



## Chitinase-producing *Salinivibrio* bacteria isolated from salt-fermented shrimp with antimicrobial and safety assessments

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Received: 23 May 2018 / Accepted: 26 July 2018 / Published Online: 30 September 2018  
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**Abstract** Chitinases are glycosyl hydrolases which cleave the  $\beta$ -1,4 linkage of chitin into oligo or monomers of *N*-acetylglucosamine. These bacterial enzymes have been used for a wide range of applications in the food and pharmaceutical industries. In this study, we isolated two potential chitinolytic strains, BAO-01 and BAO-02, from salt-fermented shrimp, which were shown to belong to the genus *Salinivibrio* through genetic characterization using 16S rRNA. These isolates were gram-positive, rod-shaped, and non-spore forming. BAO-01 showed greater growth and chitinase activity than BAO-02 after the incubation at 37 °C for 4 days. Both strains grew on a wide range of carbon and nitrogen sources, pH values, temperatures, and salt levels. However, they showed minor biochemical differences. In addition, their antimicrobial activities against foodborne pathogens and antibiotic susceptibilities were evaluated. These *Salinivibrio* spp. did not show bioamine production, hemolytic activity, and mucin degradation. Therefore, the *in vitro* screening results suggested that these bacteria could be widely used as new candidates for chitin hydrolyzation and seafood fermentation.

**Keywords** Antimicrobial · Chitinase · Food safety · *Salinivibrio* · salt-fermented shrimp

### Introduction

Jeotgal is a traditional Korean salted and fermented seafood that is used both as a food additive and as a food. Jeotgal has been shown to be a potential functional food with therapeutic properties such as has shown to be a potential bioactive functional food by antidiabetics, anticancer, antiobesity and immunomodulatory *in vivo* [1]. It is prepared from various fish, fish eggs, shellfish, fish intestines, oyster, and shrimp, which are mixed with 10–30% (w/w) salt and stored for several months at 15–25 °C [2]. Among the various types, jeotgal prepared from anchovies and small shrimp contains the highest levels of valuable chitoooligosaccharides due to the digestion of scale and shells by chitinase enzyme produced from bacteria during fermentation [3,4]. Therefore, the isolation of bacteria with strong chitinase activity is important for improving the commercial quality of fermented foods and developing novel enzymes with industrial potential.

Chitinase (EC 3.2.11.14) are glycosyl hydrolases which hydrolyze  $\beta$ -1,4 linkage of chitin have been detected in numerous bacteria, fungi, insects, plants, and animals [4]. Among them, bacterial chitinases have been implicated in the utilization of chitin as a carbon source and protection against pathogens. In recent years, chitinases produced by bacteria have been received increased attention due to a wide spectrum of properties and activities [5]. Although many bacterial chitinases have been reported, safety and efficiency issues of bacteria and their chitinase remains poorly characterized in food application [6].

Until now, the use of high salt content in shrimp fermentation is a challenge cause of the selection of salt tolerant bacteria as a starter culture. Screening for novel strains with specific properties and technologies, therefore, is still of great interest to improve the chitinase production in order to meet the increasing demand for traditional food. We recently isolated and identified a novel chitinolytic *Salinivibrio* strain from fermented salted shrimp in Yeosu, Korea. Their morphological, biochemical characteristic and 16S rDNA gene of the isolated strains were evaluated. In

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addition, the potential of these *Salinivibrio* strains for inhibiting foodborne pathogens and the *in vitro* safety were also assessed.

## Material and Methods

### Isolation and identification of chitinase producing strains

Jeotgal samples containing fresh shrimp fermented with low salt (15–20%) for 12 weeks were collected from a local market in Yeosu, serially diluted, and spread on marine agar (Difco, Detroit, MI, USA) supplemented with 0.5% (w/v) colloidal chitin. After a 5-d incubation period at 30 and 37 °C, the colonies producing a distinct clear zone were selected as chitin degrading ability. Isolates were randomly selected based on morphological differences and streak purified on marine agar. All isolates were examined by Gram staining, catalase activity, carbohydrate fermentation, and enzyme activities using the API 50CHL and API ZYM systems (bioMérieux, Durham, NC, USA).

