

## EFFECTS OF SALINITY AND SHAKING CONDITION ON THE GROWTH AND VIRULENCE OF *VIBRIO PARAHAEMOLYTICUS*

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### SUMMARY

*Vibrio parahaemolyticus* is a Gram-negative halophilic bacterium that is found in estuarine, marine and coastal environment. This organism is the major causative agent of Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) which resulted in serious damages to cultured shrimp industry. Understanding the effect of environmental factors on the growth and virulence of this potential pathogen would be beneficial for preventing its outbreak. In this study, the growth and virulence of *V. parahaemolyticus* was examined under different salinity and shaking condition. *V. parahaemolyticus* XN9 was cultured in Brain Heart Infusion (BHI) medium with different sodium chloride concentrations (2.0, 2.5 and 3.0%) and different shaking conditions (0, 120 and 240 rpm). The growth of the bacterium was recorded over 8h and six extracellular enzymes of *V. parahaemolyticus* XN9 including caseinase, hemolysin, lecithinase, lipase, gelatinase, chitinase were investigated using agar-based method. The growth of *V. parahaemolyticus* was varied among different salinity and shaking conditions. It showed the best growth at 2.0% NaCl and 240 rpm. No change in the enzymatic activity (EA) of the tested extracellular enzymes was observed while changing salinity except the significant decline of gelatinase from  $3.49 \pm 0.19$  to  $2.77 \pm 0.17$  mm following salinity increase ( $p < 0.05$ ). On the other hand, regarding shaking condition, lipase was the one to increase its activity significantly following the increase of shaking speed ( $p < 0.05$ ). While caseinase, lecithinase, gelatinase and lipase were well expressed in *V. parahaemolyticus*, no hemolytic and chitinase activity was observed in any tested conditions. In summary, our study showed that 2.0 % NaCl and 240 rpm shaking promoted the best growth of *V. parahaemolyticus* and resulted in highest activity of gelatinase and lipase in this bacterium.

**Keywords:** *Vibrio parahaemolyticus*, Acute Hepatopancreatic Necrosis Disease (AHPND); extracellular enzymes, growth, salinity, shaking condition, virulence

### INTRODUCTION

*Vibrio parahaemolyticus* is a member of *Vibrio* species from the *Vibrionaceae* family. *V. parahaemolyticus* is a Gram-negative, halophilic bacterium that can be found in estuarine, marine and coastal environment as a free organism or parasite of zooplankton, fish, shellfish and shrimp (Ceccarelli *et al.*, 2013). This motile bacterium can also be a human pathogen causing food-borne illness such as gastroenteritis with

the symptoms involving nausea, vomiting, abdominal pain and diarrhea which is often associated with consuming raw or undercooked seafood (Shinoda, 2011). Furthermore, *Vibrio parahaemolyticus* has been reported to be the major causative agent of Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) (Tran *et al.*, 2013). *V. parahaemolyticus* colonizes the shrimp gastroenteritis tract and is transmitted orally. It produces toxins causing tissue destruction,

dysfunction of the shrimp digestive organ known as the hepatopancreas and death in the first 30 days after stocking (Lai *et al.*, 2015). AHPND was firstly appeared in southern China (2009) then spread quickly over to Hainan Island (2010), Viet Nam (2010), Malaysia (2011) and Thailand (2012), Mexico (2013), the Philippines (2015) and South America (2016) which affected heavily shrimp industry leading to significant global economic loss (Li *et al.*, 2017). In general, the bacterium uses multiple virulence factors in its pathogenesis such as adhesins, hemolysin, urease, type III and type VI secretion systems (Wang *et al.*, 2015; Letchumanan *et al.*, 2014). Other extracellular enzymes such as protease, lipase, lecithinase even though are well noted as virulence factors contributing significant to the pathogenesis in *Vibrio* species, they are not well studied in *V. parahaemolyticus* (Miyoshi, 2013; Li *et al.*, 2019; Zhang *et al.*, 2016; Xiong *et al.*, 2014). So far, while hemolysins such as thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) are usually characterized as important virulence factors of *V. parahaemolyticus* in human enteritis, plasmid-encoded binary toxins PirA<sup>vp</sup>/PirB<sup>vp</sup> are the noted ones for AHPND (Letchumanan *et al.*, 2014). Besides, it is well noted that host and environmental factors have strong impact on the expression of bacterial virulence factors (Do *et al.*, 2019; Figueroa-Angulo *et al.*, 2012; Kumar *et al.*, 2019). In case of *V. parahaemolyticus*, a marine and estuarine bacterium, it is no doubt that its growth as well as virulence is greatly influenced by dissolved oxygen and salinity. Effect of salinity on growth of different *V. parahaemolyticus* strains has been long studied. Data indicated that most of the strains can grow in the range of 1-9% NaCl but have optimal salinity of 2-3% (Liu *et al.*, 2016; Whitaker *et al.*, 2010; Nishina *et al.*, 2004). Dissolved oxygen or its associate, shaking condition has not been as well studied as salinity but recent data indicated that virulence of *V. parahaemolyticus* cultured at 120 rpm was significantly higher than at 110 rpm towards *Artemia* and *Macrobrachium* larvae (Kumar *et al.*, 2019).

In this study, we investigated how *V. parahaemolyticus* XN9, an AHPND-causing isolate responded to different salinities and shaking conditions via observing its growth and production of its virulence factors including the noted six extracellular enzymes including caseinase, hemolysin, lipase, lecithinase, gelatinase and chitinase.

## MATERIALS AND METHODS

### Bacterial strain

*Vibrio parahaemolyticus* XN9 was generously provided by Nha Trang University for this study and its identity was again confirmed by our group (Vu *et al.*, 2017). Before testing, *V. parahaemolyticus* XN9 was cultured on Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Himedia, India) overnight, at 30°C.

### Growth analysis of *V. parahaemolyticus* under different NaCl concentrations and shaking conditions

*V. parahaemolyticus* XN9 was optimally maintained and preserved in Brain Heart Infusion (BHI) medium with 2.5% NaCl, 30°C, pH 8.5 and 120 rpm (Anh *et al.*, 2018). This condition (2.5% NaCl, 30°C, pH 8.5, 120 rpm) was applied in this study as standard condition to determine the effect of different salinity and shaking condition on the virulence factors of *V. parahaemolyticus*.

