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# Exploration of Respiratory Chain of *Nocardia asteroides*: Purification of Succinate Quinone Oxidoreductase

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**Abstract** *Nocardia asteroides* is a pathogenic bacterium that causes severe pulmonary infections and plays a vital role in HIV development. Its electron transport chain containing cytochromes as electron carriers is still undiscovered. Information regarding cytochromes is important during drug synthesis based on cytochrome inhibitions. In this study we explored the electron transport of *N. asteroides*. Spectroscopic analysis of cytoplasm and membranes isolated from *N. asteroides* indicates the presence of soluble cytochrome-*c*, complex-II and the modified $a_1c_1$ complex as the terminal oxidase. The molecular weight of the respiratory complex-II isolated and purified from the given bacterium was 103 kDa and was composed of three subunits, of 14, 26 and 63 kDa. Complex-II showed symmetrical α-absorption peaks at 561 nm in the reduced state. Spectral analysis revealed the presence of only one heme *b* molecule (14-kDa subunit) in complex-II, which was confirmed by heme staining. Heme *b* content was found to be 9.5 nmol/mg in complex-II. The electron transport chain of *N. asteroides* showed the presence of soluble cytochrome-*c*, cytochrome-$a_1c_1$ and cytochrome-*b*.

**Keywords** Cytochrome · Complex-II · SQR activity · Electron transport chain

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## Introduction

*Nocardia asteriodes* is a member of the family Nocardiaceae, order Actinomycetales (Bordet et al. 1972). It is the most common pathogen that can cause pulmonary disease, followed by *Nocardia brasiliensis*. It is a gram-positive, partially acid-fast, aerobic organism that causes suppurative and granulomatous lesions in humans. An increasing number of *Nocardia* infections have been noted in the past 20 years as a result of increasing numbers of immunocompromised hosts and improvement in laboratory identification techniques (Welsh et al. 1995). This enhancement has also been associated with advances in organ transplantation and increases in HIV infections in the past two decades (Brown-Elliott et al. 2006).

The respiratory electron transfer chain of bacteria is usually composed of enzyme complexes I–IV, cytochromes, ubiquinone and complex V (ATP synthase). Electrons are transferred from NADH and succinate (cytoplasmic electron carriers) to membrane-bound reductases, which in turn reduce quinone. These electrons are then transferred directly to the cytochrome-*c* oxidase pathway or through the quinol-$O_2$ oxidoreductase pathway (Dudkina et al. 2005; Megehee et al. 2006; Schultz and Chan 2001; Adams and Turnbull 1996; Shabbiri et al. 2010). Complex-I of the electron transport chain is composed of NADH-quinone oxidoreductase, complex-II is composed of succinate coenzyme O reductase (SQR), complex-III is generally composed of ubiquinone–cytochrome-*c* oxidoreductase, while cytochrome *c*-oxidase acts as complex-IV, also known as the terminal enzyme of the respiratory chain (Loskovich et al. 2005; Ferguson 2001).

The electron transfer mechanism in gram-positive bacteria is still poorly understood, whereas detailed information on gram-negative bacteria is available (Croal et al.

2004; Rabaey et al. 2007). Several types of cytochrome-*c* of low molecular mass have been reported. In gram-positive bacteria there is much less of a chance of finding soluble cytochrome because of the absence of an outer membrane or periplasmic space. The cytochrome-*c* binding surface of the oxidase is topologically outside the cell (Megehee et al. 2006). However, *N. asteroides* is a partially acid-fast bacterium and can have structural similarities to both gram-positive and -negative bacteria (Beaman 1973), so there might be a chance of finding soluble cytochrome-*c*.

