BIOACTIVE FILMS: RELEASE OF HARZIANIC ACID IN HYDROPHOBIC AND HYDROPHILIC SYSTEMS

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ABSTRACT

Bioactive coatings represent an innovative approach in food preservation. This study investigated the controlled release of harzianic acid (HA) in bioactive films composed of guar gum, carboxymethylcellulose (CMC), and pectin, in both hydrophilic and hydrophobic systems. The results revealed variations in the release rates of HA, with CMC formulations being the most effective in hydrophobic systems. The formulation containing an equal proportion of guar gum, pectin, and CMC (33.3% v/v/v) demonstrated controlled and sustained release of HA, with promising implications for antifungal applications. For bacterial inhibition, a formulation with a higher proportion of pectin and guar gum or pure CMC is recommended, providing a faster and more effective initial release. These findings underscore the potential of bioactive films as a strategy to combat undesirable microorganisms.

Keywords: Harzianic acid, Bioactive films, Controlled release, *T. harzianum*

1 INTRODUCTION

Bioactive coatings represent an innovative approach in food preservation, consisting of thin layers of safe materials that can be applied directly to the surface of food. These coatings play a crucial role in protecting and preserving food quality by acting as an effective barrier against moisture, oxygen, and solutes present in the food.[1] Currently, research is underway on the incorporation of bioactive compounds into coatings, which are natural substances or derivatives of natural sources with bioactive properties. Among them, antioxidants such as polyphenols and flavonoids are highlighted for their ability to delay lipid oxidation and nutritional degradation of foods. Additionally, antimicrobial compounds such as phenolic acids, essential oils, and plant extracts inhibit the growth of fungi and bacteria, thereby extending the shelf life of fruits.[2,3] The incorporation of these bioactive compounds into coatings poses a challenge due to their instability during the processing and storage of the coatings. It is necessary to ensure the retention of the activity of these compounds as well as their controlled release over time to maximize their beneficial effects on food quality preservation.[3]

Harzianic acid (HA) is a nitrogen-containing heterocyclic siderophore alkaloid isolated for the first time in 1994 from the *Trichoderma harzianum* SY-307 strain.[4] HA has shown promise for the biocontrol of fungi such as *Fusarium oxysporum*, Gram-positive bacteria, and antibiotic-resistant strains, owing to its antimicrobial properties.[5] Its antimicrobial and antifungal properties make it a promising bioactive compound to be incorporated into bioactive films. The study investigated the effect of adding HA to bioactive films made of guar gum, carboxymethyl cellulose, and pectin on release for hydrophilic and hydrophobic systems.

2 MATERIAL & METHODS

The production of HA occurred through liquid fermentation in potato dextrose broth (PDB) medium, where 100 mL was inoculated with 10^5 spores/mL of *T. harzianum*. The cultivation was carried out in Erlenmeyer flasks in an incubator (25°C), without agitation, for a period of 28 days. HA extraction and purification were conducted according to the method described by Pang *et al* 6. The bioactive film was prepared using a nanoemulsion of HA prepared with 80g of distilled water, 10g of HA/soybean oil (at a ratio of 2:8 w/w), and 10g of Tween 80, which was emulsified using an Ultra Turrax disperser (TECNAL, TE-147) operating at 12,000 rpm for 30 min. The nanoemulsion of HA was mixed with guar gum (1% w/v), CMC (1% w/v), and citrus pectin (1% w/v), which were separately solubilized at 70°C with 800 rpm. After cooling, glycerol (5 g/L) was added under agitation (800 rpm), and the pH was adjusted to 4.0 (using 0.1N NaOH or HCl).

Subsequently, ten bioactive mixture formulations were prepared according to a simplex centroid mixture design. The evaluated factors were guar gum, pectin, and CMC, each investigated at three levels: guar gum (0%, 50%, 100%), pectin (0%, 50%, 100%), and CMC (0%, 50%, 100%). Triplicates were conducted at the central point and axial points, totaling 12 experiments. The final concentration of HA in each formulation was 0.122 g/L. The release of HA was evaluated in the ten previously prepared bioactive coating formulations following the method described by Ranjith *et al* 7, with some modifications. A total of 9.2 mL of bioactive film was added to a petri dish (5.5 cm diameter) and dried at 30ºC for 24 hours (50% RH). To simulate water- and lipid-rich foods, distilled water and 95% ethanol were selected, respectively. Disks of bioactive films with a diameter of 17 mm from each experiment were immersed in 10 mL of distilled water or ethanol. The release of HA was quantified at 24, 48, and 72 hours at 25°C with agitation at 140 rpm.

