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# IMPROVEMENT OF β-GLUCOSIDASE PRODUCTION IN *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 and protoplast fusion techniques were 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. These isolates were tested for their CMCase and β-glucosidase productivities in comparison with the original strain. Five isolates (tow after UV application and three after EMS treatments) were selected on the basis of their highly productivity of both enzymes to be treated with colchicine (0.1% and 0.2%) as a second step of mutation induction. 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. Twenty isolates were selected to be tested for their resistance or sensitivity against four antifungal agents. Out of them four isolates were selected on the basis of their response for antifungal agents and their productivities of two enzymes to be introduced into intraspecific protoplast fusion experiments using two different methods ( PEG and electrofusion) . Three crosses were carried out among four selected isolates. The results showed that the numbers of fusants obtained after electrofusion were more than those obtained after PEG method. In addition, highly productivities of CMCase and β- glucosidase were obtained after electrofusion in three crosses. Highest DNA content and also highest amounts of CMCase and β- glucosidase were obtained after EMS-treatments followed by colchicine application

## 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). 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).

Beta-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate–limiting factor during enzymatic

hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose. 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). Neverthelese, 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).

Fungal protoplasts are an important tool in physiological and genetic research, as well as genetic manipulation can successfully be achieved through fusion of protoplasts in filamentous fungi that lack the capacity for sexual reproduction. (Lalithakumari, 2000).

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.

## MATERIALS AND METHODS

**A. MATERIALS:**

**A.1. Strain:**

*Trichoderma harzianum* NRRL 13879 strain was used in the present study, it was provided by United States Department of Agriculture, Agriculture Research Service, National Centre for Agriculture Utilization Research, USA.

**A.2. Media:**

A.2.a. Complete medium (CM): (Strauss and Kubicek 1990)
It was used for maintaining and routine culturing .

A.2.b. Minimal medium (MM): (Penttilä *et al*., 1987)
It was used for isolation of auxotrophic mutants.

A.2.c. Fermentation medium (FM): (Haapala *et al*., 1995)
It was used for testing CMCase and β-glucosidase productivities.

A.2.d. Natick medium: (Mandels and Sternberg 1976)
It was used for colchicine treatments.

A.2.e. Protoplasting medium (PM): (Kumari and Panda, 1994)
It was used for the induction of protoplasts in *T. harzianum*.

A.2.f. Hypertonic, selective and regeneration medium: (Kumari and Panda, 1994) It was used for induction of protoplasts.

**A.3. Mutagens:**

A.3.a. Physical mutagen (Ultra – Violet light, UV):

Philips T UV-30 W, WL 254 nm, lamp type no. 57413 was used for radiation treatments.

A.3.b. Chemical mutagen (Ethyl methane sulfonate, EMS)

A.3.c. Colchicine treatments

Two colchicine treatments with two different concentrations were used with *T. harzianum* .

**A.4. Reagents, buffers and solutions :-**

A.4.a. Dinitrosalicylic acid assay for reducing sugar (Miller, 1959)

A.4.b. Buffers of mutagens: (Gomori, 1955)

A.4.c. Saline solution: (0.85 % NaCl) was used in UV treatments.

A.4.d. Fermentation buffer: (Gomori, 1955)

A.4.e. Protoplast preparation and fusion buffers :( Gomori, 1955)

A.4.f. Buffer of DNA isolation: (Al-Samarrai and Schmid 2000).

**A.6. Electroporation:**

Bio –Rad CO., (USA) was used for electroporation process.

**B. METHODS.**

B.1. Mutagenic treatments.

B.1.a. UV- light mutagenicity.

B.1.b. EMS mutagenicity.

B.1.c. UV- light followed by colchicine treatments.

B.1.d. EMS followed by colchicine treatments.

**B.2. Nuclear staining:-**

A piece (2 mm × 2mm) was cut from each mycelial mat**,** stained with Giemsa stain 10 % in phosphate buffer (pH 6.8) for at least 30 min and then photographed.

**B.3. Auxanographic analysis (Lederberg 1950).**

**B.4. Fermentation and determination of CMCase and β-glucosidase activities.**

B.4.a. Fermentation procedure:

Conical 250 ml flasks, each containing 50 ml of fermentation media were inoculated with five ml of the spore suspension from eight days old slants. Flasks were incubated with shaking (200 rpm) at 28 $^{o}$C for 10 days.

B.4.b.Determinations of Carboxymethyle cellulase (CMCase) and β-glucosidase (Vaheri *et al.,* 1979).

**B.5. Protoplast fusion:**

**B.5.a. Isolation of antifungal resistant mutants:**

For the isolation antifungal resistant mutants, protoplast medium (PM) and antifungal agents were used separately, concentration of antifungal agents was added as follows:

Benomyle (0.5 and 1.0 µg/ml); miconzole (10 and 25 µg/ml) cycloheximide (75 and 100 µg/ml) and griseofulvin (250 µg/ml). A part of the mycelium of each isolate was inoculated on the surface of the antifungal medium plates, the plates were incubated at 28 $^{o}$C for six days. Colonies, which exhibited resistance or sensitivity to a specific antifungal were retested on the same antifungal dose to be sure of their stability **c**oncerning resistance or sensitivity .

**B.5.b. Electroporation process (Żukowska *et al*., 2004).**

**B.5.c. Isolation of fusants:**

Through the present study, PEG or electroporation methods, treated protoplast suspensions were plated onto an antifungal selective medium, which was supplemented with one of the antifungal agents, the plates were incubated at 28 $^{o}$C until the colonies were growen on plates surface which considered as complementary fusants. They were transplanted and subcultured several times onto selective medium before further studies.

**B.6. Isolation of total DNA from *Trichoderma* strain and their isolates (Al-Samarrai and Schmid 2000)**

## RESULTS AND DISCUSSION

**A. Induction of genetic variations:**

Induction of genetic variabilities was carried out using ultraviolet irradiation (UV) or Ethyl methan sulfonate (EMS), each of them was followed by colchicines in another experiment.

