

TRABAJOS ORIGINALES

Presentado: 21/07/2020
Aceptado: 03/01/2021
Publicado online: 25/02/2021
Editor: Leonardo Romero

Autores

Susana Sirvas*
sirvascornejo@gmail.com
<https://orcid.org/0000-0002-8095-7499>

Vanesa Buleje
vbuleje@itp.gob.pe
<https://orcid.org/0000-0002-8030-9643>

Licia Salvatierra
lsalvatierra@itp.gob.pe
<https://orcid.org/0000-0001-6865-2142>

Michael L. Jaramillo
maycol776@gmail.com
<https://orcid.org/0000-0001-5556-8793>

Correspondencia

*Corresponding author

Instituto Tecnológico de la Producción (ITP), Dirección de Investigación, Desarrollo, Innovación y Transferencia Tecnológica; Laboratorio de Biotecnología (LB); Lima, Perú.

Citación

Sirvas S, Buleje V, Salvatierra L, Jaramillo ML. 2021. Screening and identification of proteolytic, amylolytic, lipolytic, and chitinolytic bacteria from shrimp waste. *Revista peruana de biología* 28(1): e18353 (Febrero 2021). doi: <http://dx.doi.org/10.15381/rpb.v28i1.18353>

Isolation and identification of proteolytic, amylolytic, lipolytic, and chitinolytic bacteria from shrimp waste

Aislamiento e identificación de bacterias proteolíticas, amilolíticas, lipolíticas y quitinolíticas de residuos de langostinos

Abstract

Bacteria and microbial enzymes are biocatalysts and can be used as an alternative to industrial chemical processes. The present study focused on isolating and identifying bacterial strains from shrimp waste, that produce amylases, lipases, proteases and chitinases with potential use on shrimp waste treatment. Thirty-two bacterial strains were isolated, phenotypically characterized, and identified by the API system and the molecular analysis of the 16S rDNA. It was found that 28.13% of the isolated bacterial strains had amylolytic capacity, 87.50% lipolytic, 96.88% proteolytic and 28.13% chitinolytic capacity on agar plates with specific substrates. The genera *Bacillus*, *Burkholderia*, *Ochrobactrum*, *Vibrio*, *Pseudomonas* and *Shewanella* were identified. Bacteria with enzymatic capacities isolated in the present study, could be used to obtain by-products from shrimp waste as well as other industrial applications.

Resumen

Las bacterias y enzimas microbianas son biocatalizadores y pueden ser usadas como alternativa en los procesos químicos industriales. El presente estudio se centró en aislar e identificar cepas bacterianas a partir de desechos de langostinos, capaces de producir amilasas, lipasas, proteasas y quitinasas, que tuvieran potencial aplicación en el tratamiento de residuos de langostinos. Se aisló treinta y dos cepas bacterianas, caracterizadas fenotípicamente e identificadas mediante el sistema API 20 y mediante análisis molecular basado en el ADNr 16S. Se encontró que el 28.13% de las cepas bacterianas aisladas tenían capacidad amilolítica, 87.50% lipolítica, 96.88% proteolítica y 28.13% capacidad quitinolítica en placas de agar con sustratos específicos. Los géneros identificados fueron *Bacillus*, *Burkholderia*, *Ochrobactrum*, *Vibrio*, *Pseudomonas* y *Shewanella*. Las bacterias con capacidades enzimáticas aisladas en el presente estudio, podrían ser usadas para obtener subproductos de los desechos de langostinos, así como en otras aplicaciones industriales.

Palabras clave:

enzimas bacterianas; proteasas; lipasas; amilasas; quitinasas

Keywords:

bacterial enzymes; proteases; lipases; amylases; chitinases.

Introduction

The industrial uses of microbial enzymes (e.g., food, agriculture, chemicals, and pharmaceuticals) has gained interest and increased rapidly in comparison to plant and animal enzymes (Singh et al. 2016). In this context, waste could be an important source of bacteria and enzymes diversity with potential biotechnological applications.

Bacteria and their enzymes can also be used as an alternative to chemical methods in different industrial processes being an environmentally friendly technology (Singh et al. 2016). Some microbial enzymes with special characteristics of industrial importance include proteases, keratinases, xylanases, amylases, lipases and chitinases (de Souza & de Oliveira e Magalhães 2010, Rathore et al. 2015). Isolation of microorganisms of new sources would provide enzymes possessing special characteristics for use in various bio-industries requiring these enzymes (Nigam 2013).

Bacteria and microbial enzymes could also be useful to solve environmental problems caused by industrial activities (Singh et al. 2016). One of the industries in need of a strategy to reduce the environmental impact produced by the generated waste is the shrimp aquaculture industry (Páez-Osuna 2001). The use of specific microbial enzymes to address this problem could be an interesting environmentally friendly approach.

Shrimp aquaculture production provides an acceptable protein rich supplement for human consumption (Kandra et al. 2012). However, a large amount of shrimp shell-waste is also generated by shrimp farms during this activity (Dai et al. 2015). Shrimp waste is composed mainly by proteins (40%), lipids (10%), minerals (28%) and chitin (17%) (Synowiecki & Al-Khateeb 2000). Carapace (head shells) and abdominal (tail) shells constitute the shrimp shell-waste (Uno et al. 2012). This shrimp shell-waste is removed in peeling sheds near the landing or at packaging plants (Kandra et al. 2012). The cephalothorax and the exoskeleton constitute 60% of the animal (ITP & IMARPE 1996), depending on species. Countries dedicated to carniculture generate approximately up to 48 – 56% of the raw shrimp weight depending on species (Singla and Chawla 2001, Pu et al. 2010), which if not dealt with, generates a problem of environmental contamination.

Several studies have been described for chitinolytic bacteria with the ability to degrade shrimp-shell waste (Setia 2015) and lactic acid fermentation processes for shrimp head waste to be used as silage meal and for chitin recovery (Kandra et al. 2012). Isolation of bacterial strains that have specific enzymatic capacities from shrimp shell waste would have the advantage to accelerate the degradation process of this substrate to obtain products for future applications such as the recovery of chitin, pigments, essential amino acids, and fatty acids, to be used in medical, cosmetics, paper, pulp, textile, and food industries (Kandra et al. 2012). The aim of the present study was to isolate and identify bacterial strains with proteolytic, amylolytic, lipolytic and chitinolytic capacities from shrimp waste.

Material and methods

Sample collection and processing.- Shrimp (*Litopenaeus vannamei*) waste from a company dedicated to produce and commercialize hydrobiological products, located in Tumbes - Perú, was used in the present study as a source of bacterial strains. This waste, composed of

cephalothorax and abdominal exoskeletons, was collected (July, 2015) and placed into seawater broth under sterile conditions. Samples were sent in a cooler box to the Laboratory of Biotechnology in ITP, where they were immediately processed.

