Effects of Triacontanol on Mycelial Growth, Amylase Activity and Lipid Compostion of Zygomycetes

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and
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The growth of mycelia and the production of amylase in a liquid medium were accelerated in the presence of 1-triacontanol (TRIA) added to the medium and the most effective amount of TRIA to be added was 1 ppm. The ratio of triglycerides (TG) was high in the lipid compositions of the mycelia well grown in this medium and having high amylase activity.

Introduction

TRIA is straight aliphatic alcohol, $CH_3(CH_2)_{23}CH_2OH$, being one of higher alcohols and has been studied as a substance showing growth promoting effect in a paddy, corn or the like[1]. This action of TRIA is considered to be caused by the effect promoting the CO_2 fixation in a plant body and TRIA has attracted attention as a new growth promotor increasing the production of crops[2]. Further, it is said that TRIA promotes the growth of Aphyllophorales and Agaricales of Basidiomycetes, especially, *Coriolus versicolor*[3]. However, this growth promoting mechanism is not yet discussed.

We applied TRIA to Zygomycetes and investigated its effect on the growth of the mycelia, the production of amylase and the components of lipids.

Materials and Methods

1. Used strains and its preservation

Used strains are four species of the following Zygomycetes, provided from the Insti-

tute for Fermentation, Osaka (IFO).

Absidia glauca	IFO No. 4002
Actinomucor elegans	No. 6408
Cunninghamella homothallica	No. 6736
Rhizopus stolonifer	No. 4781

A part of mycelia was taken from each of the above mentioned strains and cultured on a koji agar slant medium at 28°C for a week and subsequently preserved at 4°C in a low temperature chamber. Subculture was carried out at every one month to perform culture and preservation in the same way.

2. Medium composition and culture method

① Medium composition

Basal medium		SPY medium (amylase production medium)	
Glucose	40g	Soluble starch	20g
Aspartic acid	2g	Polypepton	30g
KH_2PO_4	0.5g	Yeast extract	2g
$MgSO_4 \cdot 7H_2O$	0.25g	NH_4NO_3	1.8g
Thiamine hydrochloride	0.005g	$MgSO_4 \cdot 7H_2O$	0.3g
Distilled water	1000mℓ	Distilled water	1000mℓ
pH 5.0		pH 5.0	

② Preparation of adding solution: A surfactant is used in order to enhance solubility because TRIA is extremely low in the solubility to water.
At first, a TRIA preserving solution was prepared by using lg of the surfactant (Tween 20) per 0.1mg of pure TRIA (Okamura Seiyu K.K.). Next, distilled water was added to this solution so as to adjust the final concentration of Tween 20 to 0.1% (w/v), that is, the concentration of TRIA to 0.1ppm to prepare a solution for a basal medium.

③ Experimental media were prepared by respectively adding 0.1, 1.0 and 10.0mℓ of this solution to the basal medium 100mℓ and, separately, a control medium was prepared by adding Tween 20 0.1% to the basal medium, to carry out culture in the respective media.

④ Culture method: One hundred mililiters of each of the aforementioned experimental media was received in a 500mℓ Erlenmeyer flask and introduced into an autoclave to

be strerilized for 20min under atmospheric pressure of 1.25kg/cm². Thereafter, the medium in the flask was inoculated with each of the aforementioned preserved strains and shaking culture was carried out at 28°C in a thermostatic chamber using a rotary shaker set to 210rpm [Results 1), 2), 3), 4)]. Further, 60mℓ of each of the aforementioed medium was received in a 300mℓ Erlenmeyer flask to be subjected to sterilization and inoculation in the same way and stationary culture was performed at 26°C for 20 days in a thermostatic chamber [Results 5), 6)].

3. Enzyme preparations[4].

Culture filtrates and mycelial homogenates were used as crude preparations of extra- and intracellular amylase, respectively. Mycelia homogenates were prepared by treating 1.0g of wet mycelium in an Omnimixer (Ivan Sorvall) with 7mℓ of 0.01M potassium phosphate-sodium phosphate buffer (pH 5.6) for 10min at 4°C. The mycelial slurry was poured into a stainless-steel bottle into which 3mℓ of phosphate buffer and 20g of glass beads (0.5mm diameter) had previously been placed, and the bottle was shaken vigorously for 3min by using a Vibrogen cell mill (Edmund Bhuler). The mycelial homogenate was decanted into another container to remove the glass beads, and the volume of the homogenate was adjusted to 50mℓ with the phosphate buffer.

