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Isolation and purification of membrane-bound Cytochrome c and Cytochrome b from *Nocardia asteroides*

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In present studies, pathogenic bacterium, *Nocardia asteroides* was investigated. The growth profile of Nocardia asteroides was studied by optimizing the growth conditions by culturing 20-26 hours with maximum growth. Down-streaming process was accompanied by sonication, differential centrifugation, ultracentrifugation and detergent solubilization techniques. Partially purified membrane-bound proteins were analyzed spectrophotometricaly for membrane-bound protein contents. Reduced membrane spectra and SQR (complex II) activity showed the presence of cytochrome b while pyridine ferrohaemochrome of cytochrome c.

Keywords: Cytochrome b, cytochrome c, respiratory chain, *Nocardia asteroides*.

Nocardia asteroides is a filamentous rod-shaped gram-positive bacilli strictly aerobic in nature. Nocardia asteroides is catalase-positive and acid-fast pathogen (Beaman *et al*., 1978) belongs to family Actinomycetales is the main cause of Bronchopneumonia & Pulmonary nocardiasis in human beings. It induces oxidative burst upon contact so cannot be killed in vitro in human due to the secretion of superoxide dimutase in the medium and the cell surface (Filice *et al.,* 1980; Beaman *et al*., 1983). *Nocardia asteroids* has shown ultrastructual and biochemical alterations within the cell envelope during the growth process (Beaman *et al*., 1975; Beaman *et al*., 1969). The virulence of *Nocardia asteroides* has been studies in different forms (Gonzalez-Ochoa *et al*., 1973; Kurup *et al*., 1970; Uesaka *et al*., 1971).

The respiratory enzyme complex of *Nocardia asteroides* posses cytochrome b, the redox-active heme protein with the Fe(II) & Fe(III) substituted protoporphyrin-IX ring coordinated with the redox-active iron-atoms (Voet *et al*., 2004). The b-type cytochromes generally occurring in gram-positive bacterium functions as an essential component of the respiratory chain complexes (Kita e*t al*., 1989).

Besides iron-sulfur clusters, flavoproteins and quinone, cytochrome b, animportant carrier in the electron transport pathways. In case of gram-negative like*Escherichia coli*, several b type cytochromes have been found as a part of the enzyme complexes concerned with electron transport (Kita *et al*., 1984; Kranz *et al.,* 1983; Matsushita *et al*., 1983). Some studies have suggested the localization of the heme b in cytochrome o containing complexes (Van Weilink *et al*., 1986).

In respiratory chain studies, the activity of heme b moiety has also been studied in succinate: quinone oxidoreductase (complex II) while in *Escherichia coli* & *Bacillus subtilus* YN-2000, only one heme b has been studies which does not lose its activity during purification process but increases to four fold by elevating pH from

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7-10 during cell growth (Qureshi *et al*., 1996). Cytochrome b, a component of succinate: quinone oxidoreductase enzyme but its exact role has not so far determined (Kita *et al*., 1989).

FAD and iron-sulfer clusters have been located in membrane-bound form which is usually in soluble form. However, some gram-negative bacterium such as *Escherichia coli* has one heme b per FAD (Hederstedt *et al*., 1992).

Cytochrome c, a heme protein responsible for the electron transport to the reaction center as a photoxidized electron donor is involved in the transfer of reducing equivalents mostly associated with the reversible change in oxidation state of the prosthetic group. Mostly cytochrome c donates electrons to cytochrome c oxidase however some cytochrome c are involved in the electron donation to the cytochrome aa3 or caa3 (Yamanaka *et al.,* 1972). Not all cytochrome c react with the cytochrome aa3 but the oxidase component reacting rapidly with it (Yamanaka *et al*., 1981; Yamanaka *et al*., 1973).

Cytochrome c_1, a member of cytochrome c group acts as a donor of electron to the cytochrome c in the respiratory complex (Keilin 1966; Orii *et al*., 1962). The membrane-bound protein cytochrome c1 have a molecular weight approximately 30,000 – 65,000 in different bacteria but with almost similar sequence (Ludwig *et al*., 1983).

The NMR and X-Ray crystallography has confirmed that cytochrome c group has low spin iron in both oxidation state with six ligands, four porphyrin pyrole nitrogen, one thio-ether and one histadine (Salero *et al*. 1990). The X-Rays structure has revealed that cytochrome c1, a subunit of cytochrome bc1 complex acts by binding with cytochrome c in the respiratory enzyme complex. Mostly the electron donation to cytochrome c is accompanied by b-type cytochrome of cytochrome bc1 (Keilin *et al*.,1966; Orii *et al*., 1962).

