Evaluation of Antifungal Activity of Carbonate Solvent – Part:1

by

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Abstract

Antimicrobial agents have been used in leather manufacturing to prevent leather products from microbial contamination. In this work, the antifungal activity of green solvent such as propylene carbonate was investigated against the mixed culture of fungi isolated from wet-blue using broth dilution/well diffusion. A concentration of 5% and above (propylene carbonate) showed effective antifungal activity against the mixed culture of fungi and the efficiency of propylene carbonate on the mixed culture increased with increasing concentration/volume. Propylene carbonate exhibited fungistatic activity against the mixed culture of fungi but it lost its activity after a certain period and fungal growth was observed again. It was also found that 2% propylene carbonate in chrome tanning process effectively inhibited the fungal growth and the wet-blue can be preserved up to 30 days without any fungal attack.

Introduction

The use of fungicides in leather manufacturing is essential to prevent the damage caused by various fungal species on leather and leather products. Over the years there have been a number of changes in the fungicides used to treat leather against fungal attack. Phenyl mercury compounds like phenylmercuric acetate (PMA) were in common use and then chlorinated phenols like trichlorophenol (TCP), pentachlorophenol (PCP) and tetrachlorodibenzodioxin (TCDD) have emerged. By the early 1980s[1] the use of these compounds was restricted due to their high toxicity to humans and poor biodegradability. The fungicide 2-(thiocyanomethylthio) benzothiazole (TCMTB) became the new standard starting in the 1970s and remains in use today. In addition, other fungicides like orthophenylphenol (OPP), parachlorometacresol (PCMC), bromohydroxyacetophenone (BHAP), 4, 5,-dichloro-2-N-octyl-4-isothiazolin-3-one (DCOIT), 2-n-octyl-4-isothiazolin-3-one (OIT), 2-mercaptobenothiazole (2-MBT),[2] methylene bisthiocyanate[3] are also being used in leather making. Currently, these fungicides are slowly being replaced with carbamate (IPBC-iodopropargyl-N-butylcarbamate) and carbimate (CHED - S-Hexyl-S'-Chloromethyl-cyanodithiocarbimate) based molecules.[4] Due to the wide spectrum of activity and stability against the harsh chemical environment, CHED is finding a prominent place in the fungicide market. Besides, some attempts have been made to use environmentally and ecologically safer fungicides in leather making. Researchers studied the antifungal activity of *Anethum graveolens*/*Melaleuca alternifolia* essential oils against fungal species found on sheep lining leather[5] and also used *oregano oil* as antifungal agent in leather manufacturing.[6] Recently, an attempt has been made to develop a fungal resistant fatliquor from blended natural oils and used in leather manufacturing.[7]

Also, an attempt has been made to synthesize a novel hyperbranched polymeric antifungal agent from triazine molecules (cyanuric chloride and cyanuric acid) as an alternative to traditional fungicides.[8] Machuca et al., synthesized N-acetylated gemini surfactant and used it as an antifungal wetting agent in chrome tanning process.[9] Haibin et al., developed a new antimicrobial complex of Copper (II) with benzothiazole derivative and used in shoe lining manufacturing.[10] Recently, our research group developed a solvent selection tool and identified propylene carbonate as a potential candidate for alternative carrier medium to water.[11] Propylene carbonate (one of the cyclic carbonates) is classified as VOC exempt green solvent and also listed in the EPA (Environment Protection Agency) safer ingredients list.[12] It possesses a high flash point, low vapor pressure, high boiling point and also low toxicity (LD50: 21000 mg/kg of rat). Propylene carbonate (PC) is predominantly used as a carrier medium in ointment/cosmetic products,[13] nail polish remover,[14] dye carrier, electrolyte in Li-ion batteries[15] and CO_2/H_2S gas absorber. For the first time, propylene carbonate (PC) was successfully used as an alternative carrier medium to water in leather making and established a zero discharge chrome tanning process.[16] Further, it has also been found that there is no fungal growth on wet-blue processed in PC medium without adding any antifungal agent. In order to expand on previous research work, a systematic study has been carried out to establish the antifungal activity of propylene carbonate and its application in chrome tanning process.

Materials and Methods

Preparation of Mixed Stock Culture

Conventionally processed goat pickled pelt was converted into wet-blue without adding any fungicides in tanning. Subsequently, it has

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Manuscript received May 11, 2021, accepted for publication July 14, 2021.

been allowed for 15 days for the growth of fungi at room temperature. After the fungal growth, the inoculum was taken by cotton swab and spread over petri plates containing solidified Potato Dextrose Agar Medium (PDA). The fungi grown stock culture (Mixed culture) was stored at -18°C.

