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CELLULOSE UTILIZATION ABILITY IN *TRICHOSPORON CUTANEUM*

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The yeasts *Trichosporon cutaneum* show the ability to utilize cellulose as the only source of carbon and energy. Their cellulolytic activity may be intensified by an associated mutation (NTG + UV) and an addition of cellobiose to the culture medium.

INTRODUCTION

The best known microorganisms with high cellulolytic activity are fungi of genera *Aspergillus, Trichoderma, Chaetomium, Alternaria, Rhizopus, Penicillium,* and the bacteria *Cellulomonas, Pseudomonas, Cellvibrio, Bacillus* and *Cellulobacillus* species [3, 4, 6, 15, 16]. Yeasts belong to the group of microorganisms which are incapable to the direct utilization of celluloshe, the only exception here being strains of the genus *Trichosporon* which were found by Dennis to exhibit cellulolytic abilities [2]. *Trichosporon cutaneum* and *Trichosporon pullulans* strains in shaker cultures completely decomposed pieces of Whatman 1 filter paper in two weeks.

The *Trichosporon* yeasts were isolated from tree secretions and wood pulp [8]. Lodder [8] includes them into the family *Cryptococaceae.* They multiply by budding and through oidia, forming pseudomycelia and mycelia. They assimilate glucose, lactose, soluble starch and cellobiose.

In more recent studies Spencer [19] demonstrated that *Trichosporon cutaneum* displays amylolytic capabilities. According to Ilina et al. [7] *Trichosporon cutaneum* is resistant against higher temperatures and may develop even at 40°C. Its maximum growth rate is at pH 5.7-7.0. Neujahr [10] found that *Trichosporon* strains de grade phenols, polyphenols and

other aromatic compounds. The *Trichosporon cutaneum* biomass is characterized by high contents of protein (54-58%) and exogenous amino acids [7, 12, 22]. Given the advantageous physiological and biochemical features it is advisable to search active varieties of *Trichosporon cutaneum* capable to the direct utilization of cellulose, containing a complex of cellulolytic enzymes such as:

— glucanhydrolase 1,4-beta-D-glucan,EC.3.2.1.4.-endoglucanase (Cx),
— cellobiohydrolase 1,4-beta-D-glucan,EC3.2.1.91-exoglucanase (C1),
— beta-glucosidase,EC.3.2.1.21-cellobiase [20, 23].

MATERIAL AND METHODS

BIOLOGICAL MATERIAL

The studies were performed with *Trichosporon cutaneum* strains obtained from the Collection of Pure Cultures of the Institute of Fermentation Technology and Microbiology, Łódź Technical University, and with mutants obtained by associated mutation with NTG (N-methyl-N'-nitro-N-nitrosoguanidine) and UV. Also strains isolated from starch media — Candida tropicalis and *Candida mogii* — as well as test strains — *Endomycopsis*, *Chaetomium* and *Trichoderma viride* were used. The mutants were transfered every ten days to YPG agar slants [8], incubated at 28°C for two days and then stored at +4°C.

GROWTH MEDIA

The media used are described in Table 1. They were sterilized at 121°C for 20 min.

PRELIMINARY STUDIES OF CELLULOLYTIC ABILITY OF YEAST STRAINS

Cellulolytic activity was determined using two methods:

— The direct method of Rauted and Cowlings [1]. Analysis was performed on standard Mo medium with 2% cellulose swelled treated with 85% orthophosphoric acid. The composition of the medium is given in Table 1. 10 cm^3 of Mo medium was placed into test tubes, 18 mm in diameter, and 2 cm^3 of standardized yeast suspension (absorbance = = 0.2-0.3 at λ = 530 nm) was added. The culture was maintained at 30°C utinl 18 days. The strains with cellulolytic abilities decomposed cellulose forming transparent zones in the opaque agar slopes.

— The indicator method using the Cellulose-Azure preparation [18]. 2 cm^3 of Mo agar medium and 0.2 cm^3 of 2% Cellulose-Azure suspension

Table 1. Growth media

Media compositions	Media application
Y.P.G. [8]	strain storage, inoculations for counting
standard Mo medium (% w v): $(NH_4)_2SO_4$ 0.30; KH_2PO_4 0.10; $MgSO_4 \cdot 7H_2O$ 0.05; yeast extract 0.50; cellulose 2.00	checks of cellulolytic properties of yeasts
Mo medium + 2% swollen cellulose or 2% Cellulose-Azure	selection of cellulolytic yeasts
Mo medium + 0.1, 0.5 and 1% swollen cellulose [1]	study of growth dynamics of *T. cutaneum*
Potato extract [8] + 0.1, 0.5 or 1% of cellulose added at $\tau = 30$ h of culture	study of growth dynamics of *T. cutaneum*
Potato extract + 0.5% regenerated cellulose added at $\tau = 0$ h of culture	study of growth dynamics of *T. cutaneum*

were poured into test tubes. The medium thus obtained was inoculated with a suspension of the studied microorganisms and cultured at 30°C. During growth the cellulolytic strains utilizing cellulose from Cellulose-Azure secreted a blue stain into the medium.

