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Dunaliella salina as a Protein Expression System for the Expression of the Endolysin Lysqdvp001 Against *Vibrio parahaemolyticus*

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Abstract:

Vibrio parahaemolyticus is a gram-negative, halophilic marine pathogen that is the major cause of infections and death in aquaculture. Consumption of undercooked or raw infected seafood causes gastroenteritis in humans. Because of this rapid rise in the number of cases of vibriosis in fish and Acute hepatopancreatic necrosis disease (AHPND) in crustaceans, the dependence on antibiotics has been on the rise, leading to the occurrence of more antibiotic-resistant bacterial strains. Endolysin, a lysin derived from bacteriophage is a great alternative to antibiotics. The fact that both endolysins and the bacteriophage are both species-specific, makes them even better replacement to antibiotics. In this study, the endolysin Lysqdvp001 which has been shown to be effective against *Vibrio parahaemolyticus*, is expressed and purified from *E. coli.* Further, endolysin Lysqdvp001 is also expressed in *Dunaliella salina,* a microalgae that is a cheap, easy to culture protein expression system. *Dunaliella salina* has many nutritional benefits because of the high concentration of β-carotene. Both the purified Lysqdvp001 and the *Dunaliella salina* expressed Lysqdvp001 are shown to have good antimicrobial properties against *Vibrio parahaemolyticus*.

Keywords: Endolysin, *Vibrio parahaemolyticus, Dunaliella salina*, Lysqdvp001, Vibriosis.

Introduction

Aquaculture is one of the most rapidly growing industries which is a major source of aquatic- based food. However, due to the increase in aquaculture worldwide, diseases affecting aquatic species have also been increasing. The most prevalent and notorious one being the bacterial diseases caused mainly by bacteria belonging to the *Vibrio* species (1,2).

Vibrio parahaemolyticus is a halophilic, gram-negative bacteria that is mainly found in warm marine conditions. It is one of the leading causes of all aquatic-related infections and also infects human beings when raw or uncooked seafood is consumed. *Vibrio parahaemolyticus* is known to infect both fishes (causing vibriosis) and crustaceans (causing acute hepatopancreatic necrosis disease) (3). Like most bacterial infections, vibriosis too was traditionally combatted using antibiotics. But the over use of antibiotics has led to the development of antibiotic resistant bacteria that poses a danger to both aquatic and human health (4). This increase in antibiotic resistant bacteria has led to an urgent need for alternative ways to fight off such bacterial infections such as using plant extracts (5) and various other methods (6). One such strategy is using either bacteriophages or its derivative lysins such as endolysins, which are known to be effective against specific bacteria.

Bacteriophages and endolysins are already being used as effective control agents against many bacterial diseases (7,8). One such bacteriophage is qdvp001, which is specific against the bacteria *Vibrio parahaemolyticus.* The main endolysin derived from this phage is called Lysqdvp001 which has been characterized and is known to have strong lytic activity against *Vibrio parahaemolyticus* (9). *Vibrio parahaemolyticus* has a thick peptidoglycan outer membrane that make it difficult for endolysins to act effectively from outside, to counteract this (10) suggested adding cationic amino acids to the endolysins. In order to increase the sustainability and for easier administration of endolysin a proper expression system is required. One such system is the microalgae *Dunaliella salina.* It is a green unicellular, halophilic microalgae that thrives in salty waters like salt water lakes and many marine habitats. While many micro and macroalgae are already used for the control of various pathogens, the concept of expressing a protein in algae itself is a fairly new idea (11). *Dunaliella* is widely studied for its potential use in food and cosmetic industry because of its ability to produce high levels of beta carotene and antioxidant properties (12). *Dunaliella* is also known to be easy to cultivate and combining with its nutritional benefits, makes it a novel and effective platform for recombinant protein expression (13,14).

The current study compares the difference in efficiency between pure Lysqdvp001 and the Lysqdvp001 expressed in Dunaliella salina against Vibrio parahaemolyticus. For this purpose, Lysqdvp001 was first expressed in *Escherichia coli* BL21 DE3 and purified. It was then expressed in *Dunaliella salina* by using the vector pMDC45. Our biochemical results showed that *Dunaliella salina* expressed endolysin, Lysqdvp001 exhibit antimicrobial properties which is comparable to the *E. coli* purified endolysin.