The genomic DNA of isolates was extracted using the genomic DNA preparation Kit (SolGent, Daejeon, Korea), according to the manufacturer's instructions. PCR was performed using the universal primers [7], 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), in a Takara Thermal Cycler Dice (TP-800; Takara, Shiga, Japan). Each 25- $\mu$ L reaction contained 1 U of Taq polymerase (Takara), 0.5  $\mu$ M of each primer, 1  $\mu$ M dNTPs, 2.5  $\mu$ M MgCl<sub>2</sub>, and template DNA. PCR tubes were preheated at 94 °C for 7 min, and then the following amplification program was run: 30 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 30 s. The PCR products (5  $\mu$ L) were run on a 1% agarose gel and visualized with a UV illuminator. The amplified PCR products were purified using the AccuPrep<sup>®</sup> PCR Purification Kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Then, the purified PCR products were sequenced and assembled. The 16S rRNA sequences of two isolated strains (BAO-01 and BAO-02) were deposited in GenBank with accession numbers KX990293 and KX990294, respectively. The 27F and 1492R primers were also used for sequencing. A phylogenetic tree was constructed with MEGA v.7.0 [8] using the neighbor joining (NJ) method and 1000 replicates for the bootstrap analysis.

### Effects of pH, temperature, and NaCl concentration on cell growth

The isolated strains were cultured in marine broth (Difco) containing 0.1% (w/v) colloidal chitin (MB-C) for 6 d. To investigate the effect of temperatures and pH, the incubation was carried out at 20, 30, 37, and 50 °C in medium adjusted to pH 3, 4, 5, 6, 7, 8, 9, and 10 (with 0.1 M HCl and/or NaOH before autoclaving). To assess salt tolerance, MB-C medium was supplemented with 10–20% w/v NaCl. Growth was assessed by measuring the absorbance at 600 nm with a spectrophotometer.

### Effect of carbon and nitrogen source on chitinase production

Chitinase production was assessed after growth in 100 mL of MB-C medium containing 1% (w/v) of various carbon sources (galactose, mannitol, starch, fructose, sucrose, glucose, and dextrose) and nitrogen sources (urea, yeast extract, peptone, malt extract, gelatin, ammonium sulfate, and casein). Then, the medium was inoculated with the isolated strains (10% v/w; OD=0.5) and incubated at 37 °C for 48 h. After fermentation, cell growth and chitinase activity were assessed.

### Preparation of colloidal chitin

Colloidal chitin was prepared from shrimp shell chitin (Sigma, St. Louis, MO, USA). Briefly, 10 g of powdered chitin was suspended in 100 mL (v/w) of 10N HCl, mixed, and incubated at 4 °C overnight. Then, 1.9 L of 100% ethanol was added and incubated at –20 °C overnight to allow precipitation. The precipitates were collected by centrifuge at 8000 $\times$  g for 15 min at 4 °C. The colloidal chitin was washed with sterile distilled water until a neutral pH was achieved, and then freeze-dried to a powder and stored at 4 °C.

### Enzyme assay

A chitinase assay was carried out using colloidal chitin as a substrate according to the method of [9]. The chitinase enzyme sample was incubated with colloidal chitin (final concentration of 2 mg/mL) for 1 h at 40 °C. The reaction was stopped by incubating in a boiling water for 10 min, followed by centrifugation at 10000 $\times$  g for 5 min. The reducing sugar content was estimated using the dinitrosalicylic acid (DNS) method [10] at 540 nm using N-acetyl- $\beta$ -D-glucosamine (GlcNAc) as the standard. One unit of enzyme activity was defined as the amount of enzyme that released 1  $\mu$ mol of GlcNAc per hour.

