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Influence of Tween 80 on Lipid Metabolism of an *Aspergillus niger* Strain

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Abstract

Addition of 0.1% of nonionic surface-active Tween 80 to a medium optimized for pectolytic enzyme production of *Aspergillus niger* increased the amount of enzymes excreted by 70%. In the presence of Tween 80 the amount of sterol esters and triacylglycerols was increased. During the course of cultivation the amounts of precursors for ergosterol biosynthesis diminished with an increase of ergosterol. *A. niger* incorporated cholesterol from the medium, partly converting it to cholesterol esters. Sterol esters were formed only with selected fatty acids. Oleic acid, the hydrophobic part of Tween 80, was mainly incorporated in phospholipids and glycolipids.

Index Entries: Sterols; surface-active substances; Tween 80; phospholipids; triacylglycerols; *Aspergillus niger*.

Introduction

Supplementation of fermentation medium with surface-active substances can alter the physiological properties of microorganisms, improve metabolite production, stimulate growth and respiration, and change the organization and permeability of cell membranes *(1)*. The fatty acyl chains of phospholipids and the ratio of saturated to unsaturated fatty acids are among the most important factors modulating the fluidity and integrity of the membrane *(2)*. Sterols are also involved in maintaining the dynamic state of the membrane, and altered levels lead to a change in membrane permeability and membrane-associated functions such as the activities of membrane-bound enzymes *(3,4)*.

During studies on the production of pectolytic enzymes with *Aspergillus niger* in our laboratory, several surface-active substances were tested

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with the aim to increase enzyme excretion. Pectolytic enzyme activities were found to be highest, with the most pronounced increase in polygalacturonase activity, with the addition of the nonionic surfactant Tween 80. To elucidate the influence of Tween 80 on the metabolism of *A. niger*, lipids, particularly fatty acids, sterols, and phospholipids, were analyzed in fungal mycelia.

Materials and Methods

Microorganism and Culture Conditions

The *A. niger* strain A138, selected by screening on pectolytic enzymes after UV irradiation, was used in all experiments. It was maintained on beer-wort agar slants. Spores were suspended in sterile distilled water (approx 10^7 spores mL^{-1}) and 3 mL of this suspension were added as an inoculum to 100 mL of culture medium.

The cultivation medium *(5)* favoring pectolytic enzyme synthesis was used. It contained 14% commercial sugar, 1% dry whey, 0.2% NH_4NO_3, 0.05% $(NH_4)_2SO_4$ dissolved in distilled water with the pH adjusted to 4.5 (control medium). For testing the influence of surfactants, 0.1% of each was added to the control medium. To follow the incorporation of cholesterol, 1 g of yeast extract, 1 g of KH_2PO_4, and 20 mg of cholesterol were added to the cultivation medium instead of 10 g of dry whey.

All experiments were carried out in 500 mL baffled Erlenmeyer flasks with 100 mL of medium. Cultures were incubated on a rotary shaker at 100 rpm and 30°C for 9 d. Citric acid, polygalacturonase, and total lipids were followed on d 3, 6, and 9 with controls and with the addition of different surfactants. Detailed analyses of individual lipids were performed after 70 and 90 h of cultivation with and without Tween 80 addition.

Analysis of A. niger *Mycelium*

After determined times of cultivation, mycelia were isolated by filtration, and washed with 0.5% Tyloxapol and cold tap water. The amounts of citric acid *(6)* and polygalacturonase activity *(7)* were determined in filtrates as described previously.

A small portion of the wet mycelia was dried to a constant weight at 105°C for the determination of fungal dry biomass. The remainder of the mycelia was immediately frozen in liquid nitrogen and thoroughly crushed in a mortar. Total lipids were extracted from the crushed mycelia according to Folch et al. *(8)* and determined gravimetrically.

The total lipid extract was further separated on a silica column. Neutral lipids were eluted using chloroform, then glycolipids were eluted with acetone, and finally phospholipids were eluted using methanol. Neutral lipids were separated with petrolether–diethylether–acetic acid (20:20:0.8, v/v/v) to one-third of a thin-layer chromatography (TLC) plate and then in the same direction with petrolether–diethylether (39.2:0.8, v/v) to the

top of the plate. Amounts of free sterols, sterol esters, and triacylglycerols were determined by densitometry *(9)*.

