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Waste-Derived Antibiofilm Strategies: A Review of Eco-Enzymes and Citrus Essential Oils

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Abstract**—** *Microbial biofilms are a major cause of persistent contamination in healthcare, food processing, and agricultural systems, and they significantly contribute to antimicrobial resistance (AMR). Conventional chemical disinfectants often show reduced efficacy against mature biofilms due to the protective extracellular polymeric substance (EPS) matrix and may also raise environmental concerns. This review discusses the antibiofilm potential of eco-enzymes, which are fermentation-derived liquids produced from citrus waste and other organic substrates containing organic acids, hydrolytic enzymes, and microbial metabolites. Additionally, citrus essential oils rich in bioactive terpenoids such as limonene, citral, and linalool are reviewed as sustainable antimicrobial alternatives. Eco-enzymes disrupt biofilms mainly through acidification and enzymatic degradation of EPS components, whereas citrus essential oils primarily exert antimicrobial effects through membrane disruption and quorum sensing inhibition. Both approaches demonstrate inhibitory effects against important biofilm-forming pathogens such as Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans. Indirect evidence suggests a possible synergistic interaction between eco-enzymes and citrus essential oils, where EPS weakening by acidic fermentation products may enhance essential oil penetration into biofilms. However, significant research gaps remain, including limited standardization of eco-enzyme production, insufficient multispecies biofilm studies, and inadequate cytotoxicity evaluations. Future research focusing on optimized formulations and safety assessment may support practical applications of these waste-derived antibiofilm agents in food safety, healthcare, and environmental sanitation.*

Keywords**— *Antimicrobial resistance; Biofilm; Citrus essential oil; Eco-enzyme; Quorum sensing***

HIGHLIGHTS

- Eco-enzymes derived from citrus waste exhibit antimicrobial and antibiofilm potential.
- Citrus essential oils disrupt microbial membranes and inhibit quorum sensing.
- Combined application may enhance EPS disruption and improve biofilm eradication.
- Waste-derived antibiofilm agents support sustainable biotechnology approaches.

Global burden of microbial infections and surface-associated contamination

Microbiological contamination of biotic and abiotic surfaces, remains challenging in the healthcare, food industry and laboratory (Tamburini et al., 2015). In food processing facilities and dairy companies, biofilm development on food processing areas creates a potential for foodborne diseases and financial loss. For example *Listeria monocytogenes* biofilm established on stainless steel equipment, gained resistance to regular sanitizing processes (Lee et al., 2019). In agricultural systems, the biofilms developing within irrigation pipes and at plant microbe interfaces facilitate the survival and spread of phytopathogen (Yao & Habimana, 2019). In households, ordinary surfaces are source for surface-bound microbes that cause infection, especially in the case of immunocompromised individuals (Dancer, 2014). The surface-associated microbial contamination increases burden of antimicrobial resistance (AMR), posing a serious threat to public health and food safety. In developing world such as India, inadequate sanitation infrastructure, ineffective waste management practices, and the extensive use of chemical disinfectants further increase this problem (Aslam et al., 2018). Together, these challenges emphasize the importance of efficient, safe and environmentally friendly approaches to regulate surface-associated microbial growth (Shree et al., 2022).

Biofilms as the dominant microbial lifestyle and a major contributor to antimicrobial resistance

In environment, microorganisms are found in the form of biofilm rather than as free-living planktonic cells (Muhammad et al., 2020). Biofilm is a specialized community of microbes, encased within extracellular polymeric substance matrix composed of proteins, lipids and extracellular DNA (Di Martino, 2018). The matrix facilitates surface adhesion, nutrient retention, intracellular communication by quorum sensing (QS) signals and protection against desiccation, shear forces, antibiotics and host immunity, through initial attachment, irreversible adhesion, microcolony formation, maturation and dispersal (Karygianni et al., 2020; Samrot et al., 2021). Microorganisms found in biofilms have altered physiological conditions, lower metabolism, and high tolerance levels (Rather et al., 2021). The efflux pump activation, quorum sensing-mediated regulation and horizontal gene transfer within dense microbial communities enhances resistance against antimicrobial agents. Chronic infections attributed to biofilms being increasingly identified as major contributors to treatment failure and persistent contamination in both clinical and industrial settings (Uruén et al., 2021).

