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HURDLE APPROACH EMPLOYING MICROENCAPSULATED CARVACROL AND ENZYMES TO DISRUPT PSEUDOMONAS AERUGINOSA BIOFILMS

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Abstract

Biofilms, microbial communities enclosed in an extracellular matrix, pose a significant problem in various fields due to their resistance to stress and impeded antimicrobial penetration. They are particularly problematic in medical, food, marine, oil reservoirs, and petroleum distribution industries due to their complex structure and potential economic and health issues. Biofilm resistance to current control strategies highlights the need for new alternatives. Hurdle technology, combining two or more methods, offers an effective method for controlling biofilms effectively. In this perspective, the use of functional enzymes combined with biosourced antimicrobial such as essential oil (EO) is a promising alternative anti-biofilm approach. However, these natural antibiofilm agents can be damaged by severe environmental conditions and lose their activity. Microencapsulation of enzymes and EOs is a promising new technology that enhances their stability and biological activity. This work focuses on the problems related to biofilms in various fields, and on the use of Hurdle technology based on encapsulated enzymes with essential oils as antibiofilm agents.

Hurdle technology strategies to fight microbial biofilms

A single use of a disinfectant may be insufficient to remove the entire undesirable biofilms because of their complex structures. However, the Hurdle technology could be the solution, since it's based on a combined smart use of Hurdles such as physical-chemical, chemical-chemical, or biological-chemical disinfection methods. The goal is to achieve effective control of undesirable microbial biofilms by targeting different vital cell functions at the same time. The synergistic effect of Hurdle technology results in controlling biofilms and persistent bacterial cells on abiotic surfaces [1]. Accordingly, combining enzymes with antimicrobials would provide a promising approach to control biofilms in such a way that the enzymes destroy and destabilize the biofilm EPS matrix, so that matrix-protected cells become more effectively removed by the antimicrobials.

Enzymes weaken the biofilm's physical integrity by breaking down its multi-structural components, such as carbohydrates, polysaccharides, proteins, glycoproteins, lipids, nucleic acids, phospholipids, and glycolipids. They target these components, transforming them into smaller units [1]. In addition, natural antimicrobial substances like essential oils (EOs) have gained attention due to their high efficacy, safety, and non-toxicity [2]. Carvacrol, a major component of Origanum vulgari EO, has been shown to have significant antibiofilm activity on various surfaces [3–5]. However, essential oils are volatile, vulnerable to oxidation and photolysis, and their low solubility may reduce their efficacy [6–8]. Enzyme activity is influenced by environmental factors and optimal under restricted conditions [9]. Encapsulation can stabilize enzymes and carvacrol, improving their anti-biofilm activity, stability, and cost-effectiveness if stability is maintained for repeated use.

Whatever the sector concerned by biofilm formation, disinfection has to be undertaken economically and safely. This can be achieved by reducing disinfection frequency and in the shortest timeframe possible, reducing thus the chemicals use and energy, and the amount of waste without damaging the equipment. Thus, Hurdle technology could be more effective in controlling biofilms compared to the single use of disinfectants. Potential solutions for combined Hurdles must therefore be carefully selected to achieve an effective disinfection effect.

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Assessment of the antibiofilm activity of microencapsulated carvacrol

Carvacrol emulsions were prepared by dissolving sodium caseinate in water and shaking until hydration. The pH was adjusted to 7, and each emulsion was mixed with a maltodextrin DE 19 solution to create a feed emulsion with carvacrol (1%), sodium caseinate (0.5%), and maltodextrins (20%). The feed emulsions were shaken for 30 minutes and spray-dried using a lab-scale device with a 0.5 nm nozzle atomizer (Mini Spray-Dryer Buchi B-290, Switzerland). Powders were collected and stored at 4°C until testing. Reconstituted suspensions were prepared by scattering weighted quantities of spray-dried powders in water and shaking for 1 hour.

Circular stainless-steel coupons were used for biofilm formation. They were soaked in 95% ethanol, washed with distilled water, immersed in 1% DDM ECO detergent, washed with distilled water, and air-dried before autoclaving. Bacterial suspension of *P. aeruginosa* was added to the coupons, incubated for 1 hour, and rinsed twice with PB. The coupons were then covered with TSB medium and incubated for 24 hours at 37°C. The old TSB medium was discarded, and the coupons covered with biofilm were washed twice with PB to remove planktonic cells. Rinsed coupons were used for antibiofilm testing using free and microencapsulated carvacrol at 5 mg mL^{-1}.

The antimicrobial activity of microencapsulated Carvacrol (ME CARV) was investigated by direct analysis using fluorescence microscopy. *Pseudomonas aeruginosa* biofilm, grown on stainless steel, were stained with SYTO9 which stains live bacteria in green, and propidium iodure (PI) which stains dead bacteria in red.

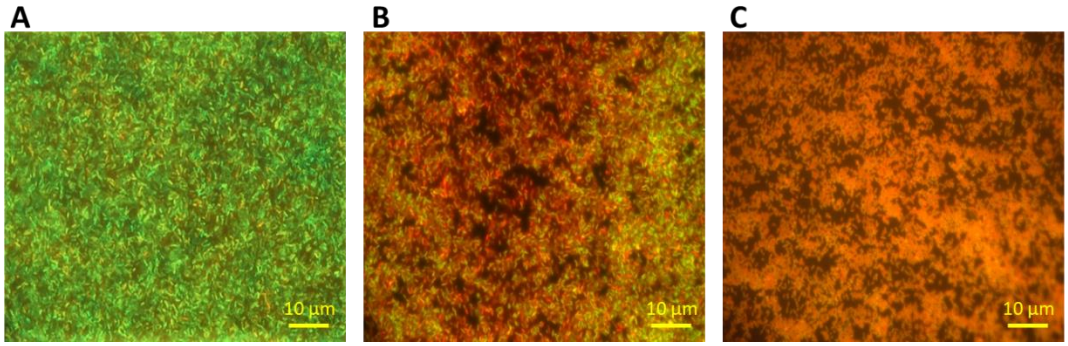

Figure 1. *P. aeruginosa biofilms images, with epifluorescence microscopy; after treatment with carvacrol at 5 mg mL^{-1} for 1 min. Cells were visualized after staining with SYTO9 (green fluorescence for living bacteria) and propidium iodide (red fluorescence for dead bacteria). Control represents biofilm treated with tryptone salt broth. A: control, B: biofilm exposed to free carvacrol, C: biofilm exposed to microencapsulated carvacrol*.

