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GENETIC TRANSFORMATION IN TRICHODERMA REESEI FOR THE IMPROVEMENT OF CELLULASES PRODUCTION

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ABSTRACT

The fungal strain *Trichoderma reesei* NRRL 12368 and three of its induced mutants, namely S/26, B/1 and 6/12, which were selected according to their cellulases activity, were used to be transformed in this investigation. The bacterial strain *E.Coli* JM 109 free plasmid was used as competent cells to host and clone the recombinant plasmid $P^{CBHI- HPh -2.2}$ which carries cellobiohydrolase 1 gene (cbh1). All transformants were isolated after growth on hygromycin B selective medium and were evaluated for cellulases activity. Results indicated that: F pase and CMCase activities were increased, while β – glucosidase did not change in all transformed isolates. After transformation of *T. reesei* NRRL 12368 with plasmid DNA, the transformants were proved to produce 20% F pase and 21.6% CMCase more than their recipient strain NRRL 12368. When S/26 isolate was used as recipient in transformation, 11 clones were isolated and their production increased with 21.1 % F pase and 23.3% CMCase more than those of 6/12. When the isolate B/1 which failed to produce any cellulases activity was transformed with plasmid $P^{CBHI-Hph-2.2}$ all transformants produced only Fpase and CMCase, giving about 24% or 16% of the original culture, Fpase and CMCase, while they did not produce any β- glucosidase like their recipient strain B/1.

Key words: *Trichoderma reesei*, Cellulase enzymes, Genetic transformation, Plasmid.

INTRODUCTION

Trichoderma reesei has been long, and still is the best studied cellulolytic organism. The techniques of genetic engineering have greatly increased our understanding not only of the cellulase enzymes but also of the molecular biology of the fungus itself and gene replacement have enabled construction of novel *Trichodeuma* strains producing altered mixtures of cellulolytic enzymes which are better suited for industrial applications than the cellulase preparations obtained with the conventional production strains. **Penttilä** *et al* (1986) cloned and sequenced the gene and the full- length cDNA coding for the major endoglucanase EG-1 and compared this to the cbh[1] gene sequence to clarify the rela-

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tionship between the EG and CBH classes of cellulases. They pointed to the fact that practically nothing is known about the catalytic sites or any other functional sites of cellulases. **Knowles et al** (1987) isolated and characterized genes coding for four major cellulases, CBH-1, CBH-11, EG-1 and EG-111 in *T.reesei.* They found that this fungus produces at least two of cellulases, each containing both endo-and exo- types of enzyme. They use the full length cDNA coding for the four major cellulases for expression in yeast; *Saccharomyces cerevisiae.* **Penttilä et al (1987)** applied transformation with plasmid carrying the dominant selectable marker and/or the arg B gene of *Aspergillus nidulans,* which was found to complement the respective arg B mutation of *T.reesei* and obtained up to 600 transformants per µg of transformed DNA. On the other hand, **Harkki et al (1991)** used genetic engineering techniques to modify the proportion of different cellulases produced by a hypercellulolytic *T.reesei* mutant strain. A general expression vector. P ^{AMHI10}, containing the promoter and terminator sequences of the strongly expressed main cellobiohydrolase 1 (cbh1) gene; was used to overexpress a cDNA coding for EG-1, the major endoglucanase .In this way the production of endoglucanase was improved by a factor of two to four. In addition, **Uusitalo et al (1991)** used genetic engineering techniques to obtain improved strain of *Trichoderma.* which secreted novel mixtures of cellulases. They pointed to the improvement of EG-1 production due to transformation of already a high producer mutant of all cellulases with a plasmid carrying the eg 11 cDNA inserted into an expression cassette be-

tween the promoter and terminator sequences of the strongly expressed cbh1 gene. **Mäntylä et al (1992)** pointed to the presence of seven separate chromosomal DNA bands in the wild type. *T.reesei* strain QM6a. In comparison with the three hyper –cellulolytic mutant strains; QM9414, RUTC-30 and VTT-D- 79125, they found that in all strains the cellulase genes cbh1,cbh2, and eg 12 were located in one linkage group (chromosome II in the wild- type), while the main endoglucanse (egII) appeared on another chromosomal DNA band (chromosome VI in the wild –type). **Abrahao–Neto et al (1995)** examined the effects of inhibition of mitochondrial function on the expression of two nuclear genes encoding the extracelluar cellobiohydrolase 1(cbhI) and endoglucanase 1 (egII) of the cellulase system of *T. reesei.* Their results showed that inhibition of the mitochondrial function results in down regulation of the two transcripts of the cellulase system, cbhI and egII. **Carle- Urioste et al (1997)** showed that the transcripts of two members of the cellulase system, cbhI and egII, are present in the uninduced *T.reesei* cells. These transcripts were induced at least 1100-fold in the presence of cellulase. They also showed that a construct containing the hygromycin B resistance encoding gene driven by the cbhI promoter confers hygromycin B resistance to *T.reesei* cells grown in the absence of cellulose. Moreover, cellulose–induced production of the cbhI transcript was suppressed when antisense RNA, against three members of cellulase system, was expressed in vivo. **Takashima et al (1998)** used *T. reesei.* cellulose genes to be expressed in *A. oryzae* as a host. In this system, the expression of *T. reesei,*

cellulase genes were regulated under the control of *A. oryzae* Taka-amylase promoter. In addition, these genes were highly expressed when maltose was used as the main carbon source for the inducer. They found also that the production of recombinant cellulases by *A.oryzae* transformants reached their maximums after 3-4 days of cultivation under an optimal temperature of 50 – 70 °C.