To separate total sterols and fatty acids, lipid extracts were saponified, extracted with diethylether, and determined gravimetrically *(10)*. Sterols were further separated by TLC in hexane–ethylacetate (60:30, v/v) and visualized with acidic ferric chloride as red-violet spots *(11)*.

GC/MS determinations of sterols were performed with a Hewlett-Packard Series II 5890 gas chromatograph connected to Auto Spec Q Mass spectrometer *(12)*.

Incorporation of cholesterol into the mycelium was tested by growing *A. niger* in the presence of 1.89 cpm/mL of ^{3}H-cholesterol. After 20 h of cultivation, 20 mg of nonlabeled cholesterol was added. After 90 h the mycelium was collected by filtration, thoroughly washed with 0.5% Tyloxapol, and finally with water *(13)*. Total mycelial lipids were extracted and neutral lipids separated by TLC as described above. TLC spots corresponding to sterols and sterol esters were scraped off the plate and transferred to 8 mL of a scintillation mixture (LSC SAFETY Cocktail, J.T. Baker) with 5% of water. Radioactivity was measured in a Tri-Carb scintillation counter.

All results presented are mean values of at least three individual experiments.

Results and Discussion

Exoprotein secretion by microorganisms is known to be stimulated by addition of surface-active substances. The mechanism of stimulation is not completely clear, but the agents appear to cause different alterations in membrane fluidity *(14)*.

We tested a number of surface-active substances (Tween 80, PEG 600, Triton X-100, CHAPS, SDS, and oleic acid) in concentrations from 0.05 to 0.20% aiming to increase pectolytic enzyme excretion. There were differences in the amounts of excreted citric acid (Fig. 1) and in polygalacturonase activity (Fig. 2) dependent on the individual surface-active substance added. The highest amounts of both metabolites were obtained with the addition of 0.1% nonionic Tween 80 to the control medium. This result is in accordance with reports for other fungi and their enzymes. Addition of Tween 80 to the culture medium of *Trichoderma reesei* resulted in increased cellulase secretion *(14)*. Higher excretion of invertase of *Neurospora crassa (15)* and higher ethanol production of *Saccharomyces uvarum (2)* have also been reported. The addition of the other nonionic detergent, PEG (polyethyleneglycol), also increased polygalacturonase production compared to the control, whereas addition of zwitterionic CHAPS or of oleic acid resulted in a small decrease. PEG condensates can permeabilize yeast cells. The extent of this effect was species dependent and may be related to membrane sterol content *(16)*. Triton X100, although a nonionic substance, hindered citric acid and polygalacturonase biosynthesis. Related studies have

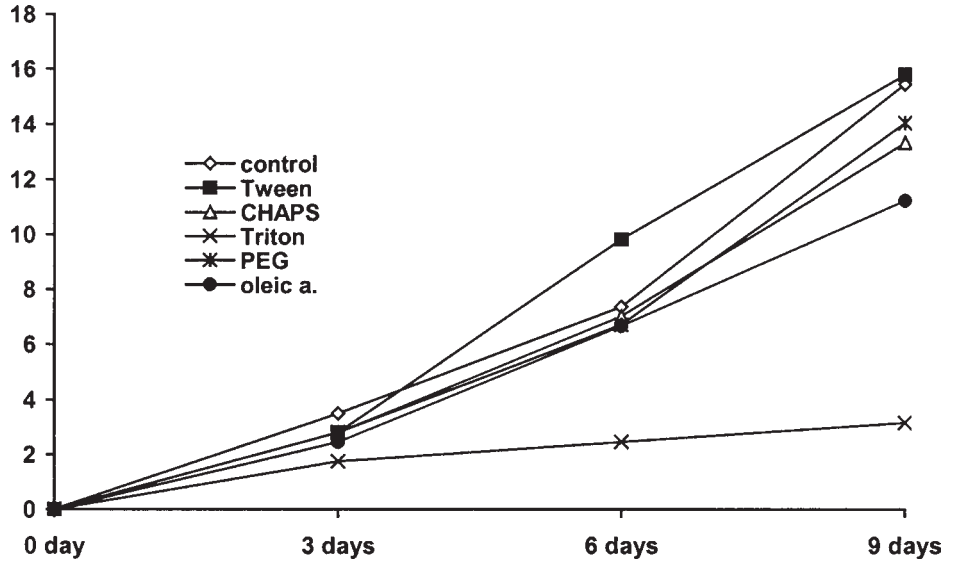

Fig. 1. Citric acid biosynthesis (g/L) during 9 d of cultivation of *A. niger* in control medium or with the addition of 0.1% of different surface active substances.

