Chapter - VI

INCLUSION OF LIMONENE INTO MODIFIED CELLULOSIC FABRICS
FOR DURABLE AND IMPROVED ANTIBACTERIAL ACTIVITY

Introduction

Natural fibers like cotton are more prone to microbial attack than synthetic fibers, since they have porous texture capable to retain moisture and supplies nutrients necessary for the growth of microbes. Antimicrobial finishing of textile materials is needed to avoid cross infection by pathogenic microorganism, to control infestation by microbes and to safeguard the textile materials from staining, odour formation and deterioration. Surface modification of fibers can change the characteristics of fabric by imparting new functionalities. Cyclodextrins are suitable auxiliaries in textile finishing because of their toxicological and ecological advantages [1].

Cyclodextrins are produced by the enzymatic degradation by means of enzyme cyclodextrin glucosyltransferase on starch [2]. They are classified as α-(six), β-(seven), and γ-(eight) cyclodextrins, based on the number of glucopyranose units present in their ring and have cavities of approximately 0.6, 0.8, and 1.0 nm in diameter respectively [3]. They play a significant role in textile industry and might be used in number of applications such as removal of surfactants from washed textiles, chemically bound to fibers to provide enhanced hydrophilicity and inclusion complex forming ability to immobilize perfumes, insect repellents, antimicrobial agents etc [4]. D-limonene (1-methyl-4-(1-methylethenyl) cyclohexene) is a monocyclic monoterpane with a lemon-like odor and is a major constituent in several citrus oils (orange, lemon, and grape
fruits). It is widely used as a flavour and fragrance additive in perfumes, soaps, foods, chewing gum, and beverages because of its pleasant citrus fragrance. D-limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) flavouring agent and well established as a chemo preventive agent against many types of cancer [5]. Microcapsules of limonene were applied in textile and food industry for fragrance release property [6, 7]. It was first registered in USA as an antimicrobial in 1971, as a dog and cat repellent in 1983 and as an insecticide in 1985. Durable insect repellent cotton is also synthesized using limonene [8]. Anti-fungal mortar and concrete are developed using microencapsulated fungus-resisting material D-Limonene [9]. The limonene in encapsulated state is used to improve the stability and aroma quality of limonene in food products [10]. Limonene as one of the constituent of essential oil has shown antibacterial activity against wide range of microorganism and is used as antimicrobial agent in food material [11, 12]. In this study limonene is incorporated into biopolished and β-CD and MCT- β-CD grafted organic cotton inorder to enhance the durability of antibacterial activity on fabric.


The unbiopolished, biopolished, unbiopolished β-CD and biopolished β-CD fabrics are loaded with limonene. The UV-Visible and FTIR analysis are performed for confirmation of β-CD and limonene on fabric. The crystalline nature and thermal stability of the modified fabrics are analyzed by XRD and TGA method. Limonene content of the various categories of fabrics is estimated from the HPLC analysis of the alcoholic extract from the respective fabrics. The antibacterial efficacy and the durability of the activity to repeated washing cycles are tested.
6.1.1. Inclusion of limonene into β-CD fabric

The grafting of β-CD takes place according to mechanism discussed in section 4.2 and inclusion of limonene into cyclodextrin cavity occurred as per the schematic representation given in figure 6.1.1.

![Figure 6.1.1: Schematic representation of limonene inclusion into β-CD fabric.](image)

6.1.2. UV-Visible analysis

UV-Visible spectrum of a) unbiopolished fabric (F₁), b) unbiopolished β-CD fabrics (F₃) are discussed in chapter 4. The spectrum of (a) limonene and (b) unbiopolished-β-CD limonene fabric (L₃) are depicted in figure 6.1.2. Limonene shows absorption maximum at 241 nm corresponding to the $\pi - \pi^*$ of exo and endo cyclic double bond in limonene. The same wavelength of absorption is observed in unbiopolished-β-CD limonene fabric (L₃) proved the existence of limonene on fabric.
6.1.3. FTIR analysis

FTIR spectrums for unbiopolished fabric (F_1), β-CD and unbiopolished β-CD fabric (F_3) are discussed in chapter 4. The spectrum of a) limonene and b) unbiopolished β-CD limonene fabric (L_3) are given figure 6.1.3. Limonene exhibits the stretching vibration of –CH in alkane at 2953 cm^{-1} and =CH at 3066 cm^{-1} as broad and small bands respectively. The stretching vibrations in –C-C and C=C are ascribe to the sharp peak at 1196 cm^{-1} and 1659 cm^{-1}. The bending vibration (-CH) of methylene and methyl groups are represented at 1470 cm^{-1} and 1380 cm^{-1}. The out of plane bending of endo and exo cyclic double bonded carbon (-CH=C) are attributed at 840 and 932 cm^{-1}.