Evaluation of Antifungal Activity by Broth Dilution Method

To verify the antifungal activity of propylene carbonate, a broth dilution test was conducted prior to the well diffusion method. The fungal inoculum was prepared from the stock culture with sterilized distilled water and vortexed. Subsequently, sterile potato dextrose broth (PDB) was prepared. Then, the mixed culture (50 µl) was added into the potato dextrose broth (Total volume - 5 ml) containing various percentages of PC viz., 0, 2.5, 5, 7.5 and 10%. Subsequently, the tubes were incubated in an incubator at 30°C.

Evaluation of Antifungal Activity by Well-Diffusion Method

Initially, the PDA medium was transferred to the petri plate and 100 µl of inoculum (mixed culture) was added into the PDA medium. Then, the mixture was uniformly mixed and allowed to solidify. On the plate, a well was made with the help of a 5 mm well cutter. Subsequently, different volumes of PC viz., 0, 25, 50, 75 and 100 µl was separately added into the each well.

The plates were incubated at 30°C for 3 days. A circular zone of inhibition is observed as a result of antifungal activity of PC and the diameter of the zone is noted.

Mode of Inhibition

The mixed culture was added into the broth containing 7.5 & 10% PC and incubated at 30°C for 3 days. Then, 100 µl of the inoculated sample was added into the broth medium (No PC added) and observed for fungal growth. On the other hand, the plating was done with the same procedure as followed in well diffusion method. Then 75 and 100 µl of PC was added into the well and incubated at 30°C. After zone formation, the plate was left undisturbed for further incubation up to 5 days to examine the recurrence of fungal growth and to determine the mode of action of PC.

Evaluation of Antifungal Efficiency on Wet-Blue

To determine the effectiveness of propylene carbonate as an antifungal agent in chrome tanning process, wet-salted goatskin was conventionally pickled and chrome tanned. After chrome tanning, the liquor was completely drained and the wet-blue was divided into 6 parts and subsequently marked. Then different percentages of PC viz., 0, 1, 2, 3, 4 and 5% w/w along with 10% water was added and agitated for 30 min. A small piece was taken from each percentage and placed in a sterilized petri plate. All the plates were kept at room temperature (30°C) for observation of notable fungal growth.

Result and Discussion

Preparation of Stock Culture

The fungal spores were taken from two different places of wet-blue and subsequently, plating was done. After 72 hrs, a dense fungal growth was observed (Figure 1). It is evident from Figure 1 that the fungal growth pattern and its morphology of the spores collected from two different places are similar.

Evaluation of Antifungal Activity by Broth Dilution Method

Preliminarily, broth dilution test was carried out to evaluate the antifungal activity of propylene carbonate. The mixed culture treated with different percentages of propylene carbonate and its growth after 72 hrs is shown in Figure 2. It is clear from Figure 2 that the fungal growth is being observed in test tubes containing 0 and 2.5% PC. However, the fungal growth in 2.5% PC treated solution is relatively low when compared to 0% PC. No fungal growth was observed in the remaining test tubes where the concentration of PC was above 2.5%.

These results suggest that the PC shows effective antifungal activity against mixed culture isolated from we-blue and its efficiency increased with increasing concentration of PC.

Evaluation of Antifungal Activity by Well-Diffusion Method

The well diffusion method was carried out and the size of zone formation was taken as a marker to analyze the antifungal activity

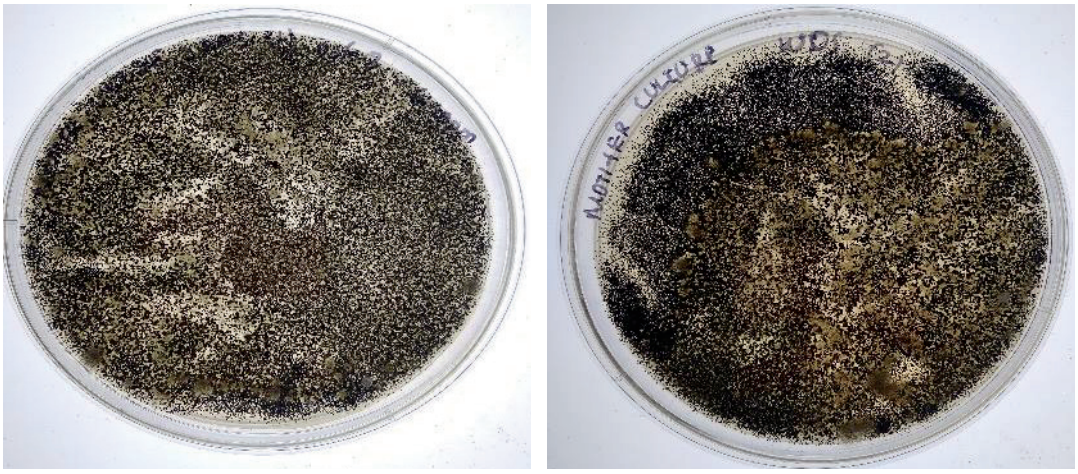

Figure 1. (a) and **(b)** Culture taken from two different position of chrome tanned leather