Limitations of conventional chemical disinfectants in biofilm control

Although there is widespread dependence on chemical disinfectants to kill microbes but their efficacy against biofilm producing microorganisms remains limited (Maillard & Centeleghe, 2023). The commonly used disinfectants, includes chlorinated compounds, quaternary ammonium compounds, aldehydes are effective on planktonic cells. The physical and chemical barrier of biofilm matrix prevents diffusion of disinfectants, enabling microbial persistent within protected microenvironment (Koti et al., 2024). The reactive disinfectants could be inactivated by biofilms and resulting in sub-lethal concentration in biofilms due to diffusion concentrations in biofilms, that allows microorganisms in biofilms to survive after several disinfection processes (Maillard & Centeleghe, 2023).

Besides the reduced effectiveness, the other factor that contributed to the intervention of newer technologies to control biofilms was the prolonged, sublethal effect of chemical-based antimicrobial agents exerted selective pressure on tolerant microorganisms, that shows cross-resistance to clinical antibiotics (Liu et al., 2024). Certain limitations linked to the safety of the environment and human health further hindered the use of traditional chemical-based antimicrobial agents, that are known to produce toxic substances, exhibit low biodegradability and accumulate in ecological systems (Basiry et al., 2022; Verlicchi et al., 2015). The use of these substances further facilitates the research activities to discover new ways for the effective removal of biofilms with limited or negligible use of chemical-based antimicrobial substances in the future (Algburi et al., 2017).

Growing interest in green, waste-derived, and sustainable antimicrobial alternatives: Eco-Enzymes and Citrus Essential Oils

The conventional chemical disinfectants have significant limitations. Their reduced efficiency combined with the rise of antimicrobial resistance and environmental concerns, has gained interest in green, waste based antimicrobial solutions. These strategies support United Nations Sustainable Development Goals (SDGs) 3(health and well-being) and 12 (sustainable production and consumption) by promoting organic waste utilization, minimizing chemical residues and developing biodegradable agents with diverse resistance breaking mechanisms (Buffet-Bataillon et al., 2012; Carey & McNamara, 2015; Edis et al., 2024; Guedes et al., 2024).

Natural products from plant materials, microbial fermentation or food waste offer alternatives to their chemical diversity and broad-spectrum activity (Burtt, 2004; Daglia, 2012; Winkelstroter et al., 2024). The fermentation-based products and plant-based essential oils offer potential leads in the area of antimicrobial and antibiofilm activity, these strategies not only reduce environmental chemical load but also convert organic waste into bioactive antimicrobial agent (Nazzaro et al., 2013; Sar et al., 2023).

This review evaluates experimental studies on eco-enzymes and citrus-derived essential oils, with particular focus on their mechanisms of action, efficacy against biofilm-forming microorganisms and limitations associated with their practical application and also explore synergistic prospective of combining eco-enzymes with citrus essential oils as a sustainable strategy for biofilm control, highlights key gaps in the current literature and outline future directions for translating these green antimicrobial systems into real-world clinical, food, and environmental applications.

1. MICROBIAL BIOFILMS:

Definition and characteristics

Microbial biofilms are structured communities of microorganisms that adhere to biotic or abiotic surfaces and become encased in a self-produced extracellular polymeric substance (EPS) matrix (Donlan & Costerton, 2002). The EPS matrix constitutes a major fraction of the biofilm biomass, accounting for approximately 50–90% of the total organic matter, and serves as a protective scaffold that provides structural stability and enhances microbial survival under adverse environmental conditions (Flemming & Wingender, 2010; Flemming et al., 2007). This matrix safeguards the embedded cells against environment stress factors such as desiccation, attacks by protozoa and phagocytosis, ultraviolet radiation, and antibiotics (Flemming & Wingender, 2010; Karygianni et al., 2020).

It differs from planktonic bacteria, contributing to 60-80% of human microbial infections according to NIH (National Institute of Health) estimates. The features include heterogenous spatial distribution with growth of microcolonies in diverse phase of metabolism, well developed water channels to enable the nutrients and waste movement and phenotypic modulation like reduced growth, increased stress resistance and multidrug resistance in microbes. Beside physical shielding, there is close cell-cell contact and transformation of competent DNA promoting horizontal gene transfer (Yang et al., 2023). Biofilms enhance microbial survival through cooperative behaviours such as resource sharing, metabolic division of labour, and protection against desiccation, predation, and antimicrobials (Hall-Stoodley et al., 2004; Hobley et al., 2015; Flemming & Wingender, 2010). This sessile lifestyle enables persistence under nutrient limitation, pH stress, and immune pressure across ecosystems from rhizospheres to catheter lumens (Costerton et al., 1999; Donlan & Costerton, 2002; Liu et al., 2024).