In bacteria, complex-II acts as a dehydrogenase and plays a role in the tricarboxylic acid cycle. It serves as the only direct link between activity in the citric acid cycle and electron transport in membranes. This complex is composed of membrane-bound and water-soluble moieties. Heme cytochrome-*b* is present in a membrane-bound moiety, while flavin adenine dinucleotide (FAD) and iron sulfur clusters are present in a water-soluble moiety (Brown 2000; Cecchini et al. 2002; Doi et al. 1983). Although cytochrome-*b* is generally present in association with SQR, the exact role of this component is yet to be determined. Previous studies have shown that complex-II can contain one or two heme *b*s per SQR (Qureshi et al. 1996; Melo et al. 2004).

Complex-IV or terminal oxidase is usually heme-Cu-oxidase, composed of two catalytic subunits, I and II. Subunit-I contains two heme clusters. The first heme, usually heme "*a*," acts as an electron input device to the second heme "$a_3$" with copper (Michel et al. 1998; Palmer 1987). Subunit-II processes the electron donation and contains a Cu center. These vertically arranged enzymes transfer charge across the membrane, and electrons are passed to $O_2$. Some variations in complex-IV have been reported, such as replacement of heme *b* or *o* with heme *a* and heme *c* in place of $a_3$ (Michel 1998; Berg et al. 2002).

In the present study, we analyzed the cytoplasmic and membrane proteins of *N. asteroides* and report the presence of soluble cytochrome-*c*, membrane-bound heme *b* and terminal oxidase $a_1c_1$. Furthermore, we also purified and characterized membrane-bound complex-II from *N. asteroides*.

## Materials and Methods

### Materials

Sodium succinate and DEAE-Sephadex were purchased from Sigma (St. Louis, MO, USA). DEAE-Bio-Gel and Bio-Gel-P100 were from Bio-Rad (Richmond, CA, USA). Commassie brilliant blue dye was from Fischer (Fairlawn, NJ, USA). Triton X-100, Tris-salt and DCPIP were from Fluka (Buchs, Switzerland). Pyridine and sodium dithionate were from BDH (Toronto, Canada). Nutrient agar, nutrient broth, SDS, EDTA and potassium dochromate were purchased from Merck (Darmstadt, Germany). All other chemicals used in this study were of extra-pure grade.

### Organism and Culture Conditions

*N. asteroides* strain NRRL-B-3828 was kindly given by Dr. Quratulain Syed (Pakistan Council of Scientific and Industrial Research Laboratories Complex, Lahore, Pakistan). *N. asteroides* was cultivated aerobically in nutrient medium at 37°C in conical flasks on a shaker incubator at 250 rpm. Cells were harvested at early exponential stage after 20 h of growth (data not shown) by centrifugation at 6,000 rpm for 20 min and suspended in 50 mM Tris–HCl buffer (pH 8.4) containing 50 mM EDTA.

### Preparation of Membrane

All steps from membrane preparation to enzyme assay were performed with some modifications according to protocols previously described (Shabbiri et al. 2010). Briefly, about 25*g* centrifugally packed frozen cells suspended in a mixture of 400 ml of 50 mM Tris–HCl buffer (pH 8.4) and 20 ml of 50 mM EDTA were disrupted with a sonic oscillator at 15 kHz at 4°C for 20 min with intervals of 2 min. After removal of unbroken cells by centrifugation at 15,000 rpm at 4°C for 15 min, supernatant-containing broken cells were ultracentrifuged at 45,000 rpm for 45 min at 4°C. After centrifugation, cytoplasm was obtained as supernatant, while a reddish brown pellet indicated cell membranes. This pellet was resuspended in a solution mixture containing 25 ml of 50 mM Tris–HCl buffer (pH 8.4), 2.5 ml of 50 mM EDTA and 4.2 ml of 20% (wt/vol) Triton X-100. This suspension was then subjected to ultracentrifugation at 45,000 rpm for 60 min at 4°C. The reddish brown supernatant of membrane proteins was collected and frozen, while the white pellet of membrane lipids was discarded.

