Identification and Characterization of Potential Biocide-Resistant Fungal Strains from Infested Leathers – A Systematic Study

by
Kavitha Sundar,[1,2] Yasmin Khambhaty[1,2] and Chandra Babu Narasimhan Kannan[1,2]*
[1]*CSIR–Central Leather Research Institute, Leather Process Technology Division*,
Adyar, Chennai-600 020, India
[2]*Academy of Scientific and Innovative Research (AcSIR),* Ghaziabad - 201002, India

Abstract

This study is aimed at identification of biocide tolerant/resistant fungal strains afflicting the leather industry. Fungal infestation occurs sometimes despite biocide treatment during leather processing. This persistent growth can be due to the development of biocide resistance which can lead to health hazards and economic loss. As no study has so far been reported to either confirm this or to identify such fungal strains, a systematic approach has been made in this study to address these aspects. Fungal strains were collected from infested leathers from tanneries to identify biocide resistant fungal strains afflicting leather industry. Phenotypic characterization revealed *Aspergillus* as the most dominant with 58% occurrence. Ten isolates were subjected to 18s rRNA sequencing and four strains were identified as *Aspergillus niger*. An *in-vitro* susceptibility to four leather fungicides was assessed to identify the biocide tolerant strains. S-6 *A. niger* strain was found to be the most tolerant as evidenced by high MIC (7.81µg ml^{-1}) against the most effective biocide, 2-(thiocyanomethylthio) benzothiazole. I*n-vivo* studies on chrome-tanned leathers also confirmed this finding. SEM studies revealed considerable morphological changes in S-6 compared to wild strain providing further evidence that it may have developed biocide resistance.

Introduction

Biocides are widely used in industries like agriculture, wood, leather etc., where the materials are prone to fungal attack.[1] The occurrence of fungal infestation on leather is a common problem, especially in conditions with high humidity.[2] Some of the fungi that are encountered in leather industry predominantly belong to genus of *Aspergillus, Penicillium, Rhizopus* and *Paecilomyces*. [3-6] The growth of fungi is controlled to a large extent with the aid of biocides. However, complaints regarding fungal growth exist despite the use of biocides during leather processing.[7] This might be due to improper use of biocides which includes insufficient dosage and concentration that may lead to poor distribution and uptake by the leathers. Insufficient contact time and inconsistency in biocide formulation can also result in reduced protection. Additionally, the most serious concern is with the probable development of biocidal resistance or tolerance in fungi. The possibility of the biocides loosing biocidal efficacy due to development of anti-microbial resistance (AMR) in some fungal species would present a difficult challenge.[8]

The infestation on leathers not only lessens the durability of the product but also decreases their commercial value.[5, 9] Fungal growth on finished leathers and products can lead to huge economic loss to the tanners as well as the buyers since remediation can be quite difficult. Hence, industries like leather sector needs to gear up to meet the emerging challenges on this front.

Resistance development towards antibiotics is well-studied and better understood than that against industrial biocides.[10] The mechanism of action pertaining to industrial biocides is also not well defined. The information available in the area of antibiotics is usually extrapolated to understand the biocidal mode of action.[11-13] Even though the concept of biocide resistance has been discussed in the leather industry, no systematic study is available either to confirm this or to identify the resistant strains of fungi.

Serious concerns regarding resistance development against biocides are expressed by various stakeholders of leather sector but the apprehension is mostly limited to protection against fungal infestation to prevent heavy economic loss. The tanners try to circumvent the problem by using biocides in rotation as well as employing new biocides in processing. But the AMR leading to serious health hazards due to possible cross resistance against antibiotics will have far reaching implications.[14, 15] Though, some chemical supply houses reject the idea of development of AMR in leather-borne fungi by arguing that it is a myth more than a fact,[16] the challenge on the front is imminent since, AMR development in clinical settings and in other areas have been proven to be real.[17] Concerted scientific efforts and studies are needed to understand the phenomenon and the mode of development in order to devise strategies for preventing the same.

Therefore, the present investigation focuses on isolation, screening and identification of biocide resistant strains. All the fungal isolates infecting leathers despite biocide treatment were collected from

* Corresponding author email: tanneryclri@yahoo.com
Manuscript received July 10, 2019, accepted for publication September 30, 2019.

various tanneries and phenotypically identified. Few strains were selected for genotypic characterization and *in vitro* antifungal susceptibility test. An *in vivo* study on biocide treated leathers was also conducted to test the growth of resistant strain. As there is no well documented study in this aspect about the leather industrial biocides, measures were taken for a systematic study in identification of a dominant fungal strain. The selected strain can be further studied in detail to understand the mode of biocide action and the possible mechanism involved in the rise of biocide resistance.

Materials and Methods

Source of media and chemicals

Media and other chemicals were purchased from Hi-Media, India. Biocide formulations based on 2-(thiocyanomethylthio)benzothiazole (TCMTB), 2-Mercaptobenzothioazole(2-MBT), 3-iodo-2-propynyl-N-butylcarbamate (IPBC) and 2,2-dibromo-3-nitrilopropionamide (DBNP) were obtained from reputed leather chemical supply houses. Wild strain *Aspergillus niger* (ATCC 6275) used in this study was procured from American Type Culture Collection (ATCC).

Collection of fungal infected leather samples from tanneries

An initiative has been taken in this study to screen and isolate fungi which infect leathers despite biocide treatment. Tanneries often facing the problem of fungal infestation were identified and a total of six tanneries were selected in the state of Tamil Nadu (Figure 1).

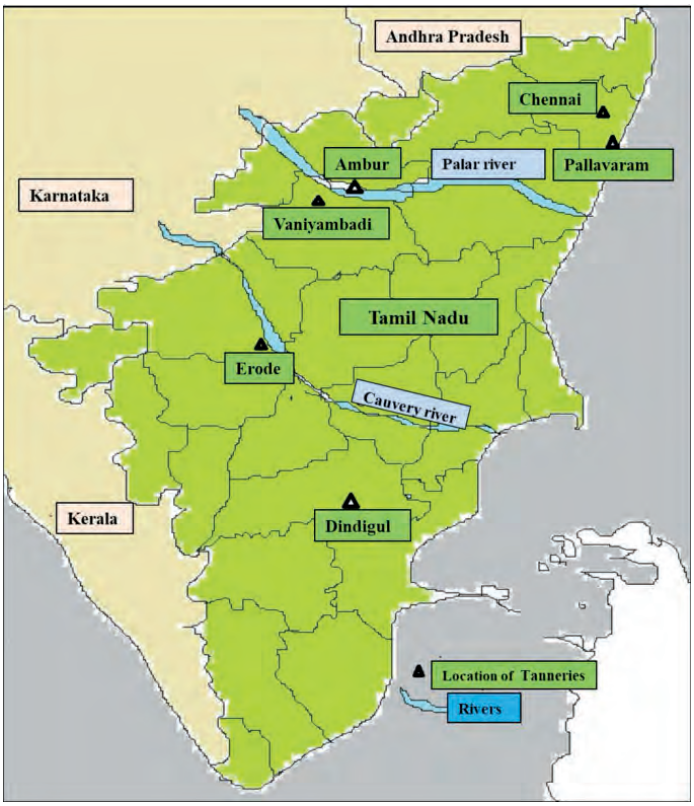

Figure 1. A map of State of Tamil Nadu, India showing the geographical location of tanneries for sample collection

Fungal strains were isolated from infested leathers, where the growth is prominently observed despite the biocide treatment. Fungal infested leathers (Figure 2) were sampled at different sampling sites within the tannery and were subsequently labelled. For example, in tannery 1, three different sampling sites were noted, and the affected leathers were collected, which resulted in a total of eight isolates. The same was followed in other tanneries and number of sites and the samples collected from each of them is presented in Table I.

