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Surfactants as Stimulants of Enzyme Production by Microorganisms

E. T. REESE AND ANNE MAGUIRE
Pioneering Research Laboratory, U.S. Army Natick Laboratories, Natick, Massachusetts 01760

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The addition of Tween 80 and sucrose monopalmitate, nonionic surfactants, to fungal cultures resulted in marked increases in yields of the enzymes cellulase, amylase, sucrase, β-1 $\rightarrow$ 3 glucanase, xylanase, purine nucleosidase, and benzoyl esterase. The action appears to be an effect of the surfactant on cell permeability.

A few years ago, we reported that cellobiose is an inducer of cellulase, providing that its concentration in the medium is kept low. The enzyme was induced when cellobiose octaacetate served as the carbon source for those fungi which also produced the necessary esterases. By this means, yields of cellulase were obtained which were much higher (4 to 100 times) than those obtained on cellobiose and of the same magnitude as those with cellulose as the inducing agent (6).

Some time later, we found that other fungi growing on sucrose monopalmitate give yields of sucrase 10 to 16 times those obtained on sucrose (9). Sucrose plus palmitic acid could not substitute for sucrose monopalmitate. As for cellulase (above), it appeared that supplying the inducer at a low level, through hydrolysis of the ester, was the basis for the increased yield.

This principle was then applied to the induction of α-galactosidase. Melibiose, an α-galactoside, and melibiose octaacetate were tried as inducers, but neither compound gave good yields. Sucrose monopalmitate, however, gave the highest α-galactosidase values. It appeared that some factor other than the ability to yield the inducer was involved in this action of sucrose monopalmitate.

When we investigated the production of nucleosidases (8), we tried adding sucrose monopalmitate and Tween 80 to media containing the inducer. Enzyme production was increased as much as fivefold by the additives in some organisms, but was not affected in other organisms. It thus became apparent that surfactants play a role in the production or in the secretion of microbial enzymes, or in both.

A search of the literature then revealed one prior example of an increase in enzyme production due to nonionic surface active agents, a 30 to 40% increase in amylase yield by *Aspergillus niger* (10). The results reported in this paper show increases of much greater magnitude than this.

MATERIALS AND METHODS

Cultures were grown in shaken flasks at 29 C on a simple salts medium (5) containing substrate (0.5 to 1.0%) plus small amounts of yeast extract (0.01%) and peptone (0.05%). Surfactants were added at 0.05 to 0.2% (usually 0.1%). Tween 80 is sorbitan polyoxyethylene monoöleate (Atlas Chem. Co., Wilmington, Del.); sucrose monopalmitate was supplied by the Colonial Sugar Co. (Gramercy, La.). Culture filtrates were assayed for the various types of enzyme activities (5, 6, 8, 9).

RESULTS

The effects of Tween 80 and of sucrose monopalmitate on the synthesis of four enzymes are shown in Table 1. Tween 80 was used more extensively than sucrose monopalmitate, chiefly because it is more soluble and easier to handle. Enzyme production by some organisms was only slightly affected by the surfactant. Most organisms were stimulated to increased enzyme production. In general, the percentage increase was greatest for organisms which produce small amounts of enzyme in the control lacking surfactant. The increase was less pronounced in the organisms which are normally good producers of the enzyme. Thus, in *Trichoderma viride* QM6a, the 13-fold increase in cellulase stimulated by Tween 80 occurred in a cellobiose culture where control yields were low; the 56% increase occurred in a cellulose culture where control yields were much higher.

The addition of Tween 80 to the culture medium gives maximum stimulation of enzyme production when added at zero-time of incubation, but later addition (3rd day) is usually still quite helpful. The results again indicate that the maxi-

mum per cent increase in yield is associated with low yield in the controls (lacking surfactant). Thus, in *Gliocladium vermoeseni*, the control produced only 3 units of esterase/ml, whereas the culture containing Tween 80 was stimulated to yield 13 units/ml. In the *Pestalotia virgatula* control, a yield of 8 units/ml was obtained, and the addition of Tween doubled the yield.

Since we postulate that the surfactant acts at the cell membrane to release the enzyme, other enzymes should be set free simultaneously. This was generally true (Table 2). In 53 enzyme com-

TABLE 1. *Effect of addition of Tween 80 and of sucrose monopalmitate on yields of enzymes*

Enzyme	Organism	Enzyme yields (units/ml)[a]		
		Control	With 0.1% sucrose monopalmitate	With 0.1% Tween 80
Cellulase (C_x)	*Aspergillus terreus* QM442	2.1	NT[b]	8.0
	Penicillium pusillum QM137g	14.0	NT	43.0
	Trichoderma viride QM6a	67.0	NT	96.0
	T. viride QM6a	3.0	NT	39.0
	T. viride QM2940	34.0	57.0	51.0
Xylanase	*A. fumigatus* QM45h	11.0	28.0	43.0
Benzoyl esterase[c] (vs. 6-benzoyl α-CH_3 glucoside)	*A. terricola* QM7418	8.6	18.5	15.2
	Gliocladium vermoeseni QM6825	3.2	6.3	13.1
	P. melinii QM1931	2.0	1.4	11.1
	Pestalotia virgatula QM479	8.6	16.5	18.5
Glucamylase	*Monascus purpureus* QM541	20.0	NT	40.0
	Penicillium hirayamae QM7885	6.1	NT	23.0

[a] All cultures were grown in shake flasks on 0.5% substrates for 2 weeks and were assayed periodically.
[b] No test.
[c] E. T. Reese, A. Maguire, and F. W. Parrish, Can. J. Biochem., *in press*.

