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Successive Construction of β-Glucosidase Hyperproducers of *Trichoderma Harzianum* Using Microbial Biotechnology Techniques

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Abstract

In an attempt to construct superior *Trichoderma harzianum* isolates for improvement β-glucosidase productivity, induction of mutants was applied. After application of UV irradiation and Ethyl methane sulfonate (EMS), 461 isolates were obtained, out of them 99 after UV application and 362 isolates after EMS treatments. Five isolates (two after UV application and three after EMS treatments) were selected on the basis of their highly productivity of both enzymes to be treated with doses of colchicine (0.1% and 0.2%) as a second step of induction mutation. After colchicine treatments, 191 isolates were obtained, out of them 40 isolates after treating the wild type strain, 70 isolates after treating the two UV induced-mutants with colchicine and 81 isolates after treating the three EMS induced-mutants with colchicine .These isolates were tested for their CMCase and β-glucosidase productivities. One isolate (D1/4) proved to be the highest producer for the two enzymes, since it produced 160% and 186% CMCase and β-glucosidase, respectively, more than the original strain. In addition, highest DNA content and also highest amounts of CMCase and β-glucosidase were obtained after EMS-treatments followed by colchicine application.

Keywords: *T. harzianum*; β-Glucosidase; Mutation; Colchicine

Introduction

Cellulose constitutes the highest proportion of municipal and wastes; it represents a major source of renewable energy and raw materials. Therefore, the utilization of cellulosic wastes to produce energy is potentially of great importance (Bhat and Bhat, 1997; Zhang and Lynd, 2004). The enzymatic conversion of cellulose is catalyzed by a multiple enzyme system. Beta-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) is one of the essential enzymes in the enzymatic conversion of cellulose. It is an important component of cellulase system and acts synergistically with endogluconase and cellobiohydrolase for complete degradation of cellulose (Szengyel et al., 2000). Thus, β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing endoglucanase and exoglucanase enzymes to function more efficiently (Harhangi et al., 2002).

Trichoderma harzianum is well known as producer of cellulolytic enzymes that extensively used for the degradation of cellulose particularly in textile and paper industries, beside its use in wastewater treatment (Prabavathy et al., 2006). Strain improvement by mutations is an age - old as a successful method. Therefore, several approaches including chemical mutations, UV irradiations and their combinations were applied to obtain enhanced cellulases producing strains (Kotchoni and Shonukan, 2002). Nevertheless, strains that are genetically improved for high level of cellulases production have been successfully used in a number of applications including animal feed, pharmaceutical and textile industries (Aristidou and Penttilä, 2000). Colchicine is known to be unsurpassed chemical agent; it has a strong mutagenetic property. Colchicine is also known as a polyploidy inducer in animals, plants, and microbes (Oenfelt and Klasterska, 1983). Colchicine binds to tubulin and then mitosis is arrested, inhibiting the normal distribution of chromosomes and yielding polyploid nuclei. Polyploids are also formed by colchicine treatment in *T. reesei* QM 9414 (Toyama and Toyama, 2001).

The aim of the present study is to construct a strain of the fungus *T. harzianum* having the genetic ability to produce the highest CMCase and β-glucosidase activities.

Trichoderma harzianum NRRL 13879 strain was used in the present study and maintained on YMGA medium slants (Strauss and Kubicek, 1990). For UV-mutagenesis, spores obtained from 8-day old slants were resuspended in 0.85% NaCl and irradiated with UV-light (254 nm) from Philips TUV-30 W lamp source at a distance of 20 cm for 3, 6, 9, 12 and 15 min. The treated conidia were put in dark for one hr and transferred to dishes with YMG agar containing 0.1% (v/v) Triton X-100. On the other hand, conidiospores of 8-day old cultures were subjected to ethylmethansulfonate (EMS) mutagenesis. Spores were collected and incubated in 0.2 M phosphate buffer pH 8.0 containing 50, 75, 100, 125 and 150µl / ml EMS (Sigma Co.,) for 30 and 60 min. Spores were then washed with sterile distilled water and serial dilutions were prepared for inoculating YMG agar.

