ITS sequence Analysis and Genome Shuffling of *Trichoderma* Sp. for Improving Cellulase Activities

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Abstract: Five isolates of *Trichoderma* Sp. were obtained and screened for cellulases activities and the highest isolate was selected and identified by amplification and sequencing of the internal transcribed spacer (ITS) region in the rDNA, the selected isolate was identified as *Trichoderma virens* AG2. The conidia of this selected strain were treated with different mutagenics, 604 isolates were obtained and screened for cellulases productivity using plate assaying technique and the promising isolates were selected for assaying in fermentation medium. The highest five cellulases producer mutants (D5, D6, D13, E3 and CC20) were selected as starting population for the first round of genome shuffling, which yielded a library of 86 colonies. The top five recombinants (R1/1, R1/3, R1/28, R1/50 and R1/55) were pooled and used as parental populations for the second round of genome shuffling which also yielded 86 colonies. After the second round of genome shuffling, nine superior isolates for cellulases activities were obtained and the highest increase was recorded by the recombinant R2/11, which reached 10.5, 7.6 and 10.8 U/ml for CMCase, FPase and β-glucosidase, respectively. These values showed increase by 308.8, 633.3 and 720% for CMCase, FPase and β-glucosidase, respectively in comparison with the original strain, *T. virens* AG2. To investigate if a change in the genetic content was evident after the mutagenesis and shuffling treatments, results of RAPD analysis revealed obvious genetic differences among mutants and shuffled strains compared with the original *T. virens* AG2.

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1. Introduction

Plants produce about 180 billion tons of cellulose per year globally, making this polysaccharide the largest organic carbon reservoir on earth (**Festucci-Buselli *et al.*, 2007**). Cellulases play an important role in cellulosic biomass hydrolysis, the high cost of hydrolyzing biomass polysaccharides to fermentable sugars remains a major obstacle that must be overcome before cellulosic ethanol can be effectively commercialized. Consequently, new studies aimed to understand and improve cellulases efficiency and productivity are at the forefront of biomass research. Enzymatic hydrolysis of cellulosic materials is achieved by a sequence of reactions with cellulase complex enzymes that work synergically in cellulose degradation, which include FPase, CMCase and β-glucosidase **(Johnvesly *et al.*, 2002).** Microbial cellulases have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, detergents and agriculture. *Trichoderma* Sp. was widely studied and industrially used (**Bhat and Bhat, 1997**).

Many strategies can be applied for reducing the cellulases production cost such as strain improvement methods e.i., mutagenesis, protoplast fusion and recombinant DNA technology **(Zhang *et al.*, 2006** and **Chen *et al.*, 2012)**. However, the relatively recent and efficient technology of genome shuffling offers a better way of improving industrially important strains. This technique combines the advantage of multi parental crossing along with the recombination of entire genomes. The genome shuffling technique has been demonstrated as an effective method for producing improved strains. Therefore, it is used to significantly improve the quality of industrially important microbiological phenotypes **(Gong *et al.*, 2009).**

Genome shuffling method offers the advantage of simultaneous genetic changes at different positions throughout the entire genome without the necessity for genome sequence information (**Petri and Schmidt, 2004**). At present, genome shuffling has been successfully applied as an effective whole-cell engineering approach for the rapid improvement of industrially important microbial phenotypes.

The (ITS) regions of nuclear rDNA have been good targets for the identification, differentiation and phylogenetic analysis of fungi using molecular techniques (**White *et al.*, 1990** and **Kubicek *et al.*, 1998**). The ITS regions are less well conserved sequences nested between the highly conserved 18S, 5.8S and 28S rDNA genes. Differences among species in the nucleotide sequence of the rDNA have been used to develop species specific primers for fungi, and this has become a common approach in molecular identification strategies **(Kularatne *et al.*, 2004)**.

The objective of the present study is molecular identification using ITS sequence analysis of

Trichoderma Sp. and strain improvement for increasing cellulases production via combination between classical and relatively recent biotechnology approaches.

2.Material and methods

Trichoderma strains and culture conditions

Five different *Trichoderma* isolates (T5, TV, AG1, A G2 and AG3) were obtained to investigate their cellulase production capacity in order to select the highest producer for further genetic analysis. Isolates T5 and TV were obtained from Burg El-Arab City of Scientific Research, Egypt, while isolates AG1, AG2 and AG3 were obtained from Faculty of Agriculture, Suez Canal University, Egypt. The isolates were maintained and routinely cultured on potato dextrose agar (PDA) at 28°C **(Chand *et al*., 2004)**.

ITS sequence analysis of potent cellulases-producing strain

The fungal strain *Trichoderma* Sp. AG2 was identified using ITS sequence analysis. Genomic DNA was extracted from mycelia of *Trichoderma* Sp. AG2 cultured for 20 h in liquid medium using EZ-spin column genomic DNA minipreps kit (plant), BIO BASIC INC. Canada, following the manufacturer's instructions. Amplification of ribosomal DNA was conducted using the ITS1 and ITS4 primers (**Table 1**) for amplification of the PCR product. The reactions were performed in a thermal cycler (Long Gene-MG96G / China). Amplification of ribosomal DNA was performed using EZ-D-PCR Master mix from (BIO BASIC INC. Canada.) in 25 µl reaction in PCR tubes. The conditions for this PCR were: initial denaturation 5 min at 94°C followed by 35 cycles of denaturation 1 min at 94°C, primer annealing 1 min at 55°C, primer extension 1 min at 72°C and a final extension phase of 5 min at 72°C, and then cooled to 4°C. PCR products were checked on 1.5% agarose gel. The PCR products were purified and determination of sequences was done at the holding company for biological products & vaccines (VACSERA, Giza, Egypt). Sequence data were edited with Lasergene Software SeqMan (DNAStar Inc.). Next, relatives were determined by comparison to rRNA genes in the National Center for Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov website) to create a matrix using MEGA5 and ClustalW programs. The tree topologies were evaluated by bootstrap analyses based on 1,000 replications with MEGA5 and phylogenetic tree were inferred using the neighbor-joining method.