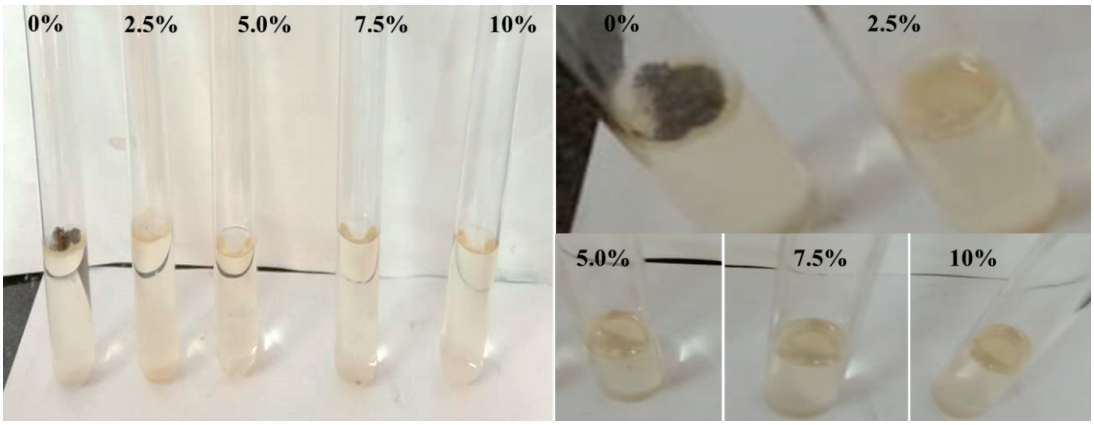

Figure 2. Mixed culture treated with different concentrations of propylene carbonate

of propylene carbonate. It is evident from Figure 3 that the zone formation was observed after 72 hrs from plating. However, the size of zone formation is varied depending upon the volume of propylene carbonate added into the well. It is also evident from Figure 3 that no zone formation was observed for 0% PC (Positive control) and no fungal growth was observed for negative control (no PC and no mixed culture). The size of zone formation for different volumes of propylene carbonate is shown in Figure 4. It is evident from Figure 4 that the zone of inhibition linearly increased with increasing the volume of propylene carbonate added into the well. The size of zone formation for 25 µl is 14 mm and it increased to 20 mm for 100 µl.

Mode of Inhibition

Generally, the antimicrobial agents used in leather manufacturing cause permanent damage to the microorganism. The phenolic based antimicrobial agents reversibly adsorbed on the cytoplasmic membrane of microorganisms which initially inhibits the transport of nutrients and eventually deteriorates the cell wall leading to microbial death.[17] On the other hand, the heterocyclic antimicrobial agent like TCMTB is an electrophilic substance that chemically reacts with the nucleophilic substance of cellular materials and causes cell death. Therefore, antimicrobial agents exhibiting static action would be safer. In order to study the mode of inhibition, the mixed culture was incubated in the presence of 7.5 and 10% PC. No fungal growth was observed up to 72 hrs. Then, incubated culture was sub-cultured (100 µl of an incubated sample taken) in the absence of PC and fungal growth was observed after 72 hrs. Further, two plates (7.5 µl and 10 µl) were incubated for 7 days and observed for recurrence at zone inhibition area. It is evident from Figure 5 that the fungal growth was observed in the zone of inhibition region. From the results, it may be concluded that the antifungal activity of propylene carbonate is fungistatic