Unbiopolished β-CD limonene fabric (L_3) exhibits the C-O stretching and OH in plane bending as small peaks between 1033 and 1415 cm^{-1}. The stretching vibration of -OH of cellulose occurs at 3357 cm^{-1}. The alkenyl group (-C=C) of limonene exhibits the stretching vibration of –C=C at 1685 cm^{-1} which confirmed the presence of limonene on fabric.

6.1.4. HPLC analysis

The limonene content of fabrics (L_1, L_2, L_3 & L_4 ) is obtained from HPLC analysis and the chromatogram is represented in figure 6.1.4. The limonene peaks are identified from their retention time obtained for limonene in separate HPLC. The response factor was calculated by running the co-chromatography with benzoic acid as internal standard. The limonene content of the fabrics L_1, L_2, L_3 and L_4 is estimated as 0.0174%, 0.0227%, 0.416% and 0.468% respectively. The enzyme treatment removes the microfibrils and provides more penetration of water and limonene into the fibers as a result of increase in pore size. Therefore more quantity of limonene is absorbed on the
fabric $L_2$, while in case of $L_4$ the prominent raise of cyclodextrin grafting yield provided more toroid cavity to accommodate limonene. Hence the eco-friendly biopolishing has played a significant role for the improvement of limonene concentration in fabrics similar to the enhancement of dyeability of reactive dyes in biopolished fabric [13].

6.1.5. XRD

The XRD pattern of unbiopolished fabric ($F_1$) and unbiopolished $\beta$-CD fabric ($F_3$) are given in chapter 4 and unbiopolished $\beta$-CD limonene fabric ($L_3$) are shown in figure 6.1.5. The crystalline reflections of fabric $L_3$ are more or less same as that of the reflections of native cotton ($2\theta = 14.8^\circ$, $16.5^\circ$, $22.8^\circ$ and $34.5^\circ$ for planes 101, 10\overline{1}, 002 and 040 of cellulose form I [14]. The prominent reflections for the planes 101, 10\overline{1}, 002 and 040 of all the three fabrics suggested that the grafting of $\beta$-CD and inclusion of limonene onto the toroid cavity of cyclodextrin has not changed cellulose to their different allomorphs such as cellulose II, III and IV form [15]. The fabrics retained their cellulose I form after grafting and inclusion of limonene. The crystallinity index of the modified fabrics ($L_3$) is 93.2% and there is no tremendous raise in crystallinity on inclusion of limonene, specifies that limonene is not chemically bonded with cellulose chain of cotton.

6.1.6. TGA

The relative thermal stabilities of a) unbiopolished fabric ($F_1$), b) unbiopolished $\beta$-CD fabric ($F_3$) and unbiopolished $\beta$-CD limonene fabric ($L_3$) are depicted in figure 6.1.6. The thermogram of the unbiopolished fabric ($F_1$) and unbiopolished $\beta$-CD fabric ($F_3$) are discussed in chapter 4 and 5. The fabric ($L_3$) has the three major stages of
weight loss and the decomposition starts 282°C with 9% weight loss. The onset depolymerisation begins at 322°C and completes at 388°C. The higher crystallinity of fabric \((L_3)\) increased the thermal stability of the fabric \([16]\). The relative weight loss proportion is less in fabric \(F_3\) and \(L_3\) in all stages of pyrolysis as compared with the reference fabric \((F_1)\), which could be the result of the presence of cyclodextrin and limonene on fabric. The final decomposition temperature has increased to 571°C as a result of intervention of the decomposition products of limonene in decomposition of cotton. The final residue content is higher than that of the other two fabrics indicates that the decomposition products of limonene have interfered with the decomposition of cotton.