4. Assay of amylase activity[5]

Soluble starch (0.56g as dry weight) was dissolved in about 80mℓ of water and boiled, cooled down to room temperature, and filled up to 100mℓ with 5mℓ of 1M acetate buffer (pH 5.0) and water. A test tube containing 0.9mℓ of the starch solution and 0.1mℓ of the eyzyme solution was incubated at 40°C for 30min. Three mililiters of 3,5-dinitrosalicyic acid (DNS) reagent was added to the tube, and the quantity of glucose formed was determined. One unit (GU/mℓ) of the enzyme activity was defined as the formation of 1 mg/mℓ glucose in the solution under the above conditions.

5. Determination of the reducing sugar with DNS[6]

To 1500mℓ of 4.5% NaOH aqueous solution, 4400mℓ of 1% DNS solution and 1,275g Rochelle salt were added in an Erlenmeyer flask. One hundred and ten mililiters of 10% NaOH solution, 45g of crystalline phenol and 340mℓ of distilled water were mixed in another flask, and filled up to 500mℓ with watar. Forty five grams of $NaHCO_3$ and the DNS solution perpared above were added sequentially to this solution.

The mixture was stirred to dissolve Rochelle salt completely, and was filtered after 2 days. About 6000mℓ of DNS reagent was prepared. Three mililiters of DNS reagent was added to 1.0mℓ of sample solution (0.2～2.0mg glucose) in a test tube. The tube was heated in boiling water for 5min, and cooled quickly under cold running water, to which 16mℓ of water was added and mixed thoroughly. The optical density of the solution at 550nm was measured.

6. Determination of wet weight of mycelia[4)]

Collected and water-washed mycelial pads on Buchner funnels were placed on three sheets of filter paper (Toyo Roshi No. 2) and covered with three sheets of filter paper. The mycelial pads were presssed by rolling a glass bottle, and this treatment was repeated three times. Then, mycelial pads were weighed. Experimental error in this method was ±10mg.

7. Lipid extraction

The procedure used to extract lipids from each sample is shown in Fig. 1. A mixture of methanol and chloroform (1:2 v/v, 50mℓ) was added to each sample; the mixture was homogenized for 2min. An extract was obtained by filtration under reduced pressure, and the residue on the glass filter or paper filter was homogenized again in the same way. The combined methanol-chloroform filtrate was washed with 0.9% NaCl solution to remove the nonlipid contaminants according to Folch's method[7)].

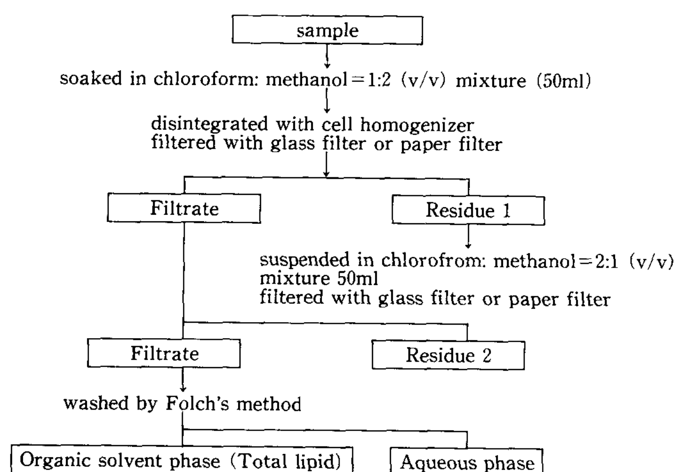

Fig. 1. Procedure for extracting lipids from mycelia.

8 . Thinchrography

The lipid contents were determined by thinchrography. Using a System Instruments Model 5000E intelligent integrator, equipped with an Iatroscan TH-10 Analyzer, a flame ionization detector, to obtain quantitative thin-layer chromatograms. The solvent system used was the same as previously described[8].

9 . Thin layer chromatography

Silica gel 60 plates (Merck Art. 5721) were used. Developing solvents, detecting reagents and standard lipid samples were the same as previously described[9].

10. Gas liquid chromatography of fatty acid methyl esters

Fatty acid methyl esters were prepared by the HCl-methanol method. Gas liquid chromatography was carried out by using a Shimadzu GC-8A gas chromatograph equipped with a ϕ0.25mm×50m capillary column and a flame ionization detector. Silor 5CP column packing was used. The column temperature was 170～230°C. The folw-rate of the N_2-carrier gas was 30mℓ/min at 170°C. The injection port and the detector were operated at 250°C.

