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NEW INDUCERS FOR CELLULASES PRODUCTION BY *TRICHODERMA REESEI* M-7

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ABSTRACT

The aim of work was to examine the influence of lactobionic acid, gluconic acid, delta-gluconolactone, lactulose and sorbitol on the production of cellulases by mutant strain *Trichoderma reesei* M-7 during batch and continuous cultivation. Positive results were achieved in the presence of lactobionic acid and lactulose as inducers. In both cases the highest inductive effect of cellulase production was observed in the presence of 1% mixtures of above mentioned substrates at the ratio 1:1 (0.5% : 0.5%) with lactose. (about 20% higher than that observed in the presence of 1% of lactose) The induction mechanism of lactobionic acid and lactulose may be attributed by slow rate of their utilisation (slower than lactose). Explanation of the inductive mechanism of lactose, lactobionic acid and lactulose on cellulases production by *Trichoderma reesei* demands further investigations among other with using a purified beta-galactosidase enzyme.

Key words: Mechanism of induction, cellulases, lactobionic acid, lactulose, *Trichoderma reesei*

INTRODUCTION

Wide range of practical application of cellulases cause interest in increasing of production above enzymes. The ability of the filamentous deuteromycete *Trichoderma reesei* to secrete greater than 40 g/l protein into culture medium has made it an important industrial source of cellulolytic enzymes. Biosynthesis of cellulases in most cellulolytic fungi is connected with the presence of inducer in cultivation medium. Cellulose has usually been considered to be the best inducer for producing well balanced cellulase system but cellulose is expensive and also cause operational and rheological problems in bioreactor. The use soluble carbon source such as lactose, cellobiose and sorbose allows lower cost of production, greater control of fermentation and simplifies the operation of the process. But new more effective inducers of cellulases production are still being searched. Bruchman et al. [1] reported on stimulation of cellulases formation in cellulose degrading *Trichoderma reesei* by cellobionolactone a component found among cellulose degradation products in culture filtrates of *Trichoderma reesei*. Concerning the origin of CBL Vaheri [14] provided indirect evidence for the existence of a cellulose oxidizing enzymes in cultures of *Trichoderma reesei* that was responsible for the formation of aldonic acid and aldonolactones. These substrates can also originate by enzymatic action of CBH I and CBH II from already naturally oxidized end chains in cellulose.

The aim of this work was to examine the influence of two other aldonic acid:lactobionic acid and gluconic acid and also delta-gluconolactone, disaccharide lactulose and sorbitol on the production of cellulases by mutant strain of *Trichoderma reesei* M-7 during batch and continuous cultivation.

MATERIALS AND METHODS

CHEMICALS: Lactose monohydrate, lactobionic acid, lactulose, salicin, azocasein, chitin, laminarine, 3,5-dinitrosalicylic acid (DNS), birchwood xylan and beta-nitrophenyl-galactoside were purchased from Sigma (St.Luis, USA). Yeast extract was purchased from BTL Company (Łódź-Poland), whereas the Whatman No. 1 filter paper was purchased from Whatman Ltd, England. All other chemicals of reagent grade were purchased from the Polish Chemical Company (POCH-Poland).

MICROORGANISM: A mutant strain *Trichoderma reesei* M-7 derived from the parent *T-reesei* QM 9414 by mutagenesis with UV-irradiation was used in this study. This mutant was obtained from the Culture Collection of the Dep. of Food Technology and Storage, University of Agriculture, Lublin, Poland, and maintained on wort-agar slant at 2°C and transferred periodically onto a fresh slant.

CULTIVATION: Batch cultivation of mutant M-7 were performed in 500 cm^3 Erlenmeyer flasks containing 100 cm^3 of medium according to Mandels and Weber [9] fortified with 1% lactose or 1% mixtures of lactose with lactobionic acid, gluconic acid, gluconolactone, lactulose and sorbitol at the ratio: 3:1, 1:1 and 1:3. This medium was sterilised by autoclaving at 0.05 MPa for 30 min, cooled and inoculated with 2% (v/v) of pre-grown cells. Cultivation were performed on a rotaty shaker (220 obr/min) at 26°C.