6.1.7. Evaluation of antibacterial activity

The antibacterial property of fabrics \((L_1, L_2, L_3\ & L_4)\) is measured from the zone of inhibition developed in and around the fabric on nutrient agar plate and represented in figure 6.1.7 for \(E.\)coli and \(S.\)aureus. The zones of inhibition developed in unwashed fabric and after ten cycles of washing are given in Table 6.1.1. Citrus products contain a wide variety of oils that are toxic to bacteria \([17, 18]\). Limonene is the major constituent of the citrus essential oil \([19]\). They contain 85-99% volatile component (limonene) and 1-15% nonvolatile component. It is well known that monoterpenes develop their antimicrobial inhibitory effects through the interaction with membrane structure and function. This is in fact due to their lipophilic and solubility properties. These interactions include membrane expansion, increase membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration and alteration of ion transport processes \([20-25]\).
The limonene loaded fabrics (L₁, L₂, L₃ & L₄) shows inhibition against the growth of E.coli and S.aureus. The exclusive nature of the fabric which has been modified with enzyme and cyclodextrin shows the highest inhibition in both bacteria compared with the all other fabrics. The gram negative bacteria is less susceptible to attack than gram positive S.aureus. Limonene as lipophilic compound is capable to attack the cell membrane (lipopolysaccharide membrane) and alter cell permeability. The presence of unsaturation in limonene has influenced the antibacterial activity. The unbiopolished and biopolished limonene fabrics exhibited antibacterial activity as a result of the absorption of limonene into void space available in fabric but their washing durability is poor. The unbiopolished and biopolished β-CD limonene fabrics developed more inhibition, but the latter one has showed pronounced effect even after repeated washing process. The presence of limonene as inclusion complex into the cavity of cyclodextrin and their controlled release are the reason for the durability of the antibacterial activity in fabric L₃ and L₅. In addition to that biopolishing boosts the grafting of β-CD and positively increased the concentration and antibacterial activity of limonene.

6.2. Biopolished MCT-β-CD limonene fabric for antibacterial activity

The unbiopolished, biopolished, unbiopolished MCT-β-CD and biopolished MCT-β-CD fabrics are loaded with limonene. The UV-Visible and FTIR, analysis are performed for confirmation of β-CD and limonene on fabric. The crystalline nature and thermal stability of the modified fabrics are analyzed by XRD and TGA. Limonene content of the various categories of fabrics is estimated from the HPLC analysis of the
alcoholic extract from the respective fabrics. The antibacterial efficacy and the durability of the activity to repeated washing cycles are tested.

6.2.1. Inclusion of limonene into MCT-β-CD fabric

The grafting of MCT-β-CD takes place according to mechanism discussed in section 4.3 and inclusion of limonene into cyclodextrin cavity occurred as per the schematic representation given in figure 6.2.1.

![Schematic representation of limonene inclusion into MCT-β-CD fabric.](image)

**Figure 6.2.1:** Schematic representation of limonene inclusion into MCT-β-CD fabric.

6.2.2. UV-Visible analysis

UV-Visible spectrum of a) limonene and b) unbiopolished MCT- β-CD limonene fabric (L_5) is depicted in figure 6.2.2. The spectrum of unbiopolished fabric (F_1) and MCT-β-CD are discussed in chapter 4. Alcoholic solution of limonene exhibits significant absorption at 241nm characteristic of π – π* in exo and endo cyclic double
bond. Unbiopolished MCT-β-CD limonene fabric (L₅) represents absorption maximum between 230-240 nm characteristic of the triazine and limonene moiety.

6.2.3. FTIR analysis

FTIR spectrums of the unbiopolished fabric (F₁), MCT-β-CD and unbiopolished MCT-β-CD fabric (F₅) are discussed in chapter 4. The spectrum of a) limonene and b) unbiopolished MCT-β-CD limonene fabric (L₅) are depicted in figure 6.2.3. Limonene exhibits the stretching vibration of –CH in alkane at 2953 cm⁻¹ and =CH at 3066 cm⁻¹ as broad and small bands respectively. The stretching vibrations in –C–C and C=C are ascribe to the sharp peak at 1196 cm⁻¹ and 1659 cm⁻¹. The bending vibration (-CH) of methylene and ethyl groups are represented at 1470 cm⁻¹ and 1380 cm⁻¹. The out of plane bending of endo and exo cyclic double bonded carbon (-CH=C) are attributed at 840 and 932 cm⁻¹. The spectrum of unbiopolished MCT-β-CD limonene fabric (L₅) exhibits stretching vibration of -OH group of cotton at 3372 cm⁻¹ and asymmetric stretching of –CH at 2876 cm⁻¹. The stretching vibration of alkenyl bond (-C=C) occurs at 1689 cm⁻¹ and the bending vibrations of methylene and methyl groups of limonene at 1369 cm⁻¹.

