THE BIOLOGICAL PRODUCTION OF ORGANIC SOLVENTS
FROM CELLULOSIC WASTES

SIX-MONTH PROGRESS REPORT - JUNE 1977
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By

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TASK 1. PRODUCTION OF CELLULASE

The effort for this task involves primarily the culturing of Thermoactinomyces YX and any derived mutants for the production of cellulase enzyme complex. The enzymes (as culture supernatants) so derived are for delivery to Dr. K. Pye for analysis of cellulase component levels.

At this time we have delivered approximately 200 ml of supernatant material derived from cultures grown in a basal mineral salts medium (Appendix) with 1% Avicel, 1% Whatman #1 filter paper (<60 mesh) or 1% CMC (degree of substitution - 0.7) as carbon source.

Future deliveries will include culture supernatants from mutant strains and those taken at selected stages in the cultural cycle in order to relate activity changes with component levels.

TASK 2. DEVELOPMENT OF MUTATION TECHNIQUES

The following techniques were developed in order to provide culture material for mutation studies with Thermoactinomyces YX. This organism is a filamentous microorganism which produces heat resistant spores on short sporophores. High density spore preparations were prepared by growth for 48 to 72 hours on predried Luria agar plates at 55°C, the plates having been seeded with broth culture of the same medium. The spores were collected by addition of water, scraping of the surface growth, filtration through glass wool, and washing by centrifugation. The final spore pellet was diluted in H₂O to a density of about 5×10^9 to-tal count units/ml.

Two mutation processes have been employed. The first procedure is ultraviolet irradiation at 254nm using a GE GT15W germicidal lamp to achieve approximately 99.9% kill. The second method employs 8-methoxypsoralen (gift of the Upjohn Company, Kalamazoo, Michigan) plus irradiation with near U.V. (365nm) to achieve a 99.9% reduction in viable cells. The spores are incubated at room temperature in 0.01% 8-methoxypsoralen for one

hour prior to irradiation to allow for uptake of this material. The surviving populations were determined by plating on a complete medium (Luria agar). Figures 1 and 2 show the survival curves for Thermoactinomyces YX using these two mutagenic agents.

The kinetics of near-UV + 8-methoxypsoralen action in Thermoactinomyces is very similar to that reported for various Streptomyces by Townsend et al (1971).

TASK 3. CELLULASE MUTANT SCREENING TECHNIQUES

A crucial element in this research program is to employ a rapid and reliable screening procedure for cellulase mutants. Two basic plating procedures are being pursued at the present time. The first employs ball-milled Whatman #1 filter paper (ball milled according to the procedure of Stewart and Leatherwood (1976)) in a basal mineral salts medium where cellulase activity is evidenced by clearing of the insoluble cellulose (CMC) as substrate. Cellulase activity is visualized as clear zones surrounding colonies after precipitating the unreacted CMC with a solution of hexadecyltrimethylammonium bromide (Hankin and Anagnostakis, 1977). In this procedure, replica plates are used, as the precipitating agent kills the cells. Screening for depressed mutants is accomplished by the addition of 0.2-0.5% glucose to either agar medium.

In addition to the above, the following cellulose substrate materials and plating variations were tried without satisfactory results.

Substrates: Avicel (pH 109), Solka floc, Schleicher & Schuell powdered cellulose, shear milled Whatman #1 filter paper (<60 mesh), HCl swollen cellulose (Whatman #1) prepared according to the method of Bose (1963), Walseth Cellulose (Whatman #1, Solka floc), shear-milled cellulose azure (<60 mesh).

Plating Variations: Cellulose Concentration (0.1 - 2.0%)
Pour Plates
Cellulose Overlay Plates

Some zone clearing was noted using Walseth cellulose and cellulose azure plates, but these two procedures are either too time consuming or expensive and the cellulase azure plates are extremely difficult to prepare due to clumping of the cellulose particles.

Figure 1. Spore U. V. survival curve - Thermoactinomyces YX
(254 nm)

Figure 2. Survival curve of *Thermoactinomyces* YX spores to exposure to near-UV (365 nm) and 8-methoxypsoralen

Attempts were also made to restrict colony size according to the technique described by Montenecourt and Eveleigh (1977), but without success. Oxgall does not retard colony growth, but instead inhibits all growth when the concentration reaches >0.1%.

