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ORIGINAL ARTICLE

Spoilage potential characterization of *Shewanella* and *Pseudomonas* isolated from spoiled large yellow croaker (*Pseudosciaena crocea*)

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Significance and Impact of the Study: Members of the bacterial genera *Shewanella* and *Pseudomonas* are widely known to be responsible for the specific spoilers in iced fish. Ten strains isolated from spoiled large yellow croaker (*Pseudosciaena crocea*) were identified as *Shewanella baltica* and *Pseudomonas* spp. *S. baltica* was demonstrated as the predominant spoiler in the refrigerated *P. crocea* due to its high metabolic activities. This work has generated baseline information for a better understanding of the role of various spoilage bacteria in chilled marine fish and for the control of contamination and growth of main spoilage bacteria to extend the shelf life of marine fish.

Keywords
16S rRNA, biogenic amine, enzyme, gyrB gene, *Pseudomonas*, *Shewanella baltica*.

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Abstract

Ten strains were isolated from a spoiled large yellow croaker (*Pseudosciaena crocea*). All of them were able to grow aerobically from 4 to 30°C, and reduce trimethylamine-*N*-oxide to trimethylamine (TMA) and produce H_2S except SB01, PF05 and PF07. Biochemical characterization and phylogenetic analysis of 16S rRNA gene showed that eight H_2S-producing isolates were closely related to *Shewanella baltica*, and two isolates PF05 and PF07 were identified as *Pseudomonas fluorescens* and *Pseudomonas fragi* respectively. However, of the eight *Shewanella*, seven isolates cluster with *S. baltica* and one with *Shewanella glacialipiscicola* based on the analysis of the *gyrB* gene. *Shewanella baltica* also had the ability to produce biogenic amines, while two *Pseudomonas* had high activities of proteinase and lipase, and failed to produce TMA and biogenic amines. In spoilage potential evaluation, the TVB-N value of *S. baltica* was significantly higher than that of *Pseudomonas* in sterile fish juice, although its growth was slower than *Pseudomonas*. Therefore, this work demonstrated that *S. baltica* was able to cause rapid and strong spoilage and was therefore identified as a specific spoilage organism in refrigerated *P. crocea*.

Introduction

Spoilage of fish is mainly caused by microbial activity, chemical oxidations and autolysis (Gram and Huss 1996). In addition, microbial activity is by far the major mechanism affecting the quality of fresh fish. Spoilage-related bacteria produce off-flavour and discolouration, resulting in large economic losses in fisheries and aquaculture. All microbiota present on the spoiled fish are not involved in spoilage (Dalgaard 1995; Gram *et al.* 2002). It is therefore important to distinguish between spoilage bacteria and microflora as only a small fraction of the bacteria actually contribute to spoilage, and are commonly referred to as specific spoilage organism (SSO). Most bacterial species related to food deterioration are able to form metabolites such as ammonia, biogenic amines, organic acid, sulphides, alcohols and ketones with unpleasant and unacceptable off-flavours (Dalgaard *et al.* 2006). Fish from cold and temperate waters contain mainly psychrotrophic Gram-negative, strict aerobic or facultative anaerobic micro-organisms of the genera *Shewanella*, *Pseudomonas*, *Aeromonas*, *Flavobacterium* or the family *Vibrionaceae* (Gram *et al.* 2002).

Shewanella species are motile Gram-negative, oxidative, H_2S-producing rod-shaped bacteria. To date, numerous *Shewanella* species have been recovered in fish, marine