Preparation of starting mutants for genome shuffling

Out of five *Trichoderma* isolates, one isolate, *Trichoderma* Sp. AG2 was found to be potent strain for celulases production and this strain was used for the strain improvement by mutations. Conidia from 8 days old PDA slants were suspended in sterile normal saline solution (0.85 % NaCl) containing a drop of Tween-80 to disperse spore clumps, spore suspensions were then treated with four different treatments of mutations. The first treatment with UV 254 nm (Philips TUV-30 W lamp) for 5, 10, 15, 20 and 25 min at distance of 25 cm. The second treatment was with 250 and 500 µg/ml N-methyl-N-nitro-N-nitrosoguanidine (NTG) for 30 and 60 min at 37ºC. The third treatment was with UV for 5 min at distance of 25 cm, then 150 µg/ml NTG were added into the suspension and treated for 30 and 60 min at 37ºC. The fourth treatment was with combination of two chemical mutagens; 150 µg/ml NTG and 250 µg/ml ethidium bromide (EtBr) for 30 and 60 min at 37ºC. The suspension of surviving spores was diluted and spread onto PDA medium containing 0.1% (v/v) triton X-100 as a restriction factor for radial colony growth plates. Plates were then incubated for 6 days at 28°C. The growing colonies were counted against the control plates and single colonies were transplanted onto PDA slants for further genetic analysis.

Plate screening method for cellulase activities

For finding the desired mutants from a large mutant library, cellulase activities screening was carried out by the examination of “halos” on solid agar plates using carboxymethylcellulose (CMC) as the substrate, followed by congo red staining and washing **(Mandel and Weber, 1969)**. Inoculated plates were incubated for 96 h at 30°C. Hyper-cellulases mutants were selected, based on the diameter of the hydrolysis zone surrounding the colonies. Based upon the ratio between the clearing zone diameter and colony zone diameter, highest cellulases mutants were chosen for enzyme submerged production **(Tearherr and Wood, 1982).**

Cellulase activities under submerged fermentation

For cellulase activities under submerged conditions, two different fermentation media FM1 (**Haapala *et al.* 1995**) and FM2 (**Toyama *et al.* 2007**) were used to test the isolates and mutants for cellulases production. For the inoculation of 50 ml of culture, 5 ml of 10^8 spore/ml of each isolate were added and the cultures were incubated on a rotary shaker (200 rpm) at 29-30°C for 10 days. The culture medium was centrifuged at 5,000 rpm using cooling centrifuge for 10 min. The clear supernatant was used as the crude extracellular enzymes source. The activities of FPase and CMCase were investigated by measuring the production of reducing sugar by the dinitrosalicylic acid reducing sugar using the method recommended by **Miller, 1959** and **Vaheri *et al.* 1979**. β-Glucosidase activity was routinely assayed with p-nitrophenyl-β-D-glucopyranoside as the substrate by using the method of **Vaheri *et al.* (1979)**. One international unit of FPase, CMCase, or β-glucosidase was defined as the amount of enzymes that released 1 µmol of glucose or p-nitrophenol per min during the hydrolysis reaction. All values given are the average of triplicates.

Protoplast fusion and genome shuffling

For protoplast fusion, the hyper-five-cellulases mutants were used, which were induced from the wild type of *Trichoderma* Sp. AG2, after different mutagenic treatments. Protoplasts were prepared through enzymatic hydrolysis of mycelia suspensions using the procedure of (**El-Bondkly, 2006; El-Bondkly *et al.* 2011 and El-Bondkly and El-Gendy, 2012)**. Equal numbers of protoplasts from different populations of these mutants were mixed, centrifuged, and resuspended in 30 % (w/v) polyethylene glycol 6000 solution containing 0.05 M glycine–NaOH buffer, pH 7.5, and 0.05 M $CaCl_2$. After gently shaking at 30 °C for 30 min to allow the protoplast fusion, the fused protoplasts were centrifuged at 3,000 rpm for 5 min. Treated protoplast pellets were resuspended in 1 ml of osmatically balanced phosphate buffer, diluted appropriately, and plated on the hypertonic regeneration medium. The hypertonic regeneration medium is the same as the protoplast medium with the addition of 10.0 g/L cellulose as carbon source instead of glucose, 0.7 M KCl, 0.1 % (v/v) Triton X-100, and 20.0 g/L agar. Plates were incubated at 30 °C until colonies grew on the surface of plates. The colonies appearing under these conditions were selected to carry out shake flask analysis and the fusants with higher cellulase activities were selected and named R1. Two successive rounds of protoplast fusion were carried out; recombinants from each round of the fused strains were saved for further analysis and used as sources of protoplasts for the subsequent round of genome shuffling, which was carried out using the same method.

Random amplification polymorphic DNA analysis (RAPD)

Randomly amplified polymorphic DNA (RAPD) analysis was carried out to detect the genetic diversity among mutants obtained from the original strain *Trichoderma* Sp. AG2 after mutagenic treatments and genome shuffling using five random primers; OPB-02, OPB-04, OPB-14, OPG-10 and OPZ-01 (Table 1). PCR was performed using EZ-D-PCR Master mix from (BIO BASIC INC. Canada.) in 25 µl volumes in thermal cycler (LongGene - MG96G-China) with the following temperature profiles: initial denaturation at 95 °C for 4 min, 35 cycles of denaturatiom at 95°C for 1 min, primer annealing at 34°C for 1 min, extension at 72°C for 1 min and final cycle of extension at 72°C for 5 min, then the reaction was hold at 4°C. The PCR products were resolved by electrophoresis on 1.5% agarose gel containing ethidium bromide, visualized under UV illuminator and then photographed. Molecular size of RAPD–PCR bands separated on gels was measured by analyzing gel images with GelAnalyzer software package version 2010a (freeware) with 100 bp DNA ladder (Larova GmbH- Germany) as DNA size marker.

3. Results and Discussion

Evaluation of collected strains for cellulases production capacity

The five different fungal strains (TV, T5, AG1, AG2 and AG3) obtained from different sources were primarily distinguished as *Trichoderma* sp. using morphological characters (**El-Bondkly *et al.* 2011** and **El-Bondkly and El-Gendy, 2012**). The five different strains of the fungus *Trichoderma* were assessed for both their cellulase activities and interaction effect of each genome with two different fermentation media (FM1 and FM2) compositions for the production of cellulases. The results clearly indicated that the isolate *Trichoderma* AG2 showed the highest FPase and CMCase activities when grown on both media FM1 and FM2 after 10 days of inoculation and fermentation (Fig. 1). This strain showed the highest production capacity for the β-glucosidase when grown on medium FM1. In spite of the isolate AG3 gave the highest β-glucosidase productivity on medium FM2; however its FPase and CMCase productivities were less than those of the isolate AG2. According to these results, the strain AG2 exhibited superior cellulase activities under the conditions of both media used in this study and the highest productivity for this strain was recorded on medium FM1. Therefore, this strain was selected for further genetic studies using the fermentation medium FM1 and all other environmental conditions applied.