Stages of biofilm formation

The development of the bacterial biofilm is a five-step cycle including reversible attachment, irreversible attachment, microcolony formation, maturation and dispersal (Ugwu et al., 2025).

Reversible attachment involves the planktonic to immobile transition phase, which occurs through reversible attachment that is mediated by weak physiochemical forces including van der waal, electrostatic forces and hydrophobic interaction (Zhao et al., 2023). The microbial cell motility depends on flagella, type lV pili or twitching motility to oppose the repulsive forces and cells attachment depends on the surface properties like hydrophobicity, roughness and charge and hydrodynamic condition and conditioning film (Saharan et al., 2024).

Irreversible attachment involves committed cells to grow on the surface through specific adhesion molecules, for example *Pseudomonas aeruginosa* contain type lV and *Staphylococcus aureus* contain MSCRAMMs. This activates c-di-GMP signaling cascades that suppress motility and stimulates EPS production (Zhao et al., 2023).

In the microcolony phase, microcolonies develop through clonal growth, aggregation of cells by surface array proteins and the production of the first EPS, which leads to the generation of 3D microcolonies (Sharma et al., 2023). The microbial cells shift from single surface colony to multi-cell aggregates results in the formation of the EPS, which provide initial biofilm structure for attachment and protection (Sharma et al., 2023). It is controlled by quorum sensing through N-acyl homoserine lactones (AHLs) in gram negative bacteria, luxl/R, rhll/R system in *Pseudomonas aeruginosa* and autoinducing peptides (AIPs) in gram positive, agr systems in *Staphylococcus aureus* (Omwenga & Awuor, 2024; Zhao et al., 2023). The Quorum sensing triggers EPS genes such as alginate, PIA, Pel/Psl for the EPS formation and reduces the motility due to increase in the c-di-GMP levels. Other QS controlled process include efflux pump activation that improves antibiotic resistance, production of virulence factors and biofilm development signals (Zhao et al., 2023). The cells produce amyloid fibers such as curli in *Enterobacteriaceae* species and Chaplin's in *Bacillus* species to maintain the structural integrity and promote eDNA (extracellular DNA) release by autolysis. At periphery microcolonies establish primary metabolic gradients by higher CO_2 consumption and sets stage for maturation (Sharma et al., 2023). This process establishes the multicellular biofilm phenotype for adherence, protection and physically distinguished from planktonic growth.

In maturation phase, the complexity in biofilm structure is maximum as EPS production increases resulting in the mushroom like morphology with stalks that bind to the surfaces and a dense cap that nurture high number of bacteria (Saharan et al., 2024; Sauer et al., 2022). The water channels interconnected penetrate throughout the structure and acts as a circulatory system for the transportation of oxygen and nutrients and removal of metabolic waste (Zhao et al., 2023). Water channel network arises due to the periodic cell movement and EPS modification catalysed by bacteria glycosidase activity. Fluid dynamics under shear force stabilizes the channel network and maintain the convective flow even under low nutrient condition (Sauer et al., 2022).

Dispersion phase is the final stage of the biofilm life cycle where the planktonic cells are released through matrix degrading enzymes such as dispersin B and rhamnolipids. The diespersin B is glycoside hydrolase that specifically degrade the PIA (polysaccharides intracellular adhesin) and rhamnolipids is a biosurfactant that reduces the EPS viscosity (Yang et al., 2023). This led to weakening the structural integrity of the biofilm matrix and reduces the cell-to-cell adhesion and widens the water channels leading to detachment of microcolonies respectively. The detached cells that are metabolically reactivated are capable of dispersing with help of motile genes for longer distance and establish biofilm colony at new site (Sharma et al., 2023)

The continuous cyclic process between biofilm and planktonic cells promotes localized persistence mediated by matrix protection and spatial dispersion through active movement This lifestyle contributes to chronic infection and frequent therapeutic failures despite of effective concentration of antibiotics (Yang et al., 2023).

