; Balasubramanian et al., 1984). The results demonstrated that in both the mesophile and the psychrotroph the pigment chromophore was similar, the pigments existed both as its *cis* and *trans* isomers and the pigments were associated with the membrane.
MATERIALS AND METHODS

Chemicals and Reagents

Methanol (spectroscopic grade) was obtained from Spectrochem (Bombay, India). Milli Q Water was prepared at the CCMB. Trypsin and DNAase were obtained from Sigma Chemical Co. (St Louis, USA). All other chemicals used were of analytical grade.

Bacterial strains and growth conditions

Maintenance and growth of psychrotrophic and mesophilic M. roseus (45R)

The maintenance and growth of psychrotrophic M. roseus was done as described in Chapter 2. The mesophilic type culture of M. roseus (NCTC 07523) was obtained from the National Collection of Type Cultures (National Chemical Laboratory, Pune) and maintained in LB medium (1 litre) containing tryptone (10 gms), yeast extract (5 gms) and sodium chloride (10 gms). The pH of the medium was adjusted to 7 with NaOH. The cells were grown in LB medium or ABM for 6 days to stationary phase at 37°C and harvested and stored as described for the psychrotrophic culture. The cultures were maintained either on ABM plates as described for M. roseus (45R) or in LB agar plates.

Photoacoustic spectra (PAS)

Principle: Photoacoustic spectroscopy (PAS) is a simple and convenient technique which can be used to obtain absorption spectra of optically opaque and light scattering samples such as biological materials, solids and surfaces (Lachaine et al., 1993). In this technique, the sample is kept in an airtight cell of constant volume. The absorption of the intensity-modulated incident electromagnetic radiation by the sample produces an intermittent temperature change in the sample as a result of nonradioactive de-excitation. The heat thus generated causes pressure fluctuations in the gas (generally air) inside the cell. The fluctuation can be monitored by using pressure sensitive detectors like microphones. Monitoring this acoustic signal over the wavelength region of interest constitutes the photoacoustic spectrum.
The intensity of photoacoustic signal depends on the absorption cross section and the thermal diffusivity. The thermal diffusivity is related to the thermal diffusion length $\mu_T$ defined below as:

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\mu_T = \sqrt{\frac{2\alpha}{\omega}}
$$

where $\alpha$ is the thermal diffusivity of the sample and $\omega$ the chopping frequency. Since only those signals generated within the thermal diffusion length will contribute towards the photoacoustic signal, the thermal diffusion length is approximately the limit of the depth that can be probed. At high modulation frequencies the thermal diffusion length is small and hence only signals from the surface are detected. The other way of monitoring the interior of a sample would be to select the signals having different phases (Mackenthun et al., 1979). The rate of transfer of the heat generated in the sample would be different at different depths than the heat generated at the surface and definite phase delays can be incorporated between these signals. Thus, by properly adjusting the phase using a lock-in analyzer, it is possible to get information about the interior of the sample at different depths. This is done by recording the inphase (surface) and quadrature (interior) components of the photoacoustic signal. This technique is therefore ideally suited for studying layered structures (Anjo and Moore, 1983) such as bacteria.

**Method:** The photoacoustic (PA) spectral measurements were made on an OAS-400 PA spectrometer (EDT Research, London, UK) in which several modifications were incorporated. Light beam from a 300 Watt Xenon arc lamp was intensity modulated using a continuously variable mechanical chopper (HMS 222, Ithaco Inc., Ithaca, USA) whose frequency could be varied between 10 Hz to several kHz depending on the chopper blades used. The PA spectra were recorded at 40 Hz. The light beam was then monochromated and passed onto the sample cell (15 mm length, 5 mm width, 1.2 mm depth, machined in an
aluminium block) to which a Bruel & Kjaer microphone (B & K 4165, Naerum, Denmark) was attached. The microphone and the sample compartment were connected through a small opening (5 mm length, 1 mm diameter). The sensitivity of the microphone was 50 mV/Pascal. The acoustic signal was passed through a preamplifier (EG&G 113, USA) and a lock-in analyser (EG&G 5206, Princeton Applied Research, Princeton, USA) and was sent to a microcomputer (IBM PC) over a GPIB interface through an A/D converter. The phase resolution of the lock-in analyser was ± 0.1° with a phase increment of 0.025°. The computer controls the spectrometer, processes the data and plots the spectrum. Normalization of the PA spectra to constant input light intensity was achieved by using the PA spectrum of carbon black.

The photoacoustic spectra of the lyophilised powder of mesophilic and the psychrotrophic *M. roseus* were recorded from a wavelength of 300 to 600 nm. The cells of psychrotrophic *M. roseus* (45R) at stationary phase or late log phase (100 ml culture) were filtered through a 0.2 μm filter (Millipore) and the filter containing the cell pellet was used directly to record the PA spectra. The depth profiling of the pigments of *M. roseus* was carried out by recording the PA spectra between 250 to 600 nm with different phase delays in the front surface illumination mode. The intensity of the PA signal was normalised at 280 nm - the wavelength at which proteins absorb.

