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Genetic Variability Studies on Cellulolytic *Aspergillus terreus* Fungi using RAPD-PCR Technique

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ABSTRACT: Forty nine fungal cultures were isolated from rural and urban waste samples from different parts of Western Maharashtra. These isolates were screened for cellulase activity and further identified as *Aspergillus terreus, Aspergillus flavus* and *Aspergillus niger* on the basis of cultural and microscopic features. *Aspergillus terreus* being a major group was selected for further investigations on genetic diversity by using RAPD-PCR technique with a aim to study the range of genetic differences between species within same genus. The PCR amplification profiles of these fungal cultures including standard MPKV reference strain of *Aspergillus terreus* with 16 random fungal primers generated 502 bands, out of which 349 bands were polymorphic, while 139 bands were unique. The average polymorphism observed was 97.21%. The genetic similarity index based on the pooled data of RAPD profiles from all the 16 primers ranged from 0.25 to 0.79 among isolates. The dendrogram and 2D PCO scatter plot constructed from the pooled data showed four major groups showing distinct genetic variability between isolates. Wide genetic variability among isolates at species level from the same ecological niche reveals that the genetic difference between population in species maintains the genetic diversity within species. This study will provoke further research for finding the minor differences within the species at genetic level and its impact on enzyme production.

Keywords: Cellulase, *Aspergillus terreus*, RAPD-PCR, polymorphism, genetic variability.

INTRODUCTION

In India rate of increase in solid waste generation is 1.3% annually. The amount of municipal solid waste generation by 2025 is expected to be 750 gms/capita per day, which presently ranges from 200 to 500 gms per capita per day. Thus, if this scenario continues, there are bound to be serious problems for waste disposal especially in urban areas (Rajput *et al.*, 2009). This rural and urban waste needs to be decomposed properly with the help of native cellulolytic microorganisms to produce good quality compost, which can in turn be incorporated into the agricultural farms for increasing crop productivity.

Cellulases are produced by several microorganisms including bacteria, actinomycetes and fungi. The latter are reported to produce maximum cellulase *in-vitro*. Among fungi various species of *Aspergillus* genus produce enzymes such as cellulase (Sridevi *et al*., 2015), lipase (Osuna *et al*., 2015) and estrase (Zhou *et al*., 2015). A few reports confirmed that *A. terreus* can carry out high cellulase production under short incubation periods using agricultural wastes (Jahromi *et al*., 2011). *A. terreus* is a common soil saprophyte that has been recovered from desert soil, grasslands and compost heaps which produces extracellular endo-β-1, 4-glucanase, exo-β-1, 4-glucanase with high levels of β-glucosidase. *A. terreus* also produces variety of secondary metabolites that are economically significant, such as lovastatin, a antihypercholesteroleic drug (Varga *et al*., 2005).

Genomes of several species of the *Aspergillus* genus often contain multiple cellulolytic enzyme genes that exhibit differential expression based on culture conditions and other factors (Ward *et al*., 2005). Genetic, transcriptomic and proteomic studies have revealed that several genes and regulatory circuits are activated during enzyme production (Al-Sheikh *et al*.,

2004). This may lead to the differences in enzyme production levels and genetic variability. PCR-based technique, involving the random amplification of polymorphic DNA (RAPD) has been used for assessing transcription patterns (Mahmood *et al.,* 2014) and genomic variability among a wide range of *Aspergillus* and related species (Megnegneau *et al.*, 1993). The utility of DNA markers as RAPD-DNA employ it as well established sample molecular marker tool for detecting genetic variability for many phytopathogenic fungi (Wostemeyer *et al.*, 2002). It is inexpensive and requires less amount of DNA (Pardo *et al.,* 2006). RAPD polymorphism results from a nucleotide base change, an insertion or deletion that alters the primer binding sites. This product can be polymorphic and may be used as genetic markers for extensive genetic variation analysis (Williams, 1990). One advantage of this technique is that the primers are universal and they can be used for genomic analysis of a wide variety of species. It is well documented in the literature that changing in the ecological niche or geographical region there is a definite genetic variability at genus level, species level, etc. But, it is interesting to study whether there is genetic variability occurs at species level from the same ecological niche. Hence, present study was designed to explore the genetic diversity in cellulolytic *Aspergillus terreus* fungi isolated from the rural and urban wastes with the help of molecular tools.