The second step mutation was done by colchicine treatments with UV-mutants and EMS-mutants. Conical 250 flasks, each containing 50 ml of Natick medium (Mandels and Sternberg, 1976) were inoculated with some UV and EMS-induced mutants. The flasks were incubated at 28°C with shaking at 120 rpm for 18 hrs, the swollen conidia were treated with 0.1 and 0.2% (w/v) colchicine and incubated with shaking (200 rpm) at 28°C for 10 days. Nuclear conditions in a mycelial mat were observed by nuclear staining; a piece (2mm × 2mm) was cut from each mycelial mat every day and stained with Giemsa solution for at least 30 min (Friend et al., 1976). After 10 days each treatment was diluted and plated onto YMG agar containing 0.1% (v/v) Triton X-100 and incubated at 28°C for six days.

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Total DNA was isolated according to Al-Samarrai and Schmid (2000) and the quantity of the obtained DNA was determined according to the UV-absorbance at 260 using spectrophotometer, model pharmacia Biotech. Ultraospec 1000.

The wild type strain and mutants were grown in fermentation medium (Haapala et al., 1995) and the medium was inoculated with 10% spore suspension from 8-day old slants and flasks were incubated with shaking (200 rpm) at 28°C for ten days. Enzyme activities were assayed in the culture supernatant according to Vaheri et al. (1979).

Results and Discussion

Induction of genetic variations

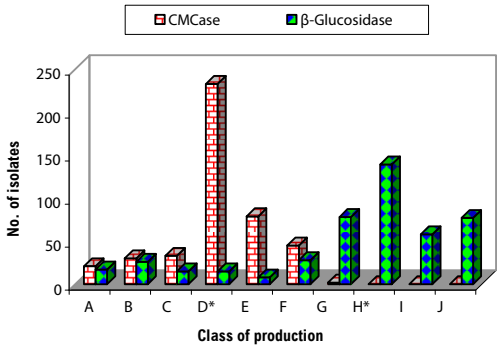
Ultraviolet light mutagenicity (UV): UV irradiation was carried out in different exposure times, i.e., 3, 6, 9, 12 and 15 min for the induction of mutations in *T. harzianum* NRRL 13879 strain. The data (not shown) indicated that, 99 colonies were isolated after the application of UV irradiation doses, out of them 24 isolates (24.24%) showed different characteristics as morphological variants according to both colony shape and color when compared with the parental strain. The highest morphological variants percentage (37.50%) was appeared as a result of 12 min, UV dose application, and the lowest percentage (11.11%) was recorded after 15 min. exposure time. Results showed that no auxotrophic mutants were obtained after application of UV light.

Ethyl methane sulfonate treatments (EMS): Fungal spore suspension of *T. harzianum* NRRL 13879 was treated with five EMS concentrations; 50, 75, 100, 125 and 150 µl/ml for 30 and 60 min. The results showed that, 362 colonies were isolated after applied EMS concentrations and incubation periods, out of them, 139 mutants (38.39%) showed different morphological variants. While, only one mutant proved to be an auxotrophic mutant, it was isolated from the concentration of 100µl/ml for 60 min. and was identified as (other mutant), since it was requiring more than three requirements tested. Complete lethalty was observed after applied the highest EMS concentration for 60 min.

Carboxymethyle cellulose and β-glucosidase activities of *T. harzianum* isolates

Figure (1) present carboxymethyle cellulase (CMCase) and β-glucosidase productivities of 461 isolates compared to the original srain, *T. harzianum* NRRL 13879. Out of 461 isolates, 99 were isolated after application of UV doses and 362 isolates were obtained following the different EMS concentrations. Results in Figure (1) indicated that, 233 (50.54%) and 139 (30.15%) of the tested isolates produced CMCase and β-glucosidase activities within the range of the parental strain (classes D and H, respectively). In the case of the 233 isolates, 48 out of them were isolated after UV application and 185 isolates after EMS treatments. But for the 139 isolates, 34 out of them were isolated after UV application and 105 after EMS treatments.

