Evaluation of Industrial Biocides on a Novel *Aspergillus versicolor* TANCK-1 and Elucidation of Their Probable Biocidal Mechanism

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Abstract In the present investigation, a novel fungus was isolated from leather watch strap and identified as *Aspergillus versicolor* TANCK-1 by 18 s rRNA sequencing. The isolated fungus was evaluated against three structurally different fungicides such as 2-(thiocyanomethylthio)benzothiazole (TCMTB), potassium dimethyldithiocarbamate (KDDC) and 2,2-dibromo-3-nitrilopropionamide (DBNP) to control the fungal growth. Among these, TCMTB was found to effectively inhibit the growth at a minimum concentration of 31.2 µg/mL as compared to 1250 and 625 µg/mL for KDDC and DBNP respectively. Increased membrane permeability in the fungicide treated samples

was evident from cellular release and decrease in cellular ergosterol content. Nevertheless, SEM analysis revealed a considerable change in fungicide treated mycelium with the collapse of hyphae structure and shrunken spores, which was observed to be very pronounced in KDDC and DBNP. Results indicated that all three biocides bring about inhibition through membrane damage with almost negligible effect on the cell wall.

Graphical Abstract Schematic diagram explaining the fungicidal action on *Aspergillus versicolor* TANCK-1

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Verticillium glaucum, *Verticillium tenerum*, *Basipetospora*, *Fusarium chlamydosporium*, *Trichosporon* and *Scopulariopsis brevicaulis* [3, 4].

Some of the fungicides used in leather industry including organo mercuric compounds like phenylmercuric acetate (PMA) and chlorinated phenols like pentachlorophenol (PCP) have been phased out due to toxicological consideration [5]. Later, fungicides such as TCMTB, ortho-phenylphenol (OPP) and p-chloro-m-cresol (PCMC) with lesser toxicity and minimum dosage levels [6] came into use for the prevention of fungal growth on leather and leather products. Currently, newer alternative fungicides like diiodomethyl-*p*-tolylsulfone (DIMPTS) and 3-iodo-2-propynyl-N-butylcarbamate (IPBC) have also been introduced for use in leather industry [7, 8]. Understanding the mode of action of fungicides and the knowledge of fungal metabolism are important for the sustained protection of leathers. The fungicidal action of the biocides is mainly due to their active functional groups such as triazoles, thiocarbamates, phenolics and propionamides. The industrial fungicides are believed to mostly target fungal cell wall, made up of chitin and glucan complex. Any hindrance in the enzymes responsible for their biosynthesis is considered as an important step in inhibition of the fungal cell proliferation.

Another important target is the cell membrane, where the enzymes responsible for sterol biosynthesis are believed to be hampered eventually, leading to depletion of ergosterol production [9, 10], thereby resulting in loss of cell membrane integrity. TCMTB based formulation belonging to triazoles and imidazoles derivatives are most effective even at very low concentrations as 10^{-11} M and they are able to disrupt the function of enzyme responsible for conversion of lanosterol to ergosterol [9]. KDDC and DBNP based formulation belongs to thiocarbamate and propionamide derivatives respectively, KDDC usually target the sterol biosynthesis resulting in the damage of cell membrane [11, 12] but DBNP mode of action is not well understood and as per the limited information available, it appears that it functions by the inhibition of membrane-bound enzymes [13].

In the present investigation, a novel fungal species hitherto not commonly encountered in leather industry has been isolated from leather watch strap. A detailed study has been performed to characterize the fungus both phenotypically and genotypically and study the susceptibility against three structurally different fungicides used in the leather industry. As there is no well documented study on the mode of action of fungicides with respect to leather fungi, a systematic study was done to identify the specific target sites of the fungicides.

Materials and Methods

Chemicals

Media and other chemicals were purchased from Hi-Media (India). Three biocide formulations namely TCMTB, KDDC and DBNP were obtained from reputed leather chemical supply houses.

