4. Results

4.1. Bacterial isolation

Based on series of biochemical tests for the identification of *V. anguillarum*, nine *V. anguillarum* isolates were isolated from estuarine finfish. All these isolates exhibited typical biochemical reactions as shown in table 10. All the isolates were identical with respect to their biochemical reactions. The isolates were recovered from Pearl-Spot fish (*Etroplus suratensis*) cultured in coastal ponds.

4.2. Molecular confirmation of *V. anguillarum* isolates

PCR amplification was carried out with crude lysate of nine isolates and one reference strain of *V. anguillarum* NB10 using the forward and reverse VaAMI-B primers (Table 2) which resulted in a product of the predicted length 429 bp while no products were obtained from other *Vibrio* strains (Figure 6).

4.3. Detection of *ompU* gene

PCR amplification with crude lysate of *V. anguillarum* NB10 using VaOMP-U F and VaOMP-U R1 primers resulted in a product of the predicted length 993 bp (Figure 7). The PCR product was cloned in pDrive Cloning Vector (Figure 8) and the positive clone was sequenced and submitted to the GenBank (Accession Number. FJ573227) and illustrated in Figure 9.

4.4. Detection of *ompK* gene

PCR amplification with crude lysate of *V. anguillarum* NB10 using VaOMP-K F1 and VaOMP-K R1 primers resulted in a product of the predicted length with 792 bp (Figure 10). The PCR product was cloned in pDrive Cloning Vector (Figure 11) and the positive clone was
sequenced and submitted to the GenBank (Accession Number. FJ705222) and illustrated in Figure 12.

4.5. Detection of \textit{ompV} gene

PCR amplification with crude lysate of \textit{V. anguillarum} NB10 using VaOMP-V F4 and VaOMP-V R3 primers resulted in a product of the predicted length of 774 bp (Figure 13). The PCR product was cloned in pDrive Cloning Vector (Figure 14) and the positive clone was sequenced and submitted to the GenBank (Accession Number. GU723694) and illustrated in Figure 15.

4.6. Deduction amino acid sequences and the predicted signal peptides

The amino acid sequence deductions revealed that signal peptide coding regions of OmpU, OmpK and OmpV are 330 amino acids, 263 amino acids and 257 amino acids respectively. The signal peptide of the OmpU and OmpK were predicted to be of 21 amino acids on the N terminal of respective proteins. However, OmpV had 20 amino acids signal peptide (Figure 16, 17 and 18).
<table>
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<th>M</th>
<th>M</th>
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<th>M</th>
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**Table 10. Biochemical reactions for V. anguillarum**

M, Motility; S, Sensitive
Figure 6. PCR amplification of *amiB* gene fragment using the forward and reverse VaAMI-B primers. Lane M: 100 bp DNA marker; Lane 1: negative control, Lane 2: *V. anguillarum* NB10; Lane 3: Va1; Lane 4: Va2; Lane 5: Va3; Lane 6: Va4; Lane 7, Va5: Lane 8, Va6; Lane 9: Va7; Lane 10: Va8; Lane 11: Va9.

4.7. Cloning in expression vector

The amplicons of *ompU*, *ompK* and *ompV* genes excluding signal peptide were cloned into pQE 30-UA vector and transformed to the competent *E. coli* SG 13009 host cells. After ampicillin–kanamycin selection, colonies were screened for the insert using the same primers and PCR conditions (details described before). Positive colonies were showed about 930 bp (Figure 19), 730 bp (Figure 20) and 714 bp (Figure 21) for *ompU*, *ompK* and *ompV* amplicons respectively.
Figure 7. PCR detection of the full length *ompU* gene in *V. anguillarum* using VaOMP-U F and VaOMP-U R1 primers. Lane M: 100 bp DNA marker; Lane 1: *E. coli* as negative control; Lane 2: *V. anguillarum* NB10 as positive control.