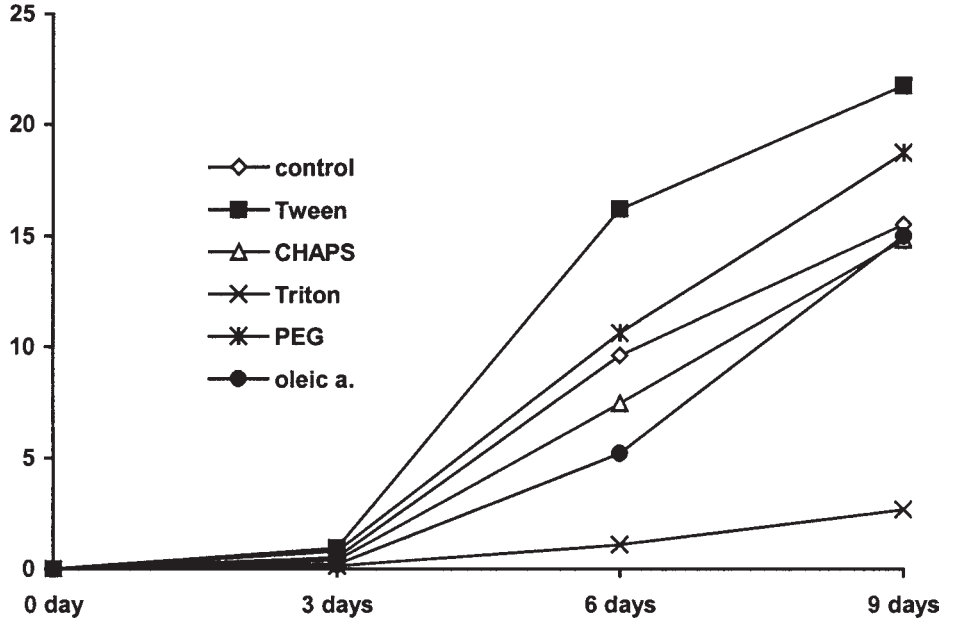

Fig. 2. Polygalacturonase activity (U/mL) during 9 d of cultivation of *A. niger* in the control medium or with 0.1% of individual surface active substances.

shown that Triton X100 was also able to increase cell permeability *(17)*, but was also recognized as a membrane disrupting agent *(18)*. Sodium dodecylsulfate, a strong anionic surface-active substance, prevented growth of the fungus in the tested range of 0.05–0.2%.

Table 1
Amounts of Total Lipids and Individual Fatty Acids in *A. niger* Mycelia with or without the Addition of 0.1% of Surface-Active Substances After 6 d of Cultivation

	Control	Tween	CHAPS	PEG	Triton	Oleic acid
% TL[a]	9.45	9.52	9.76	8.01	5.5	13.4
C14:0	5.6	1.8	6.0	6.6	3.5	3.9
C16:0	23.4	11.7	27.4	27.3	17.6	15.3
C16:1	1.7	3.4	0.3	0.9	0.7	3.6
C18:0	6.3	6.2	7.0	6.8	6.2	3.2
C18:1	36.2	52.3	32.4	32.2	38.7	58.6
C18:2	24.9	22.9	25.1	24.8	29.0	14.2
C18:3	1.8	1.8	1.8	1.4	4.2	1.3
Sat/unsat	0.54	0.24	0.67	0.67	0.37	0.28

[a]%TL, g of total lipids in 100 g of dry mycelium; C14:0, miristic acid; C16:0, palmitic acid; C16:1, pamitinoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; Sat/unsat, ratio between amounts of saturated and unsaturated fatty acids

Results of fatty acid composition of total lipids dependent on the surfactant added are gathered in Table 1. The highest amount of total lipids with the highest amount of oleic acid was detected with the addition of oleic acid to the control medium. It has been reported that oleic acid in a free state in the culture medium was absorbed and incorporated into plasma membranes of *Saccharomyces uvarum* by diffusion and was not further used as a carbon source *(19)*. With *A. niger* it is also possible that oleic acid simply diffused and represented an energy reserve as well as a storage of precursors for other lipid biomolecules, since there was no positive influence regarding citric acid and polygalacturonase activity. Addition of CHAPS and PEG resulted in an amount of total lipids as well as individual fatty acids similar to the control mycelium. Additions of Tween 80, oleic acid, and Triton X100 resulted in a lower ratio between saturated and unsaturated fatty acids as a result of an increased amount of oleic acid with a concomitant drop of palmitic acid. With the addition of Triton X100 the lowest amounts of total lipids, citric acid, and polygalacturonase were detected.

