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CELLULOLYTIC ACTIVITY OF REGENERATED PROTOPLASTS OF *TRICHOSPORON CUTANEUM* TrNU1 18

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Key words: *Trichosporon cutuneum*, cellulolytic, activity, *Trichoderma reesci*, protoplasts fusion

Clones of regenerated *T. cutaneum* protoplasts varied as regards the ability to hydrolyse cellulose. The zones of substrate hydrolysis ranged from 9.0 to 18.5 mm, with coefficient q=1.2-1.5. The regeneration of TrNU 18 protoplasts gave isolates of enhanced cellololytic abilities. Compared with the initial strain, the most active clone had a 16% greater endoglucanase activity and produced 20% more biomass.

INTRODUCTION

Protoplasts are a convenient model for various studies. They anable an assessment of enzyme secretion mechanisms and their localization [6, 9, 22], and are also used in taxonomic work, facilitating the isolation and characteristic of cell organelles [19, 20]. It is also possible to fuse protoplasts in order to obtain yeast varieties with specific properties [1, 17, 18]. In this research we strove to obtain improved yeast varieties displaying cellulolytic activity by protoplasts fusion, compdring this activity in Trichosporon protoplasts and in populations obtained after protoplasts regeneration in conditions described in [15].

MATERIAL AND METHODS

Two strains were used in the experiments:

— *Trichosporon* cutaneum TrNU 18, a mutant obtained by joint action of N-methyl-N -nitro-N-nitrosoguanidine and UV radiation [16], and

— *Trichoderma reesei* MHC 22 from the collection of the Chemicki Ustaw in Bratislava, Czechoslovakia.

The microorganisms were cultured on agar slants of standard malt wort and the YPS medium containing 2.5 g of yeast extract per dm^3, 5 g peptone per dm^3, and 10 g of soluble starch per dm^3; the culture was maintained at 28 °C for 2-5 days. Following culture, the microorganisms were stored at 4°C and reinoculated every ten days.

PREPARATION OF LYTIC ENZYME

The enzyme digesting *Trichosporon cutaneum* TrNU 18 cell walls was obtained from the *Trichoderma reesei* MHC 22 strain in a manner described by Oberman et al. in [15]. The source of lytic enzymes was an acellular postculture fluid concentrated to 1/5 of its original volume in a vacuum evaporator at 30 °C.

OBTAINING AND REGENERATION OF TRICHOSPORON *CUTANEUM* TrNU 18 PROTOPLASTS

The strain TrNU 18 was cultured in 500-cm^3 flasks for 10 h at 28 °C in 50 cm^3 of YPG medium containing (in every dm^3) 2.5 g yeast extract, 5.0 g peptone, 10 g glucose. The cells were separated from the culture medium by centrifugation at 4500 r.p.m. and washed with 0.05 M Mc-Ilvaine's buffer with pH = 4.5. The obtained biomass was pretreated for 30 min with 1% 2-mercaptoethanol at 30 °C and then suspended in 0.05 M Mc-Ilvaine s buffer stabilized osmotically with 0.8 M $MgSO_4$. The enzyme breaking up the *Trichosporon cutaneum* TrNU 18 cell walls was applied in doses of 0.12 mg protein/cm^3 of standardized yeast suspensio ($E_{550} = 0.37$). The incubation mixture was shaken gently on a shaker (80 r.p.m.) at 30 °C for 24 h.

The number of protoplasts was determined microscopically. The regeneration ability of protoplasts was expressed as the ratio of the number od colonies formed in the regeneration media to the number of protoplasts determined microscopically.

The cellulolytic activity of populations derived from the regenerated protoplasts was checked preliminarily by the indicating reaction method [7] and in shaker cultures in minimal medium M_0 containing (per dm^3) 3.0 g $(NH_4)_2 SO_4$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7 H_2O$, 3.0 g $CaCl_2$, 5.0 g of cellobiose, and 0.5 g of yeast extract; the pH of the medium was 5.0.