Figure 8. PCR amplification of full length *ompU* in recombinant QIAGEN EZ Cells using VaOMP-U F and VaOMP-U R1 primers. Lane M: 100 bp molecular marker; Lane 1: non recombinant QIAGEN EZ Cells; Lane2,3,4: positive clones of QIAGEN EZ Cells; Lane 5: *E. coli* as a negative control ; Lane 6: positive control (*V. anguillarum* NB10).
Figure 9. The complete nucleotide sequence of *ompU* gene in *V. anguillarum* strain NB10.

![Figure 9: Complete nucleotide sequence of ompU gene in V. anguillarum strain NB10.](image)

Figure 10. PCR amplification of the *ompK* gene in *V. anguillarum* using VaOMP-K F and VaOMP-K R1. Lane M: 100bp DNA molecular marker; Lane 1: *V. harveyi* as a positive control; Lane 2: *E. coli* as a negative control; Lane 3: *V. anguillarum* NB10.

![Figure 10: PCR amplification of ompK gene in V. anguillarum using VaOMP-K F and VaOMP-K R1.](image)
Figure 11. PCR amplification of full length *ompK* in the recombinant QIAGEN EZ Cells using VaOMP-K F and VaOMP-K R1 primers. Lane M: 100 bp DNA marker; Lane 1: non recombinant QIAGEN EZ Cells; Lane 2, positive clone of QIAGEN EZ Cells; Lane 3, *V. anguillarum* NB10 as a positive control.

```
001 ATGCCTAATACTTTTTAGCTCTAGGCCTAGTGGCTGCAACTTCTGCTCTGTATGGCC
061 GCTGACTATTCAGATGGCGACATCCATAAAAACGATTACAATGGAATGCAATTAAACCTA
121 ATGCCGCAATCGATGAAATTACAGGTGAATCATCGCATGATTACCTAGAGATGGAATTT
181 GCCGCCCCTCAGGGATTTTGATCTGTACGGTTACGTTGATATCTTTAACCTGCTAAGC
241 AACCAAGCAGTGACAAAGAAGGTAAAGAAAAAATCTTTATGAAATTTGCACCTCCTGTAG
300 TCACTAGATGCACTTTACAGGTAAAGATTTGTCTTTCGGGCCAGTGCAAGAGTTGTATGTC
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541 TCTTTTGAAATCTGCACTTTTACCTAAATGGAATGGGCTTAAACCTTTACGGTACTTATGAT
601 ATGGCCTGCAACATCGATGAAATTACAGGTGAATCATCGCATGATTACCTAGAGATGGAATTT
661 GCCGCCCCTCAGGGATTTTGATCTGTACGGTTACGTTGATATCTTTAACCTGCTAAGC
721 AACCAAGCAGTGACAAAGAAGGTAAAGAAAAAATCTTTATGAAATTTGCACCTCCTGTAG
781 TCACTAGATGCACTTTACAGGTAAAGATTTGTCTTTCGGGCCAGTGCAAGAGTTGTATGTC
```

800 bp
Figure 13. PCR amplification of full length \textit{ompV} gene in \textit{V. anguillarum} using \textit{vaOMP-V} F4 and \textit{vaOMP-V.R3} primers. Lane M: 100bp DNA molecular marker; Lane 1: negative control \textit{E coli}; Lane 2: positive control \textit{V. cholerae}; Lane 3: \textit{V. anguillarum} NB10 as a positive control.

Figure 14. PCR amplification full length \textit{ompV} gene in recombinant QIAGEN EZ Cells using \textit{vaOMP-V} F4 and \textit{vaOMP-V.R3} primers. Lane M: 100 bp molecular marker; Lane 1: non recombinant QIAGEN EZ Cells; Lane 2: positive clone of QIAGEN EZ Cells; Lane 3: \textit{V. anguillarum} NB10 as a positive control.
Figure 15. The complete nucleotide sequence of \textit{ompV} gene in \textit{V. anguillarum} strain NB10.
Figure 16. Deduced amino acid sequence of OMPU (signal peptide sequence is underlined).

Figure 17. Deduced amino acid sequence of OMPK (signal peptide sequence is underlined).

Figure 18. Deduced amino acid sequence of OMPV (signal peptide sequence is underlined).

