ISSN 0003-6838, Applied Biochemistry and Microbiology, 2018, Vol. 54, No. 3, pp. 301–308. © Pleiades Publishing, Inc., 2018.

Biodegradation of Gossypol by Mixed Fungal Cultures in Minimal Medium[1]

V. Mageshwaran[a], *, V. Sharma[a], M. Chinnkar[a], N. Parvez[a], and V. Krishnan[b]

[a] *ICAR-Central Institute for Research on Cotton Technology (CIRCOT), Mumbai, 400019 India*

[b] *ICAR-Indian Agricultural Research Institute, New Delhi, 110012 India*

**e-mail: mageshbioiari@gmail.com*

Received August 23, 2017

Abstract—The fungal cultures, namely – *Pleurotus sajor-caju* MTCC 1806, *Saccharomyces cerevisiae* MTCC 6933 and *Candida tropicalis* MTCC 1406 and their combinations, *C. tropicalis* + *S. cerevisiae*, *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *P. sajor-caju* were grown in minimal medium containing 100 ppm of gossypol as the sole carbon and energy source. The culture supernatants of *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* had low residual gossypol levels of 29 and 25 ppm, respectively. In the present study, we attempted to isolate gossypol-degrading enzyme and biodegraded gossypol from the culture supernatants of *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae*. The specific activity of laccase in the purified enzyme extracts of the *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* treated samples was 425 and 224 U/mg, respectively. In SDS-PAGE, the gossypol-degrading enzyme was revealed as 3 bands of molecular weights ranging from 45 to 66 kDa. The characterization of biodegraded gossypol by FTIR analysis showed a reduction in aldehydes (C-H) stretches in samples treated with fungi. Mass spectrometry analysis revealed that the monoisotopic mass of the biodegraded gossypol was 474 g/mol.

Keywords: biodegradation, *Candida tropicalis*, gossypol, *Pleurotus sajor-caju*, *Saccharomyces cerevisiae*

DOI: 10.1134/S0003683818030092

Biodegradation of phenolic/polyphenolic compounds is an oxidative process involving enzymes such as hydroxylases, oxygenases, laccases and peroxidases [1, 2]. Biodegradation of these complex aromatic structures is mediated by different microorganisms including bacteria [3–5], fungi [6–8], yeast [9] and actinomycetes [10, 11]. In addition, fungal laccases have received considerable attention because of their potential involvement in the transformation of various phenolic compounds [12].

Gossypol is a toxic polyphenolic compound present in the cotton plant. It has a molecular formula, monoisotopic 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- methylnaphthalene), respectively [13, 14]. In animals, gossypol toxicity results in reproductive diseases, growth depression and other intestinal and internal organ abnormalities. Gossypol shows a moderate acute toxicity in most species, with an oral lethal dose 50 varying in the range of 2400–3340, 500–950, 350–600, 550, and 280–300 mg/kg for rats, mice, rabbits, pigs and guinea pigs, respectively [14–16]. Microbial fermentation is a suitable method for detoxifying gossypol, because fermentation improves other nutritional properties such as the protein, and amino-acids content of cottonseed meal [17–24]. Microbial strains such as *Candida tropicalis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Aspergillus oryzae*, and *Pleurotus* sp., were effective in degrading gossypol in cottonseed meal [21, 25, 26].

A previous study revealed that the maximum detoxification rate of free gossypol by fungal combinations of *Pleurotus sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* was 92 and 75%, respectively, in cottonseed cake during solid state fermentation [27]. Yang with coworkers [23] reported that some unknown proteins were involved in *A. niger*-mediated gossypol biodegradation. Gossypol biodegradation by fungal cultures in cottonseed cake has been reported previously, however, the underlying mechanism is yet to be studied.

Therefore, the present study was aimed at isolating a gossypol-degrading enzyme and obtaining biodegraded gossypol from the culture supernatants of different combinations of fungal cultures such as *P. sajor-caju*, *S. cerevisiae* and *C. tropicalis* grown in minimal medium containing gossypol as the sole carbon and energy source.

[1] The article is published in the original.

MATERIALS AND METHODS

Microorganisms and culture conditions. The test microorganisms used in this study, namely *P. sajor-caju* MTCC 1806, *S. cerevisiae* MTCC 6933 and *C. tropicalis* MTCC 1406 were obtained from the Institute of Microbial Technology, Chandigarh, India. The cultures were grown in malt extract broth (Himedia, India) at 30°C under shaking conditions for 48 h. The cultures were maintained on malt extract slants at 4°C.

Preparation of inoculum. Cultures of *P. sajor-caju*, *S. cerevisiae* and *C. tropicalis* were grown in 100 mL of malt extract broth (pH 5.5) for 48 h at 30°C and 150 rpm. Biomass was harvested by centrifugation at 12000 × *g* for 5 min, washed twice with sterile water, suspended in 100 mL of sterile water and used as an inoculum.

Growth of fungal cultures in gossypol-containing minimal medium. The fungal cultures were grown in gossypol-containing minimal medium (pH 5.5), which was composed of (g/L): $NaNO_3$—0.5; K_2HPO_4—0.65; KH_2PO_4—0.2 and $MgSO_4$—0.1. Gossypol acetic acid (MP Biomedicals, USA) dissolved in dimethyl sulfoxide (Fisher Scientific, USA) was added to 100 mL of sterile minimal medium for attaining the final concentration of 100 ppm of gossypol as the sole carbon and energy source. Cultures of *P. sajor-caju, C. tropicalis* and *S. cerevisiae* individually (2%) and mixed cultures, *P. sajor-caju* + *S. cerevisiae* (each 1%) and *C. tropicalis* + *S. cerevisiae* (each 1%) were inoculated in separate flasks with gossypol-containing minimal medium. An uninoculated sample was maintained as the control. The inoculated flasks were incubated at 30°C and 150 rpm. Ten milliliters of broth was sampled from the flasks at different incubation periods (0, 24, 48, 72 and 96 h) and centrifuged at 12000 × *g* for 5 min to harvest the biomass. The supernatants were analyzed for their residual gossypol level by AOCS Ba 7-58. The spectrophotometric method for determining gossypol involved reaction of gossypol with aniline to form a yellow dianilino-gossypol [28]. Decrease in the residual gossypol level in the supernatants was determined using the formula (Control-Treated)/Control × 100. The dry weight of biomass (mg/10 mL) was calculated after maintaining the biomass in a hot air oven at 100°C for 4 h.