DETERMINATION OF CELLULOLYTIC COMPLEX ACTIVITY BY THE INDICATING REACTION METHOD [7]

The presence of cellulase in the cellulolytic complex is established by introducing into the M_0 growth medium 0.2% hydroxyethylcellulose (HEC) containing 13.5% covalently bonded Ostazin Brilliant Red H-3B (OBR-HEC). The initial medium for determining cellulase (endo-1,4-β-glucanase E.C.3.2.1.4) is stained red. Around colonies growing on Petri dishes there

appear light zones indicating the presence of cellulase secreted into the medium. The activity of xylanase (E.C.3.2.1.8) was determined in a medium containing 0.2% 4-o-methyl-D-glucorono-D-xylan with 13.2% Remazol Brilliant Blue R (RBB-xylan) which stains the medium blue. Xylanase secreted into the medium forms light halos around colonies. This method makes it possible to demonstrate the presence of the studied enzymes fairly rapidly (about 15 h), and was applied to preselect yeast varieties secreting extracellular hydrolases.

DETERMINATION OF CELLULOLYTIC ENZYMES ACTIVITY

The activity of endo-β-glucanase (E.C.3.2.1.4) and cellobiase (E.C.3.2.1.21) was determined by the method of Mandels et al. [12] using carboxymethylcellulose (BDH Chemicals Ltd, Poole, England) and cellobiose (Vereinigte Pharmazeumtische Werke, Praha, Czechoslovakia) as substrates. The activities of β-1,3-glucanase (E.C.3.2.1.6) and α-mannosidase (E.C.3.2.1.24) were determined according to Mann et al. [13] using laminarin and α-mannan as substrates. The amount of liberated reducing sugars was measured with the salicylic reagent [14]. The assumed unit of activity was the amount of enzymes producing 1 μmol of glucose in standard reaction conditions in 1 min.

Protein was determined by Lowry's method [11] using Serva serum albumin as standard.

The activity of acellular *Trichoderma reesei* MHC 22 postculture filtrates was determined on the basis of changes of opacity of *Trichosporon cutaneum* TrNU 18 cell suspension in reaction suspension. The unit of lytic activity of the enzyme was assumed to be the amount of enzyme reducing the opacity of the reaction mixture by 1% over 1 h [2].

The activity of cellulolytic enzymes complexes in acellular extracts and in *Trichosporon cutaneum* TrNU 18 protoplasts suspensions was determined according to Zigiel et al. [22]. The activity of endo-1.4-β-glucanase E.C.3.2.1.4 and β-glucosidase E.C.3.2.1.21 was determined together with the FPA „paper activity."

RESULTS

ACTIVITY OF LYTIC ENZYME PRODUCED BY *TRICHODERMA* REESEI MHC 22 AND FORMATION OF *TRICHOSPORON CUTANEUM* TrNU 18 PROTOPLAST

The activity of lytic enzymes produced by the *Trichoderma reesei* MHC 22 strain may be inhibited or enhanced by certain components of the culture medium such as yeast cell wall additions or cellulose (Fig. 1).

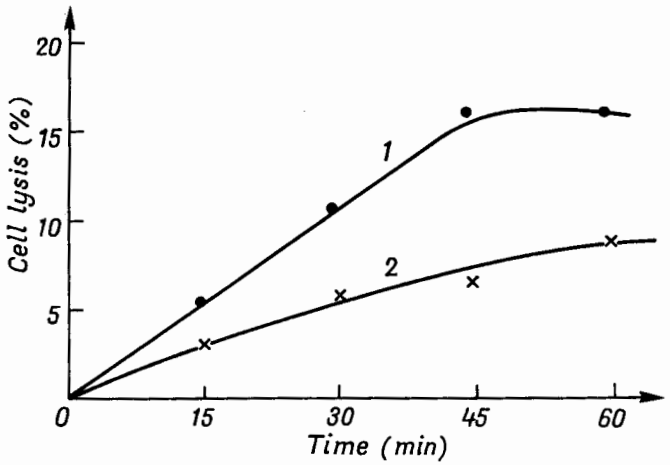

Fig. 1. Lytic activity of acellular *Trichoderma reesei* MHC 22 filtrates; 1 — *Trichosporon reesei* culture in synthetic medium with 2 g dry mass of TrNU 18/dm³ 2 — *Trichosporon reesei* culture in synthetic medium + 1%