Fed-batch (pre-cultivation) and continuous cultivation was run in a 5 dm^3 capacity bio-reactor Bioflo III- New Brunswick (USA) on the mineral medium composition described by Mandels and Weber [9], fortified either with 1% lactose or 1% lactobionic acid and lactulose alone or 1% mixture of lactose with both sugars. This medium was sterilised (at 0.05 MPa for 30 min), cooled and inoculated with previously prepared 200 cm^3 inoculum. Cultivation was performed at a constant pH value of 4 adjusted with 5% NH_4OH and 2.5% H_3PO_4 at temperature 26°C. The continuous cultivation started upon consumption of lactose in the fed-batch (pre-culture with 1% lactose) medium, followed by feeding the culture with the same mineral medium, fortified either with 1% lactose or 1% lactobionic acid and lactulose alone or 1% mixture of lactose with both sugars depending on the experimental procedure. The lactose, lactobionic acid and lactulose ratio used in different experiments were 3:1, 1:1 and 1:3, respectively, in a total of 1% final mixture of lactose with both sugars. Continuous cultivation was carried out for 96-120 h with a dilution rate of 0.024 h^{-1} (D = 0.024 h^{-1}). After cultivation, cell were separated by centrifugation (6000 x g, 10 min) at 4°C and the obtained supernatant was used for enzyme analyses.

ASSAYS: The activity of cellulases (FPU) of culture filtrates was assayed according to the method described by Mandels et al. [8] and expressed in International Unit (IU), using Whatman No.1 filter paper. The activity of beta-glucosidase in the culture filtrate was assayed according to the specifications of IUPAC [3]. One unit of enzyme activity is defined as the amount of µmol of reducing sugars equivalent liberated by 1 cm^3 of culture

filtrate per minute under the conditions of assay. The activity of endo-beta-1,4-glucanase of culture filtrates was assayed according to the method described by Targoński and Szajer [13]. The activity of xylanases of the culture filtrates was assayed accordingly: 0.9 cm^3 of 5.5% birchwood xylan solution in 0.1 M sodium acetate buffer (pH = 4.8) was used and 0.1 cm^3 of an appropriate enzyme dilution of the culture filtrate was added. Incubation was performed for 30 min at a temperature of 50°C. The reducing sugars released were measured with DNS method [10]. Activities were expressed in µmol/cm^3 x min.

The activity of protease of culture filtrates was assayed according to the azocasein method described by Lovrien et al. [5]. One unit of proteolytic activity was expressed as increased in absorbance of reaction mixture at 366 nm in 1 min per 1 cm^3. Chitinase activity of culture filtrates was assayed using colloid chitin which was obtained from a native chitin according to the method described by Lunt and Kent [7] by incubating 0.5 cm^3 of colloid chitin with 0.5 cm^3 of an appropriate dilution of the culture filtrate for 60 min. The reducing sugars liberated were measured according to the DNS method [10]. Activity was expressed in nanomol of reducing sugars / cm^3 x min. beta-1,3-glucanase activity of culture filtrates was assayed with laminarine according to the method described by Targoński [12], and expressed in µmol/cm^3 x min. Beta-galactosidase activity of culture filtrates was assayed according to the method described by Colowick and Kaplan [2], using 0.1M sodium acetate buffer (pH = 4.8) instead of 0.2 M phosphate buffer (pH = 7.25). One unit of enzyme activity was defined as increased in absorbance of reaction mixture in 1 min per 1 cm^3 of the culture filtrate.

For the estimation of lactulose and lactobionic acid in culture filtrates of *Trichoderma reesei* enzymatic kit (Boeringer Manheim, Germany) was used. Principles of method based on hydrolysis of this compounds to fructose, gluconic acid and galactose by beta-galactosidase. Then galactose was oxidised by galactose dehydrogenase in the presence of NAD to galactonic acid. NADH arising in this reaction in propotional concentration to concentration of galactose has been estimated by measurement of absorbance at λ = 365 nm. For the reaction mixture 0.2 cm^3 of citric buffer pH 6.0, 0.1 cm^3 diluted and deproteinized of sample and 0.05 cm^3 of beta-galactosidase preparation were added. Reaction mixture has been incubated by 15 min in temperature 25°C. After the incubation had finished 1 cm^3 of phosphate buffer pH = 8.6 and 2 cm^3 of disstilled water have been added. Then after mixing 0.05 cm^3 of preparation of galactose dehydrogenase enzyme was added. For estimation of lactobionic acid 2-time bigger volumes of solution of beta-galactosidase (0.1 cm^3) and 10 time extended times of incubations with this enzyme were used.