For samples immersed in ethanol, the solvent was evaporated and suspended in water (1 mL). Subsequently, the samples were acidified with 2% (v/v) HCl (4.0 N) before extraction on an SPE Stracta C18 column (55 µm, 70 A). HA was eluted from the SPE column with 1 mL of ethyl acetate (100%). The quantification of HA was performed following the methodology described by Vinale *et al* 8, using UHPLC at 360 nm based on retention time, spectrum, and calibration curve (y=0.5843x+1.4302; R^2=0.9935; 0.001-0.1 mg/mL) with purified HA from the work as an internal standard.

3 RESULTS & DISCUSSION

Ten formulations of bioactive coatings were tested for the release of HA in hydrophilic and lipophilic systems. The release rate of HA varied significantly among the different formulations within the first 24 hours (Fig. 1A and 1B). The formulation containing guar gum (100%) exhibited the highest release rate in ethanol (20.43%) and water (18.61%), indicating a faster and less controlled release of the compound. On the other hand, the formulation containing pectin (100%) showed the lowest release rate in ethanol (7.74%) and water (4.81%), e.g. a slower release of the bioactive compound. The formulation containing CMC (100%) demonstrated contradictory release rates of HA, as it obtained the highest value among the studied formulations in the hydrophobic system (29.45%), while in the aqueous medium it remained close to the rates of the formulations (5.52%). The higher release rates of the compound in ethanol may be associated with the hydrophobic chemical nature of HA[9].

