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Effect of temperature on the production of cellulases, xylanases and lytic enzymes by selected *Trichoderma reesei* mutants

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The effect of temperature in the range of 26-38°C on the production of cellulases, xylanases and lytic enzymes by four mutant strains of *Trichoderma reesei* was analysed. On the basis of these investigations three thermosensitive strains (M-7, RUT-C-30 and VTT-D-78085) which showed reduced excretion of the above mentioned enzymes as well as protein and a thermoresistant mutant (VTT-D-79124) which grew within a temperature range of 26-34°C were characterized. Higher temperature caused an increase in the level of xylanolytic enzymes produced by the four mutants. In addition, it effected the complex composition of cellulolytic enzymes secreted by VTT-D-79124 (i.e. increased and reduced excertion of β-glucosidase and β-1,4-endoglucanase respectively).

Key words: cellulases, lytic enzymes, temperature, *Trichoderma reesei*, xylanases

INTRODUCTION

Cellulose is potentially the most abundant source of carbon in the world. In view of this, most researches over the past years have focused their attention to enzymes hydrolyzing cellulose to glucose. Cellulases are multicomponent enzymatic complexes which contain three basic types of enzymes that act synergistically, thus include exo-β-glucanase, endo-β-1,4-glucanase and β-glucosidase (E n a r i and N i k u - P a u v o l a, 1987).

Fungi are the best producers of cellulases. These features were earlier observed in moulds isolated from the surface of damaged pictures painted on canvas or linen. These isolates were represented by such genera as *Aspergillus, Cephalosporium, Cladosporium* or *Stemphylium.* Subsequently thermophilic strains of fungi were screened from the traces of fecal matter of pig and were shown to be actively involved in the decomposition of cellulose in straw at 50°C. With time these strains were also isolated from other fiber – containing compounds in the environment.

The most active strains with regard to cellulolysis are species like *Aspergillus fumigatus, Chaetomium thermofile, Humicola insolans, H. lanuginosa, Mucor pusillus* and fungi from genera of *Trichoderma, Stachybotrys* and *Hypocera.* (B u j a k, T a r g o ń s k i, 1988).

Fungi species from the genus *Trichoderma* (*Deuteromycetes*) in particular *T. reesei* QM6a and its mutants (R y u , M a n d e l s, 1980), *T. harzianum* (S a d d l e r, H o g a n, 1980) and *T. viride* (F a r k a s et al., 1986) are one of the best producers of cellulosic enzymes that gained industrial application. However cellulases are not only enzymes found in the culture filtrate of the above metioned species. Auxillary enzymes synthesized in addition to cellulases such as xylanases, arabanases, pectinases and amylases have been shown to be highly important in plant biomass hydrolysis. On the other hand, lytic enzymes like β-1,3-glucanase, chitinase and proteases which act on cell walls of fungi and yeasts also play an important role. These enzymes have a negative effect on the growth-rate and productivity of cells during cultivation. Nevertheless, lytic enzymes have been found useful in protoplast formation, degradation of algae, clarification of beer and wine.

From this point of view, the mutual relationship of these enzymes in enzymatic preparation accounts for its practical application and this ration can be altered by varying the cultivation conditions such as temperature and the use of mutants or recombinant strains of *T. reesei.*

The aim of this work is to examine the effect of cultivation temperature on the production of cellulases, xylanases and lytic enzymes by four mutant strains of *T. reesei* which differ in properties.

MATERIALS AND METHODS

The following mutants strains of *T. reesei* were used in these studies: a mutant strain M-7 isolated from parental strain of *T. reesei* QM9414 after mutagenesis with UV-irridation (Dep. of Food Technology and Storage) and mutant strain designed as RUT-C-30, VTT-D-78085 and VTT-D-79124 obtained from the Finnish Culture Collection. These mutants were stored on wort-agar slant at 2°C and periodically reinnocullated.