Molecular identification of strain (AG2) by ITS region sequencing

This experiment was designed to determine for any of *Trichoderma* Sp. is the AG2 strain belonging. The ITS region of strain (AG2) was amplified with primers ITS1 and ITS4. The PCR products were verified by agarose gel electrophoresis and the amplification resulted in a fragment of approximately 560 bp (Fig. 2). The obtained fragment was purified and sequenced using ABI PRISM® 310 Genetic Analyzer (VACSERA, Giza, Egypt) and the sequence was compared with those in the NCBI Nucleotide Sequence Database by using the BLAST algorithm. Comparative analysis by MEGA5 software is shown in Fig. (3), it was demonstrated that the sequence of ITS region from the parental strain AG2 had a significant identity to a number of *Trichoderma* section. Comparison of the parental strain sequence with sequences of the reference species of *Trichoderma* contained in the bank database exhibited a similarity of 97% with 16 entries of *Hypocrea virens*. The results obtained from this experiment and presented in Fig. (3) proved that AG2 strain belongs to the fungal species *Trichoderma virens*.

Induction of genetic variations

For the induction of mutations in the selected *Trichoderma virens* AG2, spore suspensions were treated with four different treatments of mutations for strain improvement. After the application of UV irradiation doses, 219 colonies were isolated; out of them 32 isolates

(14.61%) showed different characteristics as morphological variants. The results clearly showed that the survival percentage was decreased gradually by increasing UV exposure time and the highest morphological variants percentage (21.6%) was obtained after the application of 25 min UV exposure time, and the lowest percentage (10.2%) was recorded after 10 and 20 min. exposure time (Fig. 4). After application of different NTG concentrations, 151 colonies were isolated; out of them 18 isolates (11.92%) showed different characteristics as morphological variants, and the highest percentage of the morphological variants (15.38%) was obtained after NTG treatment 250 µg/ml for 60 min. The survival percentage decreased sharply by increasing NTG concentrations and exposure time (Fig. 4).

Two combinations of mutagen treatments were used in this study for the treatment of *T. virens* AG2 conidia. The first combination was between NTG (150 µg/ml) and EtBr (250 µg/ml) for 30 and 60 min. while the second combination was between UV (5 min.) and NTG (150 µg/ml) for 30 and 60 min. The survival percentage for the EtBr-NTG combination for 30 min. (AA) was 10.35% while that for the same combination treatment but for 60 min. (BB) was 4.65%. Moreover, the survival percentage for the treatment of UV-NTG combination for 30 min. (CC) was 2.78% while that of the same combination treatment but for 60 min. (DD) yielded survival rate of 1.49%. The BB treatment showed the highest efficiency for inducing morphological variants with the rate of 27%, followed by CC, AA and DD with efficiency rate of 22.58, 19.1 and 12.8%, respectively (Fig. 4).

Cellulase activities and selection of starting mutants

Screening of isolated mutants for cellulase activities was done on CMC agar plates with plate screening medium. Mutants which showed bigger clearance zone, were the higher inducers for cellulase activities. The index of relative enzyme activity was used to determine which isolated mutants produced more cellulases, and the highest producers after each mutagenic treatment were further assayed for obtainment of full profile about cellulase activities using submerged fermentation medium.

Out of 604 isolates obtained, 219 were after application of UV doses, 151 after of NTG treatments, 142 after the combination between EtBr and NTG, and 92 after the combination between UV and NTG. Results in Table (3) indicated that out of the total tested isolates, 263 (43.54%) showed cellulase activities less than the range of the original *T. virens* AG2 strain. From these isolates, 14, 12 and 65 isolates after UV application, NTG treatments and mutagenic combinations, respectively, completely lost their abilities to produce cellulases (did not give any clear zones). On the other hand, 208 (34.43%) isolates produced cellulases within the range of the original culture. Furthermore, there were 133 (22.02%) isolates proved to produce cellulase activities in the form of clear zone, more than the range of the original culture *T. virens* AG2. These isolates were as follow: 74 (12.25%), 32 (5.30%) and 27 (4.47%) for classes E, F and G:L classes, respectively.

The highest isolates obtained after the plate assays for each mutagenic treatment were selected for fermentation analysis under submerged fermentation medium. The wild type strain (*T. virens* AG2) produced 3.4, 1.2 and 1.5 U/ml of CMCase, FPase and β-glucosidase, respectively. After application of UV treatments, 30 isolates were selected for submerged fermentation analysis. The values for this group ranged between 4.00 to 6.60, 1.29 to 4.30 and 1.60 to 7.50 U/ml for the CMCase, FPase,and β-glucosidase, respectively. In the case of NTG mutagenesis treatments, 27 isolates were selected and the values ranged between 3.30 to 5.40, 1.00 to 3.70 and 1.20 to 6.30 U/ml for the CMCase, FPase and β-glucosidase, respectively. Finally, 19 isolates after application of combination mutagenesis treatments were selected for submerged fermentation and the CMCase, FPase and β-glucosidase activities ranged between (3.60 to 5.30), (0.50 to 4.20) and (1.50 to 6.80) U/ml, respectively.These results indicated the effectiveness of plate assay method for quick qualitative screening for cellulase activities in large libraries of mutants (**Ten *et al.* 2004 and Florencio *et al.* 2012**). Results obtained after all mutagenic treatments for the improvement in cellulase activities in the form of percentage from the original strain *T. virens* AG2 are shown in Table (2). Out of these isolates, mutants with high cellulases capacity were selected as starting point for the first round of genome shuffling. As previously described, the hydrolysis of cellulose requires synergism and combinational effect of all three cellulase enzymes, exoglucanases, endoglucanases and β-glucosidases. Therefore, the selected isolates for the first round of genome shuffling should possess high activity for all measured substrates. Five mutants (D5, D6, D13, E3 and CC20) were selected as starting population for the first round of genome shuffling (Fig. 6).

