Mycologia Iranica 5(2): 53 – 61, 2018 Original Article DOI: 10.22043/mi.2019.118826

Enhancement of Xylanase Production by Protoplast Fusion of *Trichoderma* spp.

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Abstract: Filamentous fungi *Trichoderma* spp. are efficient hyper-producers of industrially important enzymes. Xylanases are essential hemicellulases having vast ranges of applications in the various industrial sectors. In this study, the protoplast fusion of two *T. virens* and *T. harzianum* strains resulted in 2.5 Uml^{-1} xylanase activity in fusant X3 showing 4.7-fold improvement in xylanase activity compared to that of parents with 0.54 Uml^{-1}. Moreover, to evaluate the influence of protoplast fusion on the xylanase activity, the expression patterns of the xylanase gene *xyn3* was analyzed in the parental strains and the using qPCR. The results demonstrated that the relative expression of the *xyn3* increased in X3 by 4.9 fold compared to that of the parents. Finally, based on the results, it could be concluded that the protoplast fusion technology is a promising approach to generate new superior fungi with high production ability of the industrially important enzymes.

Key words: Fungi, hemicellulases, enzyme activity, qPCR

INTRODUCTION

Lignocellulosic biomass is the largest source of organic materials which its conversion into fermentable sugars conducted by holocellulase enzymes, including cellulases and hemicellulases (Andreaus et al. 2008; de Siqueira et al. 2010; Song et al. 2016). Xylanase is one of the most important hemicellulases (EC3.2.1.8) catalyzing the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan, a noteworthy hemicelluloses component of lignocellulosic biomass (Kubata et al. 1994; Dashtban et al. 2009). Xylan is the second most abundant natural polysaccharide composed of β-xylopyranose residues, and its complete hydrolysis requires the action of several enzymes, including endo-1,4-β-xylanase (Polizeli et al. 2005). Xylanases have absorbed great attention due to their potential applications in the fermentation of biomass for the biofuel production, pulp and paper bleaching, improving digestibility in animal feed, juices processing in food industry and biopolishing of fabrics in textile industry (Bhat 2000; Beg et al. 2001; Harris and Ramalingam 2010; Goswami and Rawat 2015).

Fungi are the striking source of xylanases which have been extensively studied for their xylanolytic systems (Polizeli et al. 2005; van den Brink and de Vries 2011). *Trichoderma* strains are non-pathogenic filamentous fungi which are capable of producing a high yield of extracellular xylan degrading enzymes (Kar et al. 2006; Silva et al. 2015). Consequently, a great deal of effort is devoted to the strain improvement of *Trichoderma* for producing more industrial enzymes. Several studies demonstrated that genetic manipulation of fungal strains using protoplast approach could provide achieving strains with better characteristics (Dillon et al. 2008; Gong et al. 2009; Cui et al. 2014). Protoplast fusion technology can generate novel combinations of genes leading to the improvement of different character-

Submitted 8 Oct. 2018, accepted for publication 24 Dec. 2018
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ristics of a microorganism (Patil et al. 2015). Protoplast fusion is an effective tool resulting in genetic recombination to develop superior strains in filamentous fungi (Prabavathy et al. 2006). Since *Trichoderma* lacks natural mechanism for recombination of genetic material, protoplasts fusion provides an effective condition to facilitate heterokaryon formation even across species resulting in the development of interspecific and intergenetic hybrids (Savitha et al. 2010).

Holocellulases present common regulatory system in *Trichoderma*, although there are some differences between cellulases and xylanases gene expression regulation (Stricker et al. 2008; Kubicek et al. 2009; Seiboth et al. 2011). Due to the distribution of glycoside hydrolases encoding genes at different loci within the genome of *Trichoderma*, a comparative study of parents and fusants in molecular level could be informative to determine the recombination characterization (Häkkinen et al. 2012).

The objective of the present study was to achieve *Trichoderma* strains with improved xylanase production ability by protoplast fusion technique. Xylanase activity in the fusants and parental strains were evaluated and after selection of the best xylanase producer, the expression patterns of the xylanase gene *xyn3* was evaluated in the parental strains and the selected fusant using quantitative PCR (qPCR) to figure out validity and success of protoplast fusion.

MATERIALS AND METHODS

Fungal strains and culture media

Sixty *Trichoderma* isolates were obtained from the Microbial Culture Collection of Agricultural Biotechnology Research Institute of Iran (ABRII). The strains were preserved on potato dextrose agar PDA (Fluka, Germany) at 4 °C. The medium composition for plate enzyme assay was as follows: 4 g/L, K_2HPO_4; 1 g/L $MgSO_4.7H_2O$; 0.2 g/L, KCl; 0.02 g/L, $FeSO_4 \cdot 7H_2O$; 5 g/L, peptone; 1 g/L, yeast extract; 5 g/L, Oat-spelt xylan; 15 g/L, Agar; pH 7. Protoplast regeneration medium composition was as follows: 1 g/L, yeast extract; 1 g/L, N-Z amine A from bovine milk (Sigma, Germany); 1.6 M sucrose; 16 g/L, Agar. Top agar composition was 15 g/l, KH_2PO_4; 5 g/l, $(NH_4)_2SO_4$; 20 g/L, Glucose; 182.2 g/L, Sorbitol; 0.3 g/L, $MgSO_4.7H_2O$; 0.4 g/L, $CaCl_2$; 0.001 g/L, $FeSO_4.7H_2O$; 0.0008 g/L, $ZnCl_2$; 0.0005 g/L, $MnSO_4.H_2O$; 0.0005 g/L, $CoCl_2.6H_2O$; 30 g/L, Agar. Mandels and Reese medium (20 g/L, lactose; 1.4 g/L, $(NH_4)_2SO_4$; 0.3 g/L, urea; 1 g/L, proteose peptone; 2 g/L, KH_2PO_4; 0.3 g/L, $MgSO_4.7H_2O$; 0.3 g/L, $CaCl_2$; 0.001 g/L, $FeSO_4.7H_2O$; 0.0008 g/L, $ZnCl_2$; 0.0005 g/L, $MnSO_4.H_2O$; 0.0005 g/L, $CoCl_2.6H_2O$; pH 4.8) was used for xylanase production assay.