**A.1. Ultraviolet light mutagenicity (UV):**

Induction of mutations was carried out as a major tool for the induction of a wide range of genetic variations. In the present study,UV irradiation was carried out in different exposure times, i.e., 3 , 6, 9, 12 and 15 min with stirring at a distance of 20 cm 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. Total percentage of the mutants, from application of all UV doses was 24.24%, and all were morphological variants.In addition,results showed that no auxotrophic mutants were obtained after application of UV light.

**A.2. Ethyl methane sulfonate treatments ( EMS) :**

Fungal spore suspension of *T. harzianum* strain 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. Treatment with 75µ/ml for 30 min.did not appear any colonies because of the fast growth of the fungus on the medium. Complete lethalty were observed after applied the heighst EMS concentration for 60 min.

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

All selected mutants after every mutagenic treatment were tested for their CMCase and β. glucosidase activities. Tables (1 and 2) 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 Tables (1 and 2) 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 in Tables1 and 2, 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 2.5U/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 , respctively, more than the original strain ( classes E , F and G in Table 1 ), 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 in Table2 ) and 77 isolates (16.70 %) exhibited β-glucosidase at least 33.3 % more than the parental strain (Class J in Table 2 ).

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 (3.68%) 17 isolates lost completely their abilities to produce any CMCase or β-glucosidase (classe A in Tables 1 and 2).

Data in Table (3) showed a wide range of CMCase and β-glucosidase productivities of 99 tested isolates obtained after exposure to different UV doses in comparsion with the original strain, *T. harzianum* NRRL13879, which was considered as 100% CMCase and β-glucosidase producer.These results showed that some isolates lost completely their ability to show any CMCase and β-glucosidase activity ,while some other isolates showed activity less than the wild type strain in both enzymes . In addition, some isolates exhibited higher CMCase and β-glucosidase productivities more than their parental strain, since the best UV-induced mutants for the production of β-glucosidase were obtained after three and nine min exposure time, while the best activity of CMCase was appeared after 12 min. exposure time.

T1-2

**Table 3. Evaluate the efficiency of *T.harzianum* NRRL13879 UV-induced isolates for CMCase and β-glucosidase productivities.**

| UV exposure time (min) | No. of tested isolates | Ranges of CMCase and β-glucosidase (U/ml) | | | |
|---|---|---|---|---|---|
| | | CMCase | % from W.T. | β-glucosidase | % from W.T. |
| **Control** | **---** | **2.5** | **100.0** | **6** | **100.0** |
| 3 | 21 | 0.3-3.5 | 12-140 | 0.5-9.5 | 8.3-158.3 |
| 6 | 24 | 0.0-3.2 | 0.0-128 | 0.2-7.5 | 3.3-125.0 |
| 9 | 21 | 0.0-4.3 | 0.0-172 | 0.5-9.5 | 8.3-158.3 |
| 12 | 24 | 0.0-4.5 | 0.0-180 | 0.0-8.0 | 0.0-133.3 |
| 15 | 9 | 0.2-4.3 | 8.0-172 | 0.2-9.2 | 3.3-153.3 |

Mutagenic effect of EMS concentrations at 30 and 60 min on *T. harzianum* NRRL 13879 was appeared in Table (4). Results revealed that the high concentration of EMS (125 µ/ml for 30 min ) gave the best improvement of both enzymes which reached to 100 % and 125 % CMCase and β-glucosidase more than the untreated parental strain, respectively. Data showed also that some isolates lost some of their CMCase and β-glucosidase efficiency, after treating the original strain with 75 and 100 µl/ml EMS concentrations for 60 and 30 min, respectively. On the other hand, many of the rest isolates failed completely to show any productivity of both enzymes.

**Table 4. Evaluate the efficiency of *T.harzianum* NRRL 13879 EMS induced mutants for CMCase and β-glucosidase productivities.**

| EMS Conc. (µl/ml) / time (min) | No. tested isolates | CMCase | | β-glucosidase | |
|---|---|---|---|---|---|
| | | CMCase (U/ml) | % from W.T. | β-glucosidase (U/ml) | % from W.T. |
| **Control** | **---** | **2.5** | **100.0** | **6** | **100.0** |
| 50/30 | 29 | 0.0-4.5 | 0.0-180 | 0.0-9.5 | 0.0-158.3 |
| 50/60 | 26 | 0.0-4.0 | 0.0-160 | 0.0-8.0 | 0.0-133.3 |
| 75/60 | 37 | 2.0-4.2 | 80-168 | 3.5-8.5 | 58.3-141.7 |
| 100/30 | 66 | 0.2-4.5 | 8-184 | 0.5-13.0 | 8.3-216.7 |
| 100/60 | 73 | 0.0-3.5 | 0.0-140 | 0.0-8.5 | 0.0-141.7 |
| 125/30 | 49 | 0.0-5.0 | 0.0-200 | 0.0-13.5 | 0.0-225 |
| 125/60 | 50 | 0.0-4.8 | 0.0-192 | 0.0-13.0 | 0.0-216.7 |
| 150/30 | 32 | 0.0-2.8 | 0.0-112 | 0.0-10.5 | 0.0-175.0 |

Five isolates were selected on the basis of their higher CMCase and β-glucosidase productivities as shown in Table (5). 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, respectively, as well as 58.3 %, 116.7 % and 125 % β-glucosidase activity, more than the original strain.

**Table 5. The highest CMCase and β-glucosidase producer strains obtained after UV and EMS treatments.**

| 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 |

Ultraviolet irradiation and EMS, as a tool for induction of genetic variations was successfully applied by many investigators.The obtained results in this study were in agreement with those obtained by Nadalini *et al*., (1999) , Hao *et al*., (2006) and Adusl *et al*., (2007) They isolated different mutants of different *Trichoderma* species using UV-irradiation.In addition, Kotchoni and Shonukan (2002) isolated forty mutants after treating *Trichoderma* with EMS, some of them, exhibited a maximum of 10-fold improvement of cellulase production.

