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Biochemical characterization of a respiration deficient mutant of *aspergillus ochraecus*

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ABSTRACT

The sensitivity of intact mycelia of the respiration deficient mutant (rd_1) towards cyanide and azide was found to be less compared with that of the wild type and also that of the cell free extract of the mutant; where as sensitivity of the cell free extract of the rd mutant was more or same as the wild type shown. Analysis of lipid composition of the rd mutant and the wild type indicated that total lipid as well as individual lipids of the mutant were changed as compared with that of the wild type. The assay of succinate dehydrogenase and cytochrome oxidase activities in mitochondrial suspension of the respiration deficient mutant reflected that the mutant was respiratory incompetence.

KEYWORDS

Aspergillus ochraceus;
Lipid;
Mitochondrial enzymes;
Respiration deficient mutant.

INTRODUCTION

Understanding mitochondrial role in normal physiology and pathological conditions has proven to be of high importance because mitochondrial dysfunction is connected with a number of respiratory chain disorders. For many years different mutants have been isolated and used as a genetic tool for understanding the metabolic pathways and their regulation in different microorganisms[1,2,3]. Similarly, respiration deficient(rd) mutants of microorganisms have been considered as a microbial tool for understanding the mitochondrial structure, enzyme regulation[4,5] and active transport in some microorganisms mainly in yeast and to some extent in *Neurospora crassa*. A respiratory-deficient mutant isolated from *Kluyveromyces fragilis* was found to be better for ethanol production than the wild type under anaerobic condition[6,7]. A preliminary report regarding rd mutant of *Aspergillus ochraceus* was already published[8]. *Aspergillus*, a filamentus fungus of the ascomycete group has been selected for the present study as mitochondria can be isolated at different stages of fungal differentiation of this genus and *Aspergillus sp*. A lower eukaryote may be considered as a bridge between higher and lower organisms, studies on the respiration-deficient mutant of *Aspergillus ocharceus* may signify an overview idea on the structure and function of mitochondria in lower to higher organisms. In the present study,an attempt has been taken to use the isolated rd mutant of *Aspergillus ochraceus* for investigation of the respiratory activities in presence of azide and KCN, assay of mitochondrial enzymes like succinate dehydrogenase and cytochrome oxidase, and comparative study of mycelial lipid composition.

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MATERIALS AND METHODS

Organisms and growth medium

Mycelia of wild type, *Aspergillus ochraecus* and its respiratory deficient strain (rd) of the same were cultivated by germinating the fresh conidial suspension with optimal conditions in a CD broth as reported in our earlier published paper[8].

For conidial formation, culture was grown on Czapex Dox(CD) agar plates of similar composition to the CD broth solidified with 2% agar.

Measurement of respiratory activities

Preparation of mycelia

Conidial suspension of the wild type and rd_2 mutant were inoculated into 100ml of liquid CD medium in a final concentration of 10^9 conidia /ml and allowed to grow in a reciprocal shaker for 48 hours at 30^0C. The harvested mycelia of both strain was repeatedly washed separately with sterile distilled water and finally with 0.2M Na-phosphate buffer of pH 7.0.The washed mycelia was preserved for the mesurement of oxygen consumption polarographically at -20^0C and also for the preparation of cell-free extract.

Preparation of cell-free extract

The frozen mycelia was grounded with neutral alumina(1:1) in a mortal pestle and extracted with 0.2M Na-phosphate buffer of pH 7.0(5ml/g of mycelia).Cell debris was removed by centrifugation at 3000g for 15min. and preserved for the mesurement of oxygen consumption. All operations were carried out at 4^0C.

Measurement of respiration

The uptake of $O_2$was measured polarographically at 30^0C with Clark type O_2 electrode(YS1 model 55) in 3ml of medium consisting of glucose(80m mol/ L),$MgCl_2$(5m mol/L), sodium phosphate buffer(20m mol/L, pH7.0) and intact mycelia suspension/cell-free extract of wild type and rd_2 mutant. The effect of sodium azide and potassium cyanide on respiration activities were also investigated by using intact mycelia and cell-free extract separately.

Assay of respiratory enzymes

Isolation of mitochondria

Mycelia were collected after 48 hour of growth followed as before and grounded with neutral alumina(1:1) in a mortal pestle and extracted with 0.5M mannitol -4mM EDTA buffer pH 7.3(10ml buffer/1.5g of mycelia).The suspension was thoroughly homogen zied and centrifuged at 1000g for 10min for complete removal of alumina and mycelial debris .The supernatant was taken and centrifuged at 16000g for 20min .the residual pellets were washed thrice with the mannitol-EDTA buffer and the washed mitochondrial pellets was then suspended in approximate buffer for the mitochondrial marker enzymes. 0.1M Na-phosphate buffer and 0.1M K-phosphate buffer were used for the assay of succinate dehydrogenase and citocrome oxidase respectively. All operation were carried out at 4^0C.