Plate screening for xylanase production

Xylan Agar plates were inoculated with 5 mm diameter mycelial discs from seven days old culture of sixty fungal isolates on PDA, and incubated at 28 °C under a dark condition for three days. Congo Red assay (2.5 g/L) was used to determine the xylanase activity. After 15 minutes of incubation with dye solution, the plates were washed using 1 M NaCl followed by distilled water. The diameter of halos produced by each isolate was measured and used as an indicator of hydrolysis ability (Neethu et al. 2012).

Molecular characterization of selected isolates

Total genomic DNA was extracted from eighteen hyper xylanase producing isolates according to the protocol previously described by Zhong and Steffenson (2001). The quantity of extracted DNAs was analyzed by a nanodrop system (Thermo2000c, USA). PCR amplification was performed for amplification of ITS-rDNA region in 20 µL reaction volume containing 0.2 unit/µL Taq DNA polymerase (Ampliqon, Denmark); 1.5 mM, $MgCl_2$; 0.2 µM of ITS-1 primer (5'-CGTAGGTGAACCTGCGG-3') and ITS-4 primer (5'-TCCTCCGCTTATTGATATGC-3') and 10 ng genomic DNA of each isolate. PCR amplification was carried out in a PeQSTAR thermocycler (Germany) programmed as follows: an initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s, and extension at 72 °C for 1 min, and the amplification was completed with one cycle of final extension at 72 °C for 5 min. PCR amplicons were resolved by agarose gel electrophoresis and afterward, the obtained sequences (Macrogen, South Korea) were aligned using MEGA 6 and sequence analysis was performed using *Trich*OKEY 2.0 available online at http://www.isth.info/.

Protoplast isolation

Conidial suspensions of two parental strains (G25-1 and G130-4) were prepared in 0.9% NaCl from seven days fresh plates on PDA. Approximately 10^6 spores/ml were inoculated on PDA plates covered by cellophane sheets, and 20 plates were prepared for each parental strain. Plates were kept at 28 °C for 16-18 hours.

The grown mycelium on cellophane sheets incubated with 50 ml of 1.2 M $MgSO_4$-10mM Na-phosphate buffer (pH 5.8) containing 10 mg/ml Lysing Enzyme (Sigma, "Gibb.Best", Germany), 5 mg/ml of Driselase (Fluka, Germany) and 0.1 mg/ml of chitinase from *Saccharomyces griseus* (Sigma, Germany) and incubated at 28 °C with 100 rpm shaking for 3 hours and the protoplast formation was monitored by hemocytometer. After the incubation, the digested mycelium was filtered through glass filter crucible (pore size 0 and 1) and collected protoplasts were washed with 0.6 M Sorbitol- 0.1 M Tris-HCl pH 7; 1.2 M Sorbitol; 10 mM Tris-HCl pH 7.5 and finally, the pellets were

resuspended in 1 ml of STC buffer (1.2 M of Sorbitol; 10 mM of Tris-HCl pH 7.5, 10 mM of $CaCl_2$). The amount of protoplast was counted with a hemocytometer.

Protoplast fusion

Protoplast fusion of the parental strains were carried out through Stasz et al., protocol with minor modifications (Stasz et al., 1988). Equal number of each parental protoplast (10^6 protoplasts/ml) was mixed with an equal volume of 40% polyethylene glycol (PEG) (MW8000, Sigma, USA) prepared in STC buffer (0.6 M of Sorbitol; 10 mM of Tris-HCl pH 7, 10 mM of $CaCl_2$), and incubated at 28 °C for 10 min. After adding an equal volume of STC buffer, the tubes were gently inverted and centrifuged for 10 min at 100 rpm. This step was repeated; and finally, the colorless pellet was resuspended in 150 µl of STC buffer.

Protoplast regeneration

The fusion mixture was incubated on regeneration plates using the top agar method (Penttilä et al. 1987). Regeneration plates were kept at 28 °C, and colony formation was considered.

Enzyme assay of fusants

Qualitative xylanase assay: The number of seventeen regenerated colonies was transferred to PDA plates and the qualitative assay was carried out using plates containing xylan and 0.1 % Triton™ X-100 (Merck, Germany) and Congo Red method as previously described. Sampling selection was conducted based on halos formation size.

Quantitative xylanase assay: Quantitative xylanase assay was performed by XylX6 assay kit (K-XylX6-2V, Megazyme, Ireland) according to the manufacture procedure. One unit of activity is defined as the amount of enzyme required to release one micromole of 4-nitrophenol from K-XylX6-2V in one minute under the defined assay conditions.

Protein measurement of fusants

The extracellular protein content was estimated using Bradford method (1976) and bovine serum albumin was used as a standard protein. Additionally, specific enzyme activity was calculated by dividing the number of Units/ml by the protein concentration in mg/ml to get Units/mg.