**C. Colchicine treatments:**

Five isolates mentioned in Table 5 proved to give the highest CMCase and β-glucosidase activities 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 .

**C.1. Carboxymethyle cellulase (CMCase) and β-glucosidase activities of *T. harzianum* isolates after colchicine treatments**:

The grown colonies after colchicine treatments were tested for their CMCase and β-glucosidase activities.Tables (6 and 7) present the CMCase and β-glucosidase productivities of 191isolates following colchicine treatments.

**Table 6. Distribution and ranges of CMCase productivity (U/ml) in *T.harzianum* isolates after colchicine treatments.**

| Isolates class | CMCase range (U/ml) | Obtained isolates | | W.T. with colchicine | | Colchicine after UV treatments | | | | Colchicine after EMS treatments | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | W1 | W2 | L1 | L2 | P1 | P2 | E1 | E2 | R1 | R2 | D1 | D2 |
| A | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B | 0.1-0.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 1.0-1.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D* | 2.0-2.9 | 30 | 15.70 | 17 | 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E | 3.0-3.9 | 10 | 5.23 | 3 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F | 4.0-4.9 | 80 | 41.88 | 0 | 0 | 12 | 17 | 7 | 16 | 3 | 4 | 8 | 13 | 0 | 0 |
| G | 5.0-5.9 | 68 | 35.60 | 0 | 0 | 1 | 6 | 7 | 4 | 6 | 0 | 12 | 7 | 10 | 15 |
| H | 6.0-6.9 | 3 | 1.57 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| I | 7.0-7.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| J | ≥8.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | | 191 | 100 | 20 | 20 | 13 | 23 | 14 | 20 | 9 | 4 | 20 | 20 | 13 | 15 |
| | | | | 40 | | 70 | | | | 81 | | | | | |

**{W.T. / 0.1 → W1} {9/8 / 0.1 → L1} {15/4 / 0.1 → P1}**
**{50/30/17 / 0.1 → E1}{100/30/44n / 0.1 → R1} {125/30/12 /0.1 → D1}**
**{W.T. / 0.2 → W2} {9/8 / 0.2 → L2} {15/4 / 0.2 → P2}{50/30/17 / 0.2 → E2}**
**{100/30/44n / 0.2 → R2} {125/30/12 /0.2 → D2}**

**Table 7. Distribution and ranges of β-glucosidase productivity (U/ml) in *T.harzianum* isolates after colchicine treatments.**

| Isolates class | β-glucosidase range (U/ml) | Obtained isolates | | W.T. with colchicine | | Colchicine after UV treatments | | | | Colchicine after EMS treatments | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | W1 | W2 | L1 | L2 | P1 | P2 | E1 | E2 | R1 | R2 | D1 | D2 |
| A | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B | 0.1-0.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 1.0-1.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D | 2.0-2.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E | 3.0-3.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F | 4.0-4.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| G | 5.0-5.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| H* | 6.0-6.9 | 30 | 15.70 | 17 | 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| I | 7.0-7.9 | 10 | 5.23 | 3 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| J | ≥8.0 | 151 | 79.05 | 0 | 0 | 13 | 23 | 14 | 20 | 9 | 4 | 20 | 20 | 13 | 15 |
| Total | | 191 | 100 | 20 | 20 | 13 | 23 | 14 | 20 | 9 | 4 | 20 | 20 | 13 | 15 |
| | | | | 40 | | 70 | | | | 81 | | | | | |

**{W.T. / 0.1 ⟶ W1} {9/8 / 0.1 ⟶ L1} {15/4 / 0.1 ⟶ P1}**
**{50/30/17 / 0.1 ⟶ E1}{100/30/44n / 0.1 ⟶ R1} {125/30/12 /0.1 ⟶ D1}**
**{W.T. / 0.2 ⟶ W2}{9/8 / 0.2 ⟶ L2}{15/4 / 0.2 ⟶ P2}{50/30/17 / 0.2 ⟶ E2}**
**{100/30/44n / 0.2 ⟶ R2} {125/30/12 /0.2 ⟶ D2}**

Results in Tables (6 and 7) showed that after all colchicine treatments, none of the obtained isolates lost its 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 Tables 6 and 7 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 in Tables 6 and 7).

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) as shown in Table (6). 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.

Moreover, a wide range of CMCase and β-glucosidase productivities were obtained after colchicine treatments for the original strain and also UV and EMS mutants as shown in Table (8).

Regarding to the best productivities of CMCase, the results showed that , the high concentration of EMS (125 µl/ml) for 30 min followed by colchicines 0.1 % (D1) gave the highest productivity, 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.

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.

From the above results,it could be noticed that after all treatments the application of colchicine of the concentration of 0.1 % EMS induced-mutants (125µl/ml for 30 min ),D1 exhibited the highest productivity of both enzymes , since it produced 160 % CMCase and 186.7 % β-glucosidase more than the original strain. 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.

**Table 8. Evaluation of CMCase and β-glucosidase activities for *T.harzianum* mutants obtained after treatments with UV- or EMS followed by colchicine.**

| 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 |

Results summarized in Table (9) showed that, 12 out 20 isolates of them were considered as low enzymes producers and eight as highly producers were selected for protoplast fusion experiments.

Two isolates (W1/9 and W2/9) were obtained after treating the original strain with the concentration of 0.1 % and 0.2 % colchicine solution, respectively. Three isolates (L1/1, L1/9 and L1/15) were obtained after treating L (9/8) mutant with 0.1 % colchicines, while, two isolates (L2/11 and L2/16) were obtained after treating the same UV-induced mutant with 0.2 % colchicine. In addition, two isolates (P1/9 and P2/9) were obtained after

treating the P (15/4) mutant with 0.1 % and 0.2 % colchicine, respectively. Moreover, two isolates ( E1/9 and E2/3 ) were obtained after treating the E (50/30/17) mutant with 0.1 % and 0.2 % colchicines, respectively. Only one isolate (R2/10) was obtained after treating the mutant R (100/30/44 n) with 0.2 % colchicine.