Bacterial isolation and purification.- Shrimps cephalothorax (50 g) were transferred to a bottle containing 150 mL of seawater broth (SWB, 10 g meat extract, 10 g peptone, 750 mL filtered seawater and 250 mL distilled water, pH 7.5 - 8.0) and mixed in a sterile homogenizer. The mix was incubated at 25 °C for 72 h. Dilutions were made from the sample bottle using the method described by Carvajal et al. 1991. Five serial decimal dilutions were made (10^{-1} to 10^{-5}) in test tubes containing 9 mL of SWB. An aliquot of 0.1 mL of each dilution was spread onto seawater agar (SWA) plates. The plates were incubated at 25 °C for 24 h. Colonies with different morphological characteristics were selected. Isolated bacterial strains were characterized by standard microbiological procedures: Gram staining, colonial morphology, catalase test and cytochrome oxidase reaction.

Bacterial screening for enzymatic capacity.- Screening for extracellular enzymatic capacity (amylase, chitinase, lipase, and protease) was conducted on agar plates supplemented with specific enzyme substrates, such as starch, chitin, tributyrin, gelatin and casein. A transparent halo surrounding colonies would indicate enzymatic capacity. Tests were performed by duplicate.

Hydrolysis of starch.- To determine strains with amylolytic capacity, the method of Skerman (1969) was used. An aliquot of 5 µL of bacterial culture (18 – 24 h) was spread onto SWA plates supplemented with 0.2% soluble starch (w/v) and incubated at 25 °C for 5 days. Colonies were then covered with a lugol solution.

Hydrolysis of tributyrin.- Lipolytic capacity of the strains was determined by the method of Cardenas et al. (2001). An aliquot of 5 µL of bacterial culture (18 – 24 h) was spread onto tributyrin agar plates containing 1% tributyrin and supplemented with 3% NaCl (w/v); then incubated at 25 °C for 5 days.

Hydrolysis of casein.- To determine proteolytic capacity, the method described by Ryden et al. (1973) was used. An aliquot of 5 µL of bacterial culture (18 - 24 h) was spread onto milk agar plates (3% skim milk powder) and incubated at 25 °C for 5 days.

Hydrolysis of gelatin.- Proteolytic capacity was determined by the method of Frazier (1926). An aliquot of 5 µL of bacterial culture (18 – 24 h) was spread onto SWA plates supplemented with 1.5% microbiological gelatin and incubated at 25 °C for 5 days. A mercuric chloride solution ($HgCl_2$) acidified with concentrated HCl, was added onto the agar plates until colonies were completely covered.

Hydrolysis of chitin.- Chitinolytic capacity was determined by the method of Wirth and Wolf (1990). An aliquot of 5 µL of bacterial culture (18 – 24 h) was spread onto chitin agar plates (2% chitin) and incubated at 25 °C for 5 days.

Dendrogram.- A dendrogram of cluster analysis on the degradation of substrates by bacterial strains was produced by the DendroUPGMA tool (a dendrogram-construction utility, <http://genomes.urv.cat/UPGMA/>) using the Euclidean coefficient and UPGMA method.

Biochemical and molecular identification of bacterial isolates.- Biochemical tests were conducted to identify bacterial strains, by using the API 20 NE identification system according to the protocol supplied by the manufacturer. Strains with the best enzymatic capacities for each substrate were molecularly identified for future uses in industrial processes. Total DNA from those selected strains was extracted by using the PureLink Genomic DNA Mini Kit (Invitrogen). Partial 16S rDNA was sequenced by Macrogen (USA). DNA amplification by the polymerase chain reaction (PCR) method was performed using primer 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and primer 1492 R (5'-GGTTACCTTGTTACGACTT-3') according to the protocol of Reysenbach, Longnecker and Kirshtein (2000). The resulting sequences were compared with those from the GenBank database in order to determine similarities by using the BLASTN algorithm. Sequences of 16S rDNA obtained in the present work were submitted to the GenBank database: *Bacillus velezensis* RL15-42 (MK993377); *Shewanella* sp. RL15-19 (MK993378); *Shewanella* sp. RL15-10 (MK993379); *Shewanella* sp. RL15-41 (MK993380); *Vibrio parahaemolyticus* RL15-44 (MK993381); *Vibrio parahaemolyticus* RL15-22 (MK993382), and *Vibrio parahaemolyticus* RL15-50 (MK993383).

Multiple alignment of 16S rDNA sequences of selected strains from the present work and reference bacterial sequences from the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>), was performed using ClustalX2 (Larkin et al. 2007). Phylogeny trees were constructed using the Neighbor-Joining method, Kimura 2-parameter model with 1,000 bootstrap replicates on the MEGA v5 program (Tamura et al. 2011). Bacteria of different genera were used as outgroup.

Comparison between extracellular enzymatic capacity of strains isolated from shrimp waste and the predicted enzymatic capacity of strains from the GenBank database.- Amino acid sequences of proteolytic, lipolytic, amylolytic and chitinolytic enzymes were selected from the GenBank database genomes of the same species identified in the present study. Those genomes were selected due to their high number of protein annotated in relation to other strains. Those enzymes amino acid sequences were scan by sub-cellular localization using PSORTb 3.0 (Yu et al. 2010), CELLO v.2.5 (Yu et al. 2006) and Gram-LocEN (Wan et al. 2017) programs to determine extracellular localization. Results were expressed as capacity to produce extracellular enzymes found in the database genomes and compared to the capacities of strains isolated in the present study.

Results

Isolation and purification of bacterial strains.- Thirty two bacterial strains were isolated from shrimp

waste, 31 Gram negative and 1 Gram positive. All 32 strains were positive for catalase and 31 were positive for cytochrome oxidase tests (Table 1).

Enzymatic capacity.- Bacteria isolated from shrimp waste, showed different profiles for lipase, protease, amylase and chitinase capacities on agar plates (Fig. 1A and Table 1). Nine bacterial strains showed amylolytic capacity on starch agar plates. Halos diameter ranged from 12 to 35 mm; 44.44% (4) of the strains showed halos diameter \geq to 24 mm. Strains with the best amylolytic capacity were RL15-22 and RL15-42 (Table 1).

Twenty eight out of 32 bacterial strains presented lipolytic capacity on tributyrin agar plates. Halos diameter ranged from 7 to 13 mm; 25% (7) of the strains showed halos diameter \geq to 12 mm. Strains with the best lipolytic capacity were identified as *Bacillus velezensis* (RL15-42) and *Vibrio parahaemolyticus* (RL15-50, RL15-54 and RL15-55) (Table 1).