For estimation kinetic parameters of beta-galactosidase for three substrates: lactose, lactulose and lactobionic acid the crude enzyme from culture filtrates of *Trichoderma reesei* was used. Above mentioned compounds were prepared in concentrations from 2.075 mM/dm^3 to 25 mM/dm^3. Total volumes of reaction mixtures amounted 0.3 cm^3 and temperature of hydrolysis 30°C. Rates of hydrolysis were expressed in number of µmol of galactose released by 1 cm^3 of culture filtrate during 1 minute. For estimation of galactose concentration enzymatic kit (Behringer Manheim, Germany) was used. Commercial preparation of beta-galactosidase in the kit has been replaced by culture filtrates contained beta-galactosidase which has been secreted during continuous cultivation of mutant M-7 in the presence of three above mentioned substrates. Appropriate volumes of cultures filtrates were used in order to get equal activities of beta-glactosidase. In the case of lactobionic acid time of hydrolysis has been extended to 4.5 h. Reactions of hydrolysis has been carried out in 0.1 M acetate buffer pH = 4.8. The others conditions of enzymatic reactions were the same as during estimation of of concentration of lactobionic acid and lactulose in culture filtrates of *Trichoderma reesei*. Based on the obtained results Michaelis-Menten curves were plotted and values of kinetic parameters Vmax and Km of beta-galactosidase for three substrates were set.

The protein contents were assayed according to the method described by Lowry et al. [6], and expressed in mg/cm^3.

The biomass was analysed by drying to a constant weight at 105°C and expressed in mg/cm^3 dry biomass.

RESULTS

Positive results were achieved in the presence of lactobionic acid and lactulose as inducers. In both cases the highest inductive effect of cellulase production was observed in the presence of 1% mixtures of above mentioned substrates at the ratio 1:1 (0.5% : 0.5%) with lactose (Fig. 1, Fig. 2). The maximum activity of cellulases (FPU) was observed after 6th day cultivation for lactobionic acid–containing medium (Fig. 1) and 7th day for lactulose-containing medium during batch cultivations (Fig. 2) (20% higher than during cultivation with 1% of lactose). Lactobionic acid and lactulose were also used separately and as a mixtures with lactose in different ratio as source of carbon during continuous cultivation of the mutant M-7. The highest cellulolytic activity of culture filtrates has been achieved after cultivation of *Trichoderma reesei* on mixtures of lactulose and lactobionic acid with lactose in the ratio 1:1 (Table 1). On the other hand the highest activity (FPU and beta-1,4-endoglucanase) was obtained in the presence of lactobionic acid than of lactulose and this was about 20% higher than that observed in the presence of 1% lactose. Two fold increase of activity of component of cellulases complex-beta-glucosidase was also observed in this conditions in comparison to cultivation on lactose. The highest activities of lytic enzymes (chitinases and proteases) were measured in culture filtrates obtained after cultivation on medium with equal concentration of lactobionic acid and lactose (0.5% : 0.5%). Composition of the cultivation medium hadn't effect on activities of the third lytic enzyme - beta-glucanase. Feeding medium containing lactobionic acid and lactose in the ratio 1:1 and 1:3 did not affected xylanases activities of culture filtrates which were comparable to obtained after cultivation in the presence of 1% of lactose. Lactulose in 1% concentration and as a mixture with lactose was effective inducer for cellulases production but culture filtrates obtained after cultivation with this sugar as source of carbon exhibited lower FPU activities than derived from cultures with 1% of lactose (Table 2) Both lactobionic acid as well lactulose used alone proved to be worse inducers for cellulase production by *Trichoderma reesei* M-7 than lactose. Both substrates had also negative effect on the production of biomass by this strain. The induction mechanism of lactobionic acid and lactulose may be attributed by slow rate of their utilisation (slower than lactose). The presence of lactose in the medium was not already estimated after second day of each batch cultivation. After six day of batch cultivation was started from about 28% (batch cultivation with 0.5% lactose) to 61% (batch cultivation with 1% lactobionic acid) initially concentration of lactobionic acid used as the only source of carbon in cultivation medium (Fig. 3.). Lactulose used in 1% concentration as the sole source of carbon during batch cultivation of mutant M-7 was slower metabolised compare to combination with lactose (Fig. 4.). Slower rates of utilisation of both inducers were also observed during continuous cultivation of mutant M-7 (Table 1, 2). Lactobionic acid as was shown in Table 3 was low Vmax and high Km substrate of beta-galactosidase from *Trichoderma reesei*. Slower utilisation of the second mentioned above inducer – lactulose during batch and continuous cultivation was not connected with affinity of beta-galactosidase enzyme to this substrate which was even higher than to lactose.