A *Trichoderma reesei* culture with an addition of TrNU 18 yeast (2 g dry mass/dm³)obtained in a shaker culture in YPG medium produced a complex of enzymes causing a 50% greater lysis of *Trichosporon cutaneum* TrNU 18 cells than in media with a 1% addition of bracket fungus (Fig. 1). The *Trichoderma reesei* enzymatic complex featured enzymes hydrolyzing cellulose, laminarin and mannan (Table 1), which were responsible for the hydrolysis of β-1,3, β-1,4- and β-1, 6-glucosidase bonds of yeast cell walls components [8, 4].

Table 1. Activity of lytic enzymes produced by *Trichoderma reesei* MHC22

Substrate	*T. reesei* 1+			*T. reesei* 2+		
	mg protein	activity		mg protein	activity	
	(cm³)	(j/cm³	(j/mg protein)	(cm³)	(j/cm³)	(j/mg protein)
Laminarin	0.12	0.069	0.575	0.135	0.037	0.422
Mannan	0.12	0.053	0.442	0.135	0.047	0.348
Carboxymethyl-cellulose — Na	0.12	0.041	0.342	0.135	0.072	0.533

1 — culture in synthetic medium with TrNU 18 biomass (2 mg yeast dry mass/dm³ medium),
2 — culture in synthetic medium with 1% bracket fungus, j = μmol glucose/min

The cell walls of *Trichosporon cutaneum* TrNU 18 proved highly resistant to the applied lytic enzyme. A satisfactory number of protoplasts (about 81%) were obtained only after 24 h of exposure to the enzyme following pretreatment of the cells with 2-mercaptoethanol and in the presence of 0.05

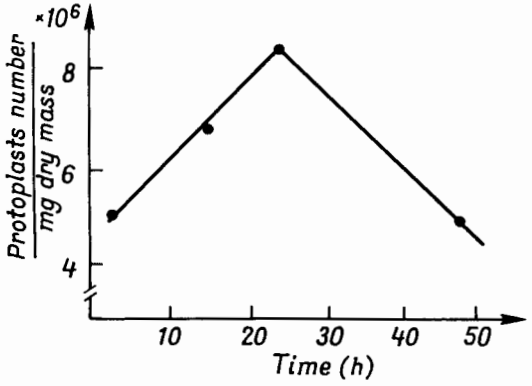

Fig. 2. Dynamics of TrNU 18 protoplasts formation in optimal conditions

M Mc-Ilvaine's buffer with pH of 4.5-5.0, 0.8 M $MgSO_4$ as osmotic stabilizer, and yeast cells from the logarithmic growth phase (Fig. 2)

When older yeast populations (48 h) were used, the protoplasts formation yield dropped to 48.6%, a figure similar to that observed by Picataggio [17. This autor linked the decreased effectiveness of the „Driselase" lytic enzyme with regard to the older (upwards of 20 h) Trichoderma reesei mycelium to changes of the chemical composition of the fungus cell wall and especially to the accumulation of chitin there.