To examine the effect of salinity, a single colony of *V. parahaemolyticus* XN9 was inoculated into BHI broth (Himedia, India) having different salt concentrations, 2.0, 2.5 and 3.0% and incubated overnight, 120 rpm at 30°C. In the next day, the bacteria suspension was used to inoculate BHI broth with corresponding salt concentrations. The starting OD<sub>620nm</sub> (0h) was adjusted to 0.05 (0h) and the flasks were incubated at 30°C, 120 rpm and measured each hour for OD<sub>620nm</sub> and dissolved oxygen (DO) concentration for 8 hours.

To examine the effect of shaking condition, same procedure was carried out on *V.*

*parahaemolyticus* but instead of varying salinity, salt concentration was kept at 2.5% and shaking conditions was varied between 0, 120 and 240 rpm. The experiments were triplicated.

#### Extracellular enzymatic activity analysis of *V. parahaemolyticus* under different NaCl concentrations and shaking conditions

Caseinase, hemolysin, lipase, lecithinase, gelatinase and chitinase were examined using agar-based method. *V. parahaemolyticus* XN9 was inoculated and cultured in either BHI broth (Himedia, India) having different salt concentrations, 2.0, 2.5 and 3.0% overnight at 30°C, 120 rpm to assess to effect of salinity on the extracellular enzyme activity or BHI broth (Himedia, India) 2.5% NaCl, overnight at 30°C at different shaking conditions, 0, 120 and 240 rpm to assess to effect of salinity on the extracellular enzyme activity. *V. parahaemolyticus* cultured was diluted at  $OD_{620nm} = 0.1$  before being applied on agar plates corresponding to each type of extracellular enzyme activity testing. All the media used were adjusted to pH 8.5. The experiments were triplicated.

- **Caseinase test:** 10  $\mu$ L suspension of *V. parahaemolyticus* was dropped onto the surface of BHI agar plates containing 1.5% (w/v) skim milk. Simultaneously, 10  $\mu$ L suspension of *Vibrio cholera* VCTC2012 was added onto another position of the plate as positive control. Then, the plate was incubated overnight, at 30°C. After incubation, observed clear halos around the colonies indicated the activity of enzyme, in this case, caseinase (Vermelho *et al.*, 1996).

- **Hemolysin test:** same procedure was carried on with few modifications. The agar used was Sheep blood agar plates which were prepared from Blood agar base (Himedia, India) and Sheep blood suspension 10% (Nam Khoa Biotech Company). *Vibrio cholera* VCTC2012 was also used as positive control. The plate was incubated for 48h.

- **Lipase test:** The agar used was tributyrin agar plate which is prepared using Tributyrin oil

(Himedia, India) and Tributyrin agar (Himedia, India). *Staphylococcus aureus* ATCC 29213 was used as positive control. The plate was incubated for 48h.

- **Lecithinase test:** the agar used was Egg-yolk agar plate containing 1 mL of Egg-yolk Emulsion (Himedia, India) and BHI (Himedia, India). *Vibrio cholera* VCTC2012 was used as positive control. The plate was incubated for 48h.

- **Gelatinase test:** the agar used was BHI agar (Himedia, India) containing 2.0% gelatin. *Vibrio cholera* VCTC2012 was used as positive control. The plate was incubated overnight. After incubation, the agar surface was immersed in saturated ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) solution. Observed clear halos around the colonies indicated the activity of gelatinase. Saturated ammonium sulfate would cloudy precipitate with the remaining gelatin and form white color plate.

- **Chitinase test:** the Coloidal Chitin was prepared following previously described procedure (Nagpure *et al.*, 2014). The agar used was BHI agar (Himedia, India) containing 5% colloidal chitin. *Vibrio cholera* VCTC2012 was used as positive control. The plate was incubated for 48h. Observed clear halos around the colonies indicated the activity of chitinase (Ohishi *et al.*, 2000).

#### Data analysis

The enzymatic activity (EA) was calculated as  $EA = \frac{D-d}{2}$ , in which D is diameter of both colony and the zone around colony (mm), d is diameter of colony (mm) (Vermelho *et al.* 1996). The EA is graded as follows: (–) when no visible halo is present, (+) when EA value is limited to 1-2 mm and (++) when EA value is equal or more than 2 mm (Vermelho *et al.* 1996). Each test was done in triplicate and the obtained data were analyzed using one-way ANOVA (Excel software, Microsoft). The means were also analyzed using t-tests ( $p < 0.05$ ) to compare the variation between the extracellular enzymatic activity of each test and corresponding standard

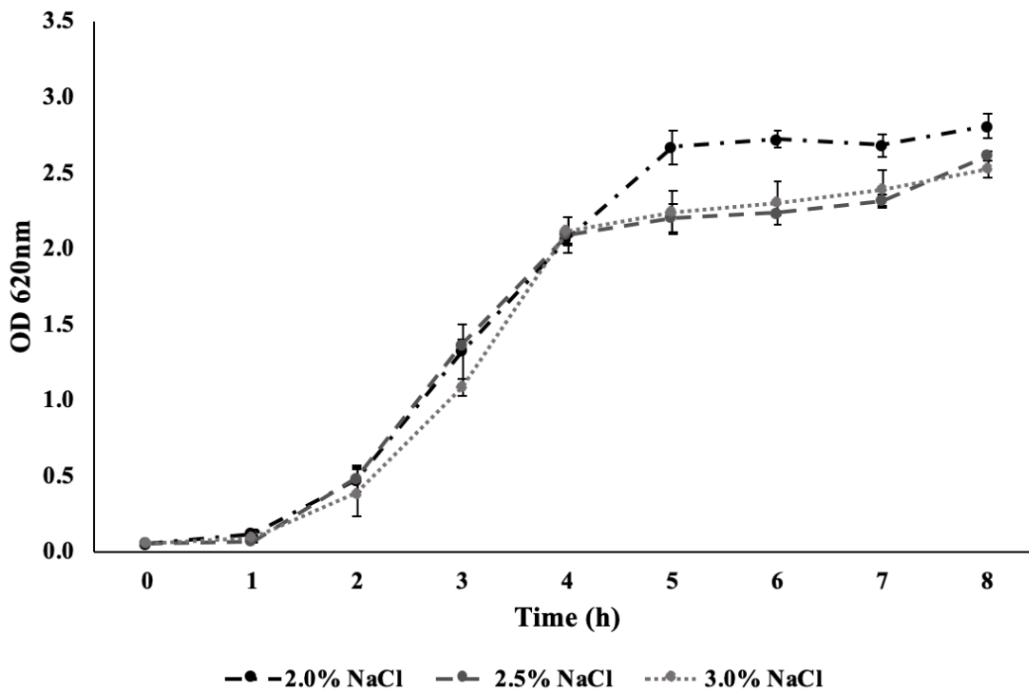
condition (2.5% NaCl, pH 8.5, 120 rpm and 30°C).