Fig.1. Effect of lactose and lactobionic acid on the production of cellulases by *Trichoderma reesei* M-7 during batch cultivation

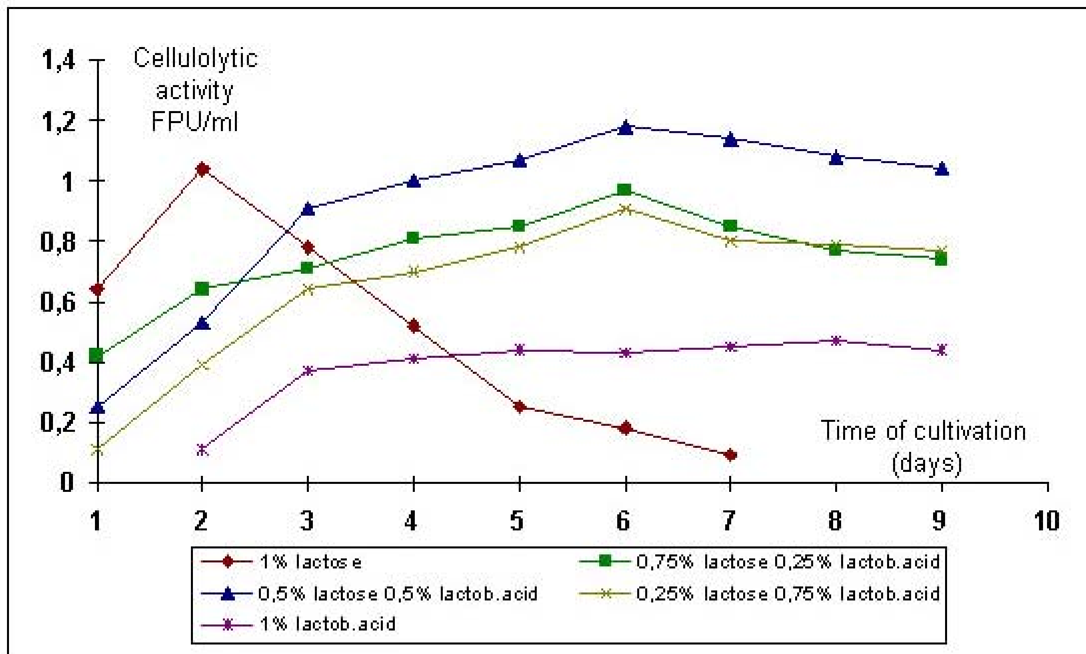

Fig. 2. Effect of lactose and lactulose on the production of cellulases by *Trichoderma reesei* M-7 during batch cultivation