On the other hand, 144 isolates (31.23%) proved to be higher CMCase producers than the original strain which produced 2.5 U/ml CMCase, these isolates were as follow : 97 (21.04%), 45 (9.76%) and two (0.43%) produced at least 20%, 60% and 100% CMCase, respectively, more than the original strain (classes E, F and G), respectively. While, 58 isolates (12.58%) produced at least 17% β-glucosidase more than the wild type strain which gave 6.0 U/ml β-glucosidase (Class I) and 77 isolates (16.70%) exhibited β-glucosidase at least 33.3% more than the parental strain (Class J). The obtained results showed that, 63 (13.66%) and 170 isolates (36.87%) exhibited CMCase and β-glucosidase activities less than the original strain, respectively. While, 21 (4.55%) and 17 (3.68%) isolates lost completely their abilities to produce any CMCase or β-glucosidase (classe A).



*D: Including the CMCase production of the parental strain (2.5 U/ml).
*H: Including the β-glucosidase production of the parental strain (6.0 U/ml).

Figure 1: A histogram representing classes of CMCase and β-glucosidase production and the number of *T.harzianum* isolates.

Enzymes / Isolates	CMCase and β-glucosidase productivities.			
	CMCase (U/ml)	% from W.T.	β-glucosidase (U/ml)	% from W.T.
Control	2.5	100.0	6.0	100.0
(L) 9/8	4.3	172.0	9.5	158.3
(P) 15/4	4.3	172.0	9.2	153.3
(E) 50/30/17	4.5	180.0	9.5	158.3
(R) 100/30/44n	4.5	180.0	13.0	216.7
(D) 125/30/12	5.0	200.0	13.5	225.0

Table 1: The highest CMCase and β-glucosidase producer strains obtained after UV and EMS treatments.

Five isolates were selected on the basis of their higher CMCase and β-glucosidase productivities as shown in Table (1). Two isolates out of them were obtained after UV-irradiation, i.e. L (9/8) and P (15/4). They showed (72% and 72%) CMCase, and (58.3% and 53.3%) β-glucosidase activities more than the original strain, respectively. Other three isolates were obtained as a result of treating *T. harzianum* NRRL13879 with (E) 50, (R) 100 and (D) 125 (µl/ml) EMS for 30 min and showed 80%, 80% and 100% CMCase activity, as well as 58.3%, 116.7% and 125% β-glucosidase activity, respectively, more than the original strain.

Ultraviolet irradiation and EMS, as a tool for induction of genetic variations was successfully applied by many investigators. Hao et al. (2006); Adsul et al. (2007) they isolated different mutants of different Trichoderma species using UV-irradiation. In addition, forty mutants after treating Trichoderma with EMS were isolated, some of them, exhibited a maximum of 10-fold improvement of cellulase production (Kotchoni and Shonukan, 2002). On the other hand, Hou (2010) found the most efficient mutagenesis in haploid Saccharomyces cerevisiae cells occurs when a mutagen confers a high frequency of mutations in the range of 50% to 90% lethality.

Colchicine treatments

Five isolates mentioned in Table 1 were treated with two concentrations of colchicines 0.1% and 0.2% (w/v) as a second step of mutations. Nuclear conditions in mycelial mat were observed by nuclear staining with giemsa solution.

CMCase and β-glucosidase activities of *T. harzianum* isolates after colchicine treatments

Results in Figure (2) showed the CMCase and β-glucosidase productivities of 191 isolates following colchicine treatments. After all colchicine treatments, none of the obtained isolates lost its