animal and sea water, including *Shewanella putrefacies*, *Shewanella baltica*, *Shewanella algae*, *Shewanella hafnienis*, *Shewanella morhuae*, *Shewanella marinintestina*. Among SSO, *S. putrefacies* were described as the spoiling agent for temperature water fish species stored in ice, whereas *S. baltica* was newly demonstrated as the main spoilage potential organism during the iced storage of marine fish due to the psychrotrophic nature and the reduction ability of trimethylamine-*N*-oxide (TMAO) to trimethylamine (TMA) causing the "fishy" off-odour. Recently, *S. baltica* was also identified as SSO involving in the spoilage of shrimp (*Litopenaeus vannamei*) stored at 4°C (Zhu *et al.* 2015). Some members of the genus *Pseudomonas* are the predominant spoilers of proteinaceous raw foods, especially aerobically chill stored beef (Doulgeraki and Nychas 2013) and seafood (Gram and Huss 1996; Macé *et al.* 2013) due to the production of high extracellular enzymes (Arslan *et al.* 2011). *Pseudomonas* spp., such as *P. fluorescens*, were determined as the predominant spoilage micro-organisms of gutted sea bream (Parlapani *et al.* 2015).

Large yellow croaker (*Pseudosciaena crocea*) is a commercially important marine farming fish species in China. However, few studies have been investigated the incidence of spoilage micro-organisms in refrigerated *P. crocea*, and related studies that assess enzymatic metabolism of spoilage bacteria in the marine fish are much less abundant. Therefore, the aims of this study were to characterize spoilage-related bacteria in raw refrigerated *P. crocea* by phenotypic and molecular methods, and to determine their spoilage potential and SSO.

Results and discussion

Biochemical characterization

Eight black colonies designated as SB01, SB03, SB06, SB07, SB08, SB09, SB11, SB12, and two white colonies as PF05 and PF07 were recovered from iron agar (IA). As shown in Table 1, all the isolates were Gram-negative rods, and eight black isolates could grow at 4, 25, 30°C, but showed no growth at 37°C, and the other two white colonies could grow at 4 to 37°C. The morphologically black isolates could grow with the 1 or 3% NaCl, and did not show activities without NaCl or high content (6%). However, two white isolates could not grow with 6% NaCl. Only one strain, SB01, was not able to produce H_2S, and seven strains displayed a clear ability to produce H_2S with a deep black colouration in IA tubes and could reduce TMAO (Table 1). Concerning enzymatic activities, the 10 strains were positive for oxidase and lysine decarboxylase activity. Except strain SB08, nine isloates could decarboxylate ornithine. Eight black strains were unable to use glucose, sorbitol, citrate and arabinose, and PF05 and PF07 could use sucrose and sorbitol. Eight black colonies and two white colonies were presumptively identified as *Shewanella* spp. and *Pseudomonas* spp., respectively, in the API 20NE database; however, they could not be assigned to a known species.

Phylogentic analysis of 16S rRNA and *gyrB* genes

The 1441 and 1141 kb nucleotide sequences of 16S rRNA genes and *gyrB* genes were used for phylogenetic analyses of *Shewanella* and *Pseudomonas* isolates respectively (Fig. 1). The six *Shewanella* strains, including SB09, SB07, SB06, SB08, SB03 and SB11, were clustered together with *S. baltica* OS155 strain. SB01 and SB12 isolates were grouped with *S. baltica* OS185 strain and *S. baltica* NCTC 10735 type strain, respectively, forming a new branch (Fig. 1a). Alignments of 16S rRNA sequence in BLAST led to 99% identity between *Shewanella* isolates and *S. baltica* type strain. *Pseudomonas* isolates PF05 and PF07 were closely related to *Pseudomonas fragi* NBRC 12049 type strain and *P. fluorescens* NBRC 15835 type strain with 99% bootstrap value respectively (Fig. 1a). In the *gyrB* phylogenetic analysis, seven *Shewanella* isolates except SB01 were clustered with *S. baltica* type strain. However, the strain SB01 was grouped with *Shewanella glacialipiscicola* NBRC 102030 type strain (Fig. 1b). All *Shewanella* isolates except SB01 shared the high sequence similarity (98–99%) with the type strains of *S. baltica*. *Pseudomonas* isolates PF05 and PF07 were clustered with the species *P. fragi* and *P. fluorescens* respectively (Fig. 1b).