### Determination of antibiotic susceptibility

The minimum inhibitory concentrations of various antibiotics were determined by the broth microdilution method in 96-well plates as described previously [11]. Briefly, isolates were grown in marine broth at 37 °C overnight to a final density of 10<sup>7</sup> CFU/mL. All tested antibiotics were serially diluted 2-fold (256 to 0.5 mg/L) using marine broth in the plate. Then, the bacteria suspension was filled up to 200  $\mu$ L. The positive controls contained no antibiotic. After 24 h incubation at 37 °C, the lowest concentration of an antimicrobial substance that visibly inhibited growth was recorded.

### Antimicrobial activity

The antimicrobial activity of the new isolates was determined by the disk diffusion method on nutrient agar [12]. The foodborne microbial pathogens used in this study were obtained from microbial culture collection centers in the Republic of Korea, such as the Korean Collection for Type Cultures, Korean Agricultural Culture Collection (KACC), and Korean Culture Collection of Microorganisms. Standard inoculum (10<sup>7</sup> CFU/mL) of each pathogen

was spread onto the surface of a nutrient agar plate. Cell-free supernatant (100  $\mu$ L) from the isolates was loaded on 8-mm paper discs, dried, and then placed on the inoculated nutrient agar plate. The plates were incubated at 37 °C for 24 h, and then the inhibitory zones were measured. The uncultured media was used as a control.

### Safety assessment

To test the safety of these newly isolated strains, biogenic amine production, mucin degradation, and hemolytic activity were examined according to the methods of [13], Zhou, Gopal and Gill [14] and Semedo, Santos, Martins, Lopes, Marques, Tenreiro and Crespo [15] respectively. To determine bioamine production, isolate colonies were streaked on decarboxylase medium prepared with or without amino acids (0.5% w/v). Mucin degradation was examined using agarose medium containing 0.3% mucin with or without glucose (g/L), and hemolytic activity was tested using blood base agar medium containing 5% defibrinated sheep blood (Thermo Fisher Scientific, Waltham, MA, USA). All plates were incubated for 24 h at 37 °C.

### Statistical analysis

The SPSS software ver. 22 (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analysis including One-way analysis of variance (ANOVA) and Duncan Post-hoc test ( $p < 0.05$ ). Results are presented as mean  $\pm$  SD (standard deviation) with at least three times replications.

## Results and Discussion

### Identification by biochemical and genetics analyses

A total of 50 morphologically different bacterial colonies were isolated from five samples of jeotgal. Two of these colonies produced a clear zone on marine agar supplemented with colloidal chitin and were selected for additional screening. These candidate isolates, named BAO-01 and BAO-02, were characterized morphologically, biochemically, and genetically. Both strains is a rod-shaped organism thought out the shape suggested that the BAO-01 cell were longer than those of BAO-02. Both strains were gram-negative and did not produce spores.

The biochemical tests were primarily analyzed to investigate the identity of the isolates, as well as the effects of pH, temperature, and NaCl concentration on growth also was estimated (Table 1). These isolates were catalase positive. Although both strains grew in the presence of high concentrations of salt, BAO-02 also grew well in the absence of NaCl. Growth was observed at 10-50 °C, with optimal growth at 37 °C. The pH range for growth was 4.0-8.0, but BAO-01 also grew at basic conditions ( $>8.0$ ), with optimal growth at pH 7.5. Although both strains showed the utilizing abilities in a wide range of carbon sources; strain BAO-01 was able to utilize xylose, mannose, cellobiose, and saccharose,

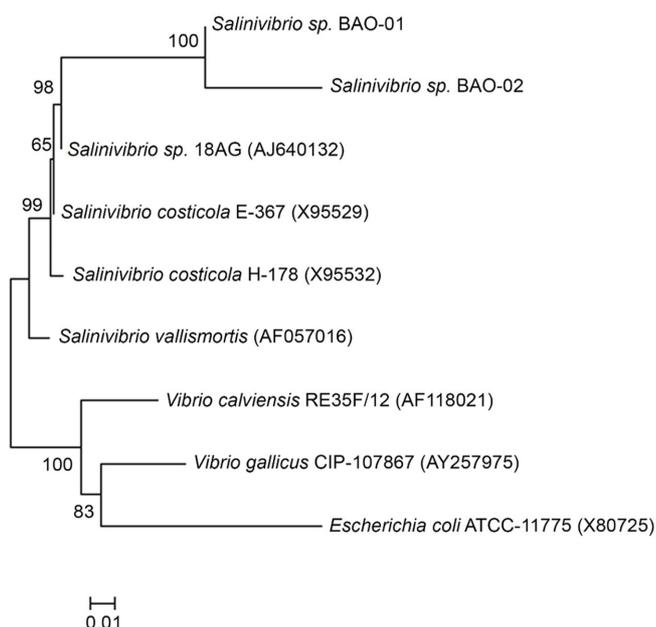
**Table 1** Physiological and biochemical characteristics of the isolated strains from salted and fermented shrimp based on the API 50 CH system and additional tests after 24 h at 37 °C