### Elution of Membrane Proteins and Purification of Complex-II

The reddish brown supernatant of membrane proteins was subjected to ion-exchange chromatography (Biologic LP system, Bio-Rad) on a DEAE-Sephadex column (4 × 16 cm), washed and equilibrated with 500 ml buffer A (50 mM Tris–HCl buffer, 50 mM EDTA and 1% Triton X-100). Membrane proteins were eluted using a linear gradient solution of 1 liter each of buffer A and buffer B (1 M NaCl and 1% Triton X-100). Elutes were dialyzed after verifying their enzymatic activity against 1 liter of buffer A. Dialyzed elutes were subjected to a second ion-exchange chromatography on a DEAE-Bio-Gel column (1.5 × 8.0 cm) and equilibrated with

buffer A. The adsorbed membrane proteins were eluted with a linear gradient of NaCl (0–1.0 M NaCl) produced in 600 ml of buffer A. Two peaks of fractions 20–25 and 32–37 (5 ml each) were collected and concentrated by lyophilization to an appropriate size. The lyophilized fraction was subjected to gel filtration with a Bio-Gel-P-100 column equilibrated with solution containing 50 mM Tris–HCl buffer (8.4), 1% Triton X-100 and 0.5 M NaCl. The yellowish red fraction of complex-II was collected and used as purified preparation.

### Spectrophotometric Measurements of Cytoplasmic and Membrane Proteins

Absorption spectra of cytoplasm and eluted membrane proteins were studied using a quartz cuvette at room temperature in the visible range (380–700 nm) using a Cecil Instruments (Cambridge, UK) UV–Visible spectrophotometer. Cytoplasm and eluted proteins were oxidized with 1 M potassium dichromate and reduced by adding 1 M of sodium dithionate. Ferrohemochrome spectra were obtained by adding 0.5 ml of 0.2 N NaOH, 1.8 ml distilled water and 0.5 ml of pyridine in 0.7 ml of purified proteins and cytoplasm, then reduced with 500 mg of sodium dithionate.

### Gel Electrophoresis of Complex-II and Molecular Weight Determination of Cytochrome-*b*

Nondenaturing polyacrylamide gel electrophoresis was performed in the presence of 0.4% Triton X-100 at 4°C, while polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed at room temperature as described by Schagger and von Jagow (1987). The presence of native heme (cytochrome-*b*) for complex-II was detected by heme staining reagent (Connelly et al. 1958). The apparent molecular weight of complex-II and cytochrome-*b* was determined with a set of protein markers (PAGE ruler prestained ladder; Fermentas; Burlington, Canada).

### Measurement of Enzymatic Activity

Complex-II (SQR) activity was measured at room temperature spectrophotometrically in time-scanned mode by following the change of absorbance due to reduction of succinate-ubiquinone 1 ($UQ_1$). Succinate-2,6-dichlorophenolindophenol (DCPIP) was used as a terminal electron acceptor, and the oxidation of succinate to fumarate was determined by monitoring the decrease in absorbance at 600 nm for 5 min. The reaction mixture contained 3 ml 50 mM Tris–HCl buffer (pH 8.4), 9 µl 0.1 M EDTA, 159 µl DCPIP/$UQ_1$ and 60 µl 0.1 M sodium succinate. The reaction was initiated by adding 40 µl isolated purified complex-II.

## Results

### Spectral Properties of Cytoplasmic and Purified Membrane Proteins

The absorption spectrum of the *N. asteroides* crude cytoplasm (Fig. 1a) showed the presence of auto-oxidizable cytochrome, while in reduced spectrum of cytoplasm, the α-peak at 549 nm appeared (Fig. 1b). In pyridine spectrum of cytoplasm (Fig. 1c), the α-peak shifted to 553.5. The absorption spectrum of crude membrane proteins at resting and oxidized stages (Fig. 2a, b) showed one peak each at 409 and 387.5 nm, respectively. Partial purification of