Genotypic characterization of the strains isolated from *P. crocea* by both 16S rRNA and *gyrB* gene sequencing revealed that seven *Shewanella* isolates were closely related to the halotolerant, psychrotolerant and nonhalophilic species of *S. baltica*. In contrast to the 16S rRNA results, SB01 formed in the *gyrB* tree a long independent branch that clustered with *S. glacialipiscicola*. It could be associated with no enough precise phylogenetic position for some *Shewanella* species based on 16S rRNA gene analysis (Satomi *et al.* 2003; Dehaut *et al.* 2014). Two *Pseudomonas* were identified as *P. fragi* and *P. fluorescens* according to similar observations of 16S rRNA and gyrB sequencing.

Protease and lipase activities

In spoilage bacteria, a number of extracellular enzymes, including protease and lipases, have contributed to food spoilage. *Shewanella baltica*, *P. fluorescens* and *P. fragi* exhibited high activities of protease and lipase after 24 h incubation (Fig. 2a,b). SB09 and SB12 isolates had the highest protease activity with the 9·7 and 10·2 IU/OD_{600}

Table 1 Phenotypic characterization of the 10 strains isolated from spoiled large yellow croaker obtained with plate cultures and API 20 NE system

Characteristics	SB01	SB03	SB06	SB07	SB08	SB09	SB11	SB12	PF07	PF05
Aerobic growth										
4°C	+	+	+	+	+	+	+	+	+	+
25°C	+	+	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+	+
37°C	−	−	−	−	−	−	−	−	+	+
Tolerance of NaCl										
0%	−	−	−	−	−	−	−	−	+	+
1%	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	+	+
6%	−	−	−	−	−	−	−	−	−	−
Production										
H_2S production	−	+	+	+	+	+	+	+	ND	ND
Trimethylamine-*N*-oxide reduction	ND	+	+	+	+	+	+	+	ND	ND
Hydrolysis										
Urea	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	−
O-nitrophenol-β-D-galactoside	−	−	−	−	−	−	+	+	−	−
Enzyme activity										
Oxidase	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	−	−	−	−	−	−	−	−	+	+
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	−	+	+	+	+	+
Tryptophanase	−	−	−	−	−	−	−	−	−	−
Use of carbon sources										
D-glucose	−	−	−	−	−	−	−	−	−	−
D-sucrose	−	−	−	−	−	−	+	+	+	+
D-mannitol	−	−	−	−	−	+	−	−	−	−
D-sorbitol	−	−	−	−	−	−	−	−	+	+
Citrate	−	−	−	−	−	−	−	−	−	−
L-arabinose	−	−	−	−	−	−	−	−	−	−

+, means growth and test positive; −, means no growth or test negative; ND, means the not detected.

($P < 0{\cdot}05$), whereas, SB03 and SB08 showed the weakest activities with only 2·9 and 3·3 IU/OD_{600} among eight *S. baltica* strains. Compared to *S. baltica*, two *Pseudomonas* PF05 and PF07 showed higher proteolytic activity with 11·5 and 10·5 IU/OD_{600} respectively (Fig. 2a). All the *Shewanella* and *Pseudomonas* strains were positive for lipase activity at 25°C (Fig. 2b). SB11 and SB12 isolates have relatively stronger ability of producing lipase than other strains ($P < 0{\cdot}05$). The lipase activities of PF07 and PF05 were about 0·09 and 0·08 IU/OD_{600}, which were significantly higher ($P < 0{\cdot}05$) than all of eight *S. baltica* isolates. Furthermore, the proteolytic and lipolytic activities of *P. fluorescens* PF07 were relatively higher than that of *P. fragi* PF05. It was in accordance with the finding of Hantsis-Zacharov and Halpern (2007), who depicted a high number of lipolytic isolates belonging to the genus *Pseudomonas*. High protease and lipase activities were detected in the supernatant of species belonging to the genus *Pseudomonas*, especially *P. fluorescens* (Baur *et al.* 2015).

