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Mitochondrial Response to Osmotic Stress in *Aspergillus candidus*

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Abstract: The respiratory activities of osmotolerant fungus *Aspergillus candidus* were studied to evaluate its response to high osmotic stress. The rate of respiration by the whole cells and mitochondrial fractions increased with elevated sucrose concentrations in the growth medium. The activity of respiratory enzymes such as, succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase, NADH oxidase and succinoxidase was higher in the cells grown in the presence of high sucrose concentrations, ranging from 1.2 -2.1 times higher than the control. Whereas, NADH dehydrogenase exhibited the highest increase at 50 and 80% sucrose, it increased 6 and 13 times respectively higher than in the control. Electron microscopic observations of *A. candidus* grown at high osmotic stress revealed a larger size of mitochondria than in the control grown cells. The mean mitochondrial diameter at 50 and 80% sucrose was approximately 2 fold larger than in the control cells grown at 3% sucrose.

Key words: *Aspergillus candidus*-respiratory, activity- mitochondria, respiratory enzymes

Introduction

Aspergillus candidus is able to tolerate high osmotic stress caused by addition of sucrose to the growth medium up to concentration of 80 (w/v). It has been found in previous work that mitochondria of osmotolerant fungi respond to high osmotic stress caused by either sucrose or NaCl by an increase in their sizes (Parekh and Chhatpar, 1989; Hefnawy, 1993; Hefnawy and Evans, 1998). These observations directed towards the study the respiratory activity of whole cells and its isolated mitochondria under osmotic stress.

Plants respond to osmotic stress by an increase or decrease in respiration rate. There is a decrease in respiration rate of wheat and gram in presence of sodium salts (Sarin, 1961), as well as algae and fresh water ascomycetes (Munda, 1964; Davidson, 1974). While, in some cases the respiration rate increased in presence of NaCl, it was observed to increase of up to 10-33% in peas as a response of NaCl stress (Livne and Levin, 1967).

In filamentous fungi, it was reported that the respiratory system of *Aspergillus sydowii* grown under salinity was more active than in absence of NaCl. The levels of respiratory enzymes were higher in the culture grown in presence of 2M NaCl than in that grown in absence of NaCl (Parekh and Chhatpar, 1989). A high levels of respiratory enzymes and more active respiratory systems were observed in the whole cells and its isolated mitochondria of *Penicillium expansum* grown at high sucrose concentrations (Hefnawy and Evans, 1998). The ability of *Aspergillus candidus* to grow in a wide range of sucrose concentrations and also in the presence of swollen mitochondria under osmotic stress, has made an attention to study the respiratory activity and the level of the respiratory enzymes in the whole cells and mitochondrial fractions and also to confirm these observations in filamentous fungi.

Materials and Methods

Organism, culture condition and medium: Aspergillus candidus was isolated from chicken feed from Egypt. The identity of the isolate was identified by International Mycological Institute UK. The organism was grown in Czapek's liquid medium supplemented with sucrose in concentrations of 3, 50 or 80% (w/v). They were grown in shaken cultures at 28°C for 7 days, harvested, washed with distilled water and blotted with tissue paper.

Mycelial disruption and mitochondrial isolation: Mycelia (2 gm fresh weight) were suspended in a chilled vial containing 0.25 M sucrose solution and sonicated with a microtip in a vibra cell ultrasonic processor at setting 5 for 10 min. at 4°C The homogenate was subjected to centrifugation at 1000 g for 10 min. The supernatant obtained was subjected to centrifugation at 10000 g for 10 min. at 4°C for isolation of mitochondria. This technique was described by (Parekh and Chhatpar, (1989).

Respiratory measurements Oxygen consumption was measured polarographically at 30°C with Clark-type oxygen electrode. A reaction chamber contained 50 mM Tris-HCl buffer (pH 7.4), 2 mM EDTA 7mM magnesium chloride, 50 mM sucrose and a mitochondrial suspension (1 mg protein) or mycelial suspension (10 mg dry. wt.). State 3 oxidation by mitochondria was measured by addition of succinate, 20 mM followed by ADP (200 μM).

The reaction system of succinate dehydrogenase (SDH) contained 250 mM sucrose, 30 mM Tris-HCl buffer (pH 7.4), 20 mM phosphate buffer (pH 7.4), 5 mM magnesium chloride, 1mM KCN, 1 mM phenazine methosulphate (PMS) and 70 μM 2,6 dichlorophenolindophenol (DCIP). The rate of reduction of DCIP was followed at 600 nm. The activity of NADH dehydrogenase was assayed with ferricyanide as electron acceptor. The reaction system was essentially the same as used for SDH except that PMS and DCIP were replaced by 1 mM potassium ferricyanide.

The reaction system for cytochrome oxidase, NADH oxidase, and succinoxidase was essentially the same as for respiratory measurement (described before) except that used for cytochrome oxidase, 3.6 mM ascorbate and 18.0 μM N, N, N, N tetramethyl p-phenylenediamine was used as substrates. In the case of NADH oxidase, 0.5 mM NADH was used as a substrate instead of succinate.

