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Isolation and Identification of Potential Gossypol Degrading Fungal Strains from Cotton Growing Soil

Vellaichamy Mageshwaran[1*], Sharmila B. Majee[2] and Kuppusamy Pandiyan[3]

[1]ICAR- Central Institute for Research on Cotton Technology, Matunga (E), Mumbai, India.
[2]Bombay Veterinary College, Parel, Mumbai, India.
[3]ICAR- National Bureau of Agriculturally Important Microorganisms, Mau, Uttar Pradesh, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author VM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SBM and KP managed the analyses of the study. Authors VM and KP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To isolate and identify gossypol degrading fungal strains from cotton growing soil of Maharashtra and Andhra Pradesh States of India.
Study Design: Experimental study.
Place and Duration of the Study: Chemical and Biochemical Processing Division, ICAR-Central Institute for Research on Cotton Technology, Matunga (E), Mumbai – 400019, India during January to December 2014.
Methodology: The isolation of fungi from soil samples was done by enrichment culture technique using gossypol as a sole carbon and energy source. The fungal isolates were inoculated in cottonseed cake (CSK) and incubated for 48 h at 30℃. The isolates were screened for gossypol detoxification by estimating free and total gossypol level in the fermented CSK. The potential gossypol degrading isolates were identified by 18S rDNA analysis.
Results: Eight isolates (F1 to F8) were identified as potential gossypol degrading strains among the fifty nine isolates obtained. The new fungal isolates from F1 to F8 were identified as

**Corresponding author: E-mail: mageshbioiari@gmail.com;*

Aspergillus terreus (KP 264957.1), *Lichithemia ramosa* (KP 264961.1), *Alternaria alternata* (KP 264960.1), *Fusarium equiseti* (KP 26459.1), *Fusarium chlamydospora* (KP 264958.1), *Fusarium* sp. (KP 209032.1), *Fusarium solani* (KP 264956.1) and *Fusarium thapsinum* (KP 264962.1) respectively, based on 18S rDNA analysis. The maximum free and total gossypol reduction (%), 65.2 and 59.8 respectively was observed in *Fusarium thapsinum* (KP 264962.1) treated CSK. The protein content was higher in fungal treated than untreated CSK.

Conclusion: The present study identifies new fungal strains capable of degrading toxic compound, gossypol in cottonseed cake.

Keywords: Degradation; fungal strains; gossypol; identification; 18S rDNA.

1. INTRODUCTION

CSK is commonly used as feed for ruminant animals. However, the use of CSK is limited for non-ruminants due to the presence of gossypol. Gossypol is a toxic polyphenolic compound present in the entire cotton plant including its seed having molecular formula, molecular mass and chemical structural formula of $C_{30}H_{30}O_{8,}$ 518.55 g/mol and 2, 2'-bis-(8- formyl-1, 6, 7 trihydroxy -5-isopropyl-3- methyl naphthalene) respectively [1,2]. Gossypol can cause reproductive disease, growth depression and other intestinal and internal organ abnormalities. Gossypol shows a moderate acute toxicity in most species, with an oral Lethal Dose (LD) 50 of 2400 – 3340, 500 – 950, 350 – 600, 550 and 280 – 300 mg/kg for rats, mice, rabbits, pigs and guinea pigs, respectively [3]. Microbial fermentation is a suitable method for detoxifying gossypol because fermentation improves other nutritional properties such as protein and aminoacids content of CSK. Microbial strains such as *C. tropicalis*, *S. cerevisiae*, *Aspergillus niger*, *A. oryzae*, *Pleurotus* sp. and *Bacillus cereus* were found effective in degrading gossypol in CSK [4-6]. Similarly, mixed fungal cultures, *Saccharomyces cerevisiae* with *Aspergillus niger*, *A niger* with *A. oryzae*, *C. tropicalis* with *S. cerevisiae* and *S. cerevisiae* with *P. sajor-caju* were found more effective in gossypol degradation in CSK compared to single culture [7,8]. The present study was aimed at isolation of gossypol degrading fungal isolates from soil samples of cotton field and identification of potential strains.

2. MATERIALS AND METHODS

2.1 Soil Sample Collection

Soil samples were collected from cotton fields of Nagpur, Rahuri, and Parbhani regions of Maharashtra, and Guntur, Andhra Pradesh, India. Approximately 100 g of soil samples were collected from the top layer of the soil (1 – 5 cm depth) in sterile poly bags.