MATERIAL AND METHODS

The fungal strain *Trichoderma reesei* NRRL 12368 and three of its induced mutants, selected according to their cellulases activity, were used to be transformed in this investigation. These mutants are S/26 (highly productive), B/1 (none productive) and 6/12 (moderately productive). The bacterial srtrain *Escherichia coli* JM 109 free plasmid strain was prepared to be used as competent cells to host and clone the recombinant, plasmid P $^{CBHI-Hph-2.2}$ which carry cellobiohydrolase 1gene. This strain was kindly provided by Stratagene Co., Germany.

Media

1- **Fermentation medium (FM):** (Haapala *et al* 1995).
2- **Protoplasting medium (PM):** (Kumari and Panda, 1994).
3- **Hygromycin B resistant medium (HBM):** Protolasting medium (PM) with the addition of cellulose, as a carbon source instead of glucose, 1M sorbitol and hygromycin B (200 µg/ml), was used as selective medium for *T.reesei* Transformants Hygromycin B (Sigma) was dissolved in distilled water.

4- **Luria medium (LB): (Sambrook *et al* 1989):** It was used for culturing and maintaining *E.coli* JM109 strain. It contained (g/L) Bacto- tryptone, 10; NaCl,10; and yeast extract, 5. To solidify, 2% agar was added. The pH of the medium was adjusted to 7.0.
5- **Ampicillin resistant medium (AM):** *Escherichia coli* JM109 strain medium (LB) was used with the addition of ampicillin (Sigma) (100µ/ml dist. water) as selective agent for the transformed cells.

- **Plasmid DNA preparation buffers (Sambrook *et al* 1989)** buffers for plasmid DNA preparation were used as follow:
- **(Glucose–Tris–EDTA)** buffer Glucose, 50 mM; Tris,pH 8.0, 25 mM and EDTA, 10 mM.
- **SDS-buffer** NaOH, 0.2 N and SDS, 1%.
- **Potassium acetate-buffer** Potassium acetate, 3.0 M and Glacial acetic acid, 2.0 M.
- **Chloroform / Isoamyl alcohol–buffer** 24 : 1 (v/v).
- **TE (10/1)–buffer** Tris- HCl (pH 8.0) 10 mM and EDTA, 1 mM.
- **Loading buffer** Sucrose, 10 % (w/v); EDTA,20 mM and Bromophenol- blue 0.04% (w/v).
- **1X TBE-buffer** Tris- HCl (pH 8.0), 0.085 M, Boric acid, 0.085 M, and EDTA, 0.002 M.
- **RNase (20 µg/ ml)**

Enzymes

Sigma Novozyme 234 and snail enzyme were used in this study for protoplast preparation.

Plasmid

The recombinant plasmid $P^{CBHI-HPh-2.2}$ was used in this study. It was kindly provided by Prof. Dr.H.El-Dorry (Biochemistry Department, Institute of Chemistry, University of Sao Paulo, Brazil). The size of this plasmid is nearly 6.2 kb. It contains a 2.2 kb DNA fragment containing the 5 -flanking region of cbh1 gene endcoding cellobiohydrolase I (CBHI) which is considered the major member of the cellulase. The coding region for the *E.coli* hygromycin β phosphotransferase (hph) gene (resistance marker for *T.reesei* transformants) and the ampicillin resistance (amp^r) gene (resistance marker for *E.coli* transformants). Figure (1) presents the chimeric construct in the correct orientation in addition to the polyadenylation signal and the origin of replication (ori).

Protoplast formation of *T. reesei*

Spore suspension from each strain was inoculated with 50 ml of PM in 250 ml flasks and incubated on a rotary shaker (120 rpm) at 28 °C for 20 hours. After incubation, the mycelia were collected by centrifugation, then washed twice with 0.7M kcl in phosphate buffer, 25mM, (pH 5.8) and then resuspended in (50mg / ml) phosphate buffer containing 0.7 M Kcl and 8 mg/ml novozyme 234.