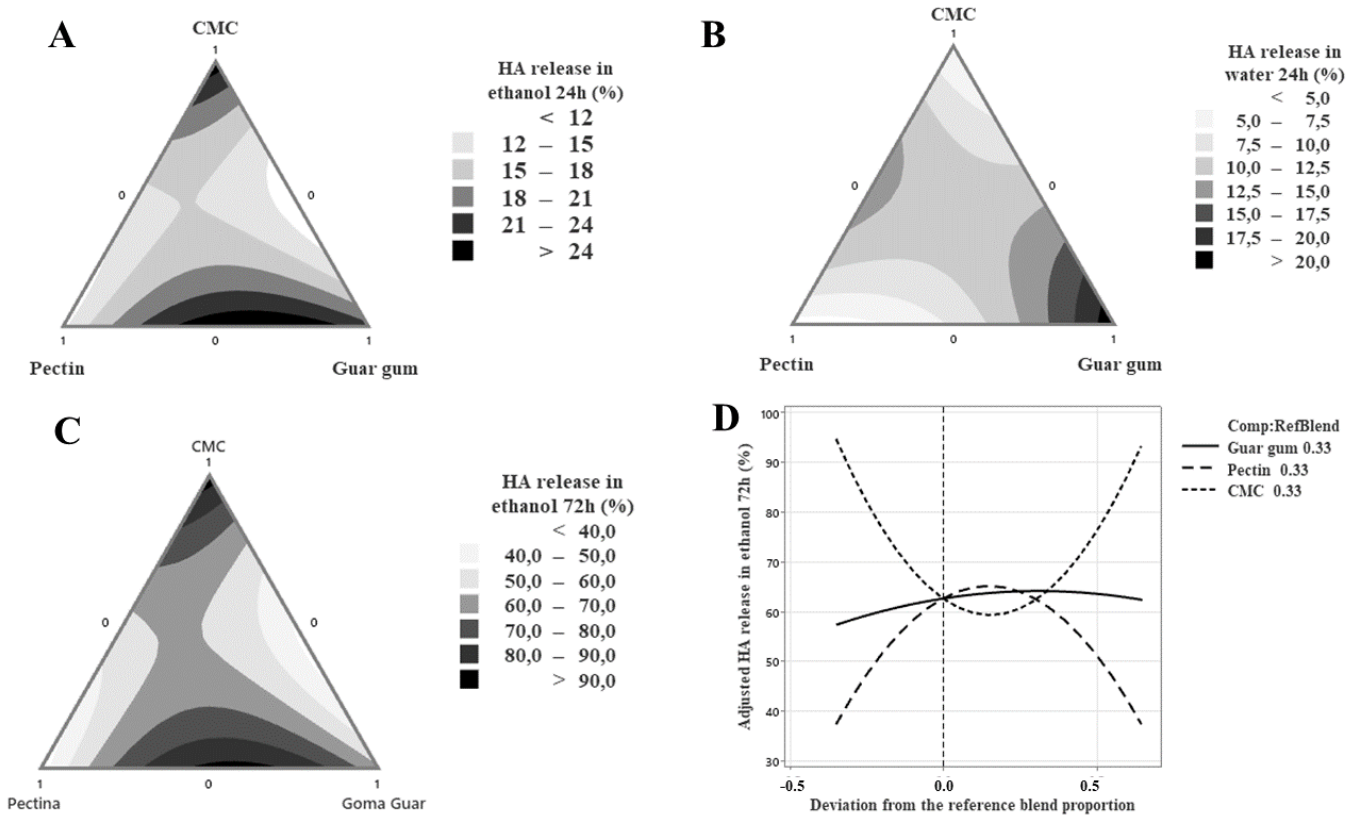

Figure 1. (A) Percentage of harzianic acid (HA) release in ethanol after 24 h, (B) HA release (%) in water after 24 h, (C) HA release (%) in ethanol after 72 h, and (D) Cox Response Trace graph, results expressed as percentage of HA release in ethanol after 72 h.

The high release rate of HA in water can be attributed to the permeability of the hydrocolloid coating due to the higher amount of guar gum in the formulation.[7] The results presented by Zhao *et al* 10, also demonstrated that the chemical nature of the bioactive compound determines the release rate behavior in bioactive coatings. In their case, thyme oil showed a higher release profile in solutions with >50% ethanol, and blood orange anthocyanins in alcoholic solutions (10%). In the study conducted by Ranjith *et al*., (2022)[7], a higher release rate of antifungal peptides was obtained in an aqueous medium (94.3±3.3 µg/mL to 113.9±3.8 µg/mL) compared to ethanol (69.4±4.5 µg/mL to 74.3±5.4 µg/mL) for coatings of CMC (1%) and pectin (3%), respectively.

After 72 hours, the release rate of HA in a hydrophobic medium for all coatings remained above 35% (Fig. 1C). The CMC formulation (100%) exhibited the highest HA release rate (>90%). In the study conducted by Sganzerla *et al* 11, a coating prepared with 2.5% CMC and 50% (v/v) blackberry extract rich in anthocyanins showed the maximum release peak of antioxidant compounds after 2 h in 10% ethanol. The significant disparity in release rates can be attributed to the unique molecular structure of CMC, characterized by its linear polysaccharide chains and high degree of substitution, which create a highly porous network that facilitates the diffusion of HA molecules.[2] Additionally, pectin with a high degree of esterification (75.7%) demonstrated a greater affinity for HA when used individually, resulting in increased compound retention. Conversely, in equimolar mixtures of pectin and guar gum, a higher compound release was observed, suggesting interactions between the carboxyl groups of the two polymers, which facilitated the release of HA.

The best formulation of the bioactive coating with a controlled and sustained release rate of HA in a hydrophobic medium was the one containing an equal proportion mixture of the polymers guar gum, pectin, and CMC (33.3% v/v/v), with values after 24 h (16.16±2.32%), 48 h (38.8±4.40%), and 72 h (59.28±2.88%), indicating a controlled and sustained release rate of the bioactive compound (Fig. 1D). This behavior is desirable for antifungal applications, as the growth of phytopathogenic fungi in food has a

critical development period within the first 72 hours. Therefore, the gradual release of HA from the coating throughout this period allows for prolonged exposure of the bioactive compound in the food matrix, resulting in effective inhibition of fungal growth.