2.2 Microorganisms and Culture Conditions

The known gossypol degrading fungal strain *Pleurotus flabellatus* M-1 was obtained from Microbiology Lab, ICAR-CIRCOT, Mumbai and maintained in malt extract agar slants at 4℃. The fungal isolates were grown in malt extract broth (1X) (Himedia, India) at 28℃ under shaking conditions for 48 h.

2.3 Basal Substrate

Cottonseed cake was procured from M/s Sri Bagyalakshmi refinery (P) Ltd., Tirupur, India. The cake was ground and passed through 10 mm sieve and stored at room temperature (25-30℃) until further use.

2.4 Isolation of Gossypol Degrading Fungi from Soil Samples

Ten mg of Gossypol acetic acid (MP Biomedicals, USA) was dissolved in one ml of dimethyl sulfoxide. Fifty µl of this solution was added to sterile minimal medium ($NaNO_3$ – 0.5, K_2HPO_4 – 0.65, KH_2PO_4 – 0.2, $MgSO_4$ – 0.1, pH-5.5) to the final volume of 5 ml in a test tube for attaining 100 ppm of gossypol. Soil sample (0.05 g) was added to the medium. The tubes were incubated at 30℃ for 48 h under shaking conditions.

The tubes containing growth was plated in rosebengal agar (Himedia, India) plates and incubated at 30℃ for 48 h. The representative fungal isolates of similar colony morphology were picked, subcultured in malt extract agar slants and pure cultures were preserved at 4℃ for till further use.

2.5 Screening of Fungal Isolates for Gossypol Degradation

Five gram of CSK with natural pH (6.0) was taken in 250 ml Erlenmeyer flask and autoclaved at 121℃ for 15 lbs pressure for 20 minutes. Four ml of 48 h old fungal culture was inoculated in CSK and thus the initial moisture content maintained in CSK was 80%. The flasks were incubated at 30℃ for 48 h. The known gossypol degrading fungal strain, *P. flabellatus* M-1 was inoculated in CSK as described above to compare the efficiency of gossypol degradation by fungal isolates. CSK with four ml of sterile water was served as a control. After incubation, the flask containing fermented substrates and control were kept in an oven at 60℃ for 4 h. The powdered samples were used for free and total gossypol analysis according to AOCS Ba 7-58 and AOCS Ba 8-78, respectively [9]. The free gossypol reduction (%) was calculated by using the formula, (Control- Treated)/ Control × 100. The protein content in the samples was estimated by kjeldahl method [10]. Each sample was replicated and the average value of each set of experiments was accounted.

2.6 DNA Extraction and 18SrDNA Sequence Analysis

The selected isolates were grown in malt extract medium (1X) for three days at 30℃ under shaking conditions and the mycelium was harvested by centrifugation. DNA extraction was performed by Benzyl chloride method [11] and the purified DNA was stored at -20℃. The polymerase chain reaction (PCR) amplification of fungal 18Sr DNA was carried out in thermal cycler using the primer set EF4 (5'-GGAAGGG[G/ATGTATTTATTAG-3') and EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') [12]. The PCR conditions were as follows: a. initial denaturation at 95℃ for 5 min b.30 cycles of denaturation at 95℃ for 1 min, annealing at 50℃ for 30 sec and an extension at 72℃ for 1 min. c. final extension at 72℃ for 10 min. The amplicons of 18S rDNA was resolved in 1.5 % agarose gel using 1X TAE buffer at 70 V and the PCR amplicons were purified and sequenced. The nucleotide sequences of fungal isolates were compared by BLAST with existing database NCBI website(http://www.ncbi.nlm.nih.gov/BLAST) to identify its closest neighbor. The phylogenetic tree of identified isolates was constructed using Neighbor-Joining method based on 18S rDNA sequences.

2.7 Statistical Analysis

The data obtained was analyzed by Web Agri Stat Package (WASP) of ICAR Research Complex Goa. A significant level of 0.05 was used.