The lytic mixtures were incubated at 30 °C with gentle shaking for up to two hours. Protoplasts were detected microscopically and the protoplast mixture was centrifuged at 4000 rpm for five min. at 4 °C, then the supernatant was discarded and the protoplasts were resuspended immediately in a sterile phosphate buffer containing 0.7 M KCl.
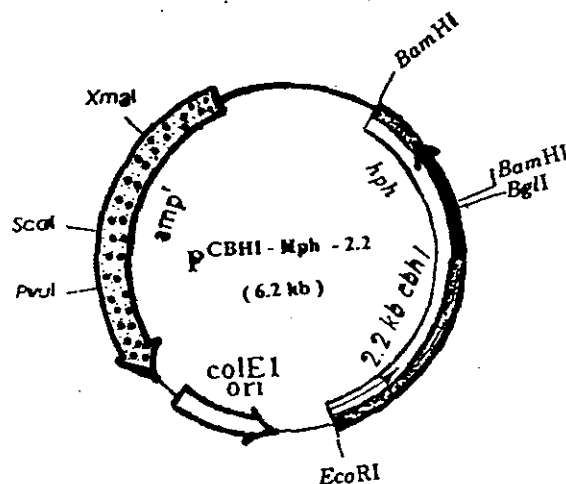

Fig 1. Suggested restriction map of plasmid $P^{CBHI-HPh-2.2}$ used in this study.

Transformation of *E. coli* JM109 strian

Transformation was applied using the plasmid $P^{CBHI-HPh-2.2}$ carrying ampicillin resistant gene as a selectable marker. The method of **Jones (1998)** for transformation of *E. coli* JM109 was used in this study.

Plasmid DNA preparation from *E. coli* JM109 strain

The method used for plasmid DNA preparation was according to **Sambrook** *et al* (1989).

Transformation of *Trichoderma reesei*

Protoplasts were prepared as mentioned before, and washed in 1 M sorbitol-10 mM Tris-HCl, pH 7.5. The mixture was centrifuged at 3000 rpm for five min., and the pellet was resuspended in 200 μl of 1M sorbitol-10 mM $CaCl_2$-10 mM tris-HCl, pH 7.5. Protoplast suspensions were mixed with 2-5 μg of transformation DNA (plasmid) in 20 μl TE buffer, then 50 μl of the mixture [25% PEG 6000 in 50mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5)] were added. The mixture was incubated on ice for 20 min, then the incubation was continued at room temperature for five min., after addition of two ml of the above mentioned PEG solution. After that, four ml of the mixture containing 1M sorbitol- 10mM $CaCl_2$- 10 mM Tris-HCl,pH 7.5 were added. Aliquotes of 500 μl from this mixture were plated on agar overley onto selective plates (HBM) and plates were incubated at 28 °C unitl single colonies appeared. The single colonies were transferred and subcultured on selective slants

several times and were tested finally for cellulases production.

Cellulases determination

Enzyme activities were assayed in the culture supernatant obtained by centrifugation for five min. at 5000 rpm under cooling in an eppendorf centrifuge. A half ml of the clear supernatant was diluted in 4.5 ml of 0.05 M citrate buffer, pH 4.8. The dilution was used to assay the enzyme activities.

Enzymatic degradation of filter paper (FPase)

A piece of filter paper (Whatman No.1,50 mg) was added to 0.5 ml of 0.5 M citrate buffer, pH 4.8, then 0.5 ml of appropriately diluted enzyme preparation was also added and the mixture was incubated for one hour at 50 °C. Since one activity unit (FPU) liberates 1μmol reducing sugar in one minute (**Miller, 1959** and **Vaheri** *et al* 1979), then reducing sugars were measured colorimeterically with the dinitrosalisylic acid (DNS) method at wavelength of 570 nm using glucose as a standard (**Miller, 1959**).

Carboxymethyl cellulose hydrolyzing activity (CMCase)

A half ml of 1% Carboxymethylcellulose in 0.05 M citrate buffer, (pH 4.8,) and 0.5 ml of diluted enzyme preparation were mixed and incubated at 50°C for 30 min. The reaction was stopped with 3 ml DNS reagent for reducing sugars, and the color can be obtained after heating for ten min and measured at wavelength of 570nm. One unit of enzyme liberates

1µmol reducing sugars in one min (Miller, 1959 and Vaheri *et al* 1979).

β- Glucosidase activity

Determination of β- Glucosidase activity was performed with P-nitrophenyl-β-D- glucoside (Sigma Co.) as substrate, 900 µl of mM P-nitrophenyl-β-D- glucoside in 0.05 citrate buffer (pH4.8) and 100 µl diluted enzyme were mixed. After incubation at 50 °C for 10 min, 1ml of 1 M NaCO₃ was added. The liberated p-nitrophenol was determined from its absorbance at 400nm. Enzyme activity unit was defined as the amount of enzyme that liberates 1 µmol p- nitrophenol under the assay conditions in one minute (Vaheri *et al* 1979).