Twenty-nine bacterial strains showed proteolytic capacity on casein and 31 on gelatin agar plates. Halos diameter ranged from 10 to 32 mm and from 20 to 46 mm, respectively; 55.17% (16) of the strains showed halos diameter \geq to 23 mm on milk agar plates and 58.06% (18) were \geq to 27 mm on gelatin agar. Strains with the best proteolytic capacity were RL15-44 and RL15-10 on gelatin and RL15-41 and RL15-19 on casein (Table 1).

Nine bacterial strains showed chitinolytic capacity after 5 days of incubation. Halos diameter ranged from 11 to 14 mm. The two strains with the best chitinolytic capacity were RL15-50 and RL15-44 (Table 1). Strains with the best activities for each substrate were chosen to be molecularly identified by 16S rDNA sequencing.

In general, 96.88% of studied bacterial strains (6 genera) showed proteolytic capacity, 87.50% lipolytic, 28.13% amylolytic and 28.13% chitinolytic capacity (Fig. 1B).

Dendrogram of cluster analysis on isolated strains enzymatic capacities.- Figure 2 is a dendrogram showing clusters of the isolated bacterial strains based on enzymatic profile similarities to degrade specific substrates. The strains were distributed into two main clusters, one with the strain *Burkholderia cepacia* that showed lipolytic capacity only, and the other cluster with the genera *Shewanella*, *Ochrobactrum*, *Pseudomonas*, *Vibrio*, *Bacillus* and 2 strains of *B. cepacia*, this last one, showed enzymatic capacity to degrade 2 and 3 substrates.

Each one of the bacterial strains studied in the present work, showed enzymatic capacity for 1, 2, 3 or all 4 of the tested enzymes (Table 1, Fig. 2). Lipolytic and proteolytic capacities were found in all studied strains, except *B. cepacia* (RL15-46) and some *Shewanella* sp. (RL15-04, RL15-09, RL15-16 and RL15-07). In contrast, amylolytic capacity was found in *Vibrio* species and *Bacillus velezensis* only. Chitinolytic capacity was shown by one *Shewanella* sp., *B. velezensis* and *Vibrio* spp. (Fig. 2).

Table 1. Enzymatic capacity of bacterial strains isolated from shrimp waste

N°	STRAIN CODE	SHAPE	GRAM STAINING			ENZYMATIC ASSAYS ON PLATE (halo diameter mm)					SPECIES
			CATALASE	OXIDASE	TRIBUTYRIN	GELATIN	CASEIN	STARCH	CHITIN		
1	RL15-01	coccus	-	+	+	10	30	22	0	0	<i>Shewanella putrefaciens</i> group
2	RL15-02	coccus	-	+	+	9	32	22	0	0	<i>Shewanella putrefaciens</i> group
3	RL15-03	rod	-	+	+	10	29	24	0	0	<i>Shewanella putrefaciens</i> group
4	RL15-04	coccus	-	+	+	0	23	22	0	0	<i>Shewanella putrefaciens</i> group
5	RL15-06	rod	-	+	+	7	35	0	0	0	<i>Burkholderia cepacia</i>
6	RL15-07	rod	-	+	+	0	23	16	0	0	<i>Shewanella putrefaciens</i> group
7	RL15-08	rod	-	+	+	11	29	25	0	0	<i>Pseudomonas aeruginosa</i>
8	RL15-09	rod	-	+	+	0	25	12	0	0	<i>Shewanella putrefaciens</i> group
9	RL15-10	coccus	-	+	+	10	35	26	0	0	<i>Shewanella</i> sp.*
10	RL15-11	rod	-	+	+	8	32	0	0	0	<i>Burkholderia cepacia</i>
11	RL15-12	rod	-	+	+	10	20	28	0	12	<i>Shewanella putrefaciens</i> group
12	RL15-16	rod	-	+	+	0	23	15	0	0	<i>Shewanella putrefaciens</i> group
13	RL15-19	rod	-	+	+	11	28	30	0	0	<i>Shewanella</i> sp.*
14	RL15-20	rod	-	+	+	8	21	24	0	0	<i>Shewanella putrefaciens</i> group
15	RL15-22	coccus	-	+	+	12	24	27	35	12	<i>Vibrio parahaemolyticus</i> *
16	RL15-24	coccus	-	+	+	10	30	27	0	0	<i>Shewanella putrefaciens</i> group
17	RL15-25	rod	-	+	+	11	32	27	0	0	<i>Shewanella putrefaciens</i> group
18	RL15-26	coccus	-	+	+	9	30	24	0	0	<i>Shewanella putrefaciens</i> group
19	RL15-28	coccus	-	+	+	10	29	23	0	0	<i>Shewanella putrefaciens</i> group
20	RL15-31	coccus	-	+	+	11	27	22	0	0	<i>Burkholderia cepacia</i>
21	RL15-39	rod	-	+	+	8	30	19	0	0	<i>Ochrobactrum anthropi</i>
22	RL15-40	rod	-	+	+	11	29	24	0	0	<i>Shewanella putrefaciens</i> group
23	RL15-41	rod	-	+	+	12	32	32	0	0	<i>Shewanella</i> sp.*
24	RL15-42	rod	+	+	-	13	26	10	27	0	<i>Bacillus velezensis</i> **
25	RL15-44	rod	-	+	+	7	46	26	23	13	<i>Vibrio parahaemolyticus</i> *
26	RL15-46	coccus	-	+	+	10	0	0	0	0	<i>Burkholderia cepacia</i>
27	RL15-47	rod	-	+	+	11	31	22	21	11	<i>Vibrio parahaemolyticus</i>
28	RL15-49	coccus	-	+	+	12	23	12	26	11	<i>Vibrio alginolyticus</i>
29	RL15-50	rod	-	+	+	13	23	21	21	14	<i>Vibrio parahaemolyticus</i> *
30	RL15-53	coccus	-	+	+	8	26	24	12	13	<i>Vibrio parahaemolyticus</i>
31	RL15-54	rod	-	+	+	13	26	20	24	12	<i>Vibrio parahaemolyticus</i>
32	RL15-55	rod	-	+	+	13	24	23	20	12	<i>Vibrio parahaemolyticus</i>

(* Strains identified by 16S rDNA sequencing and the API system. (** Strain only identified by 16S rDNA sequencing. All other strains were identified by the API system.