To date some 5500 colonies have been examined by the plating techniques described. Sixty potential mutants selected on the basis of zone size or rapid clearing on cellulose/glucose plates have or are being examined to characterize/quantitate their cellulase activity.

TASK 4. QUANTIFICATION OF CELLULASE MUTANTS

Cellulase activity is being measured in culture supernatants after 48 hours growth in 0.5% Avicel/mineral salts medium. De-repressed activity is measured during growth in 0.5% Avicel/mineral salts medium containing 0.2% glucose. In all cases, the parent strain is grown in parallel and tested in an identical manner.

Enzymatic activity is measured using 0.1M acetate (pH 6.0) as buffer, and a temperature of 65°C with a 15-minute incubation for CMC activity (endo-B1-4 glucanase) and 1 hour for filter paper activity. Protein is determined by the method of Lowry, et al. (1951), and reducing sugar is measured by the dinitrosalicylic acid (DNS) method. Data is recorded as mg reducing sugar/ml culture broth and mg reducing sugar/mg protein (specific activity).

Table 1 presents a comparison of the cellulose activity (endoglucanase and filter paper activity) of Thermoactinomyces YX and seven ultraviolet exposed isolates. Four of these isolates (#54, 56, 58 and 59) possess considerably more cellulose activity than the parent YX strain (up to 50% more). In each case, the increase in activity can be correlated with an increase in extracellular protein production as was the case with hypercellulase Trichoderma strains (Mandels, et al. (1971)).

TABLE 1. Endoglucanase and filter paper activity of Thermoactinomyces and selected ultraviolet exposed isolates

<u>Culture</u>	<u>24 Hours</u>			<u>48 Hours</u>		
	<u>Protein mg/ml</u>	<u>CMC activity mg RS/ml enzyme</u>	<u>Filter paper mg RS/ml enzyme</u>	<u>Protein mg/ml</u>	<u>CMC activity mg RS/ml enzyme</u>	<u>Filter paper mg RS/ml enzyme</u>
YX	0.270	6.75	1.00	0.390	9.75	1.64
54	0.370	9.25	1.46	0.497	12.43	2.14
55	0.243	6.08	--	0.395	9.88	--
56	0.380	9.50	1.54	0.470	11.75	2.10
57	0.254	6.35	--	0.400	10.00	--
58	0.375	9.38	1.34	0.513	12.83	1.82
59	0.405	10.13	1.20	0.529	13.23	1.92
60	0.233	5.83	--	0.400	10.00	--

TASK 5. ALTERNATE ENHANCEMENT TECHNIQUES

Studies on the effect of various cultural parameters on cellulase activity have been initiated in an attempt to maximize yields of cellulase and to understand the relationships between growth and enzymic activity.

Thermoactinomyces YX was grown in a basal mineral salts medium with 3 forms of cellulose (Avicel, Schleicher and Schuell plain cellulose powder and CMC) or a variety of mono- and disaccharides (glucose, arabinose, cellobiose, sucrose and lactose). Enzyme activity against CMC and filter paper were compared after 48 hours growth in these carbon sources. As shown in Table 2, cellulase activity is greatest with either soluble or insoluble cellulose as the growth substrate, with endoglucanase activity being highest when the organism is grown in CMC.

It appears that this organism retains some endoglucanase activity when grown on small molecular weight carbohydrates. These studies, however, are not complete enough to determine whether the levels measured are due to repression/depression or inducer/constitutive effects. High reducing sugar background prevented filter paper assays with these cultures.

Direct correlation between extracellular protein levels and enzymatic activity should not be made as growth, as determined by visual inspection, appeared to be comparable with all substrates. In addition, this organism has been found to produce a variety of non-cellulose related extracellular enzymes.

