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CO-CULTURE OF A FUNGUS TRICHODERMA AND A BACTERIUM AZOSPIRILLUM ON NITROGEN LACKING MEDIUM WITH CELLULOSE AS THE ONLY SOURCE OF CARBON. I. INDUCTION AND SELECTION OF MUTANTS OF TRICHODERMA WITH INCREASED CELLULOLYTIC ACTIVITY

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Key words: mutagenesis, UVrays, cellulolytic activity, carbon source

The strain *Trichoderma sp.* B was subject to mutagenesis using UV rays in order to obtain mutants with an increased cellulolytic activity. As a result mutants with considerably increased CMC-aşe and FP-ase activity were obtained.

During the last decade an increased interest in cellulolytic microorganisms has been observed, with a view of their future use for degradation and utilization of various cellulose waste materials. Of various microorganism groups with cellulolytic activity, such as bacteria, actinomycetes and fungi, the genus *Trichoderma* draws particular attention. In many laboratories studies are made on isolation and characteristic of cellulases produced by the fungus [3, 8, 9, 11]. Attempts are also made at isolating mutants of an increased cellulolytic activity [2, 5].

The ultimate objective of our studies is to obtain a co-culture of *Trichoderma* and *Azospirillum* in which cellulose decomposition would be associated with binding atmospheric nitrogen. A similar attempt was made by Veal and Lynch [10] who tried to connect cellulolytic activity of *T. harzianum* with that of an anaerobic azotroph *Clostridium butyricum*.

The results of their studies are encouraging. They have found, among others, a stimulation of growth of *T. harzianum* as a result of binding nitrogen by Clostridium whose growth was, in turn, stimulated by the cellulolytic

activity of the fungus. Continuing their line, we have decoded to make an attempt at associating the fungus *Trichoderma sp.* B-2 with another azotroph *Azospirillum brasilense.* Our choince of the nitrogen-binding parter was determined by the fact that according to literatura data (Bashan, Levanony, 1) it is a microaerophil of growth not limited by the presence of oxygen. We have decided, however, to go somewhat further, changing the partners with genetical methods i.e. increasing both the cellulolytic activity (Trichoderma) and the effeciency of binding atmospheric nitrogen (Azospirillum).

The induction of mutants of Azospirillum turned out to be much more difficult because of the scarcity of data on the physiology of the bacterium. This, somewhat more ambitious, thask has an advantage. In case of success of the first step (obtaining Trichoderma mutants with increased cellulolytic acitivity), it could be used to decompose cellulose waste materials, with the possibility of using the decomposition products as a source of carbon for other microorganisms executing fermentation of simple sugare.

In this paper results of studies on the induction and isolation of Trichoderma mutants with increased cellulolytic activity are presented and discussed.

MATERIAL AND METHODS

STRAINS

The following strains were used in the studies: T. harzianum - a strain coming from the collection of the Department of Food Biotechnology and Microbiology, Academy of Agriculture, Wrocław, *Trichoderma sp.* B-2 — a strain from our own collection, isolated from its natural environment by Bień. M.

MEDIA

a) Sabouraud medium according to Koch H. (4) — used for multiplying and storage of Trichoderma strains.

b) Czapek-Dox medium according to Koch H. (4) — used for testing some physiological properties of *T. harzianum and Trichoderma sp.* B-2

c) Mandels medium according to Ross A. (8), solidified with agar, supplemented with Bengal rouge (50 μg/ml) used for selection of mutants of *Trichoderma sp.* B-2 with increased cellulolytic activity. Liquid Mandels medium with 1% cellulose or 4% straw was used for determining CMC-ase and FP-ase activity of clutures of Trichoderma mutants.

CULTURE CONDITIONS

Strains of Trichoderma used in the experiments were multiplied on slopes of Sabouraud medium at 28°C till strong sporulation appeared (5 days). In case of experiments where liquid cultures were used, suspensions of conidia in physiological liquid consituted pre-cultures. The number of spores in 1 ml pre-culture was counted usind Malases chamber. Except for experiments in which physiological properties of *T. harzianum* and *Trichoderma sp.* B-2 were tested, liquid cultures were aerated by shaking.