RESULTS AND DISCUSSION

The application of genetic engineering in *Trichoderma sp.* has made it possible to modulate cellulases production in such a way that new *T.reesei* strains producing novel cellulase profiles of commercial potential are now available. Several different combinations have been made without changing the viability of the organism. Over 50% of the secreted cellulases consist of cellobiohydrolase I enzyme, the product of a single cbhI gene, and thus this gene is believed to be preceded by strong promoter- regulatory sequences. In addition, cbh1 promoter is inducible by several carbon sources including cellulose, and small oligosaccharides such as cellobiose, lactose and sophorose. The strongly inducible cbhI promoter has been used in expressing both homologous *Trichoderma* proteins

and heterologous proteins originating from other fungal species.

Plasmid P$^{CBHI-Hph-2.2}$ containing 2.2 kb 5´-flanking of cbh1 gene that expressed to cellobiohydrolase I (CBHI) protein, hygromycin B dominant selectable marker (for *T. reesei*) and ampicillin marker was used to transform *E. coli* JM109 competent cells. The *E. coli* transformants were selected on LB agar plates containing 100 µ g/ml ampicillin and subcultured several times as mentioned before. The plasmid DNA (6.2 kb) was prepared from *E. coli* JM109 and tested by 0.7 gel electrophoresis (Figure 2). This plasmid was prepared in a large scale and used to transform three isolates of *T.reesei* in addition to the original strain NRRL 12368. These isolates were selected on the basis of either extracellular proteins separation on SDS polyacrylamide gel electrophoresis and /or their productivity, as shown in Table (1).
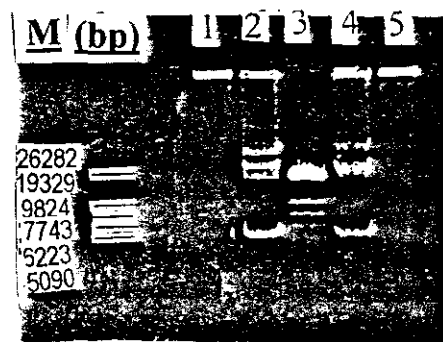

Fig: 2. Agarose gel electrophoresis representing the constructed plasmid P$^{CBHI-Hph-2.2}$
1.5 – plasmid free *E.coli* JM109.
2,4- plasmid containing *E.coli* JM109.
3- (M) marker No. 17 (Lambda/ Eco 1301 + Mlu 1) with (26282.19329.7743.6223.5090----bp).

The first transformation experiment was carried out by using plasmid $P^{CBHI-Hph-2.2}$ to transform the original culture; *T. reesei* NRRL 12368. Transformants were selected and purified on plates containing hygromycin B as dominant selectable marker. Nine transformants were selected as independent clones and tested for cellulases production in 50 ml shake flask cultivations in cellulose – containing medium (FM2) as mentioned before.

Table 1. Cellulase enzymes activity for the three selected isolates and the original culture which were transformed by the constructed plasmid $P^{CBHI-Hph-2.2}$

Isolate	Cellulase activities					
	FPase		CMCase		β- Glucosidase	
	U/ml	% From W.T	U/ml	% From W.T	U/ml	% From W.T
Original culture	2.5	100.0	3.7	100.0	5.7	100.0
S/26	7.0	280.0	7.5	202.7	7.5	131.6
B/1	0.0	0.0	0.0	0.0	0.0	0.0
6/12	1.9	76.0	4.3	116.2	2.5	43.9

Data in Table (2) indicated two main facts: a) none of the isolated transformants showed any increase or decrease in β- glucosidase activity when compared with the original isolate, b) all the isolated transformants showed increase with different degrees of both FPase and CMCase in comparison with the original culture.

Table 2. Cellulases activity for the nine transformants induced after the transformation of the original culture *T.reesei* NRRL 12368 with plasmid $P^{CBHI-Hph-}$

Transformant	Cellulases productivity					
	FPase		CMCase		β- Glucosidase	
	U/ml	% of w.t	U/ml	% of w.t	U/ml	% of w.t
Original culture	2.5	100.0	3.7	100.0	5.7	100.0
ES/1	2.7	108.0	4.2	113.5	5.7	100.0
ES/2	3.0	120.0	4.5	121.6	5.7	100.0
ES/3	2.7	108.0	4.2	113.5	5.7	100.0
ES/4	3.0	120.0	4.5	121.6	5.7	100.0
ES/5	3.0	120.0	4.5	121.6	5.7	100.0
ES/6	2.7	108.0	4.2	113.5	5.7	100.0
ES/7	3.0	120.0	4.5	121.6	5.7	100.0
ES/8	3.0	120.0	4.5	121.6	5.7	100.0
ES/9	3.0	120.0	4.5	121.6	5.7	100.0

Furthermore, results showed also that the isolated transformants are located into only two categories concerning the activities of both FPase and CMCase. The first category included isolates ES/1, ES/3 and ES/6, which showed the same FPase and CMCase activities, i,e.; 108% and 113.5 %, respectively, of those of the original parent. The second category included the other six transformants which showed the production capacity of 120% FPase and 121.6% CMCase of that of the original culture. However, results obtained from the second transformation trial including the same plasmid but with the superior cellulases producer isolate *T. reesei* S/26 as a recipient showed also that 11 transformants were obtained. These transformants were divided into two categories (Table 3), one of them included transformants AL/3, AL/5, AL/6, AL7 and AL/11, each of which produced 112.9 % FPase and 116% CMCase of that of their parental strain S/26. The other group includes AL/1, AL/2, AL/4, AL/8, AL/9 and AL/10 transformants which produced 121.4% FPase and 124% CMCase when compared with their parent S/26.