TABLE 2. Influence of carbon source on cellulase production

	Growth substrate	Extracellular protein mg/ml	Endoglucanase activity mg reducing sugar/ml enzyme	Filter paper activity mg reducing sugar/ml enzyme
	1% Avicel	0.259	7.84	0.78
	1% S+S	0.220	8.46	0.63
	1% CMC	0.117	11.70	0.87
∞	0.5% cellobiose	0.206	1.49	-- NT
	0.5% glucose	0.168	0.53	-- NT
	0.5% sucrose	0.111	1.05	-- NT
	0.5% lactose	0.056	1.03	-- NT
	0.5% arabinose	0.094	0.13	-- NT

INCUBATION: 48 hours at 55°C.

The relationship between cellulase activity in the culture broth and the initial cellulose content in the growth medium is depicted in Figure 3. These data show that cellulase activity toward CMC substrate is more responsive to growth substrate concentration in the range of 0.1 to 2% than activity towards filter paper. Clearly maximal cell-free activity is obtained with a growth substrate concentration at 0.2%. Similar type data has been reported for Thermomonospora curvata by Stutzenberger (1971). Extracellular protein levels were about 25 mg/ml in all cases.

The decrease in observed endoglucanase activity with increasing cellulase concentration may reflect binding to untreated cellulose. Studies by Sternberg with Trichoderma viride (1976) indicate that increased cellulase activity can be obtained by growth at higher cellulose concentrations (i.e. >2%) where pH, aeration and agitation are controlled.

The relationship between growth substrate concentration (0.2, 0.5 and 1.0%), incubation time and filter paper activity is shown in Figure 4. At all 3 substrate concentrations, maximum activity is attained in 48 hours, with continued incubation resulting in some loss of activity. As this organism produces an active extracellular protease, it might prove of interest to investigate the relationship of the cellulase stability to protease activity.

As Reese (1969) showed that cell-free cellulase³ activity could be increased by the addition of surfactants (Tween 80) into the growth medium of Trichoderma, a study was made of the effect of several surfactants on Thermoactinomyces cellulase production. As shown in Table 3, Tween 80, Brij 35, and Span 80 in general depressed both cellulase activity and extracellular protein production. Low levels (0.05%) of PL 61 (Wyandotte Chemical Co.) gave some stimulation of filter paper, but a reduction in CMC activity. It appears that the effects observed with Trichoderma do not hold for Thermoactinomyces.

Figure 3. Effect of growth substrate (Avicel) concentration on soluble cellulase activity

Figure 4. Relationship between filter paper activity, substrate concentration and growth of Thermoactinomyces

TABLE 3. Effect of surfactants on production of cellulases

<u>Surfactant</u>	<u>Protein mg/ml</u>	<u>CMC activity mg RS/ml enzyme</u>	<u>Filter paper activity mg RS/ml enzyme</u>
None	0.267	22.76	1.28
0.05% Tween 80	0.246	22.18	1.14
0.10% Tween 80	0.222	19.79	0.92
0.05% Brij 35	0.184	11.16	0.76
0.10% Brij 35	0.201	9.89	0.73
0.05% Span 80	0.165	12.18	0.80
0.10% Span 80	0.122	2.05	0.12
0.05% PL 61	0.279	19.68	1.55
0.10% PL 61	0.262	17.44	1.06

INCUBATION: 0.2% Avicel medium
24 hours at 55°C

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APPENDIX

1. Luria Broth/Agar

1% Tryptone
0.5% yeast extract
1.0% NaCl
0.1% glucose
*20 ml Σ salts
980 ml H₂O

For agar, add 20 gm agar/liter.
Adjust to pH 7.0 prior to sterilization.

2. Basal mineral salts medium

0.5% NaCl
0.01% yeast extract
0.31% (NH₄)₂SO₄
1.0 mg thiamine HCl
1.0 mg biotin
100 ml 0.67M sodium/potassium phosphate
buffer, pH 7.8
*20 ml Σ salts
880 ml H₂O

For agar, add 15 to 20 gm agar/liter.
Add carbohydrate (carbon source) at concentration
desired.

3. Σ salts

2.5 gm EDTA
10.0 gm MgSO₄ · 7H₂O
0.4 gm ZnSO₄ · 7H₂O
1.0 gm FeSO₄ · 7H₂O
0.76 gm MnSO₄ · H₂O
1.0 gm CaCl₂
1000 ml H₂O