Until now, induction of mutations plays a major role in genetic improvement of many characteristics in spite of the fact that the majority of selected individuals showed instabilities. This may be due to the wide range of genetic variations resulted after mutagenic treatments. Various mutagens are used as a tool for the induction of genetic variations throughout history of cellulases improvement in cellulase-producing microorganisms. **Montenecourt and Eveleigh (1977 a and b)** used ultraviolet irradiation doses and obtained 0.1% survival rate with *T. ressei* QM6a to isolate series of mutants with hypercellulase productivity. Moreover **Allen and Andreotti (1982)** isolated the mutant *T. reesei* MCG80 from the parent *T.reesei* C30 strain after its exposure to UV-light. They found that the MCG80 mutant was an excellent producer for cellulases. On the other hand,

NTG proved to be an extra ordinarily potential mutagen. Many inverstigators studied NTG effects and used different concentrations of NTG for different exposure times with *Trichoderma* Sp., they isolated many hypercellulases mutants (**Bailey and Nevalainen 1981, Mishra and Gopalkrishnan 1984, Durand *et al*. 1988**). UV and NTG individually or together were used to improve cellulase activities, **Kawamori et al. (1985)** found that the survival percentage was 50% when NTG concentration was used with *T. ressei* KY746, while when UV application dose was applied; it gave about 1% survival rate. They isolated two mutants; KDD-10 and DGD-16; which produced large clearing zone on Walseth's cellulose agar plates containing 5% glucose. As well as **Dhawan *et al.* (2003)** reported that when fungi were grown with mutagens at sublethal concentrations secretory enzyme production increased. Also **Chand *et al.* (2004)** identified two potent strains previously isolated from soil samples and subjected them to two different mutagenic treatments (MNNG), ethidium bromide (EtBr) and UV for 30 min and 1 h duration as well EtBr and MNNG were incorporated in the selective media in sublethal concentration. **Hao *et al.* (2006)** used UV light and NTG to induce mutations in *Trichoderma ressei* WX-112 and found that the ability of mutants to produce cellulases was increased 1.95 times compared with the original strain. Moreover, **El-Bondkly *et al.* (2010)** used UV irradiation and Ethyl methane sulfonate (EMS), to induce mutants in *Trichoderma harzianum*, for improvement of β-glucosidase productivity. Five of the obtained mutants were treated with colchicine and tested for their ability to produce CMCase and B- glucosidase. 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. **Shafique *et al.* (2011)** selected a strain of *Trichoderma viride* FCBP-142 to develop an over-producer of cellulases and subjected it to mutagenesis with UV and chemical ethyl methane sulfonate (EMS). Among 178 survivals after UV irradiation, bigger zones of clearing on agar plates appeared around 81 colonies of putative mutant strains of native fungus with maximum of 87 IU/ml by Tv-UV-5.6 strain in comparison to parental strain (53 IU/ml). The induction of mutation through UV irradiation promoted the cellulase activity roughly about 2-fold**.**

Genome shuffling of improved mutant populations

Genome shuffling was evaluated in this study as a tool for inducing new genetic combinations, in *Trichoderma* Sp. Genome shuffling amplifies genetic diversity by homologous recombination using protoplast fusion within the selected mutant populations. The selected mutants were subjected to two rounds of protoplast fusion. Therefore, individual protoplasts were prepared and their numbers were counted, and then, all of them were pooled together with equal number of each. The five selected mutants (D5, D6, D13, E3 and CC20) as starting populations for the first round of genome shuffling showed improved cellulases activity between 155.8% to 194%, 291.6 to 358.3% and 433.3 to 500% more than the wild type *T. virens* AG2 for CMCase, FPase and β-glucosidase, respectively (Tab. 2 and Fig. 6).

After the first round of genome shuffling, 86 colonies were selected for fermentation analysis compared with the wild type *T. virens* AG2 and the highest parent (D6) of the first round of genome shuffling. Fusants R1/1, R1/3, R1/28, R1/50 and R1/55 exhibited the most improved productivity of cellulases as illustrated in Fig. (6) ranging from 232.3% to 250.0%, 433.3% to 500.0% and 600.0% to 633.3% more than the original *T. virens* AG2 strain for CMCase, FPase and β-glucosidase, respectively. While on the same time, those fusants showed higher cellulase activities ranging from 119.6% to 128.7%, 120.9% to 139.5% and 120.0% to 126.6% more than the highest parent (D6) for CMCase, FPase and β-glucosidase, respectively. Among them, the highest increase for CMCase, FPase and β-glucosidase was found by the recombinants R1/3 and R1/28 (Fig. 6). The highest five recombinants -based on cellulase activities obtained after the first round of genome shuffling, were pooled together and used as parental populations for the second round of genome shuffling. The second round of genome shuffling yielded also a library of 86 fusants, which were screened for cellulases productivity in submerged medium. The highest increase of cellulase activities was found in the recombinants R2/11 and R2/52 (Fig. 6). Their production reached 10.5 and 10.2 U/ml for CMCase, 7.6 and 7.2 U/ml for FPase and 10.8 U/ml for β-glucosidase. These values showed increase by 308.8 and 300% for CMCase, 633.3 and 600% for FPase and 720% for β-glucosidase, in comparison with the original strain, *T. virens* AG2. In the same time, these values showed increase by 123.5 and 120% for CMCase, 126.6 and 120% for FPase and 113.6% for β-glucosidase, in comparison with the highest parental fusants, (R1/3 and R1/28).

