Acta Alimentaria, Vol. 49 (4), pp. 375–381 (2020)
DOI: 10.1556/066.2020.49.4.2

AN ACYLASE FROM *SHEWANELLA PUTREFACIENS* PRESENTS A *VIBRIO PARAHAEMOLYTICUS* ACYLHOMOSERINE LACTONE-DEGRADING ACTIVITY AND EXHIBITS TEMPERATURE-, PH- AND METAL-DEPENDENCES

Z. FANG[a], D. SUN[a], J. GAO[a], M. GUO[a], L. SUN[a]*, Y. WANG[a], Y. LIU[a]*, R. WANG[b], Q. DENG[a], D. XU[a] and R. GOONERATNE[c]

[a]College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, 524048, China
[b]College of Food Science and Engineering, Lingnan Normal University, Zhanjiang, 524048, China
[c]Department of Wine, Food and Molecular Biosciences, Lincoln University, Lincoln, Canterbury, 7647, New Zealand

(Received: 4 February 2020; accepted: 9 July 2020)

Shewanella putrefaciens supernatant was found to increase the virulence factors of *Vibrio parahaemolyticus* by efficiently degrading its acylhomoserine lactone (AHL). To further reveal the regulation mechanism and its key degrading enzyme, a potential AHL-degrading enzyme acylase (Aac) from *S. putrefaciens* was cloned, and the influences of temperature, pH, protein modifiers, and metals on Aac were tested. Aac was significantly influenced by temperature and pH, and exhibited the highest AHL-degrading activity at temperatures of 37 °C and pH of 8. Mg^{2+} and Fe^{2+} can further increase the AHL-degrading activity. 10 mM EDTA inhibited its activity possibly by chelating the co-factors (metals) required for Aac activity. Tryptophan and arginine were identified as key components for Aac activity that are critical to its AHL-degrading activity. This study provides useful information on Aac and for *V. parahaemolyticus* control.

Keywords: acylase, AHL-degrading enzyme, acylhomoserine lactone, *Shewanella putrefaciens*, *Vibrio parahaemolyticus*

As an occasional foodborne pathogen in seafood, *Vibrio parahaemolyticus* has been responsible for gastroenteritis outbreaks worldwide. *Shewanella putrefaciens* coexists with *V. parahaemolyticus* and increases virulence factors of *V. parahaemolyticus* in shrimp (WRIGHT et al., 2016; FANG et al., 2018). *S. putrefaciens* is a major food spoilage bacterium in seafood, and exhibits a higher resistance to antibiotics and metals (KANG & SO, 2016), but rarely implicated as a cause of human disease (STEINBERG & BURD, 2015). Ambient temperature, pH, and metals are also virulence factors of vibrio pathogens (GODE-POTRATZ et al., 2010; GODE, 2011; KIMES et al., 2012; GUTIERREZ et al., 2013). However, its mechanism of regulating the virulence factors of *V. parahaemolyticus* is not well understood. Quorum sensing (QS) is a bacterial communication system that regulates virulence factors and antibiotic effects via synthesis of autoinducer molecules (HAMMER & BASSLER, 2003; SHIH & HUANG, 2002). *Vibrio harveyi* AHL autoinducer is degraded by bacterial enrichment cultures of shrimp (TINH et al., 2007). The virulence gene *hlyA* is negatively regulated by the QS system in *Vibrio cholerae* (TSOU & ZHU, 2010). It is thought that QS signal degrading enzymes can alter the virulence factors.