Isolation and characterization of gossypol-degrading enzyme. ***Enzyme assay.*** Cultures of *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* were inoculated in minimal medium containing 100 ppm of gossypol. The inoculated flasks were incubated for 48 h at 30°C and 150 rpm. The supernatants were separated as described previously and evaluated for the activities of laccase, polyphenol oxidase and peroxidase. Laccase (Sigma-Aldrich, USA), polyphenol oxidase, and peroxidase were determined quantitatively by adding their respective substrates (Acros Organics, USA), namely syringaldazine (0.216 mM), catechol (0.1%) and guaiacol with hydrogen peroxide (4%), respectively following the method reported by Arnnok et al. [29]. The protein content (mg) in the supernatant was determined according to the Lowry procedure.

Purification of gossypol-degrading enzyme. One hundred milliliters of culture supernatants of *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* was precipitated with crystal ammonium sulfate 80%. Excess salt was removed by dialysis against distilled water. The purified samples were dissolved in 100 mM Na-phosphate buffer (pH 6.5). These samples were loaded in a Sephadex G-100 (Merck, Germany) column (1.5 × 35 cm) equilibrated with the same buffer. Fractions of 3 mL each were collected. The active eluted fractions were pooled and dialyzed against the same buffer. The protein content and laccase activity of the ammonium sulphate precipitates and purified enzyme extracts were determined as described earlier.

Gossypol biodegradation by purified enzyme. The culture supernatants and purified enzyme extracts, *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* were evaluated for gossypol biodegradability. The reaction mixtures were prepared in 5 mL vials; 0.5 mL of culture supernatants was added in 2.5 mL of 100 mM Na-phosphate buffer (pH 6.5). The final concentration of gossypol in the reaction mixture was 100 ppm. Futhermore, 0.1 mL of the enzyme extracts (2 mg protein/mL) was added in 2.5 mL of the same buffer containing gossypol at a final concentration of 100 ppm. Reaction tubes were incubated in a horizontal shaker at 40ºC for 1 h. Residual gossypol level in the mixture was analyzed by AOCS Ba 7-58.

UV-Vis spectrum. The purified enzyme extracts were scanned using UV-Vis spectrophotometer UV-1700 (Shimadzu, Japan) at OD in the range of 190–650 nm.

SDS-PAGE. For determining the molecular weight of the gossypol-degrading enzyme, SDS-PAGE was performed with 14% polyacrylamide gel. The SDS-PAGE reagents were purchased from Sigma-Aldrich, USA. Fifty microliters of the ammonium sulphate purified samples were run in a vertical gel electrophoresis [30] unit and stained with Coomassie brilliant blue R250 [31].

Isolation and characterization of biodegraded gossypol. Cultures of *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* were inoculated in 500 mL of minimal medium containing 100 ppm of gossypol as described earlier. Biomass was separated by centrifugation at 12000 × *g* for 5 min at room temperature. The supernatants were lyophilized in freeze dryer-lyophilizer Vir Tis 6K (Spinco Biotech., India). The lyophilized samples were dissolved in 5 mL of acetone, mixed with 100 mL of chilled ethanol and kept at −20°C overnight. The precipitates formed were removed by centrifugation at 12.000 × *g* for 10 min at 4°C. The supernatants were lyophilized and stored at −20°C for further use.

Size exclusion chromatography (SEC). The lyophilized samples were dissolved in 5 mL of acetone,

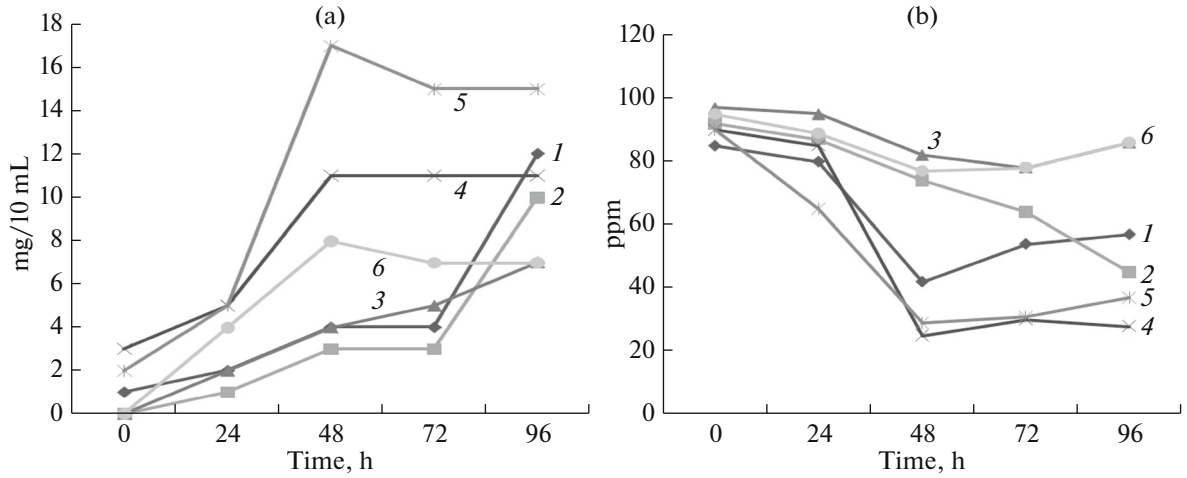

Fig. 1. Biomass (a) and residual gossypol (b) in fungal cultures grown in gossypol-containing minimal medium. *1—P. sajor-caju*; *2—S. cerevisiae*; *3—C. tropicalis*; *4—P. sajor-caju* + *S. cerevisiae*; *5—C. tropicalis* + *S. cerevisiae* and *6—C. tropicalis* + *P. sajor-caju*. Values are the means of 3 experiments.

passed through an acetone-equilibrated silica gel 60 (0.015–0.040 mm) (Merck, Germany) column (1.5 × 35 cm), and eluted with 70% acetone at a flow rate of 0.5 mL/min. Furthermore, 1 mL fractions were collected and their absorbance values in the UV-Vis range were recorded; the fractions showing peak were pooled and concentrated by lyophilization.