Cellulolytic activity of protoplasts

The protoplasts of *Trichosporon cutaneum* TrNU 18 were capable of regeneration in synthetic and natural media, with the degree of regeneration depending on the stabilizer of the regeneration medium (Table 2). In the

Table 2. Regeneration of *Trichosporon cutaneum* TrNU 18 protoplasts in various media (results after 66 h of culture)

Stabilizer	Regeneration medium*	Per cent of regenerated TrNU 18 protoplasts
0.8 M $MgSO_4$	S medium according to Maraz	19.5
	GE medium	34.1
0.8 M mannitol	GE medium	46.2
	SE medium	33.0
	SG medium	52.7
0.3 M $CaCl_2$	S medium according to Maraz	21.2

* S medium according to Maraz: 0.5% $NH_4{}_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7\ H_2O$, 2% glucose, 2% agar, 0.1% Wickerham's vitamin solution
GE medium: 0.5% glucose + 0.3% yeast extract + 1.5% agar
SE medium: 0.1% HN_4Cl, 0.62% KH_2PO_4 0.37% $Na_2HPO_4 \cdot 2\ H_2$, 0.025% $MgSO_4 \cdot 7\ H_2O$, 2% agar + 0.3% yeast extract
SG medium: 0.1% HN_4Cl, 0.62% KH_2PO_4 0.37% $Na_2HPO_4 \cdot 2\ H_2O$, 0.025% $MgSO_4 \cdot 7\ H_2O$, 2% agar + 0.5% glucose

presence of 0.8 M mannitol the number of regenerated *Trichosporon cutaneum* TrNU 18 protoplasts amounted to 33-52.7%, whereas in media with 0.8 M $MgSO_4$ the figure was 19.5-34.1%. The advantageous effect of $MgSO_4$ in the formation and regeneration of protoplasts was also reported by Jacobsen [10] and Długoński [5].

Table 3 presents results of cellulolytic activity tests of populations obtained from regenerated protoplasts of *Trichosporon cutaneum* TrNU 18. In the plate test this activity was demonstrated by 17 strains constituting 36.5% of all the studied clones. The capability to hydrolyse cellulose varied from strain to strain: the substrate hydrolysis zones ranged from 9.0 to 18.5 mm, with the coefficient q = 1.2-1.5 (Table 3).

All the tested populations derived from regenerated protoplasts *of Trichosporon cutaneum* TrNU 18 were able to metabolize xylan. The populations formed colonies of various size, with diameters ranging from 9.0 to 21.0 mm (Table 3).

Table 3. Growth of regenerated TrNU 18 protoplasts in various selective media

Growth medium	No. o tested strains	No. of colonies formed on selective media	Diameter of colonies (mm)	Diameter of substrate hydrolysis zone (mm)	q
OBR-hydroxyethyl-cellulose (OBR-HEC) + M_o	32	17	1-13	8.4-19.5	1.2-1.5
RBB-xylan + M_o	16	16	9-21	9-21	1

* $q = \frac{\text{substrate hydrolysis zone (mm)}}{\text{colony diameter (mm)}}$

OBR-HEC = Ostazin Brilliant Red H-3B-hydroxyethylcellulose

RBB-xylan = Remazol Brilliant Blue R-xylan

M_o = minimal medium (%) = 0.3 $(NH_4)_2SO_4$, 0.1 KH_2PO_4, 0.05 $MgSO_4 \cdot 7\ H_2O$, 0.05 yeast extract, 1.5 agar; pH = 5.0

Table 4. Basic growth parameters of populations derived from regenerated TrNU 18 protoplasts in M_o medium with 0.5% cellobiose

Strain	Dry mass (mg/cm*)	Dry mass yield (Y_p)*	Specific growth ratic μ (h^{-1})	Generation time T (h)	Enzyme activity**	
					CMC-ase	β-glucosidase
TrNU 18 (parent)	2.5	0.50	0.27	2.56	0.032	0.103
TrNU 18/1	2.6	0.52	0.28	2.47	0.032	0.100
TrNU 18/2	2.5	0.50	0.26	2.66	0.032	0.100
TrNU 18/3	3.0	0.60	0.24	2.88	0.037	0.124
TrNU 18/4	2.6	0.52	0.29	2.39	0.042	0.105

* $Y_p = \frac{\text{yeast dry mass (mg/cm}^3\text{)}}{\text{cellobiose content (mg/cm}^3\text{)}}$