6.2.4. HPLC analysis

HPLC chromatogram of the alcoholic extract obtained from fabrics (L₁, L₂, L₅ & L₆) are depicted in figure 6.2.4. The limonene content was evaluated from the peaks area of the sample (limonene ) and the standard benzoic acid using response factor derived from the co-chromatography run with standard compound benzoic acid. The limonene content in fabrics (L₁ = 0.0174 %, L₂ = 0.0227 %, L₅ = 0.489 %, L₆ = 0.537 % w/w) showed that enzyme treatment has improved more penetration of limonene into the
interstitial sites and also more grafting of MCT-β-CD on fabric. MCT-β-CD enriched fabric accommodated more quantity of limonene into their cavity. The unbiopolished and biopolished limonene fabric (L₁ & L₂) has lower percentage of the core material limonene as it is held by weak vanderwaals forces with cellulose chain of cotton and it can be washed out from the fabric surface. The higher content of limonene in other two fabrics (L₅ & L₆) is as result of its presence as inclusion complex into cyclodextrin cavity, which could not washed out easily from the fabric.

6.2.5. XRD

XRD pattern of unbiopolished MCT-β-CD limonene fabric (L₅) are represented in figure 6.2.5 and that of fabric F₁ and F₅ are discussed in chapter 4.

Unbiopolished MCT-β-CD limonene fabric (L₅) shows 94.4 % crystallinity and it is very close to the crystallinity of unbiopolished MCT- β-CD fabric (F₅). The limonene moiety is included into the toroid cavity of cyclodextrin and it is not involved in bonding with the cellulosic hydroxyl group. Since the grafting of MCT- β-CD and the inclusion of limonene into cyclodextrin cavity has no way hindered crystallite dimension of cellulose form I, therefore their reflection occurs only with minor deviation (±0.2°) as reported earlier [14].

6.2.6. TGA

The thermal analysis of the a) unbiopolished fabric (F₁), b) unbiopolished MCT-β-CD fabric (F₅) and c) unbiopolished MCT- β-CD limonene fabric (L₅) are represented in figure 6.2.6. The thermogram of above said fabrics shows slight variation as a result of modification. The thermogram of fabrics F₁ and F₅ are described in chapter 4 and 5.
The onset inflexion of unbiopolished MCT-β-CD limonene fabric (L₅) begins at 316°C with a weight loss of 15% which is lower than that of unmodified fabric with a weight loss of 20% at 320°C. The thermal stability of fabrics F₅ and L₅ are in close association. The increase of crystallinity and decomposition products of MCT-β-CD and limonene lowered the depolymerisation temperature and percentage of weight loss. The char pyrolysis starts at 380°C and completes at 571°C with 95% weight loss of fabric. The reduction in weight loss percentage and higher content of residue (5%) proved that the decomposition product of MCT-β-CD and limonene lowered the decomposition rate of cotton.

6.2.7. Evaluation of antibacterial activity

The agar diffusion test conducted on fabrics (L₁, L₂, L₅ & L₆) shows positive results on all fabrics towards gram negative and positive bacteria with deviation in their efficacy of the antibacterial activity. The zones of inhibition developed by fabrics are given in figure 6.2.7 for E.coli and S.aureus. Amongst all the fabrics the enzyme treated MCT-β-CD limonene fabric (L₆) developed more inhibition compared with the other three fabrics. This may be due to their higher limonene content within the toroid cavity of cyclodextrin. Another fact is that the fabrics grafted with MCT-β-CD retain nearly 60-70% of the antibacterial activity even after 10 cycles of washing, as limonene occupies hydrophobic cavity of cyclodextrin and not the interstitial site of fabric. The biopolishing has improved the antibacterial efficacy of fabric L₂ but it couldn’t help to retain the effect during washing. The antibacterial characteristics of fabrics (L₅ and L₆) have been improved and also durable as it has the limonene in protected form. Biopolishing has played a prominent role in well establishing the grafting yield of
MCT-β-CD and indirectly paved way to accommodate more percentage of limonene into the fabrics. Limonene as the major compounds of essential oils, have the ability to disrupt and penetrate the lipid structure of the cell wall of bacteria, leading to denaturation of proteins and destruction of the cell membrane [26]. When considering the antibacterial activity of limonene towards the two bacteria E.coli and S.aureus, the latter has been destroyed more compared to the former. Mostly gram negative bacteria like E.coli are less susceptible to antimicrobial agent, but limonene is capable to attack the lipopolysaccharide cell wall easily as a result of their hydrophobic or lipophilic nature. Therefore the inhibition developed in both bacteria is in close association with each other. It has been reported that the presence of alkenyl group in limonene showed more activity than p-cymene since the latter has alkyl group in their ring. The alkenyl group of limonene upon air oxidation produces ions, which disturbs the cell wall and adversely causes the leakage of ions and other cell contents [27].