**Preparation of cell membranes from *M. roseus***

The method used was essentially that described by Work (1964, 1971) with minor changes. About 100 mg of cells of *M. roseus* grown to stationary phase were suspended in 0.1 M sodium phosphate buffer (pH 7.8) and sonicated in a Branson Sonifier in which the duty control switch was set at 40% and the output knob at 3 (power output) until 90% of the cells were disrupted (5 min); they were then heated for 10 min at 60°C to inactivate lytic enzymes. The sonicated cell suspension was centrifuged at 1,000 g for 10 min and the pellet consisting of intact cells and lumps, was discarded. The supernatant was centrifuged at 25,000 g for 15 min in a Sorvall centrifuge and the sediment consisting of the cell wall
fraction enriched with cell membrane was recovered, resuspended in the buffer, spun once
again at 1,000 g for 10 min to remove cell debris and finally the cell wall fraction was
recovered from the supernatant as a pellet by centrifugation as described above (25,000 g for
15 min). Cell membranes thus obtained were once again suspended in phosphate buffer and
treated with DNase for 3 h to remove contaminating DNA and then with trypsin for 12 h to
remove contaminating proteins that were non-specifically adsorbed on the cell walls. The
suspension was then centrifuged at 1,000 g for 10 min (pellet discarded) to remove the
fragments of DNA or proteins and the cell membrane enriched fraction in the resulting
supernatant were sedimented at 25,000 g for 20 min and washed thrice with 0.9% NaCl. The
pigmented cell walls / cell membrane were lyophilised and stored as a pellet at -20°C.

RESULTS

*In vivo* characteristics and localisation of carotenoids in psychrotrophic
and mesophilic *M. roseus*

Photoacoustic spectra: The photoacoustic (PA) spectra normalised at 280 nm of the
lyophilised powder of psychrotrophic and mesophilic *M. roseus* are shown in Fig. 1. Both the
spectra showed similar features with peaks at 478, 510 and 548 nm. In addition, the
psychrotrophic strain also showed two more bands at 375 and 398 nm and the mesophilic
strain one more band at 418 nm. Freshly pelleted cells of *M. roseus* (psychrotrophic and
mesophilic) spread on a filter paper also showed the same PA spectral characteristics.

Phase dependent PA spectral studies on *M. roseus*: The in-phase (0° phase) and the
quadrature (90° phase) PA spectral profiles which are intensity normalised for the 280 nm
peak for the psychrotrophic and mesophilic strains of *M. roseus* are shown in Fig. 2 A and B.
The in-phase (surface) component is first selected by maximising the signal through adjusting
the phase so as to achieve maximum discrimination against the signal from the cell interior.
The quadrature (interior) spectrum is obtained by giving an additional 90° phase to the above
Fig. 1: Photoacoustic spectra of lyophilised cell pellets of psychrotrophic (———) and mesophilic (-----) M. roseus. The solid arrows indicate absorption at 375 and 398 nm due to the cis peak and the discontinuous arrow indicates the absorption peak at 418 nm indicative of shorter polyenes.
Fig. 2: Photoacoustic depth profile spectra of psychrotrophic (A) and mesophilic (B) *M. roseus* in phase (-----) and quadrature (--------).
maximised phase (Anjo and Moore, 1984; MacKenthun et al., 1979). In both the microorganisms the signal from the pigment was clearly seen in the surface (in-phase) spectrum while it was not very prominent in the interior (quadrature or 90°) spectrum. A 280 nm absorption peak was also prominently seen in the surface spectrum which was, however, blue shifted by about 10 nm in the interior spectrum. The ratio of the 280 nm peak to the pigment peak was taken to be indicative of the presence and extent of pigment in each phase with low values of protein to pigment ratio indicating higher concentration of the pigment. Thus, it is obvious that this ratio in both the psychrotroph and mesophile was extremely low in the in-phase but the ratio was very high in the quadrature indicating that the pigment was associated with the membrane region.

**Localisation of pigment in M.roseus (45R).** The sonicated suspension of *M.roseus* following differential centrifugation indicated the presence of the pigment in the low speed 1,000 g pellet (which consisted of unbroken cells and lumps of broken or unbroken cells), in the 1,000 g supernatant, and in the 25,000 g pellet (which is likely to contain cell walls and cell membranes). Trypsin treatment did not release the pigment from the 25,000 g pellet. These results also indicated that the pigments were associated with the cell membrane. The pigment could be easily extracted with methanol from the 25000 g pellet and the UV-visible spectrum was identical to that of the crude pigment extract from intact cells.