Production of TMA and biogenic amines

Seven *S. baltica* isolates had capacities of producing TMA from TMAO except SB01 after 12 h culture (Fig. 2c). In addition, SB12 and SB07 were shown to produce the highest level of TMA ($P < 0{\cdot}05$), followed by SB06, SB11, while SB09, SB03 and SB08 exhibited the lowest level of TMA ($P < 0{\cdot}05$). However, *P. fluorescens* PF07 and *P. fragi* PF05 isolates failed to produce TMA. As shown in Fig. 2(d), all the selected *S. baltica* strains formed putrescine, cadaverine and to a lesser extent, histamine, while *P. fluorescens* and *P. fragi* just produced low level of spermine (results not shown). Among the eight *S. baltica* isolates, SB06 and SB11 showed the highest producers of forming total biogenic amines and putrescine ($P < 0{\cdot}05$), followed by SB07, SB12, SB09, SB01 and SB03. And SB08 revealed the moderate and weakest producers of biogenic amines.

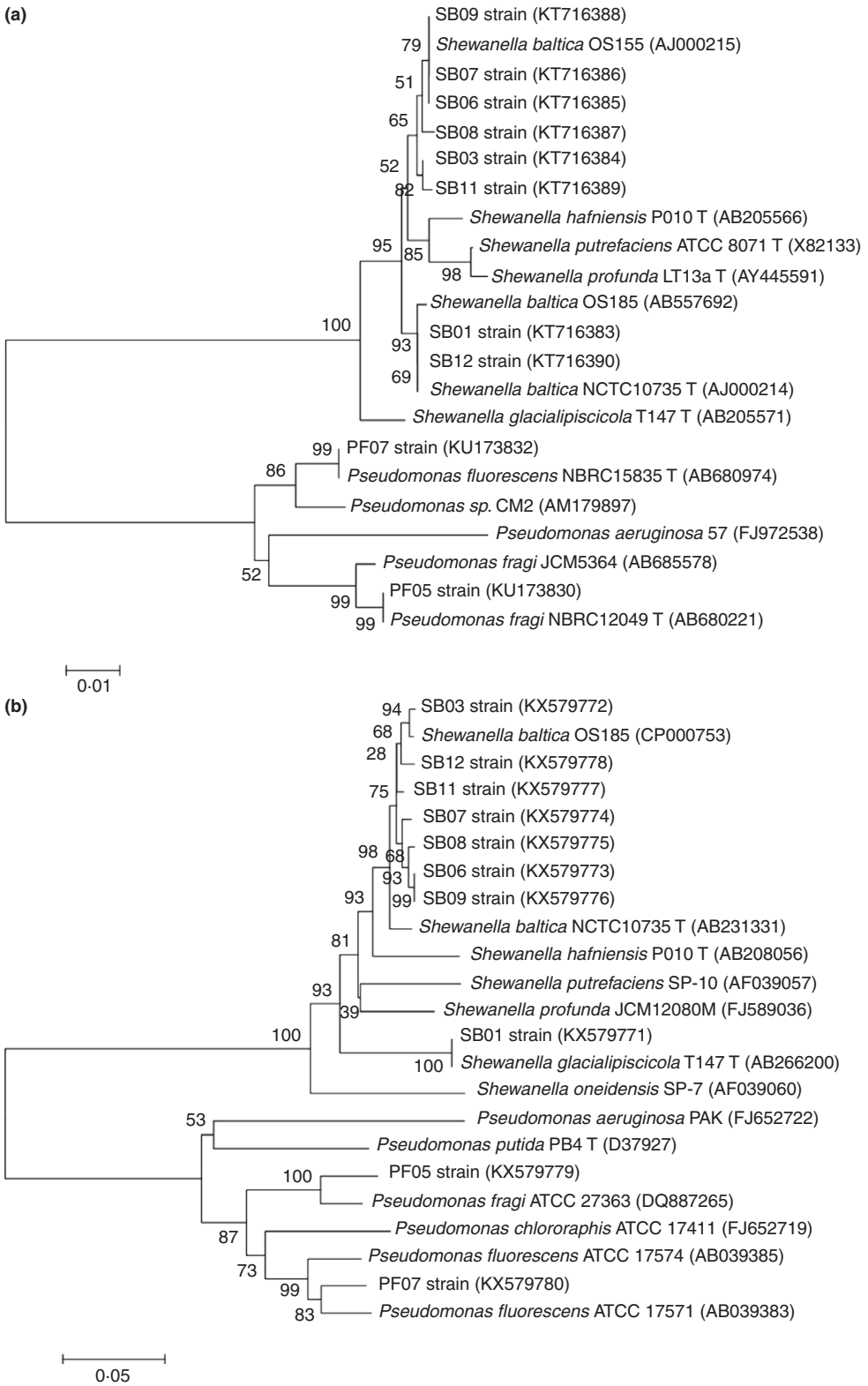

Figure 1 Unrooted neighbour joining phylogenetic tree derived from the 16S rRNA gene (a) and *gyrB* gene (b) sequences showing the relationship between the isolates and members of the genus *Shewanella* and *Pseudomonas*. Numbers at the nodes indicate bootstrap values (percentage of 1000 replicates). Bar indicated sequence divergences.