Figure 19. PCR amplification of ompU in E. coli SG13009 using VaOMP-U F1 and VaOMP-U R1 primers. Lane M: molecular marker; Lane 1: non recombinant E. coli SG13009; Lane 2: positive clone E. coli SG13009; Lane 3: V. anguillarum as a positive control.
Figure 20. PCR amplification of \textit{ompK} in \textit{E coli} SG13009 using VaOMP-K F1 and VaOMP-K R1 primers. Lane M: 100 bp DNA molecular marker; Lane 1: non recombinant \textit{E coli} SG13009; Lane 2: positive clone \textit{E coli} SG13009; Lane 3: \textit{V.anguillarum} as a positive control

Figure 21. PCR amplification of \textit{ompV} in \textit{E coli} SG13009 using VaOMP- V F 3 and VaOMP-V R 3 primers. Lane M: molecular marker; Lane 1: non recombinant \textit{E coli} SG13009; Lane 2: \textit{V.anguillarum} as a positive control; Lane 3: positive clone \textit{E coli} SG13009
4.8. Expression, purification and concentration of recombinant proteins

Three recombinant outer membrane proteins (OmpU, OmpK and OmpV) were expressed after 4 h of 1 mM IPTG induction. The molecular weight of recombinant protein encoded by the *ompU* gene was estimated to be approximately 35 kDa by 15% SDS-PAGE (Figure 22). The molecular weight of OmpK recombinant protein was estimated to be approximately 28 kDa including 3 kDa of the vector sequence by 15% SDS-PAGE (Fig. 23). The molecular weight of recombinant protein encoded by the *ompV* gene was estimated to be 27 kDa by 15% SDS-PAGE (Figure 24). Purity of the recombinant proteins obtained was about 85% and the protein concentration was around 10 and 5 mg g\(^{-1}\) of cells on wet weight basis of OmpU and OmpK. The OmpV was expressed as 2 mg g\(^{-1}\) of cells on wet weight basis.

4.9. Titration of polyclonal antibodies

The titer of rabbit hyperimmune sera against OmpU, OmpK and OmpV recombinant proteins was determined by plate-ELISA, seven days after the fifth booster injection. The titers obtained against recombinant OmpU and OmpK were 1:32,000 and 1:16,000 against OmpV proteins.

4.10. Characterization of polyclonal antibodies by Western blotting

Rabbit polyclonal antibodies against OmpU, OmpK and OmpV recombinant proteins reacted with bands corresponding to 35 kDa, 28 kDa and 27 kDa sizes respectively with the cell lysates of IPTG induced recombinant *E. coli* SG13009 cells and with the purified recombinant proteins. No bands were observed in non-recombinant *E. coli* and uninduced recombinant *E. coli* (Fig. 25, 26, and 27).
Figure 22. Expression of recombinant OmpU protein. Lane M: molecular protein marker; Lane 1: non recombinant *E. coli* SG13009; Lane 2: non induced recombinant *E. coli* SG13009; Lane 3: IPTG induced recombinant *E. coli* SG13009; Lane 4: purified OmpU.
Figure 23. Expression of recombinant OmpK protein. Lane M: molecular protein marker; Lane 1: non recombinant *E. coli* SG13009; Lane 2: non induced recombinant *E. coli* SG13009; Lane 3: IPTG induced recombinant *E. coli* SG13009; Lane 4: purified OmpK.
Figure 24. Expression of recombinant OmpV protein. Lane M: molecular protein marker; Lane 1: non recombinant *E. coli* SG13009; Lane 2: non induced recombinant *E. coli* SG13009; Lane 3: IPTG induced recombinant *E. coli* SG13009; Lane 4: purified OmpV.

Figure 25. Western blotting detection for OmpU using polyclonal antibody. Lane 1: *V. anguillarum*; Lane 2: IPTG induced recombinant *E. coli* SG13009; Lane 3: non-induced recombinant *E. coli* SG13009; Lane 4: purified OmpU.
Figure 26. Western blotting analysis of OmpK using polyclonal antibody. Lane 1: V. anguillarum crud lysate; Lane 2: non induced recombinant Ecoli SG13009; Lane 3: IPTG induced recombinant E. coli SG13009; Lane 4: purified OmpK.

