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Full Length Research Paper

Isolation and purification of membrane-bound cytochrome c from *Proteus mirabilis*

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In the present studies, respiratory chain pathogenic bacterium, *Proteus mirabilis*, was investigated. In the first phase, growth profile study was performed to optimize the *P. mirabilis* growth. Maximum bacterial growth could be obtained between 10 – 12 h of culturing time. Down-stream processing was performed by using sonication, ultracentrifugation and detergent solubilization techniques. Partially purified respiratory contents were analyzed spectrophotometrically. Pyridine-ferrochrome and redox spectra showed the presence of heme-c.

Key words: Cytochrome c, respiratory chain and *Proteus mirabilis*.

INTRODUCTION

Proteus mirabilis is facultative anaerobic, rod-shaped, gram negative bacterium. It belongs to family Enterobacteriaceae and displays swarming growth (O'Hara et al., 2000). *P. mirabilis* is an opportunistic pathogen (Griffith et al., 1976; Rosenstein et al., 1984; Griffith et al., 1973) and persist for long periods of time (Hennerr et al., 1982).

The free energy necessary to generate ATP is extracted from the oxidation of NADH and $FADH_2$ by electron transport chain, a series of four protein complexes, through which electrons pass from lower to higher standard reduction potentials. The membrane protein cytochrome c plays the role of electron donation to the photo oxidized primary donor of the reaction center in electron transport chain. Cytochrome is a hemeprotein whose characteristic mode of action involves transfer of reducing equivalents associated with reversible change in oxidation state of the prosthetic group (Agalidis et al., 1999). Formally, this redox change involves a single-electron, reversible equilibrium between the Fe(II) and Fe(III) states of the central iron atom. It is evident from literature that cytochrome C and the reaction center have a lot of differences besides these prosthetic groups. Cytochromes can be roughly classified into four groups by the kind of heme present as the prosthetic group; group a, b, c and d have heme a, protoheme, heme c and heme d or d_1, respectively (Addison et al., 1977).

It has been shown by both NMR and crystallography that in cytochrome c, the low spin iron in both oxidation states has six ligands, four porphyrin pyrole nitrogen, one thio-ether and one histidine (Salero et al., 1990). Cytochrome c acts as the electron donor for cytochrome c oxidaze and is soluble except for cytochrome c of gram positive bacteria and has a molecular weight of 8,000 - 14,000. There are many cytochrome c are known which actually function or act as the electron donor for the cytochrome aa_3 (Laemmli et al., 1970; Yamanaka et al., 1972; Yamanaka et al., 1973).

MATERIALS AND METHODS

Organism

The facultative anaerobic bacterium *P. mirabilis* strain (ATCC 29245), used in the present study was kindly provided by the PCSIR Laboratories, Lahore, Pakistan.

Growth profile of *Proteus mirabilis*

Nutrient broth was used as the growth medium for *P. mirabilis* in conical flasks and autoclaved at 121 C for 15 min. After cooling the medium, it was inoculated from the agar aseptically. It was allowed to grow at 37 C (Breitenbach et al., 1988) on shaker (C_{24} KC Refrigerated Incubator Shaker, USA). The shaker was operated at 250 rpm. Growth of *P. mirabilis* was studied after the regular

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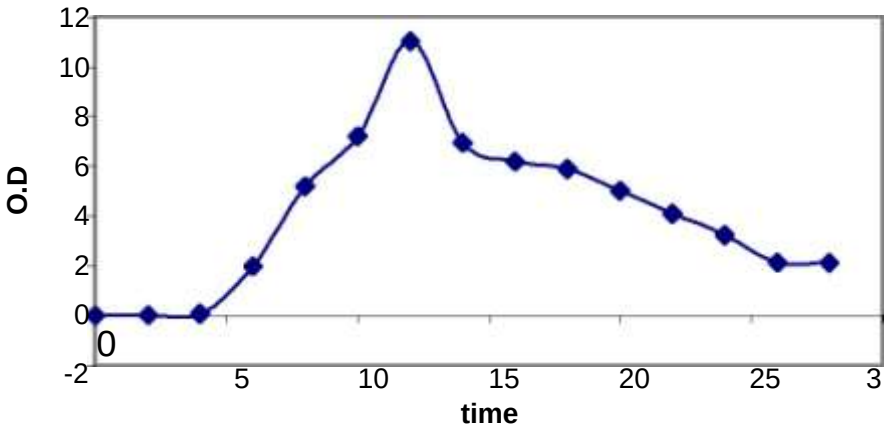

Figure 1. Growth profile of *Proteus mirabilis* measured at OD595 after regular interval of 2 h.

interval of 2 h by taking the optical density by spectrophotometer (CECIL/UV-Visible Spectrophotometer, Hitachi U-2001) at 595 nm.

