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Author(s)	TANIGUCHI, Koki; MATSUDA, Kazuo; TERAMURA, Hiroyuki; WAKE, Kazutami
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## Effect of acetic acid on growth of *Aspergillus niger*

Koki TANIGUCHI\*, Kazuo MATSUDA\*\*,  
Hiroyuki TERAMURA\*\*\* and Kazutami WAKE

Acetic acid added to the growth medium during earlier vegetative growth inhibited mycelial growth and sporulation in *Aspergillus niger*. The mycelia incubated with a non-growth acetate medium drastically decreased the dry weight and also the contents of glycogen-like polysaccharide, soluble protein and ribosomal RNA. Consequently, the concentration of amino acids and sugar in the medium increased. In the fungi, it will be noted that the breakdown of macromolecules in mycelia is caused indirectly by acetic acid (more than 0.01 M).

In general acetic acid has been used as the enhancer of sporulation in yeast (CROES, 1967a). In recent years, rapid and abundant sporulation of yeast has been obtained, with cells harvested during logarithmic growth, by employing potassium acetate rather than glucose as a carbon source (ROTH and HALVORSON, 1969). In relation to acetate metabolism, ESPOSITO *et al.* (1969) reported that acetate was consumed by respiration and a part of it incorporated various components during the sporulation of yeast. Using the acetate sporulation medium, many workers observed and quantitated as sequence of biochemical changes in the yeast cell (CROES, 1967a; CROES, 1967; b; HOOPER *et al.*, 1974; SANDO and MIYAKE, 1971). Growth and sporulation of *Aspergillus niger*, as in yeast, may be dependent upon acetate metabolism. To test this possibility, an acetate-shift culture in the fungi was examined.

We report here the inhibition of sporulation and very rapid breakdown of macromolecules in the mycelia caused by excess acetic acid.

### Materials and Methods

*Organism and cultural condition:* *Aspergillus niger* A 1015 was used in this study. Details of the culture were described in an earlier paper

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Present address:

\* Department of Hygiene, Sapporo Medical College, Sapporo, Japan.

\*\* Pola Kasei Kogyo Co., Ltd., Tokyo, Japan.

\*\*\* Kobe High School, Kobe, Japan.

Abbreviations:

PGM, potate-glucose-malt; SSC, 0.15 M NaCl/0.015 M sodium citrate; TCA, trichloro-acetic acid.

(WAKE *et al.*, 1965). A replacement culture was prepared as follows. Cultures were grown for 40 hr in 20 ml or 50 ml of basal medium with a reciprocating shaker (135 strokes per min) at 30 C. Culture medium was replaced by 20 ml or 50 ml of sodium-acetate buffer (pH 3.2, 0.01–0.04 M). Cultures were again incubated with a shaker.

*Analytical measurements:* Glycogen-like polysaccharide was carried out by the method described by ABDER-AKHER and SMITH (1951). Sugar content was measured by the phenol-sulphuric acid method (DUBOIS *et al.*, 1956) with glucose as a standard. Soluble proteins precipitated with TCA and solubilized in 1 N NaOH was measured by the method of LOWRY *et al.* (1951) with bovine serum albumin (fraction V. Armour Pharm. Co.) as a standard.

Polysaccharide, sugar, and protein were prepared with the use of 20 ml culture per determination. Amino acid was measured by the method of YEMM *et al.* (1954) with the use of 1 ml of replacement medium.