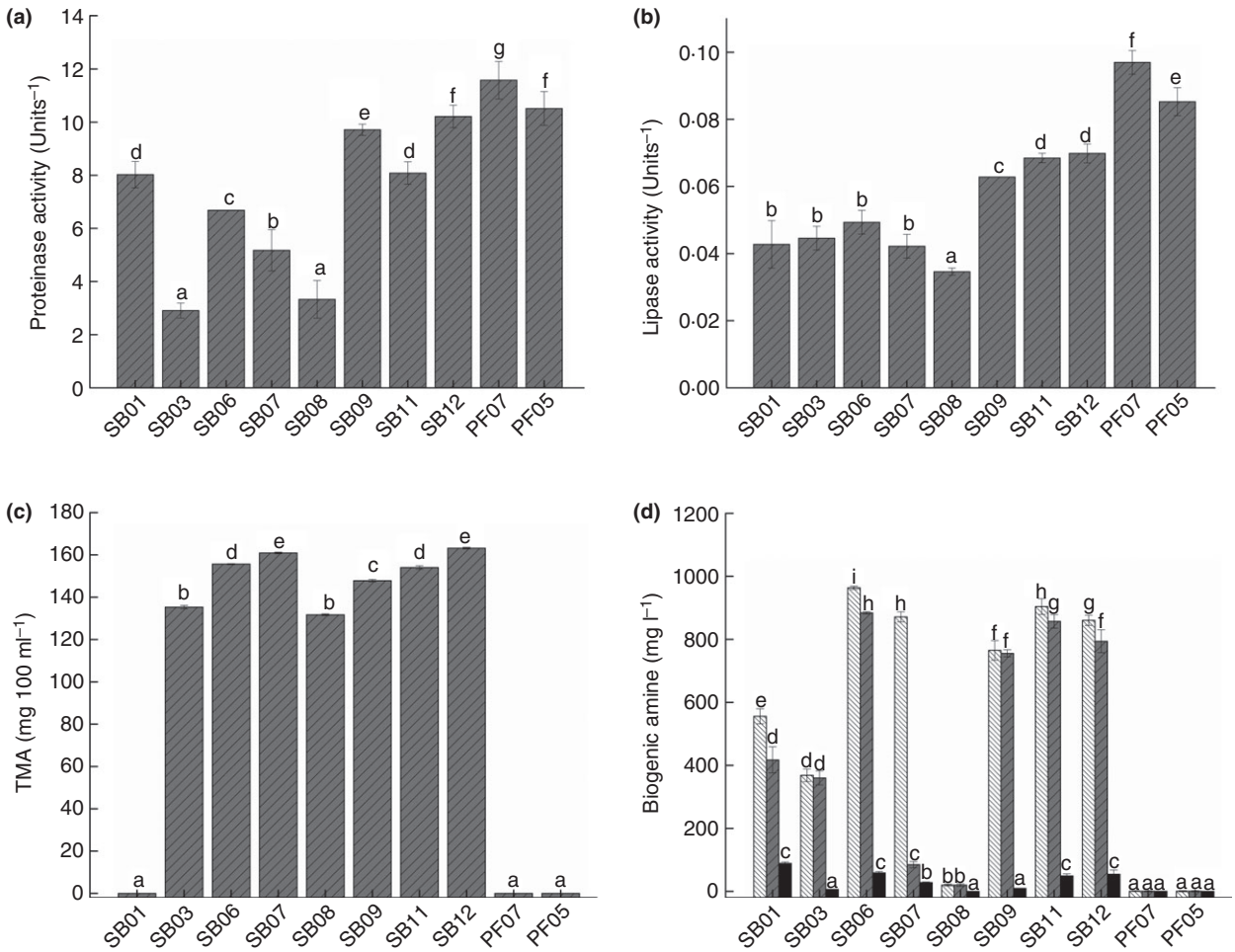

Figure 2 Protease activtiy (a), lipase activity (b) and the production of trimethylamine (c), biogenic amines (d) (▨ total BAs; ▨ putrescine and ■ cadaverine) in *Shewanella baltica* and *Pseudomonas* strains isolated from spoiled large yellow croaker. Data were expressed as mean ± standard deviations ($n = 3$). Different letters indicated significant differences at $P < 0{\cdot}05$ with regard to isolates.

Shewanella strains could produce several sulphur-containing compounds, TMA and biogenic amines, resulting in the production of strong ammonia and putrid odours in fish product (Gram and Huss 1996). In *S. baltica*, TMAO reductase and ornithine decarboxylase catalyse the production of TMA and putrescine respectively (Gram and Dalgaard 2002). In this study, it was indicated that *S. baltica* was mainly involved in the production of TMA, putrescine and cadaverine. However, *P. fluorescens* and *P. fragi* responsible for spoilage were associated with high extracellular enzymatic activities. Thus, *S. baltica* plays an important role in the spoilage of aerobically refrigerated *P. crocea* due to its high metabolic properties.

Spoilage potential in sterile fish juice

Spoilage potential of 10 strains was investigated in the sterile fish juice (*P. crocea*) stored at 4°C (Fig. 3). The control of sterile juice was detected to show no microbial growth and low TVB-N value during the storage. The initial counts in the inoculated samples were 4·9–5·2 log CFU mL^{-1}, and *S. baltica* grew rapidly and reached about 7·5–8·5 log CFU mL^{-1} at 72 h, especially SB11 and SB12. Compared with *S. baltica*, *P. fluorescens* and *P. fragi* exhibited the fastest growth rate of nearly 8·8 log CFU mL^{-1} at 72 h, and grew slowly after further incubating for 120 h. The TVB-N value increased slowly during the initial 72 h, and then rose rapidly during the 120 h ($P < 0{\cdot}05$) incubation period in the samples inoculated with *Shewanella* isolates due to the activity of spoilage bacteria and endogenous enzymes (Ruiz-Capillas and Moral 2005). When stored for 120 h, SB11 and SB12 appeared to show higher TVB-N production of about 169·7 and 157·8 mgN 100 ml^{-1}, respectively, followed by SB06, SB07 and SB09. However, two *Pseudomonas* produced the low content of TVB-N.