Isolation and Characterization

The watch strap infested with fungi as received from a reputed Indian watch strap brand has been used in the study. The fungal spores were swabbed from the surface of the infested leather watch strap using sterile scalp and placed on sabouraud dextrose agar (SDA) medium incubated for 3–8 days at 26 °C and the growth was monitored periodically. The mother culture was maintained on potato dextrose agar (PDA) medium and stored at 4 °C. The fungus isolate was cultured on SDA for radial growth by inoculating spore suspension of 10 µl ($\sim 1\text{--}5 \times 10^6$ spores/mL) on the centre of an agar plate and incubated at 26 ± 2 °C. The diameter of the growing colony was measured every 24 h in triplicate plates for 15 days. Further, the isolated fungus was identified by 18 s rRNA sequencing method [14]. The 18 s rRNA sequencing was carried out at Xcelris labs, Ahmedabad, India.

Susceptibility of Fungicides

Varying concentrations of fungicides ranging from 0.5 to 250 µg/mL for TCMTB from stock of 1 mg/mL and 0.5–2500 µg/mL for KDDC and DBNP from stock solution of 10 mg/mL was prepared using sterile distilled water [15]. Spore suspension for antifungal susceptibility test was prepared as per the standard method and spore count was determined using a haemocytometer. The minimum inhibitory concentration (MIC) was determined in vitro by macro broth dilution method (M38-A) and all fungicides were diluted using sterile sabouraud dextrose broth (SDB) to which 0.1 mL of spore suspension ($1\text{--}5 \times 10^5$ spores/mL) was added to all the vials including control. The lowest concentration that completely inhibited the growth was determined as the MIC which was assessed visually after 48 h of incubation [16].

Mode of Action of Fungicides

The fungal cells were grown in SDB at 26 ± 2 °C for 5–7 day and the mycelial mat was collected by centrifugation at 10,000 rpm for 10 min. The pellet was treated

with fungicides at twofold MIC concentration and untreated pellet was kept as control.

Estimation of Soluble Protein

The biocide treated and untreated fungal pellet was incubated with 1 M NaOH (1 mL/mg cell wall) and left for 2 h at room temperature to determine the intracellular protein by Lowry's method using BSA as standard (1 mg/mL stock) [17].

Ergosterol Assay

The effect of fungicides on the fungal cell membrane permeability was determined by ergosterol assay method [18]. The ergosterol content and sterol intermediate 24(28) dehydroergosterol (DHE) in all the samples was calculated as % of dry weight of the cell by the following equations (Detailed procedure given in supplementary file) [19].

Cellular Content Release

Fungal cells grown in 100 mL of SDB were collected by centrifugation at 8000 rpm for 10 min, washed twice with phosphate buffer (PB) (pH-7.2) and re-suspended using 100 mL of PB. The mycelial suspensions were then treated with twofold MIC of each fungicide and incubated in a shaker incubator at 28 ± 2 °C. About 2 mL of samples were drawn at different time intervals and centrifuged at 12,000 rpm for 2 min, to determine the cellular release from the mycelial cells. The absorbance for the supernatant was measured at 260 nm [20].

Determination of Chitin Content

Fungal cell walls were prepared from the mycelium pellets according to the method of Pessoni et al. [21] and its chitin content was determined and was calculated as glucosamine hydrochloride ($\mu\text{g}/\text{mg}$ of dry weight of fungal cell walls)

from a standard curve obtained with varying concentrations of glucosamine hydrochloride (5–30 $\mu\text{g}/\text{mL}$) (Detailed procedure given in supplementary file) [22].

Fourier Transform Infra-Red Spectroscopy (FT-IR) Analysis

Biocide treated and untreated samples were subjected to FTIR analysis to find the functional groups. The samples were mixed with pure KBr in the ratio of 1:9 and made into pellets. They were placed in the sample holder and the spectra was recorded in the spectral region of 400–4000 cm^{-1} with 16 scan speed and resolution of 4 cm^{-1} [23].

Scanning Electron Microscopy (SEM) Analysis

Biocide treated and untreated fungal cells were taken for SEM analysis to study the effect of fungicides on the fungal morphology [24]. The sample size of about 6×5 mm was cut from dried mycelium mat and was mounted as per standard procedure on aluminum stubs using suitable carbon tape and gold coated using an Edwards E-306 sputter coater and examined in Bruker S-3400 N at a magnification of 10000 $\times$ and a voltage of 10 kV.