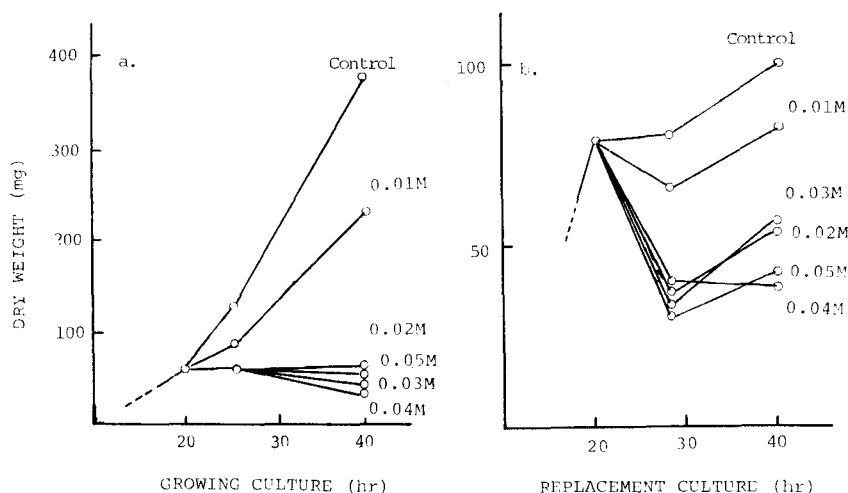
Nucleic acid was extracted from cells grown in 50 ml of the basal medium with a modification of the SDS-phenol method (PHILIPSON, 1961). The mycelia were homogenized in 20 ml of 0.1 M phosphate buffer (pH 7.2) containing 0.1 M NaCl, 2% sodium dodecylsulfate and bentonite (5 mg/ml) at 4 C, then they were stirred with an equal volume of 90% (w/v) phenol for 10 min at 4 C. After centrifugation (10,000 g, 10 min, 4 C), the aqueous phase was removed and phenol phase was re-extracted by stirring it combined with an equal volume of 0.01 M phosphate buffer (pH 7.2) containing 0.1 M NaCl for 5 min then centrifuging the whole. The aqueous phase was combined and deproteinized by combined an equal volume of 90% phenol and stirring the whole. The aqueous solution was mixed with an equal volume of chloroform-isoamylalcohol (24:1, v/v) for 10 min. The upper layer separated on standing was centrifuged at 3,000 g for 5 min at room temperature. The supernatant was dialyzed against 0.1 M SSC for 2 days at 4 C.

Nucleic acids were fractionated with MAK column chromatography (MANDELL and HERSHEY, 1960). Nucleic acids were applied to the column and eluted with a linear gradient of NaCl from 0.1 to 1.6 M. The optical density of each fraction (5 ml) was determined at 260 nm.

DNA was extracted from mycelia grown in 50 ml culture medium by SCHNEIDER's method (1946), and estimated by use of indole reagent (KECK, 1956) with Calf thymus DNA (Sigma Chemical Co.) as a standard.

## Results and Discussion

Surface culture cells were pregrown in a PGM medium. At 20 hr of growth, the culture medium was replaced by various concentration of sodium-



**Fig. 1.** Growth inhibition by acetate in surface culture.

(a) At 20 hr of growth in 50 ml of the PGM medium, various concentrations of acetate was added to the medium. Nothing was added to the control culture.

(b) At 20 hr of growth in 20 ml of the PGM medium, the culture medium was replaced by sterile deionized water (control) or various concentrations of sodium-acetate buffer (pH 3.2).

At the time intervals indicated mycelia were collected by filtration and dry weight was determined after drying at 100 C for one day.

acetate buffer (pH 3.2 acetate medium). As shown in Fig. 1, the dry weight of mycelia during the incubation decreased accompanying an increase in acetic acid concentration. A similar effect was found with butylic acid treat-

**TABLE 1** Contents of glycogen-like polysaccharide of mycelia in acetate replacement culture

Incubation time after the replacement (hr)		Glycogen-like polysaccharide	
		Total (mg)	$\mu$ g per mg dry weight
0		5.8	30.5
5	Control	4.7	27.7
	0.03 M Acetate	2.5	22.3

At 40 hr of growth in 20 ml of PGM medium with a shaker, the medium was replaced by 20 ml of sterile deionized water (control) or 0.03 M acetate. The replacement culture was again shaken for a suitable time. The procedures of glycogen-like polysaccharide extraction and assay were as described in *Materials and Methods*.

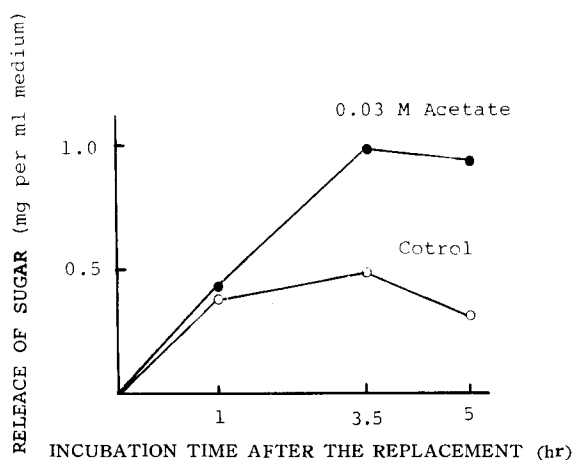


Fig. 2. Release of sugar into medium during the acetate replacement culture.

