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Preservation of large yellow croaker (*Pseudosciaena crocea*) by Coagulin L1208, a novel bacteriocin produced by *Bacillus coagulans* L1208

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ABSTRACT

Large yellow croaker (*Pseudosciaena crocea*) is a cultivated fish of great economic importance and abundant nutritional value. However, due to its high protein and water contents, it is susceptible to decomposition, leading to considerable economic loss and adverse effects on consumer health. Here, we assessed the function of the bacterial strain *Bacillus coagulans* L1208 (Bcoa) in preserving large yellow croaker during storage at 4 °C and found that Bcoa elongates the shelf-life significantly. Further investigations showed that Bcoa prolongs the storage time mainly by suppressing the growth of spoilage bacteria. Moreover, a novel bacteriocin, designated as Coagulin L1208 and produced by Bcoa, was purified and identified by N-terminal sequencing. Finally, the activity of Coagulin L1208 for suppressing spoilage bacteria during the preservation of large yellow croaker was assessed. Our results reveal the mechanism by which Bcoa aids the preservation of large yellow croaker and identify Coagulin L1208 as a potential novel antiseptic.

1. Introduction

Large yellow croaker (*Pseudosciaena crocea*) is a major marine cultured fish and is widely distributed in the coastal area of southeastern China (Zhou et al., 2016). Due to its delicious taste and high nutritional value, it is deeply loved by consumers and has become the most cultured marine fish in China. However, because of its high nutritive content, large yellow croaker is also an easy breeding ground for bacteria, causing considerable economic losses and poses a hazard to consumer health.

Multiple physical, chemical and microbiological mechanisms have been implicated in fish spoilage. Several changes, including the shift of acidity, protein degradation, lipid oxidation, ATP degradation and microbial growth, occur during this process, eventually resulting in an inferior texture (Ghaly et al., 2010). Accordingly, several indexes have been developed to measure the freshness of fish. The total volatile basic nitrogen (TVB-N) is mainly produced due to protein degradation by either bacterial or enzymatic activities and is thus usually used to evaluate fish protein degradation, especially during cold storage (Cheng et al., 2016; Woyewoda et al., 1986). ATP degradation during fish spoilage is widely assessed by the K value, which is derived from the quantification of ATP and its breakdown products, including ADP, AMP, inosine-50-monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx) (Lowe et al., 1993). In addition, the shift in acidity can be quantified by the pH value (Woyewoda et al., 1986); malondialdehyde (MDA), produced by lipid oxidation, can be measured by thiobarbituric acid (TBA) (Nishimoto et al., 1985); and the sensory level of the fish can be scored by trained adjudicators (Bremner, 1985). By combining all these indexes, the freshness of the fish can be determined accurately (Li et al., 2013b).

To prevent the serious disadvantages of fish spoilage, a number of bio-preservatives, such as tea polyphenols and the rosemary extract chitosan (Li et al., 2012; Zhao et al., 2013), chitosan (Hui et al., 2016; Li et al., 2012), lectin MCL-T (Li et al., 2015) and bayberry leaf extract (Su et al., 2014) were applied. In addition, advanced technologies such as vacuum packaging (Zhao et al., 2012), controlled freezing-point vacuum drying (Cao et al., 2016) and high hydrostatic pressure (Yang et al., 2015) have been applied to preserve large yellow croaker. However, to further prolong its shelf-life, new preservatives with high safety and effectiveness are still required.

Several special bacterial strains have been widely used in preserving food and improving human health for thousands of years (Gismondo et al., 1999). By exhausting oxygen and essential nutrition or by generating antibiotic products such as bacteriocins, lactic acid and

Abbreviations: Bcoa, *Bacillus coagulans* L1208; CFU, colony-forming units; DGGE, denaturing gradient gel electrophoresis; TBA, thiobarbituric acid; TVB-N, total volatile basic nitrogen; TVC, total viable count

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peroxide, these strains suppress spoilage bacteria and improve the preservation of food. Nisin, Natamycin and ε-poly-L-lysine are widely used antiseptics that are produced by bacteria (Delves-Broughton et al., 1996; Lucera et al., 2012; Ye et al., 2013). Recently, the bacterial species *Bacillus coagulans* was also found to produce antiseptics (Nath et al., 2015). For instance, Lactosporin produced by *Bacillus coagulans* ATCC 7050 was reported to suppress pathogenic bacteria (Riazi et al., 2012), while phenyllactic acid produced by *Bacillus coagulans* TQ33 was able to inhibit plant pathogenic fungi (Wang et al., 2013). Our previous work also found crude *Bacillus coagulans* extraction can improve large yellow croaker preservation (Liu et al., 2015), but the underling mechanism has not been revealed, and it is also not known whether a critical antiseptic that is produced by *Bacillus coagulans* can work in this regard.

In the present study, we first applied psychrotrophic *Bacillus coagulans* strain L1208 (Bcoa) to preserve large yellow croaker and found that it produces favorable results in the preservation process. It was found that Bcoa improves the preservation of large yellow croaker mainly by inhibiting the growth of spoilage bacteria. Furthermore, the present work identified Coagulin L1208 as the chief bacteriostatic ingredient in Bcoa. The Coagulin L1208 sequence was determined by N-terminal sequencing. This work demonstrates the development of a new approach for preserving large yellow croaker using Bcoa and identifies Coagulin L1208 as a novel antiseptic candidate.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Psychrotrophic *Bacillus coagulans* L1208 (Bcoa) is derived from *Bacillus coagulans* LL1103 (Li et al., 2013a; Liu et al., 2015) obtained by screening in our lab and showed enhanced ability in preserving large yellow croaker. Bcoa was subcultured in a Nutrient Broth medium at 30 °C with shaking at 150 rpm for 24 h before use. After centrifuging at 8000 × *g* for 10 min at 4 °C, Bcoa cell pellets were harvested and re-suspended in sterile NaCl solution (8.5 g/L NaCl, 1 g/L of peptone) to reach the indicated cell density.