Enzyme assays: Respiratory enzymes were assayed with mitochondrial pellets obtained after differential centrifugation. Succinate dehydrogenase and NADH dehydrogenase were assayed by the method described by King and Howard (1967). Unit of these enzymes was defined as the amount of enzyme that causes reduction of 1 μM of (DCIP) or $K_3Fe(CN)_6$ respectively. Assays of cytochrome oxidase, NADH oxidase and succinoxidase were checked polarographically. The unit

for these enzymes was defined as the amount of enzyme that causes consumption of 1 ng atom of O_2 per min. at 30 °C.

Electron microscopy: Hyphal tips from the mycelium of *A. candidus* grown in medium with 3, 50 or 80% sucrose were fixed separately for 1 h. in 0.05% sodium phosphate buffer (pH 6.9) containing 1% formaldehyde, 3% glutaraldehyde and 0.05% tannic acid and then washed with 0.1 M sodium phosphate buffer (pH 6.9) for 10 min. Mycelial pieces were treated with 0.5% osmium tetraoxide in 0.1 M sodium phosphate buffer (pH 6.9) and then left overnight at 4°C. Fixed mycelia were dehydrated in an ethanol series and transferred to propylene oxide for 30 min. and embedded in Spurr's resin.

Thin sections were cut with a glass knife and collected on uncoated grids, stained with concentrated uranyl acetate for 5 min. followed by lead citrate for 10 min and observed with a JEOL JEM 100 SX electron microscope.

Results

Aspergillus candidus, an osmotolerant fungus, could grow in concentrations of sucrose up to 80% (w/v). Respiratory activities of the whole cell and mitochondrial fraction were affected by the presence of sucrose in the growth medium. The rate of respiration in the whole cells grown in presence of 50 and 80% sucrose was 2.4 and 3 times respectively higher than in the control cells (Table 1). While, the respiration rate of mitochondrial fraction from mycelium grown at both sucrose concentrations was 1.2 and 1.4 times respectively higher than control cells grown at 3% sucrose. The rate of oxygen consumption by the mitochondrial fraction was higher than in the whole cells, this could be attributed to higher activities of mitochondrial respiratory enzymes.

The activity of mitochondrial enzymes was further checked biochemically. Higher levels and activity of succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase and succinoxidase were observed in 50 and 80% sucrose grown cells. At these sucrose concentrations the activity of these enzymes represented 1.2- 2.1 times higher than in the control. NADH dehydrogenase at 50 and 80% sucrose showed the highest increase, it was 6 and 13 times respectively higher than in the control cells (Table 2)

Electron microscopic observation of *A.candidus* grown at different sucrose concentrations is shown in Fig. 1. Mitochondria of cells grown at 3% sucrose were spherical to cup shaped structures with dense stroma and saccate cristae. Whereas, at 50 and 80% sucrose they were ovoid to spherical structures with electron-transparent stroma and baffle like cristae that were radially oriented. The size and the number of mitochondria increased with elevated sucrose concentrations in the growth medium (Table 3). The mean mitochondrial diameter at 50 and 80% sucrose was approximately 2 fold larger than at 3% sucrose. Whereas, the number of the mitochondria at both sucrose concentration was 1.5 and 1.8 times respectively higher than in the control cell.

Table 1: Respiratory activities of whole cell and mitochondrial fraction from *Aspergillus candidus* grown in Czapek's liquid medium containing 3%, 50% or 80% sucrose (w/v). Data are taken as ng atom /O /min per mg dry wt. for homogenate or mg protein for mitochondrial fraction. Values shown are means ± SE of three investigations.

Sucrose conc. %(w/v)	Whole cell	Mitochondrial fraction
3%	1.1 ± 0.18	2.8 ± 0.19
50%	2.6 ± 0.27	3.3 ± 0.12
80%	3.3 ± 0.7	3.9 ± 0.14

Table 2: The activity of some respiratory enzymes of mitochondrial fraction obtained from *Aspergillus candidus* grown in different sucrose concentrations. Values shown are means ± SE of three investigations.

Source Conc. %(w/v)	Succinate dehydrog-enase	NADH dehydro-genase	Cytochrome oxidase	NADH oxidase	Succinoxid ase
	Units/mg protein		(ng atom O consumed/min/mg protein)		
3%	12±1.04	31±1.6	3.3±0.1	2±0.3	6±0.29
50%	18±0.58	188±4.8	4±0.3	2.8±0.29	8.6±0.44
80%	24±1.2	405±7.7	5.2±0.2	2.3±0.2	12.9±0.27

Table 3: Effect of osmotic stress on the size and the number of mitochondria in *Aspergillus candidus* cells.