Materials and Methods

Strains, gene and vectors

Vibrio parahaemolyticus ATCC 17802 was purchased from American type culture collection. The lyophilized culture was stored in -80℃ deep freezer until further use. The culture was revived in a Mueller Hinton broth containing 3% NaCl and was allowed to grow overnight at 37℃. *E. coli* BL21 DE3 competent cells were purchased from TAKARA which were used for expression of the endolysin Lysqdvp001 and stored in -80℃ deep freezer. The gene sequence for the endolysin gene (ORF 60) was retrieved from Genbank-NCBI and the synthesized gene was obtained from Macrogen, Korea.

Dunaliella salina was obtained from ICAR-CIBA. Filtered seawater supplemented with Walnes/Conway medium, thiamin HCl (0.1µg/100ml) and cyanocobalamin (0.1µg/100ml) was used as a growth medium. The cells were grown at 25℃ with 12 hour lights on/off cycle in a static condition.pET-28a was used for expression in *E.coli* BL21. pENTR/D-TOPO vector (Thermofisher scientific, USA) was used as an entry vector for Gateway cloning. pMDC45 which has a CaMV35s promoter was used as a destination vector.

Cloning, expression and purification of lysqdvp001

The ORF60 gene was amplified using gene- specific primers that were obtained Xetra biosolutions, Coimbatore, India:

The underlined portions denote the *BamH1* and *Xho1* restriction sites. A 7 cationic amino acid sequence was added to the reverse primer (highlighted region in Table1). The amplified gene was cloned between the *BamH1* and *Xho1* sites of the plasmid pET -28a+. The gene containing plasmid was then transformed into *E.coli* BL21 DE3 competent cells using heat shock method and was grown in Luria Bertani broth containing 100µg/ml kanamycin. Once

Table 1: Gene-specific primers for the ORF60 gene. The underlined portions are restrictions corresponding to *BamH*1 and *Xho*1 in forward and reverse primers respectively. The highlighted region is a 7 cationic amino acid

Forward primer	5'- CGGGATCCAATGACTTTAATTCGTAAGGGTAGTCG -3'
Reverse primer	5'-CCGCTCGAGGCGCATGCGCAGGGGCGAAGATGAAGACGAATTAAGCTTCG TTATTACTAGTTACATCTGA -3'

the broth reaches 0.4-0.6 OD (log phase) at 600nm, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to make a final concentration of 500µM to induce the expression of the endolysin Lysqdvp001. After addition of IPTG, the cells were incubated at 16℃ overnight at 150rpm. The overnight culture was centrifuged and the pellet was resuspended in phosphate buffered saline (PBS). The cells were then disrupted by ultrasonication and the endolysin was purified by using the His60 Ni gravity columns (TAKARA, China) according to manufacturer's instructions. The purified protein was then stored in -80℃ deep freezer. The concentration of the purified endolysin was determined by using Bradford's assay (15). SDS-PAGE was run to verify the presence and size of the endolysin Lysqdvp001.

Cloning and expression of protein in *Dunaliella salina*

The endolysin gene ORF60 was amplified using gene-specific primer, but the forward primer has the sequence CACC at the 5' end to ensure that the PCR product is inserted into the topo vector in the right orientation. The pENTR-D-TOPO cloning kit (Thermofischer scientific) helps in inserting the PCR amplified ORF60 gene into the TOPO vector that acts as an entry vector for the gateway cloning process(16). To confirm that the ORF60 gene has been cloned into the TOPO vector in the correct orientation, M13 forward and reverse primers are used along with the gene specific primers.

Table 2: Primers used for orientation confirmation of ORF60 gene in TOPO vector.

M13 forward: Gene specific reverse:	5´-GTAAAACGACGGCCAG-3´ 5'CCGCTCGAGGCGCATGCGCAGGGGCGAAGATGAAGACGAATTAAGCTTCGT TATTACTAGTTACATCTGA -3'
Gene specific forward: M13 reverse:	5'- CGGGATCCAATGACTTTAATTCGTAAGGGTAGTCG -3' 5´-CAGGAAACAGCTATGAC-3´

The plant vector pMDC45 is used as the destination vector and by using the LR clonase enzyme (INVITROGEN, USA), the endolysin gene ORF60 is transferred from the entry TOPO vector to the destination pMDC45 vector by incubating the said mixture at 25℃ for 1 hour. The pMDC45 plasmid with the ORF60 gene is then transformed into pre-prepared electrocompetent *Dunaliella salina* cells which were prepared according to the published protocol and grown in the medium containing hygromycin (50µg/ml) at 25℃ for 15 days. To confirm the presence of the ORF60 gene, the genomic DNA was isolated by using the CTAB method (17). PCR using gene-specific primers were done to confirm the presence of ORF60 gene in the transformed *D.*

salina cells. The *Dunaliella salina* cells are then centrifuged and the pellet is resuspended in PBS for ultra sonification and extraction of protein. SDS-PAGE was run to determine the presence of the expressed Lysqdvp001 endolysin.