** Actizvity after 11 h of culture
j = μ mol glucose

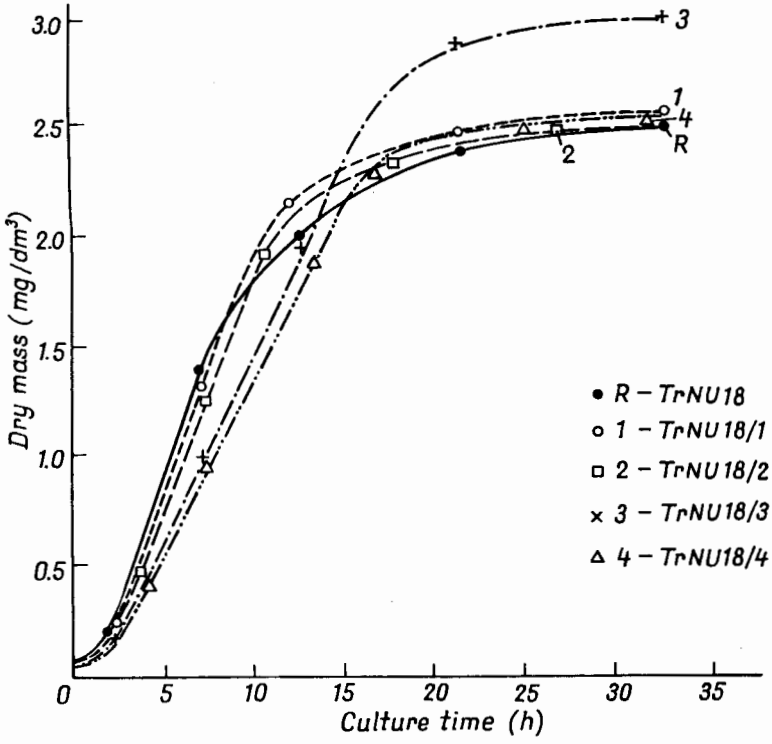

Fig. 3. Growth dynamics of regenerated TrNU 18 protoplasts in M_0 medium with 0.5% cellobiose

A comparison of growth dynamics and cellulolytic activity of four populations obtained from regenerated protoplasts of the TrNU 18 strain is shown in Table 4 and Fig. 3. In the plate test they proved capable of simultaneous hydrolysis of cellulose and xylan (Table 3). As can be seen in Table 4,the obtained varieties differed as to cellulolytic enzymes activity. In two strains, denoted TrNu 18/3 and TrNU 18/4, CM S-ase activity increased by 16-31% compared with the initial strain. The TrNU 18/3 strain also displayed a 20% higher activity of β-glucosidase. The remaining populations obtained from regenerated protoplasts demonstrated cellulolytic activities similar to those in the incitial *Trichosporon cutaneum* TrNU 18 strain. The regenerated strains in minimal medium Mo containing 0.5% cellobiose produced 2.5-3.0 g biomass per dm^3, the yield Y_P being 0.5-0.6 (Fig. 3, Table 4).

The TrNU 18/3 clone characterized by high cellulolytic enzymes activity also produced 20% more biomass than the parent TrNU 18 strain (Table 4). Thus, in the course of protoplasts production and regeneration we obtained a *Trichosporon cutaneum* strain with enhanced cellulolytic enzymes activity producing more biomass than its parent strain. Increased activity of extracel

lular β-glucosidase in regenerated Penicillium pinophilum protoplasts was also reported by Brown et al. [3].

Comparing results of cellulolytic enzymes activity in *Trichosporon cutaneum* TrNU 18 cells and protoplasts, we see that the protoplasts contain only slight amounts of cellulolytic enzymes accounting for 4.0—6.8% of total cellulolytic activity found in the initial *Trichosporon cutaneum* TrNU 18 population (Table 5). This confirms the belief that cytoplasm of *Trichosporon cutaneum* TrNU 18 cells contains only a small amount of cellulolytic enzymes.