Thin layer chromatography (TLC). TLC-plates, silica gel 60 (15 μm) coated with fluorescent indicator F254 (Merck, Germany), 10 × 4 cm dimensions were used and the SEC-purified samples dissolved in acetone were spotted 1 cm above the base. The spotted TLC plates were placed in a beaker containing tested mobile solvents namely, 65% chloroform + 35% methanol, 20% hexane + 80% ethyl acetate, 35% chloroform + 65% methanol, 100% ethyl acetate and 70% acetone. After the solvent travelled up to 90% in the TLC plate, the plates were placed in a beaker saturated with iodine vapors and the beaker was covered. After the spots developed, the Rf values were calculated.

Analytical HPLC. The SEC-purified samples dissolved in dimethyl sulfoxide were analyzed using analytical HPLC (Shimadzu LC-10 AT vp model, Japan), with a UV-Vis spectrophotometry diode array detector (spectral scan, 190–800 nm). The HPLC system was equipped with a Shimadzu (Japan) C18 column (4 × 6 × 250 mm,), packed with 5 μm particles. The mobile phase consisted of acetonitrile : water (80 : 20 vol/vol) with 0.1% phosphoric acid. The flow rate was 1 mL/min and the reading was monitored at 254 nm.

FTIR spectrum. To know the infra-red spectrum, the biodegraded gossypol was processed with KBr to make a pellet. Spectra of the pellet were recorded in the transmission mode between wave numbers of 4500 and 500 cm^{-1} using a resolution of 4 cm^{-1} 20 kHz scans speed in Shimadzu IR Prestige 21 analyzer (Japan).

Mass spectrometry. For determining the monoisotopic mass of the biodegraded gossypol, electrospray ionization-mass spectrometry (ESI-MS) was performed using a liquid chromatography quadrupole (LCQ) ion trap mass spectrometer (Finnigan MAT, USA) equipped with an electrospray ionization interface operated in the positive-ion mode. Full MS acquisition was recorded over 150–1.500 amu.

RESULTS

Growth of fungal cultures in gossypol-containing minimal medium. The biomass and residual gossypol levels in the culture supernatants of fungal cultures during different incubation periods (0, 24, 48, 72 and 96 h) were recorded (Fig. 1). The biomass increased with an increase of the incubation period. The residual gossypol level in the un-inoculated (control) flask was 100 ± 2 ppm (results not shown) irrespective of the incubation period. Although biomass was higher at 96 h, the residual gossypol level in the supernatants was after 48 h of incubation. The culture supernatants of *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* had low residual gossypol levels of 29 and 25 ppm, respectively, at 48 h of incubation.

Isolation and characterization of gossypol-degrading enzyme. The culture supernatants of the mixed fungal cultures were evaluated for activities of polyphenol oxidase, peroxidase and laccase. The specific activities of peroxidase and laccase in the samples treated with *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae*- were 3.6 and 1.5 U/mg and 12.0 and 8.0 U/mg, respectively (Fig. 2). The supernatants had negligible polyphenol oxidase activity (results not shown). The gossypol-degrading enzyme in the supernatants was precipitated using ammonium sulfate (80%) and purified through Sephadex G-100 column chromatography.

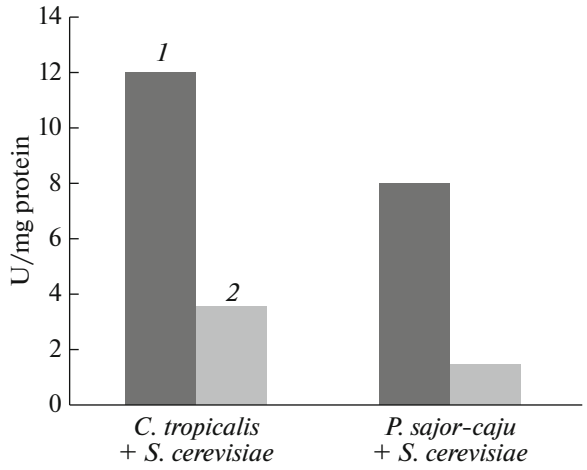

Fig. 2. Specific enzyme activity for laccase (*1*) and peroxidase (*2*) in supernatants of mixed fungal cultures. Values are the means of 3 experiments.

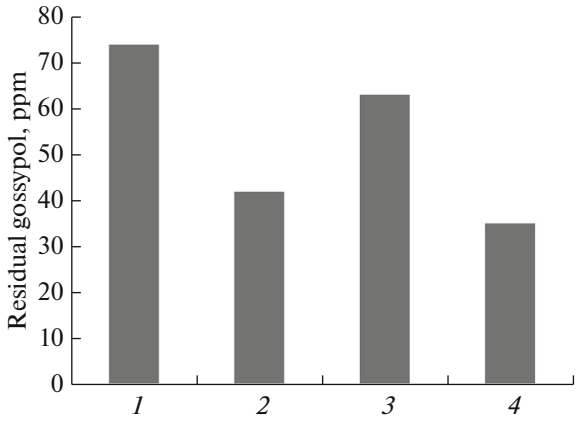

Fig. 3. Biodegradation of gossypol by fungal mixture. *1*—*P. sajor-caju* + *S. cerevisiae,* culture supernatant; *2*—*P. sajor-caju* + *S. cerevisiae,* purified enzyme; *3*—*C. tropicalis* + *S. cerevisiae,* culture supernatant; *4*—*C. tropicalis* + *S. cerevisiae,* purified enzyme. Values are the means of 3 experiments.