INDUCTION AND SELECTION OF MUTANTS OF *TRICHODERMA SP.* B-2

For the induction with UV rays 5 ml suspensions of spores were used, taken from 5 day cultures on solid Sabouraud medium, of a dinsity of conidia 4.5×10^7 conidia/ml.

Spores were rinsed from the medium with sterilized 0,1% water solution Tween 80. After centrifugation the spores were suspended in 0.1 M posphate buffer, pH 7.2.

The density of suspension was determined using Malases chamber. 5 ml suspension of a known density were placed on a sterilized Petri dish. Thus prepared 3 dishes with spores were exposed to UV rays from a distance of 56 cm during 5.8 and 10 mins. A suspension of the same density, not UV-rayed, contituted aviability control. After induction plates were kept in darkness during 3 hrs. Then the conidia suspensions were diluted properly and inoculated on Mandels medium with 1% cellulose and Bengal rouge 50 µg/ml.

Te plates were incubated at 28°C until delicate growth appeared (48 hrs). Then the plates were transferred for 20 hrs to 45°C, and after that the incubation continued at 28°C. After a week, lighter zones were visible around colonies, a result of decomposition of the cellulose contained in the medium. The colonies which gave the largest lighter zones were transferred to Sabouraud slopes. Of 12 potential mutants 9 were selected for studies in order to determine their CMC-ase and FP-ase activity.

MEASUREMENTS OF ENZYMATIC ACTIVITY

The enzymatic activity was determined in supernatants resulting from centrifugation of 5 ml culture in Mandels medium with cellulose or straw. Of 100 ml of aerated culture 5 ml samples were taken for enzymatic tests after 3, 5, and 7 days.

The cellulolytic activity of CMC-ase and FP-ase was determined using the method of Mandels and Adreotti [6].

The enzymatic activity was expressed in units — μmols of reducing sugars re-calculated to glucose, liberated during 1 minute in 1 ml sample.

RESULTS

1. Physiology of Trichoderma — range of tolerance to medium pH and effect of glucose concentration and nitrogen content in the medium on growth efficiency.

Preliminary studies on the physiology of Trichoderma were performed using two strains — *T. harzianum* obtained from the Department of Food Biotechnology and Microbiology, Academy of Agriculture in Wrocław and *Trichoderma sp.* B-2 strain from authors' own collection isolated from ist natural environment. In three consecutive tests the dependence between the fungus growth and the medium pH, glucose concentration and nitrogen content in the medium were tested. The results summarized in Table 1 show that both strains of Trichoderma are highly tolerant to pH within the range 5-7, and for *T. harzianum* the highest growth efficiency was obtained at pH 5, *Trichoderma sp.* B-2 at pH 6.

Table 1. Dependence between the growth efficiecy in Trichoderma and the medium pH/mg dry weight/100 ml medium

Duration of culture (days)	Strain	*T. harzianum*				*Trichoderma sp.* B-2			
	pH	5	6	7	8	5	6	7	8
3		66.6	66.6	46.6	26.6	238.3	273.3	277	299
4		100	53.3	36.6	23.3	335.6	422	306.6	309.6
5		73.3	66.6	40	26.6	341.3	399.6	357	325
6		123.3	60	80	36.6	412.3	459	368.3	375.3

The studied fungi were characterized by a distinct dependence betwen their growth efficiency and glucose concentration, within such broad a range as 0.1-3% the increase of dry weight, and thus the biomas in Trichoderma sp. B-2 was, however, twice higher (Table 2).Both *T. harzianum* and *Trichoderma sp.* B-2 are, to a much lesser extent, dependent on nitrogen content in their growth. Even in medium with no nitrogen 40-60 mg dry weight were obtained per 100 ml medium, i.e. only 3 times less than in the medium containing 0.4-0.9 mg/ml nitrogen (Table 3).