Turbidity reduction assay

Turbidity reduction assay was carried out for both purified Lysqdvp001 and *Dunaliella salina* expressed Lysqdvp00. An overnight culture of *Vibrio parahaemolyticus* was grown at 37℃ at 150rpm. The overnight culture was centrifuged and the pellet was treated with EDTA for 5 minutes, then washed with water and stored in a freezer. This treated pellet was suspended in a tris-HCl, pH 8.2 buffer. To 100µl of cell suspension, equal volumes of samples were added. Tris buffer was used as a negative control, lysosyme was used as a positive control, 1mg/ml of purified Lysqdvp001 and 100µl of algal expressed protein extract were used. The OD was measured at 450nm (18) for every 5 minutes for a total of 30 minutes.

Zone of inhibition

The inhibition zones of both the purified Lysqdvp001 and the algal expressed Lysqdvp001 were carried out according to (10) Lysqdvp001-5aa, Lysqdvp001-10aa and Lysqdvp001-15aa, were designed based on lysin Lysqdvp001 from Vibrio parahaemolyticus (V. parahaemolyticus. *Vibrio parahaemolyticus* was cultivated at 37℃ overnight at 150 rpm. Sterile oxford cups were used to punch even holes on MH agar. Buffer was used as negative control, gentamicin (50µg/ml) was used as positive control (19) 1 mg/ml of purified Lysqdvp001, algal expressed lysqdvp001 and protein extract of wild type *Dunaliella salina* with no recombinant protein was added as control. The plates were then incubated at 37℃ for 24hours.

Antibacterial activity against *Vibrio parahaemolyticus*

The efficiency of both the purified protein and the algal-expressed protein was analysed by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), both of which were done by broth microdilution. *Vibrio parahaemolyticus* was inoculated in its growth medium and incubated at 37℃ overnight at 150rpm. In a ratio of 1:200, the overnight culture was mixed with the growth medium containing negative control (buffer), positive control (gentamicin) (19)which is an important pathogen in aquatic animals worldwide, the antimicrobial activity of CS-GT and the effects of a CS-GT dose on the intestine histopathology and intestinal flora of V. parahaemolyticus-infected shrimps were explored. The results showed that CS-GT possessed broad-spectrum antibacterial activity, with minimum inhibitory concentration (MIC, algae expressed Lysqdvp001 and purified Lysqdvp001 in a 96 well microassay plate. Gentamicin was used from a concentration of 200-0.40µg/ml. Lysqdvp001 was used from a concentration of 2000-2µg/ml, the extracted algal protein was added serially. Since the *Dunaliella salina* expressed Lysqdvp001was a crude extract, the crude extract with the Lysqdvp001 was used to see MIC and MBC. *Dunaliella salina* expressed Lysqdvp001 was used in concentrations of 5 %(v/v) - 0.15 %(v/v). It was incubated at 37℃ for 24 hours and then the plates were read using a microtiter plate reader at 660nm. 100µl of these were then inoculated onto MH agar plates and incubated at 37℃ for 24 hours.

Results and Discussion

Synthesis, expression and purification of lysqdvp001

The 0.7 kb ORF60 gene which encodes the endolysin Lysqdvp001 was synthesised and was inserted into the vector pET 28a and transformed into *E.coli* DH5α. The presence of the ORF60 gene in *E.coli* was confirmed by PCR using the gene-specific primers. Fig 1A shows the presence of the desired gene fragment in pET-28a vector. This pET-28a vector containing the ORF60 gene was then transformed into *E.coli* BL21 DE3 cells for protein expression.

IPTG was used for protein induction. The expressed protein was purified using a His60 Ni gravity columns. SDS-PAGE shows the presence of Lysqdvp001 in both crude as well as purified sample as shown in Fig 1B. Upon doing Bradford's assay, the concentration of the purified endolysin Lysqdvp001 was calculated to be 27.3mg/ml.

