Chapter 7

Secretomics of *trh* positive strain of *Vibrio parahaemolyticus*

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7.1 Introduction

Secretomics includes protein secreted from biological units like tissue organ or cell. Bacteria secrete different types of extracellular protein in soluble condition when they try to survive or grow in a particular environment. These secreted proteins performed numerous type of biological function such as nutrient uptake, chelating metal ions catabolism, biodegradation of polymers, respiration, motility, cell attachment to the substratum and biofilm formation (Christie-Oleza et al., 2015; Islam et al., 2016; Pathak et al., 2017). Recently, it was found that secreted protein also plays an important role in the cross-talk with individual functional units facilitated the communication between cells and coordinate biological activities (Kapur and Katz, 2013; Lehr et al., 2012; Farhan and Rabouille, 2011). Pathogenic bacteria secrete different types of virulence factor and injected it into host cell by using different type of secretion system *viz*. type II, type III, type IV, type V and type VI systems (Mota et al., 2005; Michel and Voulhoux, 2009; Baron, 2009; Green and Mecsas, 2016). These injected proteins disrupt host cell functions, including cytoskeletal assembly, cytokine production, change gene expression and post-translational modifications to promote infection, modulate the host immunity and cell integrity (Gralnick and Newman, 2007; Coburn and Finlay, 2007; Deng et al., 2017). Secretome of pathogenic bacteria also contains large number of protein antigen that can be used for vaccine development and drug target site. Virulence factor and antigenic proteins were identified in many pathogenic bacteria like *Mycobacterium immunogenum* (Gupta et al., 2009; Cornejo-Granados et al., 2017), *Brugia malayi*

(Bennuru et al., 2009), *Helicobacter pylori* (Löwer et al., 2008) and *Bacillus anthracis* (Chitlaru et al., 2007) and used for drug or vaccine development.

Proteomic techniques such as SDS-PAGE, two-dimensional gel electrophoresis, MALDI-TOF-MS/MS and LC-MS/MS are promising approaches to identify different proteins, regulation of secretion and their biological function. Gel-based and non gel-based proteomics together with Mass spectrometry provide an enormous potential to separate a complex mixture of protein at high resolution and also able to identify the protein at very low concentration. In the present study, the extracellular proteins secreted by *V. parahaemolyticus* were isolated and were identified by using MALDI-TOF-MS/MS.

7.2 Material and Methods

7.2.1 Bacterial culture and isolation of extracellular proteins

V. parahaemolyticus trh (AP429) positive strain was cultured separately in two different tubes TSB supplemented with 2 % NaCl for overnight at 37 °C in shaking incubator. To induce expression of virulent gene 0.04 % bile salt was added in the media of one tube. The bacterial culture was centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was collected and filtered through 0.22 μm pore-size membranes (Millipore, Bedford, MA, USA). The protein was pelleted down by adding 2 % trichloracetic acid (TCA) in to a final concentration of 50 % (V/V) and incubate at -20 °C for overnight. The extracellular protein was collected by centrifugation at 13,000 rpm for 20 min.The pellet was washed with ice-cold acetone thrice and dried using vacuum centrifuge. Protein powder was store at -80 °C (Figure 7.1)

7.2.2 SDS-PAGE profiling

The protein powder was dissolved in PBS. The protein concentration was determined by the Bradford method (Bradford et al. 1976), using BSA as the standard. The soluble extracellular protein isolated from *trh* positive strain (AP429) was separated by 1D SDS-PAGE. SDS-PAGE was performed by using 12 % polyacrylamide gel (W/V) and 5 % stacking gel (W/V) with a stable potential of 200 V on a mini-Protean 3 electrophoresis cell (Bio-Rad) (Laemmli, 1970). A protein molecular marker (Bio-Rad) was run with the samples. After completion of the run, the gel was taken out and protein bands were visualized using Coomassie Brilliant Blue R 250 (CBB).