2. Attempts at selection of mutants with increased cellulolytic activity.

When undertaking mutant induction, ultraviolet rays were selected of safe use and comparatively effective mutagenic action. Then the dependence

Table 2. Dependence between the growth efficiency in Trichoderma and the glucose concentration in the medium dry weight

Strain	*T. harzianum*					*Trichoderma sp.* B-2				
Glucose concentration (%) / Duration of culture (days)	0	0.1	0.5	1	3	0	0.1	0.5	1	3
3	16.6	53.3	73.3	83.3	133.3	19.3	51.6	36	71.6	175.3
4	16.6	23.3	73.3	123.3	163.3	28.3	88.6	131	169	332.3
5	23.3	56.6	103.3	133.3	280	34.6	97	207	328.3	504.6
6	23.3	60	116	163.3	310	49.8	105	264.6	359	581.3

Table 3. Depedence between the growth efficiency in Triochoderma and the nitrogen content in the medium dry weight

Strain	*T. harzianum*					*Trichoderma sp.* B-2				
Duration of culture days) / nitrogen content	0	0.0198	0.0988	0.198		0	0.0247	0.0494	0.247	0.494
3	36.6	26.6	30	36.6	70	56.6	81	30.2	104.6	108
4	23.3	20	16.6	43.3	60	58.6	83.6	34.6	147.6	149
5	40	43.3	33.3	60	126	62	98.6	40.2	164.6	171.3
6	40	73.3	29	120	150	63.3	101	50.2	174.6	191

between the viability of conidia and the irradiation dose was tested, assuming that the highest efficiency should be obtained at the viability 0.1-10% conidia

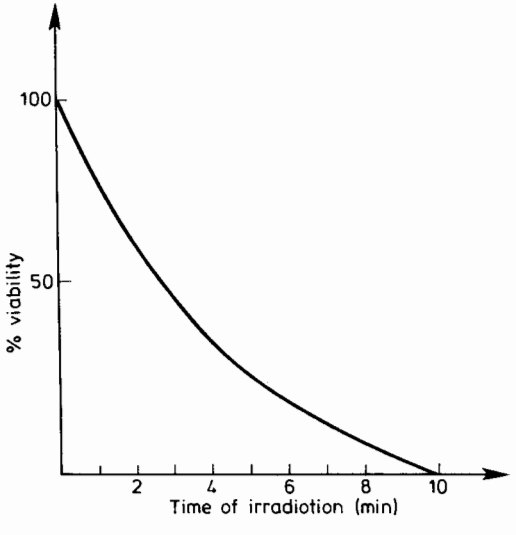

Fig. 1. Curve of viability at various time of UV irradiation

Trichoderma sp. B-2 were subject to mutagenesis. The dependence between the viability and the irradiation dose is presented in Fig. 1. Among the conidia which survived 5 and 8 minute irradiation, screening for cellulolytic activity was made, of 891 and 1286 colonies, respectively.

In the first case 2 colonies with a distinctly larger cellulose decomposition zone were isolated. In the second case 10 colonies were isolated. These variants were marked with symbols from L-1 to L-12. Nine of them were selected for further studies. Some of the selected variants differed also in their morphology