	<i>Salinivibrio</i> sp. BAO-01	<i>Salinivibrio</i> sp. BAO-02
Catalase	+	+
NaCl for growth (% v/w)		
Range	5-20	0-20
Optimum	5	5
Temperature for growth (°C)		
Range	10-50	10-50
Optimum	37	37
pH for growth		
Range	4.0-9.5	4.0-8.0
Optimum	7.5	7.5
Carbohydrates		
D-Ribose	+	+
D-Xylose	+	-
D-Galactose	+	-
D-Glucose	+	+
D-Fructose	-	+
D-Mannose	+	-
D-Mannitol	+	-
N-Acetyl-glucosamine	+	+
D-Cellobiose	+	-
D-Maltose	-	+
D-Lactose	-	+
D-Saccharose	+	-
Starch	+	+
Glycogene	+	+
Gluconate	+	-
2-keto-Gluconate	-	+

+, positive; -, negative

which could be not utilized by BAO-02 (Table 1).

The 16S rRNA gene sequence of the chitinolytic isolates was analyzed for molecular identification. The nearly complete 16S rRNA sequences of BAO-01 (1460 bases; GenBank accession no. KX990293) and BAO-02 (1473 bases; GenBank accession no. KX990294) were determined. The BAO-01 strain closely resembled *Salinivibrio costicola* E-367 (X95529) and *Salinivibrio vallismortis* (AF057016), with 92.20 and 91.78% sequence similarity, respectively (Fig. 1). BAO-02 showed 88.15 and 87.62% similarity with *S. costicola* E-367 (X95529) and *S. vallismortis* (AF057016), respectively. The *Salinivibrio* clade was also supported by high bootstrap value (99%). Based on pairwise 16S rRNA gene comparisons, the chitinolytic strains BAO-01 and BAO-02 represent the novel genus, with the proposed name *Salinivibrio*. Species belonging to the genus *Salinivibrio* have been isolated from a variety of natural sources, including lakes, marine environments, and foods [16-18]. The gram-negative bacteria in the genus *Salinivibrio* have been shown to secrete a number of degradative enzymes, including cellulase, lipase, protease, chitinase [19-22].



**Fig. 1** A phylogenetic tree generated by the neighbor joining method showing the relationships between the isolated *Salinivibrio* strains and related taxa based on 16S rRNA gene sequences. 1000 replications were used for the bootstrap analysis. Bar, 0.01 substitutions per nucleotide position

However, in the present study, we report the isolation and identification of two novel *Salinivibrio* strains with chitinase activity isolated from fermented and salted shrimp food in Korea.

### Chitinase production from isolated strains

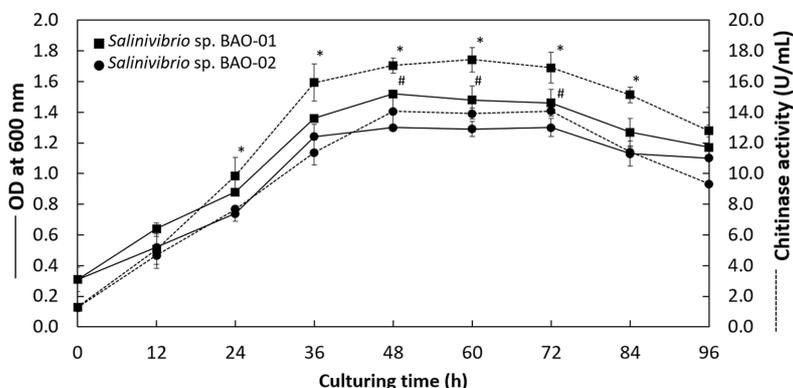
Studies were undertaken to evaluate the chitinase production of these strains during growth. The isolates were grown in marine broth containing 0.1% colloidal chitin at 37 °C for 4 d. The BAO-01 strain grew exponentially better than the BAO-02 strain after 12 h, when it reached maximum growth, and remained steady ( $OD_{600}=1.36$ ) until 48 h (Fig. 2). In a time course study, BAO-01