Sucrose Conc. %(w/v)	Size of mitochondria (μm)	Number of mitochondria
3%	0.5 ± 0.05	1.5 ± 0.64
50%	0.96 ± 0.14	2.3 ± 0.59
80%	0.95 ± 0.15	2.7 ± 0.59

The results are means of observations of 10 different fields ± SE.

Discussion

The respiratory activities of the whole cell and isolated mitochondria from *A. candidus* increased with increasing osmotic stress of the growth medium, and this could be attributed to higher activities of mitochondrial respiratory enzymes. These results are similar to that have been found in some organisms subjected to osmotic stress caused by NaCl where the rate of respiration and the level of respiratory enzymes had increased with increasing osmotic stress, (Livne and Levin, 1967; Herrera and Lopez, 1983; Emerson, 1969; Khanna *et al.*, 1984).

Levels of respiratory enzymes such as, succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase, NADH oxidase, and succinoxidase were higher in the mitochondrial fraction of *A. candidus* grown at elevated sucrose concentration. The point whether sucrose influences respiratory enzyme activity at synthesis or at activation level, needs further molecular work. Levels of these enzymes were also higher in *Aspergillus sydowii* grown in the presence of 2M NaCl than in that grown in the absence of NaCl (Parekh and Chhatpar, 1989). More active respiratory system was present in mitochondrial fractions obtained from *Penicillium expansum* grown in high sucrose concentrations (Hefnawy and Evans, 1998).

Changes in volume and shape of mitochondria occurred as a result of chemical, osmotic and mechanochemical changes. Swelling and contraction of the mitochondria occurred due to changes in the osmotic pressure (De Robertis *et al.*, 1970). Electron microscopic observations in *A. candidus* grown at high osmotic pressure, showed larger size and swollen mitochondria than control cells grown at 3% sucrose. Same observation was also found in *Aspergillus sydowii* grown in presence of 2M NaCl (Parekh and Chhatper, 1989). Also mitochondria of *Eurotium amstelodami* and *penicillium chrysogenum* exhibited an increase in their size with increasing sucrose concentrations in the growth medium (Hefnawy, 1993).

Microorganisms respond to increased osmotic pressure or reduced water activity by synthesizing and accumulation of compatible solutes such as polyols, these solutes were accumulated internally to equilibrate the cytoplasm osmotically with the surrounding medium (Brown, 1976; Yancey *et al.*, 1982; Hefnawy, 1999). These compatible solutes have been suggested as protectors of enzyme activity at low water activity (Brown, 1978).

It has been found that membrane lipid of *Zygosaccharomyces rouxii* increased in relation to salt concentration in the growth

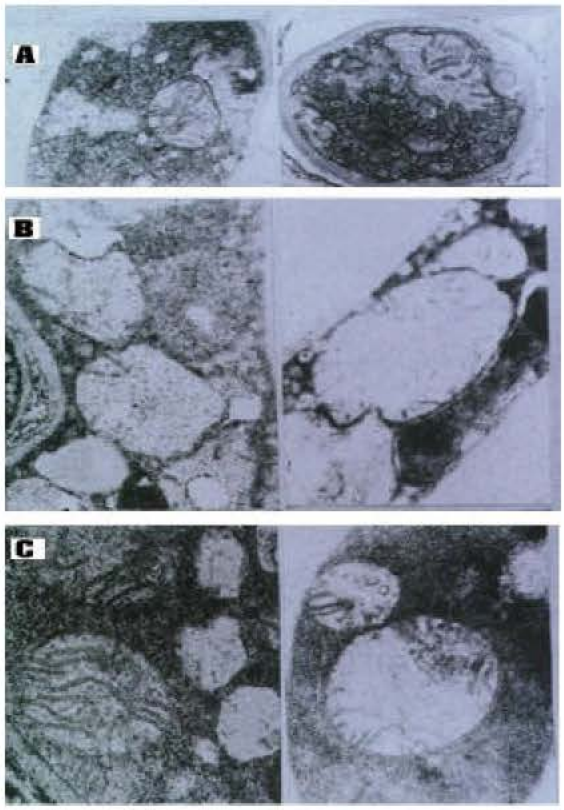

Fig. 1: Electron micrograph of *Aspergillus candidus* grown in the presence of (A) 3% sucrose, (B) 50% sucrose and(C)80% sucrose. (Magnification, X 28000).

media, and act as a protector against high osmotic pressure (Mogi *et al*., 1972; Watanabe and Takakuwa, 1987; Tunblad-Johansson and Adler, 1987). Also the membrane lipid of filamentous fungi showed an increase in its content in presence of NaCl in the growth medium (Hefnawy *et al*., 1997; Hefnawy and Evans, 2001)

It is obvious from the results that the fungal cells respond to high osmotic stress by increasing the levels and activity of respiratory enzymes present in the enlarged mitochondria. High rate of respiration may yields many energy rich molecules (ATP) which may under osmotic stress be utilized in synthesis of compatible solutes and membrane lipids to quanter balance the external osmotic pressure. We should give attention to the role of respiration in controlling osmotolerant food and feed contaminant fungi.

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