Culture procedures were the same as in Table 1. The measurements of sugar in the medium at the time intervals indicated were as described in *Materials and Methods*.

ment. However, citrate or succinate had no effect on growth at any concentration. In the surface culture, when the culture medium was replaced by sterile deionized water, the medium arrested vegetative growth such as an increase of DNA content and the dry weight of mycelia, but it did not influence sporulation. However, after replacement with the acetate medium, acetic acid (more than 0.01 M) caused the autolysis of mycelia and inhibited sporulation. A similar effect of acetic acid on growth was observed in the case of shaking the culture cells.

Using the mycelia grown in a shaking culture, we examined changes in the content of carbohydrates, proteins and nucleic acids of mycelia during incubation in acetate medium (0.03 M).

Glycogen-like polysaccharide content in mycelia was reduced to half compared to the control for 5 hr after the replacement by 0.03 M acetate medium. On the other hand, the sugar content in the medium increased (Fig. 2). The sugar content in the medium reached a plateau value in 2.5 hr after replacement. There may be a balance between the amount of released sugar and that of the uptake for reutilization of sugar.

Next we determined the content of soluble protein in the mycelia and medium. Fig. 3 shows the large decrease of soluble protein in the mycelia replaced into the acetate medium. In comparison there was little release of soluble protein into the acetate medium (Table 2). As shown in Fig. 4,

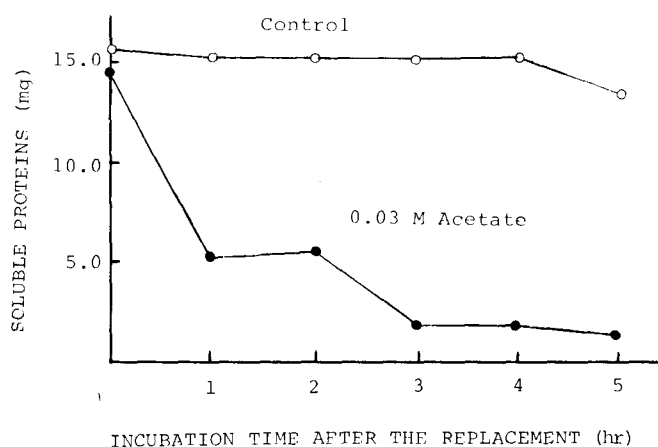


Fig. 3. Changes in the amounts of soluble protein in mycelia during the acetate replacement culture.

Culture procedures were the same as in Table 1. Frozen mycelia were homogenized in the buffer (10 mM Tris-HCl, 2 mM magnesium acetate, 25 mM KCl, pH 7.4). The homogenate was centrifuged at 10,000 g for 20 min and the precipitate was re-extracted twice. TCA was added to the combined extracts at a final concentration of 5%. After standing in the cold for 10 min, the precipitated proteins were measured as described in *Materials and Methods*.

