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Full Length Research Paper

Application and comparison of two different intraspecific protoplast fusion methods in *Trichoderma harzianum* and their effect on β-glucosidase activity

Ahmed M. El-Bondkly[1]*, Aboshosha, A. A. M.[2], Radwan, N. H.[2] and Dora, S. A.[2]

[1]Genetics and Cytology Department, National Research Center, Dokki, Giza, Egypt.
[2]Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Kaferelsheikh, Egypt.

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In an attempt to construct superior *Trichoderma harzianum* isolates for improving β-glucosidase productivity, protoplast fusion technique was applied. After application of different mutagenic treatments, twenty mutants were chosen 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 to antifungal agents and their productivities of carboxymethylcellulase (CMCase) and β-glucosidase to be introduced into intraspecific protoplast fusion experiments using two different methods (PEG and electrofusion). Three crosses were carried out among the selected four isolates. Results showed that, the number of fusants obtained after electrofusion were more than those obtained after polyethylene glycol (PEG) method. In addition, high productivities of CMCase and β- glucosidase were obtained after electrofusion in the three crosses. The applied protoplast electrofusion method proved to be a good and effective method for obtaining *T. harzianum* fusants with higher productivity of β- glucosidase enzyme.

Key words: *Trichoderma*, protoplast fusion, electroporation, β-glucosidase.

INTRODUCTION

Cellulose is one of the most abundant substrates available in nature; the potential importance of cellulose hydrolysis in the context of conversion of plant biomass to fuels and chemicals as well as cellulose hydrolysis also represents one of the largest material flows in the global carbon cycle (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 (Harhangi et al., 2002; Szengyel et al., 2000).

Members of the fungal genus *Trichoderma* are considered the main producer of extracellular cellulolytic enzymes. This fungus belongs to the fungi imperfecti and contains seven chromosomes (Mäntylä et al., 1992) or sex chromosomes (Herrera-Estrella et al., 1993). *Trichoderma harzianum* is well known as producer of cellulolytic enzymes that are extensively used for the degradation of cellulose particularly in textile and paper industries, beside its use in wastewater treatment (Prabavathy et al., 2006a and b).

Fungal protoplasts are important tools in physiological and genetic research, as well as genetic manipulation which can be successfully achieved through fusion of protoplasts in filamentous fungi that lack the capacity for sexual reproduction (Hayat and Christias, 2010; Lalithakumari,

*Corresponding author. E-mail: ahmed_bondkly@yahoo.com.

Abbreviations: CMCase, carboxymethylcellulase; **PEG,** polyethylene glycol.

2000; Mrinalini and LalithaKumari, 1998; Pe'er and Chet, 1990; Stasz et al., 1988).

The aim of the present study is to apply and compare two different intraspecific protoplast fusion methods [polyethylene glycol (PEG) and electroporation] in *T. harzianum* and to construct the strains of the fungus *T. harzianum* having the genetic ability to produce the highest carboxymethylcellulase (CMCase) and β-glucosidase activities.

MATERIALS AND METHODS

Strains of *T. harzianum*

T. harzianum NRRL 13879 strain and its mutants (EL-Bondkly et al., 2010; Table 1) were used in the present study and maintained on YMGA medium slants (Strauss and Kubicek, 1990).

Isolation of antifungal resistant mutants

For the isolation of antifungal resistant mutants, hypertonic regeneration medium (EL-Bondkly, 2006) and antifungal agents were used separately; concentrations of antifungal agents added were as follows: 0.5 and 1.0 µg/ml Benomyl; 10 and 25 µg/ml miconzole; 75 and 100 µg/ml cycloheximide and 250 µg/ml griseofulvin. A part of the mycelium of each isolate was inoculated on the surface of the antifungal medium plates; the plates were incubated at 28°C for six days. Colonies that exhibited resistance or sensitivity to a specific antifungal were retested on the same antifungal dose to be sure of their stability concerning resistance or sensitivity and used as markers to select the fusants.