On the other hand, data in Table (9) also showed that eight isolates (D1/1, D1/4, D1/8, D1/14, D2/1, D2/2, D2/4 and D2/11 ) were considered as highly efficient isolates. The frist four isolates were obtained after treatment the mutant D (125/30/12) with 0.1 % colchicine, while the rest four isolates were obtained after treated the same EMS mutant with 0.2 % colchicine.

**Table 9. CMCase and β-glucosidase productivities after colchicine treatments.**

| Parents and isolates | CMCase | % from W.T. | % from the original mutant | β-glucosidase | % from W.T. | % from the original mutant |
|---|---|---|---|---|---|---|
| **W.T.** | **2.5** | **100.0** | **100.0** | **6.0** | **100.0** | **100.0** |
| W1/9 | 3.0 | 120.0 | 120.0 | 7.5 | 125.0 | 125.0 |
| W2/9 | 3.2 | 128.0 | 128.0 | 7.8 | 130.0 | 130.0 |
| L | 4.3 | 172.0 | 100.0 | 9.5 | 158.3 | 100.0 |
| L1/1 | 4.3 | 172.0 | 100.0 | 10.0 | 166.7 | 105.3 |
| L1/9 | 4.3 | 172.0 | 100.0 | 10.5 | 175.0 | 110.5 |
| L1/15 | 5.0 | 200.0 | 116.3 | 10.5 | 175.0 | 110.5 |
| L2/11 | 5.0 | 200.0 | 116.3 | 11.5 | 191.7 | 121.0 |
| L2/16 | 5.2 | 208.0 | 120.9 | 11.5 | 191.7 | 121.0 |
| P | 4.3 | 172.0 | 100.0 | 9.2 | 153.3 | 100.0 |
| P1/9 | 4.3 | 172.0 | 100.0 | 10.5 | 175.0 | 114.1 |
| P2/9 | 5.0 | 200.0 | 116.3 | 11.0 | 183.3 | 119.6 |
| E | 4.5 | 180.0 | 111.1 | 9.5 | 158.3 | 100.0 |
| E1/9 | 5.0 | 200.0 | 116.3 | 10.0 | 165.7 | 105.2 |
| E2/3 | 4.7 | 188.0 | 104.4 | 10.0 | 166.7 | 105.2 |
| R | 4.5 | 180.0 | 100.0 | 13.0 | 216.7 | 100.0 |
| R2/10 | 4.8 | 192.0 | 106.7 | 14.0 | 233.3 | 107.7 |
| D | 5.0 | 200 | 100 | 13.5 | 225.0 | 100.0 |
| D1/1 | 6.2 | 248.0 | 124.0 | 15.5 | 258.3 | 114.8 |
| D1/4 | 6.5 | 260.0 | 130.0 | 17.2 | 286.7 | 127.4 |
| D1/8 | 5.8 | 232.0 | 116.0 | 17.0 | 283.3 | 125.9 |
| D1/14 | 6.0 | 240.0 | 120.0 | 17.2 | 286.7 | 127.4 |
| D2/1 | 5.8 | 232.0 | 116.0 | 16.5 | 275.0 | 122.2 |
| D2/2 | 5.8 | 232.0 | 116.0 | 16.5 | 275.0 | 122.2 |
| D2/4 | 5.8 | 232.0 | 116.0 | 16.8 | 280.0 | 124.4 |
| D2/11 | 5.8 | 232.0 | 116.0 | 16.5 | 275.0 | 122.2 |

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 (not shown) 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.

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.

The obtained results are in agreement with those obtained by **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.

**D. Protoplast fusion**

Protoplast fusion was the main subject to be evaluated in this study as a tool for inducing genetic recombinants especially in those fungi like, *T.harzianum*, where the sexual cycle is unknown, in order to isolate higher CMCase and β-glucosidase producing recombinants. However, the use of this technique requires labeling the parental strains before protoplasting and fusion. Some of the highest and lowest CMCase and β-glucosidase producer isolates and the original strain were used for intraspecific protoplast fusion through this study.

On the basis of the CMCase and β-glucosidase activities shown in Table (9) and resistance or sensitivity to one or more of four antifungal agents (Benomyle, Miconzole, Cycloheximide and Griseofulvin),only four mutants (L1/15, E2/3, D1/4, and D1/14 ) were selected to be used in the intraspecific protoplast fusion .Three intraspecific crosses were done using two different methods for each classical (PEG) and electropration methods.

**D.1. Intraspecific crosses**

**Cross 1**

This cross was carried out between two low CMCase and β-glucosidase producer isolates (L1/15 and E2/3) as shown in Table (10) with the application of two fusion methods. Eleven and 15 recombinants were obtained from this cross on the basis of resistance or sensitivity to both antifungal agents; miconzole and cycloheximide, and named from F1/1 to F1/11 (PEG method) and F4/1 to F4/15 (electoporation method).

The CMCase productivities of the parental isolates (E2/3 and L1/15) were 4.7 and 5.0 U/ml respectively,while their productivities of β-glucosidase were 10.0 and10.5 u/ml respectively. The highest productivity of both enzymes among 11 fusants obtained from PEG method were recorded by fusants no. F1/6 and F1/10, while they gave 20% and 14% of CMCase and β-glucosidase more than the higher parent (L1/15), respectively.

While, when CMCase and β-glucosidase efficiency were determined for the 15 fusants obtained from electroporation method , ten fusants (F4/1, F4/2, F4/3, F4/4, F4/7, F4/8, F4/9, F4/13, F4/14 and F4/15) out of them, exhibited higher productivity of both enzymes if compared with the parental isolates. CMCase productivity of these fusants ranged between 20 % produced by fusants (F4/1, F4/4, F4/7, F4/8, and F4/9 ) to 30 % produced by fusants (F4/2, F4/3, F4/13, F4/14 and F4/15) more than the higher parent (L1/15). While, fusants (F4/4, F4/7, F4/8, F4/9, F4/13, F4/14) produced 14.2 % and 19

% by fusants (F4/1, F4/2, F4/3 and F4/15) β-glucosidase more than the higher parent.