In 500 ml Erlenmeyers flasks, 100 ml of inoculum media prepared according to M a n d e l s and W e b e r (1969), fortified with 5g/l lactose were introduced. After sterilization in an autoclave at 0.05 MPa these flasks were innoculated with 1 cm part of various wort-agar slants and incubated on a rotary shaker (220 r.p.m.) at 27°C until the source of carbon was exhausted.

Trichoderma reesei mutants were cultivated in a 5l working bioreactor (New Brunswick, Scientific Co. Inc., Bioflo III). The bioreactor was filled with a mineral medium containing in addition 1 % lactose, sterilized in an autoclave at 0.05MPa for 30 minutes and innoculated with earlier prepared inoculum. Cultivation was carried out at a constant pH value of 4 and at varying temperatures of 26, 30, 34 and 38°C.

The cellulase activities (FPU) were assayed according to M a n d e l s et al., (1976) and expressed in an international unit (μmol/ml x min) using Whatman No 1 filter paper (Whatman Ltd.). β-glucosidase activity in the culture filtrate was measured according to the recommendation of International Union of Pure and Applied Chemistry (I.U.P.A.C.) (G h o s e, 1987). The xylanase activity was determined accordingly: 0.9 ml sodium acetate buffer solution (pH 4.8) and 0.1 ml diluted culture filtrate were added respectively to 50 mg xylan (Sigma). After 30 minutes of incubation at 50°C, the reducing sugars released in this mixture were analyzed using 3.5 dinitrosalicylic acid reagent method (M i l l e r, 1959). This activity was expressed in μmol/ml x min. Protease activity was assayed using azocasein (Sigma) according to the method of L o v r i e n et al. (1985). The assay of chitinase activity was carried out by the use of colloid chitin prepared from a native chitin (Sigma) according to L u n t and K e n t (1960). This enzymatic reaction was performed by incubating a mixture of 0.5 ml chitin solution and 0.5 ml culture filtrate for 60 min. and subsequently by analyzing the amount of reducing sugar released by the use of 3.5 dinitrosalicylic acid reagent method (M i l l e r, 1959). The activity was expressed in nmol/ml x min. β-1,3-glucanase was assayed using laminarine method (T a r g o ń s k i, 1991). The activity of this enzyme was expressed as μmol amount of reducing sugar released by 1 ml of the enzyme preparation in a period of 1 min. The activity of β-galactosidase was determined according to C o l o w i c k and K a p l a n (1955) using 0.1 M sodium acetate buffer at pH 4.8 instead of 0.2 M sodium phosphate buffer. All other conditions of enzymatic reaction were maintained. The protein content was assayed according to the method described by L o w r y et al. (1951). The biomass contents of fungi in the bioreactor were determined by drying to a constant weight at 105°C after centrifugation and washing of samples with distilled water and expressed in g dry biomass/l of the medium.

RESULTS AND DISCUSSION

The synthesis of cellulosic complex by *T. reesei* is well documented in literatures but the complex characterization of the acquired enzyme preparation with reference to their activities and proportion of the respective enzymes produced is lacking. Apart from the key role played by cellulolytic and xylanolytic enzymes in the hydrolysis of lignocellulosic materials, emphasis should also be directed towards the enzymes hydrolyzing polymer substances found in the cell wall of moulds and yeasts.

The composition of *T. reesei* enzyme complex has been shown to be affected by such factors as pH, temperature, aeration, medium composition and the method of cultivation (batch, continuous). Altough, there have been several reports concerning the response of cells to changes in temperature profiles in both prokaryotic and eukaryotic organism (G e t h i n g, S a m b r o o k, 1992), there are few reports involving the effect of temperature on the synthesis of extracellular enzymes by *T. reesei*, and in particular on their inter-relationship.