A total of 35 fungal strains were obtained from leathers at different stages of leather processing, which were affected despite the biocide

Figure 2. Fungal infested leather samples collected from various tanneries

Table I

Sampling and Screening of fungal Isolates from various tanneries

Tannery Identified	Sampling sites	No. Fungi isolated
Tannery 1	Site 1	3
	Site 2	2
	Site 3	3
Tannery 2	Site 4	3
	Site 5	2
Tannery 3	Site 6	3
Tannery 4	Site 7	3
	Site 8	2
	Site 9	3
Tannery 5	Site 10	2
	Site 11	2
	Site 12	1
	Site 13	2
Tannery 6	Site 14	2
	Site 15	2

treatment. The infested biocide treated leather samples viz., chrome tanned, vegetable tanned, finished leathers and leather products collected around the year from different tanneries were brought to the laboratory for further investigation. Infested areas of leathers were cut using sterile blade and all the samples were labelled individually and placed in sterile zip-lock bags. These samples were examined microscopically for the fungal growth.

Isolation and maintenance of fungal cultures

The samples confirmed for the presence of fungal growth by microscopic examination were further subjected to isolation procedure. The spores present on the surface of the infected leathers were scraped using a sterile scalpel and placed on Sabouraud Dextrose Agar (SDA) plates and incubated *in vivo* at 30± 2°C for 5 to 7 d until sufficient growth was observed. Different fungal colonies observed on the plate were further purified on SDA plates and stored at 4°C.

Phenotypic characterization

The phenotypic identification of the fungal isolates was carried out by examining the macro-morphological features by colony morphology and growth characteristics of the pure fungal cultures as described by Raper and Fennel.[18] The features like colony diameter, mycelium color, soluble pigments and presence or absence of sclerotia were assessed.[19] Additionally, the presence or absence of fruiting bodies was also examined by visual and micro morphological observation.[20-22] The isolates were further examined by wet mount technique (Lobomed, Lx-300 microscope) using Lacto Phenol Cotton Blue (LPCB) as described by James and Natalie.[23] The spore size of conidia, conidiophores, and their arrangements were assessed by traditional method.[24]

Genotypic identification and Phylogenetic analysis

After phenotypic characterization, based on the occurrence and dominance of the fungal strains on leather, ten isolates comprising seven *Aspergillus* species with the labels S-1, S-4, S-5, S-6, S-8, S-9 and S-10, one *Rhizopus* sp. (S-3), one *Penicillium* sp. (S-11) and one *Paceliomyces* sp. (S-14) were characterized genotypically. The fungal cells obtained from the isolates grown in Sabouraud Dextrose Broth (SDB) for 5 d at 30± 2°C in an incubator shaker at 125 rpm were subjected to genomic DNA extraction. The fungal biomass in each case was harvested by centrifugation at 4000 × g for 5 min and washed twice with nuclease-free water. The DNA was extracted using HiPurA fungal DNA purification kit (Hi-Media, India) as per the manufactures' protocol.

The resulting PCR amplicons were purified and sequenced at Xcelris Genomics, India, using ABI 3730 × 1 Genetic Analyser, India. The obtained nucleotide sequence data was examined using aligner software to get consensus sequence which was further subjected to BLAST analysis using Blastn site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic tree was constructed for the obtained consensus sequence using the software MEGA version 7.0 for each isolate.[25] The multiple sequence alignment was done using CLASTAL W programme[26] and neighbour joining method was adapted for tree construction.[27]

***In vitro* susceptibility of the fungal strains against selected biocides**

The minimum inhibitory concentration (MIC) was determined *in vitro* against the selected test strains by macrobroth dilution method following EUCAST methodology used for filamentous fungi (M38-A).[28] For testing the susceptibility of isolated fungi towards selected biocides, a stock solution (1 mg ml^{-1}) was prepared for all four selected biocides in sterile broth. The spore suspension (2-5x106 spores ml^{-1}) was prepared from fresh 5 d old fungal culture for inoculum.[29] Briefly, a set of 12 vials was taken for serial dilution and to each of them; 2 ml of sterile SDB was added. 2 ml of test biocide from stock of 1 mg ml^{-1} was added to the first vial and serially diluted till 10th vial to get the concentrations in the range of 0.5-1000 µg ml^{-1}. The 11th and 12th vials served as growth control and sterile control respectively. To each vial (1-11) except the sterile control, 100 µl of spore suspension was added. All the vials were incubated at 30± 2°C for 48-72 h to observe for the visible growth. The lowest concentration that completely inhibited the growth was determined as the MIC.[30] All susceptibility tests were conducted in duplicates.

Studies on the growth of resistant strains on biocide treated leather

The growth of most resistant strains of *A. niger*, S-1, S-4, S-6 and S-8, as evidenced from high MIC needed to inhibit them in the study described in the previous section, was studied in comparison with wild type ATCC 6275 on TCMTB treated leathers. The trials were conducted using pickled cow hide which was cut into pieces (18 × 10 cm), labelled and weighed. After this, the hide pieces were chrome tanned and treated with TCMTB at varying concentrations of 0.05%, 0.1%, 0.2%, 0.75% on the weight of pickled pelt. One piece served as control which was neither treated with biocide nor inoculated with any of the strains. The hide pieces were treated with biocide in a steel drum that runs at an rpm of 10 for 60 min for proper distribution of the biocide into the leathers. After this, the samples were removed from the drum and left for ageing for 48 h, maintaining them in moist condition. These test pieces were eventually subjected to tropical chamber evaluation by standard accelerated method (IS: 6191-1971) to determine the growth of the each of the fungal strains. For this, spore suspensions were prepared for all the fungi to be tested. The test leather samples were placed on petri plates and about 0.1 ml of spore suspension was smeared on the surface of the leathers. The samples were placed in a humidity chamber maintained at 30±2°C and 95-100% relative humidity and visually examined every day for the appearance of growth.