MATERIAL AND METHODS

Organism

The facultative aerobic bacterium ***Nocardia asteroides*** strain No. NRRL–B- 3828 , used was kindly supplied PCSIR Laboratories, Lahore, Pakistan.

Growth Profile of *Nocardia asteroides*

Nutrient broth was used as growth medium for ***Nocardia asteroides*** and prepared in conical flasks and autoclaved at 121oC for 15 minutes. After cooling the medium, it was inoculated from the agar slant aseptically. It was allowed to grow at 37°C on shaker (C24 KC Refrigerated Incubator shaker USA) (Beaman *et al*., 1990). The shaker was rotated at 275 rpm. Growth of *Nocardia asteroides* was studied after the regular interval of 2 hours by taking its optical density by spectrophotometer (CICIL/UV-Visible Spectrophotometer. Hitachi U-2001) at 595 nm.

Nutrient medium was prepared by dissolving 8 gram of nutrient broth in 1000 ml distilled water. The composition of nutrient broth was as followed:

- Peptone from meat 5.0 g/l
- Beef extract 3.0 g/l

Nutrient medium was sterilized at 121°C (15lbs) for 15 minutes.

Collection of Biomass

Sterilized nutrient medium was inoculated with *Nocardia asteroides* and allowed to grow at 37°C for 20 hours on shaker (Breitenbach *et al*., 1988). The shaker was rotated at 275 rpm. Then culture was prepared and it was centrifuged at 4000 rpm for 25 minutes for the collection of biomass. Centrifugation separates the cellular biomass from the culture medium. Successive batches were run in order to collect about 250gm of biomass. Then biomass was stored in refrigerator at 4°C.

Preparation of Cell Membrane Proteins

Frozen cells (about 25g wet weight kept at -20°C in a centrifugally packed state) were suspended in 100ml of 100mM Phosphate buffer (pH 7.4) and 20ml of 0.5M EDTA. The suspension was then treated with a sonic oscillator (Soniprep 150 SANYO UK) at 5-8 KHz for total period of 15 minutes with intervals of 5 minutes at 4°C. The resulting suspension was centrifuged using (HITACHI-CP 80 MX, Japan) at 15,000 rpm at 4°C for 15 minutes. Broken cells were in supernatant and unbroken cells were in the pallets and stored at – 20°C while the broken cells were discarded (Qureshi *et al*., 1998). Discard the unbroken cells. The broken cells in supernatant then ultracentrifuged at 35,000 rpm and 4°C for 60 minutes. The reddish pellet obtained was of

cell membrane whereas the supernatant was of cytoplasm. Then cell membrane pellet was suspended in 25ml of 100mM Phosphate buffer (pH 7.4) with 2.5ml 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 hour to completely solubilize the cell membrane proteins. This suspension was then ultracentrifuged at 45,000 rpm and 4°C for 45 minutes. Reddish supernatant thus obtained was of membrane proteins while pellet is of membrane lipids.

Purification of membrane-bound respiratory proteins

The membrane proteins of *Nocardia asteroides* were then purified by subjecting to FPLC (Bio Rad Laboratories, USA) on a Macro-Prep DEAE - support (ion-exchange media) equilibrated with buffer-A containing (Wt/Vol) 0.5 % Triton X-100. The proteins were then eluted with a linear gradient of buffer-A to get the pure membrane proteins for spectrophotometric determination containing 0.5% Triton X-100 and then with buffer-B containing 0.5% Triton X-100 and 0.6M NaCl. The adsorbed membrane proteins were eluted with a linear gradient of 0.3M NaCl (Qureshi *et al.,* 1998).

Absorption Spectra of Reduced Membrane Proteins

Absorption spectra were recorded with spectrophotometer (CICIL/UV-Visible Spectrophotometer. Hitachi U-2001), using a 1cm3 cuvet at room temperature. Spectral scanning was carried out between 380-650 nm. Then reduced spectrum of the membrane proteins was taken by addition of Sodium dithionite; an artificial electron donor.

Pyridine Spectra of Membrane Proteins

The reaction mixture contained 2.5ml membrane proteins, 0.5ml of 2N NaOH, 0.5ml pyridine and about 50mg of $Na_2S_2O_4$ (act as reducing agent) in the sample cuvet and took the pyridine spectrum of the membrane and cytoplasmic proteins between 380nm-650nm range. Pyridine ferrohaemochrome confirms the presence of heme c in the Cytochromes.