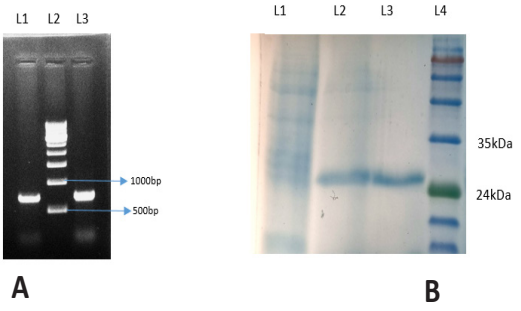

Fig 1: A- PCR confirmation for the presence of the ORF60 gene after inserting in pET-28a plasmid. L1 and L3 shows bands at ~700bp (The size of the ORF60 gene is 711bp). L2 is a 500bp ladder. B- is the SDS-PAGE where L1 is the crude protein from a non transformed *E.coli* BL21, L2 is the crude protein with Lysqdvp001 which shows the expected prtein size of 26kDa, L3 is the purified protein and L4 is the medium ranged protein ladder.

Cloning and expression of lysqdvp001 in *Dunaliella salina*:

In order to express the ORF60 gene in *Dunaliella salina,* the gene fragment was cloned into TOPO vector which acts as the entry vector for Gateway cloning. The orientation of the gene in TOPO vector is confirmed by using the M13 primers as shown in Fig 2A. With the help of LR clonase, the ORF60 gene was then transferred into the destination vector pMDC45 through homologues recombination. This was confirmed by PCR as shown in Fig 2B. The pMDC45 with ORF60 was introduced into *Dunaliella salina* through electroporation. The genomic DNA was isolated and PCR was carried out to identify the presence of transgene (ORF60 gene) in the *Dunaliella* genome (Fig 2C).

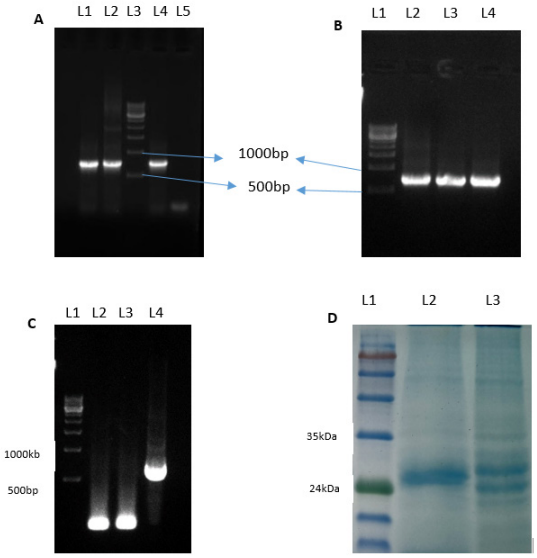

Fig 2: A- Orientation confirmation of ORF60 in TOPO using M13 primers using PCR. L1 is PCR product of M13 forward with gene specific reverse primer, L2 is gene specific forward and M13 reverse primer, L3 is a 500bp ladder, L4 is the positive control where the synthesised gene was used as template. B- PCR confirmation after ORF60 gene was was inserted into pMDC45 plasmid, L1 is 500bp ladder, L2 L3 L4 show positive bands. C- PCR confirmation of presence of ORF60 gene in genomic DNA of transformed *Dunaliella salina*. L1 is 500bp ladder, L2 and L3 show no bands, L4 shows presence of ORF60 gene in the genomic DNA of transformed *Dunaliella salina*. D- SDS-PAGE of extracted protein from *Dunaliella salina*, L1 is a medium ranged protein ladder, L2 is the crude protein extract from transformed *Dunaliella salina* which shows the expected band at ~26kDa, L3 is the crude protein extract from wild type *Dunaliella salina*

Turbidity reduction assay

The OD of the EDTA treated *Vibrio parahaemolyticus* cells with added controls, purified Lysqdvp001 and algal expressed Lysqdvp001 were measured at 450nm. Fig 3 shows the lytic activity of both the purified Lysqdvp001 and the

algal expressed Lysqdvp001were significant. This was similar to the results of the positive control, Lysosyme.