In addition, it appeared also, as was found in the first experiment, that the production activity of β–glucosidase of each of the isolated transformants was not affected due to the introduction of the plasmid DNA into the recipient strain S/26.

Results obtained from the third transformation experiment where isolate 6/12 was used as recipient are summarized in Table (4). These results showed in turn, the same trend as that of the previous ones.

It appeared that all of the obtained transformants (11) belong to two groups, the first includes the transformants; Ay/1, Ay/2, Ay/4, Ay/8, Ay/9, and Ay/11 while the second included transformants; Ay/3,Ay/5, Ay/6, Ay/7, and Ay/10.

Clones belonging to the first group showed enzyme activities of FPase and CMCase which were 21% and 23% more than that of their parent 6/12 isolate. On the other hand, transformants of the second group produced about 16% more activity of both FPase and CMCase than their parent. Also, β–glucosidase productivity for all the isolated transformants was not affected.

When B/1 isolate was used as a recipient for the same plasmid DNA, 11 transformants were isolated. They were classified into two categories concerning FPase and CMCase activities.

They produced both in equel rates, either about 16% FPase and CMCase or about 24% FPase and CMCase enzyme activities of those of the original strain as shown in Table (5).

All the results concerning transformation, could be summerised as follows:1) in all transformation experiments, it appeared that, β–glucosidase showed the same activity as the parent, 2) all isolated transformants were more FPase and CMCase producers than their parents, 3) the higher CMCase and FPase transformants in each trial were classified into two groups only.

Gene cloning, via transformation using different plasmids was carried out by many authors. **Takashima** *et al* (1998) used *T. reesei* cellulase genes to be experssed in *A.oryzae* as a host. The cellulase genes were regulated under the

Table 3. Cellulases activity for the transformants induced as a results of transforming of the *T. reesei* S / 26 isolate with plasmid P[CBHI-Hph-2.2]

| Transformant | Cellulase productivity | | | | | | | | |
| | FPase | | | CMCase | | | β- Glucosidase | | |
	U/ml	% from W.T	% from recipient	U/ml	%from W.T	% from recipient	U/ml	% from W.T	% from recipient
Original culture	2.5	100.0	35.7	3.7	100.0	49.3	5.7	100.0	76.0
S/26	7.0	280.0	100.0	7.5	202.7	100.0	7.5	131.6	100.0
AL/1	8.5	340.0	121.4	9.3	251.4	124.0	7.5	131.6	100.0
AL/2	8.5	340.0	121.4	9.3	251.4	124.0	7.5	131.6	100.0
AL/3	7.9	316.0	112.9	8.7	235.1	116.0	7.5	131.6	100.0
AL/4	8.5	340.0	121.4	9.3	251.4	124.0	7.5	131.6	100.0
AL/5	7.9	316.0	112.9	8.7	235.1	116.0	7.5	131.6	100.0
AL/6	7.9	316.0	112.9	8.7	235.1	116.0	7.5	131.6	100.0
AL/7	7.9	316.0	112.9	8.7	235.1	116.0	7.5	131.6	100.0
AL/8	8.5	340.0	121.4	9.3	251.4	124.0	7.5	131.6	100.0
AL/9	8.5	340.0	121.4	9.3	251.4	124.0	7.5	131.6	100.0
AL/10	8.5	340.0	121.4	9.3	251.4	124.0	7.5	131.6	100.0
AL/11	7.9	316.0	112.9	8.7	235.1	116.0	7.5	131.6	100.0

Table 4. The transformants resulted from the transformation of *T. reesei* isolate (6/12) with plasmid P$^{CBHI-Hph-2.2}$ and their cellulases productivity.