SQR Activity of Membrane Proteins

Succinate-Quinone-Oxidoreductase (SQR) is found in complex (II) of respiratory chain and linked with cytochrome b. SQR activity was measured spectrophotometrically (CICIL/UV-Visible Spectrophotometer. Hitachi U-2001, Japan). The oxidation of quinine to Quinol was determined by monitoring the decrease in absorbance at 600nm for 5 min. The reaction mixture contained 9ul of 0.1M EDTA, 3ml of 100mM sodium phosphate buffer (pH 7.4), 159ul of 1mM DCPIP, 60ul of 0.1M sodium succinate. The reaction was initiated by the addition of the enzyme (30-40ul of membrane proteins).

RESULTS AND DISCUSSION

Growth Profile of *Nocardia asteroides*

Effect of incubation time on the growth of *Nocardia asteroides* was checked, after 22 hours rich culture of bacteria was obtained. Before 22 hours metabolic processes were slow and cells actively grew between 20-26 hours. After 26 hours, due to consumption of nutrient medium and less availability of nutrient medium, death of bacteria occurs hence growth rate of bacteria is decreased (Fig. 1).

Absorption Spectra of Reduced Membrane Proteins

Respiratory proteins in the membrane were reduced with the addition of sodium dithionite which exhibited α peak of 561nm at which cytochrome b (cyt b 561). It should be noted in Fig. 2 that the absorbance at that wavelength not only reveals the presence of cytochrome b but also verifies by showing its β and γ peaks at 532nm and 425 in the form of prominent (characteristic peaks at 530 and 428nm respectively) in reduced state as the reduced absorptions is more reliable that the oxidized spectra and showed peaks at 428 nm, 550nm and 561nm in the Fig. 2, which told about the presence of cytochrome b.

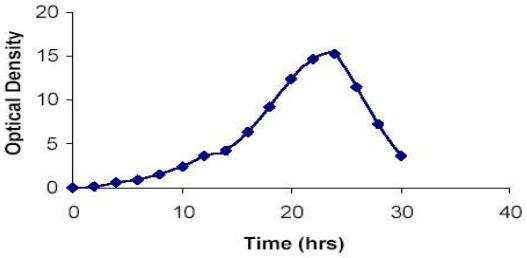

Fig. 1. Growth plot of *Nocardia asteroides*

The characteristic α peak showed the presence of cytochrome b (Otten *et al*., 2001).

The b-type cytochromes are generally occurring in gram positive bacteria function as essential component of respiratory complexes (Kita *et al*., 1989). It is linked with succinate quinone oxidoreductase of complex II of respiratory chain (Qureshi *et al*., 1996).

Pyridine Spectra of Membrane Proteins

When (2N) and a small amount of pyridine, NaOH, $Na_2S_2O_4$ are added in turn to a solution of heme or heme protein, pyridine ferrohaemochrome is formed (Fig 3). As the α peak position is a characteristic of each heme, any kind of heme can be determined by the α peak position of pyridine ferrohaemochrome.

The addition of Sodium dithionite, the reduced spectra of pyridine ferrohaemochrome shows the presence of peaks at 418.5nm, 524.5nm and 555.5nm while the α peak at 586.5nm indicates the presence of cytochrome o localized with heme b (Kita *et al*., 1989).

The β and γ peaks at 418.5nm, 524.5nm and α peak at 555.5nm in Figure-3 verifies the presence of cytochrome c1 with an approximate molecular weight 30,000 (Yu *et al*., 1984).

It showed the presence of two cytochromes having peaks at 555.5nm and 586.0nm.It showed the presence of *cytochrome c* and *cytochrome o* may be present.(Fig. 3)

SQR Activity of membrane proteins

SQR activity shows the presence of Quinone oxidoreductase enzyme complexes with cytochrome b. it shows the presence of complex II in the membrane electron transport chain. The enzymatic properties of the *Nocardia asteroides* SQR were analyzed by using the artificial electron acceptor DCPIP. When succinate was added to the oxidized enzyme, the heme b moiety of the enzyme was almost fully reduced in 5 min and showed decrease in absorbance at 660nm. The suitable detergent for solubilization and stabilization of the SQR was non-ionic detergent Triton X-100. The isolated quinone oxidoreductase enzyme showed very high stability in the presence of non-ionic detergent even at room temperature for the solubilization of the membrane proteins usually inhibited by other detergents like TTFA and HQNO (Qureshi *et al*., 1996).