**RESULTS**

**Effect of salinity on the growth of *V. parahaemolyticus***

When being cultured at different salt concentrations, *V. parahaemolyticus* XN9

behaved quite similarly, especially for 2.5 and 3.0% NaCl, with only few differences to be noted in case of 2.0% NaCl. While *V. parahaemolyticus* XN9 entered stationary phase at 4h when being cultured in 2.5 and 3.0% NaCl, it was one hour delayed in case of 2.0% NaCl. Furthermore, the growth of *V. parahaemolyticus* in 2.0% NaCl was also highest among the three tested salt concentrations, with significant difference to 3.0% NaCl ( $p < 0.05$ ) (Figure 1).



**Figure 1.** The growth of *V. parahaemolyticus* XN9 at different NaCl concentrations (2.0, 2.5 and 3.0%). The bacteria were cultured in BHI, 120 rpm, 30°C, pH 8.5. The growth was expressed via OD<sub>620nm</sub>. The experiment was triplicated.

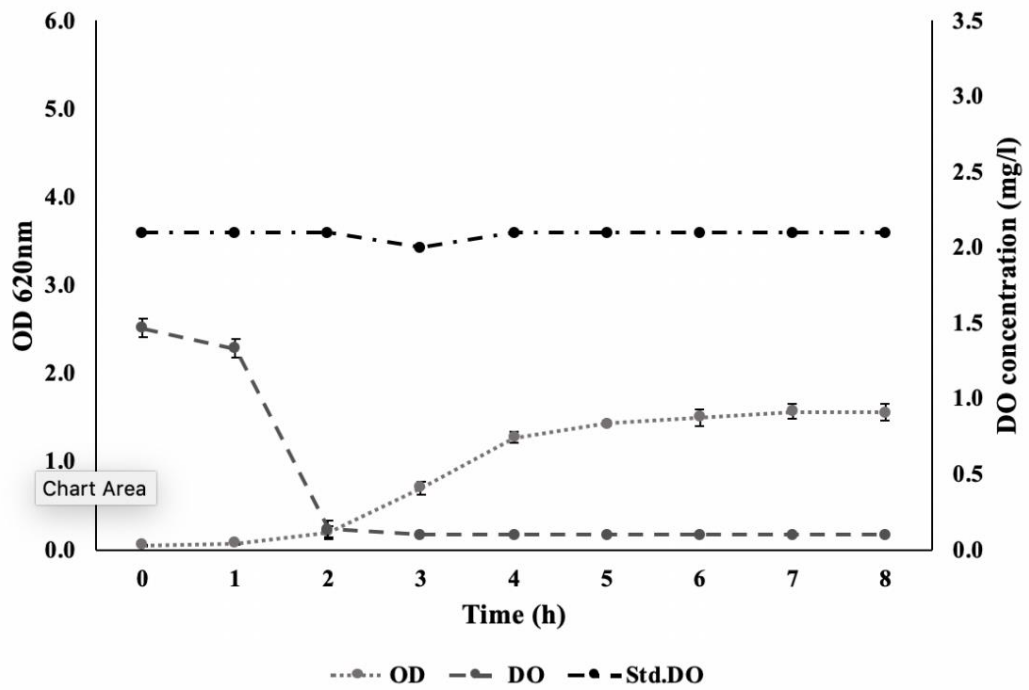
**Effect of salinity on extracellular enzymatic activity of *V. parahaemolyticus***

For caseinase and lipase, there was basically no difference in the activity of these enzymes in the three tested salt concentrations. Activity of caseinase was quite low (1.5mm, on average) while the activity of lipase was strong (2.9 mm, on average) for *V. parahaemolyticus* XN9 (Table 1).

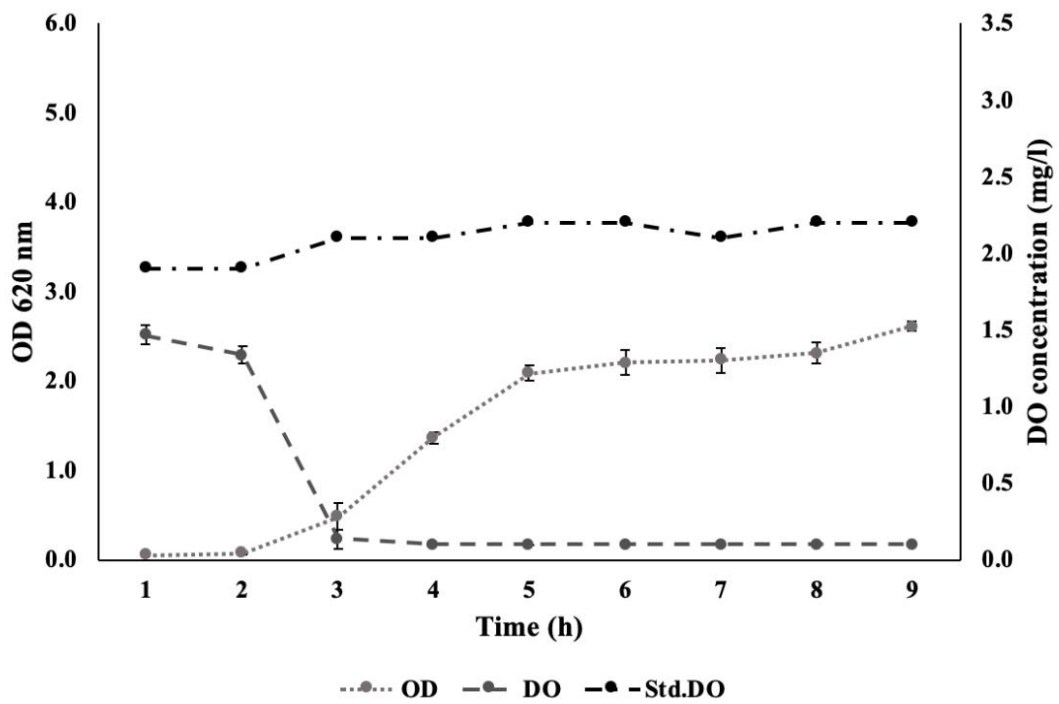
In contrast, gelatinase and lecithinase activity significantly decreased when increasing