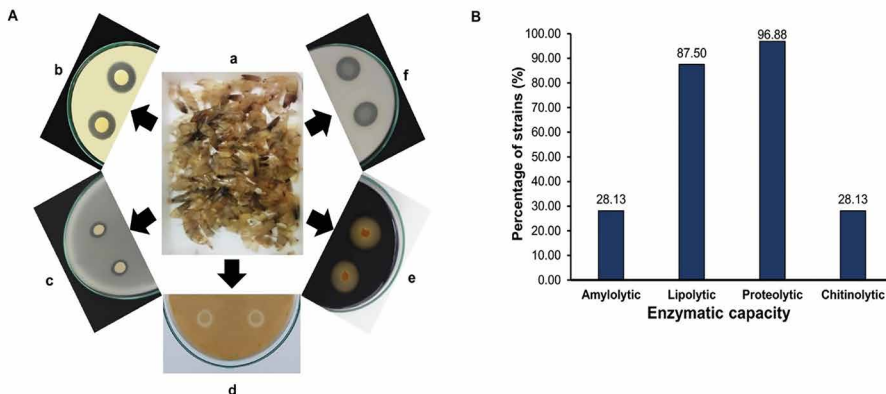


Figure 1. Lipase, protease, amylase and chitinase plate assays on bacteria isolated from shrimp waste and enzymatic percentage distribution. A: (a) Shrimp waste, (b) RL15-31: Halo of transparency on Gelatine agar, (c) RL15-49: Halo on Tributyrin agar, (d) RL15-55: Halo on Chitin agar, (e) RL15-44: Halo on Starch agar, (f) RL15-41: Halo on Casein agar. B: Percentage distribution of enzymes produced by bacterial strains isolated from shrimp waste.

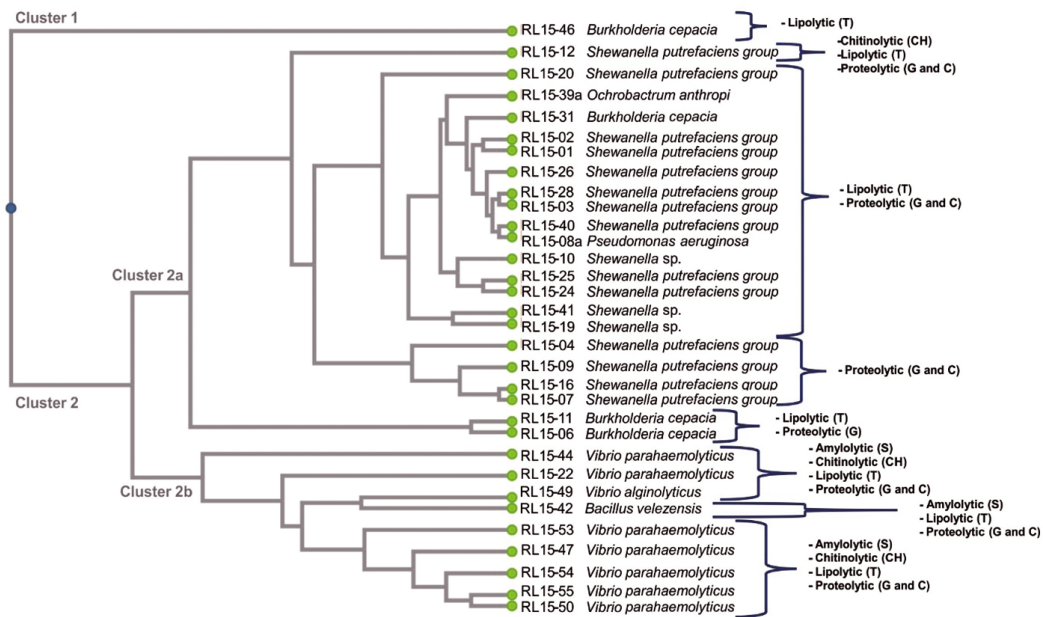


Figure 2. Dendrogram based on degradation of substrates (G: Gelatin, C: Casein, T: Tributyrin, S: Starch and CH: Chitin) by strains isolated from shrimp waste. Enzymatic profiles are indicated. Euclidean coefficient and UPGMA method were used from the DendroUPGMA tool (a dendrogram- construction utility).

Identification of bacterial species.- Out of the 32 bacterial strains isolated from shrimp waste, 53.13% (17 strains) were identified as *Shewanella* spp., 21.88% (7 strains) as *Vibrio parahaemolyticus*, 12.50% (4 strains) as *B. cepacia*, and 12.52% (4 strains) as each one of the following species: *B. velezensis*, *Vibrio alginolyticus*, *Ochrobactrum anthropi*, and *Pseudomonas aeruginosa* (Fig. 3).

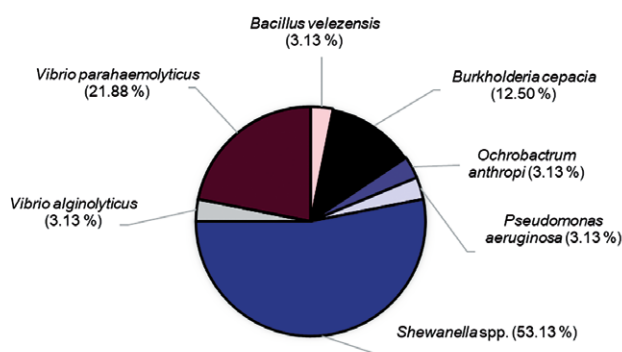


Figure 3. Percentage distribution of bacterial strains isolated from shrimp waste.

Identification of bacterial strains with the best enzymatic capacity, as well as similarity by BLASTN and phylogenetic analysis based on 16S rDNA gene sequences, revealed that strains RL15-41, RL15-10 and RL15-19 belonged to the genus *Shewanella* and were closely related to *Shewanella algae*, *Shewanella upenei* and *Shewanella halotis* with 99.15 – 100% identity percentage for these species (Fig. 4). Thus, these isolated strains were designated as *Shewanella* spp. Strains RL15-50, RL15-22 and RL15-44 were identified as *V. parahaemolyticus* with 99.7, 99.93 and

100% identity, respectively. Strain RL15-42 was identified as *B. velezensis* with 99.02% identity, which is a species synonym of *B. methylotrophicus* (Dunlap et al. 2016).

Comparison of extracellular enzymatic capacity between isolated bacterial strains and selected strains from the GenBank database.- Different extracellular enzymes were identified among bacterial strains from the GenBank database and from those isolated in the present study (Table 2). Proteolytic enzymes genes were present in all analyzed genomes from the GenBank database, except *Ochrobactrum anthropi*. Lipolytic capacity was identified in *B. cepacia*, *P. aeruginosa*, *B. velezensis* and *V. parahaemolyticus* genomes. Amylolytic capacity was identified in *B. velezensis* and *V. parahaemolyticus* genomes. Chitinolytic capacity was identified in *P. aeruginosa* and *V. parahaemolyticus* genomes. Enzymatic profiles of strains isolated in the present study were different from those from the GenBank database (Table 2).