Table 1

Characteristics of culture filtrates obtained after cultivation of *Trichoderma reesei* M-7 at different temperature ranges

Temperature of cultivation (°C)	Time of cultivation (days)	Lactose (mg/ml)	Biomass (mg/ml)	Protein (mg/ml)	Enzymatic activity of culture filtrates							
					FPU (mM/ml x min.)	β-1,4-endo--glucanase (U/ml)	β-1,4--glucosi-dase (μM/ml x min.)	xylanase (μM/ml x min.)	chitanase (nM/ml x min.)	β-1,3--glucanase (μM/ml x min.)	protease (U/ml x 10^3)	β-galacto-sidase (U/ml)
26°C	1	3.50	3.21	0.44	0.63	2.14	0	4.72	0	0	3.51	0.0076
	2	0	3.93	1.44	1.08	23.07	0.17	14.63	24.1	0.91	5.88	0.079
	3	0	3.76	1.36	0.90	21.43	0.13	11.94	36.3	1.55	7.23	0.083
	4	0	3.47	1.30	0.88	18.75	0.052	11.94	37.1	2.17	9.19	0.070
30°C	1	7.31	3.24	0.38	0.15	1.53	0	3.96	0	0	3.38	0.0086
	2	0	4.05	1.43	0.78	12	0.021	18.76	13.4	0.72	3.60	0.045
	3	0	3.96	1.25	0.71	10.35	0.084	16.13	14.8	0.99	3.80	0.047
	4	0	3.84	1.18	0.67	10.24	0.098	13.62	25.2	1.53	5.38	0.044
34°C	1	8.25	3.37	0.28	0.10	0.50	0	8.79	0	0	3.97	0.0033
	2	0	4.24	1.00	0.44	6.28	0	41.72	10.8	0.64	4.05	0.034
	3	0	3.87	1.22	0.43	6.25	0	44.75	12.2	1.14	3.90	0.036
	4	0	3.83	0.82	0.41	6.50	0	38.20	15.62	1.43	3.83	0.033
38°C	1	9.54	2.74	0.07	0	0.10	0	6.32	0	0	3.04	0.002
	2	8.15	2.78	0.08	0	0.32	0	8.25	0	0	3.19	0.0026
	3	7.75	2.81	0.10	0.015	0.34	0	9.31	0	0	3.23	0.004
	4	7.50	2.68	0.11	0.054	0.34	0	9.33	0	0	3.31	0.0066
	5	4.97	3.41	0.14	0.055	0.37	0	9.42	0	0	3.43	0.0086
	6	1.25	4.07	0.31	0.058	0.38	0	10.27	0	0.12	3.68	0.012
	7	0	4.41	0.45	0.056	0.71	0	12.22	0	0.07	3.70	0.007

Table 2

Characteristics of culture filtrates obtained after cultivation of *Trichoderma reesei* RUT-C-30 at different temperature ranges

Temperature of cultivation (°C)	Time of cultivation (days)	Lactose (mg/ml)	Biomass (mg/ml)	Protein (mg/ml)	Enzymatic activity of culture filtrates							
					FPU (mM/ml x min.)	β-1,4-endo--glucanase (U/ml)	β-1,4--glucosi-dase (μM/ml x min.)	xylanase (μM/ml x min.)	chitanase (nM/ml x min.)	β-1,3--glucanase (μM/ml x min.)	protease (U/ml x 10^3)	β-galacto-sidase (U/ml)
26°C	1	6.41	3.82	0.06	0	1.75	0	3.71	0	0	2.07	0.024
	2	3.10	3.94	0.64	0.28	3.44	0.06	8.97	23.22	0.37	4.84	0.052
	3	0	4.07	1.28	0.57	10.24	0.44	10.15	33.71	0.39	5.95	0.073
	4	0	3.84	1.12	0.44	9.36	0.39	12.44	26.52	0.48	7.28	0.070
30°C	1	6.25	3.65	0.16	0	0.94	0	3.52	0	0	2.37	0.004
	2	3.81	4.25	0.52	0.29	3.25	0.07	12.72	21.24	0.16	4.54	0.046
	3	0	4.12	1.14	0.42	6.41	0.42	15.48	21.83	0.25	4.82	0.049
	4	0	3.94	1.08	0.33	6.22	0.45	14.74	26.14	0.38	5.46	0.047
34°C	1	5.95	3.98	0.11	0	1.08	0	5.74	0	0	1.33	0.019
	2	3.21	4.51	0.45	0.12	3.62	0.04	24.82	22.87	0.14	4.39	0.043
	3	0	4.46	0.92	0.28	5.47	0.44	26.74	22.15	0.24	4.25	0.047
	4	0	4.15	0.80	0.24	5.31	0.49	26.52	27.63	0.25	4.81	0.048
38°C	low multiplication of cells											