Protoplast formation

Protoplasts were prepared through enzymatic hydrolysis of mycelium suspension using the procedure of EL-Bondkly (2002). Cultures were grown in liquid protoplast medium, containing (g/l in distilled water): glucose, 80; NH_4NO_3, 2; KH_2PO_4, 10; $MgSO_4.7H_2O$, 0.25; $FeCl_3.6H_2O$, 0.02; $MnSO_4$, 0.14 and the initial pH of the medium was adjusted to 4.5. Fifty milliliters (50 ml) of medium were dispensed into 250 ml Erlenmeyer flask for the development of mycelium. The flasks were incubated at 30°C for 20 h on a shaker maintained at 160 rpm. After incubation, the mycelium was collected by centrifugation, washed twice with 0.7 M KCl in 25 mM phosphate buffer, pH 5.8 and then resuspended in 50 mg/ml phosphate buffer containing 0.7 M KCl and 10 mg/ml Novozyme 234 (Sigma Co.). The lytic mixtures were incubated at 30°C with gentle shaking for 3 h. Incubated mixtures were filtered and protoplasts were counted in the filtered lysate.

Protoplast fusion techniques

PEG method

According to the CMCase and β-glucosidase activities and resistance or sensitivity to one or more antifungal agents, equal numbers of protoplasts from the two mutants were mixed and centrifuged at 3000 rpm for 5 min, the residue (mixture of protoplast) was suspended in 2 ml of prewarmed (30°C) solution of PEG 6000 at 30 % (w/v). The PEG containing 0.05 M $CaCl_2$ and 0.05 M glycine-NaOH buffer (pH 7.5). After incubation at 30°C for 10 min, the suspension was centrifuged at 3000 rpm for 5 min.

Electroporation process

The process of electrofusion of protoplasts was conducted in the gene pulser Bio-Rad CO., (USA) with an electrofusion chamber of 1 ml working volume. Process parameters: 1 or 2 impulses immediately following one another with a field intensity of 200 v/cm and an exposition time of 1000 min at the stage of dielectrophoresis, 1 impulse with a field intensity of 500 v/cm and an exposition time of 20 min at the stage of fusion, regulated temperature of 4°C before and after the process, rounding time of ca 20 min (Żukowska et al., 2004).

Isolation of recombinant fusants

Through the present study, PEG or electroporation methods, treated protoplast suspensions were plated onto an antifungal selective medium. It contains the same components of the protoplast medium with addition of cellulose, as a carbon source instead of glucose, 0.7 M KCl and one or more of the antifungal agents. Treated protoplast pellets were resuspended in 1 ml of asomatically balanced phosphate buffer, diluted appropriately and were plated on the hypertonic selective and nonselective regeneration media. The plates were incubated at 28°C until the colonies were grown on plate's surface. The grown colonies were considered as complementary fusants. They were transplanted and subcultured several times onto selective and nonselective media before further studies. Fusion frequency was expressed as the ratio of the number of colonies formed on selective and nonselective media.

Fermentation and determination of CMCase and β-glucosidase activities

The wild type strain, mutants and fusants were grown in fermentation medium (Haapala et al., 1995), which is optimal for CMCase and β-glucosidase productivities. 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. CMCase and β-glucosidase activities were assayed in the culture supernatant according to Vaheri et al. (1979).

RESULTS

Response of the original strain and the selected mutants to some antifungal agents

To induce new fungal recombinants through protoplast fusion, the original strain in addition to the 20 selected mutants were exposed to four antifungal agents. Table 1 summarizes the response of the original strain and the 20 selected mutants to four antifungal agents (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) as well as their CMCase and β-glucosidase activities.

Table 1. Sources, CMCase and β-glucosidase productivities and response of the selected *T.harzianum* mutants and their original strain to four antifungal agents.