Scanning electron microscopy (SEM) analysis

SEM analysis was performed to observe morphological changes, if any, in *A. niger* test strains S-1, S-4, S-6, S-8 in comparison with wild type *A. niger*. Pure cultures of all five isolates grown for 5 d in SDB were harvested to obtain the mycelial mat. The obtained mycelial mat samples were fixed in buffered 3% glutaraldehyde (0.05M phosphate buffer, pH 6.5) and incubated at 4°C for 24 h. The

samples were then washed twice with sterile water and subjected to dehydration in ethanol series (10% – 90%) for 10 min each followed by two washings in 100% ethanol. The samples were dried by transferring them to critical point drier in the presence of liquid CO_2 and stored in a desiccator for further use. The specimens were mounted on aluminum stubs using suitable carbon tape and gold coated using an Edwards E-306 sputter coater and examined in Bruker S-3400N at a magnification of 10000 × and a voltage of 10Kv.

Results and Discussion

Species of *Aspergillus* are found to be widely distributed worldwide. They grow over a wide temperature range with relatively high humidity favorable for the fungi to thrive on leather.[34] The screening study reveals the dominant occurrence of genus *Aspergillus* with 58% of infested leathers screened.

Phenotypic characterization

Identification for all the 35 fungal isolates was carried out on the basis of their phenotypic morphology by visual observation with the help of taxonomic key as reported previously[18, 31-32] as well as by microscopic characteristics as studied by the classical method widely used for identification.[18] The macro morphological features for the selected fungal species are depicted in Table II.

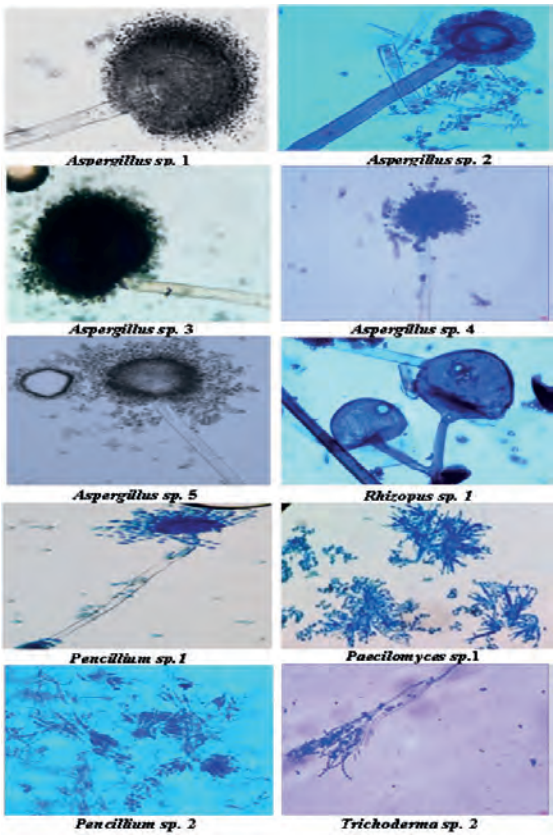

Figure 3. Lacto Phenol Cotton Blue stained fungal images observed at 40X magnification

Table II

Macro-Morphological features of the fungal isolates from tanneries

	MACRO-MORPHOLOGICAL FEATURES							
Species	**Surface/ Colony Color**	**Margin**	**Colony Reverse Color**	**Elevation**	**Growth**	**Production of Exudates**	**Soluble Pigments**	**Optimum Temperature & pH**
Aspergillums sp.1	Black (or) slightly brown to black	Entire	Creamy white to yellow/ black	Umbonate, rough surface	Moderate to rapid	Nil	Yes/some lack	32-37°C pH 5.5-7.0
Aspergillums sp. 2	Dark black	Entire	White to dull yellow	Umbonate	Moderate to rapid	Nil	Not known	32-35°C pH 5.0-6.5
Aspergillums sp. 3	White to yellow	Half or more	Pale yellow	Umbonate	Moderate	Yes	Yes	32-37°C pH 6.0-7.0
Aspergillums sp. 4	Yellow to green	Entire	Yellow to orange	Raised	Rapid and floccose	Yes/some lack	Yes	25-30°C pH 5.5-7.0
Aspergillums sp. 5	Dark black	Entire	White to pale yellow	Umbonate	Moderate	Not known	Yes	32-37°C pH 5.0-7.0
Penicillium sp.1	Olive velvety green	Half to entire vesicle	Pale to dark brown	Umbonate, velvety surface	Slow to moderate	Presence	Yes	25-35°C pH 4.5-6.5
Rhizopus sp.	Grey to black	Entire	Greyish black	Raised, even	Rapid	Yes	Yes	27-30°C pH 4.0-9.0
Paceliomyces sp.	Yellowish brown to tan color	Entire	Yellow to tan	Flat, powdery surface	Slow to moderate	Yes	Yes	30-35°C pH 4.5-6.5
Penicillium sp.2	Green with yellow border or fluffy white	Half or more	Pale yellow or dull white	Umbonate/ raised even	Rapid	Nil	Yes/some lack	32-37°C pH 4.5-5.6
Trichoderma sp.	Dark green with concentric rings	Curled	Deep yellow/ uncolored	Raised fluffy	Moderate to rapid	Not known	Yes/some lack	25-30°C pH 5.0-6.5

Table III

Micro-Morphological features of the fungal isolates from tanneries

Species	MICRO-MORPHOLOGICAL CHARACTERIZATION									
	***Aspergillums sp.* 1**	***Aspergillums sp.* 2**	***Aspergillums sp.* 3**	***Aspergillums sp.* 4**	***Aspergillums sp.* 5**	***Penicillium sp.* 1**	***Rhizopus sp.***	***Paceliomyces sp.***	***Penicillium sp.* 2**	***Trichoderma sp.***
Hyphae	Branched septate	Branched septate	Branched septate	Branched septate	Branched septate	Branched septate	Unbranched	Verticillated branched	Branched or unbranched	Unicellular with short hyphae
Conidiophore • Length • Diameter	 200-450 µm 11.5-15.0 µm	 — —	 500-2000 5.0-8.0 µm	 400-800 µm —	 950-1700 1.2-13.5 µm	 200-500 9-16	 210-300 µm 5-18 µm	 — 4.0-8.0 µm	 200-400 µm 2.6-4.0 µm	 — —
Vesicle	Globose	Globose to subglobose	Globose, radiate or columnar	Globose to subglobose	Globose or subglobose	Subglobose to ellipsoidal	Subglobose or oval	Subspherical to pyriform	Subglobose/ globus/ ellipsoid	Globose/ ovoidal shape
Conidia • Conidial heads • Diameter	 Blackish brown 3.5-6.0 µm	 Black 5.0-7.0 µm	 Rough 3.5-6.0 µm	 Smooth to rough —	 Rough 72-127 µm	 Rough walled 2.0-3.6	Black to pale brown 60-180 µm	 Smooth —	 Smooth wallled 2.1-3.2 µm	 — 2.6-3.0 µm
Ornamentation	Warty/ spiny	Echinulate	Slightly rough	Almost smooth	Rough, verrucose	Slightly Rough	Slightly rough to smooth	—	Smooth/ coarsely rough	Roughened or verrucose
Phialides	Biseriate coverentive vesicle	Uniseriate	Biseriated/ uniseriated	Mostly biseriated	Biseriated/ medulla vary	Biseriated phialides	Coenocytic	Verticillated branched/ cylindrical	Flask shaped phialides (ampulliform)	Philide sigmoid/ hooked
Fruiting bodies	Cleistothecium present	Cleistothecium present	Cleistothecium present	Cleistothecium present	Present	Cleistothecium present	Present	Present	Cleistothecium present	Present