In this study, extensive improvement in cellulases production was evident after two rounds of genome shuffling. Genome shuffling is based on the genome recombination without the knowledge of detailed genome information. The different genes, which may be associated with production, can be recombined over several rounds of genome shuffling (**Leja *et al.* 2011**). The recombinants obtained after genome shuffling in this study showed substantial improvement in cellulase activities as shown in Figure (6). **Hida *et al.* (2007)** achieved rapid improvement of hydroxycitric acid production after three rounds of genome shuffling in *Streptomyces* Sp. U121. Moreover, **Lin *et al.* (2007)** after two rounds of genome shuffling isolated several fusants. The lipase activity of shuffled strains was increased

317% over the starting strains. On the other hand, **Cheng et al. (2009)** were the first to report the use of this technique in fungi for cellulases improvement. They aimed to improve the cellulase production of the industrial catabolite-repression-resistant strain *Penicillium decumbens* JU-A10 with its mutants by genome shuffling. After two rounds of genome shuffling, they obtained three fusants with 100%, 109% and 94% increase in FPase activity than the original strain. In addition, **El-Gendy and El-Bondkly (2011)** obtained similar results when applied the genome shuffling technique to the marine derived bacterium *Nocardia* sp. ALAA 2000 strain to achieve rapid improvement of ayamycin production. After three rounds of genome shuffling, they succeeded to obtain a superior strain exhibited an improved ayamycin yield. Strain F3/22 yielded 285 µg/ml of ayamycin which was 19-fold higher than that of the initial strain and 1.9-fold higher than parental mutants. Furthermore, the genome shuffling technique was applied successfully in the marine-derived fungus *Aspergillus* Sp. NRCF5 for improving xylanase activity**.** After the application of UV irradiation, nitrosoguanidine and ethidium bromide as mutagens to construct starting mutants population, five mutants were selected as parental population for the first round of genome shuffling. After four rounds of shuffling, a high xylanase-producing recombinant, R4/31, was obtained, which produced 427.5 U/ml xylanase. This value was 6.13-fold higher than that of the starting strain NRCF5 and 2.48-fold higher than that of the parental strain **(El-Bondkly, 2012)**. **Xu *et al.* (2012)** applied genome shuffling technique to improve the cellulase activities of the wild-type strain *Trichoderma viride* TL- 124. After two rounds of genome shuffling, strain *T. viride* F161 exhibited a total cellulase activities of 1.97-fold higher than that of wild-type strain *T. viride* TL-124.

Polymorphism of genomic population by RAPD analysis

Genetic variation was expected to occur in the course of mutagenic treatments and genome shuffling as well as using the random amplified polymorphic DNA technique will reveal these genetic variations. To detect if the mutagens used in this study caused changes in the genetic information of the obtained mutants (D5, D6, D13, E3 and CC20), random amplified polymorphic DNA analysis (fingerprint) was carried out compared with the wild type *T. virens* AG2 using the primers OPG-10 and OPZ-01 (Fig. 7). The DNA fragment patterns revealed clear differences among the selected isolates and the original *T. virens* AG2 strain. The isolates D5 and D6 showed DNA fragments at molecular size of 588 bp when primer OPG-10 was used for the amplification; while this fragment was absent in the wild type *T. virens* AG2 and the other selected isolates. Furthermore, the amplification with primer OPG-10 resulted in another fragment with molecular size of 510 bp in case of isolate D13; while it was absent in the the wild type *T. virens* AG2 and the other selected isolates. Moreover, a DNA fragment with molecular size of 220 bp resulted from the amplification with primer OPZ-01 in the wild type *T. virens* AG2, while it was absent in all of the selected isolates. Results obtained from the RAPD analysis confirmed that there were genetic changes among selected isolates after mutagenic treatments. These results clearly indicated that we have achieved the diversification required for completing the genome shuffling technique.

To confirm that there were changes due to the genome shuffling, RAPD analysis was carried out using the wild type *T. virens* AG2 and the shuffled strains R2/11, R2/41, R2/51 and R2/52 of the second round of genome shuffling using primers OPB-02, OPB-04 and OPB-14. DNA banding patterns showed clear differences among the parental and the shuffled strains as illustrated in Figure (8). Based on the RAPD profile with primer OPB-02, results revealed the absence of a DNA fragment with molecular size of 434 bp in the case of the wild type *T. virens* AG2, and its presence in all of the four selected shuffled strains. Moreover, the results revealed the presence of a DNA fragment with molecular size of 274 bp in the wild type *T. virens* AG2, and its absence in all of the four selected shuffled strains (Fig. 8A). Furthermore, analysis of the DNA fragment patterns with primer OPB-04, revealed the presence of five unique DNA fragments with molecular sizes of 1350, 1235, 750 and 530 bp in the wild type *T. virens* AG2, and their absence in all of the four selected shuffled strains (Fig. 8B). Moreover, the results revealed the presence of two unique DNA fragments with molecular sizes of 835and 350 bp in the case of the shuffled strain R2/11, and their absence in some of the other three selected shuffled strains along side with the wild type *T. virens* AG2. (Fig. 8B). Furthermore, analysis of the DNA fragment patterns with primer OPB-14 revealed the presence of one unique band with molecular size of 720 bp with strain R2/11 that was absent in all of the other three selected shuffled strains along side with the wild type *T. virens* AG2 (Fig. 8C). However, this primer did not reveal any detectable differences in banding patterns among the other three shuffled strains (R2/41, R2/51 and R2/52) or between them and the wild type *T. virens* AG2. (Fig. 8C). These results indicated that with all of the three used RAPD primers, the shuffled strain R2/11 exhibited differences in the DNA banding pattern when compared with the wild type and the other shuffled strains. In addition, the DNA banding patterns were similar among the shuffled strains R2/41, R2/51 and R2/52 for all three used primers, while there were differences between them and the wild type *T. virens* AG2, except for primer OBP-14. The remarkable differences between fragment patterns of the wild type and shuffled strains can be explained due to

powerful mutagens applied in this study and the recombination process of genome shuffling.

After treatment of *T. citrinoviride* with ethyl methyl sulfonate (EMS) and ethidium bromide (EtBr), **Chandra et al. (2008)** used the RAPD technique and reported the appearance of unique DNA banding patterns in parental and mutant strains obtained. Such results confirmed the genetic changes in mutated strains after mutagenic treatments. **Xu et al. (2012)** used the RAPD technique and also reported the presence of unique DNA banding patterns in parental and shuffled strains of *T. viride* after two rounds of genome shuffling.

Table (1): Names and sequences of the used primers

Primer Name	Primer Sequence 5` ⟶ 3`
ITS1	TCC GTA GGT GAA CCT GCG
ITS4	TCC TCC GCT TAT TGA TAT GC
OPB-02	TGA TCC CTG G
OPB-04	GGA CTG GAG T
OPB-14	TCC GCT CTG G
OPG10	AGG GCC GTC T
OPZ-01	TCT GTG CCA C

Operon random primers/ Operon technologies- California, USA.

Table (2): Comparison between the highest obtained mutants after all mutagenic treatments with the original strain T. virens AG2 for cellulase activities.