Bacterial SQR have one or two heme b in the molecule (Kita *et al*. , 1989). On the other hand, it is generally accepted that SQR contains three iron-sulfer clusters, namely [2Fe-2S], [4Fe-4S] and [3Fe-4S] in the molecule (Fig. 4).

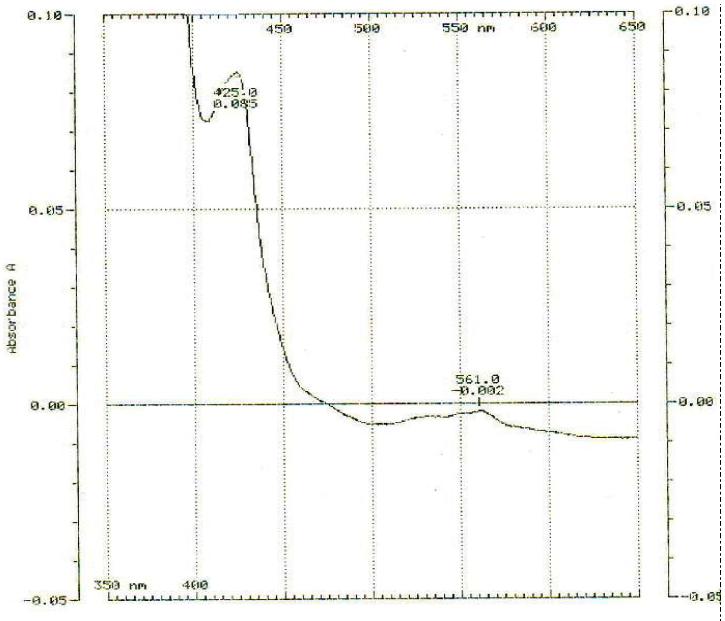

Fig. 2. Absorption Spectra of Membrane Proteins indicating the presence of cytochrome b (cyt b_{561}) in reduced form.

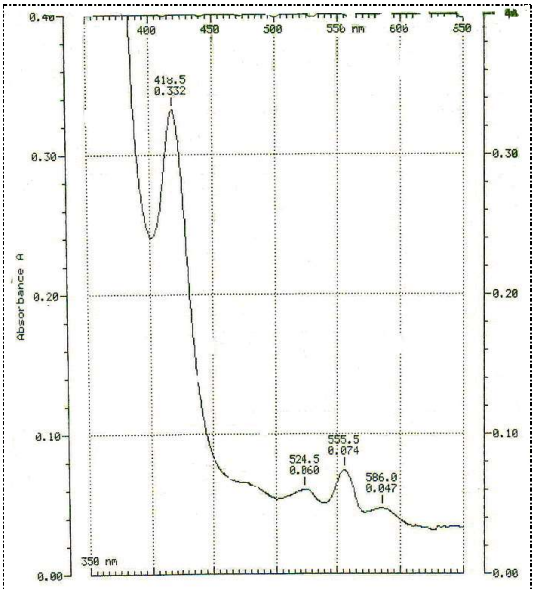

Fig. 3. Pyridine Spectra of Membrane Proteins showing the presence of cytochrome c group (cytochrome c_1) with its characteristic peaks

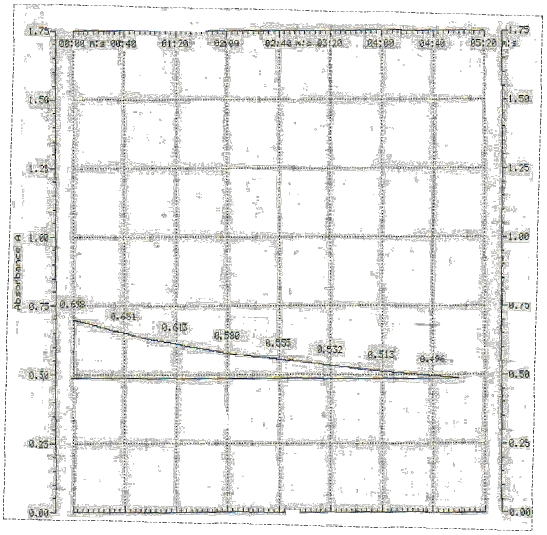

Fig. 4. SQR Activity of Membrane Proteins showing the activity of Quinone oxidoreductase enzyme in respiratory complex II

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