Discussion

Bacteria isolation from the same source in need of treatment itself, would allow to obtain strains with specific characteristics already adapted to that particular source, with the advantage of performing better than strains obtained from any other source, for a specific industrial purpose. In the present study, bacteria were isolated from shrimp waste, to be used in future shrimp waste treatment. Among them were *B. cepacia*, *O. anthropi*, *P. aeruginosa*, *V. alginolyticus*, *V. parahaemolyticus*, *B. velezensis* and *Shewanella* spp. *Shewanella* genus have been described among the predominant spoilage bacteria in refrigerated shrimp (*Litopenaeus vannamei*) (Zhu et al. 2018). *Burkholderia cepacia* has also been isolated from marine environments (Alkhunni et al. 2017). Strains of *Vibrio* are

ubiquitous in marine and estuarine aquatic ecosystems in which shrimp occur naturally and are farmed (Gopal et al. 2005), therefore, these bacteria are considered opportunistic pathogens of shrimp (Kraxberger-Beatty et al. 1990; Woo et al. 2015). Species of the genus *Bacillus* have also been isolated from shrimp waste such as *Bacillus cereus* and *Bacillus amyloliquefaciens* (Sorokulova et al. 2009; Setia 2015), as well as *B. velezensis*, isolated from the same type of source in the present work.

Isolated bacterial strains from shrimp waste showed amyolytic, lipolytic, proteolytic and chitinolytic capacities. Hydrolytic profile of microorganisms depends of the source where they have been isolated from and the composition of the substrate for the enzyme of interest (Vishwanatha et al. 2010, Muthulakshmi et al. 2011). Different substrates such as effluents of dairy and meat processing industry (Sangeetha et al. 2012, Mazzucotelli et al. 2013), soil contaminated with oil, oilseeds, and tannery effluent (Siva & Abraham 2011), have been used to obtain bacteria with hydrolytic capacity. Our results suggest that shrimp waste is a rich substrate to obtain a good diversity of bacterial enzymes (amyolytic, lipolytic, proteolytic and chitinolytic enzymes), interesting for the biotechnology industry.

Amyolytic bacteria studied in the present work, showed to be mostly *Vibrio* species (*V. parahaemolyticus*

and *V. alginolyticus*), but also *B. velezensis*. These results agree with those reported by other authors for amyolytic capacity from different *Vibrio* species (Raghul Subin & Bhat 2011). Like our results, the genus *Bacillus* has been reported with amyolytic capacity (Zhou et al. 2018). In addition, several bacteria including *B. cepacia*, *O. anthropi*, *P. aeruginosa*, *B. velezensis*, *Shewanella* spp., *V. alginolyticus* and *V. parahaemolyticus* showed the ability to produce lipolytic enzymes. Our results are in accordance with the report of a wide variety of species (sub)phyla including Betaproteobacteria (including *Burkholderia*) and Gammaproteobacteria (including *Pseudomonas* and *Vibrio*), which are known to be some of the main microbial sources of commercially available lipolytic enzymes (Hassan, Abdul-Raouf and Abdel-Rahiem Ali 2015; Narihiro et al. 2014).

Proteolytic and chitinolytic bacteria were also isolated in the present study. Brzezinska et al. (2008) also reported bacteria that produce proteases and chitinases from shrimp by-products. In our study, *Shewanella* spp., *V. alginolyticus*, and *V. parahaemolyticus* showed the ability to produce chitinolytic enzymes. *S. putrefaciens*, *Vibrio aestuarianus* and *Streptomyces* sp. isolated from crustacean waste have also been reported as chitinolytic species (Sastoque-Cala et al. 2007, Anuradha & Revathi 2013).

Table 2. Enzymatic capacity comparison between strains isolated from shrimp waste and the predicted enzymatic capacity of strains from the GenBank database

SPECIES	N° of strains	Source	Enzymatic capacity				
			Proteolytic		Lipolytic	Amyolytic	Chitinolytic
			Gelatin	Casein	Tributyrin	Starch	Chitin
<i>Shewanella</i> spp.	12	This study	+	+	+	-	-
	4	This study	+	+	-	-	-
	1	This study	+	+	+	-	+
	1	<i>S. algae</i> strain KC-Na-R1 (NZ_CP033574- NZ_CP033575)	+	+	-	-	-
	1	<i>S. upenei</i> strain 20-23R (NZ_NIJL00000000.1)	+	+	-	-	-
<i>Burkholderia cepacia</i>	1	This study	+	+	+	-	-
	2	This study	+	-	+	-	-
	1	This study	-	-	+	-	-
	1	Strain ATCCOCCUS 25416 /UCB 717 (NZ_CP012981- NZ_CP012981)	+	+	+	-	-
<i>Pseudomonas aeruginosa</i>	1	This study	+	+	+	-	-
	1	Strain RW109 (NZ_LT969519- NZ_LT969521)	+	+	+	-	+
<i>Ochrobactrum anthropi</i>	1	This study	+	+	+	-	-
	1	Strain ATCCOCCUS 49188 (NC_009672- NC_009672)	-	-	-	-	-
<i>Bacillus velezensis</i>	1	This study	+	+	+	+	-
	1	Strain CGMCOCCUS 11640 (NZ_CP026610- CP026611)	+	+	+	+	-
<i>Vibrio alginolyticus</i>	1	This study	+	+	+	+	+
	1	Strain ATCCOCCUS 33787 (NZ_CP013484- NZ_CP013488)	+	+	-	-	-
<i>Vibrio parahaemolyticus</i>	7	This study	+	+	+	+	+
	1	Strain 20130629002S01 (NZ_CP020034- NZ_CP020037)	+	+	+	+	+