7.2.3 2D gel electrophoresis

Extracellular protein was separated by 2-DE as per the manufacturer's instructions (O' Farrell, 1975). Briefly, the protein powder was dissolved in Rehydration Buffer which is composed of 8 M urea, 2 % CHAPS, 50 mM DTT, 0.2 % Bio-lyte 3/10 ampholyte, and 0.001 % bromophenol blue. Now, the IPG strips (pH 3-10, 7 cm) were rehydrated with the solution. The rehydrated IPG strips were placed inside the isoelectric focusing cells (Ettan IPGphor 3, GE Healthcare). The first dimension separation of protein was performed at a current of 50 mA/strip at the stated voltage gradient: 100 V for 3 hrs 200 V for 3 hrs, 500 V for 3 hrs, 1000 V for 2 hrs, 2000 V for 1 hrs, 4000 V for 1 hrs, 6000 V for 1 hrs and 10000 V for 1 hrs at 4 °C. After completion of the process, the focused IPG strips were placed inside the tube containing Equilibration buffers I (0.375 M tris-HCl at pH 8.8, 6 M urea, 20 % V/V glycerol, 2 % SDS, 130 mM DTT) for 15 min and again it transferred in another tube containing Equilibration buffer II (0.375 M Tris-HCl at pH 8.8, 6 M urea, 20 % glycerol, 2 % SDS, 135 mM Iodoacetamide) for

15 min. The proteins were again separated using 12 % polyacrylamide gel (W/V) and 5 % stacking gel (W/V) with a stable potential of 200 V on a mini-Protean 3 electrophoresis cell (Bio-Rad) (Laemmli, 1970). After completion of the run, the gel was taken out and protein spots were visualized using Coomassie Brilliant Blue R 250 (CBB)