All the isolates were stained with LPCB and observed at 40 × magnification under the microscope as shown in Figure 3 and the results of the microscopic characterization are detailed in Table III.

Depending on the colony color and texture, all the isolates were grouped into five different types which are presented in Table IV. However, a few of the strains from one sampling site exhibited resemblance to strains from other sites as well and hence were grouped together.

Table IV
Species-wise distribution of fungal isolates from tanneries

Groups	Unique Identification of the Isolates	Label for the Groups
Group 1	S-1, S-25, S-6, S-8, S-15, S-21, S-7, S-4	*Aspergillums sp.* 1
	S-5	*Aspergillums sp.* 2
	S-16, S-34	*Aspergillums sp.* 3
	S-10, S-24	*Aspergillums sp.* 4
	S-12	*Aspergillums sp.* 5
	S-20, S-29	*Aspergillums sp.* 6
	S-26, S-35	*Aspergillums sp.* 7
Group 2	S-17, S-2, S-30, S-14	*Paceliomyces sp.*
Group 3	S-11	*Penicillium sp.* 1
	S-23, S-27, S-31, S-13	*Penicillium sp.* 2
	S-33, S-19, S-28	*Penicillium sp.* 3
Group 4	S-3, S-18, S-32	*Rhizopus sp.*
Group 5	S-22, S-9	*Trichoderma sp.*

For example, group 1 represents *Aspergillus* species, which was further divided into seven sub types as identified by macro and microscopic examination. In the first sub type, the isolates, S-1, S-4, S-6, S-7, S-8, S-15, S-21 and S-25 were grouped under the umbrella of *Aspergillus* sp. 1 as the colonies exhibited black-brown dense colony with age and white-creamy colour at the circumference, revealing similarity amongst each other. The other sub types were grouped as follows. S-5 the lone member in the second sub type, the colony grew rapidly with black color spores and the reverse colony appeared to be wrinkled and yellow in color with age. The isolate S-12 had slow growth with white black colony with age and the mycelium appeared to be raised and rough. Though the S-12 colony morphologically resembled sub type 1 and 2, it did not have distinct conidiophore as viewed microscopically and hence was placed under third sub type. S-16 and S-34 isolates grew rapidly forming dense and homogenous colonies on the plate with pale green to brownish-yellow in color with age. S-10 and S-24 colonies appeared to be olive green in color when young and showed pale yellow in some areas with age. S-20 and S-29 colonies grew moderately, appeared initially white and exhibited velvety green-yellow color with age. S-26 and S-35 colonies grew moderately appearing green in color and reverse plate showing orange to brown with age.

The colonies of S-17, S-2, S-30 and S-14 appeared velvety powdery brown in color when young and the mycelium was flat, smooth and appeared furred with dark brown with age. All of them were grouped under *Paceliomyces* sp. (group 2). In group 3, *Penicillium* sp. isolates were again split into 3 sub-groups based on the colony morphology. S-11, the lone member in the first sub-group exhibited slow-moderate growth and the mycelium appeared white while young and showed velvety olive green with age. The second sub-group, S-13, S-23, S-27 and S-31 colonies appeared velvety to powdery with blue-green/

Table V

Fungal isolates obtained and their GenBank accession numbers

Fungal Strains	Accession Number of the Isolates (In this Study)	Closest Strain from Database and its Accession Number	% Similarity
A. niger (S-1)	MK372919	*A. niger* NJA-1 (KJ365316.1)	100%
A. niger (S-8)	MK372920	*A. niger* SF-6354 (KT185662.1)	99%
A. niger (S-6)	MK372922	*A. niger* strain F103 (MH299977.1)	99%
A. niger (S-4)	MK372923	*A. niger* strain F103 (MH299977.1)	99%
A. carbonarious (S-5)	MK372921	*A. carbonarious* strain CBS 127.49 (MH868003)	99%
Rhizopus oryzae (S-3)	MK372924	*R. oryzae* strain CBS 395.34 (MH867089)	99%
Paceliomyces variotii (S-14)	MK372925	*Paecilomyces* sp. WE3-F (KM874779.1)	99%
A. oryzae (S-9)	MK372926	*A. oryzae* strain SEMCC-3.248 (HM064501)	99%
A. nomius (S-10)	MK372927	*A. nomius* strain H5 (JF416646)	99%
A. versicolor (S-11)	KX814964	*A. versicolor* strain KBP MGU E61 (AJ9377	99%

grey-green often with white edge having red to brown exudates on surface with age. Third sub-group, S-19, S-32 and S-33 colonies grew moderately with white fluffy mycelium when young and white cottony in texture with age. The isolates of S-3, S-18 and S-32 in group 4 were labelled as *Rhizopus* sp. as the colonies showed white mycelium when young and grew rapidly with white-black furry appearance with age. The isolates of S-9 and S-22 were labelled as *Trichoderma* sp. (group 5) and the colonies in both cases appeared to have concentric ring like growth with yellowish- green with age.

From the macroscopic and microscopic observations, *Aspergillus* sp. was found to be the most dominant genus with 58% of the leather samples infested with this family of fungi followed by *Penicillium* sp. (18%), *Paceliomyces* sp. (11%), *Rhizopus* sp. (10%) and *Trichoderma* sp. (5%). Many species have been isolated and identified during various stages in leather processing by Sharma and Sharma.[33] The *Aspergillus* species was reported to be the most dominant fungi found to grow on leather, and they are known to produce asexual and sexual spores that disperse in air to long distances.[34] Among *Aspergillus*, *A. niger* was found to be the most dominant species, and this finding is in agreement with what has been reported earlier by Kanagy *et al.*[35] and Ozdilli *et al.*[36]

Genotypic characterization

Depending on the origin of the ribosomal operon (18s rRNA), gene homology (95-100%) of the species were identified. Ten isolates including some of the rare and commonly encountered fungal species were selected for identification. The obtained consensus sequence was compared for similarity with BLAST alignment search tool of NCBI Genbank dataset and evolutionary history was inferred using neighbor-joining method. The evolutionary analysis was carried out in MEGA 7.0 and distance in terms of number of base substitutions per site was computed using the maximum composite likelihood method.[25] All ten isolated strains and their sequences were deposited to Genbank database at NCBI. Their accession numbers and their closest strains from the database are given in Table V.