Assay of succinate dehydrogenase

The enzyme activity of succinate dehydrogenase was measured spectrophotometrically according to the method of Slater and Bonner[9] using potassium ferricyanide as an electron acceptor and KCN as an inhibitor of cytochrome oxidase acivity.The reaction mixture contained 0.1M Na phosphate buffer(pH 7.0)1% BSA,0.005M potassium ferricyanide,0.01M succinate and110µ g protein in mitochondrial suspension .The optical density was measured spectrophotometrically at420nm against the blank as a mixture water and mitochondrial suspension.

Assay of citochrome oxidase

Cytochrome oxidase was assayed polarographically at 30^0C with Clark type electrode using ascorbic acid as a reducing agent according to a modified method of Slater(1949)[10]. The reaction mixture contained 180µ mol K- phosphate buffer (P^H 7.5),0.15µ mol of citochrome C,60µ mol of ascorbic acid and 450µ g protein in mitochondrial suspension.

Mycelial lipid analysis

Preparation of mycelium

Freshly harvested mycelia of the wild type and rd_2 mutant grown in liquid CD medium stated as before were thoroughly washed separately with sterile distilled water and the washed mycelia was lyophilized. The lyophilized mycelia was preserved for extraction of lipid.

Procedure for extraction of lipid and estimation of individual lipids

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Lyophilized mycelia was powdered and lipid was extracted according to the method of Bligh and Dyer, (1959)[11] as described in detail in the published paper[12]. Separation of polar and neutral lipid as well as estimation of neutral lipid,phospholipid and,sterol were carried out by the methods as stated earlier[12].

Estimation of glycolipid

Total glycolipid in the total lipids was measured according to the method of Neskovic et al.(1972)[13]. The reaction mixture contained 0.5ml of chloroform solution of a known amount of total lipids and 4.5ml orcinol reagent and allowed to incubate at 80^0C for 20min. After cooling the absorbance was measured at 505nmagainst appropriate blank.

RESULTS

It has been found that% inhibition of oxygen uptake in intact mycelia both of the wild type and the respiration deficient mutant (rd_2) increases with the increase of concentration of NaN_3 and KCN; but % inhibition rate towards KCN in rd mutant was found to be approximately 2.5 less than that of the wild type (Figure 2) and towards azide it was found to be approximately 2 times less compared to the wild type(Figure 1).

The cyanide sensitivity towards cell free extract of the wild type and the mutant is shown in figure . It indicates that oxygen uptake in cell free extract of the wild type and the mutant in presence or absence of cyanide increases with the increase of time of cyanide-treatment; but the increase of oxygen uptake rate was found to be higher in cell free extract both of the strain in absence of cyanide and% inhibition of oxygen uptake in presence cyanide was more or less same in the wildtype and its mutant

Figure presents the mycelial lipid composition of the mutant and the wildtype. It was found that the total mycelial lipid of the mutant was about 17% higher than that of the wildtype. The total phospholipid, of rd mutant was about 10% ; sterol and glycolipid were found to be 41% and 58 35% less respectively, neutral lipid% higher than that of the wildtype.

The results of the assay of succinate dehydrogenase and cytochrome oxidase activities in mitochondrial suspension of rd_1 and the wildtype are presented in TABLE 1. It may be noted that the rd_1 mutant possessed 44.58% lower activity compared with that of the wild type ; whereas the activity of cytochrome oxidase in rd_1 mutant remained al most same as the wild type.

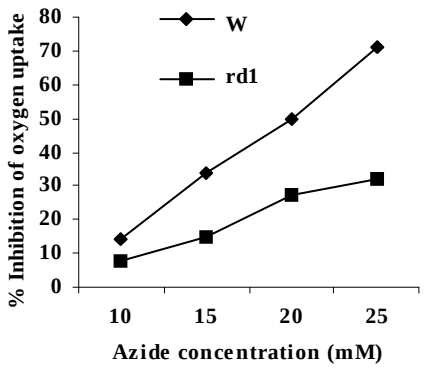

Figure 1 : Effect of sodium azide on oxygen uptake by intact mycelia of wildtype and rd mutant