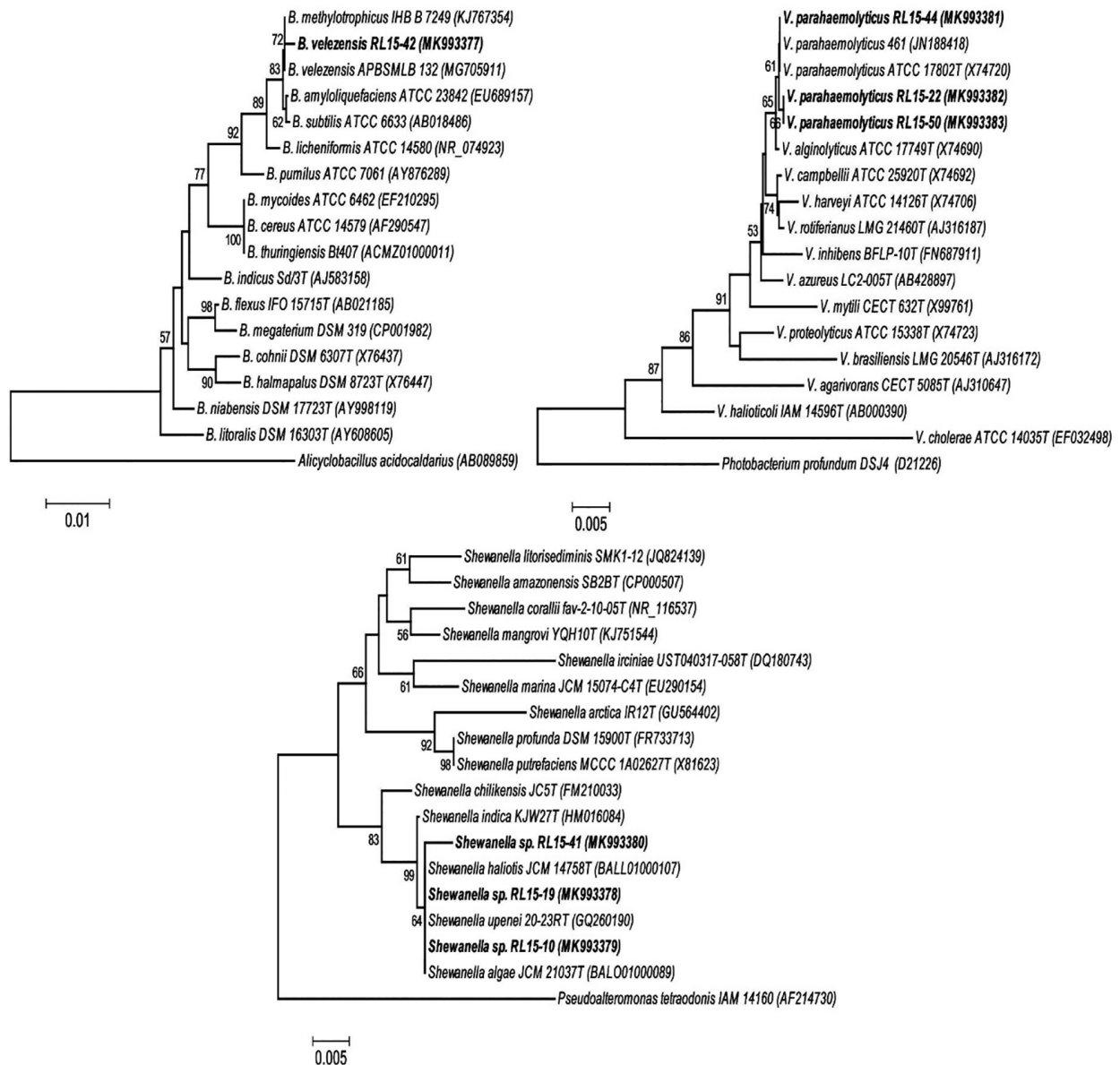


Figure 4. The tree was constructed using the Neighbor-Joining method and genetic distances were generated using the Kimura 2-parameter model. Values on the branches represent the percentage of 1000 bootstrap replicates. Bootstrap values over 50% are shown. Bacterial strains from shrimp waste are shown in bold. GenBank accession numbers are in parentheses. *Alicyclobacillus acidocaldarius*, *Photobacterium profundum* and *Pseudoalteromonas tetradonis* were used as outgroup to root the trees.

Bacterial strains with proteolytic capacity found in the present study were *Shewanella* spp., including *S. putrefaciens* group, *P. aeruginosa*, *V. alginolyticus*, *V. parahaemolyticus*, *B. cepacia*, *O. anthropi* and *B. velezensis*. *S. putrefaciens*, involved in marine fish decomposition, have shown high extracellular protease activity suggesting the presence of several proteases (Odagami et al. 1994). In addition, species of *Vibrio* including *V. alginolyticus* and *V. parahaemolyticus*, are known to produce several extracellular proteolytic enzymes (Miyoshi 2013). In a previous study, strains of *Vibrio* and *Shewanella* producing proteases were isolated from marine sediment samples (Zhou et al. 2015). We describe strains with proteolytic but no chitinolytic capacity among different genera

of bacteria, including *Bacillus*, which could be used for waste deproteinization processes. For example, chitinase-deficient *Bacillus licheniformis* strains can efficiently deproteinize shrimp shell waste, resulting in chitin of superior quality (Waldeck et al. 2006). It should be mentioned that the majority of studies on crustacean waste have been carried out to isolate only chitinolytic bacteria (Waldeck et al. 2006; Anuradha & Revathi 2013; Setia 2015).

Based on our results, bacterial strains with a diversity of extracellular enzymes were found in shrimp waste. For instance, some *B. cepacia* isolated strains were able to hydrolyze gelatin and/or casein. In addition, most of the enzymatic profiles of strains isolated in the pres-

ent study were different from those of the same species predicted from the GenBank genomes database. In this context, we did not find chitinolytic enzymes for *P. aeruginosa* in comparison to the chitinolytic potential of *P. aeruginosa* RW109 from the database, identified through bioinformatic analysis of extracellular enzymes. However, extracellular enzymes found in *B. velezensis* strain CGMCC 11640 and *V. parahaemolyticus* strain 20130629002S01 by bioinformatics analysis, showed that these strains have the potential to hydrolyze similar substrates to those hydrolyzed by our strains. Our results show an enzymatic diversity among the bacterial community isolated from shrimp waste. It has been observed that strains isolated from different environments could have different enzymatic phenotypes, although they belong to the same species.

In summary, we identified 32 bacterial strains (represented in 6 genera) with different enzymatic profiles. The most representative enzymatic capacities among the isolated strains were proteolytic (96.88%) and lipolytic (87.50%). The genus *Vibrio* showed to produce all the enzymes tested (proteases, lipases, amylases and chitinases). *V. parahaemolyticus* strains showed the highest proteolytic, amylolytic, lipolytic and chitinolytic capacity. Strains isolated from a particular substrate (e.g., shrimp waste) would have specific enzymes to hydrolyze that substrate, therefore could be used as an alternative not only to chemical methods, but also to bacterial strains obtained from a different source. They could be used in industrial processes (e.g., deproteinization process of shrimp waste to obtain chitin). Moreover, genes coding for enzymes produced by these bacteria, could be cloned and expressed in a suitable cell host, the enzymes could be characterized and used with biotechnological purposes in order to improve industrial bioprocesses.