salinity from 2.0 to 3.0% NaCl ( $p < 0.05$ ) (Table 1). At 2.0% NaCl, the enzymatic activity was highest for gelatinase ( $3.49 \pm 0.19$  mm) and lecithinase ( $5.06 \pm 0.53$  mm) whereas at 3.0% NaCl, the values were  $2.77 \pm 0.17$  mm and  $4.22 \pm 0.27$ mm for gelatinase and lecithinase, respectively.

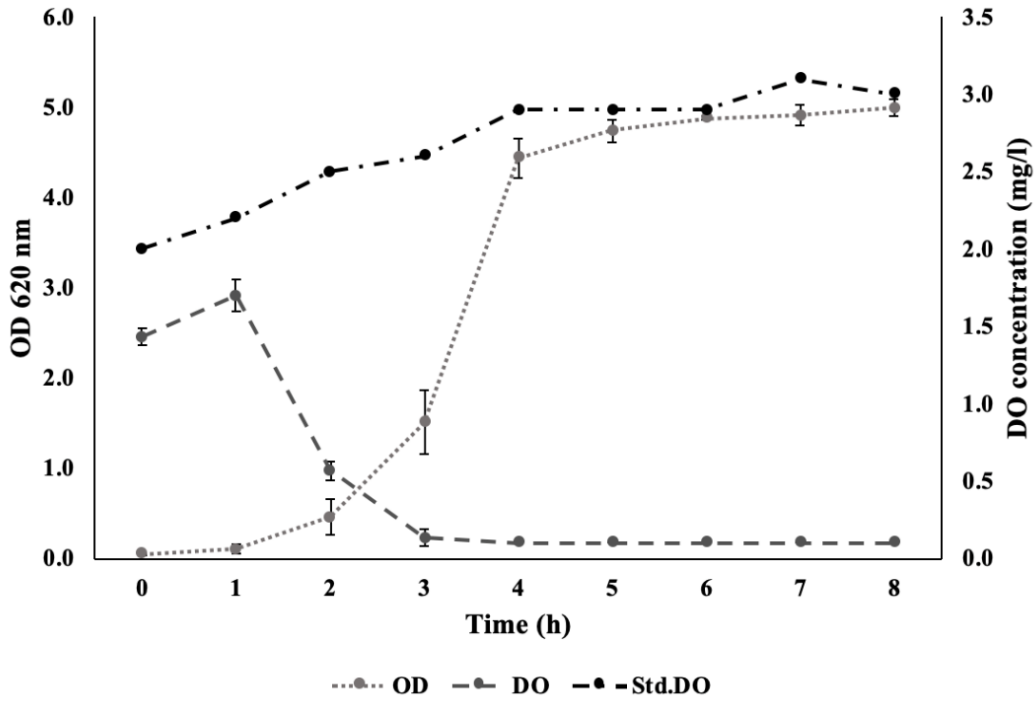
Interestingly, no activity of hemolysin and chitinase was observed for *V. parahaemolyticus* XN9 in any tested salt concentrations.



(a)



(b)



**Figure 2.** Growth of *V. parahaemolyticus* under different shaking conditions. (a). at 0 rpm, (b). at 120 rpm, (c). at 240 rpm. The bacteria were cultured in BHI at 2.5% NaCl, 30°C, pH 8.5. The corresponding dissolved oxygen (DO) concentrations were also shown (mg/L). DO measurement for the control bacteria-free medium flask was represent via Std. DO. The bacterial growth was expressed via OD<sub>620nm</sub>. The experiment was triplicated.

**Table 1.** Effect of salinity on extracellular enzymatic activity of *V. parahaemolyticus* XN9. The testing salinity was 2.0, 2.5, 3.0% and the enzymatic activity EA was shown in millimeters (mm).

Enzymatic activity (EA)	2.0% NaCl	2.5% NaCl	3.0% NaCl	T-test T <sub>2.0%</sub> vs. T <sub>2.5%</sub> (p value)	T-test T <sub>2.5%</sub> vs. T <sub>3.0%</sub> (p value)	T-test T <sub>2.0%</sub> vs. T <sub>3.0%</sub> (p value)
	1.56 ± 0.20 (+)	1.47 ± 0.31 (+)	1.55 ± 0.05 (+)	0.609	0.727	0.981
<b>Lecithinase (mm, grade)</b>	5.06 ± 0.53 (++)	4.64 ± 0.31 (++)	4.22 ± 0.27 (++)	0.084	0.335	0.211
<b>Lipase (mm, grade)</b>	2.89 ± 0.43 (++)	2.97 ± 0.27 (++)	2.81 ± 0.42 (++)	0.838	0.627	0.872
<b>Gelatinase (mm, grade)</b>	<b>3.49 ± 0.19 (++)</b>	3.03 ± 0.18 (++)	<b>2.77 ± 0.17 (++)</b>	0.072	0.218	<b>0.001</b>
<b>Hemolysin (mm, grade)</b>	0 mm (-)	0 mm (-)	0 mm (-)	None	none	none
<b>Chitinase (mm, grade)</b>	0 mm (-)	0 mm (-)	0 mm (-)	None	none	none

**Effect of shaking conditions on the growth of *V. parahaemolyticus***

The 8-hour bacterial growth in BHI 2.5% NaCl, 30°C, pH 8.5 and different shaking speeds was presented in Figure 2. As can be seen, there were significant differences in the growth of *V. parahaemolyticus* among 3 treatments of shaking conditions ( $p < 0.05$ ). The growth of *V. parahaemolyticus* increased over the increase of shaking speed (Figure 2). The DO concentration, as expected was larger in high shaking speed culture compared to the low ones. However, after 3 hours of cultures, there were no difference in

DO concentration among the 3 shaking conditions.

