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Studies on the Oxygen Uptake in Respiratory Deficient Mutants of Yeast

II. Role of Lipid Metabolism on Growth

by

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Abstract

It was reported in the previous paper that oxygen remarkably accelerates the growth of the respiratory deficient (RD) mutant of yeast. The growth rates of both RD strain and normal strain under anaerobic condition was found to be increased by supplying ergosterol and unsaturated fatty acid to culture medium and became identical to the level of aerobic growth. Without lipid supply, the cell yield of anaerobic cultures was reduced without lipid and phospholipid content of cells being conspicuously reduced, compared with aerobic cultures. When excess of ergosterol was present in the culture medium, limited supply of oleic acid (under anaerobic condition) and that of oxygen had an equivalent growth promoting effect (on a molar basis) on the RD mutant cells. Linolenic and linolic acids were found more effective than oleic acid. In the presence of excess of TW 80, less amount of oxygen or ergosterol was sufficient to give maximal cell yield than in the foregoing case. The amount of oxygen consumed by the aerobic culture of the RD strain exceeded that presumed to be incorporated into sterols and to be used for the formation of unsaturated bonds in fatty acids.

Introduction

In the previous paper (6) the author reported that the growth of respiratory deficient (RD) mutants of *Saccharomyces cerevisiae* was highly accelerated by molecular oxygen. The activity of oxygen uptake observed in those RD mutants was cyanide-insensitive, and was the largest at the beginning of the exponential growth phase. Since it did not seem to be mediated by the usual cytochrome system and since the energy to be produced by the amount of consumed oxygen was too small to account for the amount of promoted growth, the author suggested that the oxygen might be utilized for the synthesis of some factors essential to the growth.

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In 1953, ANDREASEN and STIER (1, 2) reported that unsaturated fatty acids and ergosterol added to the nutrient medium had a remarkable effect promoting the growth of yeast under anaerobic conditions. And TCHEN and BLOCH (9) and BLOOMFIELD and BLOCH (3) reported that the synthesis of these steroids and unsaturated fatty acids requires molecular oxygen.

The experiments presently reported were carried out to ascertain whether the oxygen taken up by RD mutants is utilized for the biosynthesis of these lipids. In addition, physiological significance of lipid metabolism for the growth of the mutants will be discussed.

Materials and Methods

The strains of *Saccharomyces cerevisiae* used were D 206P, a RD mutant and its parent, respiratory sufficient (RS) strain, D 206, both described in the previous paper (6). The culture medium was also the same as in the previous paper. For supplementing the medium with lipid, an alcoholic solution of ergosterol (12mg/l) and Tween 80 (polyoxymethylene sorbitan monoleate)(2.6ml/l) was added to the medium after sterilization. In case oleic, linolic or linolenic acid was used as the source of unsaturated acid, an amount of alcoholic solution neutralized with IN NaOH was added together with Tween 60 (polyoxymethylene sorbitane monolaurate) (2.6ml/l) to the medium.

Aerobic cultivation and anaerobic cultivation were carried out by bubbling air and nitrogen, respectively. For culturing with a limited amount of oxygen, a definite amount of the medium aerated vigorously at 0°C for 15 minutes was added to a bottled medium whch after being kept at 100°C for 30 minutes was cooled to 30°C under a layer of liquid paraffin. The incubation temperature was 30°C in all experiments.

The growth was measured by counting the cell number with Thoma's counting chamber or by nephelometry using Coleman nephelocolorimeter. Dry weight of cells was determined after drying for 72 hours at 98°C. Oxygen uptake was measured manometrically or polarometrically.

For the analysis of lipid content, wet cells were extracted thrice each time with ten volumes of chloroform-methanol (3:1 v/v) for 10 minutes at 52° C. Organic layers were combined and washed with 0.2 volume of 0.02M $MgCl_2$ three times and air-dried at 40°C, followed by drying in a desiccator under nitrogen at room temperature and weighing. This was regarded as the total lipid.

