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REDUCING THE COST OF CELLULASE PRODUCTION
- SELECTION OF THE HYPERCELLULOLYTIC
TRICHODERMA REESEI RUT-C30 MUTANT

A general review of the Rutgers cellulase program supported by
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DOUGLAS E. EVELEIGH

Department of Biochemistry and Microbiology
Cook College
New Jersey Agricultural Experiment Station
Rutgers University
New Brunswick, New Jersey 08903

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CONTENTS

	<u>Page</u>
I. Introduction	3
II. Procedures	3
A. Development of Screening Methodology	4
B. Evaluation of Mutant Strains	6
C. Characterization of Mutant Strains	6
C1. Hypercelluolytic mutants	6
C2. Mutants Resistant to End-Product Inhibition	7
III. Discussion	8
A. Accomplishments (Program Goals)	8
B. Accomplishments (General)	9
C. Potential New Avenues	9
IV. Summary	10
References	11
Figures	13
Publications covering the Rutgers Cellulase Program	17

I. Introduction

The concept of conversion of biomass to alternative liquid fuels received emphasis during the late 1970's. In one such approach, it was envisaged that cellulose, the major component of biomass, could be hydrolyzed to glucose and this product subsequently fermented to chemical feedstocks. The hydrolysis of cellulose was a major bottleneck to this scheme. Acid hydrolysis was relatively inefficient, yielded toxic by-products (hydroxyfurfural) and posed waste disposal problems. Enzymatic hydrolysis was potentially efficient, but was slow (1-2 days) and furthermore the cost of enzyme (cellulase) production was high. At Rutgers, we considered the enzymatic approach was the more practical avenue, in that the high price of the biomass substrate necessitated high conversion efficiency (95% yield by enzyme; 65% yield by acid). The objectives of the Rutgers program were to find an efficient microbial cellulase, and then to develop procedures for

- (A) reducing its production costs;
- (B) selection of microbial mutants with cellulase that shows greater efficiency than that of the original enzyme.

The Rutgers cellulase mutant selection program was initially supported by ERDA and subsequently by the Department of Energy.

II. Procedures

At the outset it was necessary to focus on a well defined and efficient cellulase system. The cellulase of Trichoderma reesei was selected as its enzyme complex was particularly

efficient in converting crystalline cellulose to glucose and furthermore hypercellulolytic strains QM9213 and QM9414 were available for comparative purposes (Gaden et al., 1976).

The Rutgers cellulase program can be sub-divided into the following major components:

- A. Development of Screening Methodology
- B. Evaluation of Mutant Strains
- C. Characterization of Mutant Strains.

A. Development of the Screening Methodology

Major focus was given the development of methods by which rare cellulolytic mutants could be detected amidst the wild colonies. Screening procedures were in reality, deceptively complex and thus receive emphasis in this presentation. On the one hand, microbiological textbooks present photos of microbes efficiently degrading starch, e.g., amylase production visualized by the starch-iodine reaction (Brock, 1979). In contrast, analogous demonstrations of cellulose degradation have rarely presented, even though cellulases have been studied for fifty years. The difficulty in gaining a clear demonstration of cellulose hydrolysis in a petri dish culture, results from the recalcitrant nature of crystalline cellulose. This problem had not been widely appreciated, even though a major mutational program at the U.S. Army Laboratories, Natick, MA had resulted in only limited success, e.g., mutants T. reesei QM9213 and 9414. Our approach was therefore to design a system that would guarantee visual detection of cellulose hydrolysis.

The solution was to:

- (i) use "non-crystalline" cellulose (acid swollen) as a substrate
- (ii) enhance the hydrolytic degradation (and clearing) of the cellulose around the microbial colonies, through heating overnight at 50°C, a temperature at which the cellulase acts optimally, while restricting further microbial colony growth. Clear zones of hydrolysis could be routinely obtained around cellulolytic strains by use of this methodology (Montenecourt and Eveleigh, 1977a,b).

This procedure was the key to the screening program, and in the presence of a catabolite repressor (glycerol or glucose), catabolite repression resistant mutants could be clearly visualized. Certain of these strains were also hypercellulolytic, e.g., T. reesei RUT-NG14. (Montenecourt and Eveleigh, 1977b).

With this screening basis firmly established, a range of other screening methodologies was developed based either on the action of the total cellulase enzyme complex, or on the action of individual components (endo-glucanase, β -glucosidase (Figure 1)).