Figure 27. Western blotting detection of OmpV using polyclonal antibody. Lane 1: non recombinant E. coli SG13009; Lane 2: non induced recombinant E. coli SG13009; Lane 3: IPTG induced recombinant E. coli SG13009; Lane 4: purified recombinant OmpV.

4.11. *In silico* characterization of OmpU, OmpK, and OmpV

The secondary structure model of OmpU, OmpK and OmpV proteins (Figure 28, 29 and 30 respectively) showed the dominant of beta pleated sheet form and the absence of cysteine residues from the transmembrane beta-strands. The 3D model of OmpU, OmpK and OmpV (Figure 30,31 and 32 respectively) revealed their hollow barrel structure.

Phylogenetic analysis of proteins sequences showed close relationship of OmpU, OmpK and OmpV of *V. anguillarum* with that of *V. cholerae* (Figure 34, 35 and 36 respectively). The OmpK of *V. anguillarum* was also related to that of *V. vulnificus*.

Within OmpU protein, seven B-cell epitopes and 12 antigenic sites were predicted (Figure 37). Five B-cell epitopes and seven antigenic sites were predicted in OmpK (Figure 38). Only three B-cell epitopes and ten antigenic sites were seen in OmpV (Figure 39).
The estimated molecular weight of the expressed OmpU, OmpK and OmpV were 34.39 kDa, 28.3 kDa and 27 kDa respectively.

Figure 28. Secondary structure of *V. anguillarum* OmpU. 14 transmembrane beta strands with seven external loops that lacks cysteine are shown.
Figure 29. Secondary structure of *V. anguillarum* OmpK. 12 transmembrane beta strands with six external loops and two apparent internal loops are shown.
Figure 30. Secondary structure of *V. anguillarum* OmpV predicted. 10 transmembrane beta strands that lack cysteine, six external loops and two apparent internal loops are shown.
Figure 31. 3 D structure of OmpU generated using SWISS-MODEL. A hollow barrel porin like structure (14 strands) is revealed.
Figure 32. 3 D structure of OmpK generated using SWISS-MODEL. A hollow barrel porin like structure, 12 strands are shown.
Figure 33. 3-D structure of OmpV generated using SWISS-MODEL. A hollow barrel porin like structure, 10 strands are shown.
Figure 34. Phylogenetic tree of *V. anguillarum* OmpU with *Vibrio* spp. *V. anguillarum* OmpU relatedness with that of *V. cholerae* (ZP.01983620.1), *Vibrio.* metschnikovii (ZP.05880742.1), *Vibrio.* coralliilyticus (ZP.05884332.1) *V. vulnificus* (AEE.98101.1), *V. mimicus* (ZP.06039969.1), and *V. harveyi* (ACR.22854.1).

Figure 35. Phylogenetic tree of *V. anguillarum* OmpK with *Vibrio* spp. *V. anguillarum* OmpU relatedness with that of *V. cholerae* (ZP.01951173.1), *V. vulnificus* (NP.7606911.1), *V. mimicus* (ZP.05715986.1), *V. alginolyticus* (ZP.06180292.1) and *V. harveyi* (ADB.92036.1).
Figure 36. Phylogenetic tree of *V. anguillarum* OmpV relatedness with that of *V. cholerae* (ZP.01951173.1), *V. alginolyticus* (ZP.01259420.1), *V. metschnikovii* (ZP.05880742.1), *V. harveyi* (ZP.01985307.1), *V. coralliilyticus* (ZP.05887673.1) and *V. parahaemolyticus* (EGF.41059.1).

AGELYNQDGTSMGGAERLSLKDGKADDASRVRNLFLGTKVINDSGLYGVGFYE
GEFTADEGTDNKGDLDNAORYTYAGIGGNEGEVTGYKNDGALGVITDFTDIMSYHGN
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DKTGYELAAKYTMQAVFTSTYNLESKNSGAKKDADSFADVATYFFKPNFRSYIS
YFNLLSDKVGKAKSEDELALGLRYDF

Figure 37. OmpU amino acid residues showing predicted B-cell epitopes (green highlighted) and antigenic sites (yellow highlighted), their overlapping portions (purple highlighted) and the surface exposed regions (underlined).