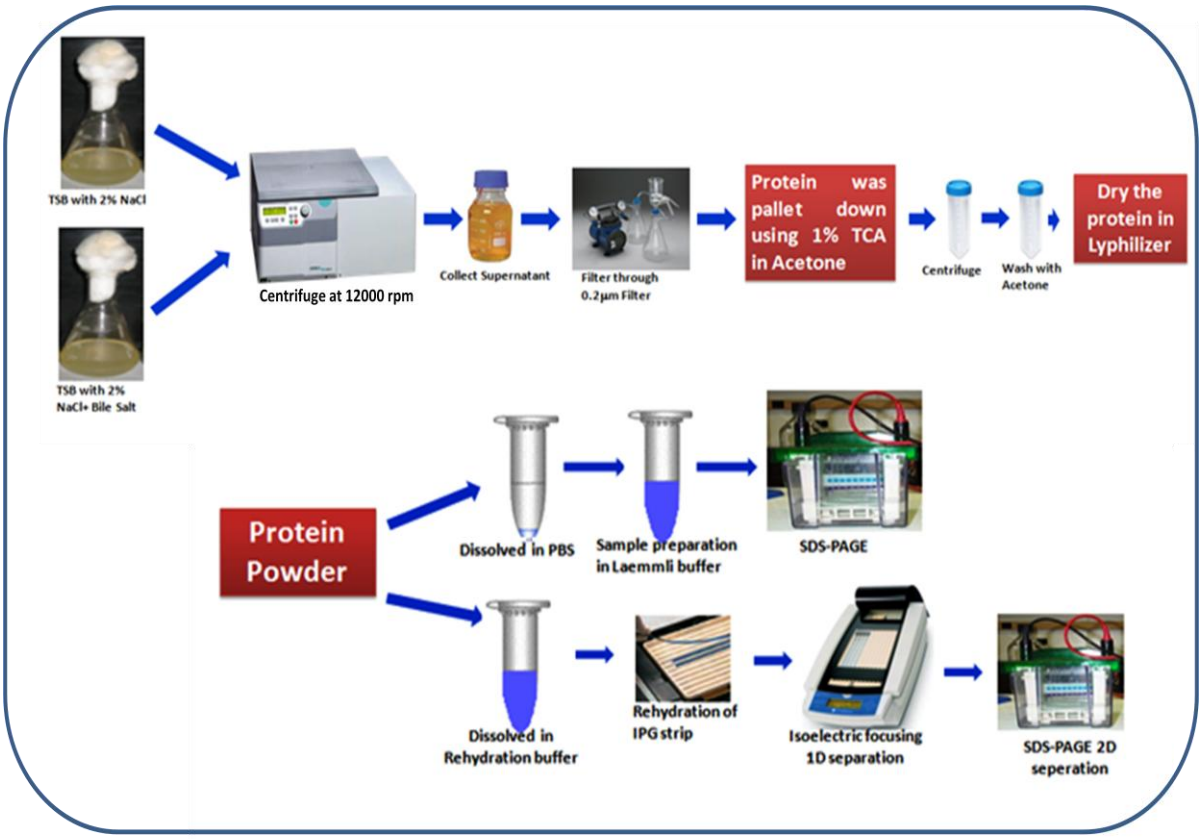

Figure 7.1 Schematic diagram of detail protocol used in secretomics study of *V. parahaemolyticus.*

7.2.4 Mass spectrometry analysis

Protein spots of interest were cut from the 2D poly-acrylamide gel, distained by using equal volumes of 100 % acetonitrile and 25 mM ammonium bicarbonate (NH_4HCO_3). After distaining the gel piece was dehydrated by using 100 % acetonitrile. The gel piece was treated with 100 mM Dithiothreitol at 56 °C for 1 hr followed by 250

mM iodoacetamide at room temperature in dark for 45 min. The peptides were extracted from the gel after completion of digestion with trypsin. The resulting peptides were extracted with acetonitrile containing 0.1 % TFA (Shevchenko et al., 2006) and dried by rotary evaporator. The dry peptides were reconstituted with 5 µl of TA buffer. The peptides obtained were mixed with α-Cyano-4-hydroxycinnamic acid (HCCA) (5 mg/mL α-Cyano-4-hydroxycinnamic acid in 1:2 ratio of 0.1 % TFA and 100 % ACN) in 1:1 ratio and the resulting 2 ul was spotted onto the MALDI plate [(MTP 384 ground steel (Bruker Daltonics, Germany)]. After air drying the sample, it was analyzed on the MALDI TOF/TOF Ultraflex III instrument (Bruker Daltonics, Germany). External calibration was done with standard peptide (Pepmix Mixture) supplied by Bruker, with masses ranging from 1046 to 3147 Da. Further analysis was done with Flex analysis software (Version 3.3) in reflectron ion mode with an average of 500 laser shots at mass detection range between 500 to 5000 m/z for obtaining the MS-MS. The masses finger print obtained in the MS-MS were submitted for Mascot search.

7.2.5 Data analysis

The mass fingerprint generated by the peptides in the MALDI-TOF/TOF-MS/MS was used for the identification protein against SwissProt database. The MASCOT sequence matching software (Matrix Science, www.matrixscience.com) was used for the analysis. The MASCOT search parameters used for the analysis of the data generated by MALDI-TOF/TOF-MS/MS were peptide mass tolerance level was set to 100 ppm, amino acids modifications like cysteine as S-carbamidomethyl-derivative and oxidation of methionine were allowed during analysis. The default search parameters like enzyme trypsin; max missed cleavages 1; fixed modification cabamidomethyl (C); variable

modifications oxidation methionine; peptide tolerance + 0.4 Da; fragment mass tolerance + 2 Da; protein mass unrestricted; instrument= Default; were used during analysis.

7.3 Results

7.3 1D SDS-PAGE

The extracellular protein isolated from control and treated samples was separated using 1-D gel electrophoresis and a representative of 1D gel profile of the protein was shown in Figure 7.2. CCB-stained 12 % SDS-polyacrylamide gels separated the protein in 24 bands in the molecular weight range of 10 to >250 kDa. The electrophoretic profiles of bacteria proteins of control (2 % NaCl) and treated (2 % Nacl + 0.04 % bile salt) show major changes in protein profiles. The two bands with molecular weight of 50 kDa were over expressed in treated where as the protein band with molecular weight 49 kDa was down-regulated in treated. The protein band with molecular weight approx 43 kDa was present in control where as it present in higher intensity in the extracellular protein of treated group. The protein band with molecular weight of 31 kDa was present in very low concentration in the control where as it was present in high abundance in extracellular protein of treated group. The protein band with molecular weight 20 kDa was present in treated group where as it absent in control group.