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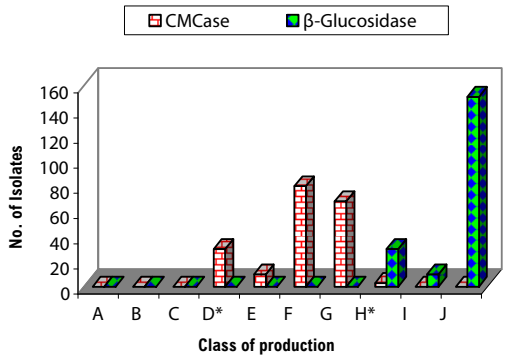
CMCase or β-glucosidase efficincy or produced less than the original strain, since the lowest productivities for the isolates in classes D and H in Figure (2) were 2.5 and 6.0 U/ml CMCase and β-glucosidase, respectively as the parental strain. The results indicated that 30 isolates produced CMCase and β-glucosidase with the same efficiency as the wild type strain, out of them 17 isolates were obtained after treating the original strain with 0.1% colchicine and 13 isolates after treating the original strain with 0.2% (classes D and H).

The rest isolates produced CMCase and β-glucosidase more than the original strain. The best improvement for CMCase activity was resulted from the three mutants isolated after treatment of EMS-mutant D (125/30/12) with 0.1% colchicine (D1). These mutants exhibited at least 140% (class H) more than the original strain T. harzianum NRRL 13879. On the other hand, 151 isolates (79.05%) were obtained after treatment of UV- treated isolates (70) or EMS-treated isolates (81) with the different doses of colchicine. These isolates proved to be higher β-glucosidase producers, since they produced at least 33.3% β-glucosidase more than the original strain.

Regarding to the best productivities of CMCase, the results showed that, the high concentration of EMS (125µl/ml) for 30 min followed by colchicines 0.1% (D1) gave the highest productivity (Table 2), since it reached to 160% and 30% more than the original strain and its original mutant, respectively. On the other hand, the same treatment showed 186.7% and 27.4 of % β-glucosidase activity more than the parental strain and its original mutant, respectively.



*D: Including the CMCase production of the parental strain (2.5 U/ml).
*H: Including the β-glucosidase production of the parental strain (6.0 U/ml).

Figure 2: A histogram representing classes of CMCase and β-glucosidase Production and the number of *T. harzianum* isolates after colchicine treatment.

For UV-induced mutants, the results showed that the best improvement in β-glucosidase efficiency reached 110% and 36.9% more than the original strain and its original mutant (15/4), respectively, after application with 0.2% colchicine. In addition, it was considered that, isolates which gave CMCase and β-glucosidase equal to or less than 5.2 and 14.0 U/ml, respectively, have low enzymes efficiency, while those which gave CMCase and β-glucosidase equal to or more than 5.8 and 15.5 U/ml, respectively, are highly enzymes producers.

The effects of the used mutagens on CMCase and β-glucosidase produced by T.harzianum NRRL 13879 strain were studied. Data in Table (3) showed the highest CMCase and β-glucosidase enzymes productivities which were more than the original strain and obtained after the applications of UV or EMS alone or followed by Colchicine treatments. Results summarized in Table (3) showed that the application of colchicine in concentration of 0.1% with EMS induced-mutants (125µl /ml for 30 min) exhibited the highest productivity of both enzymes, since it produced 160% CMCase and 186.7% β-glucosidase more than the original strain. While, colchicine treatment (0.2%) with the UV induced-mutant (15/4) produced 120% and 110% CMCase and β-glucosidase, respectively more than the parental strain. With regard to the individual effect of both mutagens EMS and UV light, it could be noticed that the EMS treatments exhibited efficiency in both enzymes more than the UV treatments.

To study the relationship between DNA contents with CMCase and β-glucosidase productivities, DNA in each of 26 isolates presented herein was isolated and the average DNA content of nucleus was measured. The results indicated that, in all cases, colchicine treated isolates proved to contain DNA amounts more than the original mutant isolates with ranges from two to five times as a result of formation of polyploidy (diploids and tetraploids). Also, most isolates which showed highly levels of CMCase and β-glucosidase productivities proved to contain higher quantities of DNA after colchicine application if compared with their original mutants. Mycelia derived from such a multinucleated conidia contain a larger number of nuclei