The specific activity of laccase in the purified extracts of the samples treated with *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* was 425 and 224 U/mg protein, respectively. The purification of the enzyme gave 35.4- and 27.9-fold increases in purity, respectively (Table 1). Gossypol degradation by the purified enzyme produced by mixture of different fungi was evaluated. The results revealed that the residual gossypol in the reaction mixtures treated with *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* was 42 and 35 ppm, respectively (Fig. 3). The UV-vis spectral analysis of the purified gossypol-degrading enzyme produced by different fungal mixtures showed an absorption maximum at 260–300 nm (Fig. 4). In SDS-PAGE, the purified enzyme was separated as 3 bands of molecular weights ranging from 45 to 66 kDa (Fig. 5).

Isolation and characterization of biodegraded gossypol. The biodegraded gossypol from the culture supernatants of *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* was separated by ethanol precipitation and lyophilized subsequently. The purification was done by SEC. The fractions from SEC showed 2 absorption peaks at 385 nm and another peak at 340 nm (results not shown). The fractions at 340 nm were collected, pooled, concentrated and subjected to further analysis. Among the different solvents tested (results not shown), TLC run with 70% aqueous acetone had distinct spots with Rf values of 0.74 and 0.77 in *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* cultures, respectively (Fig. 6).

Analytical HPLC showed major peaks at retention time of 12.5 min in the case of treatment with *P. sajor-caju* + *S. cerevisiae*, and *C. tropicalis* + *S. cerevisiae* whereas a retention time of 12.0 min was recorded for the control (Fig. 7). The FTIR analysis revealed major peaks at wavelength (cm^{-1}) 3384, 2426 and 1383 (Fig. 8a). While, in samples treated with fungal cultures (Figs. 8b, 8c), a reduction in C-H stretches (aldehydes) was observed. The MS analysis of the

Table 1. Purification of gossypol-degrading enzyme from mixed fungal cultures

Purification	Total activity,* U/mL	Protein content, mg/mL	Specific activity, U/mg protein	Purification, -fold
C. tropicalis + *S. cerevisiae*				
Supernatant	432	36	12	1
Ammonium sulfate precipitation	1688	8.5	198.6	16.6
Sephadex G-100	850	2.0	425	35.4
P. sajor-caju + *S. cerevisiae*				
Supernatant	362	45	8.0	1
Ammonium sulfate precipitation	1006	10	100.6	12.5
Sephadex G-100	560	2.5	224	27.9

* Laccase activity was measured using syringaldazine as substrate.

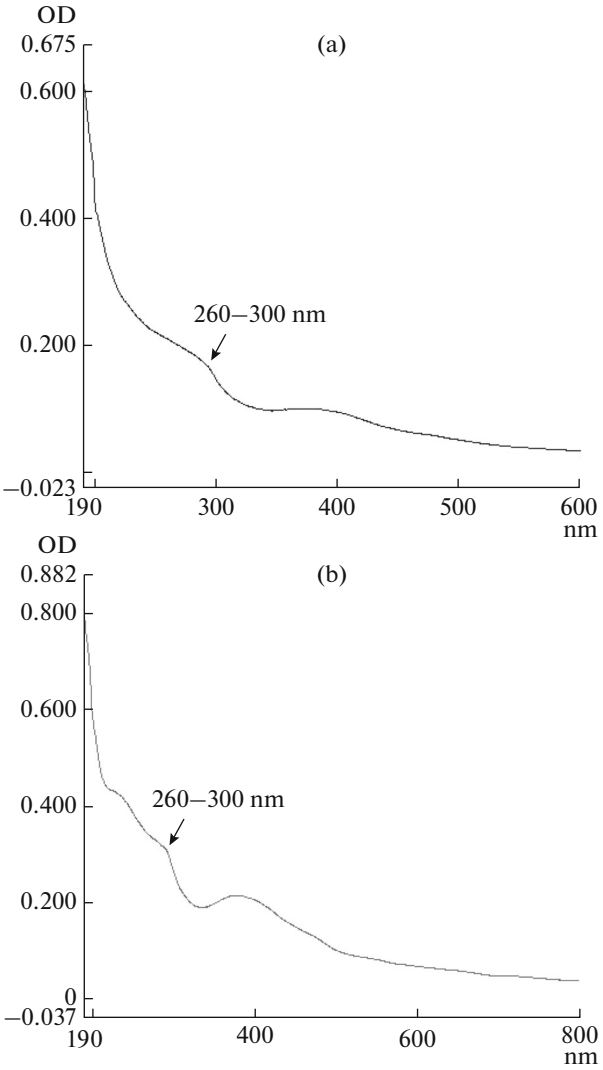

Fig. 4. UV-vis spectrum of purified gossypol-degrading enzyme produced by different fungal mixtures. (a) *C. tropicalis* + *S. cerevisiae*; (b) *P. sajor-caju* + *S. cerevisiae*.

SEC-purified biodegraded gossypol shown that the monoisotopic mass (*m/z*) of the un-inoculated (control) and samples treated with *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* were 517.3, 474.3 and 474.2 g/mol, respectively (Fig. 9).