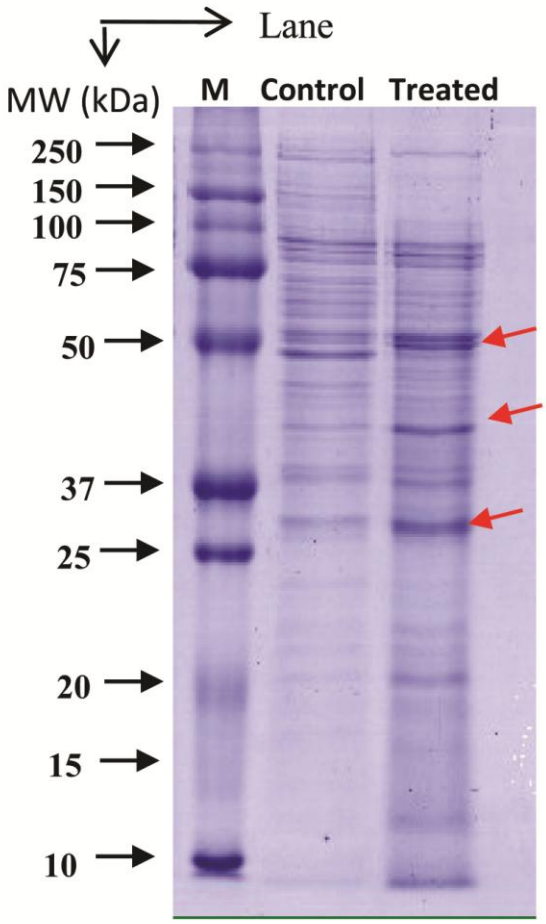

Figure 7.2 12% SDS-PAGE profile extracellular proteins of Control (TSB + 2%NaCl) Treated (TSB + 2%NaCl+ Bile salt). Red arrows indicate the bands differentially expressed protein band in control and treated sample.

7.3.2 2D gel electrophoresis

The combination of isoelectric focusing (First dimension) and SDS-PAGE (Second dimension) forms the classic separation technique in proteomics. The majority of the separated proteins fall in the range of 100-15 kDa and pI 5-9. The extracellular protein extracted from control and treated bacteria were separated *via.* 2D gel electrophoresis and the migration pattern of protein are shown in Figure 7.3. About 45 protein spots in control and 58 protein spots in exposed group were visualized using comassic brilliant blue G 250 staining. Most of the protein spots were present in

isoelectric point (pI) range of 5 to 8. Overall, 5 spots were recognized as differentially expressed and are identified with MALDI-TOF MS/MS together with aditional 5 spots.

