Biosynthesis of extracellular enzymes by isolates, mutants and recombinant strains of *Trichoderma* spp.

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The fungal genus *Trichoderma* contains species that have the potential to be of economic importance. Species in this genus are useful in the biological control of soilborne diseases (1) and tree decays. Some species produce useful enzymes and antibiotics (2) and others are involved in plant diseases (3). *Trichoderma* species have a powerful set of well – characterized exoenzymes involved in the cellulolytic pathway: cellobiohydrolase which hydrolyzes insoluble cellulose by exoattack from the non-reducing end of the cellulose chain, endoglucanase which clearly attacks soluble derivatives by an endo-type mechanism and β-glucosidase which hydrolyzes cellobiose derived from the glucanase action and cellodextrins to glucose (4). A synergistic and combined action of these three types of enzymes is necessary for the hydrolysis of crystalline cellulose. *Trichoderma* species are used as a biological control agent against the silver leaf disease of fruit trees caused by *Chondrostereum purpureum* (1). In this mode, it is a mycoparasitic action which, at least in part, should involve secretion of lytic enzymes. Lytic enzymes from *Trichoderma harzianum* are used for fungi protoplasts formation (5). Over the past 5 years, a great interest has been shown in the production of cellulase – free xylanase preparations to be applied in the pulp and paper industry. The most extensively studied xylanases are those produced by fungi, such as *Trichoderma* sp. (6).

One of the favoured organisms for production of cellulase is *Trichoderma reesei* QM6a. Cellulase production by *T. reesei* is subject of regulation i.e. induction by cellulose and repression by glucose and by other easily metabolizing sugars; consequently high productivities are obtained only in the media containing expensive and bulk cellulosic compounds as carbon source.

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Genetic improvement of *T. reesei* for cellulase production has been investigated in several laboratories and hyperproductive mutant strains were obtained by treatment with ultraviolet light, gamma irradiation, linear accelerator, diethyl sulphate and N-methyl-N-nitro-N-nitrosoquanidine. Some of the mutants have resistance to catabolite repression by glucose and very high cellulase productivity. In Table 1, the most representative results are summarized.

TABLE 1
ACTIVITIES OF FPU (FILTER PAPER UNITS) IN FERMENTATION OF DIFFERENT *T. REESEI* MUTANTS ON GLUCOSE OR CELLULOSE MEDIUM

Strain	Medium with	FPU (μmol/ml · min)	Protein (g/l)
QM 9414	4% glucose	0,4	2,0
RUT C-30	4% glucose	1,9	3,4
VTT-D-79125	4% glucose	6,4	9,6
CL 847	2% glucose	2,6	–
QM 9414	2% cellulose	1,0	–
CL 85	2% cellulose	4,1	–

Cellulase of *T. reesei* consists of two different types of enzymes, exo-cellobiohydrolases (CBH, EC 3.2.1.91) and endo-β-1,4-glucanases (EG, EC 3.2.1.4), each occurring in at least two genetically distinct isoenzyme forms, i.e. CBHI, CBH II, EG I and EG III (4). It has been shown that the first three enzymes were synthesized in approximately constant proportions, irrespective of whether cellulose, lactose or sophorose were used for induction of cellulases. Most interesting was that *T. reesei* secreted a low level of CBH II also during growth on glucose. The main cellobiohydrolase, cellobiohydrolase I (CBH I), forms the major part, about 60%, of the total secreted protein (4). The proportion of cellobiohydrolase II (CBH II) amounts to around 20% and endoglucanases, most of which is endoglucanase I (EG I), comprise 10%. β-Glucosidases account for only 1% of the total secreted protein. Cellobiohydrolase (CBH II), which comprises much lower portion of total protein, exhibits a significantly higher specific activity than CBH I. Hence, an increased proportion of CBH II in the *T. reesei* cellulase mixture should increase the overall cellulase specific activity. The application of genetic engineering to *Trichoderma* has made it possible to modulate cellulase production in such a way that new *T. reesei* strains producing novel cellulase profiles of commercial potential are now available. In an attempt to increase CBH II formation, multiple copies of the cbh 2 gene were introduced by