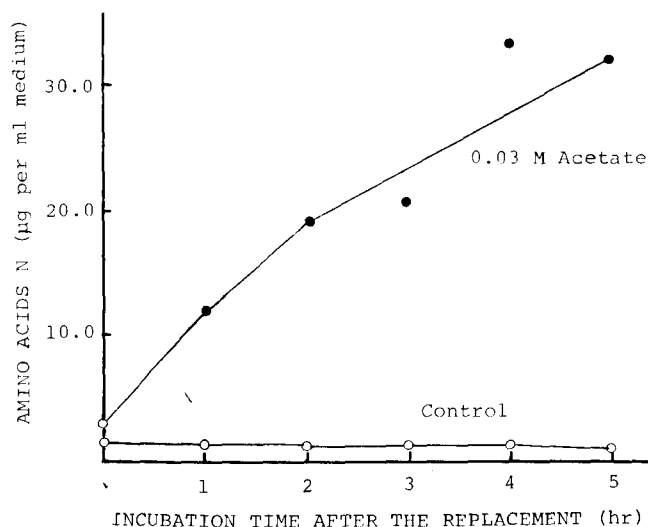
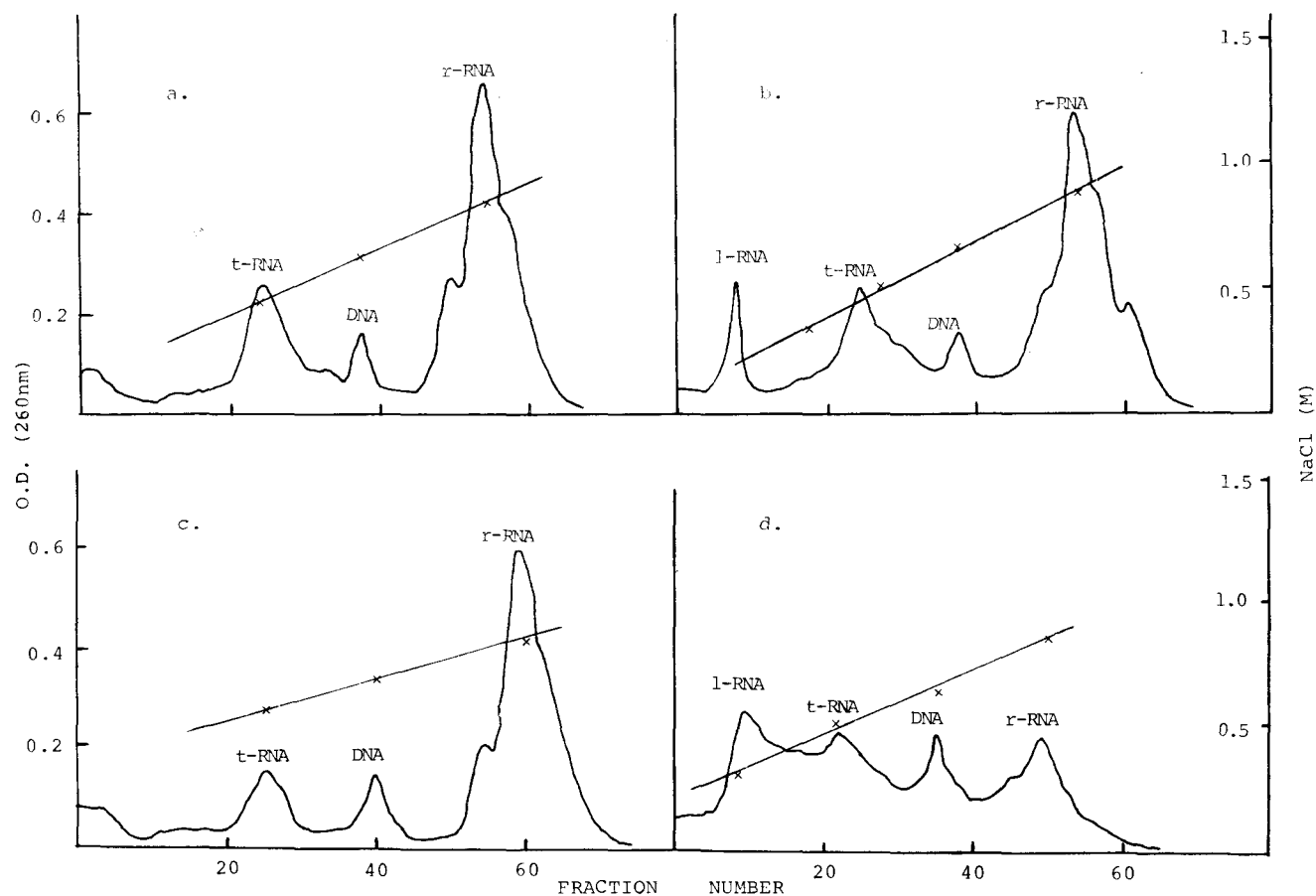


Fig. 4. Release of amino acids into medium during the acetate replacement culture.

Culture procedures were the same as in Table 1. Each 1 ml of medium was measured by ninhydrin reagent as described in *Materials and Methods*.



**Fig. 5.** MAK column fractionation profiles of nucleic acids from mycelia in acetate replacement culture.

Cultures were grown for 40 hr in 50 ml of the basal medium, culture medium was replaced into 50 ml of acetate medium. Assay procedures were described in *Materials and Methods*.

- (a) 2 hr incubation after replacement by sterile deionized water. (b) 2 hr incubation after replacement by acetate (0.03 M).  
 (c) 5 hr incubation after replacement by sterile deionized water. (d) 5 hr incubation after replacement by acetate (0.03 M).