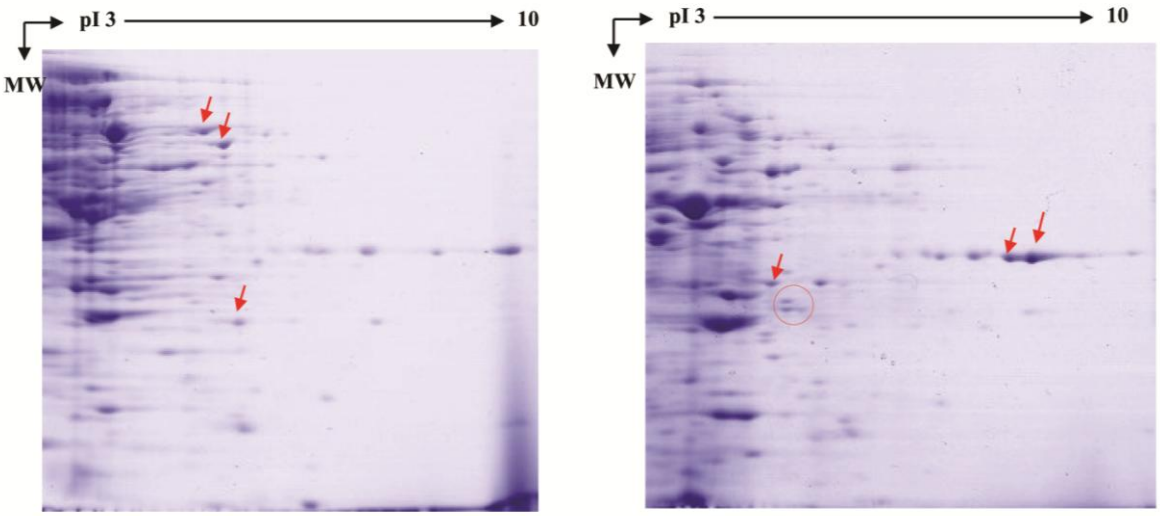

Figure 7.3 Representative 2D gels of extracellular protein of *V. parahaemoluticus* (a) Control (TSB + 2 % NaCl) (b) Treated (TSB + 2 % NaCl + Bile salt). Red arrows indicate the differentially expressed protein spots in control and treated sample. The red circle represents the protein spots present only in the treated sample.

7.3.3 MALDI-TOF analysis

10 protein spots were excised, digested with trypsin for MALDI-TOF-MS. 5 protein spots were found to be differentially expressed (Figure 7.3). These altered protein spots together with additional 5 spots were excised and submitted for identification using MALDI-TOF-MS analysis. The peptide mass fingerprint profiles generated by 10 protein spots were used in database search. The MASCOT searches resulted in nine significant identities. The peptide fragments produced were used to search against the SwissProt database and taxonomy set to Bacteria (Eubacterria) using the MASCOT search program. The identified proteins were distinguished into nine different proteins. The nine identified

proteins were tRNA-specific 2-thiouridylase, orotidine-5-phosphate decarboxylase, DNA-directed RNA polymerase subunit beta, RNA methyl transferase, NADH-quinone oxidoreductase, murein transglycosylase and disulfide oxidoreductase (Table 7.1)