Alcoholic solution of total lipid was hydrolyzed by KOH for 30 minutes on a boiling water bath and non- saponifiable lipid was extracted with dimethyl ether. The amount of steroid was analyzed with this fraction.

Saponifiable fatty acids were extracted with dimethylether from the hydrolyzate acidified with HCl. The number of double bonds was determined with pyridinic bromide by the method of YASUDA (11). During these processes loss of unsaturated fatty acids was less than 10 per cent.

For the separation of phospholipid, about 60 mg of the total lipid was dissolved

in dimethylether and poured on to a silicic acid column (2 cm x 10 cm). Phospholipid passed through the column. The column was washed with three times of its volume of dimethylether and the effluent and washings were combined and dried in a desicaator. The fraction obtained was regarded as the phospholipid. The column was further washed with more than 200 ml of petroleum ether. The eluate contained free fatty acids and neutral lipids. The amount of these lipides (non-phospholipid) was weighed after evaporation and drying in a dessicator.

Glucose content of the medium during cultivation was analyzed by the method of NELSON (7).

Results

Effect of ergosterol and Tween 80 on the growth of the RD mutant was examined under aerobic or anaerobic condition. As shown in Fig. 1, the

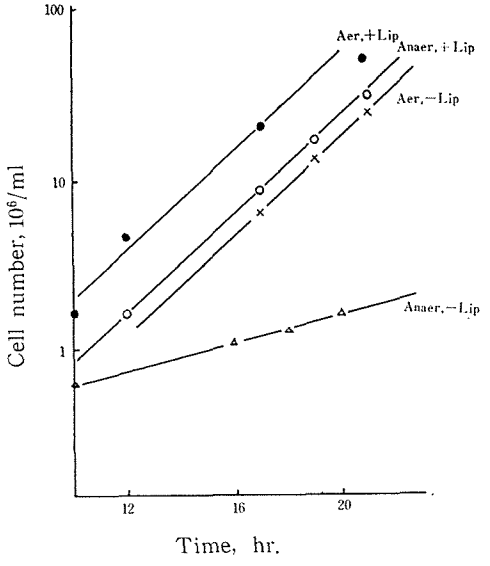

Fig. 1. Growth of RD mutant under aerobic and anaerobic conditions with and without supplementing lipids.

Ergosterol (12mg/l) and TW 80 (2.6ml/l,) as lipids. Bubbling of air and nitrogen for aerobic and anaerobic conditions, respectively. Cells were counted with Thomas' counting chamber.

aerobic growth was not affected by the supplementation of lipids, but under anaerobic condition the growth rate of RD strain was so much accelerated by the lipids as to equal that under aerobic conditions, with a doubling time approximately 1.9 hours. Both ergosterol and Tween 80 were needed for the growth promotion, single application of either one being without effect.

After repeated anaerobic cultures without lipids supply the RD strain became incapable of anaerobic growth as described in the previous paper (6). And as evident in Fig. 2, growth resumed as soon as air was introduced to such a culture. A combination of ergosterol and Tween 80 was supplemented to the anaerobic culture using injection syringe pierced through the rubber-stopper of the culture vessel. These lipids exhibited the same effect on the growth as air

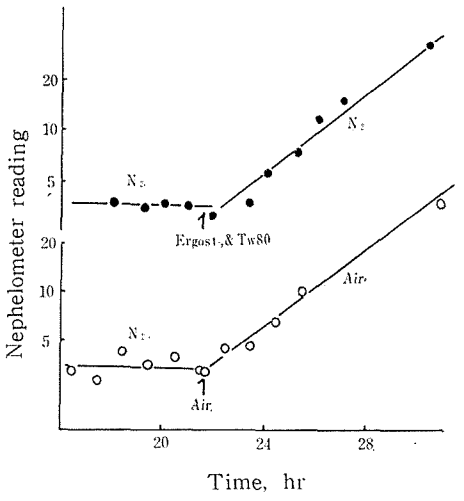

Fig. 2. Effect of oxygen and lipid on the growth of RD mutant.

Cells incapable of growth due to repeated anaerobic subculturing were inoculated. And at arrows a culture was bubbled with air and the other was supplemented with ergosterol (12 mg/1) and TW80 (2.6ml/1).

