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THE EFFECT OF SELECTED GROWING CONDITIONS OF SPOROTRICHUM PULVERULENTUM FUNGUS ON ITS CELLULOLYTIC ACTIVITY

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Key words: *Sporotrichum pulverulentum*, cellulase, potato haulm.

A stimulation of endo-β-glucanase by the *Sporotrichum pulverulentum* fungus growing in nutrient media containing selected cellulosic substrates with some biologically active substances was obtained. Some relations between pH changes and cellulase biosynthesis were observed

INTRODUCTION

In recent years there has been considerable interest in research on the utilization of cellulosic wastes as a substrate for biomass production. Microorganisms which can utilize cellulose as a carbon source demonstrate an ability to produce a strong complex of cellulolytic enzymes. Studies of Eriksson [4] demonstrated that the *Sporotrichum pulverulentum* fungus synthetizes a full complex of cellulases necessary to cellulose decomposition. Within the present study investigations were carried out aimed at increasing the synthesis of endo-1,4-β-glucanase by *S. pulverulentum* during propagation in a submerged culture.

EXPERIMENTAL

MATERIALS AND METHODS

Strain. *Sporotrichum pulverulentum* fungus was isolated from pine pulpwood chip piles by T. Nilsson at the Royal College of Forestry, Stockholm. Bergman and Nilsson (1966) tentatively called the fungus Chrysos-

porium lignorum. Novobranova [8] in Baarn, the Netherlands, named it *Sporotrichum pulverulentuum*. The strain of *S. pulverulentum* used in this study was supplied by the Swedish Forest Products Research Laboratory in Stockholm.

Growth media. Modified growth medium according to Norkrans [7] was used for cultivating the fungus both on solid and liquid substrates. The basal mineral medium contained (g/l): KH_2PO_4 0.6; $MgSO_4 \cdot 7H_2O$ 0.4; $CaCl \cdot 2H_2O$ 74×10^{-3}; ferric citrate 12×10^{-3}; $ZnSO_4 \cdot 7H_2O$ 66×10^{-3}; $MnSO_4 \cdot 4H_2O$ 5×10^{-3}; $CoCl_2 \cdot 6H_2O$ 1×10^{-3} and thiamine 0.1×10^{-3}.

As nitrogen sources were as follows:

— 2.0 g $NH_4H_2PO_4$ per 1 liter of growth medium for fungus propagation on solid substrate,

— 6.5 g/l $NH_4H_2PO_4$ + 0.43 g/l of $CO(NH_2)_2$ for propagation on liquid substrate.

The following sources of organic carbon vere added to the mineral medium:

— glucose,

— powdered cellulose, I. H. Muktell's Cellulose Powder No. 400,

— wood board liquid from a factory near Stockholm,

— waste paper ground on VIBRATOM-type mill,

— chaff made of rye, wheat and barley straw in the proportion of 1 :1 : 1 ground first on a "BAK" mill and subsequently on a VIBRATOM mill,

— dried potatoes haulm — ground as above.

Preparation of inoculum. Inoculum for the submerged culture was prepared from a mycelium obtained on Petri dishes after 8 days of *Sporotrichum pulverulentum* incubation at 25°C. In solid substrates, 10 grams of glucose per 1 liter of growth medium were used as carbon source and to solidify the substrate 15 g of agar were used pe 1 liter of medium. The obtained mycelium was homogenized with destilled water in aseptic conditions. The number of cells per 1 ml solution was determined. Inoculum containing 3.5×10^7 of cells in 1 ml was used for inoculation of liquid substrates.

Submerged culture. Propagation was carried out in Erlenmeyer flasks of 250 ml volume on a rotary shaker at 150 rpm and at 28°C for 7 days. The volume of the substrate was 100 ml at pH = 5.0. Sterile substrates were inoculated with 1 ml of the inoculum described above.