Mutant number	Source of mutant	CMCase and β-glucosidase productivities				Antifungal agents (µg / ml)						
		CMCase		β-Glucosidase		Benomyle		Miconzole		Cycloheximide		Griseofulvin
		U/ml	% from W.T.	U/ml	% from W.T.	0.5	1.0	10	25	75	100	250
W.T.	Original strain	2.5	100.0	6.0	100.0	-	-	+	+	-	-	+
W1/9	Original strain with 0.1% colchicines	3.0	120.0	7.5	125.0	-	-	+	-	-	-	+
W2/9	Original strain with 0.2% colchicines	3.2	128.0	7.8	130.0	-	-	+	-	-	-	+
L1/1	(9/8) mutant with 0.1% colchicines	4.3	172.0	10.0	166.7	-	-	+	-	-	-	+
L1/9	(9/8) mutant with 0.1% colchicines	4.3	172.0	10.5	175.0	-	-	+	-	-	-	+
L1/15*	(9/8) mutant with 0.1% colchicines	5.0	200.0	10.5	175.0	-	-	+	+	-	-	+
L2/11	(9/8) mutant with 0.2% colchicines	5.0	200.0	11.5	191.7	-	-	+	-	-	-	+
L2/16	(9/8) mutant with 0.2% colchicines	5.2	208.0	11.5	191.7	-	-	+	-	-	-	+
P1/8	(15/4) mutant with 0.1% colchicines	4.3	172.0	10.5	175.0	-	-	+	-	-	-	+
P2/9	(15/4) mutant with 0.2% colchicines	5.0	200.0	11.0	183.3	-	-	+	-	-	-	+
E1/9	(50/30/17) mutant with 0.1% colchicines	5.0	200.0	10.0	166.7	-	-	+	-	+	-	+
E2/3*	(50/30/17) mutant with 0.2% colchicines	4.7	188.0	10.0	166.7	-	-	+	-	+	-	+
R2/10	(100/30/44 n) mutant with 0.2% colchicines	4.8	192.0	14.0	233.3	-	-	+	-	-	-	+
D1/1	(125/30/12) mutant with 0.1% colchicine	6.2	248.0	15.5	258.3	-	-	+	-	-	-	+
D1/4*	(125/30/12) mutant with 0.1% colchicine	6.5	260.0	17.2	286.7	-	-	+	-	+	-	+
D1/8	(125/30/12) mutant with 0.1% colchicine	5.8	232.0	17.0	283.3	-	-	+	-	+	-	+
D1/14*	(125/30/12) mutant with 0.1% colchicine	6.0	240.0	17.2	286.7	-	-	+	+	-	-	+
D2/1	(125/30/12) mutant with 0.2% colchicine	5.8	232.0	16.5	275.0	-	-	+	-	-	-	+
D2/2	(125/30/12) mutant with 0.2% colchicine	5.8	232.0	16.5	275.0	-	-	+	+	-	-	+
D2/11	(125/30/12) mutant with 0.2% colchicine	5.8	232.0	16.5	275.0	-	-	+	+	-	-	+
D2/14	(125/30/12) mutant with 0.2% colchicine	5.8	232.0	16.8	280.0	-	-	+	+	-	-	+

L1 and L2 mutants were obtained from original strain after treatment with 9 min UV exposure time; P1 and P2 mutants were obtained from original strain after treatment with 15 min UV exposure time; E1 and E2 mutants were obtained from original strain after treatment with concentration of 50 µg/ml NTG for 30 min; R2 mutant was obtained from original strain after treatment with concentration of 100 µg/ml NTG for 30 min; D1 and D2 mutants were obtained from original strain after treatment with concentration of 125 µg/ml NTG for 30 min (EL-Bondkly el al., 2010). *Four mutants (L1/15, E2/3, D1/4 and D1/14) were selected to be used in the intraspecific protoplast fusion.

Results in Table 1 showed that, the original strain NRRL13879 exhibited resistance to both miconzole and griseofulvin antifungal agents, while it was sensitive to the other two antifungal agents. In addition, different responses appeared after exposure of the 20 selected mutants to these antifungal agents. All isolates were sensitive to the two benomyle concentrations and the high concentration (100 µg/ml) of cycloheximide, whereas they exhibited complete lethality. On the other hand, complete resistance was observed after exposure of all isolates to both miconzole (10 µg/ml) and griseofulvin (250 µg/ml). Meanwhile, different responses were noticed after exposing the 20 mutants to high concentration of miconzole

(25 µg/ml) and low concentration of cycloheximide (75 µg/ml). Five isolates (L1/15, D1/14, D2/2, D2/11 and D2/14) were resistant to miconzole in concentration of 25 µg/ml, while the rest isolates exhibited complete lethality. In addition, the results showed also that, four isolates (E1/9, E2/3, D1/4 and D1/8) were resistant to the low concentration of cycloheximide, whereas, the rest isolates were sensitive to the same antifungal concentration.

Protoplasts formation and fusion

On the basis of the CMCase and β-glucosidase activities shown in Table 1 and resistance or sensitivity to one or more of the four used antifungal agents, only four mutants (L1/15, E2/3, D1/4 and D1/14) were selected and used in the intraspecific protoplast fusion. Data in Table 1 clearly showed that, two out of the four selected isolates (L1/15 and E2/3) showed low productivity of CMCase and β-glucosidase, while the other two isolates showed high productivity. In addition, these isolates exhibited different response to the two antifungal agents that is, miconzole and cycloheximide. According to the obtained results, these four isolates were used to carry out three intraspecific crosses using two different methods, classical (PEG) and electropration method. The first cross was applied between the low CMCase and β-glucosidase producer isolates (L1/15 and E2/3), the second cross was performed between the low and high producer isolates (E2/3 and D1/14), while the third cross was carried out between the two higher isolates (D1/4 and D1/14) .