Phylogenetic analysis of all the ten strains was performed, among which four strains of *Aspergillums* sp. S-1, S-4, S-6 and S-8 belonged to *Aspergillums niger*, and they were grouped under a single tree as shown in Figure 4. Strain S-1 showed 100% identity to *A. niger* strain NJA-1 (KJ365316.1) from the dataset. Strains, S-4 and S-6 were rooted to *A. niger* strain F103 (MH299977.1) with 99% identity and the closest relative is *A. niger* isolate SB2 (MH041197.2). Strain S-8 was rooted to *A. niger* strain SF-6354 (KT185662.1) with 99% identity.

The other isolated strains of *Aspergillus* sp. S-5, S-10 and S-9, *Rhizpous* sp. S-3 and *Paecilomyces sp.* S-14 have been divided into 4 clusters as shown in Figure 5 for ease of presentation. Cluster I consisted of S-9 belonging to *A. oryzae* and S-10 to *A. nomius*, along with closest reference strains of *A. oryzae* SEMCC-3.248 (HM064501), *A. nomius* H5 (JF416646) and *A. nomius* (AB008404) respectively. Cluster II consists of S-14 strain belonging to *Paecilomyces variotii* along with its closest neighbour *Paecilomyces* sp. WE3-F (KM874779.1) and *Parcilomyces variotii* JF416647.1 with 99% identity.

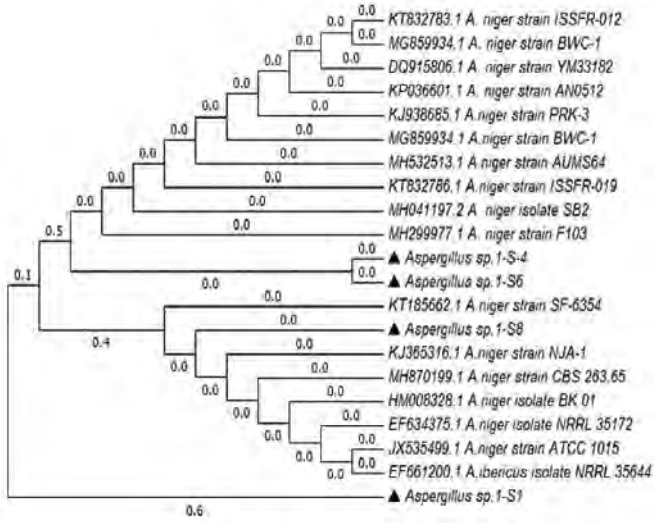

Figure 4. Phylogenetic tree constructed with 21 strains of *A. niger* nucleotide sequences, by Neighbour joining method (NJ) method for *Aspergillus sp.* (S-1, S-4, S-6, S-8). The values represented on the branch specify branch length with optimal tree length of 1.55 having 528 positions in final dataset

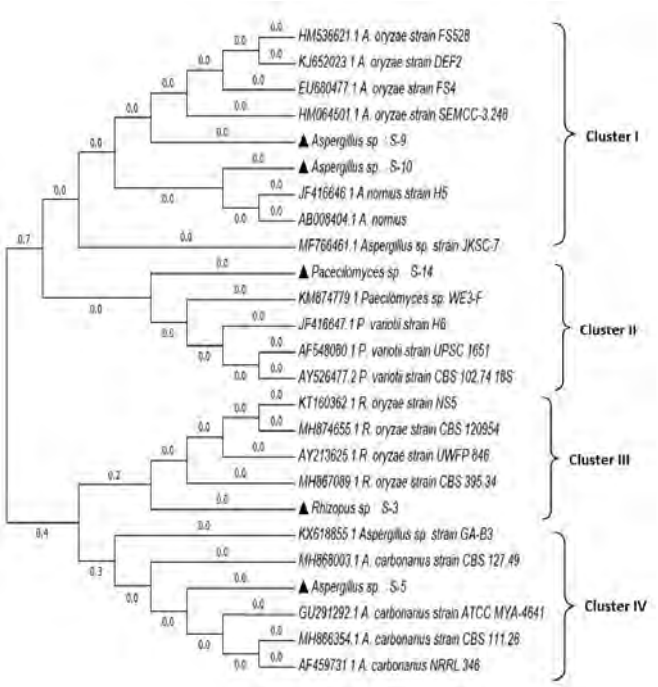

Figure 5. Phylogenetic tree constructed by Neighbor joining method (NJ) for *Aspergillus sp.* S-5, *Aspergillus sp.* S-10, *Aspergillus* sp. S-9, *Rhizopus* sp. S-3 and *Paecilomyces* sp. S-14, involving 25 nucleotides with 468 positions in the final data set. The values presented on the branch specify the length with optimum tree with the sum of branch length of 9.60

Cluster III consist S-3 strain rooted to *R. oryzae* CBS 395.34 (MH867089) with 99% similarity along with reference strains *R. oryzae* AY213625.1, MH874655.1 and KT160362.1. Cluster IV consists of S-5 strain rooted to *A. carbonarious* CBS 127.49 (MH868003) strain with 99% similarity, with other closest neighbours being strains of GU291292.1, MH866354.1 and AF459731.1 of *A. carbonarious.* Out of ten species, four strains (S-1, S-4, S-6, S-8) from *Aspergillus* sp. 1 group were identified as *Aspergillus niger.* S-5, S-9 and S-10 were identified as *Aspergillus carbonarious, Aspergillus oryzae* and *Aspergillus nomius* respectively. S-3 belonged to *Rhizopus oryzae* and S-14 to *Paecilomyces variotii.* S-11 which was grouped under *Penicillium* sp. based on phenotypic characterization earlier was found to be *Aspergillus versicolor* on gene sequencing. The results of the evaluation studies on susceptibility of this strain to commonly used leather biocides have been reported elsewhere.[37]

***In vitro* susceptibility of the fungal strains against selected biocides**

Susceptibility test for the selected ten isolates was carried out against four structurally different biocides namely, 2-(thiocyanomethylthio) benzothiazole (TCMTB), 2-Mercaptobenzothioazole(2-MBT), 3-iodo-2-propynyl-N-butylcarbamate (IPBC) and 2,2-dibromo-3-nitrilopropionamide (DBNP) (Figure 6) commonly employed in leather industry.