(Fig.2). Hence it is presumed that lack of oxygen hindered biosynthesis of lipids necessary for the cell growth.

Lipids of cells were analyzed after aerobic and anaerobic cultivation with and without supplying ergosterol and Tween 80. The results are indicated in Table 1. With vigorous aeration cultures reached the stationary phase in about 24 hours,

Table 1. Lipid content of RD mutant cells grown under aerobic and anaerobic conditions.

Culture condition	Tota lipid (mg/g dry wt)	Composition of lipid (% of total lipld) Phospholipid	Non-phospholipid	Iodine number of saponafiable lipid
Aerobic				
21 hrs	19.8	14.9	87.0	53.4
28	18,7	-	-	-
37	26.5	13.0	87.1	-
Anaerobic				
42	14.2	13.4	86.6	26.1
48	15.0	13.0	87.1	-
60	8.1	12.3*	-	-
Anaerobic, Ergosterol & TW 80				
27	18.4	-	-	-
Anaerobic, Ergosterol				
40	14.9	-	-	-

*calculated from the phosphorus content of total lipid.

when the lipid content was approximately 2 per cent of the dry weight, to increase later. Lipid content of the cells in the inciplent stationary phase (at about 45 hr) of anaerobic culture was 70 to 80 per cent of that of aerobically cultured cells at the corresponding phase of growth. Later, the lipid content decreased conspicuously whereas it increased in the aerated culture. In the cells cultured anaerobically but in the presence of both ergosterol and Tween 80, the lipid content was at the same level as in the aerobic cells, and when only ergosterol was given the lipid content was at the level of no lipid supplementation.

Although no significant difference was found between anaerobically grown and aerobically grown cells in the ratio of phospholipid to other lipid, the iodine number of saponifiable lipid differed much between the two kinds of cells. The amout of unsaturated fatty acids seems to be smaller in the anaerobic cells than the other.

The growth stimulating effect of lipids was compared with that of oxygen. Cells incapable of growth due to repeated anaerobic subculturing were inoculated to the media supplemented with a sufficient amount of ergosterol (12mg/1) and graded amounts of oxygen or oleic acid. The growth after four days incubation without additional oxygen supply is shown in Fig. 3. The growth depended

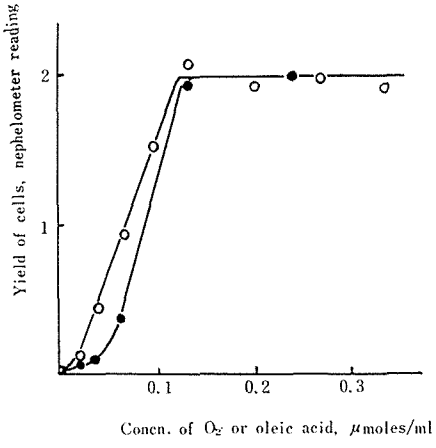

Fig. 3. Effects of amounts of oxygen and oleic acid on the growth of RD cells, given a sufficient amount of ergosterol.

After repeated anaerobic subculturing, one volume of a culture was inoculated to 1,000 volumes each of culture media containing graded amounts of oxygen or oleic acid in addition to a sufficient amount of ergosterol (12mg/1). The cultures were kept at 30° in desiccators filled with Fieser's solution. Cell yield was determined nephelometrically after 4 days' incubation.
○ : O_2, ● : oleic acid

upon the amount of oxygen or oleic acid supplied, to be saturated by about 0.12 μmoles of either oleic acid or oxygen per ml culture. One mole of oleic acid roughly corresponded to one mole of oxygen. A similar experiment was carried out as to the effects of ergosterol and oxygen, using a medium supplemented with a sufficient amount (2.6ml/1) of Tween 80. With this medium the growth promoting effects of ergosterol and oxygen were more remarkable, the cell yield being saturated with 0.03 μmoles of ergosterol or less than 0.02 μmoles of oxygen

per ml culture.