Results and Discussion

Isolation and Characterization

The novel fungus isolated from leather watch strap was subjected to morphological identification. The colonies appeared to grow moderately beginning from 2nd day reaching 11–13 mm in 7 days and 20–23 mm in 14 days of incubation at 28 ± 2 °C on SDA plate (Fig. 1). On microscopic examination, the fungus appeared to have reduced conidiogenous structures having brush-like

Fig. 1 **a** Seven days old *A. versicolor* colony on SDA showing green velvety appearance with white edges and deep red exudates. **b** The reverse of the same colony on SDA

conidial head, which indicated that this might probably be a *Penicillium* sp. (Fig. 2) but matured culture appeared as *Aspergillus* sp., with conidia arising from biserial phialides [25, 26]. Its occurrence is so uncommon in leather industry; the strain was initially mistaken for a *Penicillium* sp., which prompted us to go for genotypic identification. The consensus nucleotide sequence obtained was compared with BLAST alignment search tool of NCBI genbank dataset to identify the similarity. The results confirmed that the isolate was rooted to *A. versicolor* PSFNRO-2 with 99% similarity (Fig. 3), which is evident from the phylogenetic tree constructed using MEGA-7.0 software [27]. The 18 s rRNA sequence obtained was deposited in

GenBank database at NCBI, and the accession number of KX814964 was received. From leather industry point of view, *A. versicolor* is rarely encountered and only once *A. versicolor* F-581 strain was reported to occur on leather [3], but commonly found on adhesives in damp conditions, wall boards and airborne equipment [5].

Susceptibility of Fungicides

The inhibitory studies with three formulated fungicides proved the effectiveness in inhibiting the mycelial growth of *A. versicolor* TANCK-1. TCMTB formulation showed good inhibition at lowest concentration (31.2 µg/mL) when

Fig. 2 LPCB stained image showing morphology of *A. versicolor* with **a** radiated conidial head with biserial phialides at 100 × magnification **b** artistic illustration of the conidial head

Fig. 3 Phylogenetic tree: the evolutionary history was inferred using the neighbor-joining (NJ) method

compared with other two formulations based on KDDC (1250 µg/mL) and DBNP (625 µg/mL) (Table 1).

Understanding the Mode of Action of Fungicides

The major constituents of fungal cell walls are polysaccharides present in the form of carbohydrates, chitin is one of the main constituents of the cell wall that serves as a structural entity in order to protect the cells from any foreign agents. Another important constituent of the cell membrane is the ergosterol [28]. In this study, it is inferred from the results that only slight variation in chitin content was observed in all the three fungicide treated samples, with lowest level in DBNP (0.66 ± 0.04 µg/mg) followed by TCMTB (0.69 ± 0.05 µg/mg) and KDDC (0.77 ± 0.02 µg/mg) when compared with that of untreated sample (0.73 ± 0.05 µg/mg). A considerable decrease in intracellular protein and ergosterol contents in *A. versicolor* was observed in all three fungicide treated samples when compared with that of untreated cells. The intracellular protein content was found to be the lowest in the case of DBNP (1.117 ± 0.038 µg/mg) compared to TCMTB (1.14 ± 0.36 µg/mg), KDCC (1.42 ± 0.30 µg/mg) and untreated control sample (2.64 ± 0.59 µg/mg). Reduction in ergosterol content was the maximum in the case of DBNP with $79.39 \pm 1.11\%$, closely followed by KDDC with $63.05 \pm 0.99\%$. It was surprising that the reduction was found to be the lowest with TCMTB ($35.96 \pm 1.01\%$) considering the fact that the MIC is the lowest for this fungicide against *A. versicolor*. Therefore, overall inference from the study is that there is negligible change in the chitin content with p value > 0.05 whereas, considerable reduction was observed with intracellular protein and ergosterol contents on fungicidal treatment. This indicates that the major target of the fungicides may not be the cell wall but the target could very well be cell

membrane. The cell membrane has a significant role in maintaining the plasma membrane stability and all the three fungicides used in the present study appear to function by disruption of cell membrane integrity. This change could be attributed to the lipophilic property of fungicides and their ability to penetrate the plasma membrane which results in increasing permeability to various membrane components causing hyper-fluidity in cell membrane [10, 13].

In cellular release study, the release of cytoplasmic constituents with respect to incubation time was observed (Fig. 4). The KDDC treated cells showed maximum release of intracellular constituents with OD_{A260} of 3.67 in 5 min when compared with that of the control samples with OD_{A260} of 0.85 for the same incubation time. It is indicative of the cell membrane being affected as observed from ergosterol assay and cellular release study.