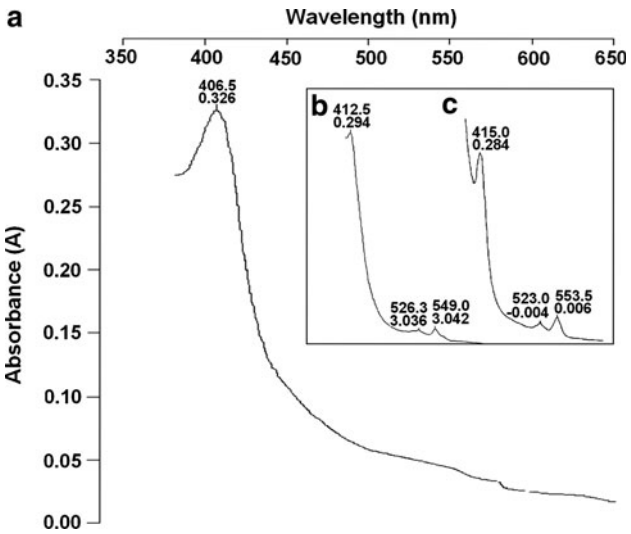


**Fig. 1** Absorption spectrum of the *N. asteroides* cytoplasm as (**a**) crude, (**b**) reduced and (**c**) with pyridine

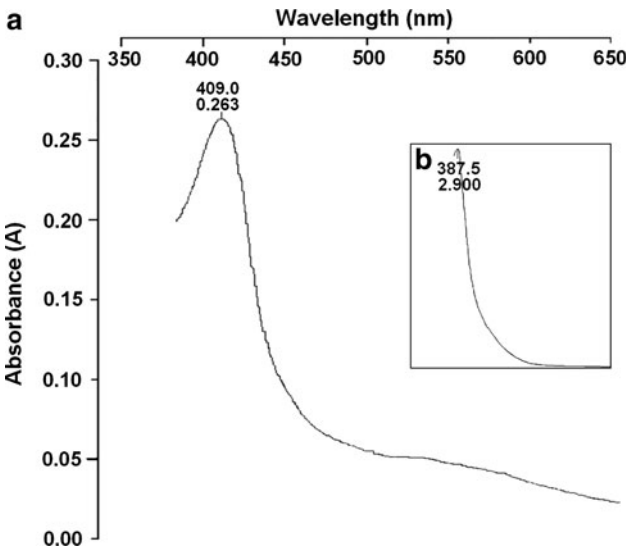


**Fig. 2** Absorption spectrum of crude membrane proteins at resting (**a**) and oxidized (**b**) stages

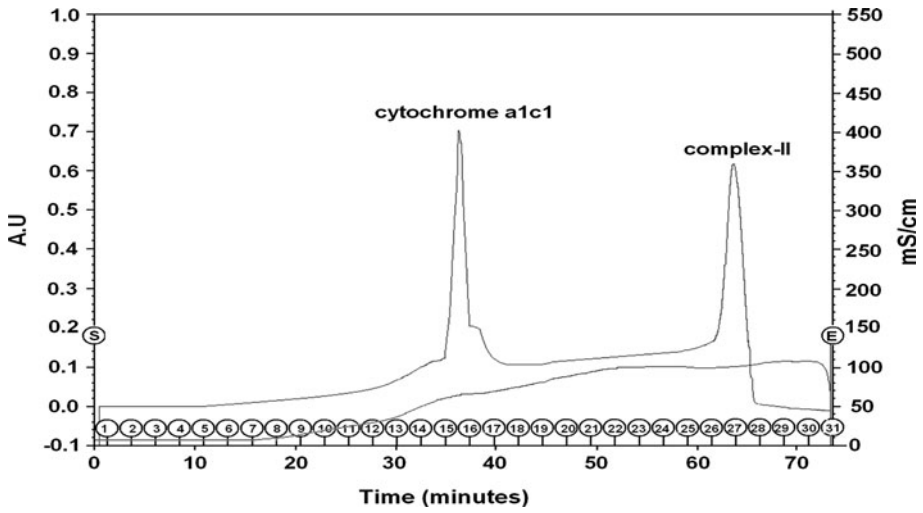


**Fig. 3** Partial purification of membrane proteins with ion-exchange chromatography. Complex-II was eluted using a linear gradient solution of 600 ml each of buffer A containing 1% of Triton X-100 and buffer B containing 1% Triton X-100 and 1.0 M NaCl