* To whom correspondence should be addressed.
Phone: +86 759 2396027; fax: +86 759 2396027; e-mails: suncamt@126.com (L. SUN); liuyingxk@sina.com (Y. LIU)

0139–3006 © 2020 *Akadémiai Kiadó, Budapest*

In a previous study, we reported that the *S. putrefaciens* supernatant exhibited a significant degradation ability of QS signal AHL of *V. parahaemolyticus* (Fang et al., 2018). *Variovorax paradoxus* can also utilise AHLs as energy sources for growth (Leadbetter & Greenberg, 2000). *Ralstonia* sp. aiiD was identified as a potent AHL acylase (Lin et al., 2003). *Shewanella oneidensis* AHL-acylase (Aac) is an aiiD homolog and contains a highly conserved Ntn_hydrolase, which has a structure similar to the common of β-lactamases superfamily (Kim et al., 2006). It is also reported to markedly degrade the AHL production of *Vibrio anguillarum* (Morohoshi et al., 2008). AHL acylase of *Shewanella algae* belongs to the penicillin acylase family, which can confer *S. algae* resistant to penicillins and other β-lactam antibiotics (Ghaneimotlagh et al., 2019). Zinc and other metal-binding motifs are common in the Ntn_hydrolase structure of (Melino et al., 1998; Ignatova et al., 2005). In this study, we investigated the Aac properties by testing the effects of temperature, pH, modifiers, and metals on its AHL degrading ability, to further reveal the mechanisms regulating the virulence factors of *V. parahaemolyticus* by *S. putrefaciens* in spoiled shrimp.

1. Materials and methods

1.1. Bacterial strains, plasmids, and growth conditions

The *V. parahaemolyticus* strain ATCC33847 (from the China Committee for Culture Collection of Microorganisms), *Escherichia coli* BL21, and DH5α (from our lab) carrying plasmids pET28a(+) (kan^r, from our lab) were grown in LB culture (10 g l^{-1} yeast extract, 10 g l^{-1} tryptone, and 5 g l^{-1} NaCl) with or without Kanamycin (50 μg ml^{-1}) at 37 °C, unless specifically noted otherwise. Kanamycin (USP Grade,), yeast extract (FMB Grade), tryptone (FMB Grade), NaCl (purity ≥99.5%), $MgCl_2$ (purity ≥99.0%), and metals and reagents were purchased from Sangon Biotech Co., Ltd (Shanghai, China).

1.2. Synthesis and expression of the aac gene in plasmid pET28a

The *S. putrefaciens aac* gene was synthesised by Sangon Biotech Co., Ltd (Shanghai, China) according to the sequence of the *aac* gene from *S. putrefaciens* strain in the database (NCBI Accession Number NC_009438). The *aac* DNA fragment and plasmid pET28a were digested with *Bam*HI and *Hind* III, and the PCR products were purified with gel electrophoresis and ligated by T4 ligase according to Fang and co-workers (2015).

The *E.coli* BL21 harbouring recombinant pET28a-aac was grown in LB culture with Kanamycin (50 μg ml^{-1}) at 37 °C for 24 h, transferred to a new LB culture, and induced with 1 mM isopropylthio-galactoside (IPTG, purity ≥99.0%, Sangon Biotech Shanghai, China) for 6 h. The recombinant protein was harvested and purified according to Morohoshi and co-workers (2008). Briefly, the cultured supernatants were ultrafiltered with an Amicon Ultra-15 membrane (10 kDa, Millipore-Sigma, USA) at 3000 *g* at 4 °C for 2 h, and the condensed supernatant was diluted with pre-cooled LB culture to 1:100 (v:v) (10 μg ml^{-1}) for further analysis.

1.3. Determination of AHL-degrading rates

V. parahaemolyticus AHL was prepared as reported by Fang and co-workers (2018). AHL was added to a 500 μl suspension of *E.coli* BL21 harbouring pET28a-aac containing 4 mM protein modifier or 5 mM metal ions or EDTA to react at 25, 30, 37, 45, 50, and 55 °C for 30

min as described by Morohoshi and co-workers (2008), at a final concentration of 5 μg ml^{-1}. The residual AHL contents were determined by liquid chromatography linked to tandem mass spectrometry (LC-MS/MS; Tandem Quadrupole LCMS-8030 (Shimadzu, Japan)) as described by Fang and co-workers (2018). For AHL analysis, the injection volume was set at 10.0 μl, and eluent A was methanol and eluent B was water at a flow rate 0.3 ml min^{-1}. The elution gradient started with 30% of eluent A, was raised to 90% in a 4 min gradient, and then was set back to the initial conditions after 3 min. For mass spectra in ESI source, the block and desolvation temperatures were 250 °C and 400 °C, respectively, desolvation gas flow rate was 15.0 l min^{-1} and capillary voltage was 4.5 kV. The ratios of LC-MS/MS peak areas of the analytes to an internal standard were measured, and the AHL samples treated without Aac supernatants were set as blank controls (0%). The data were adjusted by subtraction from the corresponding blank controls. Each experiment was performed at least 3 times.