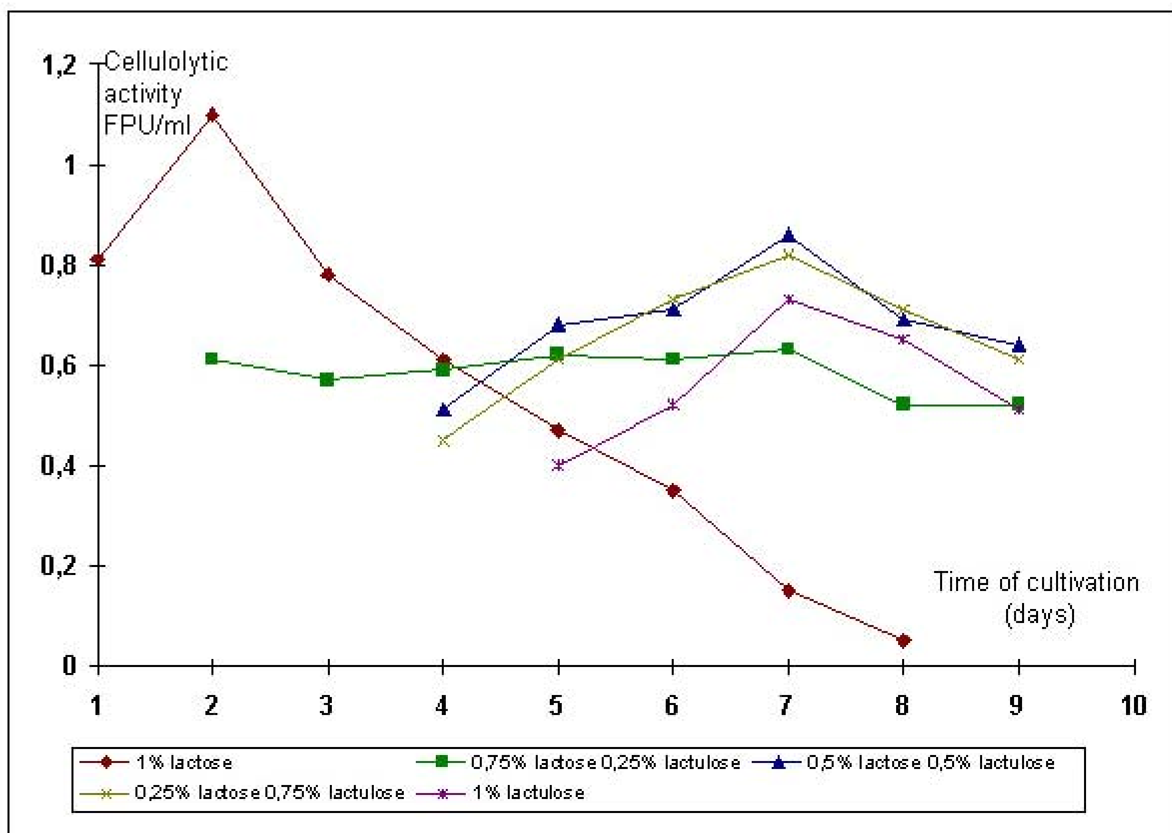

Table 1. Biomass, concentration of protein and enzymatic activities of culture filtrates obtained after continuous cultvation of *Trichoderma reesei* M-7 at different concentrations of lactose and lactobionic acid

Time of cultivation (days)	Ratio of conc. of lactose/ lactob.acid (%)	Biomass (mg/cm^3)	Protein (mg/cm^3)	Conc. of lactobionic acid in cult.medium (mg/cm^3)	Enzymatic activities of culture filtrates							
					FPU (μM/cm^3×min)	β-1,4-endoglucanase (U/cm^3)	β-glucosidase (μM/cm^3×min)	xylanase (μM/cm^3×min)	chitinase (nM/cm^3×min)	β-1,3-glucanase (μM/cm^3×min)	protease (U/cm^3×10^3)	β-galactosidase (U/cm^3)
5	1/0	4.21	2.37		1.16	25.75	0.147	22.75	23.71	2.34	8.03	0.0791
9	0.75/0.25	3.58	2.30	0.7	1.31	28.44	0.165	25.18	24.83	2.75	8.51	0.0724
13	0.5/0.5	3.25	2.27	1.1	1.42	27.52	0.300	18.65	29.44	2.48	9.95	0.0569
17	0.25/0.75	2.72	1.27	3.4	1.02	20.07	0.163	14.11	24.51	2.32	9.21	0.0347
21	0/1	1.97	0.91	5.2	0.49	9.45	0.032	5.58	24.93	2.32	10.31	0.0164

Table 2. Biomass, concentration of protein and enzymatic activities of culture filtrates obtained after continuous cultvation of *Trichoderma reesei* M-7 at different concentrations of lactose and lactulose