Literature cited

- Alkhunni SB, Gaballah MS, and Gültepe N. 2017. Pathogenic bacteria for human and fish isolated from fish farm in Kastamonu, Turkey. *Journal of Aquaculture & Marine Biology* 6(3): 157. <https://doi.org/10.15406/jamb.2017.06.00157>.
- Anuradha V, and Revathi K. 2013. Purification and characterization of bacterial chitinase isolated from crustacean shells. *International Journal of Pure & Applied Bioscience* 1(4): 1-11.
- Brzezinska MS, Lalke-Porczyk E, Donderski W, Walczak M. 2008. Occurrence and activity of microorganisms in shrimp waste. *Current Microbiology* 57:580-587. <https://doi.org/10.1007/s00284-008-9246-1>
- Carvajal G, Ayala M, Sirvas S. 1991. *Microbiología de alimentos marinos*. CONCYTEC, Lima.
- Dai JY, Cheng L, He QF, and Xiu ZL. 2015. High acetoin production by a newly isolated marine *Bacillus subtilis* strain with low requirement of oxygen supply. *Process Biochemistry* 50(11): 1730-1734. <https://doi.org/10.1016/j.procbio.2015.07.010>
- Dunlap CA, Kim SJ, Kwon SW and Rooney AP. 2016. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp. *plantarum* and *Bacillus oryzicola* are later heterotypic synonyms. *International Journal of Systematic and Evolutionary Microbiology*. , 66:1212-1217. <https://doi.org/10.1099/ijsem.0.000858>
- Frazier WC. 1926. A method for the detection of changes in gelatin due to bacteria. *Journal of Infectious Diseases* 39(4):302-309 <https://doi.org/10.1093/infdis/39.4.302>
- Gopal S, Ota SK, Kumar S, Karunasagar I, Nishibuchi M, Karunasagar I. 2005. The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *International Journal of Food Microbiology*. 102(2): 151-159 <https://doi.org/10.1016/j.ijfoodmicro.2004.12.011>
- Hassan SWM, Abdul-Raouf UM, Abdel-Rahiem Ali M. 2015. Antagonistic interactions and phylogenetic diversity of antimicrobial agents producing marine bacteria in Suez Bay. *Egyptian Journal of Aquatic Research* 41(1): 57-67. <https://doi.org/10.1016/j.ejar.2015.02.005>
- ITP & IMARPE. 1996. *Compendio biológico tecnológico de las principales especies hidrobiológicas del Perú*. Lima: Instituto del Mar del Perú.
- Kandra P, Challa MM, and Kalangi PJH. 2012. Efficient use of shrimp waste: Present and future trends. *Applied Microbiology and Biotechnology* 93(1): 17-29. <https://doi.org/10.1007/s00253-011-3651-2>.
- Kraxberger-Beatty T, McGarey DJ, Grier HJ and Lim DV. 1990. *Vibrio harveyi*, an opportunistic pathogen of common snook, *Centropomus undecimalis* (Bloch), held in captivity. *Journal of Fish Diseases* 13(6): 557-560. <https://doi.org/10.1111/j.1365-2761.1990.tb00819.x>.
- Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21): 2947-2948. <https://doi.org/10.1093/bioinformatics/btm404>
- Mazzucotelli CA, Ponce AG, Kotlar CE, Moreira MdR. 2013. Isolation and characterization of bacterial strains with a hydrolytic profile with potential use in bioconversion of agroindustrial by-products and waste. *Food Science and Technology* 33(2): 295-303. <https://doi.org/10.1590/s0101-20612013005000038>
- Miyoshi S. 2013. Extracellular proteolytic enzymes produced by human pathogenic *Vibrio* species. *Frontiers in Microbiology* 4:339 <https://doi.org/10.3389/fmicb.2013.00339>
- Muthulakshmi C, Gomathi D, Kumar DG, Ravikumar G, Kalaiselvi MK, Uma C. 2011. Production, purification and characterization of protease by *Aspergillus flavus* under solid state fermentation. *Jordan Journal of Biological Sciences* 4: 137-148.
- Narihiro T, Suzuki A, Yoshimune K, Hori T, Hoshino T, Yumoto I, Yokota A, Kimura N, Kamagata Y. 2014. The combination of functional metagenomics and an oil-fed enrichment strategy revealed the phylogenetic diversity of lipolytic bacteria overlooked by the cultivation-based method. *Microbes and Environments* 29(2): 154-161. <https://doi.org/10.1264/jisme2.me14002>
- Nigam PS. 2013. Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules* 3(3):597-611. <https://doi.org/10.3390/biom3030597>