MATERIALS AND METHODS

A total of 34 samples of decomposing rural and urban wastes were collected from different parts of Western Maharashtra to isolate cellulolytic fungi. Isolation of fungi was carried out on potato dextrose agar media. Pure cultures of 49 fungal isolates were screened for cellulase activity and the efficient cellulolytic fungal isolates were identified on the basis of cultural and microscopic features following the methodology of Subramanian (1971), Barnett and Hunter (1972); Aneja (2003). The colonial morphology of fungal isolates was examined on potato dextrose agar medium and microscopic appearance by lactophenol cotton blue staining technique which determined the morphology of reproductive mycelium i.e. conidiophores.

The molecular diversity among ten cellulolytic *Aspergillus terreus* isolates along with the MPKV standard strain (BNF/FS-68) was analyzed by employing random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique. Initially, the genomic DNA was extracted from the mycelial mat of each fungal isolate grown on PDA for 7 days at 28±2°C. For this, aerial mycelia were collected by scrapping it from the agar surface with sterile scalpel and the mycelial mat was suspended in sterile distilled water for washing. This mat was taken out and placed in aluminum foil for drying at 60°C in hot air oven for half an hour. After drying, the mycelial mat was collected in another aluminum foil and kept in refrigerator at 4°C. Further, the genomic DNA was isolated by applying the methodology described by Narasimhan and Asokan (2010) with certain modifications. The DNA pellet was dried and suspended in 100 µl of TE buffer.

Quantification of DNA was done by gel electrophoresis and NanoDrop ND-1000 USA, UV visible spectrophotometer. The DNA with ratio of OD's (260/280nm) near 1.8 was further used for amplification. The purified genomic DNA samples from the individual isolates were diluted to working concentration of 20 ng/µl and used as template DNA. The DNA sequences of sixteen fungal RAPD primers used for DNA amplification are given in Table 1. One primer at a time was used to study polymorphism within each group. The master mix required for PCR amplification of each isolate was freshly prepared. The master mix as 19 µl per tube and 1 µl of template DNA from the respective isolates was added to make total reaction volume in each tube equal to 20 µl. The PCR amplification was done by following the method proposed by Castrillo and Brooks (1998) with certain modifications as per following profiles: predenaturation at a temperature of 94°C for 5 min, followed by 40 cycles of denaturation stages at a temperature of 94°C for 1 min, annealing at a temperature of 36°C for 1 min, elongation at 72°C for 2 minutes, and finalizing at a temperature of 72°C for 10 minutes.

Twenty µl of the amplified products from each tube along with 4 µl of loading dye were separated on 1.2 per cent agarose gel using 1x TBE buffer of pH 8.0 along with l00 bp DNA ladder as DNA molecular weight marker. Electrophoresis was performed for 3 hrs at 80 volts in an submarine electrophoresis apparatus. The gel was stained with ethidium bromide (0.1%) and photographed using gel documentation system. The amplification profiles for all the primers were compared with each other and the bands of DNA fragments were scored as present (1) or absent (0) generating the binary matrices. The binary data was analyzed under the SIMQUAL module of NTSYS PC-2.0 software programme (Rohlf, 1998) using DICE coefficient (Nei and Li, 1979). A dendrogram based on the UPGMA clustering method (SAHN) (Sneath and Sokal, 1973) was used to generate a tree. Principle coordinate analysis was performed to estimate the genetic distance between each group of the isolates by using NTSYS software. Then analyzing the product matrix by EIGEN to get Eigenvectors and Eigenvalues, and finally getting its 2D scatter plot graph. The per cent polymorphism was computed by dividing total number of polymorphic bands with total number of bands and multiplying resultant with 100.