RNA extraction and quantitative PCR

Total RNA extracted from mycelium of seven days old culture of parental strains G25-1 and G130-4, and fusants in Mandels and Reese medium with TRIzol reagent (Merck, Germany). The quantity and quality of the isolated total RNA were measured by nanodrop and gel electrophoresis. First strand cDNA was synthesized using SuPrimeScript cDNA Synthesis kit (GeNet Bio, Korea) according to the manufacture procedure. The concentration of 500 ng total RNA with an anchored-oligo $(dT)_{20}$ and random hexamer primer were applied for cDNA synthesis. Gene expression was monitored by qPCR in 10 µl volume containing 5 ng of cDNA, 5 µl of SYBR green premix (Ampliqon, Denmark) with 50 mM of calibration ROX dye, and 0.2 µM of *xyn3* specific primer (xyn3 F 5'-GTACAAGGGCAAGATTCG-3', xyn3 R 5'-AAGGCAATCGAGACAAACT-3') (Rahman et al. 2009). Thermal cycling was perfor-med using StepOnePlus™ Real-Time PCR System (ABI, USA) followed by melting curve analysis with the following cycling program: 15 min at 95 °C, followed by 40 cycles of 15s at 94 °C, the 30s at 58 °C and 20s at 72 °C. The CT data achieved were analyzed by the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$) for relative quantification (Livak et al. 2001). The relative expression ratio was normalized by the actin housekeeping gene. All the experiments were conducted in triplicates, and final data analysis was performed using Microsoft Office Excel 2007.

RESULTS AND DISCUSSION

Selection of high xylanase producing isolates

To identify high xylanase producing native *Trichoderma* isolates, plate screening technique was conducted based on the formation of clear zones by each fungal isolate. According to Ten et al. (2004), the measurement of halo zone is a useful method for selection of strains with efficient capability of polysaccharides degradation. Therefore, a total number of eighteen promising isolates showing maximum clear zones were selected for next in detailed experiments (Fig. 1). Seven isolates, including G104, K2-G139-2, G130-4, G146-5, E(18-10)6, T 7-3 and Mtree1 could provide completely clear plates by degradation of all xylan substrate in the plates (Fig 1). Neethu et al (2012) identified *T. viride* strains with higher cellulase, hemicellulase and ligninase activity based on plate screening method.

Species identification of the selected isolates

An approximately 400 bp of 5.8S-ITS rDNA fragment was successfully amplified in all eighteen isolates and sequenced for *Trichoderma* identification (Fig. 2). Sequence analysis of ITS1-5.8S-ITS2 region is widely used for precise identification of *Trichoderma* species (Druzhinina et al. 2005). Using bioinformatics tools, two different species, including *T. virens* and *T. harzianum* were identified among the selected isolates (Table 1). Parental selection for protoplasting was designed to choose two different species allowing more genetic polymorphism exchange.

Quantitative xylanase assay and selection of parental strains

The quantitative xylanase assay was performed for all the eighteen selected strains by xylanase assay Megazyme kit which measured endo-Xylanase

activity. Two strains including *T. virens* G25-1 and *T. harzianum* G130-4 with the activity of 0.0568 Uml^{-1} and 1.12 Uml^{-1}, respectively, showed the maximum xylanase activity. These strains were selected as the parents for protoplast fusion, because they showed the highest xylanase activity and moreover, they were two different *Trichoderma* species, so that the creation of genetic combinations could be more accessible. Meanwhile, the lactose 2% was applied as an inducer for xylanase production, since it induces both cellulase and xylanase production pathways (Xiong et al. 2004). XYR1 is a xylanase regulator that regulates lactose metabolism in *T. reesei*, and subsequently, lactose is able to induce xylanase production (Stricker et al. 2007; da Silva Delabona et al. 2017).

Protoplast isolation, fusion and regeneration

In this study, fusion of hyper xylanase producing protoplasts was conducted with the aiming of enhancement of xylanase productivity. Three hours incubation of parental mycelium with lysing enzymes resulted in cell wall lysis and protoplasts release (Fig. 3). The high molecular weight polymer (1000-6000) of PEG functions as molecular bridges connecting the protoplasts (Verma et al. 2008) culminating in the dissolving of plasma membranes at the place of contact that provide the protoplast fusion (Fig. 4). PEG concentration is a critical factor for an efficient protoplast fusion. Here, the concentration of 40% PEG was applied that was already reported as an efficient concentration for fusion of *Trichoderma* protoplasts (Prabavathy et al. 2006; Kowsari et al. 2014; Hassan 2014). Regeneration of fusants had an approximately yield rate of 10 colonies per plate.

Table. 1. Species identification of eighteen best xylanase producing *Trichoderma* isolates based on ITS-rDNA region sequencing

Isolate	Species
G25-1	*Trichoderma virens*
G104	*Trichoderma harzianum*
G118-1	*Trichoderma harzianum*
G130-4	*Trichoderma harzianum*
G135-1	*Trichoderma harzianum*
G136-2	*Trichoderma harzianum*
G146-5	*Trichoderma harzianum*
G147-6	*Trichoderma harzianum*
GL52-5	*Trichoderma harzianum*
E(18-10)6	*Trichoderma harzianum*
K2-G139-2	*Trichoderma harzianum*
Mtree1	*Trichoderma harzianum*
T 7-3	*Trichoderma harzianum*
1 Tricho S7	*Trichoderma virens*
2 Tricho S7	*Trichoderma harzianum*
Tri 2 S8	*Trichoderma* sp.
G5	*Trichoderma harzianum*
G108-1	*Trichoderma harzianum*