- Odagami T, Morita J, Takama K and Suzuki S. 1994. Substrate specificities of extracellular proteases produced by marine putrefactive bacteria, *Shewanella putrefaciens* and *Alteromonas haloplanktis*. *Letters in Applied Microbiology* 18(1):50-52. <https://doi.org/10.1111/j.1472-765X.1994.tb00799.x>.
- Páez-Osuna F. 2001. The environmental impact of shrimp aquaculture: a global perspective. *Environ. Pollut.* 112(2): 229-231. [https://doi.org/10.1016/S0269-7491\(00\)00111-1](https://doi.org/10.1016/S0269-7491(00)00111-1)
- Pu J, Bechtel PJ, Sathivel S. 2010. Extraction of shrimp astaxanthin with flaxseed oil: Effects on lipid oxidation and astaxanthin degradation rates. *Biosystems Engineering* 107(4): 364-371. <https://doi.org/10.1016/j.biosystemseng.2010.10.001>
- Raghul Subin S, Bhat SG. 2011. Seasonal variation in the hydrolytic exo-enzyme profile of *Vibrio* sp. associated with the marine benthic environment of South India. *Indian Journal of Geo-Marine Science*, 40, 826-833.
- Rathore AS, Gupta RD. 2015. Chitinases from bacteria to human: properties, applications, and future perspectives. *Enzyme Research* 2015: 1-8. <https://doi.org/10.1155/2015/791907>
- Reysenbach AL, Longnecker K, Kirshtein J. 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Applied and Environmental Microbiology* 66(9):3798-806. <https://doi.org/10.1128/aem.66.9.3798-3806.2000>
- Ryden AC, Lindberg M, Philipson L. 1973. Isolation and characterization of two protease producing mutants from *Staphylococcus aureus*. *Journal of Bacteriology* 116(1): 25-32.
- Sangeetha R, Geetha A, Arulpandi I. 2012. Optimization of protease and lipase production by *Bacillus pumilus* SG 2 isolated from an industrial effluent. *Internet Journal of Microbiology* 5(2). <https://doi.org/10.5580/2126>
- Sastoque-Cala L, Mercado-Reyes M, Martínez-Salgado M, Quedo-Hidalgo B, Pedroza-Rodríguez A. 2007. Producción de quitinasas extracelulares con una cepa alcalófila halotolerante de *Streptomyces* sp. aislada de residuos de camarón. *Revista Mexicana de Ingeniería Química* 6: 137-146.
- Setia I. 2015. Chitinolytic assay and identification of bacteria isolated from shrimp waste based on 16S rDNA sequences. *Advances in Microbiology* 5(7):541-548. <https://doi.org/10.4236/aim.2015.57056>
- Singh R, Kumar M, Mittal A, Mehta PK. 2016. Microbial enzymes: industrial progress in 21st century. *3 Biotech* 6: 174. <https://doi.org/10.1007/s13205-016-0485-8>
- Singla AK, Chawla M. 2001. Chitosan: some pharmaceutical and biological aspects - an update. *Journal of Pharmacy and Pharmacology* 53:1047-1067. <https://doi.org/10.1211/0022357011776441>.
- Siva KM, Abraham J. 2011. A comparative analysis of protease producing microbes isolated from tannery effluent. *International Journal of Science and Nature* 2(1): 110-113.
- Skerman VBD. 1969. Starch hydrolysis detection method. Edited By I. Nueva York, John Wiley & Sons.
- Sorokulova I, Krumno A, Globa L, Vodyanoy V. 2009. Efficient decomposition of shrimp shell waste using *Bacillus cereus* and *Exiguobacterium acetylicum*. *Journal of Industrial Microbiology and Biotechnology* 36(8):1123-1126. <https://doi.org/10.1007/s10295-009-0587-y>
- de Souza PM, de Oliveira Magalhães P. 2010. Application of microbial α -amylase in industry - A review. *Brazilian Journal of Microbiology* 41(4):850-861. <https://doi.org/10.1590/S1517-83822010000400004>
- Synowiecki J, Al-Khateeb NAAQ. 2000. The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp Crangon crangon processing discards. *Food Chemistry* 68(2):147-152. [https://doi.org/10.1016/S0308-8146\(99\)00165-X](https://doi.org/10.1016/S0308-8146(99)00165-X)
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28(10):2731-2739. <https://doi.org/10.1093/molbev/msr121>
- Uno K, Higashioto Y, Chawee-pack T, Ruangpan L. 2012. Effect of chitin extraction processes on residual antimicrobials in shrimp shells. *Turkish Journal of Fisheries and Aquatic Science* 12: 89-94. https://doi.org/10.4194/1303-2712-v12_1_11
- Vishwanatha T, Jain S, Reena V, Divyashree BC, Siddalingeshwara KG, Karthic J, Sudipta KM. 2010. Screening of substrates for protease production from *Bacillus licheniformis*. *International Journal of Engineering Science and Technology* 2: 6650-6554.
- Waldeck J, Daum G, Bisping B, Meinhardt F. 2006. Isolation and molecular characterization of chitinase-deficient *Bacillus licheniformis* strains capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. *Applied and Environmental Microbiology* 72(12):7879-7885. <https://doi.org/10.1128/AEM.00938-06>
- Wan S, Mak MW, Kung SY. 2017. Gram-LoCEN: Interpretable prediction of subcellular multi-localization of Gram-positive and Gram-negative bacterial proteins. *Chemometrics and Intelligent Laboratory Systems* 162: 1-9. <https://doi.org/10.1016/j.chemolab.2016.12.014>
- Wirth SJ, Wolf GA. 1990. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *Journal of Microbiological Methods* 12(3-4): 197-205. [https://doi.org/10.1016/0167-7012\(90\)90031-Z](https://doi.org/10.1016/0167-7012(90)90031-Z).
- Woo JH, Kang KM, Kwon TH, Park NH, Lee EY. 2015. Isolation, identification and characterization of marine bacteria exhibiting complementary enantioselective epoxide hydrolase activity for preparing chiral chlorinated styrene oxide derivatives. *Journal of Industrial and Engineering Chemistry* 28: 225-228. <https://doi.org/10.1016/j.jiec.2015.02.018>.
- Yu CS, Chen YC, Lu CH, Hwang JK. 2006. Prediction of protein subcellular localization. *PROTEINS: Structure, Function, and Bioinformatics* 64: 643-651.
- Yu NY, Wagne JR, Laird MR, Melli G, Rey S, Lo R, Dao P, Cenik Sahinalp S, Ester M, Foster LJ, Brinkman FSL. 2010. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26(13):1608-1615. <https://doi.org/10.1093/bioinformatics/btq249>.
- Zhou S, Xia Y, Zhu C and Chu W. 2018. Isolation of marine *Bacillus* sp. with antagonistic and organic-substances-degrading activities and its potential application as a fish probiotic. *Marine Drugs* (16): 196. <https://doi.org/10.3390/md16060196>

Zhou Z, Wang X, Zhang H, Sun J, Zheng L, Liu H, Wang J, Shen A, Geng M, Guo Y. 2015. Chromopeptide A, a highly cytotoxic depsipeptide from the marine sediment-derived bacterium *Chromobacterium* sp. HS-13-94. *Acta Pharmaceutica Sinica B* 5(1): 62–66. <https://doi.org/10.1016/j.apsb.2014.11.001>

Zhu S, Wu H, Zhang C, Jie J, Liu Z, Zeng M, Wang C. 2018. Spoilage of refrigerated *Litopenaeus vannamei*: eavesdropping on *Acinetobacter* acyl-homoserine lactones promotes the spoilage potential of *Shewanella baltica*. *Journal of Food Science and Technology* 55(5): 1903–1912. <https://doi.org/10.1007/s13197-018-3108-z>

Agradecimientos / Acknowledgments:

The authors thank Corporación Refrigerados INYSA for providing shrimp waste samples.

Conflicto de intereses / Competing interests:

The authors declare no conflict of interest.

Rol de los autores / Authors Roles:

SS, VB, LS, MJ wrote the article. VB/MJ prepared the figures and tables; SS, VB, LS, MJ reviewed and SS approved the manuscript.

Fuentes de financiamiento / Funding:

FONDECYT (Fondo Nacional de Desarrollo Científico, Tecnológico y de Innovación Tecnológica) Convenio N° 103-2014-FONDECYT - "Obtaining the chitin biopolymer through microbial enzymatic hydrolysis of shrimp waste".

Aspectos éticos / legales; Ethics / legals:

There are no ethical or legal issues to declare in this research and work.