For bacterial inhibition, a formulation that provides a faster and more significant release within the first 24 hours is crucial, aiming for immediate action against microorganisms present in food. In this regard, a formulation with a higher proportion (50% v/v) of pectin and guar gum or 100% CMC may be recommended to achieve this goal. Pectin, with its high degree of esterification, and guar gum, known for its ability to form films and provide protective barriers, can synergistically act to achieve a more effective initial release of HA, contributing to immediate antibacterial action. This strategy may be particularly relevant in contexts where bacterial contamination poses an immediate threat to product safety. By offering a faster release within the first 24 hours, this formulation can provide robust initial protection against bacterial proliferation, thereby complementing the effectiveness of bioactive films in preventing microbiological contamination.

4 CONCLUSION

The results revealed a significant variation in the release rates of HA among different formulations of bioactive coatings, especially within the first 24 hours. Formulations based on guar gum exhibited the highest release rates, while those based on pectin showed the lowest. CMC-based formulations demonstrated higher release rates observed in hydrophobic systems. The optimal formulation for controlled and sustained release of HA in a hydrophobic medium consisted of an equal combination of guar gum, pectin, and CMC (33.3% v/v/v). This formulation displayed desirable characteristics for antifungal applications due to its sustained release profile, allowing prolonged exposure of HA in the food matrix, effectively inhibiting fungal growth. For bacterial inhibition, formulations with higher proportions of pectin and guar gum or pure CMC are recommended to achieve faster and more significant release within the first 24 hours. This strategy provides immediate protection against bacterial proliferation, complementing the effectiveness of bioactive films in preventing microbiological contamination.

REFERENCES

1 SAXENA, A., SHARMA, L., MAITY, T. 2020. Elsevier Inc. 859–880.
2 DESHMUKH, R. K., AKHILA, K. K., RAMAKANTH, D., GAIKWAD, K. K. 2022. Int. J. Biol. Macromol. 201. 1–13
3 YADAV, A., KUMAR, N., UPADHYAY, A., SETHI, S., SINGH. 2022. J Food Sci. 87 (6). 2256–2290
4 ZHANG, J. L., TANG, W. L., HUANG, Q. R., LI, Y. Z., WEI, M. L., JIANG, L. L., LIU, C., YU, X., ZHU, H. W., CHEN, G. Z., ZHANG, X. X., ZHU, Y., WANG, J., MOU, P., YAN, Y., CHEN, M., TANG, Y., SARROCCO, S., MUÑOZ, I. V. 2021. Front. Microbiol. 9. 123–129
5 OUYANG, X., HOEKSMA, J., BEENKER, W. A., BEEK, S. VAN DER, HERTOG, J. 2021. Biorxiv. 2021.12.03.471066
6 PANG, G., SUN, T., DING, M., LI, J., ZHAO, Z., SHEN, Q., CAI, F.M. 2022. J. Agric. Food. Chem. 70 (37). 11769–11781
7 RANJITH, F. H., ADHIKARI, B., MUHIALDIN, B. J., YUSOF, N. L., MOHAMMED, N. K., ARIFFIN, S. H., MEOR HUSSIN. 2022. Food Control. 135. 108789
8 VINALE, F., FLEMATTI, G., SIVASITHAMPARAM, K., LORITO, M., MARRA, R., SKELTON, B.W., GHISALBERTI, E. L. 2009. J. Nat. Prod. 72 (11). 2032–2035
9 SALVATORE, M. M., SICILIANO, A., STAROPOLI, A., VINALE, F., NICOLETTI, R., DELLAGRECA, M., GUIDA, M., SALVATORE, F., LULIANO, M., ANDOLFI, A., DE TOMMASO, G. 2022. Molecules. 27 (6). 1959
10 ZHAO, R., CHEN, J., YU, S., NIU, R., YANG, Z., WANG, H., CHENG, H., YE, X., LIU, D., WANG, W. 2023. Food Hydrocolloids. 134. 108094
11 SGANZERLA, W. G., PEREIRA R. C. P., ULIANA, N. R., CASSETARI R., M. B., DA ROSA, C. G., FERRAREZE, J. P., VEECK, A. P. DE L., NUNES, B. 2021. Agric. Biotechnol. 33. 101989

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