Biofilm-associated resistance mechanisms

The biofilm gain tolerance against the antibiotics from 10 to 1000 folds more than the planktonic bacteria due to synergistic mechanism working at multiple levels (Yang et al., 2023). Hence, they associate with 60-80% chronic infection such as implant failure, cystic fibrosis and chronic wounds (Saharan et al., 2024).

Physiological diffusion barriers: EPS is negatively charged and positively charged antimicrobials such as gentamicin, polymyxins, and quaternary ammonium compounds bind to the EPS by electrostatic interaction. The polysaccharide accumulates at higher concentration in inner biofilm due to the reduced nutrient availability and slower diffusion 10000 to 1,000,000 times higher viscosity than water, blocking the drug penetration (Saharan et al., 2024).

Persister sub-populations: Biofilm has small subpopulation of persister cells found in the inner regions of biofilm with low oxygen and limited nutrients. This cell is formed through toxin-antitoxin systems that inhibit cell metabolism and gene function. Most antibiotics depend on metabolizing bacteria to be effective and persisters acts as reservoirs of infection when treatment is stopped (Zhao et al., 2023).

Overexpression of efflux pumps: In the bacterial transport, the resistance-nodulation-cells division (RND) superfamily transporters such as the MexAB OprM system of *Pseudomonas aeruginosa*) and AcrAB-TolC *Enterobacteriaceae* species actively expel β-lactams, fluoroquinolones, and tetracyclines (Sharma et al., 2023).

Universal stress responses: The stress exerted by the biofilm lifestyle also induces a variety of other global regulatory pathways, including RpoS and two-component systems. These responses contribute to enhanced DNA repair, antioxidant defenses, cell envelope modification, and β-lactamase production that together promote general tolerance to several classes of antibiotics (Yang et al., 2023).

Immune evasion: The EPS matrix protects surface antigens from the opsonisation by preventing phagocytosis, based on size and entrapping neutrophil extracellular traps in polysaccharide matrix (Saharan et al., 2024).

2. ECO ENZYMES

Fermentation-based products produced from organic waste are being explored due to their dual advantages of biological efficacy and environmental sustainability. Eco-enzymes are produced through anaerobic fermentation of household organic waste from citrus fruits and vegetable peel, jaggery and water. These are chemically complexed liquids containing various organic acids, alcohol, enzymes and plant derived secondary metabolites that formed during the microbial metabolism (Benny et al., 2023).

The fermented product allows the conversion of roughly 30-50% of household organic waste into value added products, hence reducing the landfill disposal, methane emission and nutrient loss. At the same time, this process produces bioactive liquids that find their application in cleaning, agriculture and antimicrobial control (Varshini & Gayathri, 2023).

Ecoenzyme production

The citrus peel has numerous microorganisms such as indigenous yeast and lactic acid bacteria (Kaur et al., 2010; Razola-Díaz et al., 2024). They are widely used due to the presence of fermentable sugars, pectin, organic acids and phytochemicals that supports the microbial growth and enzymes production during the fermentation (Julinar et al., 2025). During initial fermentation, pH is close to neutral, later the pH drops to acidic level between 60-90 days due to the collection of the organic acids (Permatananda & Pandit, 2023). The longer fermentation period allows the microorganisms to degrade the complex carbohydrate and convert sugars into organic acids and active metabolites (Vidalia et al., 2023).

Chemical components of eco enzyme

The mature fermented product is rich in organic acids and secondary metabolites as citric, lactic, acetic, oxalic acids and flavonoids, alkaloids, quinones, saponins and tannins respectively. The presence of organic acids is detected by high performance liquid chromatography (HPLC) and secondary metabolites through preliminary qualitative screening methods (Ismail et al., 2024). These compounds disrupt the microbial cell membrane and alter the protein function. In addition, it contains hydrolytic enzymes as amylase, cellulase and protease, that breaks the extracellular polymeric substances and weakening biofilm integrity (Sai et al., 2023).