showed maximum chitinase production (19.02 U/mL) at 48 h of incubation, after which enzyme concentration gradually decreased (at 72 h), as shown in Fig. 2. BAO-02 produced high levels of chitinase (15.56 U/mL) as the cells grew ( $OD_{600}=0.91$ ) at 36 h, but then showed a steady decline beginning at 72 h of cultivation. In contrast, many other bacteria reached maximal chitinase activity and protease secretion during stationary phase growth, when the cell density started to decline [23,24].

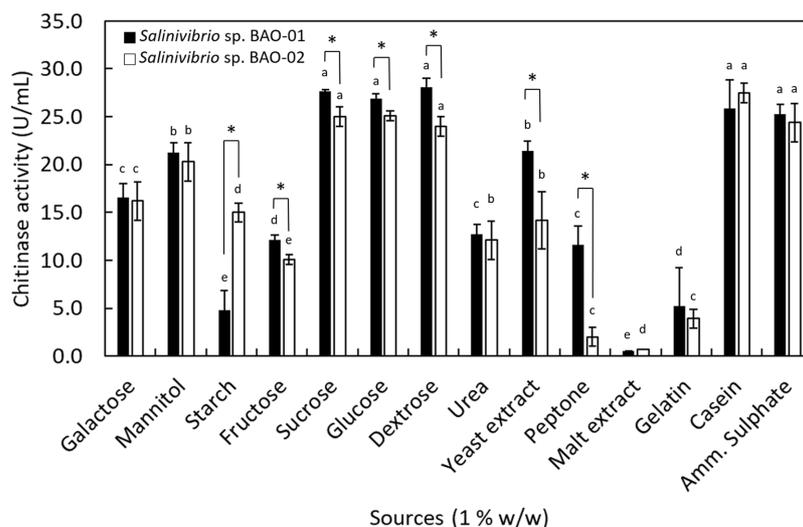
### Carbon and nitrogen sources on chitinase production

The effects of medium supplemented with fourteen carbon and nitrogen sources (1% w/w) on chitinase production by *Salinivibrio* BAO-01 and BAO-02 are shown in Fig. 3. The mechanism of enzyme production on carbon and nitrogen is still not clear, the correlation can vary depending on bacteria species. For the carbon sources, the *Salinivibrio* strains showed maximum chitinase activity during growth on sucrose, glucose, and dextrose. Kim, Ki, Lim, Vijayakumar, Park, Choi, Kim, Im and Park [25] observed the same result with glucose, sucrose, which had effective to enhance the production by *Acinetobacter parvus* HANDI 309. With all tested carbon sources tested, BAO-01 produced significantly ( $p < 0.05$ ) higher chitinase than BAO-02, except in galactose, mannitol, and starch.

Ammonium sulfate, an inorganic nitrogen source, supported significantly higher ( $p < 0.05$ ) chitinase yields in both strains. Another study showed that high concentrations (4.2 g/L) of chitin and ammonium sulfate enhanced chitinase production by *Trichoderma harzianum* [26]. Among the organic nitrogen sources tested, casein, along with a reasonable quantity of yeast extract, supported maximum chitinase production. The same findings were also reported with *Amorphoscelis punctata* HS6 [27]. In contrast, cultivation in peptone, malt extract, and gelatin drastically decreased chitinase activity but these nitrogen sources enhanced enzyme production in the case of *A. punctata* HS6 [27]. Similar observations have also been described by Singh, Mehta and Chhatpar [28] in *Paenibacillus* sp. D1.