1.4. Statistical analysis

Statistical analysis was performed with ANOVA and Duncan's multiple range test using SPSS version 19.0. The results were compared and the statistical significance P value was set at <0.05.

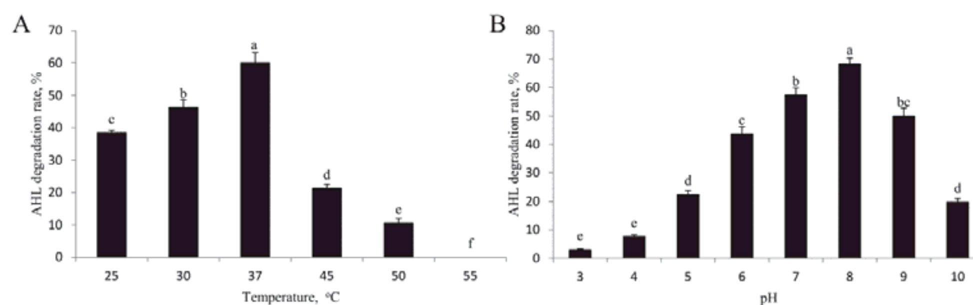

Fig. 1. Effects of temperature and pH on AHL-degrading activity of *S. putrefaciens* lactonase enzyme Aac. 500 μl supernatant of BL21 harbouring pET28a-aac was mixed with 100 μl AHL, incubated at 25, 30, 37, 45, 50, and 55 °C (A) or at pH 3, 4, 5, 6, 7, 8, 9, and 10 (B) for 30 min. the AHL contents were measured with LC-MS/MS. The data represent the means of three independent experiments. Error bars represent mean standard deviations. Different superscript letters indicate significant differences at P<0.05 according to Duncan's test.

2. Results and discussion

2.1. Effects of temperature and pH on V. parahaemolyticus *AHL-degrading activity of* S. putrefaciens *Aac*

S. putrefaciens regulates *V. parahaemolyticus* virulence factors through its AHL-degrading ability, and the temperature influences this regulation (Fang et al., 2018). To detect the potential AHL degrading activity of *S. putrefaciens* Aac and its other properties, the AHL-degrading rates of *S. putrefaciens* Aac and the effect of temperature on its activity were determined. *V. parahaemolyticus* AHL was mixed with 500 μl of Aac solution to react at different temperatures for 30 min. *S. putrefaciens* Aac exhibited a higher degrading activity at 37 °C >30 °C >25 °C with a clear positive temperature-dependent effect. However, when the temperature was >37 °C, the AHLs degrading activity of Aac decreased. Thus, the highest

AHL-degrading activity was at 37 °C. This is consistent with our previous findings that 37 °C was the optimal temperature for maximal *S. putrefaciens* activity to enhance the *V. parahaemolyticus* virulence factors (FANG et al., 2018).

Next, the effect of pH on Aac in AHL-degrading was analysed. For this, AHL was mixed with Aac solution to react at different pHs (0.1 mM Tris-HCl, pH = 3 to 10) for 30 min. At pHs <8, Aac exhibited a positive pH-dependent-effect on AHL degradation. At pHs >8, the degrading activity of Aac was suppressed. The optimal pH for Aac was 8. *S. putrefaciens* Aac showed a better pH-stability compared to that of *Bacillus* sp. (WANG et al., 2004).