The biocides were used in the range of 0.5-1000 μg ml^{-1} to test their efficacy to control the growth of fungi. The MICs for the selected isolates against biocides are presented in Table VI. All the experiments were conducted in triplicate and the two concordant inhibition concentrations were taken as MIC.

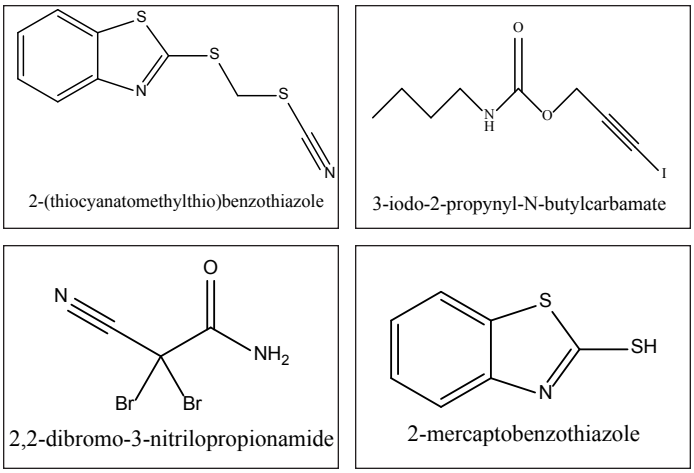

Figure 6. Chemical structures of leather biocides used in this study

Table VI

Evaluation of selected biocides against the isolated fungal strains

	Biocides (μg/ml) concentration			
Fungal Isolates	**TCMTB**	**2-MBT**	**IPBC**	**DBNP**
A. niger ATCC 6275 (Wild type)	1.95	125	3.90	125
A. niger (S-1)	3.90	125	1.95	250
A. niger (S-8)	0.97	250	1.95	250
A. niger (S-6)	7.81	250	3.90	125
A. niger(S-4)	1.95	31.25	3.90	125
A. carbonarious (S-5)	0.97	7.81	7.81	15.62
Rhizopus oryzae (S-3)	0.97	7.81	3.90	3.90
Paceliomyces variotii (S-14)	31.25	125	125	250
A. oryzae (S-9)	31.25	62.5	125	15.62
A. nomius (S-10)	1.95	125	62.5	125
A. versicolor (S-11)	31.25	250	250	62.5

From the results, TCMTB was found to be the most effective biocide in inhibiting the growth of fungi with least concentration when compared with other biocides. The MIC concentration of TCMTB was highest for strains S-9, S-11 and S-14 (31.25μg ml^{-1}) followed by S-6 (7.81μg ml^{-1}), S-1 (3.90 μg ml^{-1}), S-4, S-10 and wild type (1.95 μg ml^{-1}). The least inhibition concentration was observed for the strains S-8, S-5 and S-3 (0.97 μg ml^{-1}).

Among the four biocides, DBNP was required in high concentration for inhibition of fungal growth. The following decreasing trend of MIC for different strains was observed: S-11 (625 μg ml^{-1}) > S-1, S-8 and S-14 (250 μg ml^{-1}) > S-4, S-6 and wild type (125 μg ml^{-1}), S-5 and S-9 (15.62 μg ml^{-1}) > S-3 (3.906 μg ml^{-1}). With 2-MBT, highest MIC was observed for strains S-6, S-8 and S-11 (250 μg ml^{-1}) and

the least for S-3 and S-5 (7.8125µg ml^{-1}). In the case of IPBC, strain S-11 required highest concentration of 250µg ml^{-1} whereas S-1and S-8 required lowest concentration of 1.953µg ml^{-1}.

After the susceptibility test, the vials with TCMTB concentrations, MIC and above MIC were further subjected to identify the minimum fungicidal concentration (MFC). The MFC that required to kill the cells was the highest for S6 *(*152 µg ml^{-1}) when compared with that of the other isolates and wild type strain (31.25 µg ml^{-1}). From the results, it is concluded that S-6 is the most tolerant or resistant among all five *Aspergillus niger* strains and S-4 resembles very closely to wild type strain. TCMTB was the most effective, as the concentration required to inhibit the growth of fungi was the least compared to other biocides. The effectiveness of TCMTB on tanned leathers and its wide usage in leather industry has been reported earlier.[5, 38] From the susceptibility test, strain S-6 required four-fold increase in concentration (7.813µg ml^{-1}) of TCMTB compared to the wild strain (1.95µg ml^{-1}) and hence concluded to be the most biocide tolerant strain.

Based on the study, the *Aspergillus niger* isolates, S-6, S-1, S-4 and S-8 which required high concentration of TCMTB for inhibition were selected for further study.

SEM Analysis

Guarro *et al*.[39] have used SEM analysis to identify fungi of the same genus to their species level. Silva *et al*.[40] used the technique to identify species of *Aspergillus* genus within *Aspergillus* section *Nigri* complex. But in this study, SEM studies were carried out to understand the differences, if any, in the morphology of conidia and conidiophore due to evolutionary changes in the strains due to probable resistant development. SEM analysis revealed that all the isolates and the wild type *A. niger* have similar conidiophores, which appear to be smooth, long and narrow as shown in Figure 7. *A. niger* species S-1, S-4 and S-8 and corresponding wild type had similar morphology with round vesicle and radiated head when compared with S-6 strain, which showed conglomerated conidia (Figure 7d) with higher mycelial aggregation and sporulation.

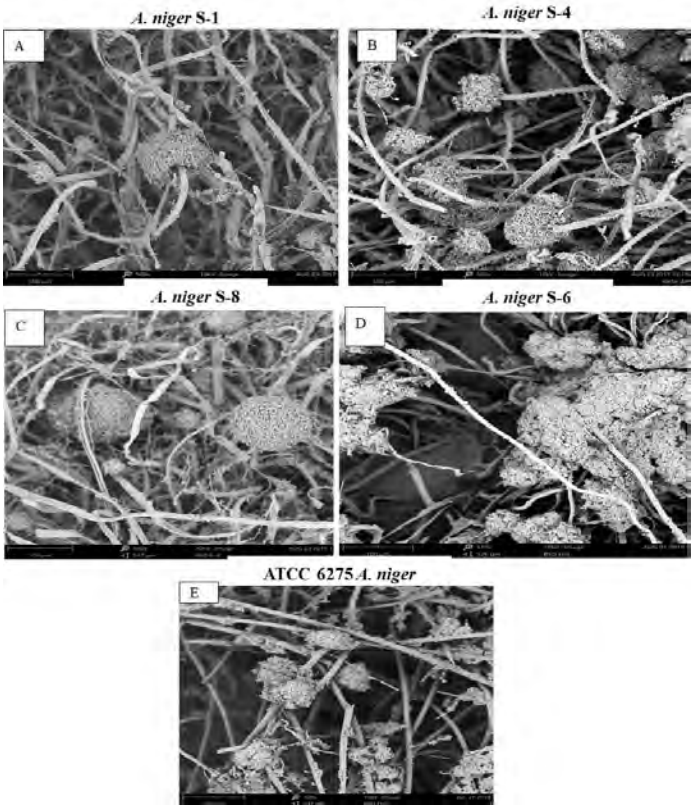

Figure 7. Scanning Electron Microscopic images of *A. niger* at 500 X magnification. (A-D) Isolated strains *A. niger* and (E) Wild type *A. niger* (ATCC 6275).