**Fig. 2** Time course of chitinase production by *Salinivibrio* sp. BAO-01 and BAO-02 in MA-C medium. \* $p < 0.05$  statistical significance between two strains in OD value and # $p < 0.05$  statistical significance between two strains in chitinase activity



**Fig. 3** Effect of carbon and nitrogen source on the chitinase activity of *Salinivibrio* sp. BAO-01 and BAO-02. Values in the same column and in same carbon or nitrogen sources with different superscripted alphabet are significantly different at  $p < 0.05$ . \* $p < 0.05$  statistical significance between two strains

**Safety use of isolated strains**

The antimicrobial activities of the isolated *Salinivibrio* strains against various foodborne pathogens were evaluated (Table 2). *Salinivibrio* BAO-01 and BAO-02 showed the highest inhibitory activities against *Escherichia coli* and *Yersinia enterocolitica*. The tests showed that these *Salinivibrio* strains have broad spectrum antimicrobial activities against both gram-positive and gram-negative species. The antimicrobial susceptibility testing showed that both *Salinivibrio* isolates strains were inhibited by penicillin (4 µg/mL), ampicillin (1 µg/mL), and streptomycin (4-8 µg/mL; Table 2). BAO-01 and BAO-02 showed resistance to vancomycin and kanamycin. Amoozegar, Schumann, Hajighasemi, Fatemi and Karbalaie-Heidari [29] showed that *Salinivibrio proteolyticus* was resistant to kanamycin (30 mg), penicillin G (10 U), and streptomycin (10 mg). In addition, a *Salinivibrio siamensis* strain isolated from fermented fish was inhibited by 10 mg of ampicillin [30]. To address the safety of these newly isolated bacterial strains before application in functional foods as a starter culture, food preservative, and probiotic, these *Salinivibrio* spp. were tested to assess various safety aspects, such as hemolytic activity, bioamine production, and mucin degradation activity. *B. cereus* KACC 11240 was used as positive control pathogen (Supplementary material 1). All safety aspects were negative results in Table 2. However further whole genome analyses of these newly isolated strains are recommended.

Two strains isolated from a fermented and salted shrimp food were selected for their chitinase-producing ability. These isolated were identified by 16S rRNA gene sequencing as *Salinivibrio* sp. BAO-01 and BAO-02. Their morphology, biochemical characteristics, and optimum growth conditions were investigated. The search for new food-safe chitinase-producing *Salinivibrio* with a wider

spectrum of antimicrobial activities for use as starter inoculum may improve food quality and reduce microbial contamination. Further explorations on developing large-scale chitinase production and effect of *Salinivibrio* in fermented food need to confirm.

**Acknowledgments** This work (Grants No. C0499996) was supported by Business for Cooperative R&D between Industry, Academy, and Research Institute funded Korea Small and Medium Business Administration in 2017.

**Table 2** Antimicrobial activity of cell free supernatant broth of isolates against common pathogens, antibiotic resistances, and safety assessment

	<i>Salinivibrio</i> sp. BAO-01	<i>Salinivibrio</i> sp. BAO-02
<i>B. cereus</i>	++	++
<i>E. coli</i> K99	+++	+++
<i>L. monocytogenes</i>	+	+
<i>S. gallinarum</i>	+	++
<i>S. aureus</i>	++	++
<i>S. choleraesuis</i>	++	+
<i>S. typhi</i>	+	+
<i>S. boydii</i>	++	+
<i>Y. enterocolitica</i>	+++	++
Penicillin	4	4
Ampicillin	1	1
(MIC) <sup>b</sup> Vancomycin	64	64
(µg/mL) Streptomycin	4	8
Kanamycin	16	8
Tetracycline	64	64
Bioamine production	ND	ND
Hemolytic activity	ND	ND
Mucin degradation	ND	ND

+: 4 mm; ++: 8 mm; +++: zone > 8 mm  
ND: non-detected

**Conflict of interest statement** We declare that we have no conflicts of interest.

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