**Table 10. CMCase and β-glucosidase productivities for the intraspecific fusants resulted from cross1**

| PEG | | | | | Electroporation | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CMCase | | β-glucosidase | | | CMCase | | β-glucosidase | |
| Parents and fusants | U/ml | % from the higher parent | U/ml | % from the higher parent | Parents and fusants | U/ml | % from the higher parent | U/ml | % from the higher parent |
| **W.T.** | **2.5** | **50.0** | **6.0** | **75.14** | **W.T.** | **2.5** | **50.0** | **6.0** | **57.14** |
| E2/3 | 4.7 | 94.0 | 10.0 | 95.23 | E2/3 | 4.7 | 94.00 | 10.0 | 95.23 |
| L1/15 | 5.0 | 100.0 | 10.5 | 100.0 | L1/15 | 5.0 | 100.0 | 10.5 | 100.0 |
| F1/1 | 5.0 | 100.0 | 10.5 | 100.0 | F4/1 | 6.0 | 120.0 | 12.5 | 119.04 |
| F1/2 | 5.0 | 100.0 | 10.5 | 100.0 | F4/2 | 6.5 | 130.0 | 12.5 | 119.04 |
| F1/3 | 4.9 | 98.0 | 11.0 | 104.66 | F4/3 | 6.5 | 130.0 | 12.5 | 119.04 |
| F1/4 | 5.5 | 110.0 | 11.5 | 109.52 | F4/4 | 6.0 | 120.0 | 12.0 | 114.28 |
| F1/5 | 3.7 | 74.0 | 10.0 | 95.23 | F4/5 | 4.7 | 94.0 | 10.0 | 95.23 |
| F1/6 | 6.0 | 120.0 | 12.0 | 114.28 | F4/6 | 4.7 | 94.0 | 10.0 | 95.23 |
| F1/7 | 4.7 | 49.0 | 10.0 | 95.23 | F4/7 | 6.0 | 120.0 | 12.0 | 114.28 |
| F1/8 | 4.9 | 98.0 | 11.0 | 104.66 | F4/8 | 6.0 | 120.0 | 12.0 | 114.28 |
| F1/9 | 5.0 | 100.0 | 10.5 | 100.00 | F4/9 | 6.0 | 120.0 | 12.0 | 114.28 |
| F1/10 | 6.0 | 120.0 | 12.0 | 114.28 | F4/10 | 4.7 | 94.0 | 10.0 | 95.23 |
| F1/11 | 5.5 | 110.0 | 11.5 | 109.52 | F4/11 | 5.0 | 100.0 | 10.5 | 100.0 |
| | | | | | F4/12 | 4.3 | 86.0 | 9.5 | 90.47 |
| | | | | | F4/13 | 6.5 | 130.0 | 12.0 | 114.28 |
| | | | | | F4/14 | 6.5 | 130.0 | 12.0 | 114.28 |
| | | | | | F4/15 | 6.5 | 130.0 | 12.5 | 119.04 |

**Cross 2**

The second cross was achieved between highly efficient CMCase and β-glucosidase producer isolate ( D1/14 ) and the lower efficient one ( E2/3 ).

Results in Table (11) revealed that only 11 fusants (F2/1 to F2/11) were resulted from the PEG method in comparison with15 fusants (F5/1 to F5/15) were obtained through electroporation method .

Concerning the 11 recombinants obtained from PEG method, it was noticed that five fusants ; (F2/1, F2/5, F2/6, F2/10 and F2/11) showed the higher activity in CMCase and β-glucosidase production , since they gave 6.8, 6.8, 6.6, 6.8 and 6.6U/ml of CMCase, respectively, as well asproduced 18.5, 18.5, 18.0, 18.5 and 18.0 U/ml of β-glucosidase, respectively.

In addition, ten fusants (F5/2, F5/3, F5/4, F5/8, F5/9, F5/10, F5/11, F5/12, F5/14, and F5/15) out of 15 obtained from electroporation method showed increase in both CMCase and β-glucosidase than the higher parental isolate (D1/14). The CMCase values recorded by these fusants ranged between 13.3 % to 20 % more than the higher parent (D1/14). While the amounts of β-glucosidase ranged between 5.8 % for F5/11 and F5/12 fusants to 7.5 % for the rest eight isolates from the higher parent.