Transformant	Cellulase activities								
	FPase			CMCase			β- Glucosidase		
	U/ml	% from W.T	% from recipient	U/ml	% from W.T	% from recipient	U/ml	% from W.T	% from recipient
W.T.	2.5	100.0	131.6	3.7	100.0	86.1	5.7	100.0	228.0
6/12	1.9	76.0	100.0	4.3	116.2	100.0	2.5	43.9	100.0
Ay/1	2.3	92.0	121.1	5.3	143.2	123.3	2.5	43.9	100.0
Ay/2	2.3	92.0	121.1	5.3	143.2	123.3	2.5	43.9	100.0
Ay/3	2.2	88.0	115.8	5.0	135.1	116.3	2.5	43.9	100.0
Ay/4	2.3	92.0	121.1	5.3	143.2	123.3	2.5	43.9	100.0
Ay/5	2.2	88.0	115.8	5.0	135.1	116.3	2.5	43.9	100.0
Ay/6	2.2	88.0	115.8	5.0	135.1	116.3	2.5	43.9	100.0
Ay/7	2.2	88.0	115.8	5.0	135.1	116.3	2.5	43.9	100.0
Ay/8	2.3	92.0	121.1	5.3	143.2	123.3	2.5	43.9	100.0
Ay/9	2.3	92.0	121.1	5.3	143.2	123.3	2.5	43.9	100.0
Ay/10	2.2	88.0	115.8	5.0	135.1	116.3	2.5	43.9	100.0
Ay/11	2.3	92.0	121.1	5.3	143.2	123.2	2.5	43.9	100.0

Table 5. Cellulases activities of clones selected after transformation of *T. reesei* (B/1) with plasmid $P^{CBHI\text{-}Hph\text{-}2.2}$

Transformants	Cellulase activities					
	Fpase		CMCase		β- Glucosidase	
	U/ml	% from W.T	U/ml	% from W.T	U/ml	% from W.T
W.T.	2.5	100.0	3.7	100.0	5.7	100.0
B/1	0.0	0.0	0.0	0.0	0.0	0.0
ME/1	0.6	24.0	0.9	24.3	0.0	0.0
ME/2	0.4	16.0	0.6	16.2	0.0	0.0
ME/3	0.4	16.0	0.6	16.2	0.0	0.0
ME/4	0.6	24.0	0.9	24.3	0.0	0.0
ME/5	0.6	24.0	0.9	24.3	0.0	0.0
ME/6	0.6	24.0	0.9	24.3	0.0	0.0
ME/7	0.6	24.0	0.9	24.3	0.0	0.0
ME/8	0.4	16.0	0.6	16.2	0.0	0.0
ME/9	0.4	16.0	0.6	16.2	0.0	0.0
ME/10	0.6	24.0	0.9	24.3	0.0	0.0
ME/11	0.6	24.0	0.9	24.3	0.0	0.0

control of *A.oryzae* Taka-amylase promoter which were induced and highly expressed when maltose was used as the main carbon source. On the other hand the cbh1 promoter was induced and highly expressed when cellulose and oligosaccharides were used as the carbon source. Furthermore, **Takashima *et al*** **(1999)** cloned a β- glucosidase gene (bgl4) and its homologue (bgl2) from *Humicola grisea* and *T. reesei*, respectively, to be expressed in *A. oryzae*. They found that the recombinant *H.grisea* BGL4 showed a significantly high level of β- glucosidase activity, while recombinant *T. reesei* BGL2 showed low β- glucosidase activity. As was mentioned before that plasmid $P^{CBHI\text{-}Hph\text{-}2.2}$ was used throughout all transformation experiments contained cbh1 gene which is expressed to cellobiohydrolase I enzyme. However, it appeared that, at least 50% of the secreted cellulases consist of CBHI (**Mäntylä *et al*** **1998 and Hazell *et al* 2000).** The same authors pointed that the dominant selectable markers such as hygromycin B and phleomycin are most preferable for industrial *T. reesei* strain and have also been used successfully. Also, it appeared

that the gene targeting has provided a mean to compare the effect of promoter change on the expression of homologous genes in order to exclude the potential differences in expression caused by distinct locations in the host genome. The cbh1 gene is believed to be preceeded by strong promoter –regulatory sequences. Also, there are some indication in *T. reesei* that the genomic site of integration of introduced genes can have profound effects on expression levels.

Results obtained by different authors led to different conclusions. For example, **Penttilä et al (1987) and Uusitalo et al (1991)** showed that the transforming DNA was integrated at several different locations in the genome indicating that probably the chromosomal site of integration may affect the level of gene expression. Moreover, **Uusitalo et al (1991)** found that, for optimal expression, which led to two fold increase, the endoglucanase 1 gene might be inserted into a specific locus in the chromosome.

On the other hand, **Cullen et al (1987)** and **Harkki et al (1991)** found that the copy number of integrated genes was correlated with the increase of enzyme activities. Also, **Harkki et al (1991)** used the promoter sequence of the strongly expressed main cellobiohydrolase 1 (cbh1) gene to overexpress a cDNA coding for EGI, the major endoglucanase. The production of endoglucanase was improved up to four folds. However, to avoid copy number and position effects, **Ilmen et al (1996)** showed that in the absence of stable autonomously replicating plasmids, the expression constructed need to be tragated to a specific locus in the genome.