Course of the experiments. In the first stage of studies the aim was to determine the effect of selected biologically active compounds on the synthesis of endo-1-4-β-glucanase and pH changes during the submerged growth of *Sporotrichum pulverulentum*. To reach this aim powdered cellulose was used as the sole carbon source in a quantity of 10 g per 1 liter of mineral growth medium. The following compounds were

applied as stimulators of initiation and synthesis of endo-glucanase enzyme:

— biotin	0.05 g/l
— giberelin acid	0.05 g/l
— yeast extract	0.1 g/l
— bowine blood serum	0.08 g/l

The culture was checked for endoglucanase production and pH level every 24 hours. The experimental results are presented in Table 1. In the

Table 1. Effect of selected substrate additives on the activity of ento-1-4-β-glucanase of the *Sporotrichum pulverulentum* fungus

Kind of substrate	Criterion investigated	Drowing time (h)						
		24	48	72	96	120	144	168
Powdered cellulose-control sample	pH*)	4.9	4.8	4.7	5.0	4.3	5.0	4.75
	unit/ml	0.0	0.13	0.18	0.58	3.04	4.56	5.04
Powdered cellulose +0.005 g/l biotin	pH	4.9	4.75	4.6	5.1	5.0	4.3	4.5
	unit/ml	0.0	0.24	0.97	1.43	3.38	4.74	7.50
Powdered cellulose +0.1 g/l yeast extract	pH	5.0	5.2	4.8	5.2	5.6	4.45	5.6
	unit/ml	0.15	1.12	5.84	4.52	4.93	14.24	8.65
Powdered cellulose +0.08 g/l cattle blood serum	pH	5.2	4.9	4.65	5.4	5.8	5.9	6.0
	unit/ml	0.08	2.30	4.04	4.61	8.70	4.80	4.43
Powdered cellulose +0.005 g/l giberelin acid	pH	5.0	5.0	4.95	5.0	5.5	5.4	4.1
	unit/ml	0.0	0.07	0.61	0.55	1.25	2.32	4.04

*) unit/ml — unit of activity of 1,4-β-endo-glucanase enzyme

second stage, tests were carried out on the utilization of some industrial and agricultural cellulosic wastes as carbon source in the propagation of *Sporotrichum pulverulentum*, using the stimulators applied in the first stage of study. Waste products were used in the following quantities:

— dried wood board liquid	30 g/l
— waste paper	10 g/l
— cereal straw	10 g/l
— dried potato haulm	20 g/l

Conditions of fungus growth were identical as in the first stage of this study. Endo-glucanase activity and pH level in the fermentation fluids were measured every 24 hours. Moreover, changes in the content of dry matter, cellulose and pure protein (after precipitations with 5% TCA) were determined in cultures in which powdered cellulose and dried potato haulm were used as carbon sources.

Analytical methods. The following determinations were carried out: dry matter [3],

cellulose [6, 9],
pure protein [5],
endo-1-4-β-glucanase activity [1].

RESULTS

The experimental findings in the first stage of the study indicate that during *Sporotrichum pulverulentum* growth on a medium with powdered cellulose as the sole carbon source the post — fermentation fluids demonstrated an increase of 1-4-β-glucanase activity with the growth of time (Table 1). However, the increase of enzyme activity was not in direct proportion to time. Enzyme activity determined as late as after 48 hrs of fungus propagation amounted to only 0.13 units/ml and a dynamic increase from 0.58 units/ml after 96 hrs to 3.04 units/ml occurred after 120 hr. Simultaneously the pH level dropped markedly and reached 5.0 after 96 hrs and 4.3 after 120 hrs (Table 1). With bovine blood serum added the maximum value (8.70 units/ml) was noticed after 120 hrs and with yeast extract it was even higher (14.25 units/ml) after 144 hrs of propagation. The maximum activity obtained after the addition of yeast extract occurred with a parallel decline of pH level from 5.6 to 4.5. However, no correlation between pH decline and increase of enzyme activity was observed when bovine bloood serum was used. The experimental results of research on the effect of the substrate on the enzymatic activity of *S. pulverulentum* demonstrate that the activity of endo-glucanase in the fluids from fungus cultures grown on natural substrates was considerably lower as compared with the usage of powdered cellulose (Table 2).