The indicator strains, including *Escherichia coli* CGMCC 1.2836, *Shewanella putrefaciens* CGMCC 1.3667, *Staphylococcus aureus* CGMCC 1.2155, *Pseudomonas aeruginosa* CGMCC 1.860 and *Salmonella enterica* CGMCC 1.1174, were originally from China General Microbiological Culture Collection Center (CGMCC). The strains were inoculated into the Nutrient Broth medium and propagated for 24 h at 30 °C before the antibacterial activity tests.

2.2. Fish preparation

Live commercial-sized large yellow croaker weighing between 400 and 500 g were purchased from Jinjiang Aquatic Market (Hangzhou, Zhejiang province, China) and delivered to the laboratory within 1 h. The fish were kept alive before being processed and were killed using slurry ice (30 min in ice-cold water) prior to processing. Then, four fragments of white muscle tissue were taken from the lateral-dorsal muscle of each fish and randomly divided into four treatment groups: Treatment 1 (T-1), treatment 2 (T-2) and treatment 3 (T-3) were dipped in sterile saline solutions (0.85% NaCl) containing different concentrations of viable Bcoa (with final concentrations of 1×10^8 CFU/mL, 1×10^7 CFU/mL and 1×10^6 CFU/mL, respectively) at 4 °C for 5 min, and the control group was dipped in a sterile saline solution without Bcoa. Then, all groups were packed into sterile plastic packs and stored at 4 °C for 20 days. Each treatment was sampled on days 0, 4, 8, 12, 16 and 20 with three parallel repeats.

2.3. Physicochemical analyses

The pH values of the filtrates were measured using a digital pH meter (FE20/EL20; Mettler Toledo, Shanghai, China), to avoid the interference of temperature shift, samples were kept for 30 min at room temperature before measurement (Chinese standard GB/T 5009.45-2003) (Ocaño-Higuera et al., 2011). The total volatile basic nitrogen (TVB-N, mg N/100 g muscle) and K values were determined by previously described methods (Li et al., 2012). ATP and its related products were determined by a reverse phase high-performance liquid chromatographic method with C18 column (Su et al., 2014). The K value was calculated as the percent rate of HxR and Hx to the sum of ATP and degradation products as follows (Saito et al., 1959): k % = [(HxR + Hx) / (ATP + ADP + AMP + IMP + HxR + Hx)] × 100. Thiobarbituric acid (TBA) was determined by spectrophotometry, according to the protocol described by Botsoglou et al. (1994).

2.4. Sensory evaluation

The overall freshness of the large yellow croaker samples was measured by a blind sensory analysis modified from a previous study (Liu et al., 2015). The appearance, odor, and texture of the flesh were evaluated by 8 trained adjudicators. The sensory evaluation was based on a 20-point (4 lowest and 20 highest) scale. The results from the 8 adjudicators were averaged to get a mean score, and a sensory score below 8 is defined as not acceptable.

2.5. Bacteriological analyses

Samples (5 g) obtained from each group of fillets were transferred aseptically into stomacher bags, each with 45 mL of sterile NaCl solution (0.85%), and homogenized for 1 min.

Then decimal dilutions were made, and 1 mL of each dilution was mixed with plate count agar (PCA, Qingdao Hope Bio-Technology), cephaloridine fucidin cetrimide agar (CFC, Qingdao Hope Bio-Technology), violet red bile glucose agar (VRBGA, Qingdao Hope Bio-Technology), basal conductance medium (BCM) (Dalgaard et al., 1996) and iron agar (IA, Qingdao Hope Bio-Technology) for the total viable count (TVC), *Pseudomonads*, *Enterobacteriaceae*, *Photobacteria* and H_2S-producing bacteria count, respectively. After setting, a 20 mL overlay of molten media was added and plates were incubated at 30 °C for 48 h for PCA and CFC plates; 30 °C for 24 h for VRBGA plates; 15 °C for 5 days for BCM plates and 20 °C for 3 days for IA plates. Three replicates of at least three appropriate dilutions on the sampling day were enumerated (Zhu et al., 2016).

2.6. Total DNA extraction

The total bacterial DNA of large yellow croaker fillets was extracted using GenElute Bacterial Genomic DNA Kit (Sigma, St. Louis, MO, USA) and analyzed according to the method of Hu et al. (2008).

2.7. DGGE analysis

The variable V3 region of the 16S rRNA gene was amplified from the bacterial DNA extracted from a fish matrix or cell pellet, using universal primers F1338 (5′-ACTCCTACGGGAGGCAGCAG-3′) with a 40-base GC clamp at the 5′ end and B1338 (5′-GTATTACCGCGGCTGCTGG-3′) (Cocolin et al., 2001). The results were qualified using 1.5% agarose gel electrophoresis and photographed under UV light in a GelDoc XR system (Bio-Rad, Hercules, CA, USA).

The PCR products were separated by DGGE analysis using the Bio-Rad DCode system (Bio-Rad, Hercules, CA, USA). The mixture of PCR products (16 μL) and 5 × loading buffer (4 μL) was applied to an 8% polyacrylamide gel with a linear gradient of urea/formamide from 35 to 55%. Electrophoresis was run in 0.5 × TAE buffer at 60 °C for 10 min at 200 V, followed by a further 12 h at 85 V. The gels were stained with $AgNO_3$ (Edenborn and Sexstone, 2007).

Prominent bands in the DGGE gels were excised with a sterile