Time of cultivation (days)	Ratio of conc. of lactose/ lactulose (%)	Biomass (mg/cm^3)	Protein (mg/cm^3)	Conc. of lactulose in cult.medium (mg/cm^3)	Enzymatic activities of culture filtrates							
					FPU (μM/cm^3×min)	β-1,4-endoglucanase (U/cm^3)	β-glucosidase (μM/cm^3×min)	xylanase (μM/cm^3·min)	chitinase (nM/cm^3 ×min)	β-1,3-glucanase (μM/cm^3 ×min)	protease (U/cm^3×10^3)	β-galactosidase (U/cm^3)
5	1/0	3.72	2.12		1.06	21.75	0.131	19.75	21.34	2.11	7.94	0.0742
9	0.75/0.25	3.65	2.17	0	0.96	21.25	0.107	22.51	19.95	2.21	8.51	0.0570
13	0.5/0.5	3.37	1.91	0	1.00	18.50	0.105	20.71	23.11	2.41	8.14	0.037
17	0.25/0.75	3.11	1.37	0.3	0.77	13.25	0.031	9.71	21.75	2.17	10.74	0.029
21	0/1	3.27	0.81	1.1	0.65	8.25	0.021	7.42	22.31	2.38	11.11	0.025

Fig.3. Utilisation rate of lactobionic acid during batch cultivations of *Trichoderma reesei* M-7

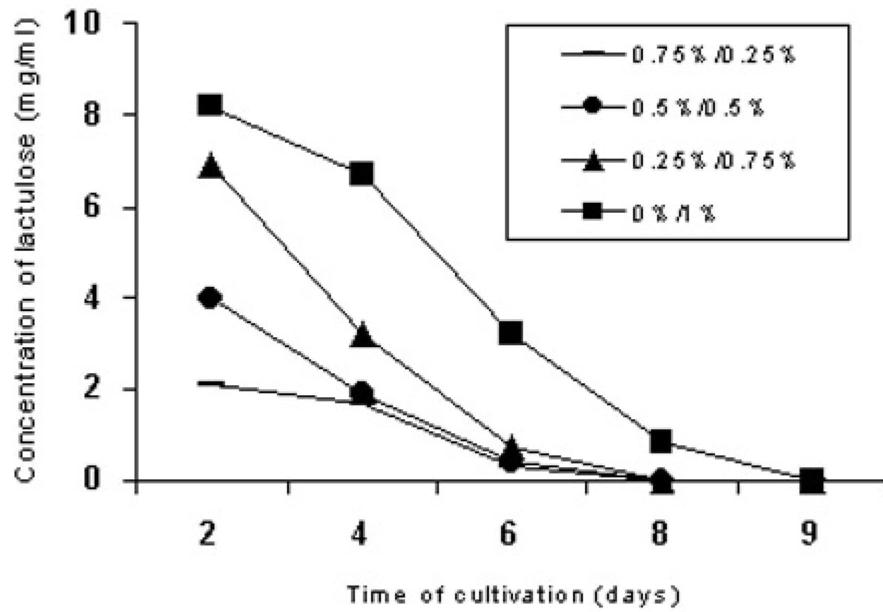

Fig. 4. Utilisation rate of lactulose during batch cultivation of *Trichoderma reesei* M-7

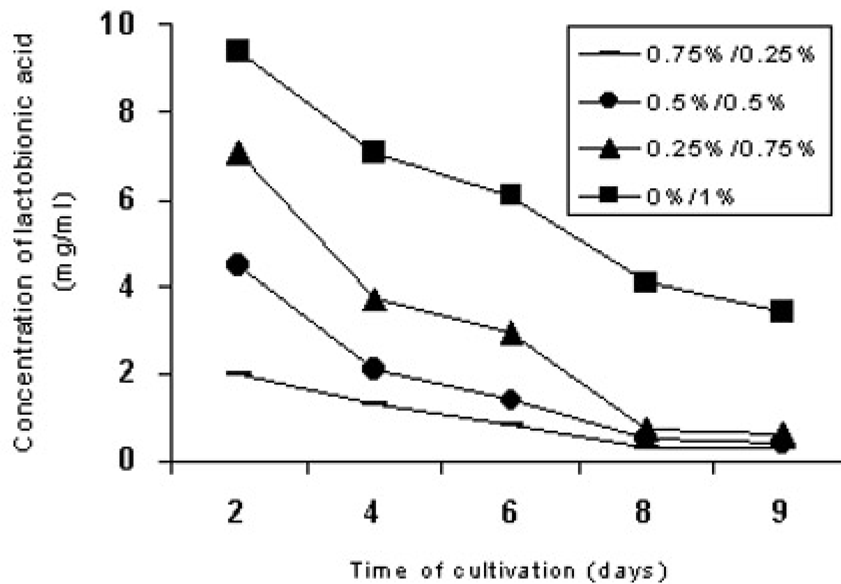

Table 3. Kinetic values of beta-galactosidase from *Trichoderma reesei* M-7 for three substrates