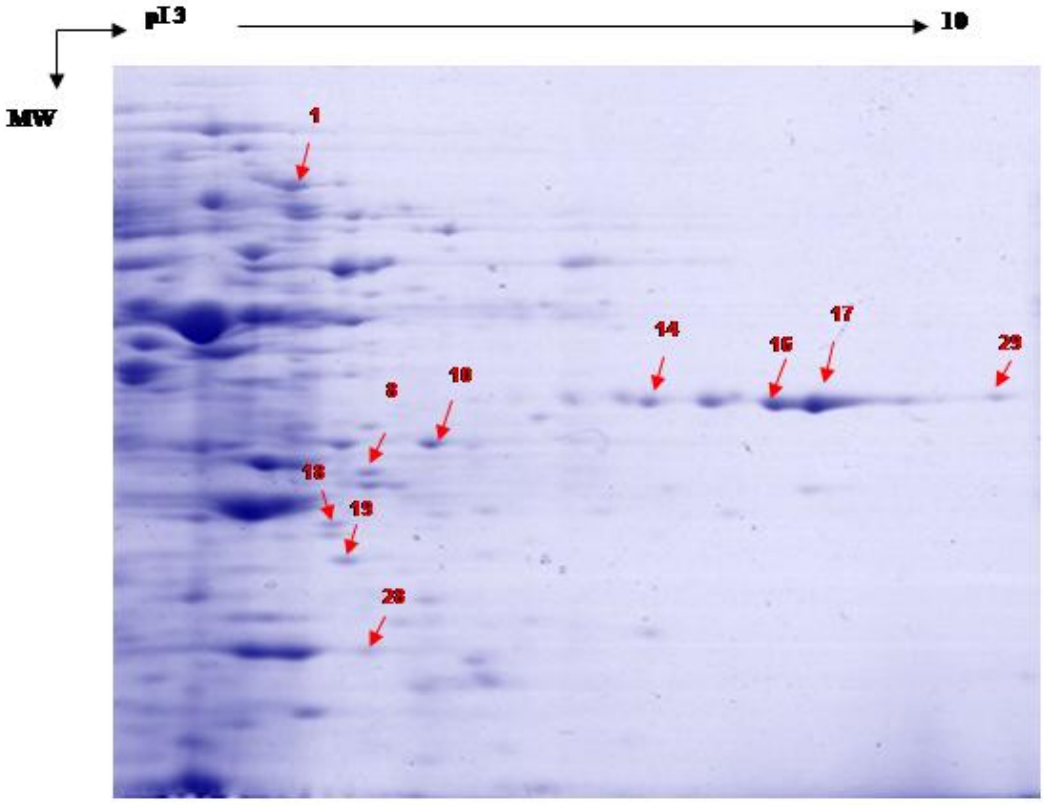

Figure 7.4 2D Gel showing the protein spots used for the MALDI-TOF MS/MS analysis.

Table 7.1 Identification of different protein spots of secretome of *V. parahaemolyticus.*

Protein spot No.	MASCOT Score	Protein	MW (Da)	pI	Species	Coverage (%)
AP 1	85	UvrABC System protein A	105987	3.5	*Staphylococcus aureus*	45
AP 8	203	Orotidine 5 phosphate decarboxylase	25453	4.0	*V. parahaemolyticus*	41
AP 10	59	Acetaldehyde dehydrogenases	32363	4.5	*Salinispora tropica*	32
AP 14	73	H-type transcriptional regulator RegA	37351	6.2	*Clostridium saccharoperbutylacetonicum*	40
AP 16	88	WhiA	36106	7.0	*Clostridium acetobutylicum*	29
AP 17	78	Glyceroldehyde 3phosphate	33433	7.4	*Bradyrhizobium diazoefficiens*	38
AP 18	46	Recombination protein RecR	21774	3.7	*Escherichia coli*	27
AP 28	39	50S ribosomal	13434	4.0	*Azoarcus* sp.	37
AP 29	61	Pantothenate kinase	37436	8.7	*Sinorhizobium fredii*	25

7.4 Discussion

Identification of extracellular effector proteins secreted by the pathogenic bacteia will help to understand the pathogenic mechanism as well as help in the development of new therapeutics against these bacteria to control the infection (Mahdavi et al., 2014). Ray et al., 2016 identified a previously uncharacterized secreted hemolysin belonging to leukocidin superfamily responsible for the *V. proteolyticus* mediated cytotoxicity in both HeLa cells and macrophages. Mahdavi et al., 2014 also identified secreted pathogen proteins from *Yersinia enterocolitica*. Three novel proteins under T6SS system were identified from the exoproteome of *Pseudomonas aeruginosa* (Hood et al., 2010).

In the present study, 5 differentially expressed protein spots together with 5 other identified by MALDI-TOF-MS/MS. Most of the identified proteins belong to "moonlighting proteins". RegA protein which is a H-type transcriptional regulator was identified in different bacteria *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Citrobacter rodentium* containing a helix-turn-helix DNA binding motif (Laguri et al., 2003; Hart et al., 2008) which was identified in the secretome of *V. parahaemolyticus*. It regulates the metabolic changes under aerobic and anaerobic environments in purple bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (Laguri et al., 2003). Studies showed that RegA is AraC-like virulence regulators and play an important role in the pathogeneciy of bacteria. In presence of bicarbonate ions within the intestine environment RegA activated the transcription of 19 different genes under 11 different operon which is involved in the intestinal colonization (Lara-Ochoa et al., 2010 Yang et al., 2008; Srikhanta et al., 2013). Similarly, WhiA and RecA proteins were identified as an extracellular protein in this study. WhiA plays an important role in cell division. Earlier the isolated proteins from *V. parahaemolyticus* were reported in *Streptomyces coelicolor*, *Bacillus subtilis* (Surdova et al., 2013). WhiA regulate the expression of different genes during sporulation including *ftsZ* (Ainsa et al., 2000; Flärdh et al., 2000). FtsZ is tubulin-like protein polymerize at the middle of the cell and initiate cell division process (Surdova et al., 2013). RecR protein is mainly involved in the homologous recombination and DNA repair process maintaining the chromosome integrity and introduce genetic variability (Rocha et al., 2005). In the present study, RecR protein was isolated in the secretome of *V. parahaemolyticus.*