3. RESULTS AND DISCUSSION

Gossypol detoxification by microbial fermentation is gaining importance among the different methods, since fermentation improves the nutritional properties especially amino acid content of CSK [4,5,7]. The replacement of soybean cake with up to 20% of fermented cottonseed cake in diet of non-ruminants such as boilers and fish resulted in increase in better feed consumption, body weight, weight gain and feed conversion ratio [13,14]. The previous results showed that the fungal strains are more effective in gossypol detoxification and nutritive quality in CSK [4-8].

In this study, attempt was made for isolation of new gossypol degrading fungal strains from cotton planted soil. A total of 108 soil samples were collected from cotton fields of Nagpur, Parbhani and Rahuri (Maharashtra) and Guntur (Andhra Pradesh) (Table 1). Minimal medium containing 100 ppm of gossypol as a sole carbon and energy source was used for isolation of gossypol detoxifying isolates. In total, fifty nine fungal isolates were obtained from 108 soil samples (Table 1). In a similar study, minimal agar plates containing gossypol was used for isolation of fungi from cotton planted soil [6,15].

Table 1. Isolation of fungal isolates from soil samples

Place	No. of Soil samples	No. of fungal isolates
Nagpur	25	7
Rahuri	23	12
Parbhani	25	20
Guntur	26	20
Total	108	59

All the fungal isolates were screened for gossypol detoxification by estimating free and total gossypol reduction (%) in CSK. Among 59 isolates, 8 isolates were selected based on their efficiency for higher reduction of free and total gossypol in CSK. The gossypol reduction and protein content improvement in CSK by selected fungal isolates (F1 to F8) has been shown in Table 2. The free gossypol (%), total gossypol

(%) and protein content (%) in raw CSK was 0.23, 2.34 and 20.1, respectively. Among the eight isolates, six isolates was showing more than 50 % reduction of free and total gossypol while the isolates, F5 and F6 showed less efficiency in reduction of either free or total gossypol. The maximum reduction of free and total gossypol was observed in F8 treated CSK and their corresponding values are 65.2% and 59.8% respectively. A known gossypol degrading fungal strain, *P. flabellatus* M-1 [16] was chosen to compare gossypol detoxification efficiency with fungal isolates. The free and total gossypol detoxification efficiency of *P. flabellatus* M-1 was found to be 69.6% and 58.1% respectively (Table 2). The results revealed that the detoxification efficiency of fungal isolate, F8 was comparable with known gossypol degrading fungal strain, *P. flabellatus* M-1.

In a similar study, *Candida tropicalis* was found more effective in free gossypol reduction among the fungal cultures such as *Candida capsuligena*, *C. tropicalis*, *Saccharomyces cerevisiae*, *Aspergillus terricola*, *A. niger* and *A. oryzae* tested [5,17,18]. *C. tropicalis* ZD-3 reported for gossypol reduction in CSK by 94.6% [5]. The protein content of fungal treated CSK was measured and compared. The fungal strain, *P. flabellatus* M-1 treated CSK had higher protein content, 27.5% followed by fungal isolate, F8 (26.4%). The results are in agreement with previous studies, where the fermentation of CSK increases the protein content by more than 10% [5,16].

The 18S rDNA nucleotide sequences of fungal isolates, F1 to F8 were submitted in the NCBI database and details of which are presented in Table 3. The fungal isolates F1 to F8 were identified as *Aspergillus terreus* (KP 264957.1), *Lichithemia ramosa* (KP 264961.1), *Alternaria alternata* (KP 264960.1), *Fusarium equiseti* (KP 26459.1), *Fusarium chlamydospora* (KP 264958.1), *Fusarium* sp. (KP 209032.1), *Fusarium solani* (KP 264956.1) and *Fusarium thapsinum* (KP 264962.1) respectively. The constructed phylogenetic tree using Neighbor-Joining method showed that *Fusarium* isolates were clustered in a common clade with *Aspergillus niger* but *Lichithemia ramosa* formed a separate clade which reveals its distant relatedness to *Fusarium* and *Aspergillus* (Fig. 1). In a similar attempt, gossypol degrading fungal strains, HQ-1, MM-2 and RP-3 isolated from cotton plant soil were identified as *Aspergillus niger*, *Fusarium oxysporum* and *Rhodotorula mucilaginosa* respectively [6,15]. The present study are in agreement with earlier studies where the predominant gossypol degrading fungi occurs in cotton cultivated soil was identified as *Fusarium* followed by *Aspergillus* and *Alternaria* based on molecular and phylogenetic analysis. Thus, our findings showed that the identified strains are promising additions to the repertoire of gossypol degrading microbial isolates.