TABLE 2 Changes in the amounts in water soluble protein in medium during the acetate replacement culture

Replacing medium	Incubation time after the replacement (hr)					
	0	1	2	3	4	5
Control	—	5.4*	—	6.3	—	5.1
0.03 M Acetate	—**	2.3	5.3	7.7	2.1	8.4

Culture procedures were the same as in Table 1. To the medium filtered, TCA was added at a final concentration of 5%. The precipitated proteins were measured by Lowry's method (*Materials and Methods*).

\*  $\mu\text{g}$  per ml medium.

\*\* no detectable.

however, the release of amino acid amounted to 30  $\mu\text{g}$  per ml of the medium. In an experiment using bovine albumin as a marker, there was no degradation of protein by acetate in vitro.

To examine the effect on the breakdown of nucleic acid, we fractionated the nucleic acids of the mycelia with MAK column chromatography. With mycelia harvested at 2 hr after the shift from the PGM medium to the acetate medium, the chromatographic profile showed a new peak of low molecular weight RNA, which did not appear in the control (Fig. 5). Moreover, at 5 hr incubation after replacement into the acetate medium, a large breakdown of ribosomal RNA occurred and the low molecular weight RNA fraction enlarged (Fig. 5). Total DNA in the mycelia was constant during incubation in the acetate medium (Table 3). The index of each RNA to DNA is calculated in Table 4. These results indicated that the increase of low molecular weight RNA was related to an increase in the degradation of ribosomal RNA.

Acetate has been routinely used to induce sporulation of yeast (CROES, 1967a). ESPOSITO *et al.* (1969) reported concerning acetate utilization during sporulation of yeast. However, our experiment showed that when the pre-grown cells of *Aspergillus niger* were transferred to an acetate medium, both growth and sporulation were inhibited with acetate. As the degradation of cellular macromolecules and the release of low molecules into the medium were apparent, acetate might play a role in autolysis of *Aspergillus niger*.

On autolysis in fungi, VOSTI and JOSLYN (1954) indicated that there was a meaningful relation between the release of enzymes and autolysis. ARNOLD (1972) investigated p-toluenethiole as an initiator autolysis in baker's yeast and reported that it was useful in the preparation of a cell-wall enzyme. WOOLY and PETERSON (1942) observed that in the autolysis of *Aspergillus sydowi* 63 % of the proteins in the mycelium were degraded into amino



TABLE 3 Changes in DNA contents of mycelia during acetate replacement culture

Replacing medium	Incubation time after the replacement (hr)			
	0	2	3.5	5
Control	835*	877	—	834
0.03 M Acetate	—**	795	788	763

Culture procedures were the same as in Fig. 5. The mycelia per flask at the time intervals indicated was used for assay. The DNA assay was described in *Materials and Methods*.

\*  $\mu$ g.      \*\* not tested.

TABLE 4 Levels of r-RNA, t-RNA, and l-RNA in mycelia after the replacement by acetate

Nucleic acid	Incubation time after the replacement (hr)				
	0	2		5	
	Cont.	Cont.	0.03 M Acetate	Cont.	0.03 M Acetate
DNA	1.0	1.0	1.0	1.0	1.0
l-RNA*	—	—	0.8	—	2.2
t-RNA	3.6	2.9	2.1	1.9	1.9
r-RNA	8.1	6.2	4.9	5.0	2.0

The ratio of each RNA to DNA was expressed on a weight basis. The levels of r-RNA, t-RNA, and l-RNA are calculated from the data of the MAK column chromatography analysis.

\* low-molecular-weight RNA.

acids and that they were released into the medium. In HORIKOSHI's experiment (1958), toluole induced the release of sugar and amino acids into the medium in *Aspergillus oryzae*. He suggested that the process of autolysis induced by toluole was to be divided into two steps; the increase of the permeability of the cell membrane and the degradation of macromolecules by the action of enzymes. In *Aspergillus niger* the release of soluble proteins was small, so acetate may act more as an activator of degradative enzymes than as a modifier of cell wall structure.

Recently some reports on fungal lysosomes have been presented (WILSON *et al.*, 1969). It is well known that denaturation of the lysosomal membrane injured chemically with acetate results in the activation of the lysosomal enzymes. However, we have no information on the lysosome in *Aspergillus niger*.

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