Antimicrobial and antibiofilm evidences

Eco-enzymes are not only effective on planktonic bacterial growth but also with biofilm formation in both Gram positive and Gram-negative bacteria (Sai et al., 2023). The antimicrobial activity of eco enzymes have been reported based on invitro studies against wide range of microorganism including *Streptococcus mutans, Enterococcus faecalis, Lactobacillus acidophilus, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Enterococcus species,* and fungal species such as *Aspergillus niger* and *Fusarium species,* (Julinar et al., 2025; Lubis et al., 2024; Sai et al., 2023; Vidalia et al., 2023). Eco enzyme effect on planktonic bacteria has been studied using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, agar well diffusion methods, and time-kill studies (Mavani et al., 2020; Sai et al., 2023).

Even though studies are limited on the antibiofilm activity of eco enzymes, existing evidence suggests that eco enzyme can disrupt the biofilm effectively. In *streptococcus mutans, Enterococcus faecalis* and *Lactobacillus acidophilus* biofilm is disrupted on exposure to eco enzymes in comparison to sodium hypochlorite. Antibiofilm activity of eco enzymes due to the presence of organic acids, hydrolytic enzymes and phytochemicals that degrades extracellular polymeric matrix and inhibit microbial growth (Sai et al., 2023). The reported antimicrobial and antibiofilm evidence of eco-enzymes derived from organic waste is summarized in Table 1

Table 1. Reported antimicrobial and antibiofilm activity of eco-enzymes derived from organic waste

Eco-enzyme substrate/source	Target microorganisms	Assays	Key findings	Proposed mechanism	Reference
Fruit peel eco-enzyme	*Enterococcus faecalis*	MIC, MBC, time-kill assay	Significant antibacterial effect observed	Organic acids + enzymatic degradation	Mavani et al., 2020
Citrus/mixed peel eco-enzyme	Oral pathogens (*S. mutans*, *L. acidophilus*)	Biofilm assay	Biofilm reduction reported	Acidification + EPS degradation	Sai et al., 2023
Household organic waste eco-enzyme	*E. coli*, *S. aureus*, *S. typhi*, *P. aeruginosa*	Agar well diffusion	Broad inhibition reported	Organic acids + phytochemicals	Vidalia et al., 2023
Organic waste eco-enzyme	*Fusarium* spp.	Antifungal growth inhibition	Growth suppression reported	Acidic metabolites	Lubis et al., 2024
Fruit peel eco-enzyme mixture	Gram-positive and Gram-negative bacteria	Antibacterial assay	Antimicrobial activity reported	Bioactive acids and metabolites	Tallei et al., 2023

3. CITRUS ESSENTIAL OILS

Plant essential oils overview

Plant essential oils are volatile and aromatic secondary metabolites synthesized by plants. These oils are extracted from leaves, flowers, seeds, bark and fruit peels of plants by physical method such as steam distillation, hydro distillation, cold pressing or supercritical CO2 extraction (Karne et al., 2023). Essential oils contain mixture of compounds such as terpenes, terpenoids and phenylpropanoids that are responsible for antimicrobial activity (Masyita et al., 2022). Due to their broad-spectrum antimicrobial activity and natural origin, essential oils are widely being researched and considered as future source of antimicrobial compounds and it is widely used in food preservation, cosmetics and healthcare and effective in preventing and controlling persistent biofilm (Rossi et al., 2022).

Citrus essential oil components

Citrus essential oils are mainly obtained from the fruit peel in Rutaceae family (*Citrus sinensis* for sweet orange, *Citrus limon* for lemon and *Citrus paradisi* for grapefruit) that constitute about 0.5-5% fresh fruit weight and contain high levels of monoterpene and oxygenated compounds (Lu et al., 2019). The bioactive compounds in oils are D-limonene, linalool, γ-terpinene, β-pinene, and citral, that are identified by gas chromatography and mass spectrometry (GC-MS)

(Nuryandani et al., 2024). Citrus essential oils are hydrophobic in nature hence easily diffuses into microbial lipid layer, show excellent antimicrobial and antibiofilm activity (Peng et al., 2024a). The effectiveness of the citrus oil can be increased against the multi drug resistant strains by combining with other antibiotics or other essential oils (Sreepian et al., 2022).

Antimicrobial mechanism and evidences

Citrus essential oils exhibit antimicrobial properties against broad range of microorganisms through membrane disruption, protein coagulation and oxidative stress induction (Song et al., 2020; Zhong et al., 2022). The hydrophobic molecules limonene and citral penetrates into the phospholipid bilayers, increases membrane fluidity and promote pore formation (Zhong et al., 2022). This results in leakage of intracellular components such as K+ and ATP, leading to the loss of membrane potential and cell death (Song et al., 2020). Membrane depolarisation and permeability changes is quantified by propidium iodide assays (Zhong et al., 2022). Several studies show 85-95% membrane depolarization at 1X minimum inhibitory concentration (MIC) (Song et al., 2020).