RESULTS AND DISCUSSION

On the basis of cultural and microscopic features the fungal cultures were identified as *Aspergillus* species. Amongst the different groups of *Aspergillus* species,

the major group of *Aspergillus terreus* comprising ten isolates was further studied for molecular diversity within group by employing RAPD-PCR technique. Sixteen random primers generated a total of 502 bands, where the number of bands produced per primer varied from 9 to 46 (Plate 1).

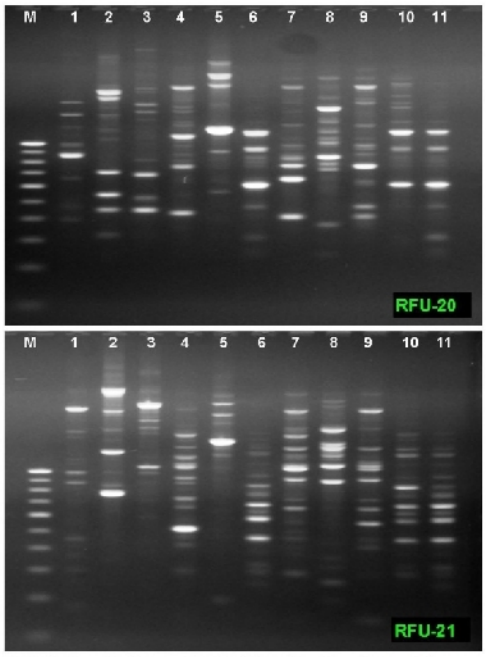

Plate 1. Amplification profiles of different isolates of *Aspergillus terreus* with RFU-20 and RFU-21 primer

Lane M : Marker 100 bp (StepUp Low Range DNA Ladder)
Aspergillus terreus isolates:
1. F-3 2. F-4 3. F-13 4. F-17 5. F-19 6. F-24
7. F-27 8. F-34 9. F-35 10. F-44 11. BNF/F8-88 (MPKV strain)

On an average 31.37 bands were produced per primer. Out of 502 bands, 349 bands were polymorphic, 139 were unique while, 14 bands were monomorphic. Most of the primers produced polymorphic bands varied from 2 to 28. Maximum polymorphic bands (28) were observed with primer RFU-8, while minimum polymorphic bands (2) were observed with RFU-7. The number of polymorphic loci amplified varied with different primers and isolates. The amplified DNA fragment ranged from 227 to 2157 bp. Among all the primers the maximum amplification was observed with RFU-20, RFU-21 and RFU-3 primers which produced 46, 38 and 38 bands, respectively, whereas least banding pattern was generated by RFU-7 with 9 bands. All the primers showed the genetic polymorphism between the *A. terreus* isolates tested. Average polymorphism observed was 97.21% (Table 1).

Table 1: RAPD analysis of *Aspergillus terreus* isolates.

Primer Name	Primer sequence	Total No. of bands	No. of monomorphic bands	No. of unique bands	No. of polymorphic bands	% poly -morphism
RFU 1	5' CCT GGG CCA G 3'	34	0	10	24	100.00
RFU 2	5' CCT GGG CGA G 3'	30	1	2	27	96.66
RFU 3	5' CCT GGG CTG G 3'	38	1	12	25	97.37
RFU 4	5' CCT GGG CTA T 3'	32	0	11	21	100.00
RFU 5	5' CCT GGG CTT G 3'	32	1	4	27	96.88
RFU 6	5' CCT GGG CTA C 3'	33	0	11	22	100.00
RFU 7	5' CCT GGG CTT A 3'	9	5	2	2	44.44
RFU 8	5' CCT GGG TCG A 3'	34	0	6	28	100.00
RFU 9	5' CCT GGG TGC A 3'	31	0	8	23	100.00
RFU 10	5' CCT GGG TGA C 3'	33	1	5	27	96.97
RFU 11	5' CCT GGC TTA C 3'	23	3	11	9	86.96
RFU 14	5' CTC CCT GAC C 3'	31	0	7	24	100.00
RFU 20	5' GAG GGC ATG T 3'	46	0	22	24	100.00
RFU 21	5' CCG GCC CCA A 3'	38	1	10	27	97.37
RFU 23	5' CCG GCC ATA C 3'	27	1	7	19	96.30
RFU 25	5' CCG GCT GGA A 3'	31	0	11	20	100.00