Some metabolic enzymes which are mainly present inside the cells were also identified in the secretome of *V. parahaemolyticus*. Acetaldehyde dehydrogenases identified in the secretome of *V. parahaemolyticus* oxidize acetaldehyde to acetate (Edenberg et al., 2007). This enzyme is present in both eukaryotic and prokaryotic systems. Besides, its enzymatic function also plays an important role in the pathogenicity (Jagadeesan et al., 2010). During infection with *Listeria monocytogenes*, acetaldehyde dehydrogenase and Listeria adhesion protein (Lap) interact with Hsp 60 receptor of host cell which promote the attachment of bacteria in the intestine (Jagadeesan et al., 2010). Many intercellular protein or enzyme function as adhesins molecule on bacterial cell surface though they do not contain any signal sequences for secretion (Jeffery, 2018). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is another important glycolytic enzyme present in cytoplasm of bacterial cells. This enzyme was also present in the cell surface of many bacteria and fungi though it is lacking of secretion signals (Aguilera et al., 2012; Finlay et al., 1995; Alvarez et al., 2007). Egea et al. (2007) reported that GAPDH is released in the culture media by enterohemorrhagic and enteropathogenic *Escherichia coli* which might be helping in the adhesion of bacteria by interacting with human plasminogen and fibrinogen. Similarly, GAPDH plays a significant role in adhesion of *Neisseria meningitides* within host cell and promotes bacterial colonization and infection (Tunio et al., 2010). Orotidine-5-phosphate decarboxylase is an important enzyme in pyrimidine biosynthesis and also present in the secretome of *V. parahaemolyticus.* This enzyme catalyzed decarboxylation of orotidine monophosphate to uridine monophosphate (Appleby et al., 2000). But to date, no search information is available on the association of this protein with the pathogenicity of the bacteria.

In the present study, 50S ribosomal protein was identified in the secretome of *V. parahaemolyticus.* The presence of different ribosomal proteins was identified in the secretome of many different bacteria like *Granulicatella adiacens*, *Haemophilus parasuis Streptococcus suis* and *V. parahaemolyticus* (Wu et al., 2008; Wei et al., 2014; He et al., 2015; Karched et al., 2019). The presence of 50S ribosomal protein in the secretome of *V. parahaemolyticus* was also reported by He et al. (2015). 50S ribosomal protein belongs to the moonlighting proteins which play different biological function when they are present in the extracellular medium after secretion (Henderson and Martin, 2014; Karched et al., 2019). Ribosomal protein (L7/L12) of *Brucella abortus* showed antigenic and immunogenic property which was used for the development of vaccine against brucellosis (Oliveira and Splitter, 1996; Ribeiro et al., 2002).

He et al., 2015 also carried out gel-based secretomics of *V. parahaemolyticus* and identified sixteen protein from the secreted protein out of which six protein are virulence-associated factors involved in the pathogenicity. The majority of other identified were house keeping proteins play an important role in the normal metabolic pathway.

7.5 Conclusion

Overall in the present study five "moonlighting proteins" were identified which normally present in the cell and play a major role in the metabolic pathway but also act as a virulent factor when they were secreted out from the cell. These proteins mainly help in cell proliferation, cell attachment, receptor binding protein and transcription regulator. If the activity of the identified such important virulent protein could be inhibited, it would help in the development of new therapeutics against this pathogen.