Two types of mutant could now be screened for:

- (i) hypercellulolytic strains (e.g., RUT-NG14, RUT-C30).
- (ii) strains with more efficient cellulase (e.g., strains with β -glucosidase resistant to end-product inhibition)

A range of mutants were isolated and their genealogy is outlined in Figure 2.

A corollary of these successful mutant screens was the world wide attention given the development of new T. reesei mutants (Table 3). Prior to our work, studies of at the U.S. Army Laboratories, Natick

had yielded limited success, and deterred further investigation. An exception was in Finland where a cellulase screening program paralleled our studies (Nevalainen and Palva, 1978).

B. Evaluation of the Mutant Strains

Productivity: The initial focus was to gain mutants that rapidly produced high yields of cellulase. Evaluation at Rutgers was based on cellulase production in shake flasks. Potentially useful mutants were then sent to the U.S. Army Laboratories, Natick, MA, and the Department of Chemical Engineering, University of California, Berkeley, CA for evaluation in larger scale fermentors. There was a fairly good correlation between the productivities of the mutants in "flask" and "fermentor" culture. Cultures RUT-NG14 and RUT-C30 were outstanding cellulase producers (Montenecourt and Eveleigh, 1978, 1979).

The co-ordination between the laboratories supported by DOE deserves special mention as the successful production of high yields of cellulase by RUT-C30, was through mutational screening combined with large scale fermentation expertise. For instance the cellulase production by the wild strain QM6a in flask culture was 0.5-1.0 filter paper units (fpu)/ml in 7-10 days culture. In comparison RUT-C30 could yield 10-14 fpu/ml in 4-5 days in a fermentor (for review see Mandels, 1982).

C. Characterization of the Mutant Strains

C1. Hypercellulolytic Mutants: In order to gain some insight into the bases of the hypercellulolytic mutations, considerable study was devoted to more detailed physiologic characterization. In brief the salient points are:

a). Hypercellulolytic mutants produce a cellulase complex with individual components essentially in the equivalent proportions to that found in the wild strain. These analyses have been through purification of the components (e.g., via high precision liquid chromatography and antibody characterization). Thus, a major point is that presumably all of the enzymes of the cellulase complex are subject to control via a single mutation, i.e., in T. reesei there is one regulatory control mechanism that governs the production of all "cellulase components" simultaneously. Regulation of the individual components has also been demonstrated in that certain cellulase negative strains can revert to β -glucosidase positive strains. Individual regulation of the other major component (cellobiohydrolase, endoglucanase) has yet to be demonstrated.

b). A major morphogenic change has been noted in RUT-C30 as it contains considerably enhanced amounts of endo-plasmic reticulum (Ghosh et al., 1982 - D.O.E. support; Washington office). Whether or not the greater amount of endoplasmic reticulum is the direct result of a mutation on its synthesis per se, or is a result of some other regulatory control has not yet been determined.

C2. Mutants Resistant to End-Product Inhibition (E.P.I.)

Cuskey (1982) spent considerable effort in the adaptation of the basic mutational screening techniques for application to selection of mutants to end-product inhibition. The basic approach was to screen for such mutants using the previously developed visual assays but combining an end-product inhibitor into the assay medium, e.g., 10% glucose in the case of β -glucosidase screening. Limited success in selection

of end-product resistant mutants was achieved (e.g., the glucose inhibition constant wild strain QM6a (K_i) - 0.35 mM: for RUT-EPI 13 - 1.05 mM).

Several other points were clearly made from this study.

a) The selection of end-product inhibition resistant mutants presumably involves a substitution of amino-acids within the active site of the enzyme. This type of mutation is far rarer, than for instance, the general inactivation of a regulatory protein limiting cellulase production, i.e., hypercellulolytic strains occur far more frequently than EPI mutants.

b) The screening protocol for selection of E.P.I. resistant mutants does not clearly eliminate hypercellulase producing strains. On this basis a large number of other hypercellulolytic strains were also isolated concomitantly in the E.P.I. screening (L-series, Table 2).

III. Discussion

III.A. Accomplishments (Program goals)

1. A range of screening protocols were developed that permitted selection of hypercellulolytic mutants.
2. Several hypercellulolytic strains were isolated, e.g., RUT-C30.
3. Culture of RUT-C30 under selective fermentor conditions yielded up to 15 fpu/ml. The extracellular protein concentrations achieved were particularly high (up to 2%). Productivities of 120-130 fpu/1/hr have been demonstrated.

4. Regulation of the total cellulase complex is, in part through a general control system. Discrete control of β -glucosidase has also been demonstrated.