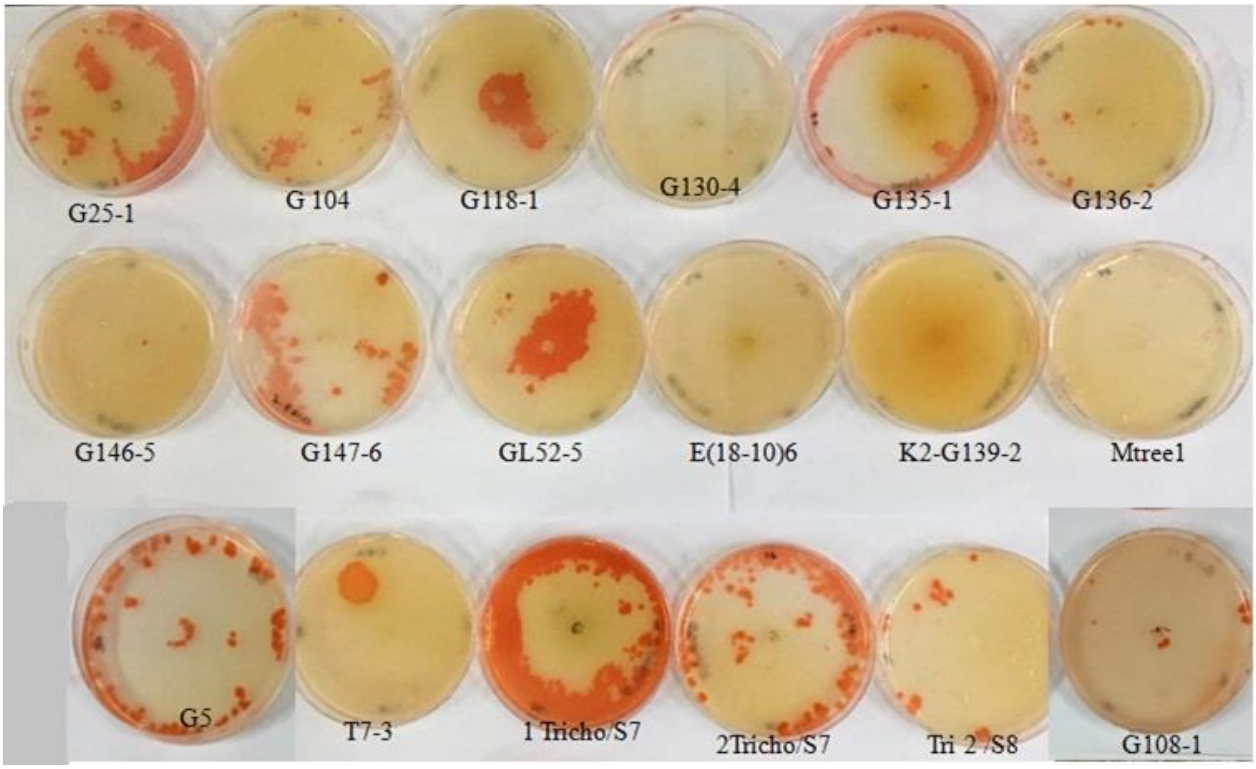

Fig. 1. Congo red assay of the eighteen selected isolates presenting highest ability to use xylan as a sole carbon source in plate medium after 3 days of incubation at 28°C.

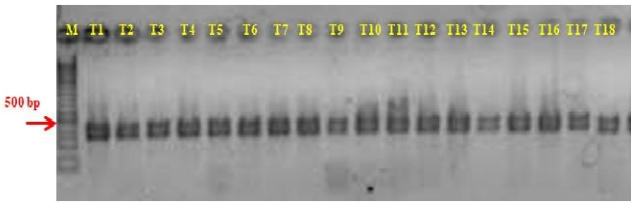

Fig. 2. Amplification of ITS rDNA fragment in the selected *Trichoderma* isolates. **M:** 1 kb DNA ladder, **T1-T18:** eighteen selected *Trichoderma* isolates.

Comparative xylanase activity and protein content in the parental strains and fusants

The regenerated colonies were transferred to the xylan plates to compare xylanase activity between the parents and fusants. Our results demonstrated that the percentage of halo formation in the parental strains including G25-1 and G130-4 were 50% and 65%, respectively, while that of the fusant named as X3 was 80%. Therefore, xylanase activity improvement was qualitatively confirmed.

Moreover, quantitative enzyme assay showed that xylanase activity was significantly enhanced in the fusant X3 (Fig. 5). Compared with the average enzyme activity of the parental strains, G25-1 and G130-4, with 0.54 Uml^{-1} xylanase activity, the X3 strain showed 2.5 Uml^{-1} of xylanase activity, with amount of 4.7 fold improvement. Holocellulase improvement through protoplast fusion has been reported by other researchers. Strain improvement of *T. reesei* xylanase was achieved through UV mutagenesis followed by protoplast fusion resulting in 3.1 fold increase in enzyme production over the wild type strain (Soroor et al. 2014). Total cellulase activity improvement was achieved through genome shuffling in *T. viride* with 1.97-fold higher enzyme activity in comparison with wild-type strain (Xu et al. 2012). Meanwhile, EL-Bondkly and Talkhan carried out intra-strain *T. harzianum* protoplast fusion resulting in two fold increase compared to the parental strains (EL-Bondkly and Talkhan 2007). Moreover, two fold increase in β-1,4-endoxylanase activity compared to the native strain was obtained through γ-ray induced mutagenesis in *Aspergillus niger* (Ottenheim et al. 2015).