Table 2. Effect of the kind of substrate with 0.1 g/l of yeast extract added on the activity of 1,4-β-endo-glucanase during *Sporotrichum pulverulentum* fungus growth

Kind of substrate	Criterion investigated	Growing time (h)						
		24	48	72	96	120	144	168
10 g/l of powdered cellulose — control sample	pH*)	5.3	5.5	4.9	5.4	5.7	4.6	5.1
	unit/ml	0.14	1.70	5.06	4.04	4.08	12.2	8.1
30 g/l — wood board liquid dried	pH	5.4	6.1	6.25	4.56	4.9	5.6	6.1
	unit/ml	0.0	0.0	0.0	2.50	1.24	0.55	0.785
10 g/l waste paper	pH	5.1	5.6	4.6	5.8	5.9	6.0	6.05
	unit/ml	0.0	0.45	1.67	0.31	0.33	0.38	0.74
10 g/l cereal straw	pH	5.2	5.1	5.6	5.6	5.7	5.7	5.8
	unit/ml	0.0	0.65	1.23	1.73	1.265	0.99	0.85
20 g/l dried potato haulm	pH	5.5	5.9	5.25	5.2	5.0	5.0	5.8
	unit/ml	0.05	1.05	2.5	2.68	2.75	2.65	1.20

*) unit/ml — unit of activity of 1,4-β-endoglucanase enzyme

It should be emphasized here that the maximum activity of the enzyme in the experiments with powdered cellulose, wood board liquid, and waste paper occurred after a decrease of the pH level, similarly as in the first stage of study. However, in the experiments in which cereal straw and potato haulm were used, such considerable changes of pH were not observed. Among the used substrates the highest enzymatic activity was noticed in culture fluid with potato haulm.

In the study, the suitability of potato haulm as a substrate for endoglucanase production as well as the degree of cellulose utilization, loss of dry matter and increase of pure protein content were investigated. The results are given in Table 3. *Sporotrichum pulverulentum* during 7 days

Table 3. Output of pure protein from the culture of *Sporotrichum pulverulentum* fungus

Kind of substrate	Growing time h	Dry matter		Cellulose			Pure protein	
		g/l	loos %	g/l	% in dry m.	loss %	g/l	% in dry m.
Powdered cellulose	0	10.225	—	9.928	97.09	—	not determined	
	168	8.072	21.05	5.590	69.25	43.69	5.363	6.15
Potato haulm	0	20.918	—	5.99	28.2	—	1.742	8.33
	168	18.094	13.50	3.70	20.4	38.20	2.037	11.25

— 12.36 g of pure protein was obtained from 100 g of utilized cellulose in the culture on a powdered cellulose substrate;

— 10.45 g of pure protein was obtained from 100 g of utilized dry matter in the culture on potato haulm substrate

of propagation utilized 43.69% of powdered cellulose and 38.2% of cellulose from potato haulm. The less extensive decomposition of cellulose from potato haulm may probably be due to the utilization by the fungus of some other more easy available carbon sources in the medium. The biomass produced by the fungus grown on powdered cellulose contained 6.15% d.m. of pure protein but 11.25% d.m. of pure protein when grown on potato haulm. The balance of dry mass loss over 7 days of propagation demonstrated that 10.45 g of pure protein were produced from 100 g of utilized dry matter of potato haulm. When powdered cellulose was used, protein output attained 12.36 g from 100 g of utilized cellulose.

DISCUSSION

The addition of selected biologically active compounds to increase the synthesis of endo-glucanase by *Sporotrichum pulverulentum* grown in a submerged culture on powdered cellulose demonstrated differentiated action. The activity of endoglucanase in samples with added giberelin acid

was lower than in the control sample. In both substrate samples a marked increase of enzyme activity was accompanied by a simultaneous decline in pH level. Experimental findings show that the application of biotin, yeast extract and bovine blood serum had a positive influence on the synthesis of endo-glucanase by *Sporotrichum pulverulentum* grown on powdered cellulose substrate. Yeast extract and bovine blood serum were found to be the most efficient stimulators of biosynthesis initiation and endo-glucanase production. When these substances were used the occurrence of the enzyme was determined as early as after 24 h of propagation. The increase of activity during propagation time was more dynamic, too. Taking into consideration all the experimental findings of the first stage of study it was interesting to notice a correlation between pH changes and the dynamics of endo-glucanase biosynthesis during the propagation period. It was difficult to report this correlation in earlier experiments. However, except for blood serum additive in all other samples, the activity of endo-glucanase increased with the decline of pH level in the propagation fluid. The experimental findings of the first stage of this study indicate that yeast extract should be used for stimulation of the endo-glucanase biosynthesis in the submerged culture of *Sporotrichum pulverulentum* grown on natural cellulosic substrates. Considerably lower endo-glucanase activity in media with other natural cellulose substrates was observed in the second stage of study. This can be explained by the fact that the fungus was given in the substrate a sufficient amount of easily available carbon required for its growth and so the biosynthesis of endo-glucanase was not needed. From the industrial and agricultural cellulosic wastes used as a carbon source for *Sporotrichum pulverulentum* growth, potato haulm seemed to be the most efficient in the production of endo-glucanase enzyme. The possible utilization of potato haulm is a problem of considerable importance in countries having high potato crops.