5. Over production of cellulase in T. reesei RUT-C30 is related in some manner to enhanced production of endoplasmic reticulum. This type of mutation has rarely been observed in other secretory systems.

6. A limited range of β -glucosidase mutants resistant to inhibition by glucose has been obtained. However the K_i of these strains is only 1.05 mM.

III.B. Accomplishments (General)

1. Worldwide stimulation in screening for hypercellulolytic strains (Figure 3).

2. Submission of a patent application covering the development of the screening methodology (under review as of July, 1982).

3. Hypercellulolytic mutants (RUT-NG14 and RUT-C30) have been made available to researchers on a worldwide basis.

III.C. Potential New Avenues

1. The screening protocols permit the isolation of a wide range of cellulase mutants. We have isolated strains that specifically hyper-secrete β -glucosidase. Other mutants include constitutive, temperature sensitive, and secretion strains. Thus the basic screening methodology allows new methods of probing and gaining further insights into the cellulase complex.

2. Increased cellulase productivity. Productivities of 120 fpu/1/hr have been achieved in the fermentor. By immobilization of cells we have gained 135 fpu/1/hr and predict productivities of 200 fpu/1/hr (Frein et al., 1982, Frein in preparation).

3. We have still been unable to resolve the question of which is the limiting component of the cellulase complex, i.e., is it cellobiohydrolase, endoglucanase or β -glucosidase. There is evidence that endoglucanase is the limiting component. Our newer screening methodologies (e.g., use of Congo Red) permits selection of specific endoglucanase mutants.

4. Secretion. The mutants permit study of secretion of an industrially useful enzyme. In spite of the industrial application of enzymes, this type of study has received scant attention.

5. Cellulase of Trichoderma reesei has been focussed on. Other microbes merit study, e.g., bacteria may have completely distinct systems. From an applied viewpoint, thermostable cellulases and also their application in conjunction with partial acid hydrolysis deserves attention.

6. Enhanced cellulase production should be considered via recombinant DNA methodologies. This approach can be significant for a range of industrially useful enzymes.

IV. Summary

A program was developed with focus on the selection of hyper-cellulolytic strains of the fungus Trichoderma reesei. Several strains were isolated, the most effective being T. reesei RUT-C30. This strain produces upto 15 filter paper units/1/hr. Extracellular protein yields

are at 2%, a level equivalent to that obtained in industrial enzyme production. Increased productivities of 135 fpu/l/hr and maintained for 15 days, have been achieved through cell immobilization techniques. The high productivities are related in some manner to enhanced endoplasmic reticulum production in the mutant RUT-C30. Selection of mutants with greater resistance to end-product inhibition of β -glucosidase were made.

The results of this program should result in lowering of the cost of cellulase production and also permit the synthesis of more effective enzymes. The program stimulated worldwide interest in screening for hypercellulolytic strains.

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Figure 1. Selective screening methodologies for the isolation of high yielding cellulolytic regulatory mutants

Enzyme	Substrate	Repressor/Inhibitor	Visualization ^{1,2}
Total cellulase complex	Cellulose Powder [Acid swollen cellulose] ³ [Dyed cellulose]		Clearing zone formation
Catabolite repression resistant synthesis			
Total cellulase complex	Acid swollen cellulose ³	5% Glycerol 2-Deoxyglucose	Clearing zones after several days incubation at room temperature 50°C overnight.
Endoglucanase	Carboxymethyl cellulose	5% Glycerol or 5% Glucose 2-Deoxyglucose	Clearing zones after flooding with quaternary ammonium compounds or congo red Large colonies growing in the presence of 2 DG.
β-Glucosidase	(1) Esculin	5% Glucose	Black rings in the presence of ferric ammonium citrate
	(2) Cellobiose	2-Deoxyglucose	Large versus pin-point colonies
	(3) MUG ⁴	5% Glucose or 5% Glycerol	Fluorescent aglycone under ultra-violet illumination
	(4) X.glu ⁵	Same as above	Blue aglycone

Figure 1 (Continued)

Enzyme	Substrate	Repressor/Inhibitor	Visualization ^{1,2}
End-product inhibition resistant	Any of the above substrates	High concentrations of end product inhibitor (e.g., glucose, cellobiose, δ -gluconolactone)	Appropriate visualization
Enhanced secretion	Any of the above substrates	Membrane modifying agents (e.g., polyene antibiotics - nystatin) ⁶	Increased enzyme action specifically in the agar medium
Constitutive	Any non-inducing substrate	None	Overlay with any selective substrate

¹For references see text.