ADYSGDGIHKNDYKWMQFNLMAAAIDELPGESSHDYLEMEEFGGRSIFDLYGYVDIFN
LNSPSDKEGKEKFMKFAPRMSLDALTGKDLSGFPVQELYVSTLMEEWGNSGVNTJ
QKVGLGSDVNVPFVKVGLNLYGTYDNEKDWNGFQISTNWFKPFYFFENGFSFISYQ
GYIDYQFGMDDKNTALKTSNGGAMFGNYWHSDRFAVGYGLKGYKDVYGLKDEGL
AGKTTGFHYLAVTYKF
Figure 38. OmpK amino acid residues showing predicted B-cell epitopes. (green highlighted) and antigenic sites (yellow highlighted), their overlapping portions (purple highlighted) and the surface exposed regions (underlined).

V. anguillarum count was obtained on TSA plates after incubation with serum of both vaccinated and non-vaccinated fish at different time points (Figure 40). The largest reduction was seen in case of fish vaccinated with heat killed bacteria after 15 days of boosting followed by the sera of the fish group that was vaccinated with OmpV.
Figure 40. Vaccinated fish serum inhibitory effect on the bacteria. *V. anguillarum* count (expressed as log10 CFU ml\(^{-1}\)) upon incubation with sera from *Labeo rohita* vaccinated OmpV, OmpK, OmpU, heat killed bacteria and control at different days post-vaccination. Column bars with asterisk indicate significant difference (p < 0.05) from the control at each sampling point.

### 4.12.2. Fish protection

The fish challenged with *V. anguillarum* showed 90% of mortality in the control fish group. The relative percentage of survival in the vaccinated fish was 44%, 62%, 67% and 67% for the fish vaccinated with OmpU, OmpK, OmpV and heat killed bacteria respectively.

### 4.13. Effect of culture conditions on the expression of *ompK* gene

#### Effect of incubation time

The growth curve study of *V. anguillarum* NB10 showed that it takes 5 h to reach log phase and 30 h to enter decline phase. On the basis of these results, 4 time points (5 h, 10 h, 20 h and 30 h) were taken to determine *ompK* gene expression. The highest significant
expression fold (11.36) of \textit{ompK} gene was found at 10 h time point. There was no significant difference seen in expression at 20 h and 30 h when compared to 5 h (Table 11).

**Effect of salt concentration**

Study on the effect of varying salt concentration on growth and OmpK expression of \textit{V. anguillarum} NB10 showed that NaCl up to 3\% has minimal effect (Table 12). Expression of \textit{ompK} at 2\% NaCl (2.74 fold) was higher than at 1\%, 3\% and 4\% of NaCl.

**Effect of bile salt concentration**

While growth of \textit{V. anguillarum} NB10 seemed to decrease with increasing bile salt concentration in the medium. Expression of \textit{ompK} gene was highest at 0.4\% bile salt concentration (5.8 fold) (Table 11).

**Effect of iron chelating agent and blood supplementation**

Supplementation of iron chelating agent 2, 2’-bipyridine slightly suppressed growth, but the expression of \textit{ompK} was increased to 5.7 fold (Table 12). On supplementation of culture medium with fresh human blood, the growth increased but expression of \textit{ompK} decreased (Table 12). When both 20 m mol\textsuperscript{-1} 2, 2’-bipyridine and blood were supplemented, \textit{ompK} expression was increased 2.3 fold compared to 5.7 fold in the presence of 2, 2’-bipyridine alone (Table 12). \textit{ompK} expression was 10.004 fold when grown in medium with supplementation of 100 m mol\textsuperscript{-1} 2, 2’-bipyridine and blood.

Table 11. \textit{ompK} expression and cell count of \textit{V. anguillarum} under varying growth conditions in the medium.
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### Table 12. Expression of ompK and cell count of *V. anguillarum* under varying concentration of 2, 2'-bipyridine and blood (% v/v) in the medium.
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