**DISCUSSION**

Ascenzi and Cooney (1975) used the method of subcellular fractionation to demonstrate that in mesophilic *M.roseus* the pigment was associated with the membrane fraction. Our earlier studies in psychrotrophic *M.roseus* using similar techniques demonstrated the association of pigments with the membrane fraction (Jagannadham et al., 1991). However, our earlier studies did not rule out the possibility of the carotenoid pigments associating with the membranes as an artifact of the isolation procedure. PAS allows the localisation of the pigments in intact bacterial cells, thus overcoming the artefacts associated
with the subcellular fractionation method. In the present study we have used PAS to localise pigments in both the mesophilic and psychrotrophic *M. roseus*.

The PA spectra unambiguously demonstrated that the pigments in the lyophilised cell pellets of psychrotrophic and mesophilic *M. roseus* contained similar chromophores with multiple absorption peaks at 478, 510 and 548 nm characteristic of carotenoids (Fig. 1). Further, the two additional peaks at 375 nm and 398 nm in the PA spectrum of psychrotrophic *M. roseus* suggest that the cells contained *cis* carotenoids *in vivo*. In fact, in psychrotrophic *M. roseus* the *trans* carotenoids (P1 to P4) could be separated from the *cis* isomers with P1 to P4 eluting before the corresponding *cis* isomers. The *cis* isomers showed absorption peaks at 365 and 385 nm in addition to peaks at 460, 489 and 524 nm (See Fig. 2 in Chapter 2). The *cis*-isomers of carotenoids were reported earlier to be present in bacteria such as *M. roseus*, *Brevibacterium* sp., *Erwinia herbicola* and *Erythrobacter longus* (Nelis and De Leenheer, 1989; Sandmann, 1990; Takaichi, 1990; Jagannadham *et al.*, 1991). Further, an absorption peak at 418 nm indicative of the presence of shorter polyenes (Schwartzel and Cooney, 1974a; Davies, 1976) was distinctly obvious in the mesophilic *M. roseus*.

In general, carotenoids have been implicated in various physiological functions but so far nothing is known with regard to whether these functions are performed by both or only a particular isomeric form of a carotenoid. Our results indicate that in psychrotrophic *M. roseus*, the synthesis of both *cis* and *trans* carotenoids increased when the cells were grown at low temperature (5°C) compared to cells grown at 25°C (See Chapter 5). Since these carotenoids were associated with the membrane it would be logical to assume that *cis* carotenoids would further increase the fluidity of the membrane, an event which normally occurs due to increased synthesis of unsaturated fatty acids in cells grown at low temperature (Russell, 1984, 1992; Sutari and Laakso, 1992; Nichols and Russell, 1996).
It was shown earlier that in mesophilic *M. roseus* carotenoids were associated with the cell membrane (Ascenzi and Cooney, 1975). Thus, this system could be well approximated to a two layer model consisting of the surface (which would include the pigments and the proteins) and the rest (which would include proteins and other cell constituents). In the present investigation, PAS was used to ascertain the localisation of carotenoid pigments in vivo in bacteria. The information sought to be obtained by PAS could be acquired by two different methods. For instance by varying the chopping frequency it is possible to record the depth profiles of specimens provided the specimen is large and thermal diffusivity is accurately known. Thus, this approach is not convenient to bacterial cells which are very small (the diameter of the psychrotrophic *M. roseus* is 1-2 \( \mu \text{m} \)) and very high chopping frequencies would be required. Further, the accurate determination of thermal diffusivity of bacterial cells is extremely difficult. Due to these difficulties intrinsic to the specimen being analysed in the present study, phase resolved monitoring of PA signals was carried out and the in-phase (surface) and quadrature (interior) spectra were recorded. This, latter approach has been effectively used for biological samples (Adams and Kirkbright, 1977; O'Hara et al., 1983; Nery et al., 1987; Baesso et al., 1994).

From the PA spectra it is apparent that the pigments were distributed anisotropically with most of the pigments being distributed in the cell membrane as evidenced from the fact that the surface component (0\(^{\circ}\) phase) of the PA signal showed all the characteristic absorption peaks of the pigments and a peak at 280 nm due to the proteins, while the quadrature (90\(^{\circ}\) phase) component showed only one major band at around 270 nm which was blue shifted by about 10 nm with respect to the in phase protein absorption peak at 280 nm. This shift might be due to the composite absorption between proteins, nucleic acids and other cellular components. These results clearly showed that the bulk of the absorbing chromophores are distributed in the cell membrane and the pigments were present at almost the same depth in both the mesophilic and psychrotrophic species. This, in vivo localisation of carotenoids in the cell membrane of *M. roseus* confirmed the earlier in vitro studies by Ascenzi and Cooney (1975) and our own observations (Jagannadham et al., 1991).