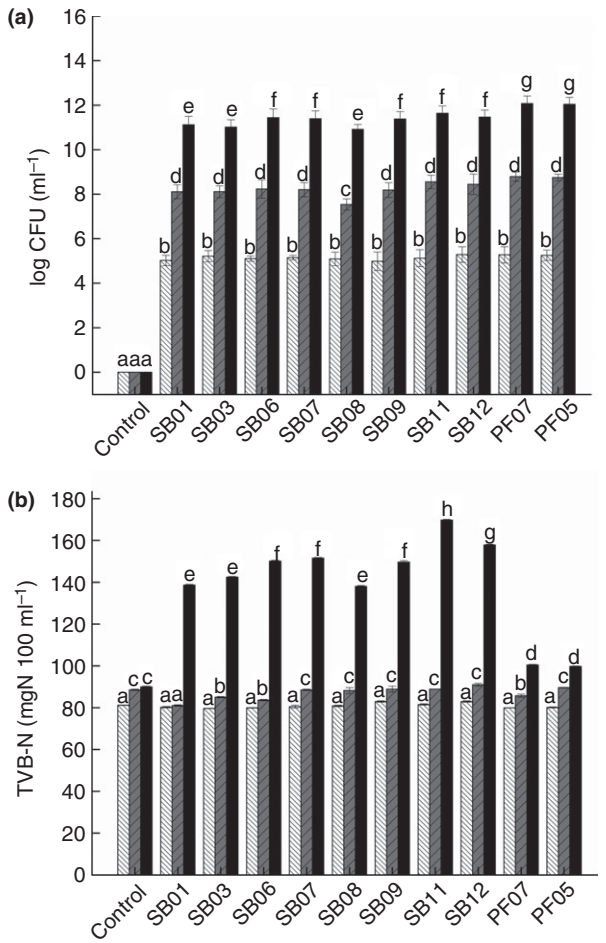

Figure 3 The total bacterial count (a) and TVB-N value (b) of 10 isolates in sterile fish juice of large yellow croaker stored at 4°C (▨) 0 h; (▨) 72 h and (■) 120 h). Data were expressed as mean ± standard deviations (n = 3). Different letters indicated significant differences at $P < 0{\cdot}05$ with regard to storage time and isloates.

In this study, spoilage potential in sterile fish juice significantly differed ($P < 0{\cdot}05$) between *Shewanella* and *Pseudomonas*. *Shewanella baltica* SB11 and SB12 tested were two strong spoilers, identified as SSO in the spoiled *P. crocea*. Compared to *S. baltica*, *P. fluorescens* and *P. fragi* were nondominant microbiota in *P. crocea* according to the change in TVB-N in the sterile juice, although faster growth was observed. The high spoilage potential of *S. baltica* in sterile juice could be associated with the production of many metabolites, such as the fishy compound TMA, putrescine and cadaverine, and enzymatic activity. In addition, there was not a good correlation between bacterial population and TVB-N value in sterile juice inoculated with various isolates. Compared to bacterial growth, bacteria require longer time to produce some metabolites, leading to accumulation of TVB-N over the storage period. Furthermore, the slow increase in TVB-N level could also be explained by the fact that except microbial metabolic pathways, enzymatic reactions originating from the spoilage organisms could also be involved in spoilage compound production in fish products (López-Caballero *et al.* 2007). It was hypothesized that *Pseudomonas* with high proteolytic and lipolytic activities could promote the microbial spoilage. Similarly, Dabadé *et al.* (2015) reported that some nonspecial microbiota also had effect on the spoilage of aquatic product. This study further demonstrated that *Shewanella* are typical spoilers of fresh *P. crocea*, and *Pseudomonas* with high extracellular enzymes may affect spoilage processes.

Materials and methods

Isolates from the spoiled fish

A 10-g fish sample was taken from spoiled *P. crocea* stored at 4°C and homogenized with 90 ml of sterile peptone saline solution for 1 min in a Stomacher 400 Laboratory Blender (Steward Company, London, UK). Then, 10-fold serial dilutions were made and were inoculated on IA (Hope Bio-Technology Company, Qingdao, China). After incubation at 25°C for 48 h, 43 bacteria were picked randomly and then the isolates were further purified on another IA plate at 25°C (Zhu *et al.* 2016). Spoilage potential of those strains were preliminary investigated in the sterile fish juice stored at 4°C (results not shown), and the different spoilage capabilities of eight black colonies and two white colonies were taken and stored at −80°C in broth with 20% glycerol for further characterization.

Phenotypic characterization

The ability of these isolates to aerobically grow at 4°C (72 h), 25°C (24 h), 30°C (24 h) and 37°C (24 h) was tested on LB broth (Hope Bio-Technology Company). Tolerance of the isolates to NaCl was tested by incubating the isolates aerobically at 25°C for 24 h in LB broth supplemented with 0, 1, 3 and 6% NaCl. The ability of the isolates to reduce TMAO was tested on LB broth supplemented with 20 mmol l^{-1} of TMAO (Sigma–Aldrich, Shanghai, China) for 24 h at 25°C. Confirmation of H_2S production was obtained by inoculation of IA tubes incubated at 25°C for 24 h. The commercial identification system API 20 NE (BioMérieux, Marcy I Etolle, France) was used for phenotypic characterization.