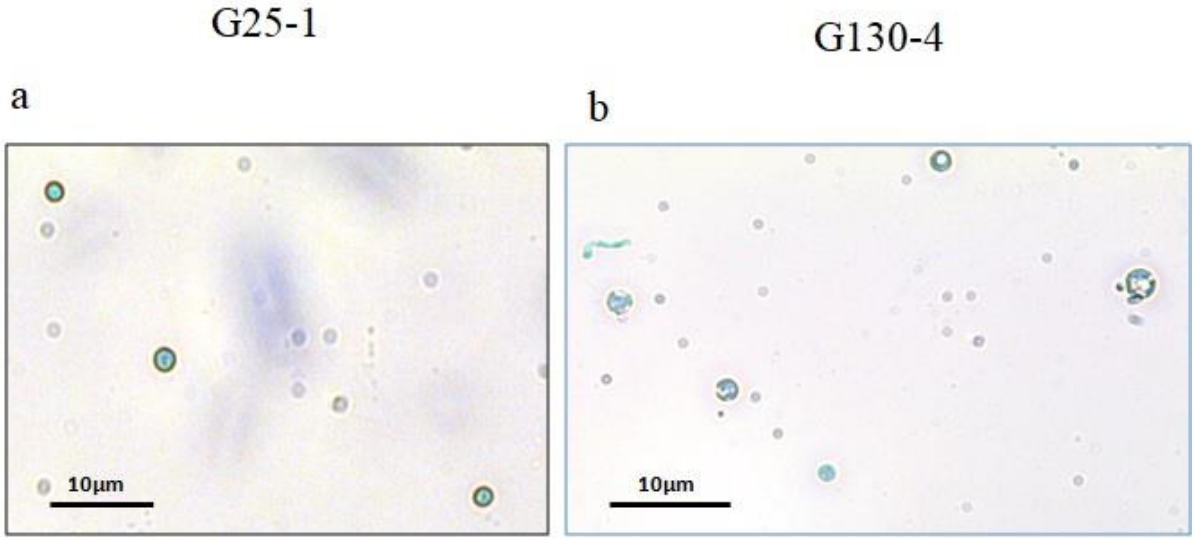

Fig. 3. The released protoplasts of the parental *Trichoderma* strains. a. *T. virens* G25-1, b. *T. harzianum* G130-4).

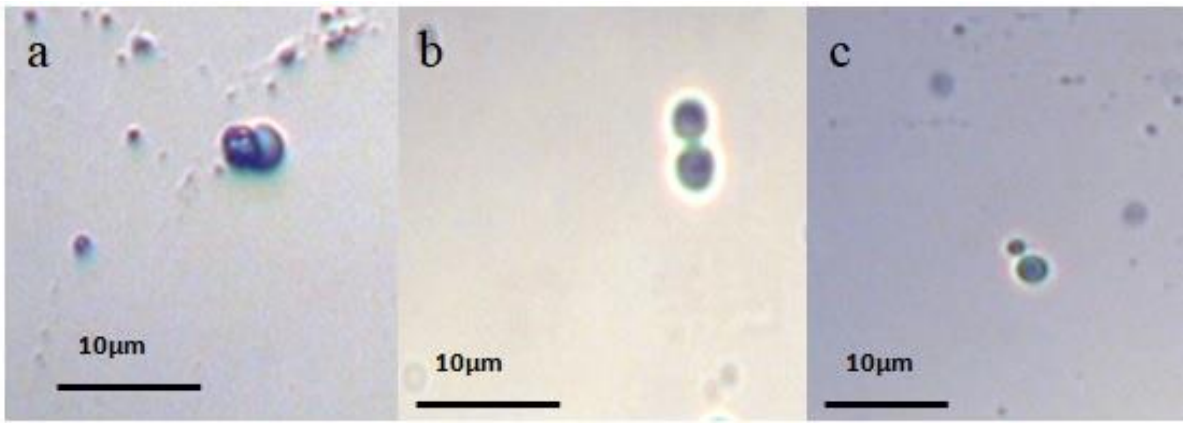

Fig. 4. a-c. Protoplast fusion monitoring of the parental strains *Trichoderma virens* G25-1 and *T. harzianum* G130-4 by sampling from fusogen solution during 10 minutes incubation of parental protoplasts with PEG at 28 °C.

The protein content in the obtained crude enzyme of the fusant X3, was 134.63 µg ml^{-1} as against 5.6 µg ml^{-1} in G25-1 and 109.33 µg ml^{-1} in G130-4 as parental strains (Fig. 6). Moreover, specific enzyme activities of the G25-1, G130-4 and X3 strains were 6.07, 9.63 and 18.88, respectively. These results clearly demonstrated that the fusant X3 not only has the ability of producing high protein levels, but also its enzymatic capability to convert substrate is higher than that of parents. Prabavathy et al. study of intra-strain protoplast fusion of *T. reesei* resulted in fusants formation with highest amount of 78 µg ml^{-1} protein content (Prabavathy et al. 2006).