STUDY OF GROWTH DYNAMICS OF *TRICHOSPORON CUTANEUM*

The growth dynamics of Trichosporon cutaneum was determined in a shaker culture in the following media:

— standard Mo+0.1, 0.5 or 1% of swollen cellulose,

— potato extract+0.1, 0.5 or 1% of swollen cellulose added at time $\tau = 30$ h of culture,

— potato extract+0.5% cellulose added at $\tau = 0$ h of culture.

Every 24 h during the 96-h culture cell content was determined by the plate method; the results were expressed as the number colony furminf of units capable of growth in the form of colonies (CFU/cm^3); sugars were determined by paper chromatography [13].

MUTATION OF *TRICHOSPORON CUTANEUM*

The yeasts were subjected to associated mutation with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and UV radiation. Strain *Trichosporon cutaneum* Tr1 was cultured in YPG medium for 48 h, a cell suspension of denusity $2.75 \cdot 10^7$ CFU/cm^3 was prepared in phosphate buffer of pH 0.7. The suspension was treated with 800 μg/cm^3 NTG for 30 min. After mutation the yeasts were centrifuged, washed and suspended in

sterile distilled water. The resultant suspension was UV-irradiated for 30 and 90 seconds. Following mutation the yeasts were stored in darkness for 2 h and then centrifuged and washed with a buffer of pH 7.0. The number of viable cells was determined by inoculation on the YPG medium. All mutants obtained in this medium were isolated on agar slants and used for study after 24 h of culture.

METHODS OF ESTIMATIONS

— The plate method was used to determine the number of colony forming units in the YPG medium; the results were given as CFU/cm^6.

— Endoglucanase activity was determined as the activity of CMC-ase in cell-free culture agains carboxymethylcellulose sodium salt (Na-CMC). The amount of enzyme forming a 1 mg of glucose at 40°C during 1 h was taken as the unit of CMC-ase cellulolytic activity.

— Beta-glucosidase activity was determined by measuring the amount of glucose obtained during 30-min hydrolysis of 10 mmol solution of cellobiose at 50°C in 0.1 M phosphate buffer of pH 5.5. The activity was expressed in μmol glucose min [9, 16, 21].

RESULTS AND DISCUSSION

CELLULOLYTIC ABILITY OF THE YEASTS

The cellulolytic ability of yeast strains isolated from starch media and stored in pure cultures collection was investigated. The results of the study are shown in Table 2. The performed tests revealed that only

Table 2. Cellulose utilization by yeasts after 18 days of culture

Microorganism	Height of cellulose hydrolysis zone in agar slopes (mm)	Indicator method with Cellulose-Azure
Candida tropicalis Sd33	0	–
Candida tropicalis R37	0	–
Candida mogii	0	–
Trichosporon cutaneum	6	+ + +
Trichosporon sericeum	n.d.	+ +
Trichosporon pullulans	n.d.	–
Endomycopsis fibuligera	0	–
Endomycopsis capsularis	0	–
Trichoderma viride	7	+ + +
Chaetonium globosum	7	+ + +

+ + + — strong blue colour (cellulose decomposition)
– — absence of blue colour (no cellulose decomposition)
n.d. — not determined

two of the examined strains, *Trichosporon cutaneum* and *Trichosporon sericeum*, displayed cellulolytic capabilities. The strain *Trichosporon pullulans* and the remaining *Candida* and *Endomycopsis* yeasts failed to produce the cellulolytic enzyme complex. *Trichosporon cutaneum* exhibited a distinct ability to decompose cellulose: after 18 days of culture the height of the cellulose hydrolysis zone in agar slopes was 6 mm, there was also the intense staining in the culture with Cellulose-Azure (Table 2). Strains of *Trichoderma viride* and *Chaetomium globosum*, typical species known as cellulolytic Ones cultured in identical conditions produced 7 mm cellulose hydrolysis zones (Table 2).