**Table 11. CMCase and β-glucosidase productivities for the intraspecific fusants resulted from cross 2**

| PEG | | | | | Electroporation | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Parents and fusants | CMCase | | β-glucosidase | | Parents and fusants | CMCase | | β-glucosidase | |
| | U/ml | % from the higher parent | U/ml | % from the higher parent | | U/ml | % from the higher parent | U/ml | % from the higher parent |
| **W.T.** | **2.5** | **41.66** | **6.0** | **34.88** | **W.T.** | **2.5** | **41.66** | **6.0** | **34.88** |
| E2/3 | 4.7 | 78.33 | 10.0 | 58.13 | E2/3 | 4.7 | 78.33 | 10.0 | 58.13 |
| D1/14 | 6.0 | 100.0 | 17.2 | 100.0 | D1/14 | 6.0 | 100.0 | 17.2 | 100.0 |
| F2/1 | 6.8 | 113.33 | 18.5 | 107.55 | F5/1 | 4.0 | 66.66 | 10.0 | 58.13 |
| F2/2 | 4.7 | 78.33 | 10.0 | 58.13 | F5/2 | 6.8 | 113.33 | 18.5 | 107.55 |
| F2/3 | 4.7 | 78.33 | 10.0 | 58.13 | F5/3 | 7.0 | 116.66 | 18.5 | 107.55 |
| F2/4 | 4.0 | 66.66 | 9.0 | 52.32 | F5/4 | 7.0 | 116.66 | 18.5 | 107.55 |
| F2/5 | 6.8 | 113.33 | 18.5 | 107.55 | F5/5 | 6.0 | 100.0 | 17.2 | 100.0 |
| F2/6 | 6.6 | 110.0 | 18.0 | 107.55 | F5/6 | 5.5 | 91.66 | 15.2 | 88.37 |
| F2/7 | 5.2 | 86.66 | 15.2 | 88.37 | F5/7 | 4.7 | 78.33 | 10.0 | 58.13 |
| F2/8 | 6.0 | 100.0 | 17.2 | 100.00 | F5/8 | 7.2 | 120.0 | 18.5 | 107.55 |
| F2/9 | 4.7 | 78.33 | 10.0 | 58.13 | F5/9 | 7.2 | 120.0 | 18.5 | 107.55 |
| F2/10 | 6.8 | 113.33 | 18.5 | 107.33 | F5/10 | 7.2 | 120.0 | 18.5 | 107.55 |
| F2/11 | 6.6 | 110.00 | 18.0 | 104.65 | F5/11 | 6.8 | 113.33 | 18.5 | 105.81 |
| | | | | | F5/12 | 6.8 | 113.33 | 18.5 | 105.81 |
| | | | | | F5/13 | 6.0 | 100.0 | 17.2 | 100.0 |
| | | | | | F5/14 | 6.8 | 113.33 | 18.5 | 107.55 |
| | | | | | F5/15 | 7.2 | 120.0 | 18.5 | 107.55 |

**Cross 3**

This cross was done between highly CMCase and β-glucosidase producer isolates (D1/14 and D1/4).Fourteen fusants were obtained from this cross using PEG method and 15 after application of electroporation method on the basis of antifungal test as shown in Table (12).

All tested fusants showed variable levels of CMCase and β-glucosidase activities. Out of the 14 tested fusants obtained, six (F3/4, F3/5, F3/8, F3/9, F3/10 and F3/14) proved to have higherproductivity of CMCase and β-glucosidase if compared with their parents. CMCase productivity of these fusants ranged from 7.5 U/ml (produced by the fusants F3/4, F3/9, F3/10 and F3/14) to 8.0 U/ml ( produced by the fusants F3/5 and F3/8). While, β-glucosidase productivity ofthese fusants ranged from 19.0 U/ml ( produced by the fusants F3/4, F3/5, F3/9, F3/10 and F3/14 ) to 19.5 U/ml (produced by the fusant F3/8 ).

In addition nine fusants out of the 15 which obtained through electroporation technique proved to have higher productivity of CMCase, out of them four fusants (F6/4, F6/9, F6/10 and F6/14) produced 33 % more than the higher parent (D1/4). While (F6/5, F6/6, F6/11, F6/13 and F6/15) exhibited about 42% CMCase more than D1/4. Furthermore, ten fusants (F6/2, F6/4, F6/5, F6/6, F6/9, F6/10, F6/11, F6/13, F6/14 and F6/15) proved to have high productivity of β–glucosidase, their products ranged from 4% to 16% more than both parents (D1/4 and D1/14), respectively.

**Table 12. CMCase and β-glucosidase productivities for the intraspecific fusants resulted from cross 3 .**

| PEG | | | | | Electroporation | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Parents and fusants | CMCase | | β-glucosidase | | Parents and fusants | CMCase | | β-glucosidase | |
| | U/ml | % from the higher parent | U/ml | % from the higher parent | | U/ml | % from the higher parent | U/ml | % from the higher parent |
| **W.T.** | **2.5** | **41.66** | **6.0** | **34.88** | **W.T.** | **2.5** | **38.46** | **6.0** | **34.88** |
| D1/14 | 6.0 | 92.30 | 17.2 | 100.0 | D1/14 | 6.0 | 92.30 | 17.2 | 100.0 |
| D1/4 | 6.5 | 100.0 | 17.2 | 100.0 | D1/4 | 6.5 | 100.0 | 17.2 | 100.0 |
| F3/1 | 6.0 | 92.30 | 17.2 | 100.0 | F6/1 | 6.5 | 100.0 | 17.2 | 100.0 |
| F3/2 | 6.5 | 100.0 | 17.2 | 100.0 | F6/2 | 6.5 | 100.0 | 18.0 | 104.65 |
| F3/3 | 5.3 | 81.53 | 15.2 | 88.37 | F6/3 | 6.3 | 96.92 | 17.2 | 100.0 |
| F3/4 | 7.5 | 115.38 | 19.0 | 110.46 | F6/4 | 8.0 | 133.33 | 19.5 | 113.37 |
| F3/5 | 8.0 | 123.07 | 19.0 | 110.46 | F6/5 | 8.5 | 141.66 | 20.0 | 116.27 |
| F3/6 | 6.2 | 95.38 | 17.2 | 100.0 | F6/6 | 8.5 | 141.66 | 20.0 | 116.27 |
| F3/7 | 6.0 | 92.30 | 17.2 | 100.0 | F6/7 | 6.0 | 92.30 | 17.2 | 100.0 |
| F3/8 | 8.0 | 123.07 | 19.5 | 113.37 | F6/8 | 6.0 | 100.0 | 17.2 | 100.0 |
| F3/9 | 7.5 | 115.38 | 19.0 | 110.46 | F6/9 | 8.0 | 133.33 | 19.5 | 113.34 |
| F3/10 | 7.5 | 115.38 | 19.0 | 110.46 | F6/10 | 8.0 | 133.33 | 19.5 | 113.34 |
| F3/11 | 6.0 | 92.30 | 17.2 | 100.0 | F6/11 | 8.5 | 141.66 | 20.0 | 116.27 |
| F3/12 | 6.5 | 100.0 | 17.2 | 100.0 | F6/12 | 5.4 | 90.0 | 15.3 | 88.95 |
| F3/13 | 6.5 | 100.0 | 17.2 | 100.0 | F6/13 | 8.5 | 141.66 | 20.0 | 116.27 |
| F3/14 | 7.5 | 115.38 | 19.0 | 110.46 | F6/14 | 8.0 | 133.33 | 19.5 | 113.34 |
| | | | | | F6/15 | 8.5 | 141.66 | 20.0 | 116.27 |

Comparing both methods of protoplast fusion used in this study (PEG and electroporation), the obtained results clearly showed that the number of fusants obtained after application of electrofusion were more than those obtained after application of PEG method.