The isolates S-1, S-4, S-8 and wild type appeared to have round and radiated vesicle with biseriated phialids which was not distinctly visible in the case of S-6 strain. These observations might give further support that S-6 may have developed some sort of tolerance/ resistance to the biocide.

Evaluation of growth of *A. niger* strains on biocide treated wet-blue Leathers

Study was conducted to identify the *A. niger* strain which could resist biocide action the most (Figure 8). The wild type *A. niger* showed slight growth on 32nd dwith 0.2% TCMTB as shown in Figure 8D.

Among the test strains, *A. niger* S-6 was found to be the most dominantly growing which showed a slight growth on the grain

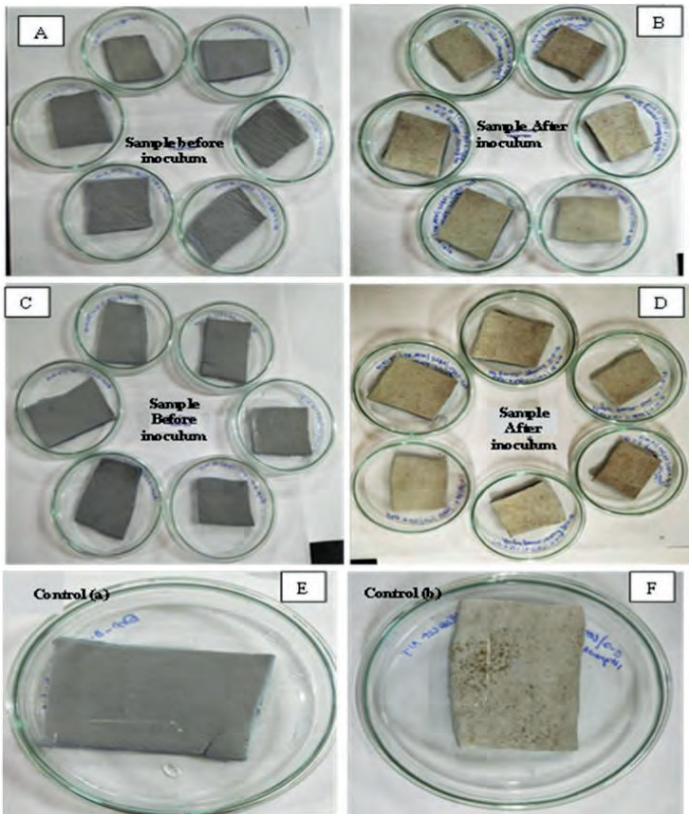

Figure 8. Leathers treated with TCMTB and evaluated for the growth of fungal resistance. A & C are biocide treated leathers before inoculum, B & D are treated leathers inoculated with *A. niger* S-6 and *A. niger* wild type respectively. Piece treated with 0.2% TCMTB served as control (a) without fungal inoculum and (F) is the growth control (b) without any biocide treatment

surface on 8thd of inoculum with 0.05% TCMTB, 11th d with 0.1%, 18th d with 0.2% as shown in Figure 8B. With S-6, the growth was the highest with the grain area being completely covered and the flesh side showing sparse growth. Even with high concentration of biocide (0.75%), growth was seen in the cross section of the leather on 45th d. S-1, S-4 and S-8 isolates showed slight growth on 11th d for 0.05% TCMTB. S-4 showed moderate growth on 40th d for 0.2% TCMTB and no growth with 0.75%, whereas, S-1 and S-8 showed growth on 25th and 30th d respectively for 0.2% TCMTB. As per the published literature, about 0.2% offering of TCMTB could give adequate protection for the wet blue leathers stored for 3-4 months.[5] But this accelerated susceptibility study indicates that all the isolates studied in this investigation have gained some sort of tolerance against TCMTB and among them S-6 seems to be the most resistant as it was found to grow even at very high concentration of 0.75%, which is not usually employed in leather processing. Therefore, *in vivo* growth studies on chrome tanned biocide treated leathers provided further support to the finding that the strain S-6 was the most resistant with persistent growth.

Conclusion

From the present screening study, it is revealed that *Aspergillus* sp. was the most dominant fungal species found to grow despite biocide treatment with an occurrence on 58% of the leather samples screened. Among *Aspergillus genus, Aspergillus niger* was found to be the most common and dominant species growing on leathers. From the results of *in vitro* and *in vivo* studies carried out with wild type *A. niger* as control, the isolate *A. niger* (S-6) was found to be the most biocide tolerant. It is proposed to carry out detailed studies with this particular strain to understand the resistant mechanism.

Acknowledgement

One of the authors, S. Kavitha, gratefully acknowledges Department of Science & Technology (DST), Government of India for financial support vide reference No.SR/WOS-A/LS-1415/2015 (G) under Women Scientist Scheme to carry out this research work.