Genetic similarity index was computed based on pooled data of RAPD profiles for *Aspergillus terreus* as DICE coefficient using the similarity routine of NTSYS PC 2.0 software (Table 2). Highest genetic similarity index of 0.79 was between the isolates F-44 and BNF/FS-68. The minimum genetic similarity index of 0.25 was observed between isolates F-44 and F-13. Dendrogram representing the genetic relationship among the isolates based on RAPD analysis was developed and presented in Fig. 1. On the basis of RAPD analysis, *Aspergillus terreus* isolates were classified into four broad groups. Group I consisted of two subgroups. Subgroup Ia consisted of isolate F-3 and F-34 with similarity coefficient index 0.38, Ib consisted of isolates F-17, F-27 and F-35. The group II further classified into two subgroups. The subgroup IIa consisted of F-24 isolate and BNF/FS-68 strain having the genetic similarity coefficient 0.83. The subgroup IIb consisted of single F-44 isolates having genetic similarity coefficient 0.79. The third group consisted of single isolate F-19. The fourth group consisted of isolates F-4 and F-13 isolate having genetic similarity coefficient 0.40. Similar results were reported by Aiat (2006) who used RAPD fingerprints for analysis of genetic relationships among *A. niger, A. flavus and A. parasiticus*. Four arbitrary 5-base primers were successfully used to amplify DNA extracted from mycelium. RAPD fingerprints of *A. niger, A. flavus and A. parasiticus* revealed polymorphism in 37, 59, 51% of the analyzed strains. The similarity percent was 37% in *A. niger*, 58% in *A. flavus* and 51.5% in *A. parasiticus*. Irshad and Nawab (2012) characterized seven species of *Aspergillus* at molecular level, using RAPD. RAPD- PCR conditions were optimized for two primers of series B, GL Decamer B-09 and GL Decamer B-10 out of 10 total primers. GL Decamer B-09 showed 38 bands and GL Decamer B-10 gave 46 bands, showing 50% and 57% similarity respectively, among species.

Table 2: Genetic similarity as DICE coefficient based on pooled data of RAPD profiles for *Aspergillus terreus.*

Isolate	F-3	F-4	F-13	F-17	F-19	F-24	F-27	F-34	F-35	F-44	BNF/ FS-68
F-3	1.00										
F-4	0.39	1.00									
F-13	0.30	0.40	1.00								
F-17	0.39	0.32	0.38	1.00							
F-19	0.35	0.37	0.35	0.39	1.00						
F-24	0.39	0.34	0.27	0.31	0.33	1.00					
F-27	0.41	0.35	0.35	0.63	0.41	0.37	1.00				
F-34	0.38	0.39	0.40	0.39	0.37	0.40	0.46	1.00			
F-35	0.39	0.38	0.40	0.58	0.36	0.38	0.70	0.48	1.00		
F-44	0.34	0.36	0.25	0.32	0.36	0.70	0.44	0.42	0.40	1.00	
BNF/FS-68	0.39	0.37	0.28	0.34	0.36	0.83	0.41	0.41	0.41	0.79	1.00