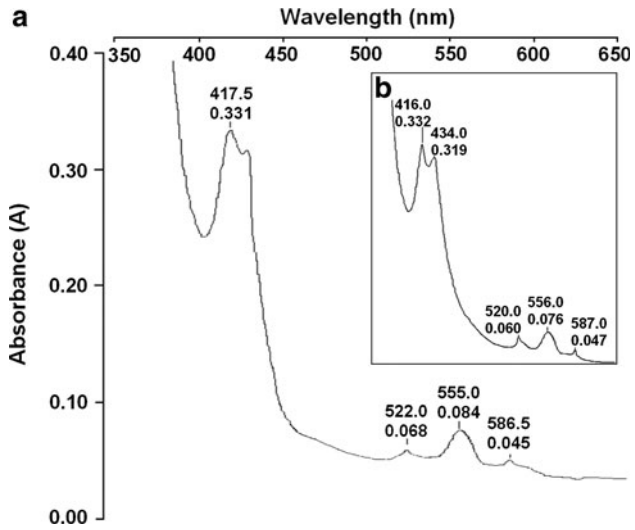


**Fig. 4** Reduced (**a**) and pyridine (**b**) spectra of cytochrome-$a_1c_1$

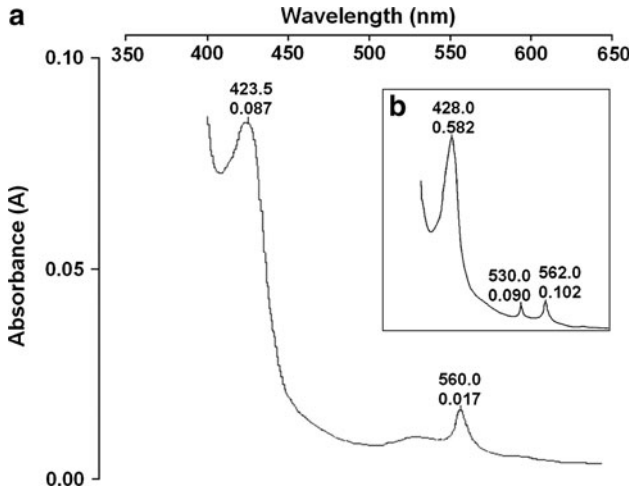


**Fig. 5** Reduced (**a**) and pyridine (**b**) spectra of complex-II

membrane proteins with ion-exchange chromatography showed the presence of two membrane-bound cytochromes (Fig. 3). When purified membrane protein fraction 1 was reduced with sodium dithionate, it showed prominent peaks at 586.0, 555.5, 524.5 and 418.5 nm (Fig. 4a). In pyridine spectra shifting of peaks was observed at 587.0, 556.0, 520 and 416.0 nm, while a new peak at 434.0 nm also appeared (Fig. 4b). Reduced spectra of fraction 2 (Fig. 5a) showed peaks at 561.0 and 425.0 nm. It also showed a trough at 525–535 nm. Pyridine spectra showed three peaks, at 562.0, 530.0 and 428.0 nm (Fig. 5b).

## Purification of Complex-II from *N. asteroides* Strain NRRL-B-3828

The Triton X-100 solublized *N. asteroides* membrane-bound complex-II in active form was purified by ion-exchange chromatography and gel filtration. The representative purification factors and purification yield are summarized in Table 1. Complex-II was found to be purified to an electrophoretically homogenous state when subjected to PAGE and stained with Coomassie brilliant blue, and one single band was observed after heme staining.

## Enzymatic Properties of Complex-II

The enzymatic properties of the complex-II isolated and purified from *N. asteroides* were analyzed spectrophotometrically at 660 nm using artificial electron donor DCPIP. The heme *b* moiety of oxidized complex-II was fully reduced in 5 min when succinate was added to complex-II (Fig. 6). The optimal pH of the reaction was 8.4.