cotransformation with the Neurospora crassa pyr 4 gene using a pyr G qauxotrophic mutant of *T. reesei* QM 9414. The crude cellulase mixture of one transformant exhibited roughly 1,5 fold increase in specific cellulase activity (FPU) and displayed a 3 to 4 fold elevated CBH II formation (7). Table 2 lists several novel strains of *Trichoderma* constructed by genetic engineering.

TABLE 2

SEVERAL NOVEL *TRICHODERMA* STRAINS PRODUCING ALTERED OF CELLULASES, CONSTRUCTED BY GENETIC ENGINEERING. CELLULASE PROFILES HAVE BEEN CHANGED FOR INSTANCE BY INSERTIONAL INACTIVATION OF THE GENE RESPONSIBLE FOR THE SYNTHESIS OF CBH I AND BY REPLACEMENT OF THE GENE CODING FOR CBH II

T. reesei strain	Special feature
VTT-D-88358	Improved endoglucanase I production
VTT-D-87312	No cellobiohydrolase
Alko 2466	Improved endoglucanase I production, no cellobiohydrolase I
Alko 2566	No cellobiohydrolase II

Trichoderma strains producing altered mixtures of cellulases, constructed by genetic engineering. Cellulase profiles have been changed, for instance by insertional inactivation of a gene responsible for the synthesis of CBH I and by replacement of a gene coding for CBH II (8). It is therefore concluded that transformation with individual cellulase genes can be a useful and simple tool to alter the quantitative pattern of cellulase produced by *T. reesei*. The production of large amount of cellulase by *Trichoderma* indicates that this fungus has an excellent secretion capacity. One copy of each of the major cellulase genes in fungal genome indicates that the cellulase promoters are very strong. The production of calf chymosin with *Trichoderma* shows that this fungus is a useful organism not only for production of cellulases but also of proteins of heterologous origin (8). However, some proteins might be difficult to express in heterologous host (heme requiring ligninase), *Trichoderma* has proven promising alternatives to other existing production organisms.

Trichoderma sp. produce not only a set of powerful extracellular enzymes which can degrade carbohydrate fractions of lignocellulose but also, in addition to antibiotics, they produce lytic enzymes which enable them to destroy many plant pathogens. Elad et al. (9) suggested that strains of *Trichoderma* spp. may be selected on the basis of their biocontrol effectiveness by screening for 1,3-β-D-glucanase and chitinase activities with chitin and glucan; these are the most obvious targets to be attacked in the fungal pathogen cell walls. Thus, total protein profiles were obtained from a range of *Trichoderma* sp. and no correlation between those patterns and biocontrol effectiveness against *Rhizoctonia solani* was determined. Hovever, it has been shown

(10,11) that a broad range of fungi is inhibited in their growth *in vitro* by chitinases and that some specific 1,3-β-D-glucanase act synergistically with those chitinases in fungal growth inhibition. Kitamoto et al. (12) have shown that an enzyme from *T. harzianum* dissolved cell walls of a wide range of filamentous fungi belonging to *Basidiomycotina Ascomycotina*, *Deuteromycotina* and *Zygomycotina* and could be used to make protoplasts. A lyophilized preparation of the *Trichoderma* enzymes had about 0,3 units/mg 1,3-β-D-glucanase activity and 0,36 units/mg chitinase activity. The ability of this enzyme to digest cell walls of mycelia varied depending on the species. Other enzymes which could influence cell-wall degradation are protease. Cell walls of many fungi containing protease activity could be important in the biocontrol action of fungi.