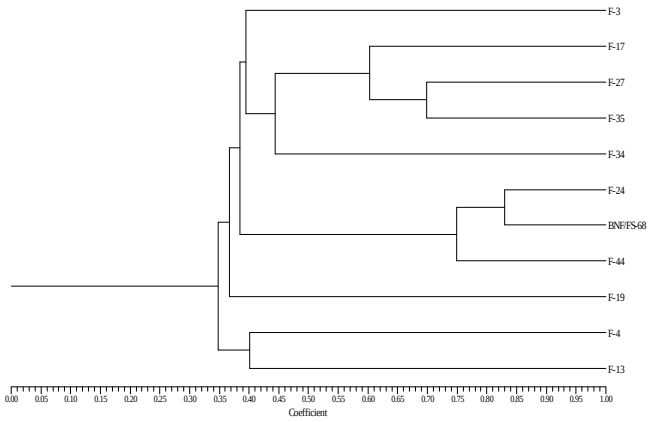

Fig. 1. Dendrogram representing the clustering among different isolates of *Aspergillus terreus*.

The dendrogram clustering was reflected on grouping in 2D PCO scatter plot (Fig. 2) which represents the phylogenetic relationship among different isolates of *A. terreus*. The *A. terreus* strains were grouped in four groups. The isolates F-13, F-4 and F-19 were in one group, while second group consisted of F-3 and F-34 isolates. Third group was found distinct from first two and include isolate F-17, F-27 and F-35, however, the remaining isolates F-24, F-44 and BNF/FS-68 were genetically diverse and placed in fourth group.

From the present study it is revealed that there was wide genetic variability among *Aspergillus terreus* isolates at species level though they were from same ecological niche, which may be due to the minor genetic differences in the strains within same species. Mahmoud *et al*. (2016) found that within species genes

were differentially expressed in the two fermentation systems for three isolates during cellulase enzyme production. Lass-Florl *et al.*, (2007) analyzed *Aspergillus terreus* isolates using random amplification of polymorphic DNA-PCR with three different primers R108 (5'-GTATTGCCCT-3'), CII (5'-GCGCACGG-3') and P4 (5'-GATAGATAGATAGAT-3'). By combining the results of analysis using these three primers, 33 and 26 distinct profiles in the two different collections were identified. No strain similarity in either location was detected, indicating great genetic diversity of *A. terreus*. Similar results indicating great genomic diversity of *A. terreus* are reported by Narasimhan and Asokan (2010). Genetic analysis using RAPD is attractive as no prior knowledge of the DNA sequence is available and primers are designed randomly with the sole constraint being GC contents. It permits simultaneous investigation of multiple loci in a single PCR reaction (Bhattacharya and Ranade 2001). Like all other techniques, the RAPD-PCR has limitations; the prominent shortcoming of RAPD is its low band repeatability and occurrence of pseudo bands. But it can be improved by applying suitable conditions to remove the impurities in extracted DNA and keeping the amplification conditions stable. It is concluded that the use of DNA marker can increases the efficiency of analyzing genetic diversity among different fungal isolates, as described by Rath (2002).

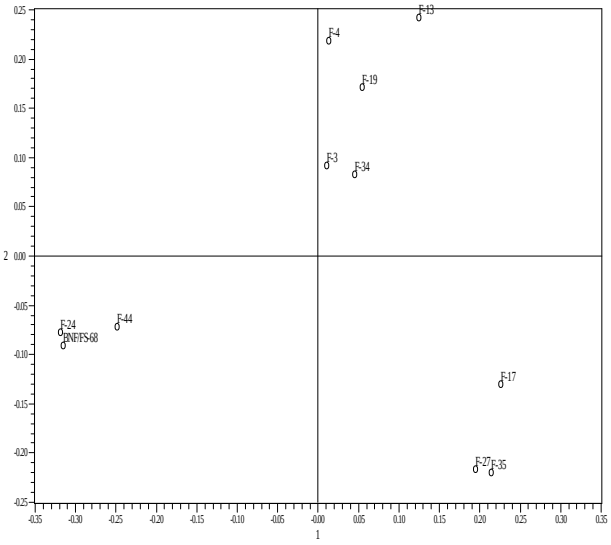

Fig. 2. RAPD 2D PCO scatter plot representing the phylogenetic relationship among different isolates of *Aspergillus terreus*.