Each peak was identified by comparing the retention time with those of the authentic standards (Nippon Oil & Fats Co.): methyl esters of even numbered straight chain saturated acids (C_{14}～C_{18}), palmitoleic acid, oleic acid, linoleic acid and linolenic acid. The rate of the peak areas was calculated with a System Instruments Model 5000A intelligent integrator.

11. Chemicals

The organic solvents used were of reagent grade and were freshly distilled before use. The other organic and inorganic reagents, of analytical grade or the highest quality commercially available, were used as was, that is without distillation.

Results and Discussion

1) Comparison in amylase production of four strains grown on SPY medium: The amylase activity of after the culture on the medium with initial pH 5 for 4 days are shown in Table 1. As a result, the lowering of pH was confirmed in two strains, *A. glauca* and *R. stolonifer*. From this, it is estimated that acids were formed with growth. The mycelia were well grown in all of four strains and the almost same value was

obtained in amylase activity.

Table 1. Amylase production by four species of Zygomycetes

	Final pH	Growth (g wet wt./100mℓ)	Amylase activity (μg/mℓ)	
			Extra-	Intra-
A	5.01	0.70	566.1	16.0
B	3.56	0.65	595.0	18.8
C	3.61	0.66	587.5	13.8
D	4.99	0.51	542.5	14.4

A : *Actinomucor elegans* No. 6408
B : *Rhizopus stolonifer No*. 4781
C : *Absidia glauca* No. 4002
D : *Cunninghamella homothallica* No. 6736

2) Effect of the addition of TRIA on the growth and amylase activity: On the basis of the results shown in Table 1, 10mℓ of TRIA was added to 100mℓ of the SPY medium and cultured for 4 days. The results are shown in Table 2. Compared with the growth on the SPY medium, mycelia were found well-grown for all of four strains, while amylase activity showed no difference.

Table 2. Effect of TRIA on amylase production

	Final pH	Growth (g wet wt./100mℓ)	Amylase activity of culture broth (μg/mℓ)
A	5.01	0.74	586.2
B	3.51	0.72	598.4
C	3.84	0.61	541.0
D	3.62	0.81	591.0

A : *Actinomucor elegans* No. 6408
B : *Rhizopus stolonifer No*. 4781
C : *Absidia glauca* No. 4002
D : *Cunninghamella homothallica* No. 6736

3) Effect due to concentration of TRIA on amylase activity: Next, the effect due to the concentration difference of TRIA was investigated. 0.1, 1.0 and 10.0mℓ of TRIA were added to 100mℓ of the SPY medium to carry out culture for 4 days. The results are shown

in Fig. 2. As shown by Fig. 2, the amylase activity of *A. glauca* gradually decreased with an increase in the addition amount, and a relatively gentle motion was obtained in *R. stolonifer*. Amylase activity was markedly obstructed in *A. elegans* and *C. homothallica* with an increase in the addition amount. However, in the proper addition amount, it was confirmed that amylase activity increased by 10—30% as compared with the growth only on the SPY medium. The best result was obtained when 1.0mℓ of TRIA was added to 100mℓ of the SPY medium.

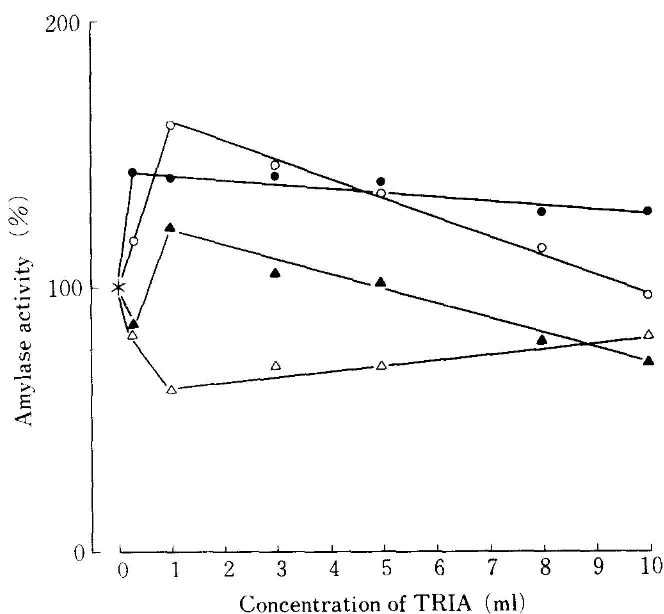

Fig. 2. Effect of Concentration of TRIA on Amylase Production

Symbols: △*Absidia glauca* No. 4002
○*Actinomucor elegans* No. 6408
▲*Cunninghamella hamothallica* No. 6736
●*Rhizopus Stolonifer* No. 4781
*Control