Isolate	CMCase		FPase		β-glucosidase	
	U/ml	% from W.T.	U/ml	% from W.T	U/ml	% from W.T
W.T	**3.4**	**100**	**1.2**	**100**	**1.5**	**100**
B 35	4.1	120.5	2.8	233.3	6.0	400.0
B 40	5.0	147.0	2.8	233.3	6.0	400.0
C 12	4.2	123.5	2.7	225.0	6.0	400.0
D 5*	6.1	179.4	3.5	291.6	6.5	433.3
D 6*	6.6	194.0	4.3	358.3	7.5	500.0
D 12	5.2	153.0	2.7	225.0	6.0	400.0
D 13*	6.0	176.4	3.6	300.0	6.5	433.3
D 14	5.2	153.0	3.4	283.3	4.0	266.6
E 3*	6.4	188.0	4.0	333.3	7.0	466.6
E 14	5.0	147.0	3.3	275.0	3.0	200.0
E 19	5.2	153.0	2.2	183.3	6.2	413.3
E 20	5.3	155.8	3.4	283.3	6.2	413.3
E 21	5.1	150.0	1.8	150.0	6.0	400.0
E 23	5.4	158.8	3.6	300.0	4.5	300.0
E 32	5.9	173.5	3.5	291.6	6.6	440.0
E 36	5.3	155.8	3.3	275.0	3.0	200.0
E 37	5.4	158.8	3.4	283.3	6.3	420.0
NTA 23	5.4	158.8	3.6	300.0	6.3	420.0
NTA 25	3.7	108.8	3.7	308.3	6.0	400.0
NTA 40	4.3	126.4	3.7	308.3	6.0	400.0
NTA 41	5.3	155.8	3.4	283.3	6.2	413.3
NTB 4	4.4	129.4	2.9	241.6	6.0	400.0
NTB 17	4.1	120.5	2.6	216.6	6.0	400.0
AA 15	5.3	155.8	3.1	258.3	1.8	120.0
BB 69	5.3	155.8	1.9	158.3	4.5	300.0
BB 71	5.2	153.0	3.4	283.3	4.6	306.6
CC 20*	5.3	155.8	4.2	350.0	6.8	453.3

***Five hupercellulotyic mutants were used in the first round of genome shuffling.**

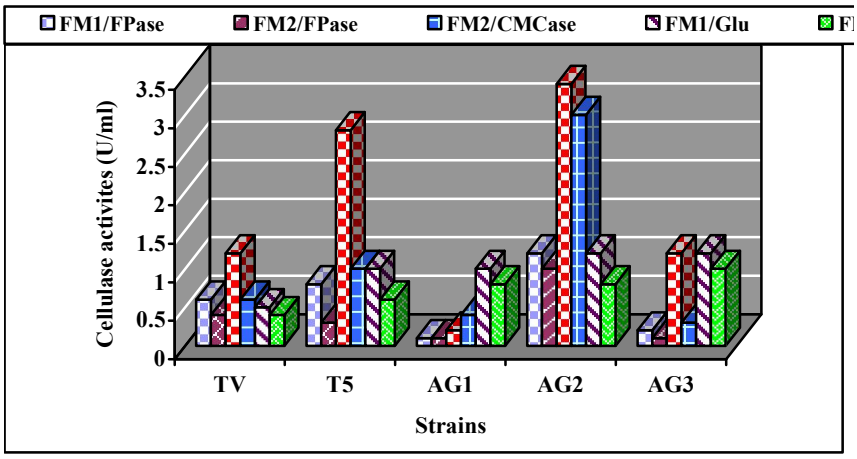

Fig. (1): Cellulases productivity (U/ml) of the obtained *Trichoderma* strains under two different fermentation media (FM1 and FM2).

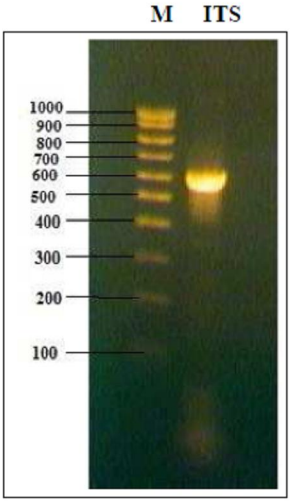

Fig. (2): Agarose gel showing the PCR amplified band of the ITS region (ITS1, ITS2, and 5.8S), M; DNA 100 bp ladder as marker.

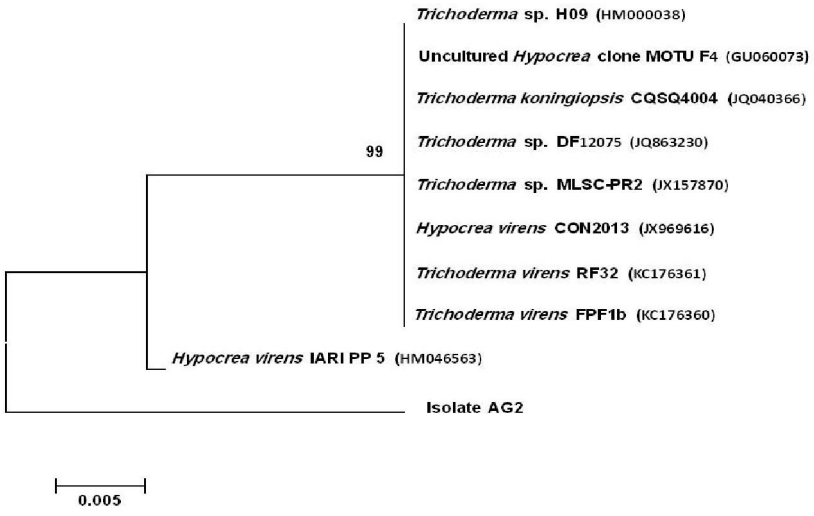

Fig. (3): Phylogenetic tree of ITS sequences in *Trichoderma* Sp. AG2 sequences were retrieved from GenBank and aligned in MEGA5 software.