DISCUSSION

The results from previous studies have indicated that the fungal strains such as *C. tropicalis*, *S. cerevisiae*, *Pleurotus* sp and *A. niger* and their combinations can biodegrade up to 90% gossypol in cottonseed meal [19–21, 25, 26]. In this study, we attempted to isolate a gossypol-degrading enzyme and obtain biodegraded gossypol from the culture supernatants of fungal cultures grown in gossypol-containing minimal medium. The experimental results revealed that the cultures of *C. tropicalis*, *S. cerevisiae*, *P. sajor-caju* and their combinations, *P. sajor-caju* + *S. cerevisiae*, *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *C. tropicalis*, could grow in minimal medium containing 100 ppm of gossypol as the sole carbon and energy source. In another study, biodegradation of phenanthrene, a three-ring polycylic aromatic hydrocarbon, by white rot fungus, *Trametes versicolor* was investigated in a medium containing 100 mg/L of phenanthrene [7]. The residual gossypol level in the cultures of *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* was the lowest at 48 h of incubation (Fig. 1). A slight increase in the residual gossypol level of the culture supernatant after 48 h might be due to the release of negligible levels of gossypol bound with fungal protein during the earlier stages of growth. In a similar study, mixed cultures of *C. tropicalis* ZD-3 + *A. niger* ZD-8 and *S. cerevisiae* + *A. niger* had a higher gossypol detoxification rate in cottonseed meal than individual cultures [20, 32]. Yang et al. [22] reported that the optimum conditions for gossypol-degrading fungal cultures grown in minimal medium are 72 h at 30°C. The fungal cultures, such as *Candida*, *Fusarium*, *Aspergillus*, *Phanerochaete*, *Saccharomyces*, and *Pleurotus*, have the ability to degrade phenol and phenol-related compounds and utilize them as the sole carbon and energy source [1, 2].

The fungal enzymes involved in the degradation of polycyclic aromatic compounds are peroxidases, laccases and polyphenol oxidases [7, 12, 33]. In the present study, laccase activity (syringaldazine used as substrate) of the culture supernatants of mixed fungal cultures was higher than their peroxidase activity; polyphenol oxidase activity of these cultures was negligible. The specific activity of laccase in the purified

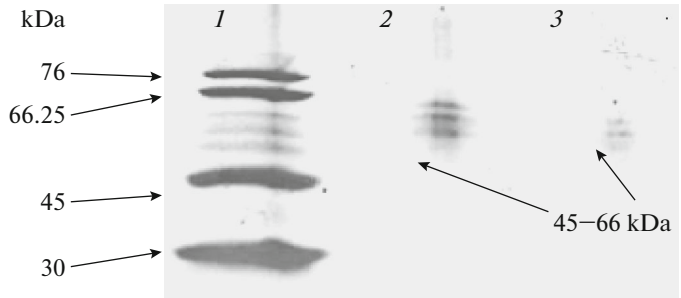

Fig. 5. SDS-PAGE of gossypol-degrading enzyme. *1*—Protein markers; *2*—enzyme produced by *C. tropicalis* + *S. cerevisiae*; *3*—enzyme produced by *P. sajor-caju* + *S. cerevisiae*.

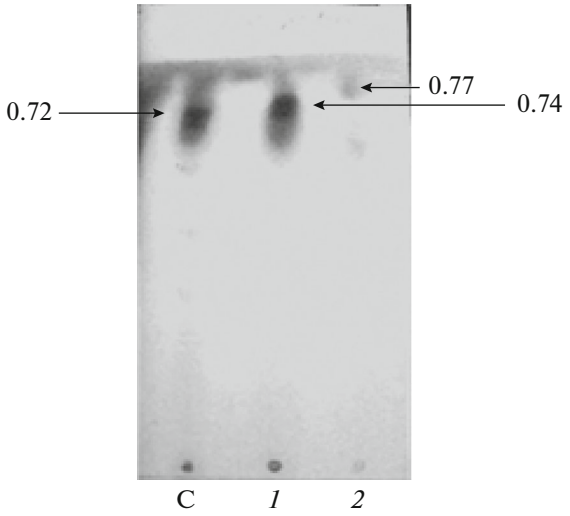

Fig. 6. TLC chromatogram of biodegraded gossypol in 70% acetone. C—control; *1*—treatment with *C. tropicalis* + *S. cerevisiae*; *2*—treatment with *P. sajor-caju* + *S. cerevisiae*.

enzyme was 29–35 fold higher than that in the culture supernatants. The culture supernatants and purified laccase were evaluated for their ability to degrade 100 ppm of gossypol in phosphate buffer. The culture supernatants and purified enzyme extracts reduced the residual gossypol levels by 30 and 60% respectively, in the reaction mixtures, demonstrating that extracellular laccase produced by mixed fungal cultures is involved in gossypol degradation in the culture medium. In a similar study, gossypol degradation by an extracellular laccase produced by *Pleurotus florida* grown on chopped rice straw substrates was demonstrated [34]. In the present study, characterization of the purified enzyme by UV-vis spectral analysis, confirmed its proteinaceous nature. The molecular mass of the purified enzyme ranged from 45 to 66 kDa, as determined by 14% SDS-PAGE. In a similar study, laccase isozymes with a molecular weight of 60 and 65 kDa with a pI value of 3.0 were isolated from *Pleurotus ostreatus* [35]. The molecular weight of laccase produced by wood rotting fungi was estimated to be 60 kDa [33]. Proteomic analysis of the enzyme extract obtained from the gossypol-degraded medium indicated the presence of protein spots with molecular weights ranging from 25 to 66 kDa [23]. Therefore, previous studies substantiated the present study results, which indicate that laccase is produced by mixed fungal cultures in gossypol-containing minimal medium.