As shown in Table 2, higher polygalacturonase activity and higher amounts of citric acid were secreted when Tween 80 was present. This result is in line with previous results where yeast demonstrated higher protein excretion in the presence of nonionic surface-active substances *(17,20)*. Tween 80 can stimulate enzyme production in many fungi by monitoring both the entrance and exit of compounds from the cell. It was the most effective surfactant in stimulating the release of enzymes of the cellulase complex in *Neurospora crassa (21)*.

Because oleic acid is a part of the Tween 80 molecule, we expected that the lipid composition of *A. niger* cells grown in its presence might be different to that growing without the surfactant. To elucidate the effect of Tween 80 the lipid composition of *A. niger* mycelium grown with or without Tween 80

Table 2
Amount of Fungal Biomass, Citric Acid, and Polygalacturonase Activity in the Presence or Absence of Tween 80

	Control		0.1% Tween 80	
	70 h	90 h	70 h	90 h
Dry biomass (g/L)	3.31 ± 0.39	7.06 ± 0.56	3.09 ± 0.22	5.41 ± 0.50
Citric acid (g/g db)	0.42 ± 0.15	0.99 ± 0.20	0.91 ± 0.13	2.00 ± 0.26
PG activity (U/mg db)[a]	0.13 ± 0.12	0.60 ± 0.04	0.14 ± 0.03	1.02 ± 0.11

PG, polygalacturonase activity; results are the mean of at least three experiments in duplicate, g/g db, gram per gram dry biomass; [a]U/mg db, units per milligram dry biomass.

Table 3
Amount of Total Lipids, Total Sterols, and Total Fatty Acids in *A. niger* Mycelia Grown for 70 or 90 h in the Control Medium or with the Addition of 0.1% of Tween 80

	mg/g dry mycelium					
	Control[a]			0.1% Tween 80[a]		
	TL	ST	FA	TL	ST	FA
70 h	91.7	20.1	48.3	104.8	25.5	58.1
90 h	97.3	21.1	34.1	112.5	29.5	45.5

[a]TL, total lipids; ST, sterol fraction; FA, fatty acid fraction.

was followed. The amounts of total lipids, sterols, and fatty acids are shown in Table 3. Mycelia grown in the presence of Tween 80 synthesized more total lipids with more sterols and fatty acids. Results of separation of lipids into sterols, sterol esters, and triacylglycerols are shown in Table 4. Sterol esters and triacylglycerols accumulated in lipid particles with triacylglycerols representing energy reserves and storage of precursors for membrane biosynthesis *(9)*. In the presence of Tween 80 there were more sterols and triacylglycerols synthesized in mycelia. When Tween 80 was present, biosynthesis of sterols, sterol esters, and triacylglycerols was active during cultivation of 70–90 h, whereas in the absence of Tween 80 the sum of sterols and sterol esters remained constant with a small increase in triacylglycerols. Esterification of sterols is an important step in ergosterol metabolism. With yeasts, esters are made from ergosterol precursors, which are less necessary for active metabolism when there is a lower formation of membranes. Esters are stored in lipid particles and hydrolized when cells need ergosterol, serving as an additional mechanism for free sterol regulation *(22)*. With *A. niger* the amount of sterols needed could also be regulated through the synthesis of sterol esters.

GC/MS analysis of sterol fractions showed the presence of individual intermediates of the ergosterol pathway (Fig. 3). Ergosterol increased in time and comprised over 85% of total sterols with other intermediates in

Table 4
Sterols, Sterol Esters, and Triacylglycerols
in Mycelia of *A. niger* after 70 or 90 h of Cultivation
in the Absence (Control) or Presence of 0.1% of Tween 80

	mg/g dry mycelium					
	Control[a]			0.1% Tween 80[a]		
	ST	StE	TAG	ST	StE	TAG
70 h	13.5	6.6	2.0	16.9	8.6	4.2
90 h	10.8	10.3	3.1	18.7	10.8	7.8

[a]ST, sterols; StE, sterol esters; TAG, triacylglycerols.