Table 3. Effect of TRIA on amylase production in *C. homothallica* No. 6736

	Final pH	Growth (g wet wt./100mℓ)	Amylase activity (μg/mℓ) Extra-	Intra-
SPY medium	4.88	0.78	522.0	72.5
Tween 20 added medium	4.30	0.87	515.0	50.4
TRIA added medium	3.98	1.14	528.0	67.9

4) Effect of TRIA and Tween 20 on *C. homothallica*: Table 3 shows the results after the culture for 4 days in the case only of the SPY medium and in such a case that 10mℓ of TRIA and 0.1mg of the surfactant (Tween 20) were respectively added to 100mℓ of the SPY medium. The yield of mycelia in the case of the addition of TRIA to the SPY medium was about one point decimal four times as much as that in the case only of the SPY medium. The yield of mycelia in the case of culture in the presence of Tween 20 was almost equal to that in the case only of the SPY medium. Judging from these results, it was cleared that growth of the mycelium was promoted by the addition of TRIA to the SPY medium. On the other hand, amylase activities were almost same in the respective case and an increase in the weight of mycelia and the production of enzyme were not accompanied.

5) Lipid compositions of *C. homothallica* cultured on basal medium containing TRIA or Tween 20: The lipid compositions of mycelia obtained by the 20 days stationary culture only on the basal medium and that on the medium prepared by respectively

Table 4. Lipid compositions of *C. homothallica No.* 6736

	amount of mycelium (wet weight)	TG	FFA	PL	unidentified
			(% of total lipids)		
Basal medium	4.2 g	51.6	14.1	32.1	2.2
Tween 20 added medium	5.4	57.3	11.2	30.5	1.0
TRIA added medium	7.6	66.6	7.6	24.0	2.5

TG : Triacylglycerides
FFA : Free fatty acid
PL : Polar lipid

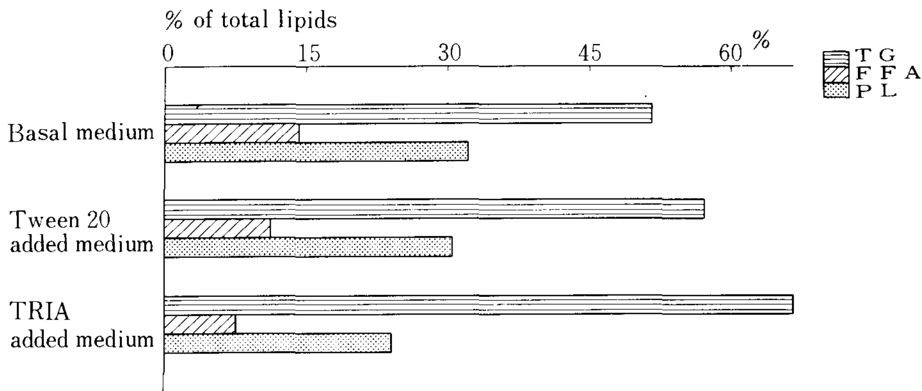

Fig. 3. Lipid compositions of *C. homothallica* No. 6736

adding 0.1mℓ of TRIA and 0.01mg of Tween 20 to 100mℓ of the basal medium are shown in Table 4 and Fig. 3. The amount of mycelia are reduced in the order of TRIA > Tween 20 > only basal medium, and it is estimated that TRIA has growth promoting effect. Further, the TRIA added group is high in the ratio of TG contained in the lipid compositions and different from the other. On the other hand, the basal medium group and the Tween 20 added group were higher than the TRIA added group in the ratios of polar lipid (PL) and free fatty acid (FFA) in the lipid compositions.

6) Fatty acid compositions of *C. homothallica* cultured on medium containing TRIA or Tween 20: The respective mycelia subjected to the analysis of fatty acid was cultured under the same condition as that adapted when the lipid compositions of the above 5). The fatty acid compositions of mycelia were fractionated into TG, FFA and PL to be respectively calculated with respect to TG, FFA and PL. The results are shown in Table 5 and Fig. 4. The main fatty acid compositions of TG, FFA and PL were long chain fatty acids of 16:0, 18:0, 18:1, 18:2, and 18:3. The fatty acid compositions of respectively cultured mycelia were similar each other. Further, the fatty acid compositions of PL is low in the ratio of 18:0 in respective mycelia as compared with the fatty acid compositions of FFA and TG, and different in such a pont that the ratio of 18:3 is high. The fatty acid compositions of FFA is similar to that of TG and it is estimated that the fatty acid compositions of FFA results from TG being a preparatory carobn source.