**Effect of shaking condition on extracellular enzymatic activity of *V. parahaemolyticus***

It was observed that lipase activity significantly increased when increasing the shaking condition from 120 to 240 rpm ( $p < 0.05$ ) while caseinase, lecithinase and gelatinase did not exhibit any significant variations following an increase the shaking speed (Table 2). Just like in the case of salinity, *V. parahaemolyticus* XN9 did not express any hemolysin and chitinase activity (Table 2).

**Table 2.** Effect of shaking condition on extracellular enzymatic activity of *V. parahaemolyticus* XN9. The testing shaking speed was 0, 120, 240 rpm and the enzymatic activity EA was presented in millimeter (mm).

Enzymatic activity (EA)	0 rpm	120 rpm	240 rpm	T-test between T <sub>0 rpm</sub> and T <sub>120 rpm</sub> (p value)	T-test between T <sub>120 rpm</sub> and T <sub>240 rpm</sub> (p value)	T-test between T <sub>0 rpm</sub> and T <sub>240 rpm</sub> (p value)
Caseinase (mm, grade)	1.42 ± 0.17 (+)	1.47 ± 0.31 (+)	1.33 ± 0.05 (+)	0.826	0.565	0.523
Lecithinase (mm, grade)	4.27 ± 0.55 (++)	4.64 ± 0.31 (++)	4.08 ± 0.38 (++)	0.137	0.199	0.648
Lipase (mm, grade)	3.09 ± 0.18 (++)	<b>2.97 ± 0.27 (++)</b>	<b>3.41 ± 0.27 (++)</b>	0.592	<b>0.008</b>	0.177
Gelatinase (mm, grade)	3.22 ± 0.13 (++)	3.03 ± 0.18 (++)	2.98 ± 0.08 (++)	0.388	0.765	0.06
Hemolysin (mm, grade)	0 mm (-)	0 mm (-)	0 mm (-)	none	none	none
Chitinase (mm, grade)	0 mm (-)	0 mm (-)	0 mm (-)	none	none	none

**DISCUSSION**

With ability to live either freely, parasitically or pathogenically, *V. parahaemolyticus* seems to be very flexible and responds actively to its surrounding environment. Understanding how this pathogen responds to different conditions is crucial to understand its pathogenesis. Culture conditions, no doubt, can highly affect the growth as well as the virulence of *V.*

*parahaemolyticus*.

Salt is an absolute requirement for the bacterial survival and production of virulence factors, especially the capacity of causing EMS/AHPND disease in shrimps. Due to its halophilic habitat. *V. parahaemolyticus* is capable of growth at 1.0 to 9.0% NaCl (Whitaker *et al.*, 2010). Our previous data indicated that *V. parahaemolyticus* can optimally survive in the

media (TSB, BHI, LB, MB) containing 2.0 to 3.0% NaCl (Anh *et al.*, 2018). In this work, we investigated different salt concentrations for BHI only and it was observed that the growth of *V. parahaemolyticus* was similar between 2.5 and 3.0% NaCl condition while significantly different to 2.0% NaCl. At 2.0% NaCl, *V. parahaemolyticus* grew a bit more slowly but reached higher density. Interestingly, at 2.0% NaCl, *V. parahaemolyticus* also expressed significantly high activity of gelatinase than in higher salt concentrations ( $p < 0.05$ ). Other tested extracellular enzymes did not show significant correlation with salinity. Lipase, caseinase and particularly lecithinase were expressed constantly and strongly for *V. parahaemolyticus* in any salinity. Therefore, it can be suggested that the three enzymes are kind of important for the growth and survival of *V. parahaemolyticus*.

The growth and biosynthesis of *V. parahaemolyticus* XN9 were also strongly influenced by different shaking conditions. As expected, the growth of *V. parahaemolyticus* was most limited in case of no shaking. It was quite understandable as suitable shaking speed created more liquid surface area, increasing oxygen transfer to the bacteria culture medium, resulting in the escalation of bacteria growth rate. However, it would be interesting to look at the corresponding DO concentration data in correlation with bacterial growth. It was shown that the differences in DO concentration were remarkable in the first 3 hour of cultures among three shaking conditions, however diminished afterward. It suggested that once the bacteria reached high density, the DO consumption was super quickly and shaking condition could not provide extra visible difference.

Although growth of *V. parahaemolyticus* was clearly different among different shaking condition, the enzymatic activities, in contrast, did not change much except for lipase. Caseinase, gelatinase and lecithinase were still well expressed in different shaking conditions. Lipase activity increased significantly when shaking speed increased from 120 rpm to 240 rpm. In short, shaking at 240 rpm gave the best

growth as well as highest lipase activity of *V. parahaemolyticus*.

We did not observe in any case the production of hemolysin and chitinase. That *V. parahaemolyticus* XN9 did not show hemolytic characteristics was predictable as hemolytic activity is much more frequently found in clinical isolates but not environmental ones (Bhowmik *et al.*, 2014). Chitinase, on the other hand, generally plays an important role in the degradation of the crustacean rich biopolymer chitin, converting it into nutrients for marine bacteria (Frederiksen *et al.*, 2013). It is of course not present in all *V. parahaemolyticus* strains (Hirano *et al.*, 2019). However, the absence of chitinase in AHPND *V. parahaemolyticus* XN9 indicated that chitinase might not be essential in shrimp pathogenesis or induced *in vitro*.

## CONCLUSION

Our data suggested that salinity and shaking condition are two crucial factors affecting not only the growth but also extracellular enzymatic activity of *V. parahaemolyticus*. For the salinity, 2.0% NaCl concentration gave the best growth as well as gelatinase activity of *V. parahaemolyticus* XN9. For the shaking condition, 240 rpm showed the best growth and lipase activity of the bacteria. In general, while caseinase, gelatinase, lecithinase and lipase were constantly expressed in *V. parahaemolyticus*, activity of hemolysin and chitinase was not observed.