Fig. 4 Release of cytoplasmic cellular constituents in control and fungicide treated cells

Table 1 Susceptibility of fungicides against *Aspergillus versicolor* at various incubation hours

Fungicides	Concentrations (µg/mL)														
	24 h					48 h					72–120 h				
	2500	1250	625	31.25	15.62–0.5	2500	1250	625	31.25	15.62–0.5	2500	1250	625	31.25	15.62–0.5
TCMTB	–	–	–	–	+	–	–	–	–	++	–	–	–	–	+++
KDDC	–	–	–	+	+	–	–	+	++	++	–	–	++	+++	+++
DBNP	–	–	–	+	+	–	–	–	++	+++	–	–	–	+++	+++
Sterile control	–					–					–				
Growth control	+					++					+++				

– No Growth

+ Slight Growth around the circumference

++ Moderate Growth

+++ Full Growth

FTIR Analysis

FT-IR analysis of all the samples shows peaks above 3000 cm^{-1} corresponding to large O–H and N–H

Fig. 5 FT-IR spectra of control (CB) and fungicide treated culture extracts of DBNP, TCMTB and KDDC

stretching vibrations and peaks in the region of $3000\text{--}2800\text{ cm}^{-1}$ corresponding to aliphatic alkanes often associated with hydrocarbon region of lipids. The shift in the region of $2350\text{--}2364\text{ cm}^{-1}$ was observed with larger transmittance in the case of both KDDC and DBNP compared to control but for KDDC, the peak was sharp and larger (Fig. 5). This peak corresponds to N–H stretching vibrations indicating fragments of amino acids. Further a shift in $1450\text{--}1476\text{ cm}^{-1}$ corresponding to $-\text{CH}_2$ bending was observed in KDDC, which could be attributed to lipid $-\text{CH}_2$ bending signals in either protein or chitin present as chitin-glucan complex. Such a shift from 1460 to 1452 cm^{-1} of $-\text{CH}_2$ was reported earlier for chitin-glucan complex [29].

The many additional peaks were observed with KDDC. The peaks at 3388 , 1722 , 1631 and 1511 cm^{-1} may probably be protein related corresponding to amide I, 1466 cm^{-1} due to $-\text{CH}_2$ bending corresponding to lipids and/or polysaccharides, and 1367 and 1214 cm^{-1} due to C–O stretching. The lipid content as evidenced by C–H stretching vibrations in $-\text{CH}_3$ and $-\text{CH}_2$ at $2854\text{--}2852$ and $2924\text{--}2918\text{ cm}^{-1}$ respectively was observed in all treated samples [23]. Therefore, the spectral analysis indicates that there is pronounced release of lipids and other cell components with KDDC when compared with that of DBNP

Fig. 6 Scanning electron microscopic images of *A. versicolor* TANCK1 **a** control **b** treated with TCMTB, **c** treated with DBNP and **d** treated with KDDC

and TCMTB as a result of extensive cell membrane damage due to the interaction of all the three fungicides.

SEM Analysis

SEM analysis revealed the effect of fungicides on hyphal morphology (Fig. 6). The control sample had normal mycelial growth with biserial conidial structures and tubular homogenous hyphae (Fig. 6a). There is a considerable change in the mycelium showing collapse of hyphae structure with reduction in thickness, probably due to the loss of cytoplasmic contents leading to collapse of cell membrane with fungicidal treatment and the effect was observed to be very pronounced in the case of KDDC and DBNP (Fig. 6c, d) compared with TCMTB (Fig. 6b). The spores also seem to have shrunk in size with biocide treatment. Similar effect was also reported by Bajpai et al. [24] in the case of *P. italicum*, which was concluded to be due to increase in cell permeabilization.

Conclusion

The novel *A. versicolor* TANCK-1 was effectively inhibited by all three leather fungicides namely TCMTB, KDDC and DBNP by the disruption of the cell membrane as evidenced by reduced ergosterol content. TCMTB was found to be effective at lower concentration. However, the disruption of cell membrane was found to be relatively more in the case of KDDC and DBNP with higher extent of release of ergosterol, which was also supported by SEM analysis. Further investigation on gene expression is being carried out to understand the complete mode of fungicide action and the changes brought about in *A. versicolor* with the potential to lead to development of biocidal resistance.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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