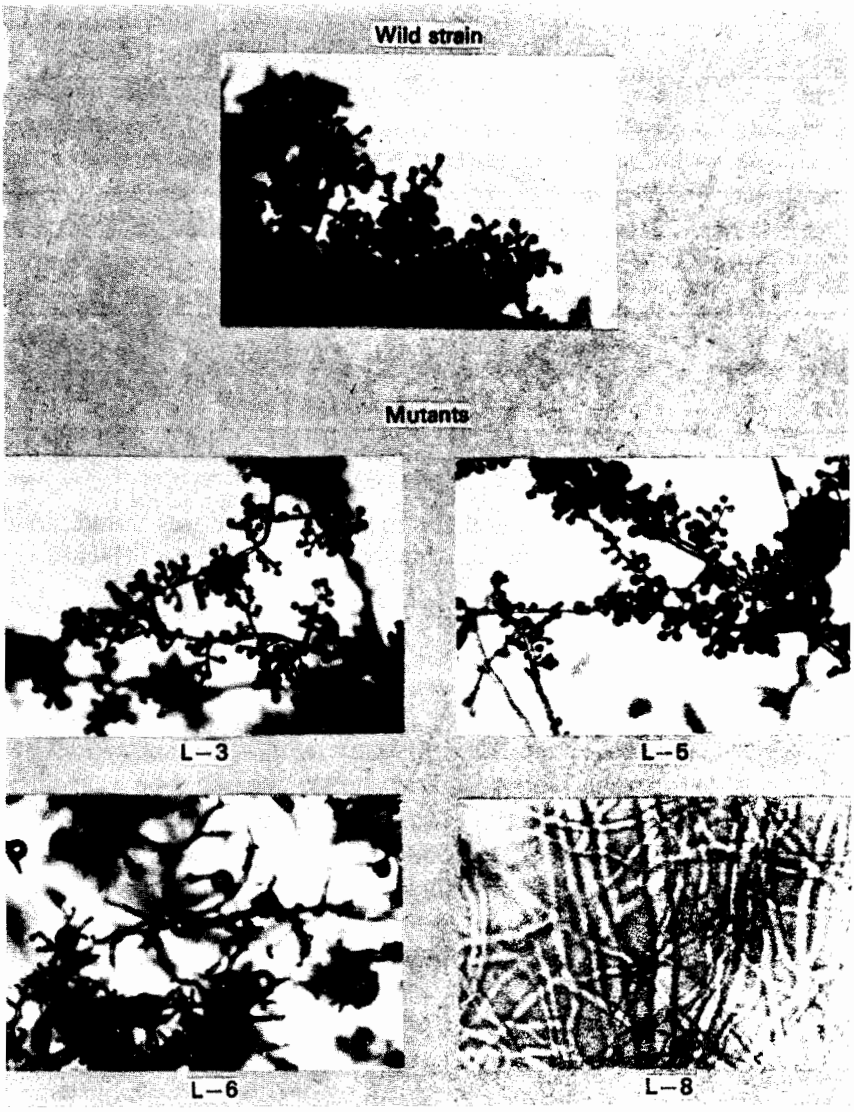

Fig. 2. Mycelium morphology of selected mutants and wild strain of *Trichoderma sp.* B-2

from the wild strain. Thus the variant L-6 sporulated much later, and the diamenter of its colonies was c. 5 × smaller. Around the colonies in Mandels medium large lichter zones appeared which suggested a considerable cellulolytic activity. The L-8 variant formed a sterile mycelium with no distinct lighter zone.

However, its abundant growth could suggest an increased use of cellulose. A comparison of mycelium morphology on microscope slides of particular variants and the wild is presented on photographs (Fig. 2). Having preliminarily selected variants of an increased cellulolytic activity, the activity of CMC-ase and FP-ase was tested. Five day cultures were tested, in liquid Mandels medium, assuming that they should be culture at stationary phase.

The results of tests of CMC-ase activity are presented in Fig. 3. Of 9 variants, 5 showed a distinctly increased activity of this enzyme, as compared to the original strain, and the mutant L-6 seems to be the most promising. Its CMC-ase activity was 3× higher than that of the wild strain. What more interesting, this variant showed also the highest FP-ase activity.