Other unsaturated fatty acids found in yeast cells, such as linolenic acids, had the growth promoting effect when a sufficient amount of ergosterol was present in the medium. The results are represented in Fig. 4. Linolic acid,

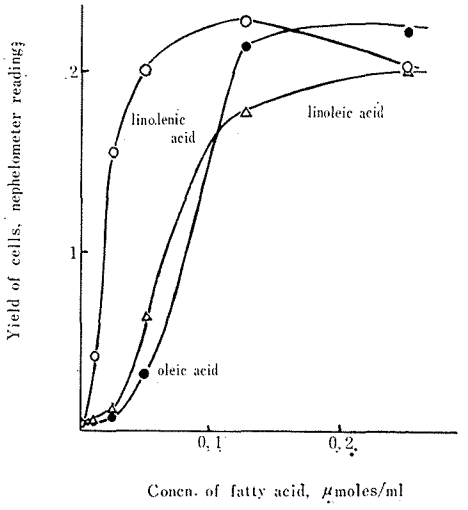

Fig. 4. Effect of various acids on the anaerobic growth of the RD strain, given a sufficient amount of ergosterol.

having two double bonds in a molecule, was more effective than oleic acid at low concentrations and linolenic acid, with three double bonds, was very effective.

In order to see the relation of the amount of growth to the amount of oxygen consumed during the growth, cells were cultured in Warburg vessls. Glucose content of the medium was also analyzed at intervals. As illustrated in Fig. 5, oxygen was consumed as growth proceeded, and approximately 1.1 μmoles oxygen had been consumed per ml culture when the culture reached the stationary phase. Some oxygen uptake was observed at the stationary phase, although glucose in the medium had been completely lost.

Cells were cultured in the same way as in the above but using larger vessels, and the steroid content and the total double bond number of saponifiable lipids in the cells were determined. After 45 hours' cultivation, the double bond content of the lipid was 6.9×10^{-8} moles (consumption of Br_2) per total cells in ml of medium, and the sterol content amounted to 7×10^{-7}g per cells in ml of medium.

The growth promoting effect of ergosterol and unsaturated fatty acids was also observed in the respiratory sufficient strain of yeast. As reproduced in Fig. 6, normal cells grew as well under anaerobic conditions as under aerobic conditions when the medium was supplemented with ergosterol and Tween 80. The RD strain showed the same growth rate as the normal strain when their cultures were vigorously aerated. As shown in Table 2, no remarkable difference

was observed between RD strain and the normal strain in doubling time and cell number, dry weight and mean cell weight at the stationary phase, when the former strain was cultured with vigorous air bubbling or cultured anaerobically with supplementation of proper lipids.