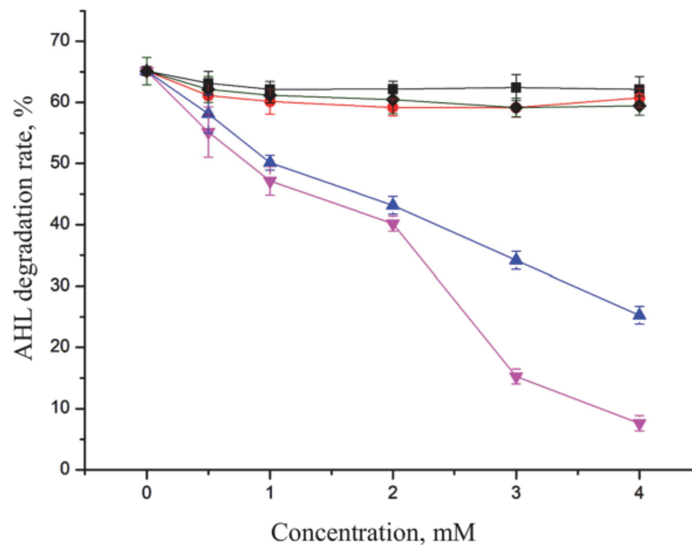

Fig. 2. Effects of protein modifiers on AHL-degrading activity of *S. putrefaciens* lactonase enzyme Aac. The mixture of Aac and AHL was incubated with or without indicated concentrations of DTT, BrAc, Acac, NBS, and PMSF at 37 °C for 30 min. The AHL contents were measured with LC-MS/MS. The data represent the means of three independent experiments. Error bars represent mean standard deviations.
—■—: DTT; —●—: BrAc; —▲—: Acac; —▼—: NBS; —◆—: PMSF

2.2. *Effects of protein modifiers on* S. putrefaciens *Aac*

The Aac activity is usually ascribed to its protein structure. Ntn_hydrolase is a key conserved structural domain of Aac and is also found in ATP-dependent proteases (KIM et al., 2000). Such structural domains are usually rich in arginine, serine, lysine or histidine residues. Besides, disulphide bonds offer structural stability but can be broken by dithiothreitol (DTT). Arginine residues are modified by acetylacetone (Acac), which is used to confirm the presence of arginine residues in proteins (DIKLER et al., 2015). BrAc was used to analyse the histidine active site residues (MORGAN & MULLEREBERHARD, 1976), and N-bromo succinimide (NBS) was used to analyse tryptophan based active site residues (TENG et al., 2006). 5-methylphenazinium methyl sulphate (PMSF), as a routine protease inhibitor, was used to modify serine based active site residues. In order to analyse and confirm the Aac enzymatic properties and key active site residues, DTT, BrAc, PMSF, NBS, and Acac were used to test their effects on AHL degrading activity of Aac. Compared to DTT, BrAc, and PMSF, addition of 1 mM or higher doses of NBS and Acac significantly reduced the AHL degrading activity

of Aac (Fig. 2), suggesting that tryptophan and arginine residues are the key active sites of Aac. The results showed that Aac was significantly inhibited by NBS and Acac, which is highly specific for tryptophan and arginine residues (Teng et al., 2006; Dikler et al., 2015). AHL acylase and GL-7-ACA acylase both belong to family of β-lactam acylases with a conserved Ntn_hydrolase (Kim et al., 2000; Kim et al., 2006). In previous reports, tryptophan and arginine were also found to be involved in the substrate binding and catalytic activity of GL-7-ACA acylase in *Pseudomonas* sp. (Lee et al., 2000). These results suggest that tryptophan and arginine residues play important roles in the AHL degradation activity of Aac.