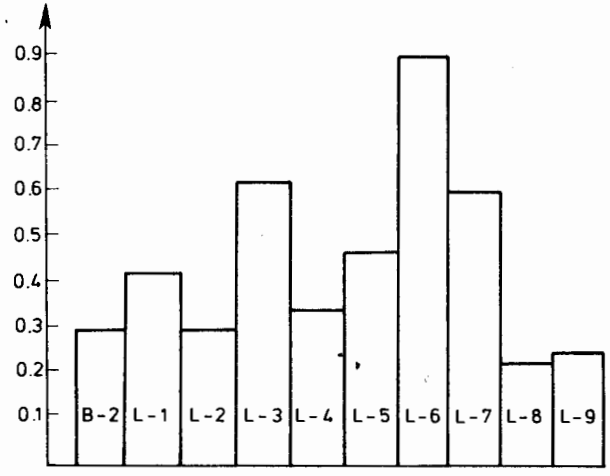

Fig. 3. CMC-ase activity in 5 day aerated cultures of mutants and wild strain *Trichoderma sp.* B-2

It appears significant that the same variants which show increased CMC-ase activity have also an increased FP-ase activity (Fig. 4). The variant mentioned (L-6) is also 3× more active with respect to FP-ase.

Because the ultimate goal of our studies is to use Trichoderma for biodegradation of cellulose waste materials (straw), the measurements of CMC-ase and FP-ase activity were repeated on Mandels medium with straw as a source of carbon. As a control, Mandels medium was used with cellulose. The measurements were taken in cultures 3, 5 and 7 days old. The results of CMC-ase activity are presented in Table 4. The measurements were made

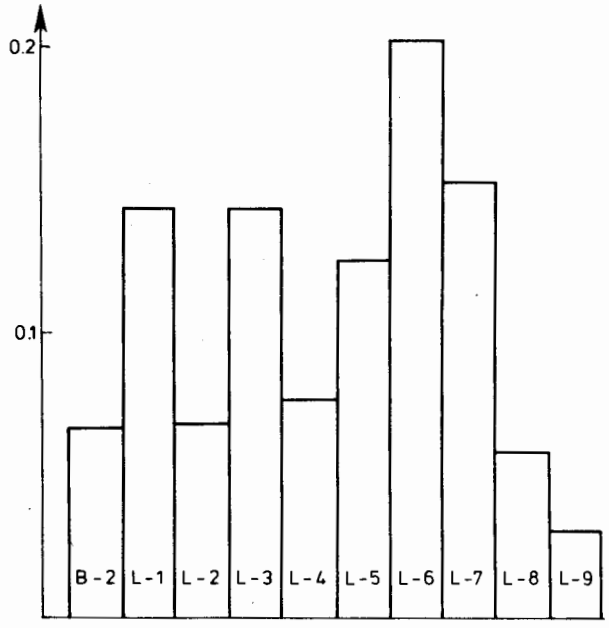

Fig. 4. FP-ase activity in 5 day aerated cultures of mutants and wild strain *Trichoderma sp.* B-2

Table 4. CMC-ase activity in aerated cultures mutants and wild strain *Trichoderma sp.* B-2 in Mandels medium with. a) with cellulose, b) with straw

Media		Mandels medium with cellulose			Mandels medium with straw		
Strain	duration of culture (days)	3	5	7	3	5	7
	L-1	0.050	0.46	1.14	0.10	1.90	4.39
	L-3	0.046	0.46	1.11	0.11	2.49	5.28
	L-5	0.048	0.43	1.05	0.10	2.30	4.64
	L-6	0.069	0.97	2.14	0.15	3.47	6.42
	L-7	0.047	0.59	1.17	0.12	2.88	5.38
	B-2	0.037	0.27	0.7	0.09	1.17	2.42

on variants which in the previous studies had show increases CMC-ase activity.

On the medium with cellulose as a substrate all the variants tested showed an increased CMC-ase activity as compared to the wild strain, the L-6 variant showing again a 3 × higher activity. The activity of the enzyme was the highest in 7 day cultures. It is worth noting that the values of CMC-ase activity of 5 day cultures were similar in this and in the preceding experiment.

As far the most important matter is concerned, i.e. the CMC-ase activity on straw as a substrate, as early as in 3 day culture it is already 2× higher than on the medium with cellulose. In 7 day cultures the differences in activity are at least 4×. Finally, the L-6 mutant also in this situation showed the highest CMC-ase activity.