The greatest potential for improving the production of extracellular enzymes appears to be in the use of a fed-batch mode of fermentation. In addition to the greatly enhanced production of enzymes Hendy et al. (11), reported that fed-batch operation allowed maintenance of lower levels of mycelial mass, thereby reducing the cost of aeration and agitation. Although extracellular enzymatic complexes of *Trichoderma reesei* mutants have been extensively studied, little is known about secretion of lytic enzymes over longer period of cultivation.

The primary results, obtained from fermentation runs at constant pH values on cellulose being the source of carbon, are shown in Fig. 1, 2. In the first fed-batch with *T. reesei* M-7 when the medium pH value was 3,5,

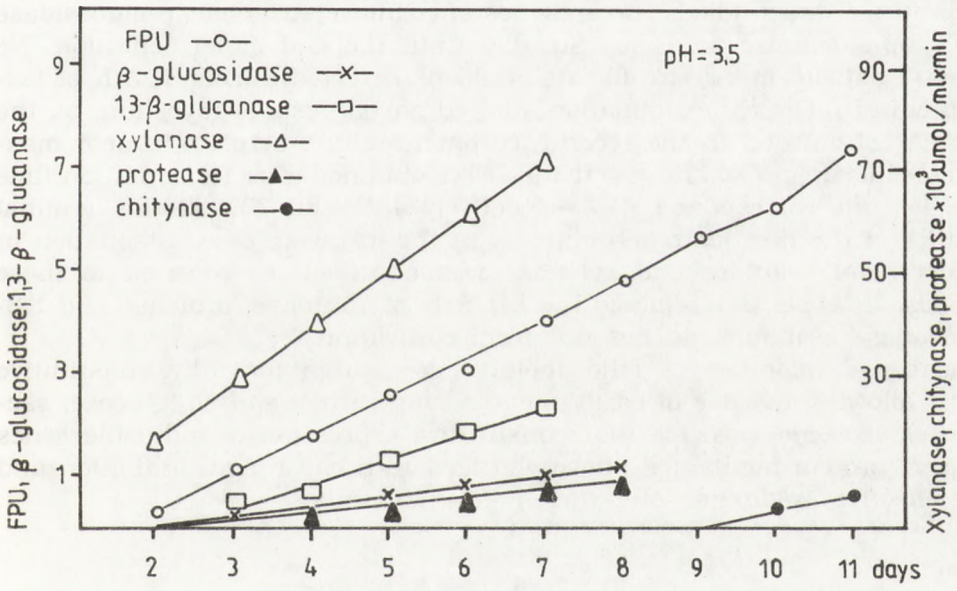

Fig.1. Fed-batch culture profile of *T. reesei* M-7 on cellulose, at pH 3,5.