Also noteworthy is the fact that after the regeneration of protoplasts, about 64% of the clones which grew on plates did not metabolize cellulase as the sole carbon source (Table 3). This would support the view that the cellulolytic activity of *Trichosporon cutaneum* TrNU 18 is not regenerated in tune with the regeneration of cell walls.

Table 5. Compariсon of cellulolytic activity in Trichosporon cutaneum TrNU 18 cells and protoplasts

Cellulolytic enzyme	Activity in acellular extracts of parent strain strain (j/mg protein)	Activity in TrNU 18 protoplasts lysata	
		(j/mg protein)	compared with cellulotytic activity of cells (%)
endo-1.4-β-glucanase	0.30	0.012	4.0
β-glucosidase	0.19	0.013	6.8
FPA „paper activity"	0.19	0.010	5.3

CONCLUSIONS

1. *Trichosporon cutaneum* protoplasts regenerated in both natural and synthetic media. The degree of protoplasts regeneration, ranging from 19.5 to 52.7%, depended on the stabilizer of the regeneration medium. Regeneration was best in media stabilized with 0.8 M mannitol (Table 2).

2. The plate test combined with suitable indicators may be used to rapidly select regenerated TrNU 18 protoplasts and assess their activity. This test revealed that, compared with the regenerated populations, 36.5% of the clones hydrolysed cellulose, and 100% metabolized xylan (Table 3).

3. By regenerating protoplasts and subjecting them to a two-stage selection in indicator media, it is possible to obtain *Trichosporon cutaneum* strains biosynthesizing cellulolytic enzymes better than the initial strain. The selected TrNU 18/3 strain produced 20% more biomass in minimal medium with cellobiose, and exhibited a 16% higher endogucanase activity and 20% higher β-glucosidase activity (Table 4).

4. *Trichosporon cutaneum* protoplasts display slight cellulolytic enzymes activities, amounting 4.0—6.8% of tatal cellulolytic activity of the parent cells (Table 5).

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Aktywność celulolityczna zregenerowanych protoplastów drożdzy *Trichosporon cuteneum* TrNU18

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Streszczenie

Protoplasty *Trichosporon cutaneum* TrNU18 cechowały się zróżnicowaną zdolnością regeneracji na zastosowanych podłożach (tab. 2). Najkorzystniejszym środowiskiem dla odtwarzania ścianki komórkowej drożdży *Trichosporon cutaneum* TrNU18 okazało się podłoże syntetyczne z dodatkiem 0,5% glukozy (SG) stabilizowane za pomocą 0,8 M mannitolu. Liczba zregenerowanych tu proto-plastów wynosiła 52,7% (tab. 2). W podłożach stabilizowanych 0,8 M mannitolem proto-plasty *Trichospon cutaneum* odznaczały się wyższą o ok. 35% zdolnością regeneracji niż w obecności 0,8 $MgSO_4$ (tab. 2). Stosunkowo słabo, bo ok. 21% protoplastów regenerowało się w podłożu syntetycznym wg Maraz, w którym stabilizatorem smotycznym był 0,3 M $CaCl_2$. Zregenerowane protoplasty tworzyły w podłożu minimalnym M_0 z dodatkiem 0,5% celobiozy plon biomasy z wydajnością $Y_p = 0{,}4-0{,}6$ (tab. 4).

W wyniku regeneracji protoplastów otrzymano klon TrNU18/3, który charakteryzował się korzystniejszymi właściwościami niż szczep rodzicielski TrNU18, mianowicie: podwyższeniem o 16% aktywności endoglukanazy i o 20% β-glukozydazy, wyższą o 20% produkcją biomasy (tab. 4).

W protoplastach *T. cutaneum* zlokalizowane są niewielkie ilości enzymów celulolitycznych. Ich aktywność stanowiła od 4% do 6,8% całkowitej aktywności oznaczanej w komórkach populacji wyjściowej (tab. 5).