The antimicrobial potential varies across different citrus varieties. Lime essential oil exhibits greater potential against Gram-positive bacteria with larger inhibition zones (>17mm) than orange and grapefruit essential oils (14-16mm) in comparison to 78% alcohol-based sanitizers against *Staphylococcus aureus. Bacillus subtilis* has also proven to be highly susceptible to finger citron *(Citrus* medica) essential oil with 95% inhibition at 2x MIC (Li et al., 2019). Similarly, *Citrus sinensis* essential oil over 90% suppression of E. coli at concentration ranging from 25ug/ml, along with synergistic action against multidrug resistant bacteria using efflux pump inhibitory activity (Ambrosio et al., 2019).

The Gram-negative bacteria such as *Pseudomonas aeruginosa* exhibit moderate susceptibility to citrus essential oil with reported zone of inhibition of 12-13 mm. This reduced sensitivity is due to the presences of an outer membrane rich in lipopolysaccharide, limiting the diffusion of hydrophobic antimicrobial compounds. The enhanced activity against *Pseudomonas aeruginosa* has been observed at higher citral concentrations or due to oxidative stress–inducing mechanisms such as the production of reactive oxygen species as well as the inhibition of membrane-associated ATPase (Tang et al., 2023).

Citrus essential oils also demonstrate notable antifungal activity. Lime essential oil has shown strong efficacy against *Candida albicans*, producing large zones of inhibition and up to 92% suppression of ergosterol biosynthesis. Essential oil derived from Citrus limon peel at concentrations around 1.56% (v/v) has been reported to completely eliminate *Candida* species biofilms, accompanied by inhibition of hyphal morphogenesis and disruption of yeast-to-hyphal transition in clinical isolates. Citral appears to play a central role in this activity by selectively targeting the ergosterol biosynthetic pathway, achieving fungicidal effects at minimum fungicidal concentrations (MFCs) near 32 µg/mL and inducing reductions of approximately 3 $\log_{10}$ CFU/mL within two hours of exposure (Li et al., 2019).

Antibiofilm evidences

Citral compound has shown to inhibit acyl-homoserine lactone-mediated quorum sensing in *Pseudomonas aeruginosa,* resulting in down regulation of biofilm associated genes such as pel and psl. In *Staphylococcus aureus,* it interferes with quorum sensing systems and agr pathway. The biofilm disruption in *S. aureus* and *P. aeruginosa* has been observed on exposure to orange essential oil, fragmentation of the biofilm matrix was observed by confocal microscope and reduction up to 60% in alginate content. Linalool reduces extracellular DNA release and bacterial motility that play an important role in biofilm maturation and stability (Zhong et al., 2022). The antibiofilm evidence of citrus essential oils and their major bioactive compounds is summarized in Table 2.

Table 2. Antimicrobial and antibiofilm evidence of citrus essential oils and major bioactive compounds

Citrus oil source	Major compounds	Target microorganism(s)	Assay/biofilm model	Key findings	Mechanism	Reference
Citrus reticulata peel oil	Limonene-rich	*Listeria monocytogenes*	Antibiofilm assay	Strong antibiofilm activity	Membrane disruption	Peng et al., 2024
Mandarin essential oil	Limonene	*Staphylococcus aureus*	MIC + membrane depolarization	Strong bactericidal effect	Leakage + membrane damage	Song et al., 2020

Citrus unshiu oil	Terpenoids	*Aeromonas hydrophila*	Membrane integrity assay	High antibacterial activity	Membrane disruption	Zhong et al., 2022
Lemon peel oil	Citral	*Candida albicans*	Biofilm inhibition assay	Significant reduction of fungal biofilm	Ergosterol inhibition	Li et al., 2019

4. Combined Antibiofilm Potential of Eco-Enzymes and Citrus Essential Oils

Though eco-enzymes and citrus essential oils reported antimicrobial and antibiofilm activities individually, their combined effect remains underexplored strategy for the biofilm control. Based on the accumulated experimental data from the indirect fermentation and enzymatic assisted antimicrobial strategies the synergistic effect may be effective biofilm targeting agent (Ranaldi et al., 2025; Sereia et al., 2025). Citrus fruit waste, essential oils include limonene, citral, linalool and terpinene which are well reported for their antimicrobial, antibiofilm and quorum sensing inhibitory properties (Gupta et al., 2025; Peng et al., 2024). Indirect evidence from analogous fermentation-derived antimicrobials and enzyme–essential oil systems provide useful information for synergistic mechanisms in the absence of direct studies of eco-enzyme–citrus essential oil combinations.