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## TÁC ĐỘNG CỦA NỒNG ĐỘ MUỐI VÀ ĐIỀU KIỆN LẮC TỚI SỰ PHÁT TRIỂN VÀ ĐỘC TÍNH CỦA *VIBRIO PARAHAEMOLYTICUS*

Trần Ngọc Mỹ Hạnh, Trần Vân Nhi, Nguyễn Thị Thu Hoài

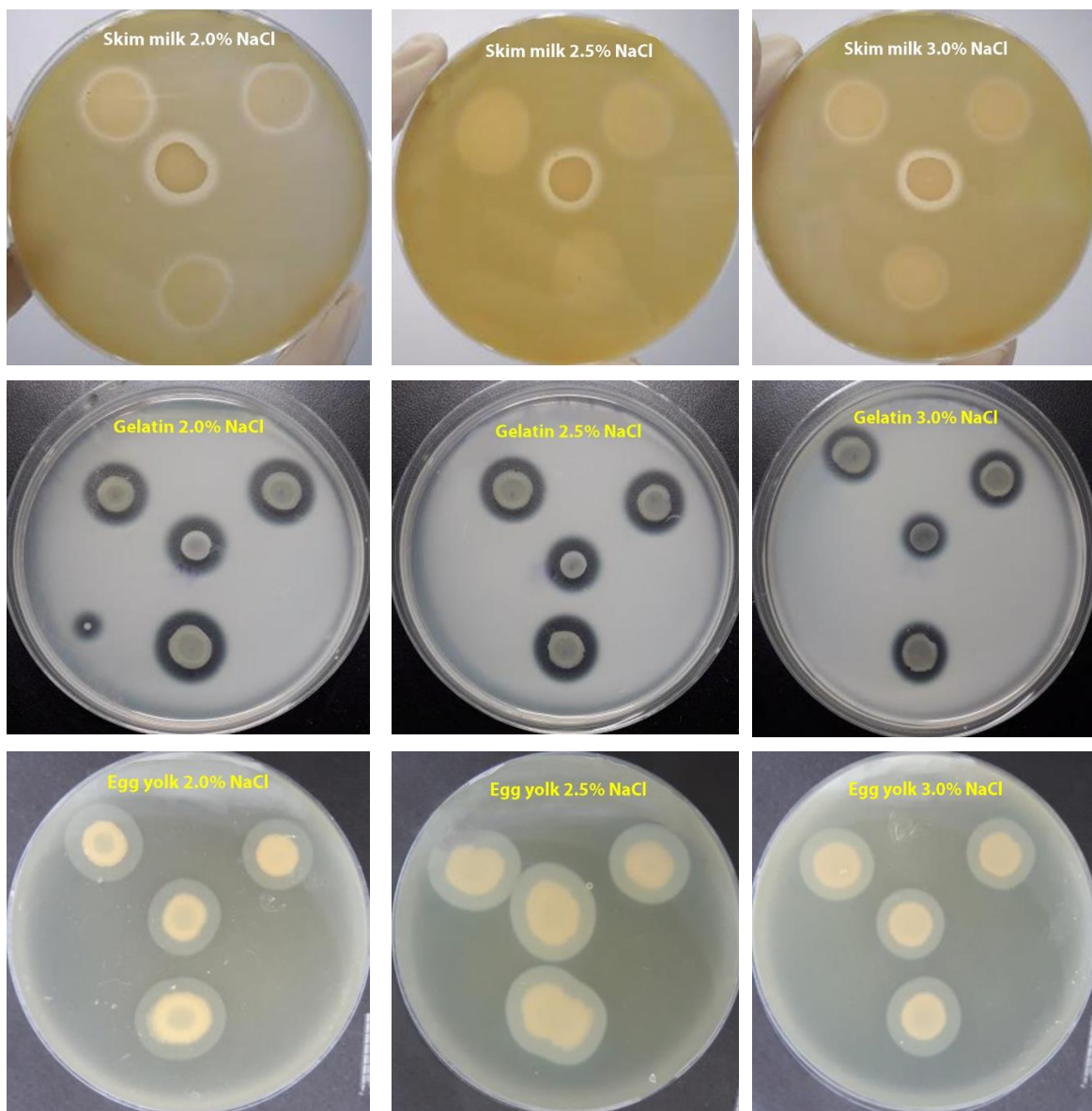
Trường Đại học Quốc tế, Đại học Quốc gia Thành phố Hồ Chí Minh