**Conclusion**

Eco-friendly enzyme modification was applied as tool to improve the fixation yield of β-CD and MCT-β-CD. Limonene, a biodegradable nontoxic compound was incorporated into fabric and their quantity was estimated by HPLC. UV-Visible and FTIR spectral studies confirmed the fixation of β-CD, MCT-β-CD and the inclusion of limonene into fabric. XRD analysis of fabrics explained the increase of crystallinity of fabrics after β-CD and MCT-β-CD fixation. The thermal stability analysis by TGA showed improvement in thermal stability of β-CD and MCT-β-CD fabrics. Limonene is active against gram negative and gram positive bacteria and the inhibition is fairly higher in S.aureus compared to E.coli. The durability of the antibacterial agent in
biopolished β-CD limonene fabric (L₄) and biopolished MCT-β-CD limonene fabric (L₆) was remarkably high and withstands the ten cycles of washing process. The biopolishing technology played a significant role for the improvement of performance properties in modified fabrics.


References


**Table 6.1.1:** Antibacterial activity of fabrics against E.coli and S.aureus.

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<td>S.aureus</td>
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Figure 6.1.2: UV spectra of a) Limonene and b) unbiopolished β-CD limonene fabric (L₃).

Figure 6.1.3: FTIR spectra of a) Limonene and b) unbiopolished β-CD limonene fabric (L₃).
Figure 6.1.4: HPLC chromatogram for alcoholic extract of limonene a) unbiopolished limonene fabric (L₁), b) biopolished limonene fabric (L₂), c) unbiopolished β-CD limonene fabric (L₃) and d) biopolished β-CD limonene fabric (L₄).
Figure 6.1.5: XRD spectra of unbiopolished β-CD limonene fabric (L₃)

Figure 6.1.6: TGA curves of a) unbiopolished fabric (F₁), b) unbiopolished β-CD fabric (F₃) and c) unbiopolished β-CD limonene fabric (L₃).
Figure 6.1.7: Antibacterial activity of a) unbiopolished limonene fabric (L₁),
b) biopolished limonene fabric (L₂), c) unbiopolished β-CD limonene
fabric (L₃) and d) biopolished β-CD limonene fabric (L₄) against E.coli
and S.aureus.
Figure 6.2.2: UV spectra of a) Limonene and b) unbiopolished MCT-β-CD limonene fabric (L₅)

Figure 6.2.3: FTIR spectra of a) Limonene and b) unbiopolished MCT-β-CD Limonene fabric (L₅)
Figure 6.2.4: HPLC chromatogram for alcoholic extract of limonene a) unbiopolished limonene fabric (L₁), b) biopolished limonene fabric (L₂), c) unbiopolished MCT-β-CD limonene fabric (L₅) and d) biopolished MCT-β-CD limonene fabric (L₆).
Figure 6.2.5: XRD spectra of unbiopolished MCT-β-CD limonene fabric (L₅).

Figure 6.2.6: TGA curves of a) unbiopolished fabric (F₁), b) unbiopolished MCT-β-CD fabric (F₅) and c) unbiopolished β-CD limonene fabric (L₅).
Figure 6.2.7: Antibacterial activity of a) unbiopolished limonene fabric (L₁), b) biopolished limonene fabric (L₂), c) unbiopolished MCT-β-CD limonene fabric (E₃) and d) biopolished MCT-β-CD limonene fabric (E₃) against E.coli and S.aureus.