According to the conditions described under materials and methods, enzymatic treatments and subsequent examination of the treated mycelia from the selected parental strains with a phase-contrast microscope showed that, gradual degradation of fungal mycelia started after the addition of 10 mg/ml novozyme 234 enzyme. The whole cell wall digestion was achieved following incubation at 30°C with gentle shaking for 3 h. Maximum release of protoplasts differed from one mutant to the other. From the mycelium of mutant L1/15, the highest yield of protoplasts (2.8 x 10^7/ml) was obtained after the 3 h incubation period. The maximum release of protoplasts was obtained with mutant E2/3 that yields 1.9 x 10^7 protoplasts per ml. On the other hand, 3.0 x 10^7 and 3.5 x 10^7/ml protoplasts were released from mutant D1/4 and D1/14 mycelium after the 3 h incubation time, respectively. Two different protoplast fusion techniques were the main subject to be evaluated in this study. They are tools for inducing genetic recombinants especially in fungi like, *T. harzianum*, where the sexual cycle is unknown, in order to isolate higher CMCase and β-glucosidase producing recombinants (Hayat and Christias, 2010). However, the use of these techniques requires labeling the parental strains before protoplasting and fusion (EL-Bondkly, 2006). Some of the highest and lowest CMCase and β-glucosidase producer isolates were used for intraspecific protoplast fusion through this study.

Fusion frequency, estimated as the ratio of the number of colonies regenerating on the nonselective medium to the number of colonies formed on the selective medium, was found to be different from one cross to another. The fusion frequencies were 1.8 x 10^{-3} and 2.5 x 10^{-3} in the case of fusion between isolates L1/15 and E2/3 when PEG and electrofusion methods were applied, respectively. On the other hand, the frequencies of intraspecific protoplast fusion between E2/3 and D1/14 were increased to 2.0 x 10^{-3} (PEG method) and 2.6 x 10^{-3} (electrofusion method). The highest frequencies were between mutants D1/4 and D1/14 yielding 2.5 x 10^{-3} for PEG method and 2.8 x 10^{-3} for electrofusion method. Other investigators mentioned that, the formation, regeneration and fusion of protoplasts are affected by different factors, for example, enzymes, time of treatments, mycelial age, regeneration medium, protoplast fusion method (EL- Bondkly and Talkhan, 2007; Lalithakumari, 2000; Prabavathy et al., 2006b).

Evaluation and comparison of PEG and electroporation methods

Cross 1

This cross was carried out between the two low CMCase and β-glucosidase producer isolates (L1/15 and E2/3) as shown in Table 2 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 were marked from F1/1 to F1/11 (PEG method) and from F4/1 to F4/15 (electoporation method). The CMCase productivity of the parental isolates (E2/3 and L1/15) was 4.7 and 5.0 U/ml, while their productivities of β-glucosidase were 10.0 and 10.5 U/ml, respectively. The highest productivity of both enzymes among the 11 fusants obtained from PEG method was recorded by fusants number F1/6 and F1/10, where they gave 20 and 14% of CMCase and β-glucosidase more than the higher parent (L1/15), respectively.

Concerning CMCase and β-glucosidase activities 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 when compared

Table 2. CMCase and β-glucosidase productivities for the intraspecific fusants resulted from cross 1.

Parent and fusant	PEG				Parent and fusant	Electroporation			
	CMCase		β-glucosidase			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	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

with the parental isolates. CMCase productivity of these fusants ranged from 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). In the meantime, fusants (F4/4, F4/7, F4/8, F4/9, F4/13 and F4/14) produced 14.28%, whereas fusants (F4/1, F4/2, F4/3 and F4/15) gave 19.04% β-glucosidase more than the higher parent.

Cross 2

The second cross was achieved between the highly efficient CMCase and β-glucosidase producer isolate (D1/14) and the lower efficient one (E2/3). Results in Table 3 revealed that, only 11 fusants (F2/1 to F2/11) resulted from the PEG method, in comparison with 15 fusants (F5/1 to F5/15) which 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 higher activity in CMCase and β-glucosidase production, since they gave 6.8, 6.8, 6.6, 6.8 and 6.6 U/ml of CMCase, respectively, and also produced 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 the15 obtained from electroporation method showed increase in both CMCase and β-glucosidase than the higher parental isolate (D1/14). The CMCase activity recorded by these fusants yielded between 13.33 and 20% more than the higher parent (D1/14). Productivity of β-glucosidase ranged from 5.81% for F5/11 and F5/12 fusants to 7.55% for the rest eight isolates over the higher parent.