In this study, we also attempted to isolate and characterize biodegraded gossypol from the culture supernatants. The culture supernatants of *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* were subjected to ethanol precipitation for eliminating the residual protein according to the method reported by

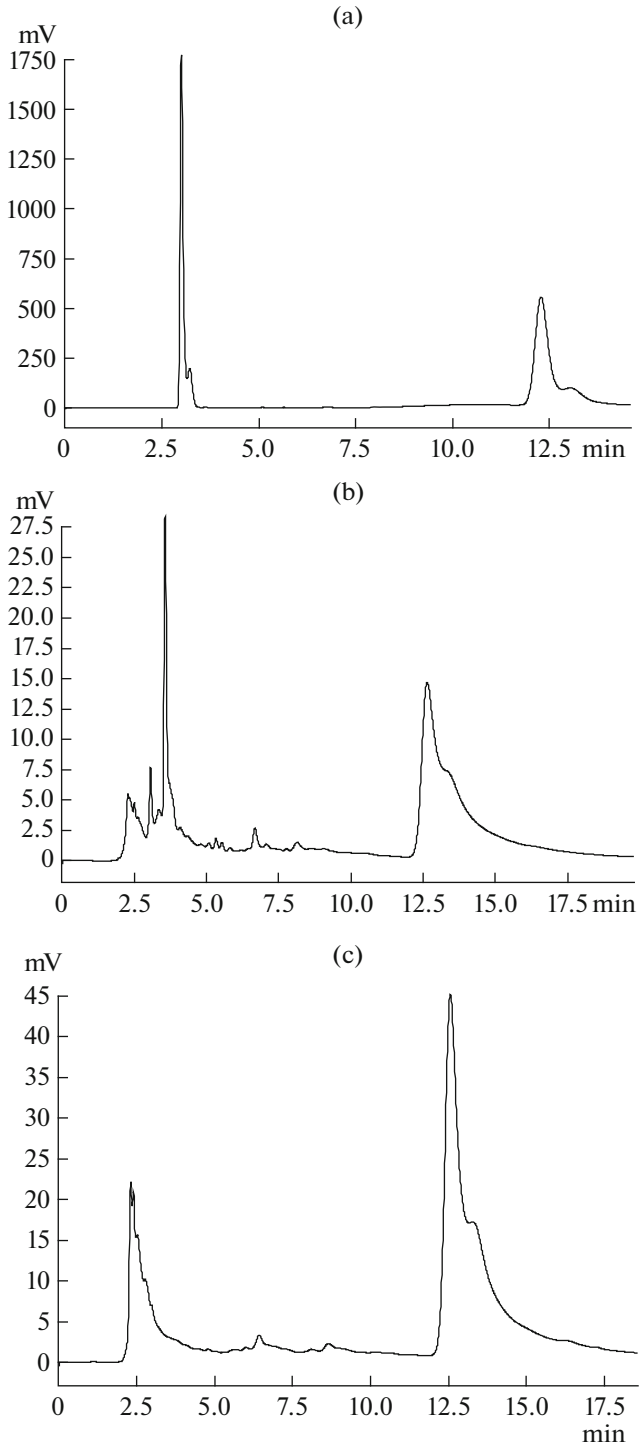

Fig. 7. HPLC chromatogram of biodegraded gossypol. (a) Control (un-inoculated); (b) treatment with *P. sajor-caju* + *S. cerevisiae*; (c) treatment with *C. tropicalis* + *S. cerevisiae*.

Van Oss [36]. The protein-free extracts were purified by SEC. The purified fractions having absorption maxima at 340 nm, referred to as SEC purified fractions, were collected and pooled. Fractions with

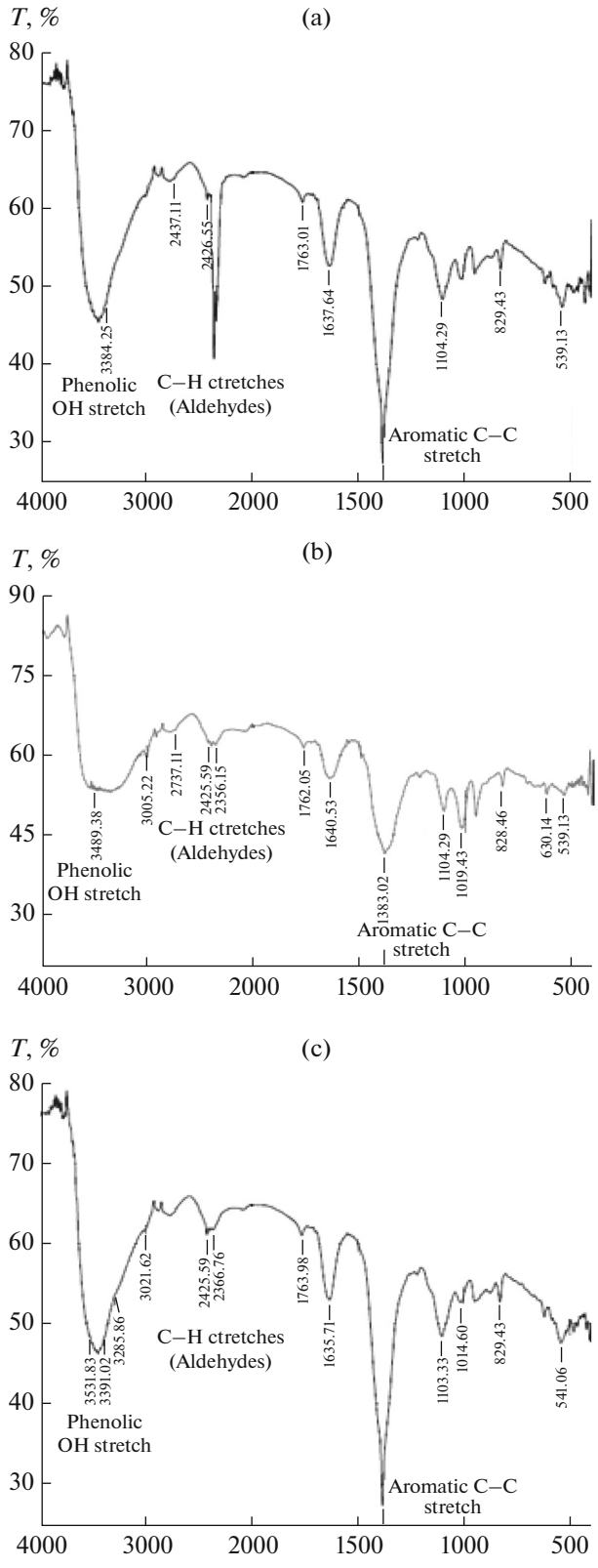

Fig. 8. FTIR spectrum of biodegraded gossypol. (a) Control (gossypol in mineral medium); (b) treatment with *P. sajor-caju* + *S. cerevisiae*; (c) treatment with *C. tropicalis* + *S. cerevisiae*.