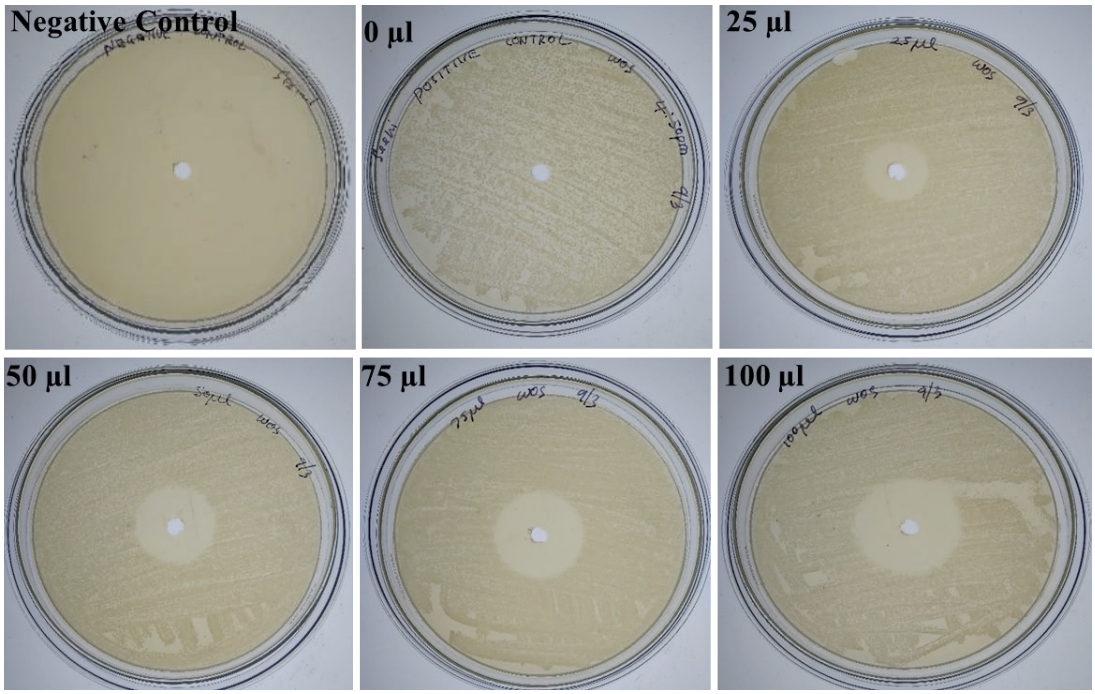

Figure 3. Antifungal activity of propylene carbonate – well diffusion method

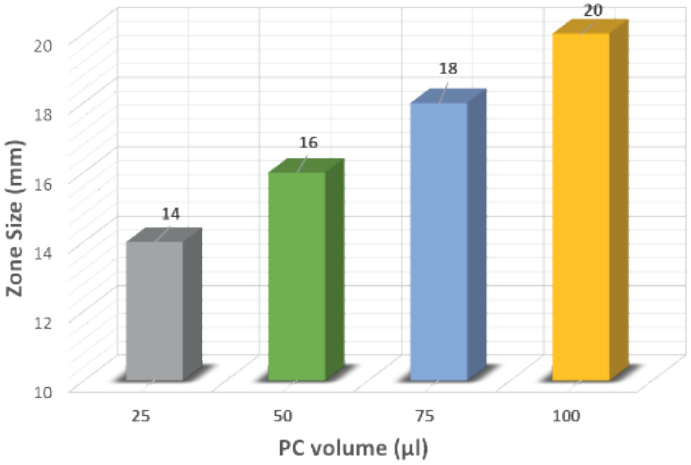

Figure 4. Zone of inhibition for different concentrations of PC

and it only arrests the fungal growth for a particular period of time without killing them.

Evaluation of Antifungal Efficiency on Wet-Blue

In order to study the antifungal efficiency of different percentages of propylene carbonate viz., 0, 1, 2, 3, 4 and 5% w/w were employed in chrome tanning. The wet-blue treated with each percentage was monitored for a period of 30 days and shown in Figure 6. On day 5 fungi formation was observed for 0% PC and no fungal growth for the remaining percentages. However, on day 14 growth was observed for 1% treated wet blue. No fungal growth was observed for remaining concentrations up to 30 days. Thus, it is clear from this observation that fungal growth shows resistance to lower concentrations of PC (1%) and the growth is inhibited with high concentration of PC (2% and above).

