82. **Tatsuzo Fujii**: Biochemical Studies on Pathogenic Fungi. IV.* Submerged Culture for the Respiratory Study of Dermatophytes.

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In the studies on the metabolism of filamentous fungi many difficulties are encountered which are not experienced in the case of bacteria. The main obstacles frequently encoutered are: a) Cultivations of most of the fungi except yeast-like ones, do not give homogenous single cell suspension and subsequently quantitative handling of the culture becomes difficult; b) the cell wall of the fungus frequently shows a comparatively low permeability to some of the substrates externally added; and c) fungus cells usually contain large amount of reserve material to be oxidized within the cell, thus showing a very high rate of endogenous respiration which makes it difficult to interpret the results of respiratory experiments on the fungus.

To overcome some of the above-mentioned difficulties, Foster[1] recommended submerged culture, in place of the common surface culture, in obtaining fungus cells suitable for metabolic studies. By this culture method, very fine pellets distributed uniformly throughout all parts of the culture fluid are readily obtainable. The pellets thus formed can be brought into suspension in a suitable medium and handled by pipetting just as in the case of cell suspensions of bacteria. Furthermore, the submerged pellets can be regarded as homogenous with respect to metabolic reactions carried out within the cell, as Foster pointed out, whereas in the mycelial pad obtained by surface culture there exists non-homogeneity of biochemical mechanism in some cases, because the surface pad consists of a dense mass of tightly intertwined and meshed hyphal cells and the lower portion is in direct contact with the nutrient fluid but not with air (oxygen) while the upper half is exposed to air but depleted of nutrients.

In an attempt to investigate the metabolic reactions, particularly respiratory systems, functioning within the hyphal cells of dermatophytes, it becomes necessary to find a suitable living cell preparation of the fungus for the experiments. However the author could not find any reports available concerning the submerged culture of dermatophytes. Such a situation led the author to investigate the applicability and the feature of the submerged culture, comparing it with the ordinary surface culture on fluid medium.

Because of simplicity, a shaking machine which accommodates culture flasks was employed to obtain submerged cultures. *Trichophyton gypseum* was used as test organism, and the culture medium was Sabouraud's glucose broth. The details of the method and preparation are described in the experimental part of this paper.

After four days' shaking in an incubator, heavy growth was obtained. Fine white particles of smooth texture appeared throughout the culture fluid. It should be mentioned here that in a surface culture, that is, if the culture flask is left to stand still in an incubator, visible growth is perceived as late as about one week after inoculation, and it takes two weeks or more for the mycelial pad to cover the entire surface of the culture fluid. Thus, in view of the rapidity in harvesting

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1) J. W. Foster: "Chemical Activities of Fungi," 1949.

fungus cells as well as the fineness of the mycelial particles formed, the shaking culture proved to be preferable to surface culture.

When oxygen consumption by the slice of surface pad in the presence of glucose was measured by the conventional Warburg's manometry, it was discovered that the lag phase which appears before the increase in rate of oxygen uptake is exceptionally long, being as long as 9 to 10 hours. However, if the mycelial suspension of the shake culture was employed, the stimulation in oxygen uptake appeared considerably earlier, namely at about 2 to 3 hours after the addition of glucose, as seen from Fig. 1. This shortening of the lag phase, possibly due to the altera-

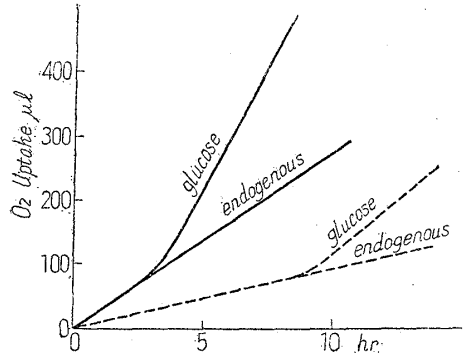

Fig. 1. Difference in Lag Phase of Glucose Oxidation Between Surface and Submerged Culture

Solid line : submerged pellet suspension
Broken line : surface pad slice

tion in cell permeability to substrate, is practically of advantage for the prevention of contamination by other microörganisms as well as for economy of time.

The stimulation of oxygen uptake in the presence of various substrates was measured using both slices and discs of the surface pad respectively. When discs were used, the increase in oxygen uptake in the presence of glucose, acetate, or pyruvate was not great enough to distinguish it from endogenous respiration. All these substrates increased the oxygen consumption of the slice to about two to three folds, indicating clearly that these substances could be oxidized by the fungus cells (Table I). Other sugars and members of the tricarboxylic acid cycle used were

Table I. Oxidation of Various Substrates by Surface and Submerged Culture

Substrates	Oxygen uptake Q_{O_2} (N)		
	Surface culture		Submerged culture suspension
	Disc	Slice	
None	61	135	155
Glucose	82	311	458
Galactose		166	171
Fructose		131	397
Arabinose		123	
Gluconate		113	438
Glucuronate		123	132
Acetate	81	458	415
Pyruvate	67	332	456
Citrate		98	338
Aconitate		133	412
α-Ketoglutarate			422
Succinate		112	489
Fumarate		134	
Malate		121	428
Lactate		153	387
Oxalate		126	131

without stimulative effect. However, the situation became quite different when the pellet suspension of submerged culture was employed. In addition to the above-mentioned three metabolites, fructose, gluconate, lactate, and all the tricarboxylic

acid cycle members tested stimulated the oxygen consumption notably.