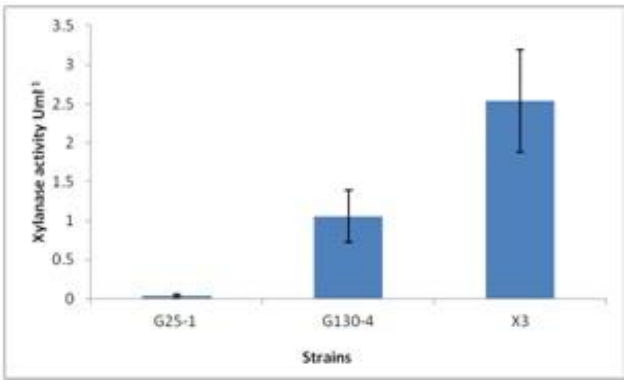

Fig. 5. Comparative analysis of xylanase production in the parental strains including *T. virens* G25-1 and *T. harzianum* G130-4 and the fusant X3. Averages from three replicates of each strain are shown with standard deviations.

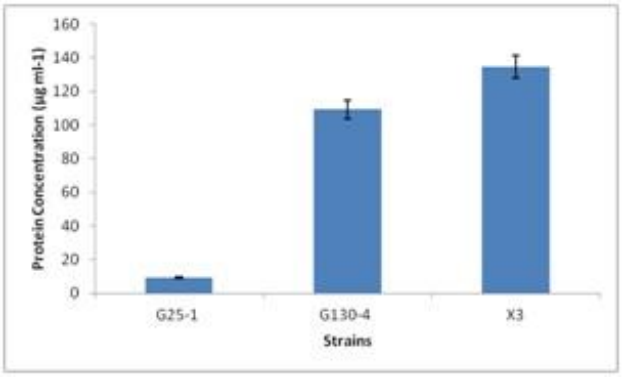

Fig. 6. Extracellular protein concentration of supernatants obtained from parents and fusants of *Trichoderma* after 7 days of spore inoculation in Mandels and Reese medium at 28 °C. Averages from three replicates of each strain are shown with standard deviations.

Gene expression analysis of the parental and fusants strains

To further validate the impact of protoplast fusion on the analyzed fusant, the expression level of the gene *xyn3* involved in xylanase activity was monitored by qPCR. In the present study, comparative expression analyses were conducted for each sample relative to the parent's average expression levels. Our expression analysis showed that protoplast fusion resulted in the up-regulation of *xyn3*. The results revealed that the relative expression of *xyn3* was increased in X3 by 4.9 fold in comparison with that of parents (Fig. 7). According to the findings, out of the five identified xylanolytic genes expressed by *Trichoderma* termed as *xyn1* to *xyn5* (Ramoni et al. 2017), *xyn3* plays a critical role in the conversion of xylan-containing biomass (Nakazawa et al. 2016; Rahman et al. 2009; Herold et al. 2013). Indeed, *xyn3* expression is closely related to the cellulase expression and is exclusively induced by typical cellulase inducers such as lactose (Xu et al. 2000). Additionally, it is reported that in *T. reesei* PC-3-7, the highest expression of xylanase genes in response to cellulosic carbon was attributed to *xyn3*. Therefore, expression analysis of this gene provided valuable data to consider the xylanase activity in the parental strains and relative fusants (Furukawa et al. 2008; Hirasawa et al. 2018). The results of expression analysis besides xylanase activity assays revealed that the obtained fusant X3 has the promising potential for industrial applications.

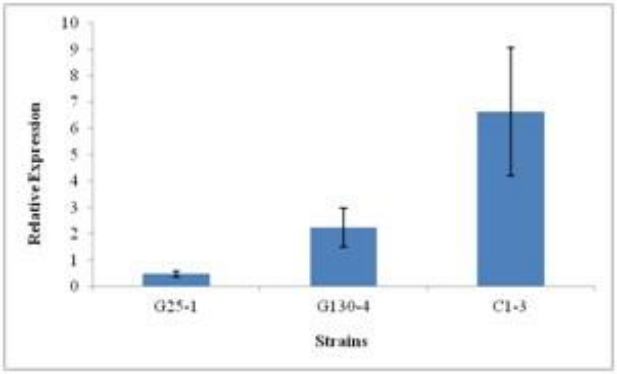

Fig. 7. Expression analysis of the gene *xyn3* in the *Trichoderma* strains. G25-1 and G130-4, as the parents and X3, as the selected fusant. Transcriptional levels for *xyn3* were analyzed by comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$). Transcriptional level of *actin* was used as an endogenous control.

Conclusion

In summary, the outcome of the current study demonstrated the possible employment of protoplast fusion between different species as a successful strategy to generate enzyme-super producing strains of filamentous fungi lacking inherent sexual reproduction. The higher xylanase activity of the X3 confirmed through enzymatic activity assays, and the expression analysis of *xyn3* provided a firm evidence for our claim to apply this technology in industry triggering the high level of the interest enzymes.

ACKNOWLEDGEMENTS

We thank Prof. Javier Avalos and Genetic department of University of Seville, Spain, for their kind supports.

REFERENCES

Andreaus J, Ferreira Filho EX, Bon EPDS. 2008. Biotechnology of holocellulose–degrading enzymes. In: Biocatalysis and Bioenergy. (CT Hou & JF Shaw): 197–229. John Wiley and Sons Inc., New Jersey, USA.

Beg Q, Kapoor M, Mahajan L, Hoondal GS. 2001. Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56: 326–338.

Bhat MK. 2000. Cellulases and related enzymes in biotechnology. Biotechnol Adv 18: 355–383.

Bradford, MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72: 248–254.

Cui YX, Liu JJ, LiuY, Cheng QY, Yu Q, Chen X, Ren XD. 2014. Protoplast fusion enhances lignocellulolytic enzyme activities in Trichoderma reesei. Biotechnol Lett 36: 2495–2499.

da Silva Delabona P, Rodrigues GN, Zubieta MP, Ramoni J, Codima CA, Lima DJ, Seiboth B. 2017. The relation between xyr1 overexpression in Trichoderma harzianum and sugarcane bagasse saccharification performance. J Biotechnol 246: 24–32.