2.3. Effects of metals on S. putrefaciens *Aac*

Most beta-lactam acylases are metal-dependent enzymes. Zinc-binding motif is conserved in β-lactam acylases family, and is influenced by zinc (Melino et al., 1998). As an aiiD homolog, *S. putrefaciens* Aac may have a common structure to β-lactamases superfamily (Kim et al., 2006). In order to further analyse the properties of Aac, its metal dependence was tested. 5 mM Mg^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, and Mn^{2+} were added to Aac and AHL mixed solution to react at 37 °C for 30 min (Fig. 3A). 5 mM Mg^{2+} and Fe^{2+} significantly enhanced the AHL-degrading activity of Aac compared to the control (without metals). This explains why Fe^{2+} could enhance the *V. anguillarum* virulence in Japanese eels and ayu (Nakai et al., 1987). However, 5mM Ca^{2+}, Cu^{2+}, Ba^{2+}, and Mn^{2+} significantly suppressed the AHL-degrading activity of Aac. The effect of Zn^{2+} on Aac was slightly negative but not obvious. However, a significant decrease in AHL-degrading activity was observed in Aac following the addition of 10 mM or higher EDTA (a metal chelator) concentration (Fig. 3B). In other bacteria, some typical acylase activities have been found to be increased by Mg^{2+} (Zou et al., 2016). The lactonases from *Burkholderia* sp. and *Bacillus* sp. were inhibited by Cu^{2+} and Zn^{2+} but not by ion-chelating EDTA (Mochizuki, 2001), which did not explain that Zn^{2+} is required for catalytic activity of lactonase with a zinc-binding motif (Crowder et al., 1997). When the concentration of ion-chelating reagent EDTA was increased to remove the residual Zn^{2+} from Aac, the AHL degradation activity significantly decreased. These results together indicate that metals are also required for the AHL degrading activity of Aac, confirming that metals increase *V. parahaemolyticus* virulence factors by enhancing the Aac activity of *S. putrefaciens* or other symbiotic bacteria in shrimp.

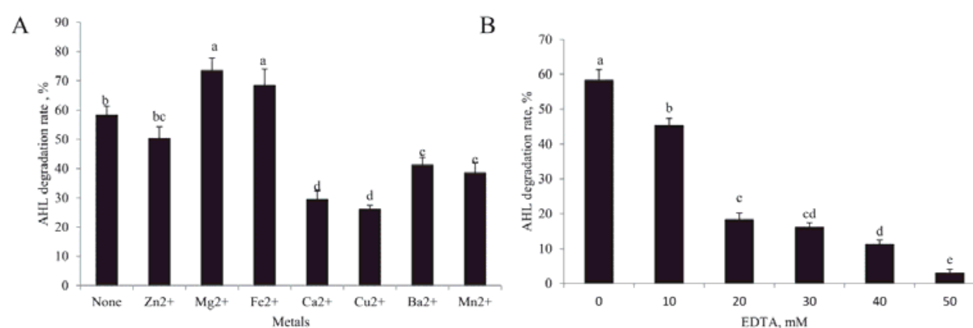

Fig. 3. Effects of metals on AHLs degrading activity of *S. putrefaciens* Aac. The mixture of Aac and AHL was incubated with or without 5 mM Mg^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, and Mn^{2+} (A) or indicated concentration of EDTA (B) at 37 °C for 30 min. The AHL contents were measured with LC-MS/MS. The data represent the means of three independent experiments. Error bars represent mean standard deviations. Different superscript letters indicate significant differences at $P<0.05$ according to Duncan's test.

3. Conclusions

In this study, we cloned a putative AHL acylase gene *aac* from *S. putrefaciens* and found that Aac could degrade QS autoinducer molecule AHL and increase virulence factors of *V. parahaemolyticus*. *S. putrefaciens* Aac was sensitive to environmental temperature, pH, and metals. It is noteworthy that Acac, NBS, and EDTA significantly impaired Aac. The key amino acid residues, such as tryptophan and arginine, and metals also contribute to its AHL-degrading activity, and Mg^{2+} and Fe^{2+} can further enhance it. These findings further confirmed the AHL degradation activity of *S. putrefaciens* Aac as an AHL acylase. More importantly, the Aac contributes to the increase in *V. parahaemolyticus* virulence factors. Lowering the activity of Aac using inhibitors could be an effective strategy to control the *V. parahaemolyticus* virulence in shrimp preservation.