On media with the same substrates measurements were also taken on FP-ase activity. The results are presented in Table 5. They indicate that also the FP-ase activity is higher with straw than with cellulose. In increases in time from 3 to 7 days and the L-6 mutant is the most efficient with respect to the activity of this enzyme.

Table 5. FP-ase activity in aerated cultures of mutants and wild strain *Trichoderma sp.* B-2 in Mandels medium with. a) cellulose, b) straw

Media		Mandels medium with cellulose			Mandels medium with straw		
Strain	duration of culture (days)	3	5	7	3	5	7
	L-1	0.020	0.094	0.34	0.044	0.46	1.06
	L-3	0.022	0.130	0.44	0.060	0.57	1.29
	L-5	0.024	0.125	0.44	0.065	0.48	1.34
	L-6	0.047	0.160	0.58	0.082	0.63	1.75
	L-7	0.026	0.140	0.50	0.057	0.56	1.39
	B-2	0.011	0.07	0.25	0.034	0.33	0.92

DISCUSSION

Our studies were aimed at defining basic growth parameters of Trichoderma. We have also undertaken an attempt at obtaining mutants of Trichoderma with an increased cellulolytic activity. The attempts at inducing mutants of B-2 of an increased cellulolytic activity were fairly successful. The increase in activity obtained, in spite of the single-step mutagenesis, does not depart from that of mutants isolated by Bland et al. (2) or Ostrikowa and Konovalova (9). What more, our mutants show a distinctly higher cellulolytic with straw than with pure cellulose. The L-6 mutant combining the highest CMC-ase and FP-ase activity appears to be especially interesting.

CONCLUSIONS

1. The growth efficiency of strains of *T. harzianum* and *Trichoderma sp.* B-2 is not drastically limited by such parameters of medium as nitrogen content and pH. There is, however, a distinct dependence between the growth efficiency of Trichoderma and the glucose concentration in the medium.

2. A single-step mutagenesis of the strain *Trichoderma sp.* B-2 allows obtaining of a considerable increase in the CMC-ase and FP-ase activity.

3. All the mutants of *Trichoderma sp.* B-2 show a considerably higher activity of cellulolytic enzymes in Mandels medium with straw than in the same medium with cellulose avicel as the only source of carbon.

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KOKULTURA GRZYBA TRICHODERMA I BAKTERII AZOSPIRILLUM NA PODŁOŻACH BEZAZOTOWYCH Z CELULOZĄ JAKO JEDYNYM ŹRÓDŁEM WĘGLA

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Streszczenie

Szczepy *T. harzianum* i *Trichoderma sp.* B-2 przebadano w celu określenia zależności między wydajnością wzrostu tych grzybów, a zawartością w podłożu azotu i glukozy pH podłoża. Wyniki przeprowadzonych doświadczeń pozwalają na stwierdzenie, że wydajność wzrostu mierzona w mg suchej masy/100 ml podłoża, jest w niewielkim stopniu uzależniona od stężenia azotu w podłożu, a także od pH podłoża. Wzrost Trichoderma jest wyraźnie limitowany stężeniem glukozy.

Szczep *Trichoderma sp.* B-2, izolowany ze środowiska naturalnego, poddano mutagenezie w celu uzyskania mutantów o wyższej aktywności celulolitycznej. Jako czynnik mutagenny zastosowano promieniowanie ultrafioletowe. Wstępną selekcję mutantów przeprowadzono kierując się wielkością strefy przejaśnienia wokół kolonii na podłożu Mandelsa z celulozą avicel jako jedynym źródłem węgla. Mając wstępnie wyselekcjowane potencjalne mutanty przeprowadzono

testy ich aktywności CMC-azowej i FP-azowej. Najwyższą aktywnością zarówno CMC-azową jak i FP-azową charakteryzował się mutant L-6. Przeprowadzono również badania w celu określenia efektywności wykorzystywania słomy jako jedynego źródła węgla w podłożu. Wszystkie mutanty wykazywały wyraźnie wyższą aktywność celulolityczną w podłożu Mandelsa ze słomą niż w tym samym podłożu z celulozą avicel.