## Gel Electrophoresis of Complex-II

Three protein bands appeared on the 12.5% SDS-PAGE; when isolated, complex-II was analyzed for subunit

**Table 1** Purification of complex-II

| Step | Total vol. (ml) | Total protein (mg) | Total heme *b* (nmol) | Heme *b* protein (nmol/mg) | Yield (%) |
|---|---|---|---|---|---|
| Solublized membranes | 80.0 | 235 | 310 | 1.3 | 100 |
| DEAE-Sephadex | 25.0 | 39 | 119 | 3.05 | 38.34 |
| DEAE-Bio-Gel | 10.0 | 8.7 | 41.3 | 4.08 | 13.3 |
| Gel filtration | 8.0 | 4.2 | 35.6 | 8.5 | 11.48 |
| Purified enzyme | 1.0 | 2.9 | 27.4 | 9.5 | 8.7 |

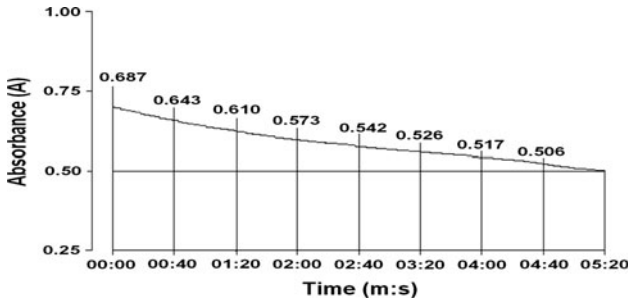


**Fig. 6** Enzyme assay of complex-II from *N. asteroides*. The enzymatic reaction was time-scanned for 5 min at 660 nm to observe the decrease in absorption of DCPIP dye, which acts as artificial electron acceptor in vitro

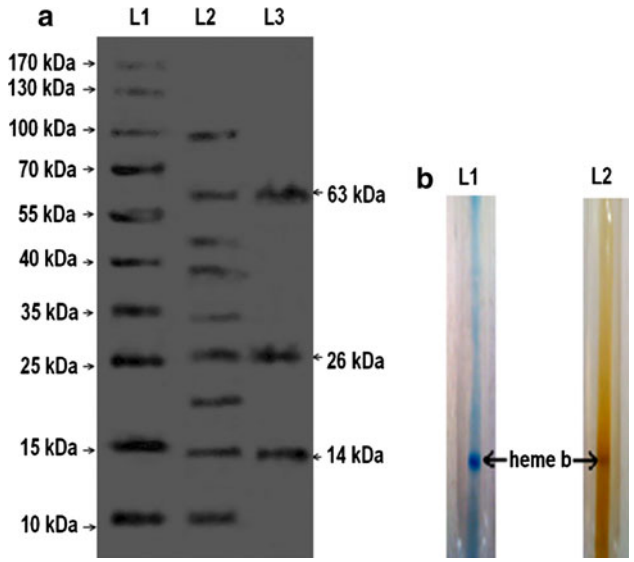


**Fig. 7** SDS-PAGE (**a**) and Native PAGE (**b**) of complex-II isolated from *N. asteroides*. Purified cytochrome-*b* was loaded on a 12.5% gel. The gel was stained with Coomassie brilliant blue after electrophoresis. *Lane 1*, molecular mass marker proteins; *lane 2*, sample 1, partially purified complex-II after ion-exchange chromatography; *lane 3*, sample 2, purified complex-II. Total approximate size of the complex-II was 113 kDa. The molecular masses of three subunits of the *N. asteroides* complex-II were estimated to be 63, 26 and 14 kDa. Purified cytochrome-*b* was run on Native PAGE and stained. **a** Coomassie brilliant blue staining of heme *b*, **b** heme staining of heme *b*

composition and denatured at room temperature for 15 min in 5% mercaptoethanol and 2% SDS (Fig. 7a). The molecular masses of the subunits were estimated to be 63, 26 and 14 kDa, respectively. This purified complex-II was also subjected to Native PAGE, followed by Coomassie brilliant blue and heme staining. A single band corresponding to a molecular mass of 14 kDa was stained (Fig. 7b).