Table 3

Characteristics of culture filtrates obtained after cultivation of *Trichoderma reesei* VTT-D-79124 at different temperature ranges

Temperature of cultivation (°C)	Time of cultivation (days)	Lactose (mg/ml)	Biomass (mg/ml)	Protein (mg/ml)	Enzymatic activity of culture filtrates							
					FPU (mM/ml x min.)	β-1,4-endo--glucanase (U/ml)	β-1,4--glucosi-dase (μM/ml x min.)	xylanase (μM/ml x min.)	chitanase (nM/ml x min.)	β-1,3--glucanase (μM/ml x min.)	protease (U/ml x 10^3)	β-galacto-sidase (U/ml)
26°C	3	9.02	1.94	0.26	0	0	0	0	0	0	0	0
	5	5.52	2.48	0.44	0.20	8.11	0	2.78	0	0	13.68	0.0086
	6	5.00	2.92	1.48	0.67	11.45	0.15	5.18	0	0	14.22	0.038
	7	0.25	2.86	1.90	0.80	15.00	0.34	11.08	13.4	3.75	15.18	0.041
	8	0	2.59	2.21	1.01	18.07	0.52	11.60	35.1	9.08	25.85	0.039
30°C	3	9.52	2.04	0	0	0	0	0	0	0	6.09	0
	5	6.04	2.58	0.30	0.15	8.43	0	3.46	0	0	19.38	0.015
	6	4.15	2.47	0.49	0.46	12.50	0.34	9.63	0	4.51	21.73	0.034
	7	1.25	2.51	1.24	0.70	15.31	0.68	15.83	14.3	11.95	24.27	0.038
	8	0	2.64	1.90	1.03	16.66	0.70	19.26	46.1	15.72	27.26	0.039
34°C	3	9.25	1.72	0.06	0	0	0	0	0	0	0	0
	5	8.75	2.22	1.13	0	0	0	0	0	0	8.44	0.0016
	6	7.05	2.14	0.14	0.82	1.68	0	1.78	0	0	21.31	0.0093
	7	6.27	2.34	0.42	0.12	7.50	0.15	8.41	0	0	22.79	0.013
	8	4.26	2.42	0.67	0.53	9.04	0.46	21.97	28.1	4.74	25.63	0.034
	9	2.55	2.31	1.28	0.80	12.04	0.66	30.74	59.3	34.20	28.29	0.032
	10	0	2.22	1.92	1.00	13.36	0.94	32.09	87.0	55.15	28.86	0.031
38°C	low multiplication of cells											

Table 4

Characteristics of culture filtrates obtained after cultivation of *Trichoderma reesei* VTT-D-78085 at different temperature ranges