TABLE 2. *Effect of Tween 80 (0.1%) on enzyme production of fungi*

Organism	Enzyme (units/ml)[a]									
	Glucanases						Glucosidase			
	β-1,4-		β-1,3-		α-1,4-		Salicinase		Cellobiase	
	No Tween	With Tween	No Tween	With Tween	No Tween	With Tween	No Tween	With Tween	No Tween	With Tween
Aspergillus luchuensis QM873	0.7	3.7	23	32	21.0	35.0	4.6	4.9	13.0	15.2
A. terreus QM72f	5.0	6.3	0.5	2.4	0.3	1.1	0	1.2	0.2	1.9
A. terreus QM442	3.7	10.0	1.8	3.8	0.6	1.0	1.5	1.9	3.7	5.4
Basidiomycete QM806	2.1	3.1	60	125	14	31	0	0	NT[b]	0.2
Penicillium brasilianum QM6947	4.9	3.6	8.8	14	1.2	1.7	0	0.6	0.3	1.3
P. funiculosum QM474	0.4	0.4	41	31	12.0	7.6	0.7	1.1	7.8	14.1
P. ochrochloron QM477	1.0	2.9	24	32	0.5	1.5	2.8	4.6	10.6	16.8
P. parvum QM1878	8.5	5.3	25	64.0	0.9	1.7	1.6	2.4	6.4	9.8
P. pusillum QM137g	7.6	17+	18	18	3.6	21.0	0	0.8	1.8	2.6
Trichoderma viride QM6a	9.0	4.0	2.7	3.3	15	16	0	0	NT	0.1

[a] Units (40 C, 1 hr) = 0.50 mg of reducing sugar per ml. The above fungi were grown on lactose (0.3%) plus cellobiose (0.2%) on a shaker at 29 C. In all cases, there was <0.3 unit of lactase/ml. Tween 80 (0.1%) was used.
[b] No test.

parisons (cultures ± Tween 80), only five exceptions were observed, three of which involved cellulase (*T. viride*, *Penicillium brasilianum*, *P. parvum*). The data indicate that the value of Tween 80 in increasing enzyme yields varies (i) from organism to organism and (ii) for different enzymes of the same organism. For example, *Basidiomycete* QM806 showed increased enzyme production of all three enzymes produced by it, and *A. terreus* showed increased enzyme production of all five enzymes produced by it. In other organisms, Tween 80 may cause a general increase of most enzymes, but an inhibition of one or two. If Tween 80 operated only by promoting leakage or release of the enzymes, one might expect an increase in all enzymes and in other intracellular components. The interpretation may therefore be more complex than only a change in permeability of the membrane.

The increase in enzyme yields as a result of the surfactant was not very spectacular in some of these examples (Table 2), probably because all fungi were grown under the same conditions. Cellulase production of *T. viride*, for instance, was actually inhibited by Tween 80 in this test; yet when grown under more favorable conditions (1% cellobiose, 0.3% Tween 80), cellulase production was stimulated eightfold by the surfactant. The improvement in yields, therefore, also reflects the conditions of growth.

If surfactants increase the amount of enzyme produced and secreted into the medium by acting on the membrane, they might also cause other internal components to be similarly released. One of the obvious products for examination is pigment. Eighteen strongly pigmented fungi were grown in shaken flasks on starch with and without Tween 80 for 15 days at 29 C. Only one, *A. amylovorus*, produced appreciable pigment under these conditions. Filtrates from the culture containing Tween 80 had an optical density (550 nm) six times that of the culture lacking Tween. There was also an increase of red pigment in the mycelium. Thus, this effect may be one of production and release of pigment.

DISCUSSION

The purpose of this paper is to emphasize the value of surfactants (e.g., Tween 80) for increasing enzyme yields. Seven different enzyme systems have been tested, and all responded favorably to the surfactant. The enzymes used were those normally present in the extracellular solution, and no data are available for intracellular enzymes. The fact that yields of pigment and enterotoxin (3) are similarly increased indicates that the surfactant effect is not limited to enzymes. The way now seems open to a general improvement in yields of those fungal products normally secreted by the organism.

The explanation of how the surfactants act to increase enzyme yields is largely conjectural. We know that the agents we are using are generally not toxic to the organisms. We also know that the release of enzyme occurs some time (days) after the complete consumption of substrate; i.e., probably from old cells, but not from cells undergoing autolysis since there is no accumulation of autolysis products.

Tween 80 and related surfactants have been used for some time in bacterial cultures to assist in growth. They have also been found to promote the entrance of compounds into cells. Thus, mycobacterial cells grown with Tween 80 have an increased permeability to triphenyl tetrazolium (7), and *Pseudomonas aeruginosa* cells grown in a medium containing Tween 80 are much more suceptible to inhibition by benzalkonium chloride and polymyxin B (1). In animals, amphibian larvae (2) are much more responsive to anaesthetics (cocaine, xylocaine, etc.) in the presence of surfactants. These data indicate that surfactants promote both entrance and exit of compounds from the cell.

Surfactants have effects on fungi other than those reported above. Our results confirm those of Takahashi et al. (10), who reported that there is a tendency for growth in shaken flasks to change from pellet form to mycelium of a more disperse type. Krzeminski et al. (4) reported an increased production of carotene in *Neurospora*. In this instance, the product remained inside of the cells. Apparently, surfactants do not always work in the same way.

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