*

This work was supported by funds from the National Natural Science Foundation of China (grant number 31371746, 31371777.) and Higher Educational Cultivation Program for Major Scientific Research Projects of Guangdong Ocean University (grant number GDOU2013050205, 2014050203).

References

Crowder, M.W., Maiti, M.K., Banovic, L. & Makaroff, CA. (1997): Glyoxalase II from *A. thaliana* requires Zn(II) for catalytic activity. *FEBS Lett., 418*(3), 351–354.

Dikler, S., Kelly, J.W. & Russell, D.H. (2015): Improving mass spectrometric sequencing of arginine-containing peptides by derivatization with acetylacetone. *J. Mass Spectrom., 32*(12), 1337–1349.

Fang, Z., Kuang, X., Zhang, Y., Shi, P. & Huang, Z. (2015): A novel HAC1-based dual-luciferase reporter vector for detecting endoplasmic reticulum stress and unfolded protein response in yeast *Saccharomyces cerevisiae*. *Plasmid, 79*, 48–53.

Fang, Z., Sun, D., Li, C., Sun, L., Wang, Y., Guo, M., … & Liu, Y. (2018): Regulatory effects of *Shewanella putrefaciens* isolated from shrimp *Penaeus orientalis* on the virulence factors of *Vibrio parahaemolyticus* and evaluation of the role of quorum sensing in virulence factors regulation. *FEMS Microbiol. Ecol., 94*(7), fiy097.

Ghaneimotlagh, R., Mohammadian, T., Gharibi, D., Menanteauledouble, S., Mahmoudi, E., … & Elmatbouli, M. (2019): Quorum quenching properties and probiotic potentials of intestinal associated bacteria in Asian sea bass *Lates calcarifer. Mar. Drugs, 18*(1), 23.

Gode, C.J. (2011): Vibrio parahaemolyticus *responds to growth on a surface by initiating a program of gene control that is regulated by calcium, iron, and quorum sensing.* (PhD Thesis), University of Iowa, Iowa, US. 208 pages.

Gode-Potratz, C.J., Chodur, D.M. & Mccarter, L.L. (2010): Calcium and iron regulate swarming and type III secretion in *Vibrio parahaemolyticus*. *J. Bacteriol., 192*(22), 6025.

Gutierrez West, C.K., Klein, S.L. & Lovell, C.R. (2013): High frequency of virulence factor genes tdh, trh, and tlh in *Vibrio parahaemolyticus* strains isolated from a pristine estuary. *Appl. Environ. Microb., 79*(7), 2247–2252.

Hammer, B.K. & Bassler, B.L. (2003): Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol. Microbiol., 50*(1), 101–104.

Ignatova, Z., Wischnewski, F., Notbohm, H. & Kasche, V. (2005): Pro-sequence and Ca^{2+}-binding: Implications for folding and maturation of ntn-hydrolase penicillin amidase from *E. coli*. *J. Mol. Biol., 348*(4), 999–1014.

Kang, C. & So, J. (2016): Antibiotic and heavy metal resistance in *Shewanella putrefaciens* strains isolated from shellfishes collected from West Sea, Korea. *Mar. Pollut. Bull., 112*(1), 111–116.

Kim, J.K., Yang, I.S., Shin, H.J., Cho, K.J., Ryu, E.K., Kim, S.H., . . . & Kim, K. H. (2006): Insight into autoproteolytic activation from the structure of cephalosporin acylase: A protein with two proteolytic chemistries. *P. Natl. Acad. Sci. USA., 103*(6), 1732–1737.

Kim, Y., Yoon, K.-H., Khang, Y., Turley, S. & Hol, W.G. (2000): The 2.0 A crystal structure of cephalosporin acylase. *Structure, 8*(10), 1059–1068.

Kimes, N.E., Grim, C.J., Johnson, W.R., Hasan, N.A., Tall, B.D., Kothary, M.H., . . . & Morris, P.J. (2012): Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. *ISME J, 6*(4), 835–846.

Leadbetter, J.R. & Greenberg, E.P. (2000): Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax parado*xus. *J. Bacteriol., 182*(24), 6921–6926.