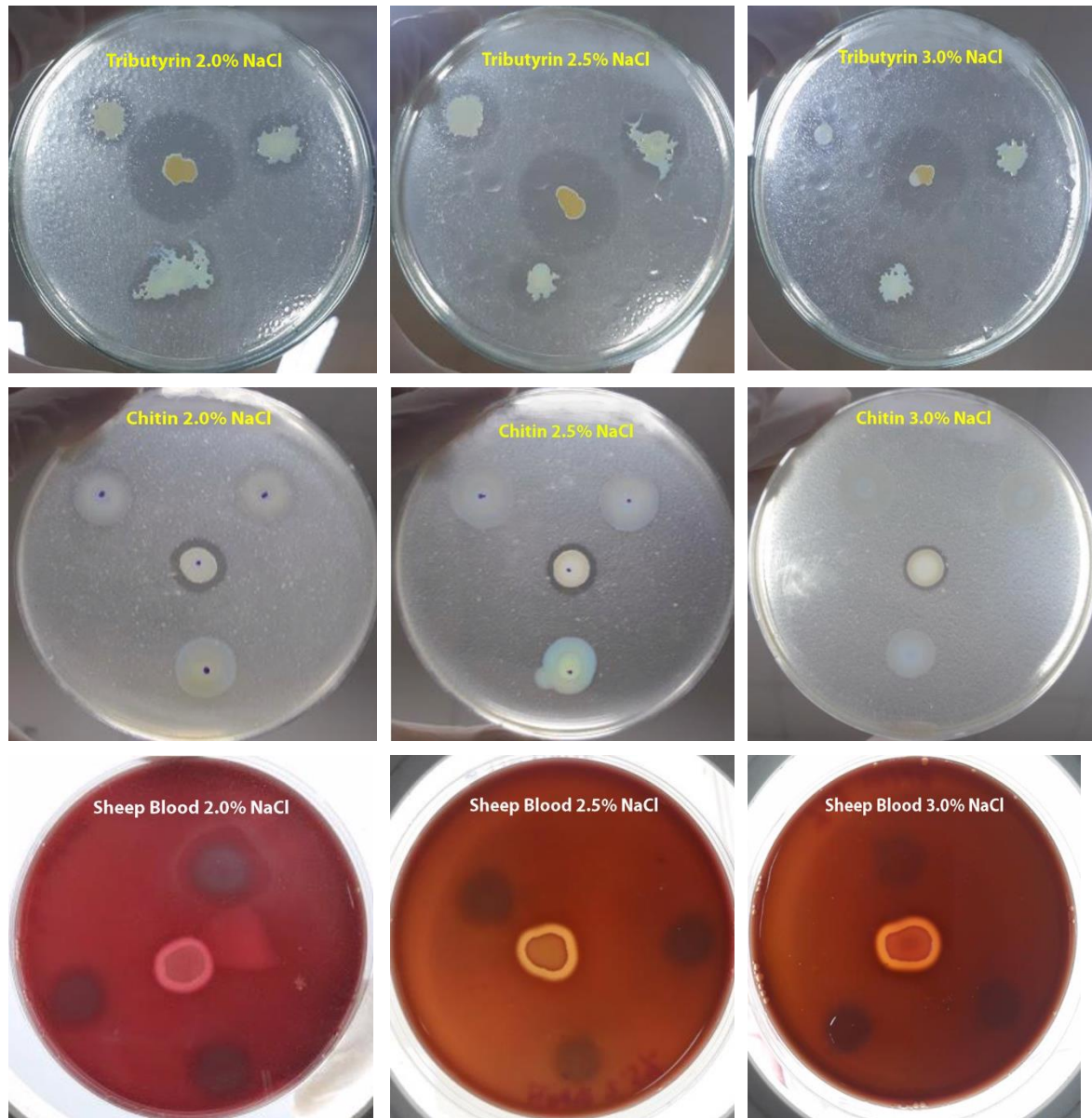
### TÓM TẮT

*Vibrio parahaemolyticus* là vi khuẩn Gram - âm, ưa muối, rất phổ biến ở vùng nước mặn, nước lợ và ven biển. Vi khuẩn này là tác nhân chính gây hội chứng chết sớm hay còn gọi là bệnh hoại tử gan tụy cấp tính, gây ra thiệt hại lớn cho nuôi trồng tôm. Để phòng chống hiệu quả các đợt bùng phát bệnh do vi khuẩn này gây ra thì rất cần thiết có những hiểu biết về ảnh hưởng của môi trường sống tới sinh trưởng và độc tính của loài vi khuẩn này. Vì vậy, chúng tôi tiến hành nghiên cứu sự tác động của tốc độ lắc và các nồng độ muối khác nhau tới sự sinh trưởng và độc tính của *V. parahaemolyticus*. *V. parahaemolyticus* XN9 được nuôi cấy trong môi trường dinh dưỡng BHI chứa các nồng độ muối 2,0, 2,5 và 3,0% với các điều kiện lắc 0, 120 và 240 rpm. Sự sinh trưởng của vi khuẩn được theo dõi trong 8 giờ và 6 chất tiết ngoại bào gồm caseinase, hemolysin, lecithinase, lipase, gelatinase và chitinase được phân tích sử dụng phương pháp khuếch tán đĩa thạch. Kết quả cho thấy, sự phát triển của *V. parahaemolyticus* bị tác động lớn bởi tốc độ lắc và nồng độ muối. Vi khuẩn sẽ phát triển tốt nhất ở điều kiện muối 2,0 % và lắc 240 rpm. Không có sự thay đổi lớn về các hoạt tính enzyme ngoại bào trong các điều kiện muối, ngoại trừ sự suy giảm đáng kể của enzyme gelatinase khi nồng độ muối tăng, thay đổi từ  $3.49 \pm 0.19$  tới  $2.77 \pm 0.17$  mm ( $p < 0.05$ ). Mặt khác, với tốc độ lắc, lipase là enzyme có sự tăng cường hoạt tính khi tốc độ lắc tăng lên ( $p < 0.05$ ). Trong khi các enzyme caseinase, lecithinase, gelatinase và lipase thể hiện hoạt tính tốt ở *V. parahaemolyticus*, hoạt tính hemolysin và chitinase lại không quan sát được ở chủng nghiên cứu. Như vậy, dữ liệu thu được cho thấy nồng độ muối 2,0 % và tốc độ lắc 240 rpm cung cấp điều kiện tối ưu cho sinh trưởng của *V. parahaemolyticus* và cho kết quả hoạt tính gelatinase và lipase cao nhất.

**Từ khóa:** *Vibrio parahaemolyticus*, bệnh hoại tử gan tụy cấp tính, độc lực, enzyme ngoại bào, nồng độ muối, sinh trưởng, tốc độ lắc

