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CONSTRUCTION OF A CRISPR/Cas12a COLORIMETRIC SENSOR FOR MONITORING MEAT FRESHNESS

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I. INTRODUCTION

Spoilage microorganisms in food systems, particularly meat and poultry, are directly responsible for freshness, food losses, and outbreaks [1]. Currently, *Pseudomonas* prevails in aerobic packaging atmospheres as an important spoiler for representing total bacterial counts and further evaluating meat freshness [2]. Conventional approaches for detecting *Pseudomonas* spp. are primarily based on selective growth media. However, these approaches are both time-consuming (more than 24 h) and inaccurate (false negative) for specific subspecies [3].

This work presents a novel colorimetric sensing strategy based on magnetic enzyme-labeled nanoparticles combined with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a without nucleic acid amplification. Cleaving the independently synthesized magnetic enzyme-labeled probe allows translation of *Pseudomonas* spp. count into a colorimetric signal for monitoring meat freshness.

II. MATERIALS AND METHODS

2.1 Bacterial culture, plating, and DNA extraction

All tested *Pseudomonas* and non-*Pseudomonas* strains isolated from meat and meat-related sources, previously stored at -80°C, were streaked and cultured on specific agar media for 24 h. The target DNAs were extracted and purified according to the method of the DNA extraction kit (Vazyme Biotech Co., Ltd., China).

2.2 Preparation of magnetic enzyme-labeled probes

First, the 100 μL (1 mg/mL) of magnetic nanoparticles (MNPs) were washed in MEST buffer (100 nM; pH = 5; 0.05% Tween 20). Subsequently, a solution containing 100 μL of N-hydroxysuccinimide (10 mg/mL) was introduced to activate the MNPs. After activation, a solution containing a single-stranded DNA (ssDNA) (4 nmoL) ligand was combined for 2 h at room temperature. Finally, the MNPs-ssDNA complexes were incubated with the streptavidin-horseradish peroxidase (SA-HRP) (1 mg/mL) for 2 h at 37°C. The products of magnetic nanoparticle-ssDNA-horseradish (M-S-H) were washed using PBST (0.01 M; pH = 7.4; 0.05% Tween 20) and then stored at 4°C.

2.3 Quantitative analysis and Practical evaluation of the CRISPR/Cas12a sensor

The CRISPR/Cas12a sensor method included 200 nM Cas12a-crRNA, 0.2 mg/mL probe, 1 U/μL RNase inhibitor, 1× NEBuffer R2.1, and 2 μL target DNA. Then, this mixture was incubated at 37°C for 1 h. Following magnetic separation, 40 μL of 3,3',5,5'-tetramethylbenzidine was incubated at 37°C. Finally, a termination solution was added, resulting in a yellow signal. All results were obtained from at least three independent experiments as the mean ± SD.

A solution containing the *Pseudomonas* strains was prepared, with initial bacterial concentrations of 8.7 log CFU/mL. Then, a 10-fold gradient dilution was prepared for the bacterial template. The templates of *Pseudomonas* were analyzed using the CRISPR/Cas12a sensor to evaluate the quantitative sensitivity of this sensor method. Subsequently, three representative concentration gradients (3.9 log CFU/g, 4.8 log CFU/g, and 6.3 log CFU/g) were selected to evaluate the practical efficacy in intricate meat matrixes. The artificially contaminated chicken breast samples were analyzed using the CRISPR/Cas12a sensor method.

III. RESULTS AND DISCUSSION

3.1 Characterization of the magnetic enzyme-labeled probes

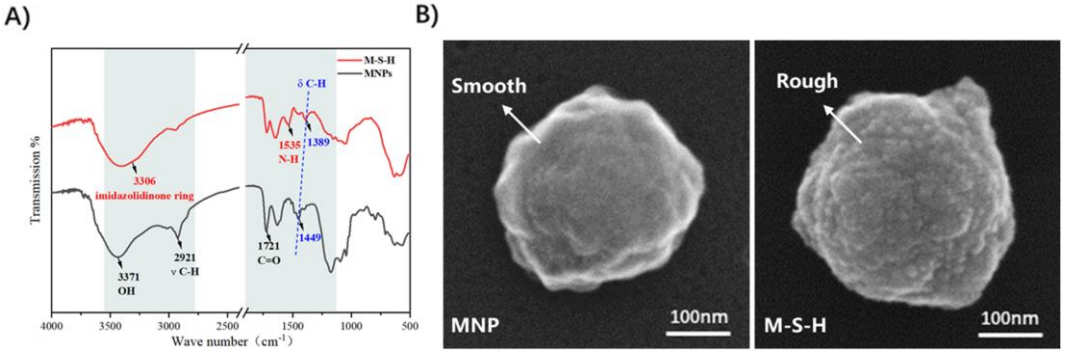


Figure 1. (A) Fourier transform infrared (FTIR) spectroscopy. (B) Scanning electron microscopy (SEM).