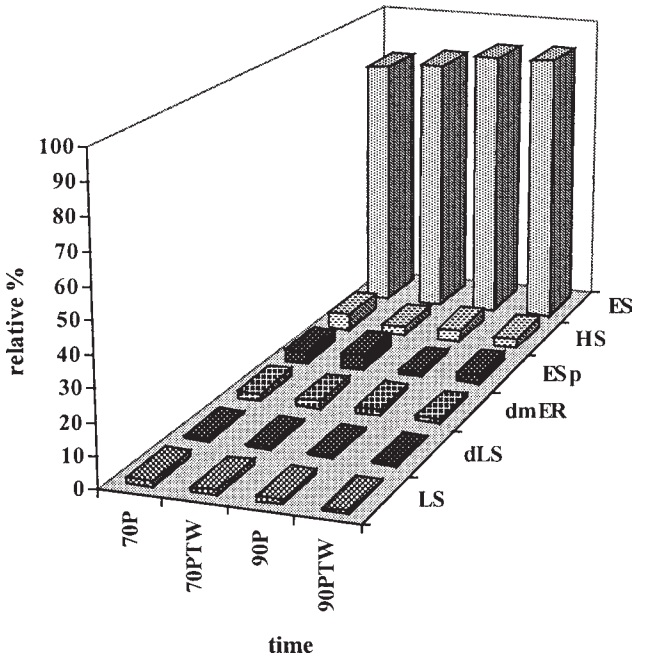

Fig. 3. Relative amounts of individual intermediates of ergosterol biosynthesis in *A. niger* mycelia grown with (PTW) and without (P) addition of Tween 80. LS, lanosterol; dLS, 24-methylene dihydrolanosterol; dmER, 4,4-dimethyl-ergosta-8,24(28)-dienol; Esp, ergosterol peroxide; HS, cholesterol; ES, ergosterol.

low amounts, suggesting active synthesis. Altered levels could contribute to a changed membrane permeability as well as membrane-associated functions, such as membrane-bound enzymes *(3,4)*.

Surprisingly cholesterol, which was not synthesized by fungi, was detected in mycelia of *A. niger*. Analysis of dry whey revealed the presence of 1.8 mg cholesterol per gram. After addition of ^{3}H-cholesterol to the medium, ^{3}H-cholesterol and ^{3}H-cholesterol esters were detected in lipid extracts of the mycelia. In the extract, ^{3}H-cholesterol comprised 2.52% and

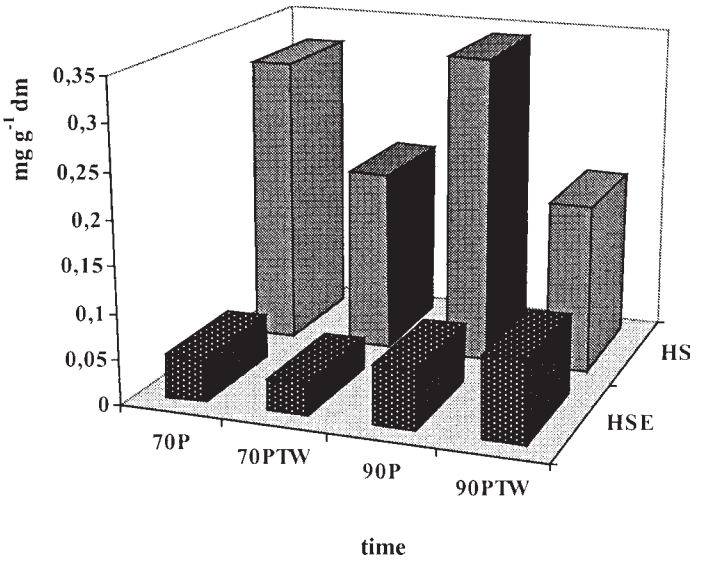

Fig. 4. Amounts of cholesterol (HS) and cholesterol esters (HSE) in *A. niger* mycelia in the absence (P) or with the addition (PTW) of Tween 80 after 70 or 90 h of cultivation.

sterol esters 0.55% of total ^{3}H-cholesterol added to the medium. Formation of sterol esters is known to be a microsomal process *(23)*, which confirmed the incorporation of exogenous cholesterol into fungal cells.