²Fast growing cultures can be controlled to give small discrete colonies to aid visualization, through incorporation of inhibitors into the culture medium (e.g., Ox-gall, Rose bengal, Triton X-100 or deoxycholate). Physical restriction of colonies has also been successfully used by Durand and Tiraby (1980b - see text).

³Modified substrates have been used to facilitate detection of mutants. However, the use of these substrates, which are often showed increased susceptibility to cellulase action, does not guarantee selection of mutants for the total cellulase complex, as synergism between the cellulase components is most evident with truly crystalline substrates.

⁴4-Methylumbelliferyl- β -D-glucoside

⁵5-Bromo-4-chloro-3-indolyl- β -D-glucoside

⁶Mutants with enhanced secretion have been obtained through selection of polyene resistant strains. Polyenes can also be used for enrichment of mutants (Nevalainen and Palva, 1978).

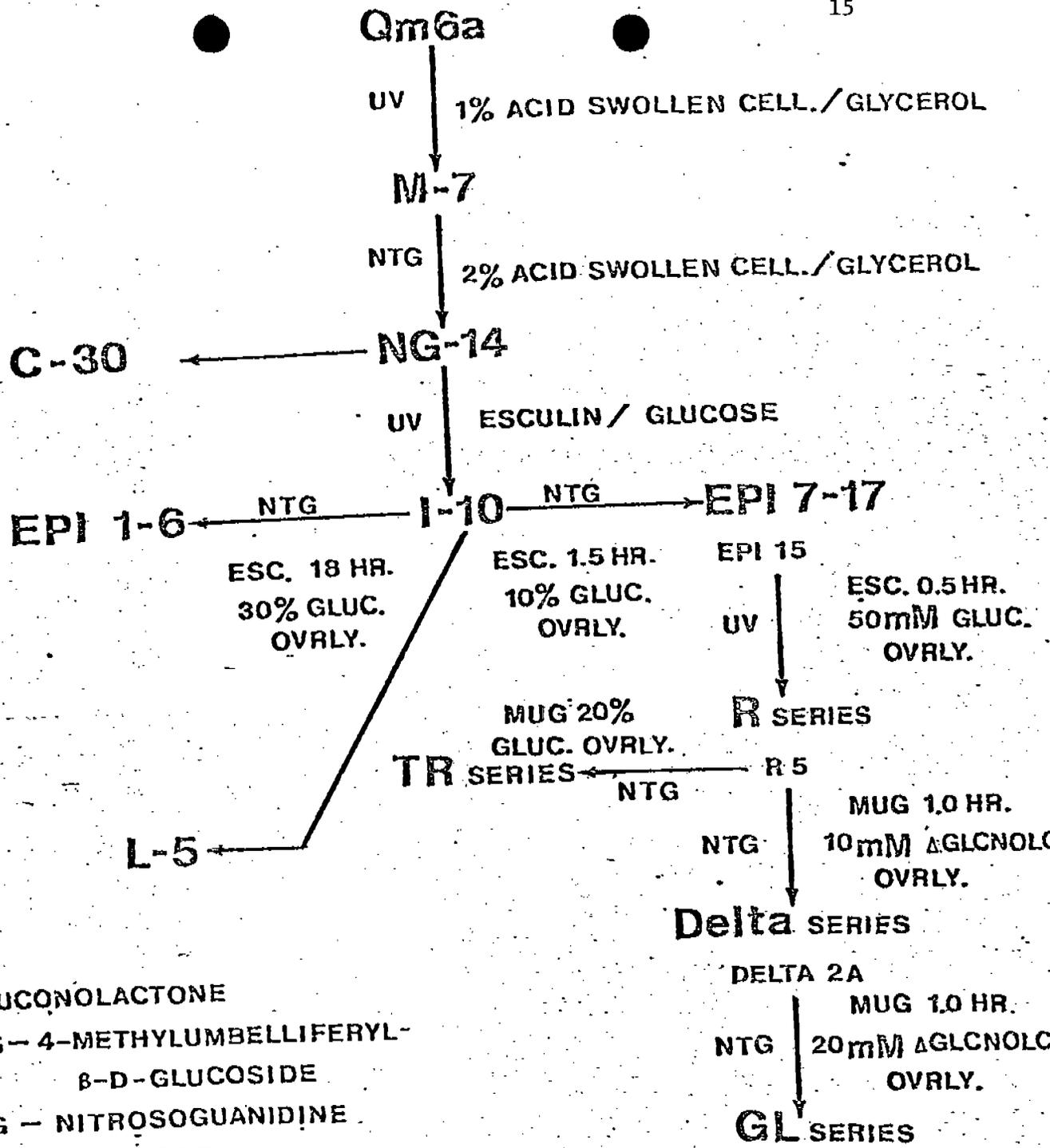
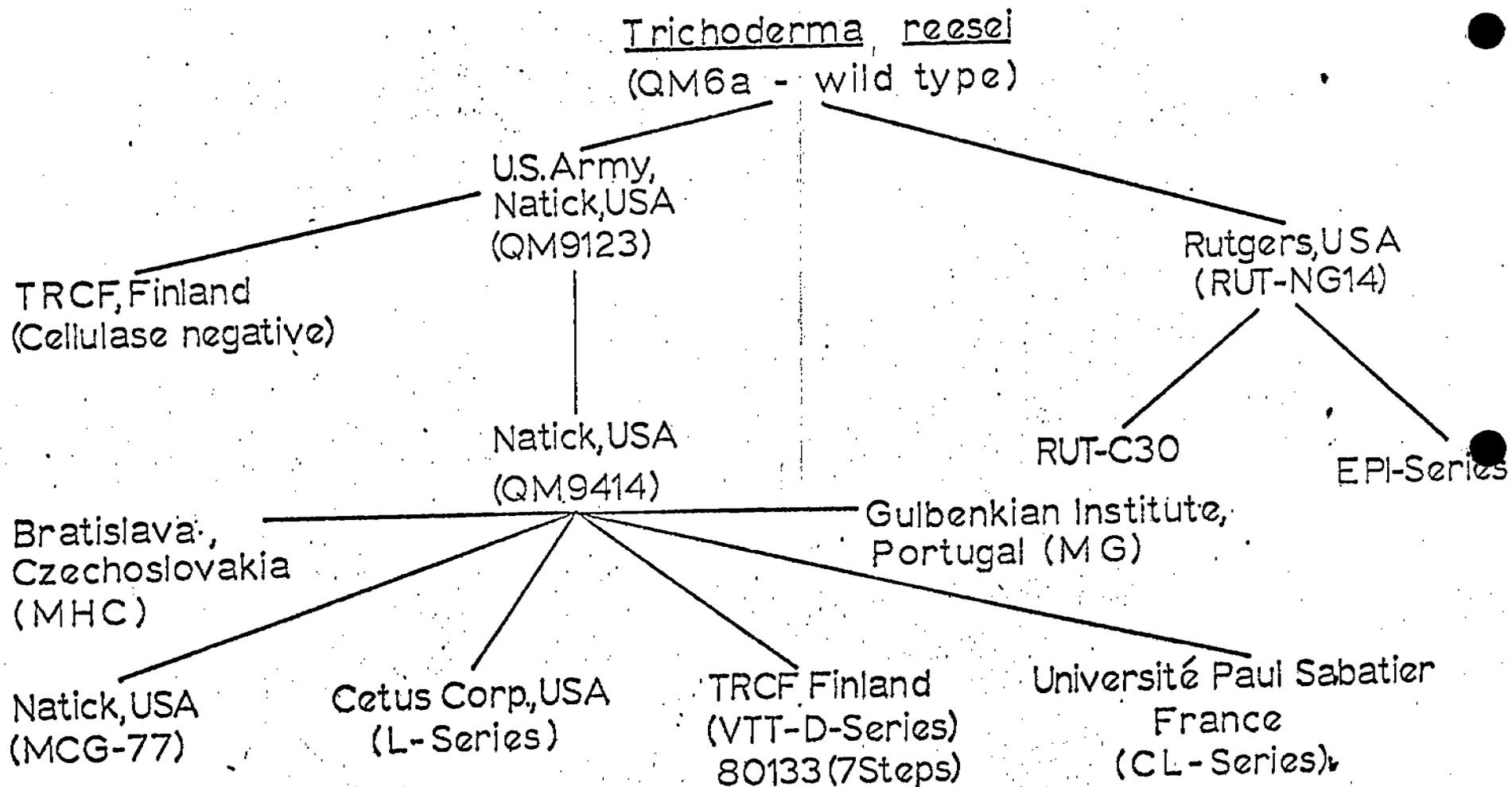


Figure 2. Genealogy of Rutgers Trichoderma reesei cellobiase mutants.

Figure 3. Worldwide Programs (1982) for the development of hypercellulolytic Trichoderma reesei strains.

CELLULASE PROGRAMS (Trichoderma reesei)



MUTANT PROGRAM

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