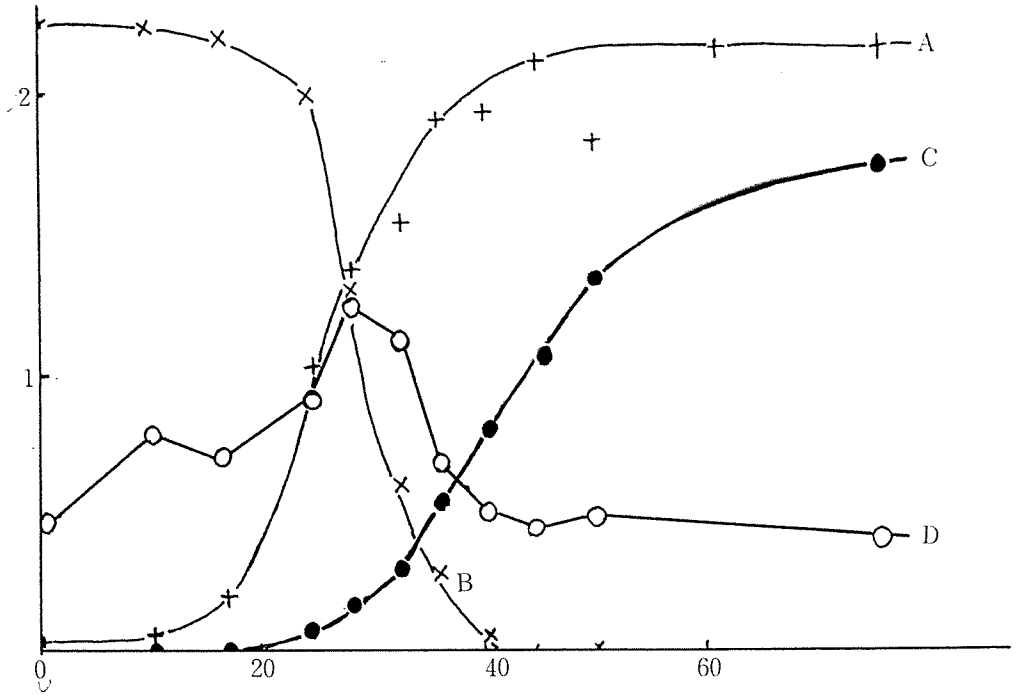

Fig. 5. Growth, glucose consumption, oxygen consumption, and Q_{O_2} of the RD strain in shaking culture.

Ordinate is read by multiplying 10^8/ml for cell count(A), 10^{-1}M for glucose concentration in medium(B), 10^{-6}moles/ml for amount of oxygen consumed(C), and μl O_2/mg dry wt. /hr for Q_{O_2} (D).

Aerobically cultured RD cells were inoculated in 2.0 ml of culture medium contained in Warburg vessel, and shaken. Oxygen consumption was determined manometrically, and cell count, glucose concentration of the medium, and dry weight of cells (as the base for Q_{O_2}) were determined by using up content of a time.

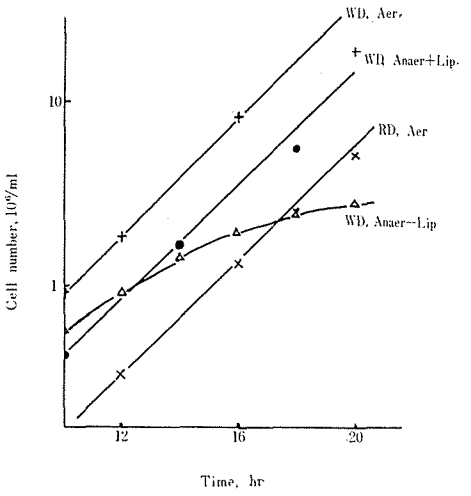

Fig. 6. Effect of lipid enrichment on growth of the normal strain under aerobic and anaerobic conditions.
Air and nitrogen gas were bubbled for aerobic and anaerobic cultures, respectively. Lipid enrich ment, as for Fig. 1.

Table 2. Growth patterns of normal and RD strains cultured for 48 hr aerobically and anaerobically with and without lipid enrichment.

	Respiratory Deficient Strain		Normal Strain		
	Anaerobic		Aerobic		Aerobic
	-Lipid	+Lipid	-Lipid	+Lipid	-Lipid
Doubling time, hr	6.5	2.0	2.0~2.2	1.9	1.9~2.0
Final cell number/ml	4.0×10^6	2.2×10^8	1.8×10^8	-	2.0×10^8
Final cell dry weight mg/ml	0.3	3.7	3.6		3.7
Cell dry weight mg/cell	8×10^{-3}	1.7×10^{-8}	$1.2\sim2\times10^{-8}$		1.9×10^{-8}

Discussion

Experimental results showed that, for the growth promoting effect on the RD yeast strain used, oxygen was replaceable by a combination of ergosterol and an unsaturated fatty acid. Taking into account the facts that oxygen is required for the biosynthesis of these lipids (3, 9) and that the RD strain is capable of synthesizing unsaturated fatty acids under aerobic conditions (9), it may be presumed that at least some portion of oxygen taken up by RD cells are utilized for the synthesis of those lipids.