Cross 3

This cross was done between the highest CMCase and β-glucosidase producer isolates (D1/4 and D1/14). Fourteen fusants were obtained from this cross using PEG method and 15 immediately after the application of electroporation method on the basis of antifungal test as shown in Table 4. These 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 higher productivity of CMCase and β-glucosidase when 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 of these 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 obtained through electroporation technique proved to have higher productivity of CMCase, in which four fusants (F6/4, F6/9, F6/10 and F6/14) produced 33.33% more than the higher parent (D1/4). While five fusants (F6/5, F6/6, F6/11, F6/13 and F6/15) exhibited about 41.66% 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 giving from 4.65 to 16.27% more than their parents (D1/4 and D1/14), respectively.

DISCUSSION

Protoplast fusion is an effective tool for inducing genetic recombinations and developing superior hybrid strains in filamentous fungi (Mrinalini and LalithaKumari, 1998; Pe'er and Chet, 1990; Stasz et al., 1988). Genetic recombination is a powerful method for developing superior industrial strains. Comparing both methods of protoplast fusion used in this study (PEG and electroporation), the obtained results clearly showed that, the number of recombinant fusants obtained after application of electrofusion was more than that obtained after application of PEG method. On the other hand, higher productivity of CMCase and β-glucosidase was recorded after electrofusion compared with the PEG method in the three crosses carried out through of this study.

Regarding the first cross (Table 2) carried out between the 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 14.28 to 19.04% 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 4.66 and 14.28% more than the higher parent (L1/15) as shown in the same table.

The second cross was carried out between the lower producer isolate (E2/3) and the higher producer (D1/14); results in Table 3 showed that, ten fusants obtained after electrofusion method showed higher productivity of the two enzymes. The CMCase productivity of these fusants was ranged between 13.33 and 20% over the higher parent (D1/14). While, β-glucosidase productivities was ranged from 5.81 to 7.55% over 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 13.33% CMCase productivity more than the higher parent (D1/14), as well as, produced from 4.65 to 7.55% β-glucosidase more than the higher parent (Table 3).

In the case of the third cross between the two high producer isolates (D1/4 and D1/14), 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 33.33 to 41.66% 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 4.65 to 16.27% 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 15.38 to 23.07% CMCase more than the higher parent (D1/4), as well as showed from 10.46 to 13.37% β-glucosidase productivity more than the two parents when PEG method was applied.

Fifteen (15) self-fusant strains using PEG in STC buffer from *T. harzianum* PTh18 strain were isolated (Prabavathy et al., 2006a). Among them, the strain SFTh8 produced maximum chitinase with two-fold increase when compared with the parental strains. Furthermore, all the self-fusants exhibited increasing of antagonistic activity against *Rhizocotonia solani* than the parents. On the other hand, EL-Bondkly and Talkhan (2007) applied the intraspecific protoplast fusion in *T. harzianum* and they selected eighteen self fusants, four of them (ATh1/9, ATh1/12, ATh1/14 and ATh1/17) produced high chitinase activity, while fusant (ATh1/7) produced 94.3% more chitinase activity than the original strain. Moreover, Prabavathy et al. (2006b) used intraspecific protoplast fusion to enhance carboxymethyl-cellulase activity and they found that, most of the fusants exhibited fast growth and abundant sporulation compared to non-fusant and parental strains. Furthermore, two fusants ($SFTr_2$ and SFT_3) recorded more than two-fold increase in enzyme activity. They suggested that, protoplast fusion can be used to develop superior hybrid strains of filamentous fungi that lack inherent sexual reproduction.

In conclusion, there are two main advantages of electro-poration method over the traditional PEG method, the first one is its 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 *T. harzianum* fusants with higher productivity of β-glucosidase enzyme.

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Table 3. CMCase and β-glucosidase productivities for the intraspecific fusants resulted from cross 2.

PEG					Electroporation				
Parent and fusant	CMCase		β-glucosidase		Parent and fusant	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

Table 4. CMCase and β-glucosidase productivities for the intraspecific fusants resulted from cross 3.

PEG					Electroporation				
Parent and fusant	CMCase		β-glucosidase		Parent and fusant	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
D1/4	6.5	100.0	17.2	100.0	D1/4	6.5	100.0	17.2	100.0
D1/14	6.0	92.30	17.2	100.0	D1/14	6.0	92.30	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

Table 4 Cont.

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

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