So, results obtained in the present study were nearly in partial agreement with the conclusion reported by **Ilmen et al (1996)** and (**Mäntylä et al (1992)**, who found that cellulase genes are located in two groups, the genes cbh1, cbh2 and egl2 were located in one linkage group on chromosome II, while endoglucanase 1(egl1) gene is located on another chromosomal band (chromosome VI).The introduction of the plasmid P$^{CBH1-Hph-2.2}$ might be targated either to the first group.i.e., chromosome II or to chromosome VI. In the case of the first proposal,cbh1 carried by the plasmid added another copy of cbh1 to the same group containing cbh1,cbh2, and egl2. On the other hand, if this plasmid was integrated into the other group,i.e., chromosome VI, this means that it was integrated in a different position, which may lead to another expression of the gene and this is in complete accordance with the results obtained in this study.

Furthermore, in case of the first proposal, the increase of FPase and CMCase activities may be due to the replication of the gene cbh1,i.e., dose effect. On the other hand, **Karhunen et al (1993)** studies the effect of cellobiohydrolase 1 (cbh1) promoter on the expression of the endoglucanase 1 cDNA (egl1). They found that the amount of the egl1 mRNA produced under the control of the cbh1 promoter in the transfomants was 8 to 16 fold higher which indicated that cbh1 promoter was about 10 times more efficient than the egl1 promoter. While, **Abrahao-Neto et al (1995)** examined the effect of inhibition of mitochondrial function on the expression of the cbh1 and egl1 genes of *T.reesei*. They showed that inhibition of the mitochondrial function results in down- regulation of the two transcripts of the cellulase system.

Therefore, it is possible to suggest a role of the mitochondria in regulating the expression of the cellulase transcripts.

REFERENCES

Abrahao-Neto, J.; C.H.B. Rossini; S. El-Gogary; F. Henriquesilva; O. Crivellaro and H. El-Dorry (1995). Mitochondrial functions mediate cellulase gene expression in *Trichoderma reesei*. *Biochemistry, 34, (33): 10456-10462.*

Carle–Urioste, J.C.; J. Escobar-Vera; S. El-Gogary; F.Henrique-Silva; E.Torigoi; O.Crivelaro; A. Herrera-Estrella and H. El-Dorry (1997). Cellulase induction in Trichoderma reesei by cellulose requires its own basal expression. *J. Biol. Chem., 272(15): 10169-10174.*

Cullen, D.; S.A. Leong; L.J. Wilson and D.J. Henner (1987). Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, hph. *Gene, 57: 21-26.*

Haapala, R.; E. Parkkinen; P. Suominen and S. Linko (1995). Production of extracelluar enzymes by immobilized Trichoderma reesei in shake flask cultures. *Appl. Microbiol. Biotechnol., 43: 815-821.*

Harkki, A.; A. Mäntylä; M. Penttilä; S.Muttilainen; R. Bühler; P. Suominen; J. Knowles and H. Nevalainen (1991). Genetic engineering of Trichoderma to produce strains with novel cellulase profiles. *Enzyme Microb. Technol., 13: 227-233.*

Hazell, B.W.; V.S.J. Teo; J.R. Bradner; P.L. Bergquist and K.M.H. Nevalainen (2000). Rapid transformation of high celluase-producing mutant strains of Trichoderma reesei by microprojectile

bombardment. *Applied Microbiology, 30 (4): 282-287.*

Ilmen, M.; M.L. Onnela; S. Klemsdal; S. Keränen and M. Penttilä (1996). Functional analysis of the cellobiohydrolase 1 promoter of the filamentous fungus *Trichoderma reesei*. *Mol. Gen. Genet., 253:303-314.*

Jones, P. (1998). Vectors: cloning applications. *Essential Techniques Series,* Published in association with BIOS Scientific Publishers Limited.

Karhunen, T.; A. Mäntylä; K.M. Helena Nevalainen and P.L. Suominen (1993). High frequency one-step gene replacement in *Trichoderma reesei*. I. Endoglucanase I overproduction. *Mol. Gen. Genet., 241:515-522.*

Knowles, J.; P. Lehtovaara; T. Teeri; M. Penttilä; I. Salovuori and L. Andre (1987). The application of recombinant-DNA technology to cellulases and lignocellulosic wastes. *Phil. Trans. R. Soc. Lond. A 321:449-454.*

Kumari, J.A. and T. Panda (1994). Intergeneric hybridization of *Trichoderma reesei* QM9414 and Saccharomyces cerevisiae NCIM 3288 by protoplast fusion. *Enzyme Microb. Technol., 16:870 – 882.*

Mäntylä, A.L.; K.H. Rossi; S.A. Vanhanen; M.E. Penttilä; P.L. Suominen and K.M. Helena Nevalainen (1992). Electrophoretic karyotyping of wild-type and mutant *Trichoderma longibrachiatum* (reesei) strains. *Curr. Genet., 21: 471-477.*

Mäntylä, A.; M. Paloheimo and P. Suominin (1998). Industrial mutants and recombinant strains *of Trichoderma reesei*. Trichoderma and Gliocladium Volume 2: 291-309. Edited by Gary E. Harman and C.P. Kubicek.