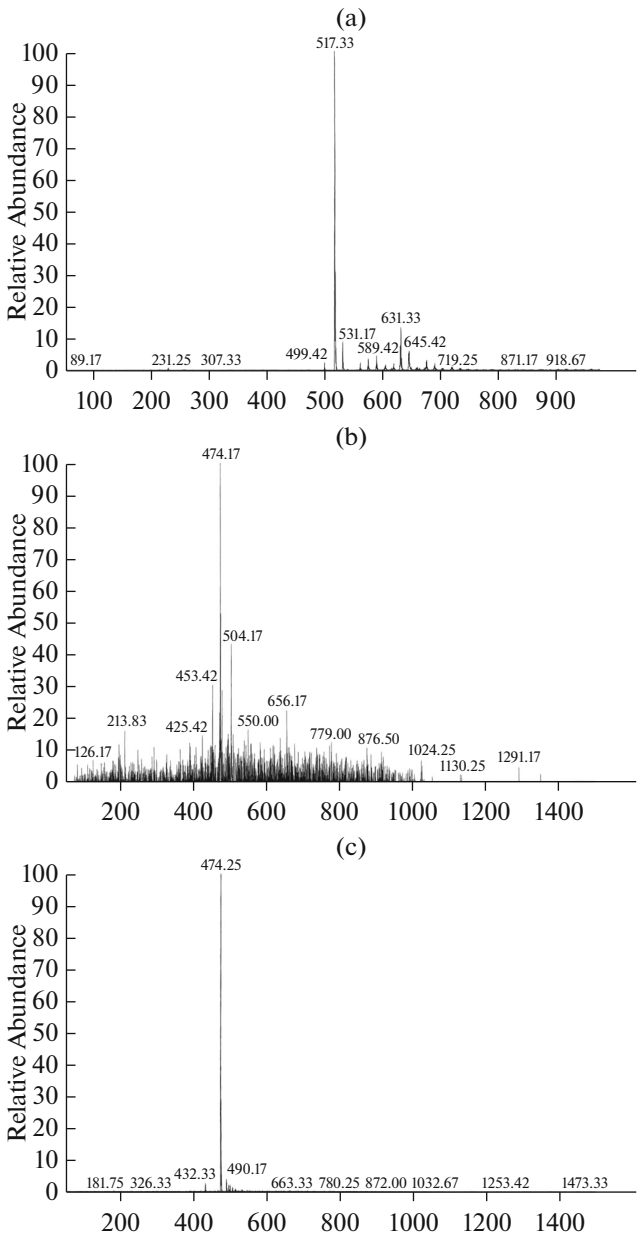

Fig. 9. Mass chromatogram of biodegraded gossypol. (a) Control; (b) treatment with *C. tropicalis* + *S. cerevisiae*; (c) treatment with *P. sajor-caju* + *S. cerevisiae*.

absorption maxima at 385 nm corresponded to residual gossypol in the supernatant [15]. The TLC analysis revealed a small change in the Rf values of SEC-purified fractions compared with that of gossypol (control) in the aqueous acetone (70%) solvent phase. Furthermore, analytical HPLC analysis revealed a difference of 0.5 min in the retention time values of the SEC purified fraction and control (gossypol). In a similar study, biodegraded compound, anthroquinone with retention time of 4.4 was observed in the HPLC analysis during biodegradation studies of anthracene by *Bjerkandera* sp. [16]. The results of TLC and HPLC

analyses demonstrated that gossypol is structurally modified in samples treated with fungi. The aldehyde groups present in gossypol's tautomeric forms and are suggested to be the main contributors to gossypol's toxicity [13]. The FTIR analysis revealed that there is a reduction in aldehyde stretch in samples treated with fungal cultures suggesting detoxification of gossypol took place during biodegradation process. The MS analysis revealed that the monoisotopic mass of the biotransformed gossypol was 474 g/mol compared with that of gossypol (517 g/mol). The results are consistent with those of previous studies [18–23], suggesting that the mixed fungal cultures used in this study transform gossypol into a non-toxic metabolites in the gossypol containing-minimal medium.

Cultures of *C. tropicalis* + *S. cerevisiae* and *P. sajorcaju* + *S. cerevisiae* showed maximum gossypol degradation (71 and 75%, respectively) in gossypol-containing minimal medium. The mixed fungal cultures produced laccase with molecular weights ranging from 45 to 66 kDa. The isolated biodegraded gossypol had a monoisotopic mass of 474 g/mol. In conclusion, the results of this study revealed that mixed fungal cultures produce laccase and transform gossypol into non-toxic metabolites. Further research elucidating the gossypol biodegradation pathway with participation of laccase is warranted.

ACKNOWLEDGMENTS

The financial assistance provided by the National Agricultural Science Fund (NASF), ICAR, India (grant no. PHT 3026) is greatly acknowledged.