Table 2. Gossypol degradation in cottonseed cake by the selected fungal isolates

Isolate	Free gossypol (%)	Free gossypol reduction (%)	Total gossypol (%)	Total gossypol reduction (%)	Protein content (%)
F1	0.09	60.9	1.04	55.6	24.2
F2	0.09	60.9	1.16	50.4	24.2
F3	0.09	60.9	1.00	57.3	25.3
F4	0.11	52.2	1.16	50.4	23.2
F5	0.16	30.4	1.12	52.1	24.2
F6	0.15	34.8	1.30	44.4	23.7
F7	0.09	60.9	1.17	50.0	24.5
F8	0.08	65.2	0.94	59.8	26.4
P. flabellatus M-1 (Reference strain)	0.07	69.6	0.98	58.1	27.5
Raw cottonseed cake (Control)	0.23	-	2.34	-	20.1

Table 3. Similarity percentage of 18s rDNA sequences of the selected isolates compared to those obtained from database

Isolate	Accession no. of submitted nucleotide sequence	Accession no. from available database	Identity (%)	Identified species
F1	KP 264957.1	KM 924436.1	93	*Aspergillus niger*
F2	KP 264961.1	-	-	*Lichithemia ramosa*
F3	KP 264960.1	JQ907485.1	96	*Alternaria alternata*
F4	KP 264959.1	JF819150.1	99	*Fusarium equiseti*
F5	KP 264958.1	KU571483.1	99	*Fusarium chlamydospora*
F6	KP 209032.1	KX 06001.1	100	*Fusarium sp.*
F7	KP 264956.1	JX535014.1	93	*Fusarium solani*
F8	KP 264962.1	KX 944297.1	99	*Fusarium thapsinum*

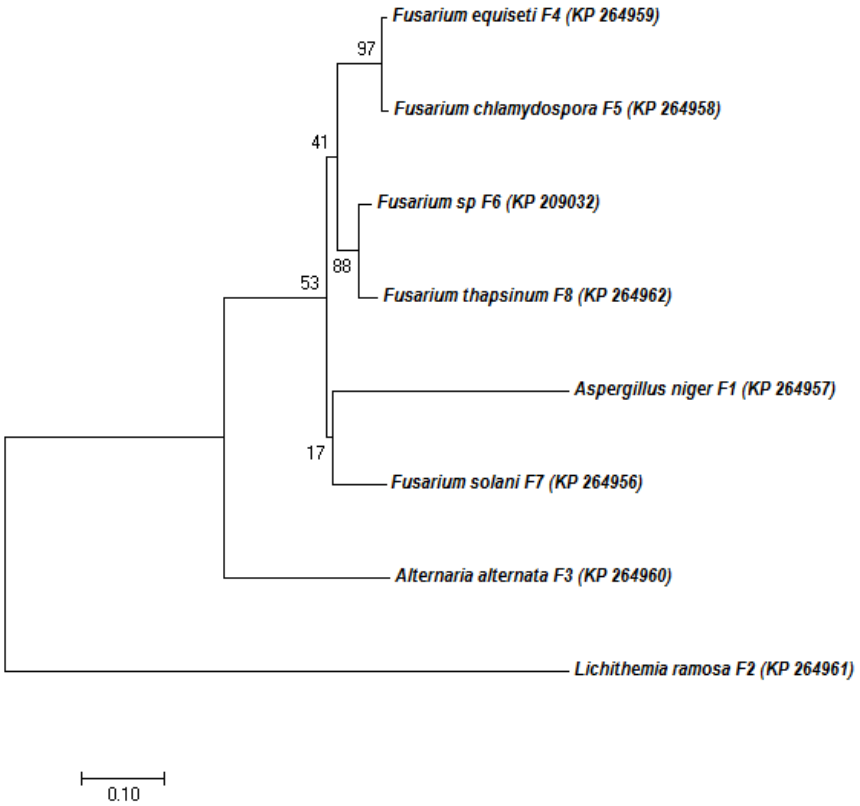

Fig. 1. Phylogenetic tree of gossypol-degrading fungal isolates based on 18S rDNA sequences