Lee, Y.S., Kim, H.W., Kang, B.L. & Park, S.S. (2000): Involvement of arginine and tryptophan residues in catalytic activity of glutaryl 7-aminocephalosporanic acid acylase from *Pseudomonas* sp. strain GK16. *Biochim. Biophys. Acta, 1523*(1), 123–127.

Lin, Y., Xu, J., Hu, J., Wang, L., Ong, S.L., Leadbetter, J.R., Zhang, L.H. (2003): Acyl-homoserine lactone acylase from *Ralstoni*a strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol. Microbiol., 47*(3), 849–860.

Melino, S., Capo, C., Dragani, B., Aceto, A. & Petruzzelli, R. (1998): A zinc-binding motif conserved in glyoxalase II, beta-lactamase and arylsulfatases. *Trends Biochem. Sci, 23*(10), 381–382.

Mochizuki, K. (2001): Purification and characterization of a lactonase from *Burkholderia* sp. R-711, that hydrolyzes (R)-5-oxo-2-tetrahydrofurancar-boxylic acid. *Arch. Microbiol., 175*(6), 430–434.

Morgan, W.T. & Mullereberhard, U. (1976): Chemical modification of histidine residues of rabbit hemopexin. *Arch. Biochem. Biophys., 176*(2), 431–441.

Morohoshi, T., Nakazawa, S., Ebata, A., Kato, N. & Ikeda, T. (2008): Identification and characterization of N-acylhomoserine lactone-acylase from the fish intestinal *Shewanella* sp. strain MIB015. *Biosci., Biotech., Bioch., 72*(7), 1887–1893.

Nakai, T., Kanno, T., Cruz, E.R. & Muroga, K. (1987): The effects of iron compounds on the virulence of *Vibrio anguillarum* in Japanese eels and ayu. *Fish Pathol., 22*(4), 185–189.

Shih, P.C. & Huang, C.T. (2002): Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J. Antimicrob. Chemoth., 49*(2), 309–314.

Steinberg, J.P. & Burd, E.M. (2015): 238 - Other Gram-negative and Gram-variable bacilli. -in: Bennett, J.E., Dolin, R. & Blaser, M.J. (Eds): *Mandell, Douglas, and Bennett's principles and practice of infectious diseases* 8th. Saunders Elsevier, Philadelphia. pp. 2667–2683

Teng, L.R., Fan, H., Zhang, Y.Y., Qi, Y.U., Huang, Y.F. & Liu, L.Y. (2006): Chemical modification and fluorescence spectrum of tryptophan residues in pullulanase. *Chem. Res. Chinese U., 22*(01), 69–72.

Tinh, N.T.N., Gunasekara, R.A.Y.S., Boon, N., Dierckens, K., Sorgeloos, P. & Bossier, P. (2007): N-acyl homoserine lactone-degrading microbial enrichment cultures isolated from *Penaeus vannamei* shrimp gut and their probiotic properties in *Brachionus plicatilis* cultures. *FEMS Microbiol. Ecol., 62*(1), 45–53.

Tsou, A.M. & Zhu, J. (2010): Quorum sensing negatively regulates hemolysin transcriptionally and posttranslationally in *Vibrio cholerae*. *Infect. Immun., 78*(1), 461–467.

Wang, L.H., Weng, L.X., Dong, Y.H. & Zhang, L.H. (2004): Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *J. Biol. Chem, 279*(14), 13645–13651.

Wright, M.H., Matthews, B., Arnold, M.S.J., Greene, A.C. & Cock, I.E. (2016): The prevention of fish spoilage by high antioxidant Australian culinary plants: *Shewanella putrefaciens* growth inhibition. *Int. J. Food Sci. Tech., 51*(3), 801–813.

Zou, C., Duan, X. & Wu, J. (2016): Magnesium ions increase the activity of *Bacillus deramificans* pullulanase expressed by *Brevibacillus choshinensis*. *Appl. Microbiol. Biot., 100*(16), 7115–7123.