Miller, G.L. (1959). Use of denitrosalicylic acid reagent for determination of

reducing sugar. *Anal. Chem., 31: 426-428.*

Penttilä, M.; P. Lehtovaara; H. Nevalainen; R. Bhikhabhai and J. Knowles (1986). Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene, 45: 253-263.*

Penttilä, M.; H. Nevalainen; M. Rätt; E. Salminen and J. Knowles (1987). A versatile transforamtion system for the cellulolytic filamentous *fungus Trichoderma reesei. Gene, 61: 155-164.*

Sambrook, J., E.F. Fritsch, and T. Maniatis (1989). *Molecular cloning:* A laboratory Manual, 2nd Ed, pp. 20-89, Coid Spring Harbor Laboratory, Cold Spring Harbor, Ny.

Takashima, S.; H. Likura; A. Nakamura; M. Hidaka; H. Masaki and T. Uozumi (1998): Overproduction of recombinant *Trichoderma reesei* cellulases by *Aspergillus oryzae* and their enzy-enzymatic properties. *J. Biotechnol., 65:(2-3): 163-171.*

Takashima, S.; A. Nakamura; M. Hidaka; H. Masaki and T. Uozumi (1999). Molecular cloning and expression of the novel fungal β-glucosidase genes from Humicola grisea *and Trichoderma reesei. J. Biochem., 125: 728-736.*

Uusitalo, J.M.; K.M.H. Nevalainen; A.M. Harkki; J.K.C. Knowles and M.E. Penttilä (1991). Enzyme production by recombinant *Trichoderma reesei* strains. *J. Biotechnology, 17:35-50.*

Vaheri, M.P.; M.E.O. Vaheri and V.S. Kauppinen (1979). Formation and release of cellulolytic enzymes during growth of *Trichoderma reesei* on cellobiose and glycerol. *Eur.J. Appl. Microbial. Biotechnol., 8:73-80.*

التحول الوراثي في فطر تريكوديرما ريساي لتحسين إنتاج أنزيمات السليلوليز

[٥١]

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استخدمت فـي هـــذه الدراســـة السلالة NRRL-12368 من فطر تريكوديرما ريساي المنتجة لإنزيمات السـليلوليز مـع ثلاثة طافرات مستحدثـة منها لتحويلـها وراثياً باستخـدام البلازميـد CBHI–HPH- 2.2 والحامل للجين cbh1 الـذي ينتـج إنزيم Cellobiohydrolase I.

وهذه الطافرات الثلاثة تم اختيارها علـى أساس إنتاجيتها لإنزيمات السـليوليز وهـي السلالة S/26 (عالية الإنتاج)، السـلالة B/1 (غير المنتجة) والسـلالة 6/12 (متوسـط الإنتاج).

وبعد التحول الوراثي تم تقييم العـزلات المتحولـة وراثيـاً مـن حيـث إنتاجيتهـا للسليوليز.

وتشير النتائج الى مايلى

أمكن عزل تراكيب وراثية جديـدة مـن جميع التحولات الوراثية الناتجة وكانت تقـع في طرازين أحدهمـا يـؤدى الـى زيـادة الإنتاجية اكثر من الطرز الآخر .

كانت الزيادة فـي إنتاجيـة السـليلوليز مقصورة على كلا مـن FPase, CMC ase حيث لم تتغير انتاجية انزيم β-gluosidase في جميع المتحولات المتحصـل عليـها . وأدى التحول الوراثي للسلالة الأصلية مـن الفطر الى تحسين في الإنتاجية يصـل الـى ٢٠% من أنزيم FPase و٢١,٦% من أنزيم CMCase مقارنة بإنتاجية السـلالة غـير المحولة وراثياً، وعندما تم التحول الوراثـي للطافرة العالية الإنتاجية S/26 تم اختيار ١١ عزلة محولة وراثياً تحسنت فيها الإنتاجيـة بمقدار ٢١,٤ % FPase و٢٤% CMCasa زيادة على انتاج تلك الطافرة غير المحولـة وراثياً.

وفى حـــالة اسـتخدام الطـافرة 6/12 متوسطة الإنتاجية وتحولها وراثياً تم انتخاب ١١ عـزلة محـولة وراثياً تحسـن فيـها الإنتاج بمقدار ٢١,١% FPase ، ٢٣.٣%

زيادة على إنتاج تلك الطافرة غير CMCase   استعادت قدرتها على إنتاج كلاً من أنزيمــى
المحولة وراثياً.   FPase ، CMCase منتجة حوالـى ٢٤% أو
وعندما تم التحول الوراثى للطافرة B/1   ١٦% من إنتاجية سلالة الفطر الأصلية مــن
غير المنتجة لأي من أنزيمـــات الســليوليز   هذين الأنزيمين بالتســاوي ولكنـــها ظلـت
الثلاثة أمكن اختيار عزلات محولة وراثيـــاً   فاقدة القدرة على إنتاج أنزيم β-glucosidase.