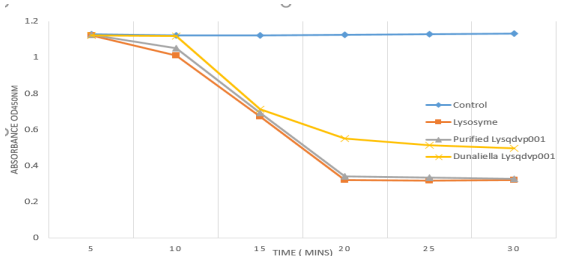

Fig 3: The graph shows the lytic activity of both Lysqdvp001 and *Dunaliella salina* expressed Lysqdvp001 against EDTA treated *Vibrio parahaemolyticus*. The lytic activity was measured for 30 minutes at 25℃.

Zone of inhibition

The antimicrobial activities of both purified Lysqdvp001 and *Dunaliella salina* expressed Lysqdvp001 were evaluated by measuring the zone of inhibition. As shown in Fig 4 both purified endolysin and algal expressed endolysin, both showed significant lytic activity. Their zones of inhibition were similar to the zone of inhibition of the positive control gentamicin.

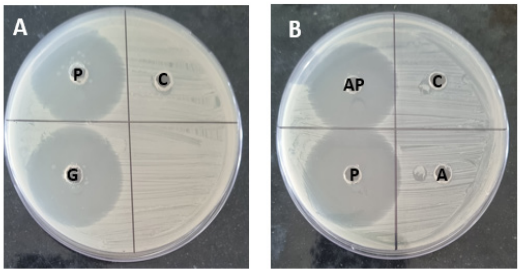

Fig 4: A- shows the zone of inhibition of G-gentamicin, P-purified protein and C- control(water). B- Zone of inhibition of AP- *Dunaliella* expressed Lysqdvp001, P- purified Lysqdvp001, A- wild type *Dunaliella salina* extract , C- control

Anti-microbial activity against *Vibrio parahaemolyticus* (MIC and MBC)

MIC and MBC were measure to calculate the antibacterial activity of both purified Lysqdvp001 and algal expressed Lysqdvp001. The minimum concentration at which no growth of new cells were found is known as the minimum inhibitory concentration. The MIC for purified Lysqdvp001 was 0.25mg/ml and when these were plated, the minimum concentration at which no colonies are formed is called the minimum bactericidal concentration. The MBC for the purified Lysqdvp001 was found to be to 1mg/ml. The MIC of *Dunaliella salina* expressed Lysqdvp001 was 1.25 % (v/v) and MBC was found to be 2.5 % (v/v) as shown in Table 3.

Table 3: Shows the MIC and MBC values of the positive control gentamicin, Purified Lysqdvp001 and *Dunaliella salina* expressed Lysqdvp001.

Sample	MIC	MBC
Gentamicin (control)	12.5µg/ml	25µg/ml
Purified Lysqdvp001	250µg/ml	1mg/ml
Dunaliella salina- Lysqdvp001	1.25%(v/v)	2.5%(v/v)

Discussion

Endolysins, for quite some time have been considered an alternative to antibiotics, as over exposure to antibiotics can lead to the emergence of multi-drug resistant bacteria (20). One such pathogen is *Vibrio parahaemolyticus*, which is the cause of majority of the bacterial infections in aquaculture and also a major cause of food borne illnesses. The specificity of bacteriophages against specific bacteria make its endolysins all the more important and effective as an alternative to antimicrobials (21). One such endolysin is Lysqdvp001 which is known to be very effective against *Vibrio parahaemolyticus* as shown by (9,10,22)However, unlike gram-positive bacteria, gram-negative bacte-

ria tend to have a thicker peptidoglycan outer membrane. In this study, a 7 cationic amino acid was added to the Lysqdvp001 to increase the hydrolytic activity. The cells were also treated with EDTA to increase the cell permeability and increase the effectiveness of Lysqdvp001. Apart from using EDTA there are also other methods such as adding hydrophobic peptides or by using organic acids (23,24)Using microalgae such as *Dunaliella salina* as an expression system has its advantages like low cost, ease of culturing and easy to manipulate transgenically (25). In this study, the gene ORF60 was transformed into electrocompetent *Dunaliella salina* cells (17) and then grown in a hygromycin supplemented media. The protein extracted from this *Dunaliella salina* containing Lysqdvp001 showed similar antimicrobial activity as the purified Lysqdvp001 as shown by the Fig 4B.

Collectively, this study shows that *Dunaliella salina* can be used as a very good eukaryotic system to express recombinant protein and the Lysqdvp001 endolysin expressed in *Dunaliella salina* has shown to have similar activity to the purified Lysqdvp001.

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