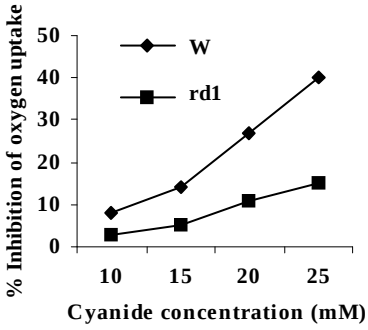

Figure 2 : Effect of KCN on oxygen uptake by intact mycelia of wildtype and rd mutant

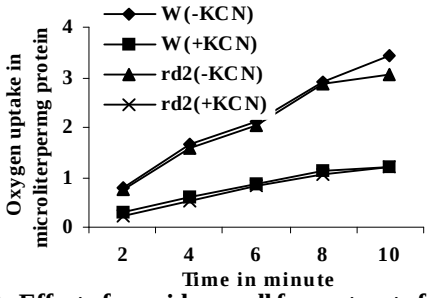

Figure 3 : Effect of cyanide on cell free extract of wildtype and rd mutant

TABLE 1 : Specific activities of succinate dehydrogenase and cytochrome oxidase in mitochondrial suspension of the wildtype and its rd mutant.

	Specific activities of enzymes in cell free extract	
Strain	**Succinate dehydrogenase[a]**	**Cytochrome oxidase[b]**
Wild type	3.14	78.75
rd_1	1.74	74.25

[a]µmole/mg protein/hr, [b]µl O_2 consumed /mg protein /hr

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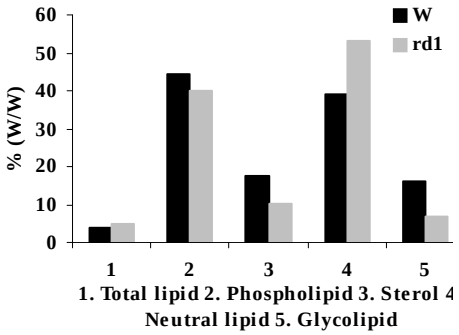

Figure 4 : Mycelial lipid composition of wild type and its rd1 mutant

DISCUSSION

In the paper published earlier presented an account of the isolation of N- methl N'-nitro N-nitroso guanidine (MNNG) induced rd mutants of *Aspergillus ochraceus* showing poor respiratory activity which exhibited small growth on CD medium containing glucose as a source of carbon and negligible or no growth on acetate medium. The sensitivity of intact mycelia of the rd mutant towards cyanide and azide was found to be less compared with that of the wild type and also that of the cell free extract of the mutant; where as sensitivity of the cell free extract of the rd mutant was more or same as the wild type shown. These findings indicated that alteration of membrane permeability of the rd mutant may create insensitivity towards cyanide and azide. The results obtained from the studies of mycelial lipid analysis supported the same as reported in *Aspergillus niger*[14], since the rd mutant possessed 41% and 58.35% less sterol and glycolipid respectively, neutral lipid was found to be about 35% higher than that of the wildtype. Ggycolipids and sterols have already been identified as the important component of the membrane in eukaryotic cells for maintenance of membrane dynamicity[15,16]. The diversity in structures and physical properties of lipids provides a wide variety of possible interactions with proteins that affect their assembly, organization, and function either at the surface of or within membranes[17,18,19]. The assay of succinate dehydrogenase and cytochrome oxidase activities in mtichondrial suspension of rd_1 and the wildtype signified that the rd_1 mutant possessed 44.58% less activity of succinate dehydrogenase whereas citochrome oxidase activity of the mutant remained almost same as the wild type. As observed earlier by Ghosh and Das, (1993)[8] in unchanged color of the rd_1 mutant in the 2,3,5 triphenyl tetrazolium chloride -overlay technique may possibly be due to defect in the synthesis of flovoproteins associated with the rd_1 mutant reflected the results obtained from the assay of mitochondrial enzymes as the rd_1 mutant of *A.ochraceus* showed less activity compared with that of the wild type . It was reported that the rd mutant of *Saccharomyces cerevisiae*[20] showed normal cytochrome *c* oxidase (complex IV) activity and four fold increased lysophospholipid content. All these properties showed by the rd_1 mutant of *Aspergillus ochraceus*,a non-lethal respiratory deficient mutant may be similar to the petite mutant of yeast as reported by Colson et al.[21]. Such type of mutants may be used for understanding genetics and biochemistry of mitochondria in organisms and may also be applied in production of organic acids like citric acid under oxygen limited condition as a respiratory-deficient mutant of *Kluyveromyces fragilis* was used for better production of ethanol under anaerobic condition[6,7].

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