TRICHOSPORON CUTANEUM GROWTH DYNAMICS

The results of experiments are illustrated in Figs 1-3. These show that culture in potato extract with various doses of cellulose 0.1, 0.5 and 1%) added after 30 h of growth did not cause significant change in cell number in subsequent periods of culture (Fig. 1).

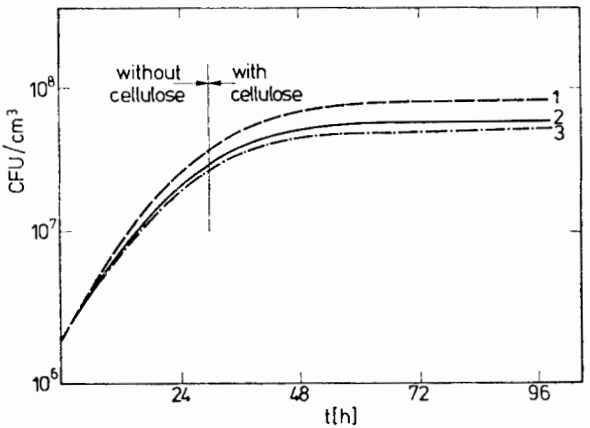


Fig. 1. Growth of *Trichosporon cutaneum* in potato extract with various doses of cellulose (1 — 0.1%, 2 — 0.5%, 3 — 1%) added after 30 h of culture

On the other hand, in the media with 0.5% cellulose added to the potato extract at the beginning of culture ($\tau = 0$) there was a distinct increase in cell number to $3.17 \cdot 10^8$ CFU per cm³ (Fig. 2). This may be due to the induction of cellulolytic enzymes by cellulose, their its activity becoming apparent after the exhaustion of carbon components in the potati extract.

Additions of 0.5% and 1% cellulose to the potato extract after 30 h of culture did not cause the increase in cell number. In the presence of 0.1% cellulose the cell number was $8.5 \cdot 10^7$ CFU/cm³ while at 1% cellulose added to the medium $6.95 \cdot 10^7$ CFU/cm³ were formed (Fig. 1).

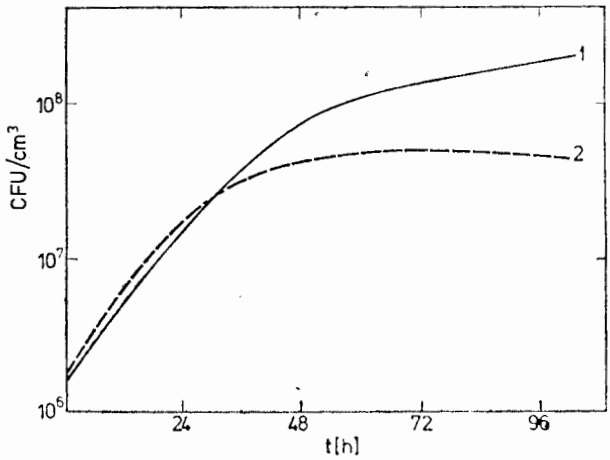
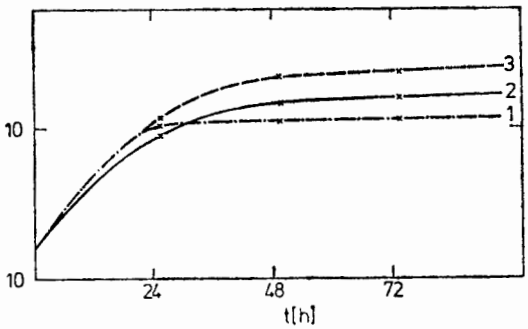


Fig. 2. Growth of *Trichosporon cutaneum* in potato extract with 0.5% cellulose added at 0 h (1) and after 30 h of culture (2)



Concentration	μ (h^{-1})	T (h)	μ_{max}	$K_s(g)_{dm^3}$
0.1	0.067	10.3		
0.5	0.073	9.76	0.09	1.56
1.0	0.082	8.45		

Fig. 3. Growth of *Trichosporon cutaneum* in Mo medium with various doses of cellulose (1 — 0.1%, 2 — 0.5%, 3 — 1%)

As we see in Fig. 3 the increase of cellulose concentration from 0.1% to 1% in the standard Mo medium stimulated an increase in cell number from $2.46 \cdot 10^7$ CFU/cm³ to $4.25 \cdot 10^7$ CFU/cm³. The growth rate μ was in the range 0.067-0.082 h^{-1} (Fig. 3). The values of the Ks constant and μ max determined from the Lineweaver-Burk equation were 1.56 g/dm³ and 0.09 h^{-1}, respectively.