The lipid content of RD cells did not decrease very much when the cells were cultured under anaerobic conditions (Table 1). However, since the cell yield was markedly smaller than under aerobic conditions, lipid synthesis in the whole culture must have been slow and limited the growth under anaerobic conditions.

when limited amounts of unsaturated fatty acids or ergosterol were given together with a sufficient amount of counterpart, the cell yield of anaerobic cultures depended on the amount of limiting lipid. Similarly, when limited amounts of oxygen were given the cell yield was proportional to the amount of oxygen. When the minimal amounts of oxygen and of oleic acid which could support the maximal cell yield was compared, one mole of oxygen roughly corresponds to one mole of oleic acid. This might suggest that one mole of oxygen is used to synthesize one mole equivalent of double bond in fatty acids, and, if so, that nearly all the oxygen taken up by RD cells under these conditions is utilized for the synthesis of unsaturated fatty acids. When linolic or linolenic acid was used instead of oleic acid in the above experiments, linolic acid was a little more effective than oleic acid and linolenic acid was approximately three times more effective than oleic acid, in the range of their amounts limiting the growth. Unlike these results ANDREASEN et al (2) reported that linolenic acid was less effective than linolic acid or oleic acid at their suboptimal concentrations. Since they used respiratory competent cells for the experiment, difference in strains may be a plausible reason for the different results. Other possibilities can not be excluded, however. The results that the linolenic acid is more effective than other fatty acids on the anaerobic growth of RD strain is favorable for the presumption that molecular oxygen is necessary for the synthesis of unsaturated fatty acids.

Unsaturated fatty acids are components of phospholipids which are contained in the membrane systems of cells. Ergosterol also is contained in membrane systems. The importance of phospholipid is inferred from the fact that the anaerobic growth without supply of suitable lipids was restricted without remarkable reduction of phospholipid content of cells.

When Tween 80 was given sufficiently, a very small amount of ergosterol or oxygen was necessary to cause maximal yield in anaerobic culture. A smaller amount of ergosterol sufficed the need than unsaturated fatty acid.

HAYAISHI (4) found using ^{18}O that, even in RD mutant of yeast, 0.17 per cent of the oxygen atom in the cell material was derived from molecular oxygen. The oxygen bound to position 3 of ergosterol is reported to originate from molecular oxygen. However, the content of steroids in the cells amounted approximately to 7 $\times 10^{-7}$g/3.6mg cells and its oxygen content is calculated to be at most 3.6 $\times 10^{-8}$g, namely 0.001 per cent of dry weight, which seems to be too little to account for the above mentioned ^{18}O content. The amount of oxygen required to cause the maximal cell growth was found to be 0.12 μmole or 0.02 μmole/ml of medium when excess of ergosterol or excess of Tween 80 was respectively present in the medium. Hence 0.14 μmole of oxygen/ml of medium is seemingly sufficient, but the amount of double bond and ergosterol actually found in the cells was far less. From these reasons the oxygen consumed by RD mutant might participate in some other biochemical processes besides, although these processes have not been specified.

According to KATOH (5), the recognition of oxygen by anaerobically grown wild type yeast to trigger respiratory adaptation is not inhibited by KCN and some other

respiratory inhibitors except carbon monoxide, and the Michelis constant, Km for oxygen in that case is about 10^{-4}M, namely much higher than that of cytochrome oxidase for oxygen. Similar situation was observed for the oxygen uptake of RD strains (6). Therefore, at least some portion of the oxygen taken up by RD cells is presumably utilized through systems similar to those capable of working in the wild strain cells which have become devoid of competent cytochrome system owing to anaerobiosis. Reversion of carbon monoxide inhibition by exposure to light might imply existence of oxygenase containing heme. An oxygenase activity possibly belonging to the cytochrome b type was observed in a segregational RD mutant as reported in the previous paper (6). This oxygenase might participate in the oxygen uptake in the RD mutant. In the wild strain as well as in the RD strain, an addition of suitable lipids to the culture medium enhanced anaerobic growth to the level of aerobic growth under the experimental conditions. And also in the wild strain the absence of lipid supply limited the anaerobic growth but not remarkably the lipid content of cells. Therefore, in both wild and RD strains, the growth retardation under anaerobic conditions may be ascribed to an inhibition of lipid biosynthesis essential to the growth.

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