Dashtban M, Schraft H, Qin W. 2009. Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 5:578–595.

de Siqueira FG, de Siqueira EG, Jaramillo PMD, Silveira MHL, Andreaus J, Couto FA, Batista LR, Ferreira Filho EX. 2010. The potential of agro–industrial residues for production of holocellulase from filamentous fungi. Int Biodeterior Biodegradation 64: 20–26.

Dillon AJP, Camassola M, Henriques JAP, Fungaro MHP, Azevedo ACS, Velho TAF, Laguna SE. 2008. Generation of recombinants strains to cellulases production by protoplast fusion between Penicillium echinulatum and Trichoderma harzianum. Enzyme Microb Technol 43:403–409.

Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakacs G, Kubicek CP. 2005. An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. Fungal Genet Biol. 42: 813–828.

El–Bondkly AM, Talkhan FN. 2007. Intra–strain crossing in Trichoderma harzianum via protoplast fusion to enhance chitinase productivity and biocontrol activity. Arab J Biotechnol 102:233–240

Furukawa T, Shida Y, Kitagami N, Ota Y, Adachi M, Nakagawa S, Ogasawara W. 2008. Identification of the cis–acting elements involved in regulation of xylanase III gene expression in Trichoderma reesei PC–3–7. Fungal Genet Biol 45:1094–1102.

Gong J, Zheng H, Wu Z, Chen T, Zhao X. 2009. Genome shuffling: progress and applications for phenotype improvement. Biotechnol Adv 27:996–1005.

Goswami GK, Rawat S. 2015. Microbial Xylanase and their applications–A review. Int J Curr Res Aca Rev 3:436–450.

Häkkinen M, Arvas M, Oja M, Aro N, Penttilä M, Saloheimo M, Pakula TM. 2012. Re–annotation of the CAZy genes of Trichoderma reesei and transcription in the presence of lignocellulosic substrates. Microb Cell Fact 11:1–26.

Harris AD, Ramalingam C. 2010. Xylanases and its application in food industry: a review. J Exp Sci 1:1–11

Hassan MM. 2014. Influence of protoplast fusion between two Trichoderma spp. on extracellular enzymes production and antagonistic activity. Biotechnol Biotechnol Equip 28:1014–1023.

Herold S, Bischof R, Metz B, Seiboth B, Kubicek CP. 2013. Prepared for the possible: xylanase gene transcription in Trichoderma reesei is triggered by different inducers representing different hemicellulosic pentose polymers. Eukaryot Cell EC.00182–12.

Hirasawa H, Shioya K, Furukawa T, Tani S, Sumitani JI, Kawaguchi T, Ogasawara W. 2018. Engineering of the Trichoderma reesei xylanase3 promoter for efficient enzyme expression. Appl Microbiol Biotechnol 102:2737–2752.

Kar S, Mandal A, Mohapatra PKD, Mondal KC, Pati BR. 2006. Production of cellulase–free xylanase by Trichoderma reesei SAX3. Braz J Microbiol 37:462–464.

Kowsari M, Motallebi M, Zamani RM. 2014. Construction of new GFP–tagged fusants for Trichoderma harzianum with enhanced biocontrol activity. J Plant Prot Res 54:122–131.

Kubata BK, Suzuki T, Horitsu H, Kawai K, Takamizawa K. 1994. Purification and characterization of Aeromonas caviae ME–1 xylanase V, which produces exclusively xylobiose from xylan. Appl Environ Microbiol 60:531–535

Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. 2009. Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. Biotechnol Biofuels 2:1–14.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real–time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Nakazawa H, Kawai T, Ida N, Shida Y, Shioya K, Kobayashi Y, Morikawa Y, et al. 2016. A high performance Trichoderma reesei strain that reveals the importance of xylanase III in cellulosic biomass conversion. Enzyme Microb Technol 82:89–95.

Neethu K, Rubeena M, Sajith S, Sreedevi S, Priji P, Unni KN, Sarath Josh MK, Jish VN, Paradeep S., Benjamin S. 2012. A novel strain of Trichoderma viride shows complete lignocellulolytic activities. Adv Biosci Biotechnol 3:1160–1166.

Ottenheim C, Werner KA, Zimmermann W, Wu JC. 2015. Improved endoxylanase production and colony morphology of Aspergillus niger DSM 26641 by γ–ray induced mutagenesis. Biochem Eng J 94:9–14.

Patil NS, Patil SM, Govindwar SP, Jadhav JP. 2015. Molecular characterization of intergeneric hybrid between Aspergillus oryzae and Trichoderma harzianum by protoplast fusion. J Appl Microb 118:390–398.

Penttilä M, Nevalainen H, Rättö M, Salminen E, & Knowles J. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 6: 155–164.

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: properties and industrial applications. Appl Microb Biotechnol 67:577–591.

Prabavathy VR, Mathivanan N, Sagadevan E, Murugesan K, Lalithakumari D. 2006. Intra–strain protoplast fusion enhances carboxymethyl cellulase activity in Trichoderma reesei. Enzyme Microb Technol 38:719–723.

Rahman Z, Shida Y, Furukawa T, Suzuki Y, Okada H, Ogasawara W, Morikawa Y. 2009. Application of Trichoderma reesei cellulase and xylanase promoters through homologous recombination for enhanced production of extracellular β–glucosidase I. Biosci Biotechnol Biochem 73:1083–1089.