CONCLUSION

There was wide genetic variability among *Aspergillus terreus* isolates at species level though they were from same ecological niche, which may be due to the minor genetic differences in the strains within same species or differential expression of genes. RAPD PCR is very useful to detect similarities and differences in different fungal species. Data generated by RAPD study is useful in estimating distances between and within same species and might help in further genomic research on differences in enzyme production levels within closely related population. Closely related species of other microorganisms can be similarly characterized at molecular and biochemical level.

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Conflict of Interest. None.

REFERENCES

Aiat, N. (2006). Genetic variability among three species of *Aspergillus* 2. Random amplified polymorphic DNA (RAPD) markers for genetic analysis. *J. Appl. Sci. Res.*, *2*(10): 709-713.

Al-Sheikh, H., Watson, A. J., Lacey, G. A., Punt, P. J., MacKenzie, D. A., Jeenes, D. J., Pakula, T., Penttila, M., Alcocer, M. J. C. & Archer, D. B. (2004). Endoplasmic reticulum stress leads to the selective transcriptional down regulation of the glucoamylase gene in *Aspergillus niger*. *Mol. Microbiol.*, *53*: 1731-1742.

Aneja, K. R. (2003). Experiments in Microbiology, Plant Pathology and Biotechnology. New Age International Publishers, New Delhi, India. pp. 1-607.

Barnett, H. L. & Hunter, B. B. (1972). Illustrated Genera of Imperfect Fungi. 2nd Ed. Minneapolis Burgess Publishing Co., Minneapolis. pp. 1-241.

Bhattacharya, E. & Ranade, S. A. (2001). Molecular distinction amongst varieties of Mulberry using RAPD and DAMD profiles. *BMC Plant Biol.*, *1*(1): 1-8.

Castrillo, L. A. & Brooks, W. M. (1998). Differentiation of *Beauveria bassiana* isolated from the darkling beetle *Alphitoblus diaperinus* using isozyme and RAPD analysis. *J. Invertebr. Pathol., 72*(3): 190-196.

Irshad, S. & Nawab, R. (2012). Molecular characterization of seven different species of *Aspergillus* through random amplified polymorphic DNA (RAPD) and enzyme analysis. *J. Microbiol. Res., 2*(3): 47-50.

Jahromi, M. F., Liang, J. B., Rosfarizan, M., Goh, Y. M., Shokryazdan, P. &. Ho, Y. W. (2011). Efficiency of rice straw lignocelluloses degradability by *Aspergillus terreus* ATCC 74135 in solid state fermentation. *Afr. J. Biotechnol., 10*(21): 4428-4435.

Lass-Florl, C., Grif, K. & Kontoyiannis, D. P. (2007). Molecular typing of *Aspergillus terreus* isolates collected in Houston, Texas, and Innsbruck, Austria: evidence of great genetic diversity. *J. Clin. Microbiol., 45*(8): 2686–2690.

Mahmood, N., Ahsan, M., Muhammad, I., Afzal, S., Qureshi, Z., Naqvi, S. A. R., Yar, M., Khan, Z. A. & Shahzad, S. A. (2014). Molecular expression profile of different cellulolytic enzyme genes in *Aspergillus niger* in response to UV radiation and chemical mutagenesis. *Cellul. Chem. Technol., 48*: 529-533.

Mahmoud, M. A., Al-Othman, M. R., Abd-El-Aziz, A. R. M., Metwaly, H. A. & Mohamed H. A. (2016). Expression of genes encoding cellulolytic enzymes in some *Aspergillus* species. *Genet. Mol. Res., 15*: 15048913.