The ability to decompose cellulose by *Trichosporon cutaneum* was also confirmed by paper chromatography (Table 3). Glucose spots appeared in the potato extract at the initial periods of culture, and their presence was related to the glucose content in potaoes [10]. In later hours of culture

Table 3. Evaluation of cellulolytic ability of *Trichosporon cutaneum* by the method of paper chromatography

Medium	Glucose				
	0 h	24 h	48 h	72 h	96 h
Mo medium + 0.1% cellulose	—	—	–	–	+–
Potato extract + 0.1% cellulose, τ = 30 h	++	–	–	–	+
Mo medium + 0.5% cellulose	–	–	–	–	+–
Potato extract + 0.5% cellulose, τ = 30 h	++	–	–	–	+
Mo medium + 1% cellulose	–	–	–	–	+–
Potato extract + 1% cellulose, τ = 30 h	++	–	–	–	+
Potato extract + 0.5% cellulose, τ = 0 h	++	–	–	–	+

++ — distinct glucose spot
+ — perceptible spot
+– — glucose trace

this component disappeared and then reappeared in the 96th hour of culture as of a distinct glucose spot. Traces of glucose as the terminal product of cellulose metabolism also appeared in the Mo standard medium after 86 h of culture. The performed studies confirmed the cellulolytic abilities of *Trichosporon cutaneum.*

SELECTION OF CELLULOLYTIC MUTANTS OF *TRICHOSPORON CUTANEUM*

T. cutaneum mutants obtained by associated action of NTG and UV irradiation were cultured in Mo medium with 0.5% of Cellulose-Azure for 8 days at 27°C. The cellulolytic abilities of the mutants were assessed by measuring absorbance at $\lambda = 610$ nm which is an indicator of colour intensity of the culture medium as well as of the cellulolytic activity of the strains [14].

The obtained results presented in Table 4 show that already after 3 days of culture exo-cellulase appeared in 6 mutants. Their absorbance values determined in Cellulose-Azure containing media were in the range 0.035-0.155. All the 6 strains were taken for further study. Their activity was assessed in Mo medium with 2% cellulose Whatman 1. Bio-

Table 4. Selection of cellulolytic *Trichosporon cutaneum* mutants with Cellulose-Azure

Strain	Extinction (λ = 610 nm)		
	3rd day	5th day	8th day
Trichosporon cutaneum Tr 1	0	0.01	0.07
Trichosporon cutaneum Tr 1 (six strains)	0.035-0.155	—	—
Trichosporon cutaneum Tr 1 (24 strains)	0	0.01-0.23	0.025-1.2

mass yield (CFU/cm^3), beta-glucosidase activity and CMC-ase activity were determined during the 9-day culture.

Table 5 shows values characterizing the activity of strains The *Trichosporon cutaneum* mutants obtained by joint UV and NTG mutation demonstrated various activity of the cellulolytic complex. The beta-glucosidase activity in the mutants ranged from 0.07 to 0.21 units/cm^3, while CMC-ase activity from 0-0.12 units/cm^3. The most active mutant, Tr 8, exhibited a three-fold increase of beta-glucosidase activity and a two-fold increase of CMC-ase activity in comparison to the parent strain (Table 5).

Table 5. Growth and cellulolytic activity of *Trichosporon cutaneum* mutants in Mo medium with 2% cellulose Whatman 1 (9th day of culture)

Mutant	CFU/cm^3	beta-glucosidase (units/cm^3)	CMC-ase (units/cm^3)
Tr 1	$2.5\ 10^7$	0.07	0.06
Tr 8	$5.4\ 10^7$	0.21	0.12
Tr 15	$1.3\ 10^7$	0.09	0.05
Tr 17	$3.0\ 10^6$	0.15	0.00
Tr 18	$5.0\ 10^6$	0.18	0.00
Tr 23	$5.2\ 10^7$	0.16	0.06
Tr 24	$5.2\ 10^7$	0.18	0.05

EFFECT OF SELECTED SUBSTRATES ON CELLULASES BIOSYNTHESIS AND BIOMASS PRODUCTION BY *TRICHOSPORON CUTANEUM*

As it is shown in Table 5, *Trichosporon cutaneum* produces enzymes acting hydrolytically against carboxymethylcellulose and cellobiose. The activity of these enzymes is induced by various carbon sources in the culture medium [5, 21]. This was checked in another series of experiments in Mo medium containing 0.5% cellobiose (Table 6). The results show that when cellobiose was present in medium the strains produced about