On the other hand, higher productivity of CMCase and β-glucosidase was recorded after electrofusion compared to the PEG method in the three crosses carried out through this study.

Regarding the first cross (Table 10) carried out between two low producer isolates (L1/15 and E2/3), ten fusants obtained after electrofusion method produced from 20 % to 30 % CMCase more than the higher parent (L1/15)). Also, they produced from 14 % to 19 % β-glucosidase more than the higher parent. On the other hand, four fusants (F1/4, F1/6, F1/10 and, F1/11) obtained after PEG method produced from 10 % to 20 % CMCase, while other five fusants ( F1/3, F1/4, F1/6, F1/8, F1/10 and F1/11 ) produced β-glucosidase ranged between 40.66 % and 14.28 % more than the higher parent (L1/15) as shown in the same Table.

For the second carried out between the lower producer isolate (E2/3) and the higher producer one (D1/14); results in Tabel 11 showed that ten fusants obtained after electrofusion method showed higher productivtity of the two enzymes. The CMCase productivity of these fusants was ranged between 13.3 % and 20 % from the higher parent (D1/14). While, β-glucosidase productivities was ranged between 5.8 % to 7.5 % from the higher parent. On the other hand, five fusants (F2/1, F2/5, F2/6, F2/10 and F2/11) obtained after PEG method showed from 10 % to 13.3 % CMCase

productivity more than the higher parent (D1/14) , as well as, produced from 4.6 % to 7.3 % β-glucosidase more than the higher parent (Table 11).
In the case of the third cross between the two highly producer isolates (D1/4 and D/14), nine fusants obtained from electroporation method ( F6/4, F6/5, F6/6, F6/9, F6/10, F6/11, F6/13, F6/14, and F6/15 ) recorded 33.3 % to 41.6 % CMCase more than the higher parent (D1/4 ) and ten fusants ( F6/2, F6/4, F6/5, F6/6, F6/9, F6/10, F6/11, F6/13, F6/14, and F6/15 ) showed from 4.6 % to 16.2 % β-glucosidase more than both parents. On the other hand, six fusants (F3/4, F3/5, F3/8, F3/9, F3/10 and F3/14) produced 15.3 % to 23 % CMCase more than the higher parent (D1/4) , as well as, showed from 10.4 % to 13.3 % β-glucosidase productivity more than the two parents. These results were in agreement with those obtained by,Prabavathy *et al*. (2006) and EL-Bondkly and Talkhan (2007)

In conclusion, there are two main advantages for electroporation method over the traditional PEG method. The first one is simplicity and the second advantage, this method is more reproducible than the classical method (PEG).The improvement of microbial strains was conducted in many research centers and most commonly involve the introduction of additional genes into the cell genome, or an increase in the number of existing genes. The applied protoplast electrofusion method proved to be a good and effective method for obtaining a *Trichoderma harzianum* fusants with higher productivity of β-glucosidase enzyme.

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# ...تحسين إنتاجية إنزيم بيتا جلوكوسيديز في فطر التريكودريرما هيرزينيوم باستخدام تقنيات التكنولوجيا الحيوية الميكروبية

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تهدف هذه الدراسة الى الحصول على سلالات من فطر *Trichoderma harzianum* ذات كفاءة انتاجيه عالية من إنزيم بيتا جلوكوسيديز عن طريق استحداث تباينات وراثية باستخدام المطفرات الكيماوية أو الطبيعية وكذلك الحصول على تراكيب وراثية جديدة عن طريق الدمج الخلوي باستخدام طريقتي PEG و electroporation والمقارنة بينهما. وقد أظهرت النتائج انة تم الحصول على ٤٦١ عزلة منها ٩٩ عزلة بعد المعاملة بالأشعة فوق البنفسجية و ٣٦٢ عزلة بعد المعاملة بالمطفر الكيماوي (EMS) . تم اختبار هذه العزلات بالنسبة لإنتاجيتها من انزيمى CMCase و β- glucosidase ومقارنتها بإنتاجيه السلالة الابويه. أوضحت النتائج أن هناك اختلافات كبيرة من حيث إنتاجيه هذه العزلات.تم انتخاب خمس عزلات منها للمعاملة بالكولشيسين على اساس انتاجيتهما العالية من كلا الأنزيمين.أثنان (L9/8 andP15/4) بعد المعاملة بالاشعة فوق البنفسجية وثلاث (L50/30/17,R100/30/44 and D125/30/12) بعد المعاملة بالمطفر الكيماوى (EMS) .نتيجة المعاملة بالكولشيسين تم الحصول على ١٩١ عزلة منها ٤٠بعد معاملة السلالة الأبوية و٧٠بعد معاملة العزلات الناتجة من الأشعة فوق البنفسجية و٨١ بعد معاملة العزلات الناتجة من المعاملة بالمطفر الكيماوى EMS .تم اختبار جميع العزلات الناتجة من المعاملة بالكولشيسين بالنسبة لانتاجيتها من كلا الأنزيمين وكانت هناك تباينات عالية فى انتاجيتها وكانت اعلى انتاجية للعزلة (D1/4) حيث وصلت الى ١٦٠و١٨٦% اعلى من السلالة الابوية فى كلا الأنزيمين .تم اختيار ٢٠ عزلة من هذة العزلات منهم ١٢ منخفضة الأنتاجية وثمانية ذات كفاءة عالية لمعرفة استجابتهما لاربع مضادات فطرية لايجاد دليل وراثى فى تجارب الدمج الخلوى. وبناء على ذلك تم اختيارأربعة عزلات أثنان منها منخفضة الأنتاجية (E2/3and L1/15) وعزلتين ذات انتاجية عالية (D1/4 and D1/14) لإجراء تجارب الدمج الخلوي. من خلال طريقة PEG وelectroporation حيث تضمنت كل طريقة نفس التهجينات (ثلاث تهجينات مختلفة منخفض × منخفض- منخفض × مرتفع – مرتفع × مرتفع ) وعند المقارنة بين كل من طريقتي الدمج الخلوي أوضحت النتائج إن عدد المندمجات الخلوية التي تم الحصول عليها من طريقه الدمج الكهربي اكبر من تلك التي تم الحصول عليها بطريقه الدمج الخلوي العادي (PEG) حيث تم الحصول على ١١، ١١، ١٤ مندمجة خلوية عند إجراء الثلاث تهجينات بالطريقة العادية بينما تم الحصول على ١٥ مندمجة خلوية لكل تهجين من التهجينات السابقة بطريقه الدفع الكهربي .كذلك تم الحصول على الانتاجيه العالية من كلا الإنزيمين CMCase وβ- glucosidase بطريقه الدفع الكهربي مقارنه بالطريقة العادية. في محاوله لدراسة العلاقة بين محتوى DNA وإنتاجيه كلا الإنزيمين أمكن استنتاج أن المعاملة بالكولشيسين بعد المطفر الكيماوي كانت أكثر كفاءة حيث أن العزلة D1/4 أظهرت محتوى أعلى من DNA وكذلك إنتاجيه عالية من CMCase و β-glucosidase