Collection of biomass

Sterilized nutrient medium was inoculated with *P. mirabilis* and allowed to grow at 37 C (Breitenbach et al., 1988) for 10 h on shaker. The shaker was rotated at 250 rpm. Then the culture was prepared and it was centrifuged at 4000 rpm for 25 min for the collection of biomass.

Preparation of cell membrane proteins

Sample cells (25 g, wet weight) kept at 0 C (Franks et al., 1993) were suspended in 100 ml of 100 mM phosphate buffer (pH 7.4) and 20 ml of 0.5 M EDTA. There have been many methods developed for the mechanical disruption of cell to isolate the cell membrane from *P. mirabilis*. Here we used the method of sonication (Kaback et al., 1969). For it, the suspension was then treated with a sonic oscillator (Soniprep 150 SANYO UK) at 10 - 12 KHz for total period of 15 min with interval of 1 min at 4 C. The resulting suspension was centrifuged by centrifuge machine (HITACHI-CP 80 MX) at 15,000 rpm at 4 C for 15 min. Supernatant obtained was consisted of membrane fraction as well as cytoplasmic fraction where unbroken cells were collected as pallets. Unbroken cells (pallets) were stored at -20 C. The supernatant was then ultarcentrifuged at 35,000 rpm at 4 C for 60 min. The reddish pallet obtained was of cell membrane whereas the supernatant was of cytoplasm (Qureshi et al., 1998). Then cell membrane pallet was suspended in 25 ml of 100 mM phosphate buffer (pH 7.4) with 2.5 ml EDTA and 4.2 ml of 20% (W/V) Triton-X 100 with final concentration of detergent is 3% (Penefsky et al., 1971; Helenius et al., 1975; Neugebaver et al., 1990; Jones et al., 1987). The resulting mixture was stirred using a magnetic stirrer for 1 h to solubilize the total cell membrane proteins. This suspension was then ultra-centrifuged at 45,000 rpm at 4 C for 45 min. Reddish supernatant thus obtained was total membrane proteins while pallet was consisted of membrane lipids.

Pyridine spectrum of membrane proteins

The reaction mixture contained 2.5 ml membrane protein solution, 0.5 ml of 2 N NaOH, 0.5 ml pyridine and about 50 mg of $Na_2S_2O_4$ (acting as a reducing agent) in the sample cuvette and scanned the sample between 380 – 650 nm ranges. Pyridine ferroheamchrome confirms the presence of covalently bound heme c in the cytochromes.

Redox spectra of membrane proteins

Absorption spectra were recorded with spectrophotometer (CICIL/UV-Visible Spectrophotometer, Hitachi U-2001), using a 1 cm light path cuvette at room temperature. Spectral Scanning was carried out between 380 – 650 nm. Reduced spectrum was taken by addition of a small amount of sodium dithionite.

RESULTS AND DISCUSSION

Growth profile of proteus mirabilis

Effect of incubation time on the growth of *P. mirabilis* was checked with every two hours up to total growth time. Log- phase was found between 6 - 12 h. Maximum growth was observed at around 12^{th} h. Afterwards, there was a sharp decline in growth profile. Decline phase was spread up to 12 h duration, which shows decline in the nutrient component and the tension of growth condition as shown in Figure 1. We routinely collected the biomass at the 12^{th} h of growth with maximum growth actively.

Reduced spectrum showing presence of cytochrome c

Figure 2 showed the absorption peak at 416.5 nm, 524 nm and 550.5 nm (Otten et al., 2001) . These peaks showed the presence of cytochrome c in the membrane proteins. The membrane-bound c type cytochrome has a molecular weight of approximately in the range of 12 – 30 kDa. However, this can be confirmed by the electrophoretic data.