## Discussion

*N. asteroides* belongs to a subgroup of aerobic nocardioform actinomycetes and causes severe human urinary tract diseases. The electron transport chain of *N. asteroides* is still undiscovered. In this study, for the first time, we report the presence of certain cytochromes and purification of complex-II containing cytochrome-*b*.

Absorption spectra of cytoplasm showed the presence of cytochromes (Fig. 1a), while reduced spectra of cytoplasm confirmed the presence of soluble cytochrome-*c* having an α-peak at 549 nm (Fig. 1b). Cytochrome-*c* acts as the direct electron donor for cytochrome-*c* oxidase or for the enzyme which performs functions similar to those performed by oxidase. However, in pyridine spectra, shifting of the α-peak to 553.5 nm indicated the presence of heme *c* (Fig. 1c) (Connelly et al. 1958; Trudinger et al. 1985; Basu et al. 2008).

Absorption spectra of membrane at resting stage showed the presence of some autoredox state cytochromes (Fig. 2a), while the absorption spectra of oxidized membrane revealed that all the respiratory redox proteins were in oxidized stage as the characteristic α-peak of cytochromes was absent (Fig. 2b). The characteristic peak at 586 nm in reduced spectra showed the presence of cytochromes *a* and *c* in combined form. Cytochrome-$a_1c_1$ has been purified from *N. winogradskyi* by Tanaka et al. (1983). Its reduced form shows absorption peaks at 416, 521 and 556 nm (attributed to heme *c*) and at 434 and 587 nm (attributed to heme *a*). This cytochrome was not autoxidizable, so it is not a terminal oxidase as earlier believed. Furthermore, its spectral properties due to heme *a* are really those of $a_1$ (Mogi 2009; Tanaka et al. 1983).

The presence of SQR and any type of cytochrome-*b* has been reported in many bacteria. Cytochrome-*b* is a component of complex-II and is required for electron transfer

from succinate to ubiquinone. Many workers have reported the presence of complex-II and many types of cytochrome-*b* in several bacteria (Shabbiri et al. 2010; Qureshi et al. 1996; Tamegai et al. 1993). In this study we isolated and purified complex-II from *N. asteroides*. Complex-II was highly active and best solubilized in non-ionic detergent Triton X-100. The α-peak at 561.0 nm that appeared during the reduced spectrum of purified fraction 2 revealed the presence of the respiratory bound complex of oxidoreductase (complex-II), which was confirmed by pyridine spectra. These absorption peaks were very close to the isolated complex-II from other bacteria.

Bacterial SQR can contain one or two heme *b* molecules in the complex (Qureshi et al. 1996). *Proteus mirabilis* and *Escherichia coli* complex-II showed only one heme *b*, while *Bacillus subtilis* contains two heme proteins (Shabbiri et al. 2010; Qureshi et al. 1996). A symmetrical α-peak at 562.0 nm revealed the presence of a single cytochrome-*b*, which was confirmed by gel electrophoresis. The molecular weight of the purified complex-II was 103 kDa, very near the purified complex-II by other bacteria (Qureshi et al. 1996; Xin et al. 2009; Yariv et al. 1981). Heme staining of only the 14-kDa subunit confirmed the presence of one heme *b* molecule in *N. asteroides* complex-II. Recovery in the purification on the basis of total heme *b* content was calculated to be about 9.5 nmol/mg, which yields 8.6% of the total solubilized protein. SQR was also very active when analyzed using the artificial electron acceptor DCPIP. A decrease in absorbance of DCPIP in 5 min confirmed the reduction ability of enzyme.

In conclusion, our study revealed that the electron transport chain of *N. asteroides* contains highly active complex-II, while the presence of terminal oxidase $a_1c_1$ and soluble cytochrome-*c* was also observed.

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