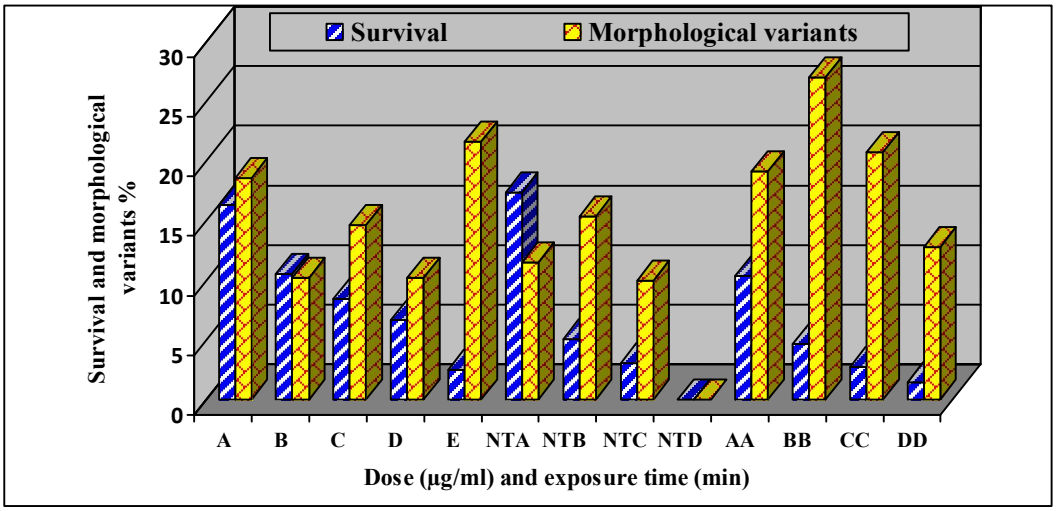

Fig. (4): Survival and morphological variants rates of *T.virens* AG2 after application of different mutagenic treatments for different concentrations and exposure times. A, B, C, D and E (5, 10, 15, 20 and 25 min UV exposure time, respectively); NTA, NTB, NTC and NTD (250µg/ml/30 min, 250µg/ml/60 min, 500µg/ml/30 min and 500µg/ml/60 min NTG, respectively); AA, BB, CC and DD (250µg EtBr/150µg NTG/ml/30 min, 250µg EtBr/150µg NTG/ml/60 min, 5 min UV/150µg NTG/ml/30 min and 5 min UV/150µg NTG/ml/60 min, respectively).

Table (3): Distribution and ranges of cellulase activity (clear zone ranges) obtained after different mutagenic treatments

Isolates class	Clear zone range	No. and % of obtained isolates	UV exposure time (min.)					NTG concentration			Combination			
								250 µg/ml	250 µg/ml	500 µg/ml	EtBr 250 µg/ml+ NTG 150 µg/ml		UV 5 min. + NTG 150 µg/ml	
			5	10	15	20	25	30 min.	60min.	30 min.	30 min	60 min	30 min	60 min
A	0.0	91 (15.0%)	1	0	0	6	7	4	2	6	25	27	8	5
B	1-1.09	13 (2.1%)	8	4	1	0	0	0	0	0	0	0	0	0
C	1.1-1.19	159 (26.3%)	22	13	18	8	2	20	8	27	14	10	6	11
D*	1.2-1.29	208 (34.4%)	10	17	16	17	8	21	27	17	12	26	20	17
E	1.3-1.39	74 (12.2%)	2	12	3	8	5	4	0	7	13	7	8	5
F	1.4-1.49	32 (5.2%)	0	2	2	7	7	2	1	2	1	2	6	0
G	1.5-1.59	9 (1.4%)	0	0	1	2	4	0	0	0	0	0	2	0
H	1.6-1.69	12 (1.9%)	0	1	0	1	3	1	1	1	0	1	2	1
I	1.7-1.79	1 (0.2%)	0	0	0	0	0	0	0	0	1	0	0	0
J	1.8-1.89	1 (0.2%)	0	0	0	0	1	0	0	0	0	0	0	0
K	1.9-1.99	2 (0.3%)	0	0	0	0	0	0	0	0	0	1	1	0
L	2-2.09	2 (0.3%)	0	0	0	0	0	0	0	0	2	0	0	0
Total		604	43	49	41	49	37	52	39	60	68	74	53	39
			219					151			234			

* Including original strain which produced 1.2 in the form of clear zone range.

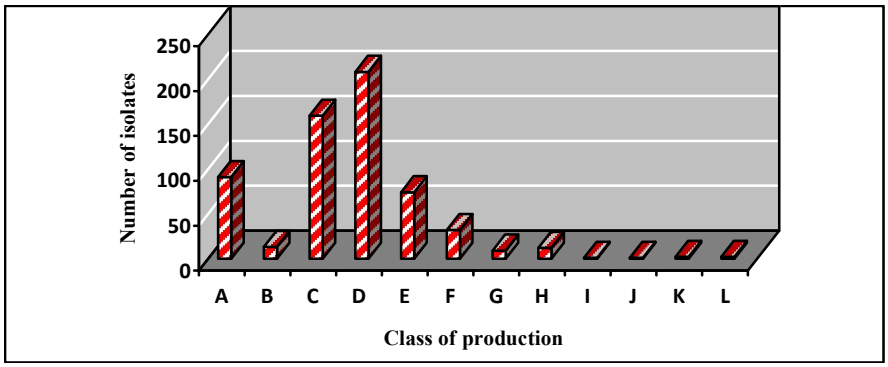

Fig. (5): A histogram representing classes of cellulases production in the form of clear zone.

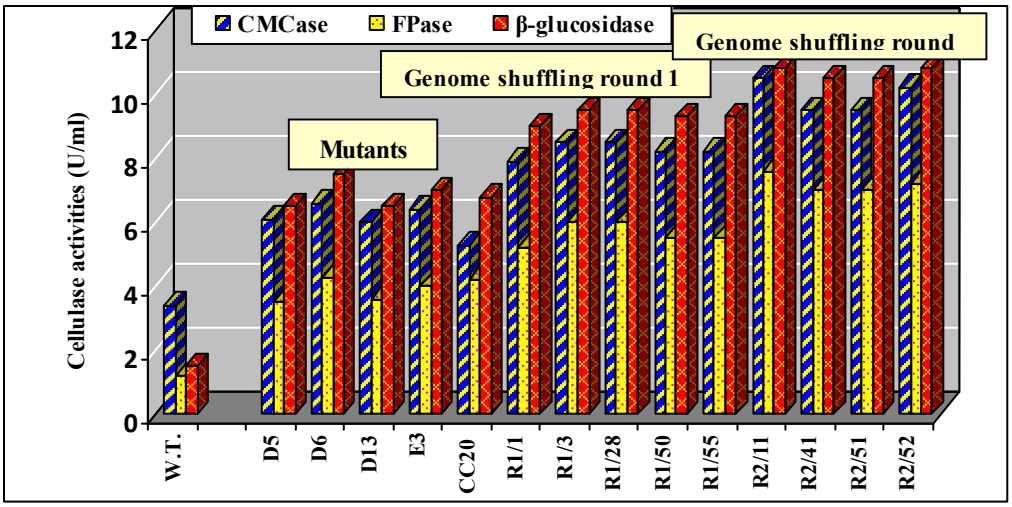

Fig. (6): Genetic improvement of cellulase activities through induction of mutation with different mutagenic treatments followed by two rounds of genome shuffling compared to the original strain *T. virens* AG2.