In the functional sense cytochrome c is a group c cyto-

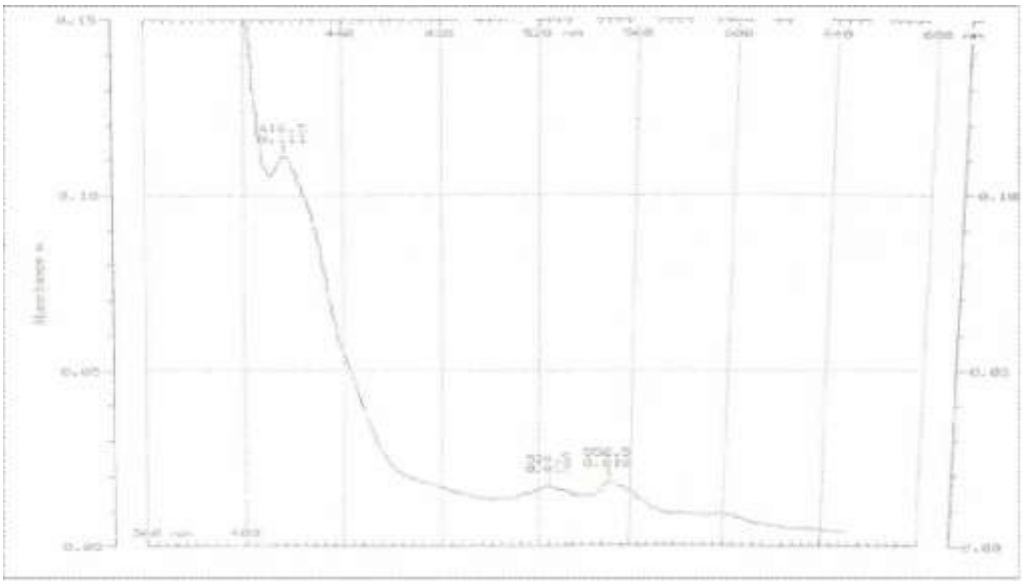

Figure 2. Reduced spectrum showing presence of cytochrome c in membrane proteins.

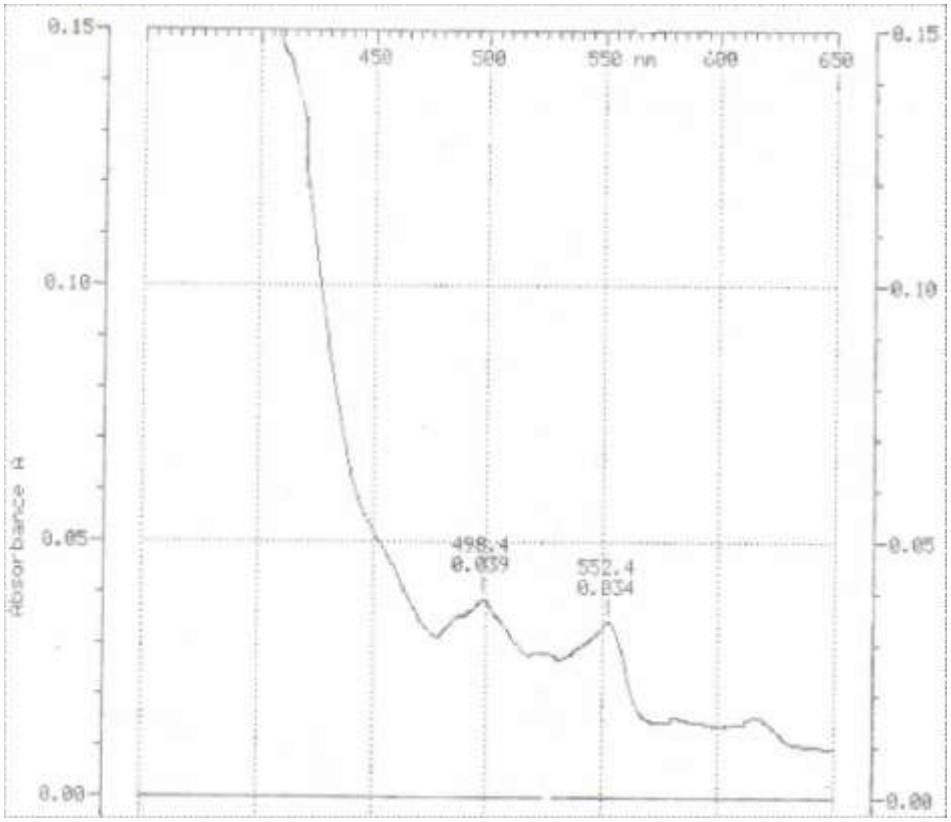

Figure 3. Pyridine spectrum of membrane proteins showing the presence of heme c.

chrome which acts as the direct electron donor for cytochrome c oxidaze or for the enzyme which perform functions similar to those by the oxidase (Yamanaka et al., 1992).

Pyridine spectrum of membrane proteins

When pyridine, NaOH (2 N) and a small amount of $Na_2S_2O_4$ are added in turn to a solution of heam or heam

protein, pyridine ferroheamchrome is formed. As the α peak position is characteristic of each kind of heam, we can identify the kind of heam by determining the α peak position of pyridine ferroheamchrome. Reduced with dithionite pyridine spectra showed the peak at 498.4 nm, a small shoulder at 525 and 552 nm in Figure 3. These spectral properties suggest that the membrane proteins contain heam c (Qureshi et al., 1998).

It is possible that cytochrome c-552 may serve as an electron donor for cytochrome c oxidase. Generally the c-type cytochromes function as physiological electron donors to the terminal respiratory enzyme complexes (Qureshi et al., 1996).

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