Eco enzyme produced by fermentation, have low acidic pH because of their high content of organic acids like citric, acetic and lactic acids (Vidalia et al., 2023). The acidic environments have been reported to alter the EPS of biofilm by breaking the ionic bond cross links within polysaccharide network, thus increasing porosity of matrix and reducing diffusion barrier. This weakened EPS could be advantageous for penetration of hydrophobic citrus oil components into biofilm (Sereia et al., 2025; Touati et al., 2025). Indirect experimental evidence obtained from antimicrobial systems using fermentation has confirmed this mechanism (Ranaldi et al., 2025). Fermentation products from *Lactobacillus* species and orange essential oil against *Bacillus cereus* biofilm (Ammar et al., 2025), while fermented vegetable by-products enhanced cinnamon oil through pH-driven sensitization (Touati et al., 2025) were demonstrated to have a potential effect on essential oils in terms of antibiofilm activity. The softening effect on the biofilm caused by organic acids increased the diffusion of terpene compounds, thus demonstrating increased biofilm disruption compared with individual activity (Ranaldi et al., 2025; Touati et al., 2025). Despite being indirect evidences, the mechanistic implications of the observations strongly support the potential synergistic antibiofilm action of eco-enzymes and citrus essential oils.

5. Cytotoxicity, Research Gaps and Future Directions

The eco enzyme and citrus essential oils have shown potential antimicrobial and antibiofilm properties but their large applications require careful evaluation of safety and cytotoxicity. The systematic evaluations of toxicological studies remain limited as the current studies focus on antimicrobial efficiency. Eco enzymes produced through fermentation of organic waste, generally exhibit low environmental toxicity. However, their composition can vary depending on the raw material used, fermentation conditions and duration that influence their biological effects and highlights the need for better standardisation. Citrus essential oils often considered as safe because of natural origin, but their bioactive terpene components can cause irritation when applied at higher concentration, largely due to their membrane disruption properties. Studies using mammalian cell model have reported concentration dependent toxicity, highlighting the importance of optimising dosage, particularly for application in food contact surfaces, agricultural environment or household settings. Therefore, combining citrus essential oil with eco enzyme could be advantageous, as synergistic interaction could be effective on biofilm at lower concentration of essential oils. Despite of growing interest in both substances, there are still knowledge gaps in the combined use of eco-enzymes and citrus essential oils. There is a lack of direct research on the synergistic antibiofilm properties of eco-enzymes and citrus essential oils, as well as a lack of standardized approaches to the production and analysis of eco-enzymes. Most of the studies focuses on the single type of microbes, not on the multispecies biofilms in clinical and industrial settings.

Future studies should focus on filling these knowledge gaps by working on reproducible eco-enzymes and comprehensive cytotoxicity and environmental safety evaluations. Studies that use mature multispecies biofilm models, in addition to synergy analyses and advanced imaging, will play a pivotal role in validating the efficacy of these eco-enzymes. Addressing these issues will be a significant step forward in translating this sustainable, waste-based antibiofilm strategy into safe and effective solutions for food safety, agriculture, and environmental sanitation.

CONCLUSION

Eco-enzymes and essential oil from citrus provide effective way to manage biofilms. Eco-enzymes through hydrolysis and acidification break the structure of extracellular polymeric substance (EPS). In addition, citrus terpenoids affect the cellular membrane and disrupt quorum sensing. In vitro studies work well on the important pathogens and using them together may improve antibiofilm properties but challenges remain in standardizing formulation, using multispecies models and safety evaluations. Further research on consistent production, effective mixture and toxicity assessment will help to overcome these obstacles. This approach provides a sustainable method for managing biofilms in sanitation, food safety, agriculture, and healthcare, while reducing antimicrobial resistance and using organic waste.

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