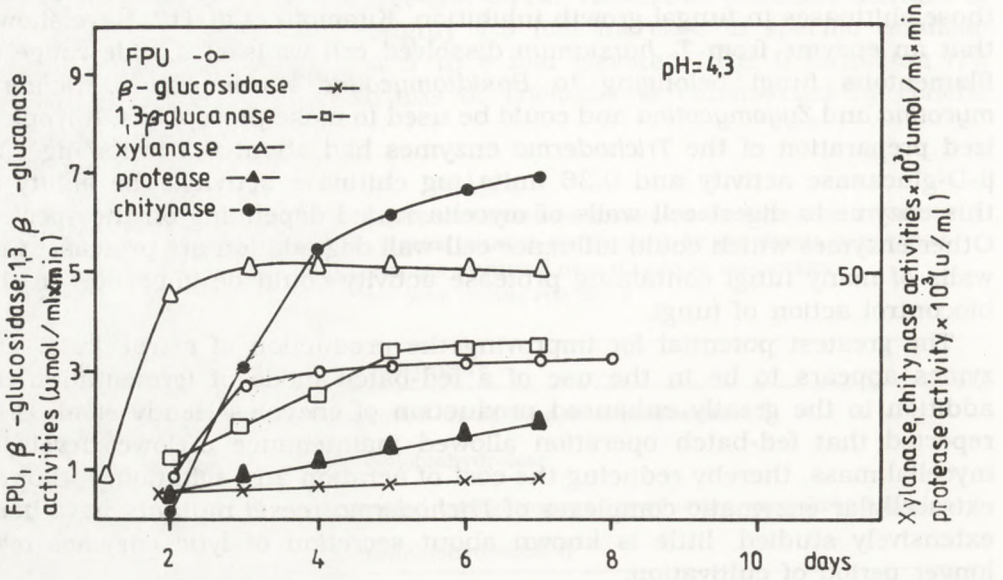

Fig.2. Fed-batch culture profile of *T. reesei* M-7 on cellulose, at pH 4,3.

the time course of FPU, xylanase, β-glucosidase, 1,3-B-glucanase, chitinase and protease was studied. The activities of cellulase, xylanase, β-glucosidase and 1,3-β-glucanase increased steadily until the end of fermentation. No chitinase activity in culture filtrate could be detected during 192 h of fed-batch culture. Optical examination showed partial lysis of mycelium on the sixth day of culture. In the second fed-batch culture at pH 4,3, near maximum cellulase and xylanase activities were obtained after fermentation time of 96 h, thus corresponding to respectively 45% and 70% of the terminal activities of the first fed-batch culture. In the following days, stagnation in production of cellulase and xylanase was observed. In contrast to these enzymes, *T. reesei* M-7 retained the capacity of chitinase, protease and 1,3-β-glulcanase formation during long-term cultivation.

Further development should include the search for fully constitutive strains allowing the use of easily metabolizing carbon source (glucose, saccharose). It seems possible that constitutive expression of inducible genes encoding protein mentioned above will lead to broad fungal inhibition and more effective production of extracellular enzymes.

Literature

1. Papavizas G. C., (1985), Ann. Rev. Phytopathol., 23, 23.
2. Domsch K. H., Gams W., Anderson T., (1980), Compendium of soil fungi, 1, Academic Press, London.
3. Shurtleff M. C., (ed), (1980), Compendium of corn diseases, 2nd ed. American Phytopathological Society, St. Paul, Minn.
4. Enari T-M., Niku-Paavola M-L., (1987), Critical Reviews in Biotechnology, 5, 67.
5. Toyama M., Shinmyo A., Okada H., (1983), J. Ferment. Technol., 61, 409.
6. Senior D. J., Mayers P. R., Seddlęr J. N., (1989), Appl. Microbiol. Biotechnol., 32, 137.
7. Kolbe J., Kubicek C. P., (1990), Appl. Microbiol. Biotechnol., 34, 26.
8. Unsitolo J. M., Nevalainen K. M. H., Harkki A. M., Knowles J. K. C., Penttilo M. E., (1991), J. Biotechnology, 17, 35.
9. Elad Y., Chet I., Chet L., (1982), Can. J. Microbiol., 28, 719.
10. Schlumbaum A., Mauch F., Vogeli U., Boller T., (1986), Nature, 324, 365-396.
11. Mauch F., Mauch-Mani B., Boller T., (1988), Plant Physiol., 88, 936-942.
12. Kitamato Y., Mori N., Yamamoto M., Ihiwa T., Ichikano Y., (1988), Appl. Microbiol. Biotechnol., 28, 445.
13. Hendy N., Wilke C., Blanch H., (1984), Biotechnol. Letters, 4, 785.

Biosynteza enzymów pozakomórkowych przez izolaty, mutanty i rekombinaty *Trichoderma* spp.

Streszczenie

Dokonano szerokiego przeglądu badań biochemicznych nad enzymami otrzymanymi z hodowli *Trichoderma reesei*. Podkreślono również rolę enzymów *Trichoderma harzianum* w rozpuszczaniu ścian komórkowych wielu gatunków grzybów patogenicznych. Ustalono zwiększenie aktywności enzymów pozakomórkowych przez właściwy dobór warunków fermentacji, związanych głównie z wartością pH.

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