These differences in the behavior of the fungus cell to the aerobic breakdown of various substrates may possibly be due to the differerences in cell permeability to substrates externally added, or alternatively may depend upon the degree of exhaustion of the inner reserve materials which are considered to be used by the cell itself more preferentially than the substrates externally added. At any rate, this apparent difference in the utilization of added substrates depending upon the culture method would be significant in selecting fungus preparations for respiratory experiments, and in this respect, too, submerged culture is advantageous in giving a satisfactory preparation.

Table II. Effect of Cyanide upon Endogenous and Glucose Respiration of Surface and Submerged Cultures

KCN concentration (M)	Oxygen uptake Q_{O_2} (N)			
	Surface culture		Submerged culture	
	endogenous	glucose	endogenous	glucose
0	115	254	150	368
10^{-4}	110	232	61	68
10^{-2}	112	238	58	60

This probable alteration in cell permeability was also noted in the inhibition experiments with cyanide and other inhibitors upon respiration. As shown in Table II, cyanide in a 10^{-4} *M.* concentration inhibited the oxygen uptake of endogenous respiration of submerged culture pellet to about one-third of its normal rate, and of glucose respiration to the same level, while in the case of the slice of surface pad no appreciable effect was found even in the presense of higher concentrations of cyanide. The similarity was also found in the case of several other inhibitors. Oxygen uptake of pellet suspension in the presence of glucose decreased to below one-half by the addition of azide (5×10^{-3} *M.*), iodoacetate (5×10^{-3} *M.*) or arsenite (2×10^{-3} *M.*), while these inhibitors were without effect when the slice was used. Thus the slice of surface pad was proved to be inadequate for the inhibition study of respiration, probaly due to the impermeability of its cell wall to some inhibitors.

All these results led the author to conclude that the shake culture (submerged culture) is an excellent method for obtaining cell preparations of *Trichophyton gypseum* suitable for the study of respiration of the fungus, while surface culture failed to produce a suitable mycelial preparation.

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Experimental

Organism—A pleomorphic strain of *Trichophyton gypseum* provided by Prof. Yamamoto, University of Kyoto, was used.

Culture Medium—Though nutritional studies to obtain chemically defined medium for dermatophytes were attempted by many workers as reviewed by Georg,[2] there is no satisfactory synthetic medium to support good and rapid growth of the trichophyton comparable to that on complex medium such as those containing peptone or other natural products. Because of this, Sabouraud's glucose broth as described below was employed :

Glucose	40 g.
Peptone (Polypeptone "Takeda")	20 g.
Water, to make	1,000 cc.

2) L. K. Georg : Trans. N. Y. Acad. Sci., Ser. II, **11**, 281(1949).

Culture Method—One hundred-cc. portion of the fluid medium was placed in a 500-cc. Erlenmeyer flask and after autoclaving at 120° for 15 mins., inoculation was made from a stock culture. These flasks were left to stand in the incubator at 25° for about 2 weeks. This was a surface culture and the mycelial pad which covers the surface of the fluid was used for the experiments. To obtain submerged growth, the 500-cc. flasks which was specially made suitable for shaking and which contained 100 cc. of the medium and small pieces of inocula were accommodated on a shaking machine placed in a room at a constant temperature of 25°. The rate of shaking was 100 strokes per min. and the amplitude was 7.5 cm. Usually after 96 hrs.' cultivation, fine pellets suspended throughout the medium were removed and supplied for the experiments.

Mycelial Preparations for Respiratory Experiment—1) Surface culture: The mycelial pad, after repeated washing and dehydration by pressing, was floated on Krebs-Ringer-phosphate solution for about 24 hrs. at room temp.(starvation culture), thus allowing the cells to exhaust the reserve materials. The part of the pad with comparatively uniform thickness was then bored with a cork borer of 5 mm. diameter, and five "discs" thus obtained (740 ± 63 μg. N) were used per one Warburg flask. Another pad was cut into width of 10 mm. and sliced vertically with a razor blade. Ten such "slices" (174 ± 11 μg. N) were used for one flask.

2) Submerged culture: The pellets, after being subjected to starving culture in just the same way, was ground lightly in a mortar and brought into suspension in Krebs-Ringer-phosphate solution (pH 7.4). Two cc. of this suspension was used for one flask. Nitrogen content was 302 ± 10 μg. per cc. suspension, so the suspension may be considered to be almost homogeneous for practical purposes.

Warburg Manometric Method—Main chamber: 2 cc. pellet suspension (or five discs or ten slices plus 2 cc. Krebs-Ringer-phosphate). Side arm: 0.5 cc. 0.1 *M.* substrate solution (50 μ mole). Center well: 0.3 cc. 10% KOH.

The oxygen uptake was measured by reciprocal shaking at the rate of 90 strokes per min. in a thermostat of 37°.

Summary

Applicability of submerged culture was tested in order to obtain a suitable mycelial preparation of *Trichophyton gypseum* for respiratory study.

The submerged culture supplied a very fine mycelial suspension, which could make quantitative handling of the preparation possible, as early as 4 days after inoculation. The suspension thus obtained was found to be more preferable than the slice or bored disc of the mycelial pad obtained by surface growth, in respiratory experiments, because the former, which showed a considerably shorter lag phase in the oxidation of glucose, was permeable to many substrates which were not apparently oxidized by the surface pad preparation, and was susceptible to respiratory inhibitors such as cyanide, azide, iodoacetate, and arsenite.

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