References

1. Hawksworth, D.L; Global species numbers of fungi: Are tropical studies and molecular approaches contributing to a more robust estimate. *Biodiver Conserv.* **21**, 2425-2433, 2012.
2. Tancous, Jean, J.; Skin, Hide and leather defects. 2nd ed. Libraries Australia, USA: pp. 363, 1986.
3. Lindner, W. and Neuber, H.U., Preservation in the tannery. *Int. Biodeterior. Biodegrad*. **26,** 195-203, 1990.
4. Rother, H.J.; Microorganism: the scourge of the leather industry. *World Leather*. 48-50, 1965.
5. Orlita, A.; Microbial biodeterioration of leather and its control: a review. *Int. Biodeterior. Biodegrad*. **53**, 157-163, 2004.
6. Ravindran, J. and Rajeshwari, N.; Assessment of paracholorometacresol (PCMC), a fungicide against three common fungal species in leather manufacturing. *Int. J. Pharm. Bio. Sci*. **7**, 127-136, 2016.
7. Dalton, D.L.; New generation fungicide for the leather industry. *JALCA* **107**, 21-30, 2012.
8. Rivero-Menendez, O., Ana Alastruey-Izquierdo., Emilia Mellado. and Manuel Cuenca-Estrella.; Trizole Resistance in Aspergillus Spp.: A worldwide Problem. *J Fungi (Basel)*. **21**, 1-20, 2016.
9. Rathore, D.S., Neha, S. and Shashi, C.; Isolation, screening and relative capacity of fungi which causes infestation of finished leather. *Int. J. Curr Microbiol Appl. Sci*. **2**, 74-83, 2013.
10. Ghannoum, M.A. and Rice, L.B.; Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **12**, 501-517, 1999.
11. Poole, K.; Mechanisms of bacterial biocide and antibiotic resistance. *J Appl Microbiol Symp Suppl*. **92**, 55-64, 2002.
12. Maillard, J.Y.; Bacterial target sites for biocide action. *J. Appl. Microbiol. Symp. Suppl*. **92**, 16-27, 2002.
13. Russell, A.D.; Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infect Dis*. **3**, 794-803, 2003.
14. Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Melchers, W.J., Verweiji, P.E., Cuenca-Estrella, M. and Rodriguez-Tudela, J.L.; A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob Agents Chemother*. **51**, 1897-1904, 2007.
15. Faria-Ramos, I., Sofia Farinha., Joao Neves-Maia., Tavares P.R., Isabel M Miranda., Leticia, M, Estevinho., Cidalia Pina-vazl., Acacio, G, Rodrigues.; Development of cross-resistance by *Aspergillus fumigatus* to clinical agriculture azoles following exposure to prochloraz, an agriculture azole. *BMC Microbiology*. **155**, 1-5, 2014.
16. Vanja, M.K., Stephen, D.B. and Mainul, Haque.; Fact and Fantasy Regarding Resistance to Microbicides', Presented at the 96th Annual Meeting of the American Leather Chemists Association, 2010.
17. Chowdhary, A., Kathuria, S., Xu, J., Sharma, C., Sundar, G., Singh, P.K., Gaur, S.N., Hagen, F., Klaassen, C.H. and Meis, J.F.; Clonal Expansion and Emergence of Environmental Multiple-Triazole-Resistant Aspergillus fumigatus Strains Carrying the TR34/L98H Mutations in the cyp51A Gene in India. *PLoS One*. **7**, e52871. doi:10.1371/journal.pone.0052871, 2012.
18. Raper, K.B. and Fennell, D.I.; The genus Aspergillus. Baltimore, Maryland, USA: Williams and Wilkins: p1-686, 1965.
19. Gilman, J.C.; A Manual of soil fungi. Oxford & IBH Publishing Corporation, New Delhi, India, 2001.
20. Watanabe, T.; Pictorial atlas of soil and seed fungi morphologies of cultured fungi and key to species. 2nd ed. Boca Ratón: CRC Press, 2002.
21. Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C. and Varga, J.; Diagnostic tools to identify black aspergilli. *Stud Mycol*. **59**, 129-145, 2007.
22. Pinto, F.C.J., De-Lima, D.B., Agustini, B.C., Dallagassa, C.B., Shimabukuro., Márcio Chimelli, M.F., Brand, D., Fadel-Picheth, C.M.T. and Bonfim, T.M.B.; Morphological and molecular

identification of filamentous fungi isolated from cosmetic powders. *Braz Arch Biol Technol*. **6**, 897-901, 2012.

23. James, C. and Natalie, S.; Microbiology - A Laboratory Manual (Ed.), p. 211-23, 2001.
24. Kawato, M. and Shinobu, R., On Streptomyces herbari colour, nov sp. supplement: a simple technique for the microscopic observation, Memoirs of the Osaka University of Liberal Arts and Education. B. *Natural Sciences*, **8**, 114-119, 1960.
25. Kumar, S., Stecher, G. and Tamura, K., MEGA7: molecular evolutionary genetics analysis version -7.0 for bigger datasets. *Mol. Biology. Evol*. **33**, 1870-1874, 2016.
26. Tamura, K., Nei, M. and Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) **101**,11030-11035, 2004.
27. Saitou, N. and Nei, M.; The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molec Biol Evol*. **4**, 406-425, 1987.
28. Lass-Florl, C., Cuenca-Estrella, M., Denning, D.W. and Rodriguez-Tudela, J.L. Antifungal Susceptibility testing in Aspergillus spp. According to EUCAST methodology. *Medical Mycology*I. **44**, S39-S25, 2006.
29. Petrikkou, E., Rodriguez, T.J.l., Cuenca-Estrella, M., Alicia Gomez., Molleja, A. and Mellado, E.; Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. *J. Clin Microbio*. **39**, 1345-1347, 2001.
30. Salem, M.Z.M., Zidan, Y.E., Mansour, M.M.A., El-Hadidi, N.M.N. and Abo Elgat, W.A.A.; Antifungal activities of two essential oils used in the treatment of three commercial woods deteriorated by five common mold fungi. *Int. Biodeterior. Biodegr*. **106**, 88-96, 2016.
31. Peterson, S.W.; Phylogenetic analyses of Aspergillus species using DNA sequences from four loci. *Mycologia*. **100**, 205-226, 2008.
32. Schoch, C.L., Aime, M.C., De Beer, W., Crous, P.W., Hyde, K.D. and Penev, L.; Using standard keywords in publications to facilitate updates of new fungal taxonomic names. *IMA Fungus*. **8,** 70-73, 2018.
33. Sharma, O.P. and Sharma, K.D.; Deterioration of chrome tanned leather by *Aspergillus niger*, *Agra Univ. Jour. Res. (Sci)* **27**, 81-82, 1978.
34. Bennett, J.W.; An overview of the genus *Aspergillus*. In: *Aspergillus:* Molecular Biology and Genomics. (Machida M, Gomi K, eds) Caiser Academic Press, Portland. pp. 1-17, 2010.
35. Kanagy, J.R., Seebold, R.E., Charles, A.M. and Cassel, J.M.; Deterioration of leather under optimum mildew growing conditions. *JALCA* **44**, 270-282, 1949.
36. Ozdilli, K., Issever, H., Ozyildirim, B.A., Hapeloglu, B., Ince, H., Yegenoglu, Y., Susever, S., Erelel, M., Isik, E. and Gedikoitlu, G.; Biological Hazards in Tannery workers. *Indoor and Build Environment*. **16**, 349-357, 2007.
37. Kavitha, S., Khambhaty, Y. and Chandra Babu, N.K.; Evaluation of industrial biocides on a novel Aspergillus versicolor TANCK-1 and elucidation of their probable biocidal mechanism. *Indian. J. Microbiol*. **58**, 51-59, 2017.
38. Hauber, C. and Germann, H.P.; The addition of fungicides in chrome tannage and their penetration, absorption and distribution in the wet blue. *World Leather*. 75-82, 1997.
39. Guarro, J., Josepa, G. and Stchigel, A.M.; Developments in fungal taxonomy. *Clin. Microbiol. Rev.* **12**, 454-500, 1999.
40. Silva, D.M., Batista, L.R., Rezende, E.F., Fungaro, M.H.P., Sartori, D. and Alves, E. Identification of fungi of the genus *Aspergillus* section *nigri* using polyphasic taxonomy. *Braz. J. Microbiol*. **42**, 761-773, 2011.