**Table 1. Distribution and ranges of CMCase activity (U/ml) in *T.harzianum* isolates after mutagenic treatments .**

| Isolates class | CMCase range (U/ml) | Obtained isolates | | UV exposure time (min) | | | | | EMS ( µl / ml ) / time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | 3 | 6 | 9 | 12 | 15 | 50/30 | 50/60 | 75/60 | 100/30 | 100/60 | 125/30 | 125/60 | 150/30 |
| **A** | 0.0 | 21 | 4..55 | 0 | 1 | 1 | 2 | 0 | 4 | 2 | 0 | 0 | 6 | 1 | 2 | 2 |
| **B** | 0.1-0.9 | 30 | 6.50 | 5 | 1 | 2 | 5 | 1 | 2 | 1 | 0 | 4 | 3 | 2 | 0 | 4 |
| **C** | 1.0-1.9 | 33 | 7.15 | 0 | 1 | 2 | 0 | 0 | 0 | 1 | 0 | 15 | 8 | 2 | 2 | 2 |
| **D*** | 2.0-2.9 | 233 | 50.54 | 7 | 13 | 14 | 12 | 2 | 15 | 15 | 17 | 34 | 52 | 16 | 12 | 24 |
| **E** | 3.0-3.9 | 97 | 21.04 | 9 | 8 | 1 | 4 | 2 | 6 | 6 | 13 | 10 | 4 | 12 | 22 | 0 |
| **F** | 4.0-4.9 | 45 | 9.76 | 0 | 0 | 1 | 1 | 4 | 2 | 1 | 7 | 3 | 0 | 14 | 12 | 0 |
| **G** | 5.0-5.9 | 2 | 0.43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| **H** | 6.0-6.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **I** | 7.0-7.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **J** | ≥ 8.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total** | | 461 | 100 | 21 | 24 | 21 | 24 | 9 | 29 | 26 | 37 | 66 | 73 | 49 | 50 | 32 |
| | | | | 99 | | | | | 362 | | | | | | | |

**D* including the original strain which produced 2.5 U/ml**

**Table 2. Distribution and ranges of β-glucosidase productivity (U/ml) in *T.harzianum* isolates after mutagenic treatments.**

| Isolates class | β-glucosidase range (U/ml) | Obtained isolates | | UV exposure time (min) | | | | | EMS ( µl / ml ) / time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | 3 | 6 | 9 | 12 | 15 | 50/30 | 50/60 | 75/60 | 100/30 | 100/60 | 125/30 | 125/60 | 150/30 |
| A | 0.0 | 17 | 3.68 | 0 | 0 | 0 | 1 | 0 | 2 | 1 | 0 | 0 | 6 | 1 | 2 | 4 |
| B | 0.1-0.9 | 26 | 5.63 | 2 | 1 | 3 | 4 | 1 | 6 | 4 | 0 | 1 | 2 | 0 | 0 | 2 |
| C | 1.0-1.9 | 15 | 3.25 | 3 | 0 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 1 | 4 | 3 |
| D | 2.0-2.9 | 15 | 3.25 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 5 | 1 | 3 | 2 | 0 |
| E | 3.0-3.9 | 8 | 1.73 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 0 | 1 | 1 | 0 |
| F | 4.0-4.9 | 28 | 6.07 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 9 | 8 | 6 | 0 | 1 | 1 |
| G | 5.0-5.9 | 78 | 6.91 | 4 | 3 | 0 | 4 | 1 | 5 | 5 | 14 | 16 | 16 | 3 | 6 | 1 |
| H* | 6.0-6.9 | 139 | 30.15 | 2 | 14 | 4 | 10 | 4 | 9 | 6 | 9 | 18 | 33 | 10 | 13 | 7 |
| I | 7.0-7.9 | 58 | 12.58 | 2 | 5 | 9 | 4 | 1 | 0 | 5 | 1 | 10 | 4 | 4 | 7 | 6 |
| J | ≥ 8.0 | 77 | 16.70 | 7 | 0 | 3 | 1 | 1 | 1 | 4 | 3 | 4 | 5 | 26 | 14 | 8 |
| **Total** | | 461 | 100 | 21 | 24 | 21 | 24 | 9 | 29 | 26 | 37 | 66 | 73 | 49 | 50 | 32 |
| | | | | 99 | | | | | 362 | | | | | | | |

**H* including the original strain which produced 6.0 U/ml**