REFERENCES

1. Basha, K. M., Rajendran, A., and Thangavelu, V., *Asian J. Exp. Biol. Sci.*, 2010, vol. 1, no. 2, pp. 219–234.
2. Krastanov, A., Alexieva, Z., and Yemendzhiev, H., *Eng. Life Sci.*, 2013, vol. 13, no. 1, pp. 76–87.
3. Saber, D.L. and Crawford, R.L., *Appl. Environ. Microbiol.*, 1985, vol. 50, no. 6, pp. 1512–1518.
4. Gurujeyalakshmi, G. and Oriel, P., *Appl. Environ. Microbiol.,* 1989, vol. 55, no. 2, pp. 500–502.
5. Khan, S.A., Hamayun, M., and Ahmed, S., *Enzyme Microb. Technol.,* 2006, vol. 38, nos. 1–2, pp. 10–13.
6. Field, J.A., Jong, E.D., Costa, G.F., and Bont, J.A. M., *Appl. Environ. Microbiol.*, 1992, vol. 58, no. 7, pp. 2219–2226.
7. Han, M.-J., Choi, H.-T., and Song, H.-G., *J. Microbiol.*, 2004, vol. 42, no. 2, pp. 94–98.
8. Bishnoi, K., Kumar, R., and Bishnoi, N.R., *J. Sci. Ind. Res.*, 2008, vol. 67, pp. 538–542.
9. Sunil, S.A., Chen, M.-Y., Lee, D.-J., and Ren, N.-Q., *Biotechnol. Bioeng.*, 2007, vol. 96, no. 5, pp. 844–852.
10. Hanne, L.F., Kirk, L.L., Appel, S.M., Narayan, A.D., and Bains, K.K., *Appl. Environ. Microbiol.*, 1993, vol. 59, no. 10, pp. 3505–3508.
11. Jaweria, R., Peshwe, S.A., and Ingale, A. G., *Ind. J. Appl. Res.*, 2013, vol. 3, no. 6, pp. 241–243.
12. Baldrian, P., *FEMS Microbiol. Rev.*, 2006, vol. 30, no. 2, pp. 215–242.
13. Kenar, J.A., *JAOCS*, 2006, vol. 83, no. 4, pp. 269–302.
14. Gadelha, I.C.N., Fonseca, N.B.S., Oloris, S.C.S., Melo, M.M., and Blanco, B.S., *Sci. World J.*, 2014, vol. 1, pp. 1–11.
15. *EFSA Journal,* 2008, vol. 908, pp. 1–55.
16. Ozdogan, M., Wellmann, K., and Paksuz, E., *J. Anim. Physiol. Anim. Nutr.*, 2012, vol. 96, no. 1, pp. 95–101.
17. Weng, X.-Y. and Sun, J.-Y., *Process. Biochem.,* 2006, vol. 41, no. 7, pp. 1663–1668.
18. Weng, X.-Y. and Sun, J.-Y., *Biochem. Eng. J.*, 2006, vol. 32, no. 3, pp. 226–232.
19. Zhang, W.-J., Xu, Z.-R., Zhao, S.-H., Jiang, J.-F., Wang, Y.-b., and Yan, X.-H., *Toxicon*, 2006, vol. 48, no. 2, pp. 221–226.
20. Zhang, W.-J., Xu, Z., Sun, J., and Yang, X, *Asian–Aust. J. Anim. Sci.*, 2006, vol. 19, no. 9, pp. 1314–1321.
21. Zhang, W.-J., Xu, Z.-R., Zhao, S.-H., Sun, J.-Y., and Yang, X., *Animal Feed Sci. Tech.*, 2007, vol.135, no. 1, pp. 176–186.
22. Yang, X., Guo, J.-L., and Sun, J.-Y., *Afr. J. Microbiol. Res.*, 2011, vol. 5, no. 19, pp. 3066–3072.
23. Yang, X., Sun, J.-Y., Guo, J.-L., and Weng, X.-Y., *J. Sci. Food Agric.,* 2011. doi 10.1002/jsfa.4675
24. Wang, X., Tang, U.-W., Yao, X.-H., Wu, Y.-F., Sun H., and Xu, Y.-X, *Afr. J. Microbiol. Res.*, 2012, vol. 6, no. 36, pp. 6537–6544.
25. Khalaf, M.A. and Meleigy, S., *Int. J. Agric. Biol.,* 2008, vol. 10, no. 2, pp.185–190.
26. Mageshwaran, V. and Kathe, A.A., *Indian J. Anim. Nutr.,* 2013, 30, no. 3, vol. pp. 313–319.
27. Shaikh, A., Kathe, A.A., and Mageshwaran, V., *KKU Res. J.*, 2014, vol. 19 (suppl.), pp.67–73.
28. *AOAC, Free and Total Gossypol Methods, Official and Tentative Methods of the AOCS*, American Oil Chemist's Society, 4th ed., Chicago, 1989, pp. 1–5.
29. Arnnok, P., Ruangviriyachai, C., Mahachai, R., Techawongstien, S., and Chanthai,S., *Int. Food Res. J.,* 2010, vol. 17, pp. 385–392.
30. Laemmli, L.K., *Nature,* 1970, vol. 227, pp. 680–685.
31. *Protein Electrophoresis*, Kurien, B. and Scofield, R., Eds., Totowa, N.J.: Humana Press, 2012, vol. 869, pp. 471–479.
32. Atia, A.I. and Rahim, G.A.A., *J. Radiat. Res. Appl. Sci.*, 2009, vol. 2, no. 2, pp. 397–415.
33. Heinzkill, M., Bech, L., Halkier, T., Schneider, P., and Anke, T., *Appl. Environ. Microbiol.,* 1998, vol. 64, no. 5, pp. 1601–1606.
34. Rajarathnam, S., Shashirekha, M.N., and Bano, A., *World J. Microbiol. Biotechnol.*, 2001, vol. 17, no. 3, pp. 221–227.
35. Mansur, M., Arias, M.E., and Flardh, M., *Mycologia,* 2003, vol. 95, no. 6, pp. 1013–1020.
36. Van Oss, C. J., *J. Protein Chem.,* 1989, vol. 8, no. 5, pp. 661–68.