Temperature of cultivation (°C)	Time of cultivation (days)	Lactose (mg/ml)	Biomass (mg/ml)	Protein (mg/ml)	Enzymatic activity of culture filtrates							
					FPU (mM/ml x min.)	β-1,4-endo--glucanase (U/ml)	β-1,4--glucosi-dase (μM/ml x min.)	xylanase (μM/ml x min.)	chitanase (nM/ml x min.)	β-1,3--glucanase (μM/ml x min.)	protease (U/ml x 10^3)	β-galacto-sidase (U/ml)
26°C	3	7.75	2.25	0.03	0	0	0	0	0	0	0	0
	5	6.55	2.47	0.15	0.05	1.72	0	0	0	0	7.53	0.009
	6	5.77	2.58	0.44	0.27	6.41	0	1.87	8.21	0.62	8.47	0.022
	7	3.74	2.87	0.56	0.47	7.53	0.031	3.27	10.20	0.65	9.35	0.032
	8	0.54	2.72	1.10	0.84	7.87	0.035	6.94	47.00	0.78	9.30	0.038
	9	0	2.98	1.04	0.86	10.22	0.048	6.42	55.00	0.83	8.74	0.034
30°C	3	7.91	2.41	0.11	0	0	0	0	0	0	0	0
	5	6.43	2.23	0.32	0.07	0.51	0	0	0	0	0	0.012
	6	6.41	2.34	0.41	0.28	5.72	0.010	4.42	15.40	0.58	7.51	0.028
	7	5.48	2.68	0.53	0.31	8.41	0.028	10.42	18.20	0.59	11.90	0.0284
	8	2.25	2.94	0.73	0.41	8.57	0.040	11.87	42.00	0.68	12.29	0.0293
	9	0	3.12	0.95	0.44	8.92	0.044	11.84	47.00	0.68	12.25	0.0283
34 i 38°C	low multiplication of cells											

The result of enzyme activities obtained in the culture filtrates during cultivation of *T. reesei*; (M-7, RUT-C-30, VTT-D-78085, and VTT-D-79124) at different temperature profiles using lactose as the source of carbon are presented in Tables 1, 2, 3 and 4. On the basis of these results it was demonstrated that different temperatures affected both the dynamics of enzyme secretion as well as their activities in M-7 and RUT-C-30 mutants. In these two cases higher cultivation temperature reduced the enzyme activities in all the culture filtrates examined with the exception of xylanase (M-7 and RUT-C-30) and β-glucosidase (RUT-C-30). The production of the former enzymes (xylanases) as higher at increased temperature than at low temperature. At this stage these results are consistent with the observations made by S u h et al., (1985) who reported an increased biosynthesis of xylanases at 37°C after 3 days of cultivation compared to cultivation at low temperature, i.e. between 25 and 30°C. Similar observations were reported by M e r i v u o r i et al. (1990) and T a r g o ń s k i (1991). The authors examined in addition the effect of temperature in the secretion of enzymes by two strains of *T. reesei*. However, our results showed that no trace of β-glucosidase activity was found in the culture filtrate of M-7 mutant cultivated at 34°C. On the other hand, RUT-C-30 mutant showed a similar enzyme activity profiles at 26, 30 and 34°C. The reduced activity of chitinase, protease and β-1,4-glucanase and the soluble protein contents found in the culture filtrates of *T. reesei* M-7 were associated with an increased cultivation temperature, but contrary to this higher biomass content was observed when it was assumed that the point of comparison is the moment of total consumption of lactose. Similar tendency was observed in the case of *T. reesei* mutant RUT-C-30. However, growth inhibition, evident decline of protein secretion and low of all enzymes were observed at of 38°C in all the culture filtrates examined with the exception of xylanase activity in M-7 and a slight increase in the mass of mycelium of RUT-C-30 cells. The low rate of lactose consumption observed in these mutants explains the reduced β-galactosidase activity found in the culture filtrates at this temperature profile. Higher temperature however, had a different effect on the behaviour of VTT-D-79124 mutant which showed the lowest lactose consumption rate, and prolonged the cultivation time and exposed the cells to a long-time interaction with lytic enzymes. Furthermore, this also explains the relative low biomass content found in the culture filtrate and the higher activities of chitinase, protease and endo-β-1,4-glucanase in the final phase of analysed culture filtrates. Contrary to the former obseved mutants, an increased activity of the above mentioned lytic enzymes was observed as the temperature increased with a marginal variation of (filter paper unit) cellulolytic activity. This activity was almost similar to that observed in the last three temperature profiles and about 1 FPU was attained. This behaviour could be associated with respective changes in the proportion of cellulosic enzyme produced e.g. reduction of endo-β-1,4--glucanase secretion and increased secretion of β-glucosidase. The relationship between higher levels of the latter enzyme and the increased activity of lytic enzymes was confirmed by the results of K u b i c e k (1981) who examined the effect of β-1,3-glucanase on the secretion β-glucosidase during cultivation of *T. reesei*. Extensive progression of fungal lysis and secretion of lytic enzymes by