Substrate	V_{max} (μ mol/cm^3 x min)	K_m (mM)
lactose	9.09	16.66
lactobionic acid	0.4	66.6
lactulose	6.66	13.51

DISCUSSION

Inspiration for searching for new inducers for cellulase production by *Trichoderma reesei* M-7 were experiments performed by Vaheri [14, 15, 16] and continued by other researchers. Vaheri provided evidence for an oxidative cell-wall bound enzyme participating in cellulose breakdown. She found in culture filtrates of *Trichoderma reesei* among the soluble reaction products of enzymatic hydrolysis aldonic acid and its lactons i.e. cellobionic acid and cellobiono-1,5-lactone. These compounds can be formed by oxidizing end chains of cellulose and cellobiohydrolases action. Recently evidence for an induction of cellulases in *Trichoderma reesei* by cellobiono-1,5-lactone has been presented by Iyayi et al. [4] and Szakmary et al. [11]. They showed that cellobiono-1,5-lactone induces cellobiohydrolases I and II and endoglucanase I but not xylanases so it is more specific than one of the most efficient inducers for cellulases production – sophorose. This compound exhibited optimal induction at lower concentration and acted synergistically with sophorose and cellobiose. Induction of cellulases, based of estimation of cellobiohydrolase I activity, was three times higher when CBL was added to cellobiose as a carbon source than it was supplied alone. The levels of cellulases formed under these conditions were comparable to those obtained when *Trichoderma reesei* was grown on cellobiose plus delta-gluconolactone or nojirimycin respectively, which are both inhibitors of beta - glucosidase. These data suggest an indirect effect of CBL on cellulase induction, by inhibiting cellobiose hydrolusis. Cellobino-1,5-lactone is a low Vmax and low Km substrate of beta-glucosidase [Kubicek – Pranz – unpublished material] and inhibits beta-glucosidase activity with higher Km substrates (sophorose, cellobiose). This phenomenon is similar to observed in our work during batch and continuous cultivations of mutant *Trichoderma reesei* M-7 in the presence of lactose and lactobionic acid in the ratios 3:1 and 1:1. Lactobionic acid was a low Vmax substrate of *Trichoderma reesei* beta-galactosidase hence could indirectly caused decrease of hydrolysis rate of lactose during cultivation. On the other hand this compounds was high Km substrate of beta - galactosidase. Both CBL and lactobionic acid when were used as a sole source of carbon provoked poor growth because were very slowly metabolized by the fungus. Effect of CBL can be explained by the gluconolactone arising from it by the action of beta - glucosidase which is not metabolised by the fungus. According to the model proposed for CBL, cellobiose and beta-glucosidase, it may be postulated that the compounds provoking cellulase formation are also poor substrates of beta-galactosidase. This matter demands further investigations using a purified beta-galactosidase from *Trichoderma reesei*.

CONCLUSIONS

Lactobionic acid and lactose proved to be good inducers for cellulases production by *Trichoderma reesei* M-7. In both cases the highest inductive effect on cellulolytic enzymes production during batch and continuous cultivation has been observed in the presence of the mixtures of these compounds with lactose in the ratio 1:1. Enzymatic activities of cellulases of culture filtrates obtained after cultivation of *Trichoderma reesei* M-7 in the presence of mixture of lactobionic acid and lactose were about 20% higher than measured after cultivation of mutant in the presence of lactose in 1% concentration as a source of carbon. Lactulose and lactobionic acid were slower than lactose metabolised by *Trichoderma reesei* M-7. It was probably caused by slower rate of hydrolysis of both substrates by beta-galactosidase related to lower activities of this enzyme in culture filtrates from cultivation of mutant M-7 and lower affinity of beta-galactosidase enzyme to lactobionic acid in comparison to lactose. Explanation of the inductive mechanism of lactose, lactobionic acid and lactulose on cellulases production by *Trichoderma reesei* demands further investigations among other with using a purified beta-galactosidase enzyme.

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