The fourier transform infrared spectroscopy showed a significant peak in the amide II band at 1535 cm^{-1} (N-H) and a broader peak in the 3000-3500 cm^{-1} range at 3306 cm^{-1}, indicating the presence of the imidazolidinone ring in biotin (Figure. 1A). The scanning electron microscopy images demonstrated that the M-S-H probe exhibited a more uneven surface structure. In addition, a higher level of granularity was confirmed compared with that of the original MNP (Figure. 1B). Thus, magnetic enzyme-labeled probes were successfully constructed, with nanomagnetic nanoparticles as carriers, single-stranded DNA as the cleavage site, and HRP as the signaling molecule.

3.2 Sensitivity and Application Validation of the CRISPR/Cas12a sensor

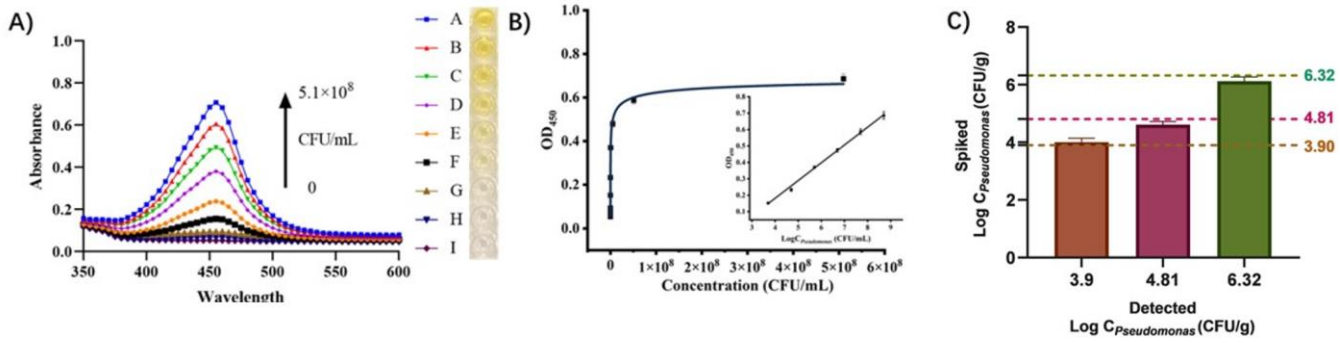


Figure 2. (A): UV-visible absorption spectral response, (A~H: 5.1×10^8~5.1×10^1 CFU/mL; I: 0 CFU/mL). (B): Relationship between OD_{450} and logarithm of bacterial concentration. (C) Consistency between *Pseudomonas* spp. spiked and detected.

The target was quantified based on the bacterial concentration using the CRISPR/Cas12a sensor method. The OD value at 450 nm increased with an increase in bacterial concentration from 0 to 5.1×10^8 CFU/mL (Figure. 2A). A linear relationship was observed between the OD_{450} value and logarithmic value of bacterial concentrations, ranging from 3.7 to 8.7 log CFU/ml (Figure. 2B). The linear equation was y = 0.1084x - 0.2527 (x = log C *Pseudomonas*, and R^2 = 0.9939), with the limit of detection (LOD) for detecting *Pseudomonas* spp. established as 3.6 log CFU/mL. The effective quantification range of this sensor can encompass bacterial count fluctuations throughout its shelf life, thus realizing an accurate real-time assessment of chilled meat freshness.

Three concentration gradients of *Pseudomonas* (3.9 log CFU/g, 4.8 log CFU/g, and 6.3 log CFU/g) were spiked into blank chicken breast samples. The results demonstrated average recoveries ranging from 95.96% to 102.59% in artificially contaminated chicken breasts, with a relative standard deviation (RSD) ranging from 3.93% to 8.61% (Figure. 2C). The satisfactory recoveries and low RSD values indicated that the CRISPR/Cas12a sensor method could effectively detect *Pseudomonas* for assessing freshness in complex meat samples.

IV. CONCLUSION

In this work, a novel CRISPR/Cas12a sensor was developed to determine meat-borne *Pseudomonas* spp. contamination without pre-amplification for visually and quantitatively assessing meat freshness. This sensor utilized magnetic nanoparticles as carriers and enzymatically labeled molecules as probes for signal detection. The synthesized magnetic nanoparticles were successfully characterized using the FTIR and SEM. This sensor could quantitatively detect *Pseudomonas* spp. within the linear range of 3.7 log to 8.7 log CFU/mL. Overall, this work extends the application of the CRISPR/Cas12a system in the field of colorimetric sensing and contributes new insights into the exploration of reliable tools for monitoring meat freshness.

The developed sensor is also expected to facilitate its widespread use in microbial monitoring across different scenarios, including environmental monitoring, clinical diagnosis, and food safety.

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