Phylogentic analysis

DNA was extracted from single colonies after growing in trypticase soya broth at 25°C for 24 h using the bacterial

DNA isolation kit (Bioer Technology Company, Guangzhou, China). The phenotypically identified *Shewanella* and *Pseudomonas* isolates were genetically identified by sequencing the 16S rRNA and the *gyrB* genes as previously described (Fox *et al.* 1992; Yamamoto and Harayama 1995). The 16S rRNA and the *gyrB* sequences were aligned with sequences of type strains of members of the genus *Shewanella* and *Pseudomonas* that were available at the GenBank. Genetic distances and clustering were obtained using Kimura's two-parameter model, phylogenetic trees were constructed using the neighbour joining method and pairwise similarities of the 16S rRNA and *gyrB* were calculated with MEGA5 software (Tamura *et al.* 2011). The 16S rRNA gene partial sequences of 10 strains were submitted to GenBank (KT716383–KT716390, KU173830, KU173832), and the *gyrB* gene partial sequences generated in this study were deposited in Genbank (KX579771–KX579780).

Protease and lipase assay

After the isolates were incubated in LB broth for 24 h, the cultures (0·4–0·8 OD at 600 nm) were collected by centrifugation at 5000 ***g*** for 15 min and the supernatant was used as a source of crude enzyme. Proteolytic and lipolytic activities were examined with bacterial protease assay kits (catalogue no. 15021, Genmed Scientifics, Plymouth, MN, USA) and bacterial lipase assay kits (catalogue no. 15020, Genmed Scientifics), respectively, according to the manufacturer's instructions. The absorbance was measured at 412 nm compared with the blank. One unit (IU) of protease or lipase activity was defined as the amount of enzyme required to liberate 1 μmol of tryptophan or dimercaptopropanol tributyrate equivalent per min at 37°C.

TMA and biogenic amine assay

For the TMA analyses, *S. baltica* was cultured in LB media containing 10 mmol l^{-1} TMAO at 25°C on a rotary shaker at 180 rev min^{-1}. After incubating for 12 h, trichloroacetic acid was added and the mixture of bacterial culture was centrifuged at 6000 ***g*** for 10 min. The quantity of TMA in the supernatant was determined spectrophotometrically by following the colorimetric formation of the picric acid salt of TMA (Dyer 1945). Biogenic amine analyses in bacterial cultures were performed according to the procedures developed by Kim *et al.* (2009) with a slight modification. Bacterial cultures were taken for quantifying the amounts of biogenic amines by HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) based on the precolumn dansyl chloride derivatization method as described in previous report (Proestos *et al.* 2008).

Spoilage potential evaluation

The preparation of sterile fish muscle juice of *P. crocea* was performed according to the method of Dalgaard (1995). The isolates were inoculated into sterile juice to reach the inoculated level of 4 log CFU mL^{-1}, and sterile water was used as control. All batches of inoculated fish juice and the control were stored at 4°C. After 72 and 120 h, the juice samples inoculated with 10 isolates were subjected to determine the bacterial population and TVB-N. The samples were used for microbiological count using IA as described by Macé *et al.* (2013). TVB-N was measured using steam distillation with FOSS Kjeltec 8400 Automatic Nitrogen Determination apparatus (Foss, Hillerød, Denmark) in triplicates (Li *et al.* 2012).

Statistical analysis

Three replicate trials were done for each sample, and all the experiments were repeated for three times. Analysis of variance (ANOVA) and Duncan's multiple-range test for mean separation ($P < 0{\cdot}05$) were conducted using SPSS software (ver. 18.0) (SPSS Inc., Chicago, IL).

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Conflict of Interest

No conflict of interest declared.

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