The results of Figure 1 showed that the ME CARV 5 mg mL^{-1} has a strong antibacterial effect against *P. aeruginosa* biofilms (Figure 1C), when compared with the effect of free carvacrol 5 mg mL^{-1} (Minimal Inhibitory Concentration, MIC in 2% dimethyl sulfoxide) (Figure 1B). This is highlighted by the increase of the number of dead cells (red cells) stained by PI when compared to the control biofilms (Figure 1A). Figure 1A showed that *P. aeruginosa* control biofilm treated with Tryptone Salt Broth presented a dense biofilm composed by a viable biomass (green cells) stained by SYTO9.

These results demonstrated the strong antimicrobial effect of the microencapsulated carvacrol against the embedded biofilm cells at the same carvacrol concentration (5 mg mL^{-1}) when compared to free carvacrol. These findings are in accordance with previous studies which shown that encapsulating essential oils, such as carvacrol, within microcapsules can significantly enhance their antimicrobial properties against a range of resistant microorganisms, including biofilms. The microencapsulation process protects the active compounds from degradation, allows for controlled release, and facilitates better penetration and interactions with the target microbial cells and biofilms [10–12].

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Assessment of the antibiofilm activity of sequential treatment with pepsin and trypsin and microencapsulated carvacrol

The sequential treatment with both pepsin, trypsin (1 mg mL^{-1}) and carvacrol at ½ MIC (2.5 mg mL^{-1}) of *P. aeruginosa* biofilms, grown on stainless steel, resulted in a significant synergistic inactivation of biofilm. The results of figure 2C showed the strong effect of the applied Hurdles. Pepsin and trypsin treatment result in a dispersion of the biofilm matrix (Figure 2B) with significant decrease in biofilm biomass following pepsin / trypsin treatment, showing scattered bacterium and non-dispersed clusters for *P. aeruginosa* biofilm. *P. aeruginosa* cells are thus exposed to carvacrol.

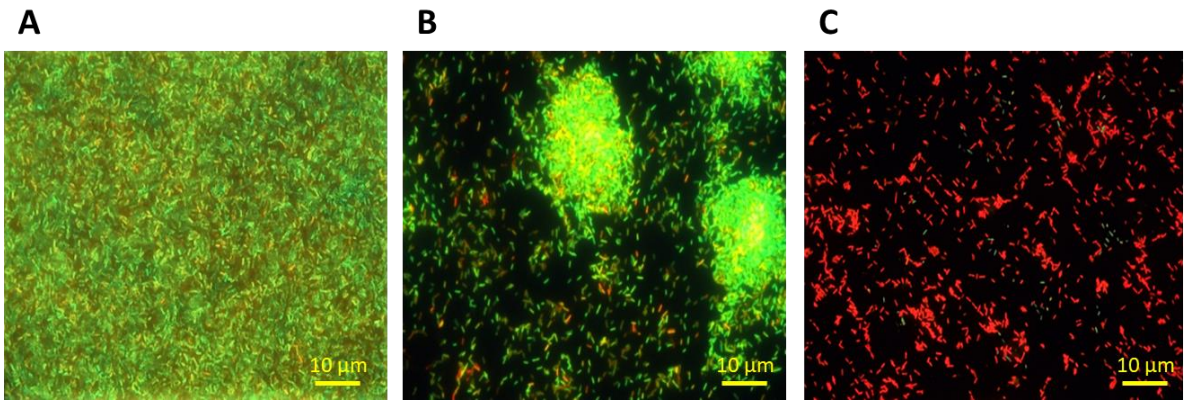

Figure 2. *P. aeruginosa biofilms epifluorescence microscopic images after treatment with a sequential treatment with both pepsin, trypsin (1 mg mL^{-1} for 1 h each) (B), and followed by treatment with carvacrol at ½ MIC (2.5 mg mL^{-1}) for 1 min (C). Cells were visualized using SYTO9 (green fluorescence for live bacteria) and propidium iodide (red fluorescence for dead bacteria). Control represents biofilm treated with tryptone salt broth (A).*

Figure 2C showed that after sequential enzymatic treatment and microencapsulated carvacrol, the biomass of both biofilms and the number of viable cells were significantly decreased, and the clusters were predominantly stained by PI. In addition, this combined treatment allowed to reduce the amount of carvacrol to ½ MIC (2.5 mg mL^{-1}). These finding showed that a combined treatment of enzymes and microencapsulated carvacrol provide a good demonstration of the efficiency of Hurdle technology to tackle bacterial biofilms.

Conclusion:

This work provides that the combined treatment using enzymes and carvacrol could be a promising sustainable and ecofriendly strategy for the eradication of microbial biofilms. The use of these biobased agents would further reduce the use of chemical agents, energy costs, and water consumption needed for cleaning and disinfection. In addition, the combination of enzymes and essential oils showed that these Hurdles have synergistic effect and results in a reduction of the concentrations required to yield the same antimicrobial activity when compared to non-combined treatment.

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