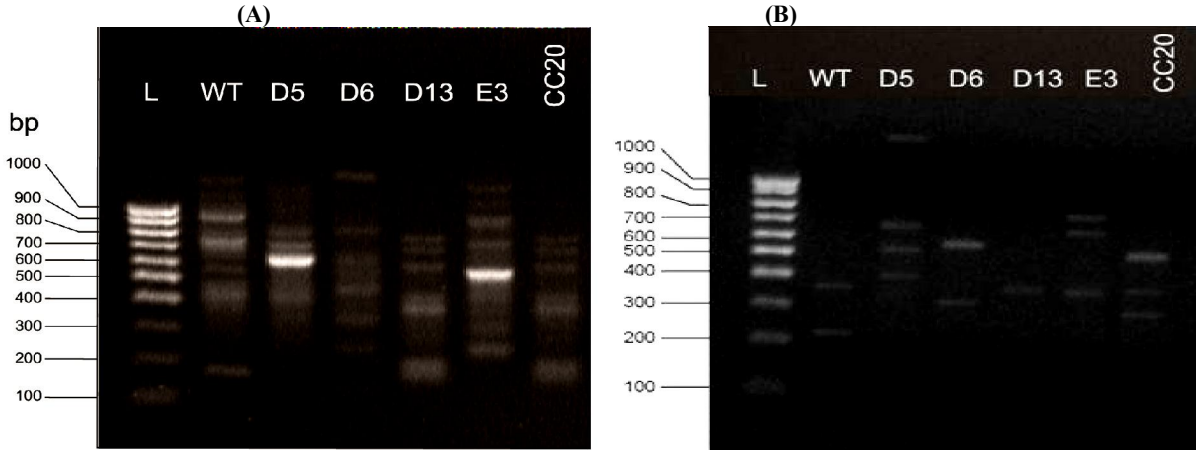

Fig. (7): Agarose gel showing the banding patterns using primers OPG-10 (A) and OPZ-01 (B) of the selected isolates after mutagenic treatments in comparison with the original strain *T. virens* AG2 (WT).

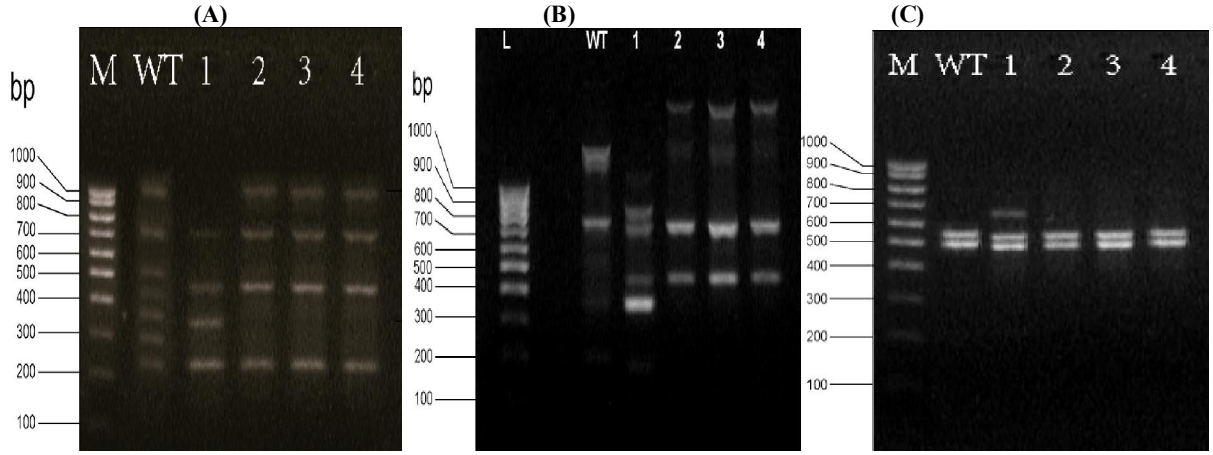

Fig. (8): Agarose gel showing DNA banding patterns of selected shuffled strains after the second round of genome shuffling. A, B and C are primers OPB-02, OPB-04 and OPB-14, respectively. Lanes: (M) 100 bp ladder; (WT) wild type; (1) R2/11; (2) R2/41; (3) R2/51 and (4) R2/52 shuffled strain.

Conclusion

After application of all mutagenic treatments, five isolates D5, D6, D13, E3 and CC20 proved to have the highest cellulases productivity. The isolates D5, D6 and D13 were isolated after UV irradiation time of 20 min, E3 was isolated after UV irradiation time of 25 min. and isolate CC20 was isolated after the combination of UV+NTG /30 min. These five isolates were selected as starting populations for the first round of genome shuffling which yielded five isolates (R1/1, R1/3, R1/28, R1/50 and R1/55) showed the highest improve for cellulase activities and used for the second round of genome shuffling. After the second round of genome shuffling , significant increase in cellulase activities was appeared by fusants (R2/11, R2/41, R2/51 and R2/52) when compared to the wild type strain. Based on the RAPD analysis, the selected mutants and shuffled strains showed differences in DNA fragment patterns when compared to the wild type, indicating that the genetic information had been altered or transferred from the parental strains to shuffled strains by genome shuffling and that the genetic information of the shuffled strains had been changed. Results obtained in this study clearly demonstrated that the whole genome can be efficiently improved by genome shuffling for the enhancement of the cellulase activities.

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