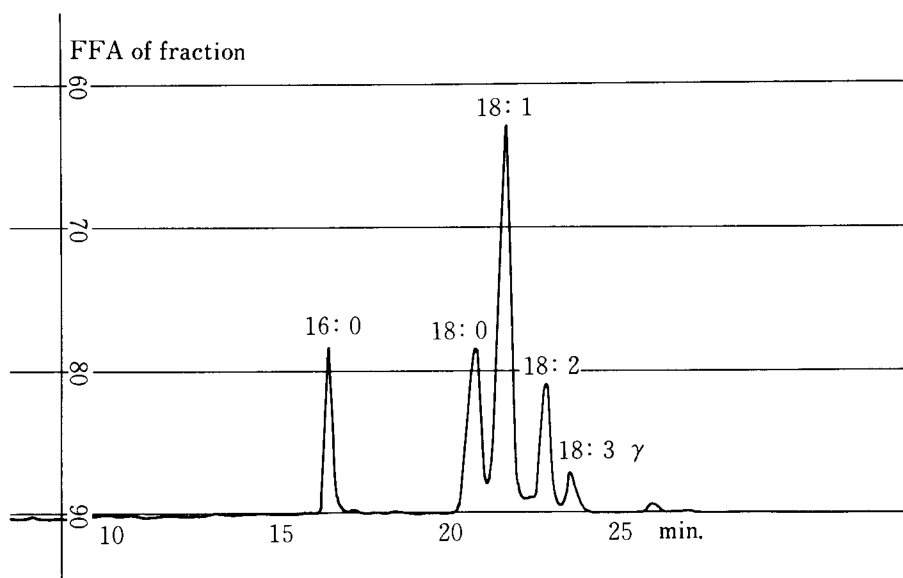

Fig. 4. GLC of fatty acids from the mycelia growth on the TRIA added medium of *C. homothallica* No. 6736

Table 5. Fatty acid compositions of fractionized in *C. homothallica* No. 6736

	fraction	14 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	18 : 3γ
		(% of total fatty acid)						
Basal medium	FFA	0.4	10.3	0.4	18.6	41.8	13.7	4.8
	TG	0.9	13.6	1.1	16.4	37.3	12.7	8.6
	PL	0.5	8.3	2.1	3.9	34.7	14.7	13.5
Tween 20 added medium	FFA	0.6	11.4	0.2	20.2	45.1	11.5	3.7
	TG	0.9	15.6		15.6	38.1	10.9	7.9
	PL	0.8	9.9	1.4	3.3	42.7	14.2	14.4
TRIA added medium	FFA	0.2	11.6	0.1	19.5	43.5	12.2	4.3
	TG	1.1	19.0	1.2	15.9	37.8	10.1	6.4
	PL	0.6	12.4	1.3	0.1	53.5	17.3	11.9

In this experiment related to Zygomycetes, the amount of the mycelia cultured on the TRIA added basal medium was more than that of the mycelia cultured only on the basal medium, in both of shaking culture and stationary culture. This result shows that TRIA has effect on the growth of mycelia, and is considered to be caused by the promotion of CO_2-fixation due to Wood-Werkman reaction in the same way as the case of CO_2 in the cell containig no chlorophyll of plants. That is, the oxidation of an organic acid is actively carried out as the energy source of CO_2-fixation, and oxaloacetic acids, malic acid, fumaric acid or the like are vigorously formed from pyruvic acid being a CO_2 -acceptor and CO_2. By the vigorous production of these acids being the materials of the TCA cycle, it is considered that cell wall constituting substances, proteins and lipids are vigorously formed and growth is promoted.

Further, there is γ-type linoleic acid in the fatty acids of Zygomycetes[10]. This acid is called vitamin F and the precursor of prostaglandin related to fatty acid metabolism. This acid is absent in Basidiomycetes and Ascomycetes.[11] Furthermore, fungi forming strong saccharifying amylase such as *Mucor roxii*, *Rhizopus japonicus* or the like are known in Zygomycetes. Especially, *R. japonicus* is applied to the saccharification of the starchy raw material used in the production of alcohol according to the amylo process or the production of saccharifying enzyme used in the production of glucose according to an enyzymatic saccharifying process, and extremely important fungi in food industry.

Presently, TRIA is investigated with respect to the adding period for obtaining the best effect in the growth, the others of Zygomycetes, and the kind or amount of a surfactant.

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