Treatments	No. of tested isolates	CMCase			β-glucosidase		
		CMCase (U/ml)	% from W.T.	% from UV or EMS induced mutant	β-glucosidase	% from W.T.	% from UV or EMS induced mutant
Control		2.5	100.0	------	6.0	100.0	------
W1	20	2.5-3.0	100.0-120.0	100.0-120.0	6.0-7.5	100.0-125.0	100.0-125.0
W2	20	2.5-3.2	100.0-128.0	100.0-128.0	6.0-7.8	100.0-130.0	100.0-130.0
L		4.3	172.0	100.0	9.5	158.3	100.0
L1	13	4.3-5.0	172.0-200.0	100.0-116.3	9.5-10.5	158.3-175.0	100.0-110.5
L2	23	4.3-5.2	172.0-208.0	100.0-120.9	9.5-11.5	158.3-191.7	100.0-121.0
P		4.3	172.0	100.0	9.2	153.3	100.0
P1	14	4.3-5.5	172.0-220.0	100.0-127.9	9.2-12.5	153.3-208.3	100.0-135.9
P2	20	4.3-5.5	172.0-220.0	100.0-127.9	9.2-12.6	153.3-210.0	100.0-136.9
E		4.5	180.0	100.0	9.5	158.3	100.0
E1	9	4.5-5.2	180.0-208.0	100.0-115.6	9.5-13.5	158.3-225.0	100.0-142.1
E2	4	4.5-4.8	180.0-192.0	100.0-106.7	9.5-12.5	158.3-208.3	100.0-131.6
R		4.5	180.0	100.0	13.0	216.7	100.0
R1	20	4.5-5.2	180.0-208.0	100.0-115.6	13.0-14.5	216.7-241.7	100.0-111.5
R2	20	4.5-5.2	180.0-208.0	100.0-115.6	13.0-14.5	216.7-241.7	100.0-111.5
D		5.0	200.00	100.0	13.5	225.0	100.0
D1	13	5.0-6.5	200.0-260.0	100.0-130.0	13.5-17.2	225.0-286.7	100.0-127.4
D2	15	5.0-5.8	200.0-232.0	100.0-116.0	13.5-16.8	225.0-280.0	100.0-124.4

Table 2: Evaluation of CMCase and β-glucosidase activities for *T. harzianum* mutants obtained after treatments with UV- or EMS followed by colchicine.

Enzymes / Treatment	CMCase and β-glucosidase productivities %(more than the W.T.)	
	CMCase	β-glucosidase
UV	80 (12 min.) 72 (15 min.)	58 (3 min.) 58 (9 min.)
EMS	100 (125 μg/ml / 30 min.)	125 (125 μg/ml / 30 min.)
UV + colchicines	120 (15 min / 0.1) 120 (15 min / 0.2)	110 (15 min. / 0.2)
EMS + colchicines	150 (125 μg/ml / 30 min./ 0.1)	187 (125 μg/ml / 30 min./ 0.1)

Table 3: The highest CMCase and β-glucosidase enzymes productivities more than the W.T. strain obtained after the applications of all treatments.

compared with that of the original strain. The nuclear diameter of the mycelia of multinucleated conidium does not increase, although the DNA content of the mycelia increases (Toyama et al., 2007). The multinucleate conidia were produced from the green mature conidia of *Trichoderma reesei* Rut C-30 strain by colchicine treatment. The strain with higher Filter paper degrading ability was selected among those conidia using a double layer selection medium (Toyama et al., 2008). Toyama and Toyama (2001), who treated EMS treated isolate of *T. reesei* (M14-2) with 0.1% colchicine. They found that the cellulase production and growth rate of new isolate (M14-2B) were increased. They also concluded that, M14-2B might be constructed using gene sources amplified by additional autopolyploidization from a low growing cellulase hyperproducer, M14-2.

It can be concluded that EMS-treatments followed by colchicine application were more effective in inducing superior isolates such as D1/4 which showed the highest DNA content, as well as, the highest amounts of CMCase and β-glucosidase at all.

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