Table 6. Comparison of growth and cellulolytic activity of *Trichosporon cutaneum* strains in Mo medium supplemented with 0.5% cellulose and 0.5% cellobiose (5th day of culture)

Strain	Medium	Biomass		Yp+	Beta-glucosidase (units/cm³)	CMS-ase (units/cm³)
		CFU/cm³	g/dm³			
Trichosporon cutaneum Tr 1	Mo + 0.5% cellulose	$1.4\ 10^7$	—	—	0.170	0.06
	Mo + 0.5% cellobiose	$1.7\ 10^8$	3.7	0.74	0.198	0.34
Tr 8 mutant produced by UV + NTG treatment	Mo + 0.5% cellulose	$4.3\ 10^7$	—	—	0.18	0.16
	Mo + 0.5% cellobiose	$2.6\ 10^8$	3.72	0.74	0.18	0.44

$$^{+}Yp = \frac{\text{biomass (g/dm}^3\text{)}}{\text{cellobiose (g/dm}^3\text{)}}$$

10 times more cells, their number increased to 10^8 CFU/cm³. The biomas level in the presence of this substrate amounted to about 3.7 g/dm³ with the yield coefficient Yp = 0.74. Cellobiose increased the CMC-ase activity of the strains 3-5-fold but had no effect on beta-glucosidase activity.

CONCLUSIONS

1. The yeasts *Trichosporon cutaneum* display cellulolytic abilities; these were demonstrated in indicator media in cultures with cellulose as the only carbon source (Tables 2 and 3, Fig. 3).

2. The applied indicator methods for cellulolytic activity control, namely measurements the height of the cellulose hydrolysis zone in agar slopes and of staining intensity in the medium with Cellulose-Azure, may be used only in preliminary asssessments of yeasts' usefulness in cellulose utilization.

3. Associated mutation (NTG+UV) provided mutants of *Trichosporon cutaneum* displaying various activity of the cellulolytic complex. In the most active mutant, Tr 8, the increase of beta-glucosidase activity comparing to the parent strain one was 3-fold, and CMC-ase activity was about twice higher (Table 5).

4. Cellobiose supplying in the culture medium stimulated the cellulolytic activity of *Trichosporon cutaneum* strains much better than cellulose; it led to a 10-fold increase in cell number in culture and to a 3-5-fold increase in the activity of CMC-ase (Table 6).

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BADANIA ZDOLNOŚCI WYKORZYSTYWANIA CELULOZY PRZEZ *TRICHOSPORON CUTANEUM*

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Streszczenie

Zdolność wykorzystywania celulozy przez drożdże *Trichosporon cutaneum* oceniano metodami wskaźnikowymi przez pomiar: wysokości słupa hydrolizy celulozy oraz intensywności zabarwienia podłoża z Cellulose-Azure. Kontrolowano dynamikę wzrostu szczepu w podłożu standardowym Mo, w którym celuloza stanowiła jedyne

źródło węgla oraz w wyciągu ziemniaczanym w obecności różnych stężeń celulozy (0,1, 0,5, 1%) dodanej w 30 h hodowli i przy 0,5% dawce tego substratu wprowadzonej na początku hodowli.

Przeprowadzone próby pozwoliły stwierdzić, że badany szczep charakteryzował się właściwościami celulolitycznymi. Najaktywniej drożdże rozwijały się w próbie zawierającej wyciąg ziemniaczany i 0,5% celulozy dodanej na początku hodowli. Namnożenie ich wynosiło $3{,}1 \cdot 10^8$ komórek w 1 cm^3 podłoża. Intensyfikację zdolności celulolitycznych *T. cutaneum* uzyskano przez zastosowanie skojarzonej mutacji. Czynnikami mutagennymi były: nitrosoguanidyna i promienie U^v. W wyniku tych badań otrzymano mutanta Tr 8, który wykazał wyższą aktywność celulolityczną niż szczep rodzicielski. Jego namnożenie wynosiło $5{,}4 \cdot 10^7$ CFU/cm^3, a w kompleksie enzymatycznym wykazał aktywność beta-glukozydazy równą 0,21 jedn./cm^3 i aktywność CMC-azy na poziomie 0,12 jednostek.

Przeprowadzone badania pozwoliły również stwierdzić, że końcowy plon biomasy produkowanej przez szczepy *Trichosporon cutaneum* był związany z rodzajem substratu węglowego. Obecność 0,5% celobiozy w podłożu Mo spowodowała 10-krotny wzrost namnożenia drożdży w odniesieniu do hodowli z celulozą oraz indukowała od 3 do 5 razy aktywność CMC-azy.