VTT-D-79124 mutant is implicated by increased level of soluble protein secreted into the culture filtrate, which was higher than that found in the other mutants. The level of β-galactosidase produced by this mutant was lower than that found in both RUT-C-30 and M-7 mutants, however at higher temperature decreased activity of this enzyme was not observed. Analogically as in the case of RUT-C-30 and M-7 mutants, the temperature ranging from 26 to 30°C favoured higher secretion of xylanase by VTT-D-79124 mutant. On the other hand, the constant temperature profile at 34°C inhibited generally the growth and biosynthesis of enzymes by the strain VTT-D-78085. The results obtained for of endo-β-1,4-glucanase and total cellulolytic activities (expressed in FPU) (Tables 1-4) indicated that the former is particularly sensitive to higher temperature profiles when shifted from 26-38°C. This effect is more visible in M-7 mutant and was relatively low in the case of VTT-D-79124 mutant.

On the basis of the results obtained in these studies the termosensitive *T. reesei* mutant (i.e. VTT-D-78085) as well as thermostable mutant (VTT-D-79124) in the ranges of 26 to 34°C were selected. The behaviour of these examined mutants will be the subject of our further studies and thus might contribute to understanding the regulatory mechanism involved in the biosynthesis of cellulases by *T. reesei.*

From the relevant literature, one can infer that the effect of high cultivation temperature is similar to the effect of ethanol on protein secretion by *T. reesei* (M e r i v u o r i et al., 1987). The mechanism of ethanol activity and temperature could be similar to that of tunicamycin, and antibiotic which is a specific inhibitor of postranslational glycosylation of polypeptides in the endoplasmic reticulum (N-glycosylation) (M e r i v u o r i et al., 1985). It is assumed that both N- or O-glycosylation is responsible for the cellulosic enzyme secretion as well as increased thermostability and resistance to proteolysis. The effect of ethanol has been compared to thermal shock effect destruction of endoplasmic reticulum found in hypersecretive aleuronic cells of barley granules (B e l a n g e r et al., 1985).

Although, the regulatory mechanism of temperature in gene expression of fungi has not been fully explained, suitable selection of strains and cultivation temperature could lead to the control of composition of synthesized enzyme complex by *T. reesei.* This can be useful, for example in acquiring a cellulase free xylanolytic preparation which could be applied in bleaching of cellulosic pulps and paper.

These studies represent a preliminary work towards continuous production of cellulase in which lytic enzymes will play a more important role than in the batch cultivation.

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Wpływ temperatury na produkcję enzymów celulolitycznych, ksylanolitycznych i litycznych przez wybrane mutanty *Trichoderma reesei*

Streszczenie

Zbadano wpływ temperatury w zakresie od 26°C do 38°C na produkcję enzymów celulolitycznych, ksylanolitycznych i litycznych przez 4 mutanty *T. reesei*. W wyniku przeprowadzonych badań wyselekcjonowano trzy temperaturowrażliwe mutanty (M-7, VVT-D-78-085, RUT-C-30) charakteryzujące się zmniejszeniem wydzielania enzymów i białka oraz temperaturoopornego w zakresie 26-34°C mutanta VTT-D-79124. Podwyższenie temperatury hodowli powodowało zwiększenie produkcji enzymów ksylanolitycznych przez 4 mutanty. Dodatkowo temperatura powodowała zmiany w składzie kompleksu enzymów celulolitycznych u VTT-D-79124, a mianowicie zmniejszenie wydzielania endo-β-1,4 glukanazy a zwiększenie β-glukozydazy.

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