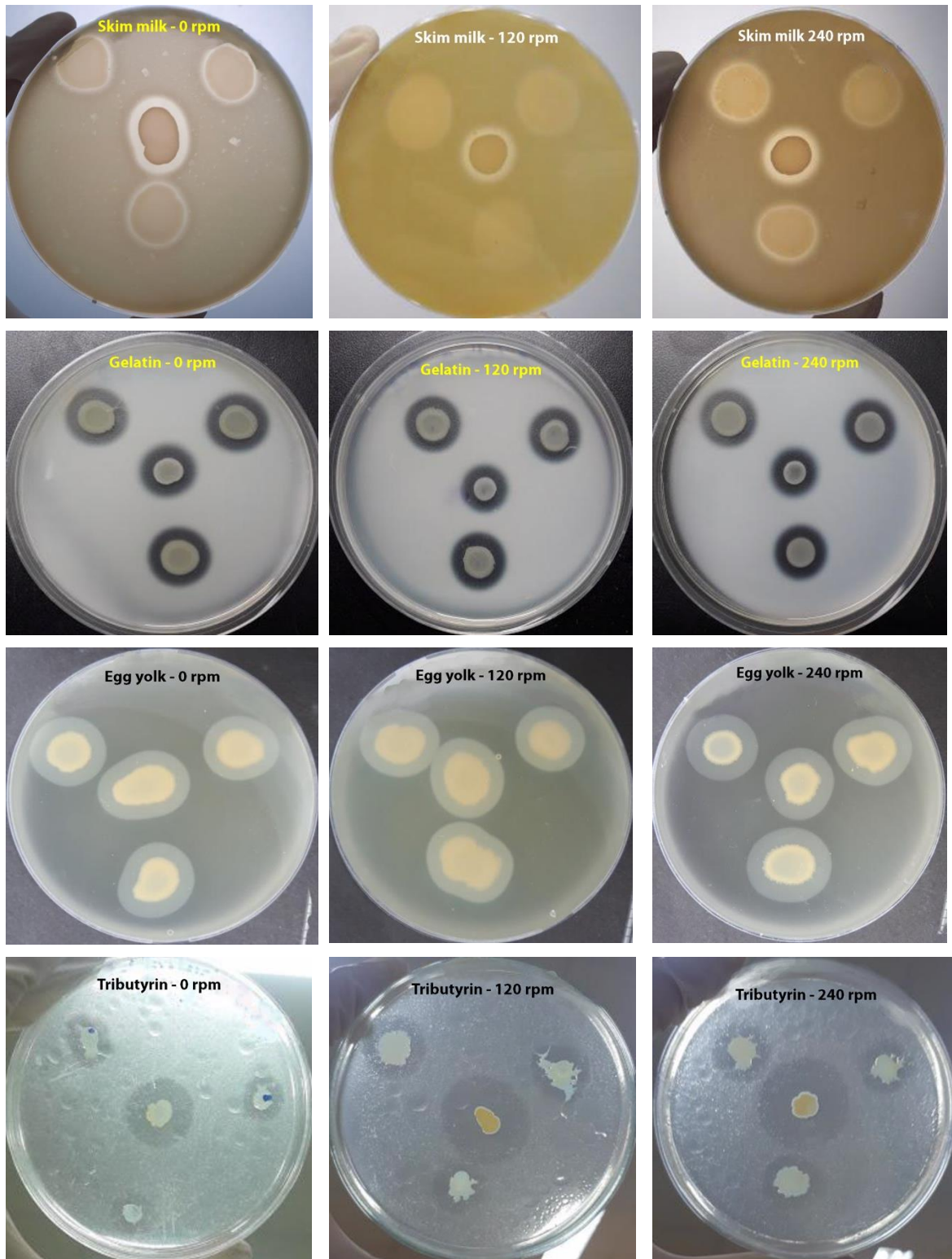
**Supplementary figures**

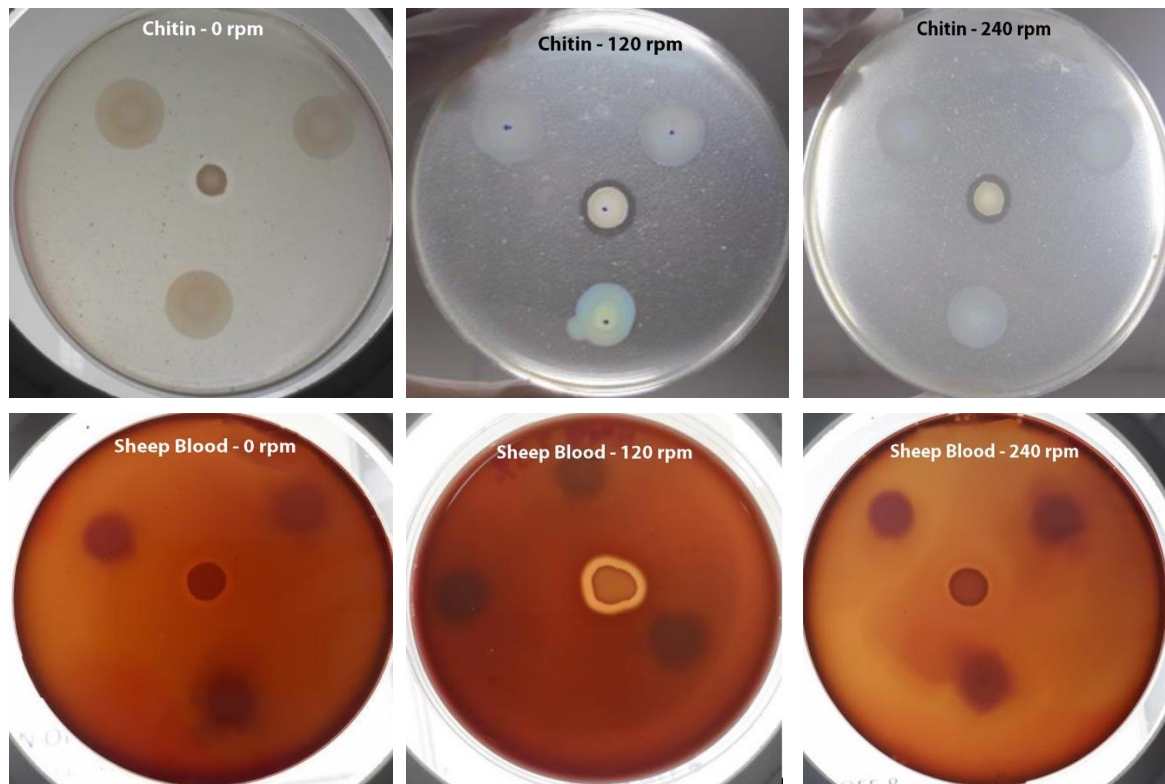




**Figure S1.** Effect of salinity on extracellular activity of *V. parahaemolyticus*. Representative images of agar-based testing for: from up to down: caseinase, gelatinase, lecithinase, lipase, chitinase and hemolysin; from left to right, 2.0; 2.5 and 3.0% NaCl. On each agar plate, positive control is loaded in the middle surrounded by 3 technical replicated droplets of *V. parahaemolyticus* XN9.







**Figure S2.** Effect of shaking speed on extracellular activity of *V. parahaemolyticus*. Representative images of agar-based testing for: from up to down: caseinase, gelatinase, lecithinase, lipase, chitinase and hemolysin; from left to right, 0; 120 and 240 rpm. On each agar plate, positive control is loaded in the middle surrounded by 3 technical replicated droplets of *V. parahaemolyticus* XN9.