The mycelium grown in medium without Tween 80 incorporated more cholesterol than did mycelium grown in the presence of Tween 80 (Fig. 4). The amount of cholesterol incorporated decreased slightly in time with a concomitant increase of cholesterol esters.

Separation of total lipids into a neutral lipid, glycolipid, and phospholipid fraction showed a large difference between the control mycelium and that grown in the presence of Tween 80 (Table 5). The control contained more neutral lipids and less glycolipids and phospholipids with a higher ratio of saturated to unsaturated fatty acids. These results point to lower membrane permeability of the control compared to Tween 80 grown mycelia. Palmitic acid, oleic acid, and linoleic acid comprised the bulk of fatty acids (Table 5). Linoleic acid prevailed in the mycelium grown in the control medium, whereas with Tween 80 the increase in the amount of oleic acid was significant. Oleic acid, a part of Tween 80 molecules, was incorporated into different lipid fractions. Fatty acid analysis of glycolipids, phospholipids, triacylglycerols with free fatty acids, and sterol esters showed that the oleic acid of Tween 80 was preferentially incorporated into glycolipids and phospholipids. Triacylglycerols and free fatty acids as lipid depots did not contain high amounts of oleic acid compared to amounts obtained when Tween 80 was absent. As with triacylglycerols, sterol esters were selectively formed with palmitic acid, oleic acid, and linoleic acid. With the addition of Tween 80, the percentage of oleic acid bound to sterols increased at the expense of linoleic acid. For *S. cerevisiae* it has been reported

Table 5
Fatty Acid Composition of Total Lipids and Individual Lipid Fractions from Mycelia Grown in Medium without and with the Addition of 0.1% of Tween 80[a]

		% TL	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	Sat/uns
TL	70P	100	5.5	24.9	6.1	28.0	33.8	1.6	0.59
	70Tw	100	2.8	16.4	3.6	45.1	30.9	1.2	0.29
NL	70P	61.2	3.5	26.1	4.8	32.6	32.1	0.9	0.52
	70Tw	47.6	3.6	19.8	4.2	42.5	29.2	0.7	0.38
GL	70P	7.2	0.7	22.6	5.1	33.5	38.2	0.0	0.40
	70Tw	11.4	2.3	12.7	3.9	63.1	18.0	0.0	0.23
PL	70P	31.6	0.7	29.5	1.7	27.6	40.0	0.0	0.47
	70Tw	41.0	1.6	24.5	1.0	42.8	29.4	0.0	0.37
TAG	70P		2.7	23.7	Tr	36.7	36.6	0.2	0.36
FFA	70Tw		0.0	20.4	0.0	40.6	38.8	0.3	0.26
StE	70P		0.0	6.8	0.0	22.5	70.7	0.0	0.07
	70Tw		0.0	8.4	0.0	35.6	56.0	0.0	0.09

[a]C14:0, miristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; Sat/uns, ratio between saturated and unsaturated fatty acids; Tr, traces; 70P, 70 h of cultivation in control medium; 70Tw, 70 h of cultivation with Tween 80 addition; TL, total lipids; NL, neutral lipids; GL, glycolipids; PL, phospholipids; TAG+FFA, triacylglycerols plus free fatty acids; StE, sterol esters.

that synthesis of sterol esters exhibited high specificity for unsaturated C18 fatty acids *(24)*, and this also appears to be the case with *A. niger*.

As with previous studies *(2,16,25)*, oleic acid, as a part of Tween 80, was incorporated into fungal cells after enzyme liberation, most probably by esterases and thus increased the amount of unsaturated fatty acids. In glycolipids and phospholipids as well as sterol esters oleic acid was substantially increased by Tween 80 supplementation. The nature of the fatty acyl chain of phospholipids is one of the most important factors modulating the fluidity and integrity of the membrane. The ratio of saturated to unsaturated fatty acids may greatly influence membrane properties. A lower ratio can give rise to better permeability *(2)*. The results clearly show the effect of Tween 80 on the lipid composition of *A. niger* mycelia. However, it is beyond the scope of the present article to speculate which mechanisms are involved in the increased activity of polygalacturonase. Different lipid contents might effect the metabolism in a number of ways and perhaps changed lipid composition of membranes can increase protein transportation as well. Yet the confirmation of such a hypothesis will have to await further experiments.

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