4. CONCLUSION

The fungal strains, F1 to F8 had higher free and total gossypol reduction in CSK among the fifty nine isolates tested. These strains were identified as *Aspergillus terreus*, *Lichithemia ramosa*, *Alternaria alternata*, *Fusarium equiseti*, *Fusarium chlamydospora*, *Fusarium* sp., *Fusarium solani* and *Fusarium thapsinum* respectively, based on molecular characterization. The fermented CSK had improved protein content as well. Thus, the identified fungal strains would add the list of gossypol degrading microbial strains for its effective utilization in solid state fermentation process for gossypol detoxification and nutritive quality improvement of CSK.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kenar JA. Reaction chemistry of gossypol and its derivatives. JAOCS. 2006;83(4): 269-302.
2. Gadelha ICN, Fonseca NBS, Oloris SCS, Melo MM, Blanco BS. Gossypol toxicity from cottonseed products. The Sci. World J. 2014;1:1-11.
3. EFSA. Gossypol as undesirable substance in animal feed. The EFSA Journal European Food Safety Authority. 2008;908:1-55.
4. Weng XY, Sun JY. Biodegradation of free gossypol by a new strain of *Candida tropicalis* under solid state fermentation: Effects of fermentation parameters. Process Biochemistry. 2006;41:1663-1668.
5. Zhang W, Xu Z, Sun J, Yang X. A study on the reduction of gossypol levels by mixed culture solid substrate fermentation of cottonseed meal. Asian –Aust. J. Anim. Sci. 2006;19(9):1314-1321.
6. Yang X, Guo J, Sun J. Biodegradation of free-gossypol by a new fungus isolated from cotton planted soil. Afr. J. Microbiol. Res. 2011;5(19):3066-3072.
7. Atia AI, Rahim GAA. Detoxification treatments of free gossypol in CSK by microbial treatment of mixed cultures and biochemical evaluation on rabbits. J Rad. Res. Appl. Sci. 2009;2(2):397-415.
8. Vellaichamy M. Optimization of solid state fermentation process for gossypol detoxification in heat sterilized CSK by mixed fungal cultures. Intl. J Food Ferment. Technol. 2016;6(1):97–102.
9. AOCS. Free and total gossypol methods. In: Official and tentative methods of the AOCS. 4th Ed. American Oil Chemist's Society: Chicago; 1989.
10. AOAC. Official methods of analysis. 16th Ed. Association of Official Analytical Chemists: Washington DC; 1999.
11. Zhu H, Qu F, Zhu LH. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. Nucleic Acids Res. 1993;21(22):5279-5280.
12. Gontia-Mishra I, Tripathi N, Tiwari S. A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies. Indian J Biotechnol. 2014;13:536-539.
13. Kanyinji F, Sichangwa M. Performance of broilers fed finishing diets with fermented cottonseed meal as partial replacement for soybean meal. J. Anim. Sci. Adv. 2014;4(7):931-938.
14. Lim SJ, Kim SS, Pham MA, Song JW, Cha JH, Kim JD, Kim JU, Lee KJ. Effects of fermented cottonseed and soybean meal with phytase supplementation on gossypol degradation, phosphorus availability and growth performance of olive flounder (*Paralichthys olivaceus*). Fish Aqua Sci. 2010;13(4):284-293.
15. Xia Y, Xiao-Yan W, Jian-Lin G, Jian-Yi S. Screening and Identification of gossypol-degraded strains isolated from a soil microcosm. Cotton Science. 2010;22(6): 539-546.
16. Mageshwaran V, Kathe AA. Detoxification of gossypol in cottonseed meal by *Pleurotus fabellatus* strain M-1 under solid state fermentation. Indian J. Anim. Nutr. 2013;30(3):313-319.
17. Khalaf MA, Meleigy SA. Reduction of free gossypol levels in cottonseed meal by microbial treatment. Int. J. Agri. Biol. 2008;10(2):185-190.
18. Zhang WJ, Xu ZR, Zhao SH, Sun JY, Yang X. Development of a microbial fermentation process for detoxification of gossypol in cottonseed meal. Anim. Feed Sci. Technol. 2007;135(1-2):176-186.

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