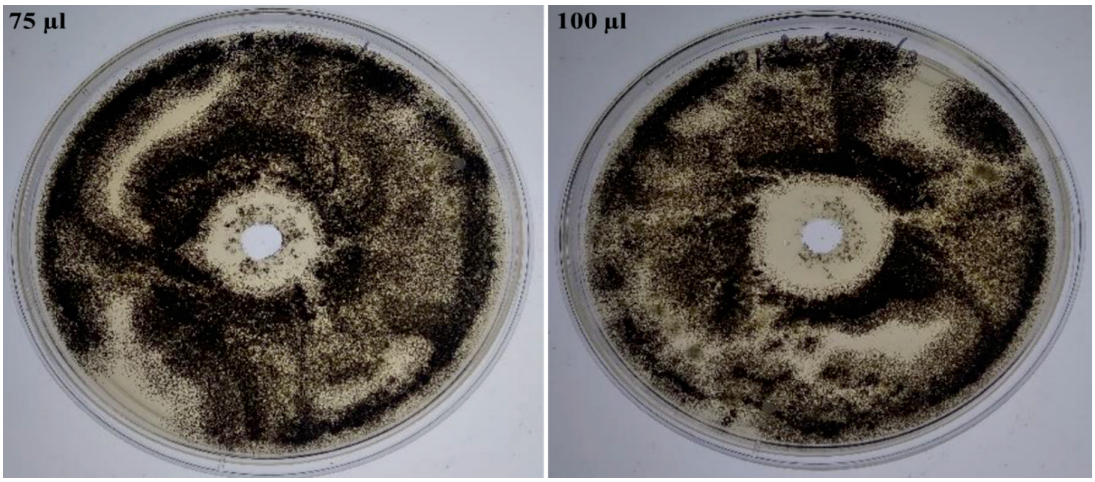

Figure 5. Mode of inhibition of propylene carbonate — Recurrence Test

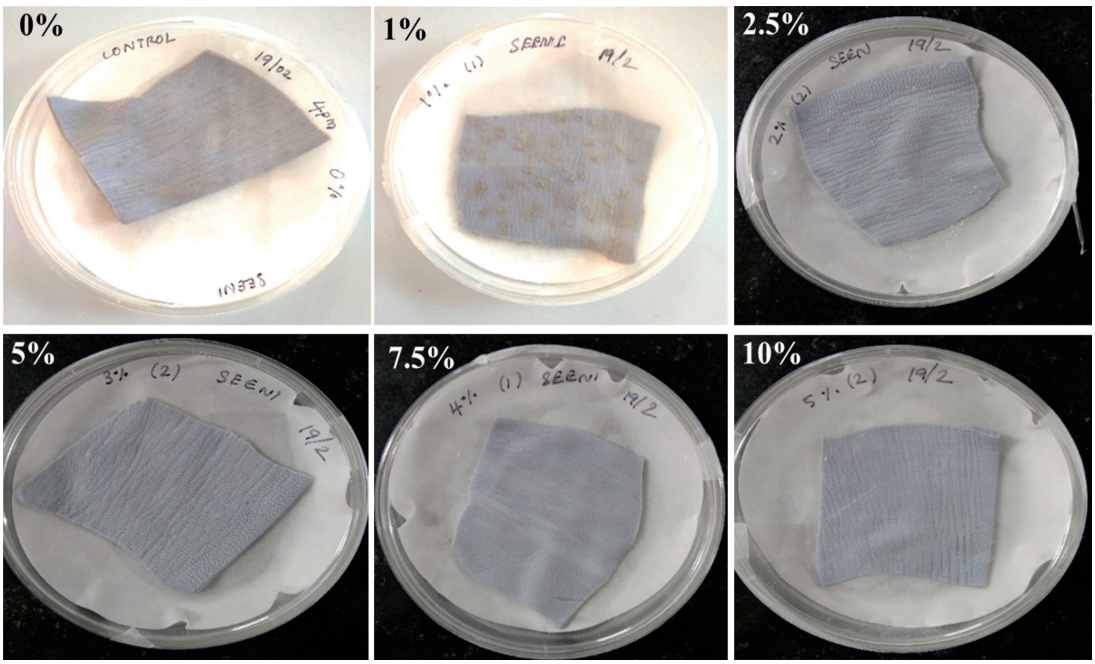

Figure 6. Wet-blue treated with different concentration of PC (After 30 days).

Conclusions

In this work, the antifungal activity of propylene carbonate against a mixed culture isolated from wet-blue was successfully evaluated. The results from the broth dilution method and well diffusion method suggest that the efficiency of propylene carbonate increased with increasing concentration/volume. It was also found that the mode of inhibition of propylene carbonate is fungistatic action and recurrence occurred after a certain period. The effective concentration of propylene carbonate in chrome tanning process is 2% and wet-blue may be preserved 30 days without fungal growth. Therefore, the propylene carbonate (green solvent) would be an ideal alternative for the conventionally used harmful antimicrobial agents.

Acknowledgement

The authors thank Council of Scientific and Industrial Research (CSIR), India for financial support. CSIR-CLRI's Communication Number - 1586

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