Megnegneau, B., Debets, F. & Hoekstra, R. F. (1993). Genetic variability and relatedness in the complex group of black Aspergilli based on random amplification of polymorphic DNA. *Curr. Genetics, 23*(4): 323-329.

Narasimhan, B. & Asokan, M. (2010). Genetic variability of *Aspergillus terreus* from dried grapes using RAPD-PCR. *Adv. Biosci. Biotechnol., 1*(4): 345-353.

Nei, M. & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA., 76*: 5269-5273.

Osuna, Y., Sandoval, J., Saade, H., Lopez, R. G., Martinez, J. L., Colunga, E. M., de la Cruz, G., Segura, E. P., Arevalo, F. J., Zon, M. A. & Fernandez, H. (2015). Immobilization of *Aspergillus niger* lipase on chitosan-coated magnetic nanoparticles using two covalent-binding methods. *Bioprocess and Biosystems Engineering, 38*(8): 1437-1445.

Pardo, J., Urban, C., Galvez, E. M., Ekert, P. G., Muller, U., Kwon-Chung, J., Lobigs, M., Mullbacher, A., Wallich, R., Borner, C. & Simon, M. M. (2006). The mitochondrial protein Bak is pivotal for gliotoxin-induced apoptosis and a critical host factor of *Aspergillus fumigatus* virulence in mice. *J. cell boil., 174*(4): 509-519.

Rajput, R., Prasad, G. & Chopra, A. K. (2009). Scenario of solid waste management in present Indian context. *Caspian J. Env. Sci., 7*(1): 45-53.

Rath, P. M., Petermeier, K., Verweij, P. E. & Ansorg, R. (2002). Differentiation of *Aspergillus ustus* Strains by Random Amplification of Polymorphic DNA. *J. Clin. Microbiol., 40*(6): 2231-2233.

Rohlf, F. J. (1998). Numerical taxonomy and multivariate analysis system, Version 2.0 (Exeter Software, New York).

Sneath, P. H. A. & Sokal, R. R. (1973). Numerical taxonomy: the principles and practice of numerical classification (H. Freeman and Co., San Francisco).

Sridevi, A., Narasimha, G., Ramanjaneyulu, G., Dileepkumar, K., Rajasekhar Reddy, B. & Suvarnalatha Devi, P. (2015). Saccharification of pretreated sawdust by *Aspergillus niger* cellulase. *3 Biotech, 5*(6): 883-892.

Subramanian, C. V. (1971). Hypomycetes on account of Indian species, except *Cercospora*. ICAR Publication, New Delhi, India. pp. 1-929.

Varga, J., Toth, B., Kocsube, S., Farkas, B., Szakacs, G., Rigo, K., Teren, J. & Kozakiewicz, Z. (2005). Evolutionary relationships among *Aspergillus terreus* isolates and their relatives. *Antonie van Leewenhoek, 88*(2): 141-150.

Ward, O. P., Qin, W. M., Dhanjoon, J., Ye, J., & Singh, A. (2005). Physiology and biotechnology of *Aspergillus*. *Adv. in appl. Microbiol., 58*: 1-75.

Williams, J. G. K., Hanafey, M. K., Rafalski, J. A. & Tingey, S. V. (1990). Genetic analysis using the random amplified polymorphic DNA marker. *Methods in Enzymology, 218*: 705-740.

Wostemeyer, J. & Kreibich, A. (2002). Repetitive DNA elements in fungi (mycota): impact on genomic architecture and evolution. *Curr. Genet., 41*: 189-198.

Zhou, M., Huang, Z., Zhou, B., Luo, Y., Jia, G., Liu, G., Zhao, H. & Chen, X. (2015). Construction and expression of two-copy engineered yeast of feruloyl esterase. *Electronic J. Biotechnol., 18*(5): 338-342.

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