Ramoni J, Marchetti–Deschmann M, Seidl–Seiboth V, Seiboth B. 2017. Trichoderma reesei xylanase 5 is defective in the reference strain QM6a but functional alleles are present in other wild–type strains. Appl Microb Biotechnol 101: 4139–4149

Savitha S, Sadhasivam S, Swaminathan K. 2010. Regeneration and molecular characterization of an intergeneric hybrid between Graphium putredinis and Trichoderma harzianum by protoplasmic fusion with some modification. Biotechnol Adv 28:285–292.

Seiboth B, Ivanova C, Seidl–Seiboth V. 2011. Trichoderma reesei: a fungal enzyme producer for cellulosic biofuels. Biofuel Production–Recent Developments and Prospects, Dr. Marco Aurelio Dos Santos Bernardes (Ed.) ISBN: 978–953–307–478–8, InTech.

Silva LAO, Terrasan CRF, Carmona EC. 2015. Purification and characterization of xylanases from Trichoderma inhamatum. Electron J Biotechn 18:307–313.

Song HT, Gao Y, Yang YM, Xiao WJ, Liu SH, Xia WC, & Jiang ZB, et al. 2016. Synergistic effect of cellulase and xylanase during hydrolysis of natural lignocellulosic substrates. Bioresour Technol 219:710–715.

Soroor MA, Ghazy AEHM, Khattab AENA, El–Aziz AMA, El–Shatoury EH, Al Zahraa A. 2014. Enhancement of xylanase production from rice straw by Trichoderma reesei through uv–mutagenesis and protoplast fusion. Egypt J Exp Biol (Bot) 10:125–134

Stasz TE, Harman GE, Weeden NF. 1988. Protoplast preparation and fusion in two biocontrol strains of Trichoderma harzianum. Mycologia 80:141–150.

Stricker AR, Steiger MG, Mach RL. 2007. Xyr1 receives the lactose induction signal and regulates lactose metabolism in Hypocrea jecorina. FEBS Lett 581:3915–3920.

Stricker AR, Mach RL, De Graaff LH. 2008. Regulation of transcription of cellulases–and hemicellulases–encoding genes in Aspergillus niger and Hypocrea jecorina (Trichoderma reesei). Appl Microb Biotechnol 78:211–220.

Ten LN, Im WT, Kim MK, Kang MS, Lee ST. 2004. Development of a plate technique for screening of polysaccharide–degrading microorganisms by using a mixture of insoluble chromogenic substrates. J Microbiol Meth 56: 375–382.

van den Brink J, de Vries RP. 2011. Fungal enzyme sets for plant polysaccharide degradation. Appl Microb Biotechnol 91:1477–1492.

Verma N, Bansal MC, Kumar V. 2008. Protoplast fusion technology and its biotechnological applications. Chem Eng Trans 14:113–120.

Xiong H, Turunen O, Pastinen O, Leisola M, Von Weymarn N. 2004. Improved xylanase production by Trichoderma reesei grown on L–arabinose and lactose or D–glucose mixtures. Appl Microb Biotechnol 64:353–358.

Xu J, Nogawa M, Okada H, Morikawa Y. 2000. Regulation of xyn3 gene expression in Trichoderma reesei PC–3–7. Appl Microb Biotechnol 54:370–375.

Xu F, Jin H, Li H, Tao L, Wang J, Lv J, Chen S. 2012. Genome shuffling of Trichoderma viride for enhanced cellulase production. Ann Microb 62:509–515.

Zhong S, Steffenson BJ. 2001. Virulence and molecular diversity in Cochliobolus sativus. Phytopathology 91:469–476.

بهبود تولید آنزیم زایلاناز از طریق امتزاج پروتوپلاست دو گونه‌ی *Trichoderma*

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چکیده: گونه‌های قارچ *Trichoderma*، از مهمترین منابع تولید آنزیم‌های صنعتی در جهان به شمار می‌روند. آنزیم‌های زایلانازی، گروهی از آنزیم‌های همی سلولازی هستند که در صنایع مختلف، طیف وسیعی از کاربردها را دارا هستند. در تحقیق حاضر، امتزاج پروتوپلاست دو گونه ی *T. virens* و *T. harzianum*، منجر به فعالیت زایلانازی ۲/۵ واحد در میلی لیتر در استرین امتزاج یافته‌ی X3 شد که در مقایسه با میانگین فعالیت زایلانازی والدین که ۰/۵۴ واحد در میلی لیتر بود، میزان ۴/۷ برابر افزایش نشان داد. علاوه بر این، به منظور ارزیابی اثر امتزاج پروتوپلاست در افزایش فعالیت زایلانازی، آنالیز بیان ژن *xyn3* با استفاده از qPCR، انجام شد. نتایج نشان داد که بیان نسبی ژن *xy3* در امتزاج یافته‌ی X3، به میزان ۴/۹ میانگین بیان نسبی این ژن در والدین بوده است. بر اساس نتایج به دست آمده، می‌توان اظهار داشت که فناوری امتزاج پروتوپلاست می‌تواند به عنوان ابزاری امیدبخش برای تولید میکروارگانیسم‌هایی که دارای خصوصیات برتر برای کاربردهای صنعتی هستند، به کار گرفته شود.

کلمات کلیدی: قارچ ها، همی سلولازها، فعالیت آنزیمی، qPCR

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تاریخ دریافت: ۱۳۹۷/۰۷/۱۶ تاریخ پذیرش: ۱۳۹۷/۰۹/۱۷