CONCLUSIONS

1. *Sporotrichum pulverulentum* grown by submerged technique on liquid substrates with powdered cellulose as the sole carbon source demonstrated its good utilization for propagation and synthesis of the 1.4-β-glucanase enzyme.

2. By adding 0.05 g/l biotin, 0.08 g/l bovine blood serum and particularly 0.1 g/l yeast extract to the standard substrate, the initiation of endo-glucanase biosynthesis was accelerated by 24 hrs and the cellulolytic activity of the post-fermentation fluids increased 3-fold. E. g. after 144 h of growing, the control samples demonstrated 4.56 units/ml and the samples with 0.1 g of yeast extract added demonstrated 14.25 units/ml.

3. Some correlation was observed between pH level and endo-glucanase biosynthesis. In a predominant number of samples the activity of endo-glucanase increased with the decline of pH level in the fermentation fluid. Since pH measurements can be done quickly and easily, this criterion should be subjected to further extensive biochemical investigations aimed at future practical applications.

4. It was found that cellulosic wastes used in this study i.e. wood board liquid, waste paper, cereal straw and potato haulm can be utilized as carbon source for *Sporotrichum pulverulentum* growth.

5. It was demonstrated that the biosynthesis of 1,4-β-endoglucanase on cellulosic wastes from industry and agriculture even with the use of pure, powdered cellulose. The highest values of enzyme activity with the use of waste cellulosic materials varied from 1.67 units/ml to 2.75 units/ml, while with the use of pure, powdered cellulose they ranged from 12.2 units/ml to 14.25 units/ml.

6. The employed fungus *Sporotrichum pulverulentum* decomposed cellulose in 43.69% in the medium containing powdered cellulose and in 38.20% in the medium with potato haulm during 7 days of propagation period. Pure protein output by the fungus grown on potato haulm amounted to 10.45 g from 100 g of utilized dry matter, and 12.36 g from 100 g of utilized cellulose when powdered cellulose was contained in the substrate.

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WPŁYW WYBRANYCH WARUNKÓW HODOWLI GRZYBA *SPOROTRICHUM PULVERULENTUM* NA JEGO AKTYWNOŚĆ CELULOLITYCZNĄ

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S t r e s z c z e n i e

Hodowle wgłębne grzyba *Sporotrichum pulverulentum* na podłożach zawierających odciek z produkcji płyt wiórowych, odpady papiernicze, słomę zbożową i łęty ziemniaczane wykazały dobre wykorzystanie substratów do produkcji biomasy grzybowej i syntezy 1,4-β-endoglukanazy.

Stwierdzono, że wprowadzenie do pożywki 0.05 g/l biotyny, 0,08 g/l surowicy z krwi bydlęcej, a przede wszystkim 0,1 g/l ekstraktu drożdżowego pozwalało na przyspieszenie inicjacji biosyntezy endoglukanazy o 24 h oraz na trzykrotne zwiększenie aktywności celuloilitycznej w płynach pohodowlanych. Zaobserwowano także pewną zależność między zmianami wartości pH podłoża, a biosyntezą endo-β-glukanazy (tab. 1.).

Najwyższe aktywności enzymatyczne przy użyciu odpadowych surowców celulozowych wynosiły od 1,67 jedn./ml do 2,75 jedn./ml, natomiast przy stosowaniu czystej, sproszkowanej celulozy wynosiły od 12,2 jedn./ml do 14,25 jedn./ml (tab. 2).

Stwierdzono, że *S. pulverulentum* w przypadku stosowania czystej celulozy, rozkładał substrat w 43,7%, natomiast rozkład celulozy w łętach ziemniaczanych wynosił 38,2%. Wydajność białka właściwego w przeliczeniu na zużytą celulozę wahała się od 10,4% do 12,3% (tab. 3).