V. parahaemolyticus is a major causative agent of food-borne gastroenteritis and is also responsible for wound infections and septicaemia in immunocompromised individuals (Fujino *et al.* 1953; Barker and Gangarosa 1974; Morris and Tenney 1985; Yeung and Boor 2004). *V. parahaemolyticus* induced gastroenteritis were mostly related to the consumption of undercooked sea foods (Barker and Gangarosa 1974). This pathogen requires serious concern due to increase in number of outbreaks in recent years, emergence of pandemic strains and its expansion with rise in water temperature in response to the global warming (Nair *et al.* 2007; Okuda *et al.* 1997; Gil *et al.* 2007; Boyd *et al.* 2008). It is clear that a better understanding of the mechanism of *V. parahaemolyticus* pathogenesis and associated virulence factors were not understood completely. The genome sequencing of a highly virulent *V. parahaemolyticus* strain, *O3:K6* had shed some light on its pathogenicity machineries with the indication of two type III secretion systems (T3SS), along with thermostable direct haemolysins (TDH) in the organism (Makino *et al.* 2003; Hiyoshi *et al.* 2010). Since then, intense research has focused on characterizing these T3SSs.

It has been evidenced that T3SS delivers barrage of effector proteins harbouring eukaryotic domains and motifs that usurp various signalling pathways, thereby promoting survival and replication of pathogens within host (Mattoo *et al.* 2007). By employing tissue culture and animal model system, a myriad of T3SS effector proteins have been identified from *V. parahaemolyticus*. These factors are not only unique in terms of their structural and functional aspects but also target and modulate distinct

cellular processes to evade host surveillance system (Broberg *et al.* 2011). It has been demonstrated that the T3SS1 has been involved in cytotoxicity whereas T3SS2 is responsible for enterotoxicity and cytotoxicity of *V. parahaemolyticus* (Hiyoshi *et al.* 2010). For example, T3SS1 elaborates a spectrum of effectors namely VPA0450, VopS, VopQ and VopR which are targeting different cellular pathways and their consorted action leads to cell death involving induction of autophagy, cell rounding and then cell lysis. Contrarily to T3SS1, the cumulative action of T3SS2 effectors notably VopA, VopL, VopT, VopC, VopV and VopZ are responsible of cytotoxicity and enterotoxicity in the infected cells (Burdette *et al.* 2008; Zhang and Orth 2013; O'Boyle and Boyd 2014).

Deciphering the mechanism of action of these virulence proteins is vital to advance our understanding of these pathogenic bacteria. The lack of tractable genetic systems among higher eukaryotes limits the functional analysis of these T3SS effectors. In recent time, *Saccharomyces cerevisiae*, the budding yeast has gained much attention as a non-mammalian model system to identify and evaluate functionality of diverse arrays of virulence factors not only for its easy cultivation but also due to presence of cellular pathways that are well conserved in mammalian systems (Valdivia 2004; Curak *et al.* 2009; Siggers and Lesser 2008).

Although the significance of T3SS of *V. parahaemolyticus* in mediating host-pathogen interaction is becoming clearer but still the contribution of many effector proteins in infection and their targets in eukaryotic cells is not well understood.

The work presented in this thesis first investigated the screening of several *V. parahaemolyticus* putative effectors that had been predicted *in silico* by PSI-BLAST homology search using yeast as a model system. By employing homology search analysis, over 55 virulence associated *V. parahaemolyticus* proteins available in CSGID database were identified and 14 T3SS effectors including both putative and confirmed have been selected. A yeast cell model was employed to investigate the effect of effectors over yeast growth. Expression of effectors, VopL, VopA, VopQ, VPA0450 and VopT was toxic to yeast and inhibited yeast growth. This indicated that yeast can be exploited further for investigating the function and mechanism of these effectors. The screening of selected putative *V. parahaemolyticus* effectors in yeast model system resulted in identification of two new candidate T3SS effectors, VP1683 (VopR) and VPA1380 for the first time. The presented yeast screening highlighted the fact that yeast can provide a simple model for effectors screening.

The following chapter deal with the functional and structural characterization of VopR, a new effector identified in chapter-3 and it's *V. alginolyticus* homologues (VAVopR). The results of yeast viability plating assay demonstrated VopR mediated yeast growth inhibition was due to cytotoxic nature of VopR over yeast cells. In order to find out the region important for VopR mediated yeast toxicity, yeast spotting analysis of yeast cells expressing different truncated constructs of VopR was performed. Spotting assay revealed the cruciality of intact C-terminus in VopR mediated yeast toxicity, as the deletion of 25 residues from C-terminus resulted in

complete loss of activity. In contrast, 100 N-terminus residues found dispensable for VopR toxic activity. Western blot analysis of truncated versions of VopR expressed in yeast cells ruled out the possibility that loss in activity was due to the expression or *in vivo* stability of truncated VopR constructs in yeast cells. Confocal microscopy of GFP tagged VopR and its truncated variants revealed that VopR was primarily localized to yeast plasma membrane and the membrane localization signal was found to be localized at N-terminus of VopR. Yeast MAPK kinase western blot analysis suggested that ectopic expression of VopR in yeast cells leads to activation of HOG MAPK signalling pathway, which need further confirmatory investigation. Haploid yeast deletion library used to identify the VopR targets has not resulted in any positive hit, suggested that other screening methods like over-expression yeast library or multicopy suppressor screens to be employed for such highly toxic effectors.

VopR BLAST analysis leads to identification of VopR homologue in *V. alginolyticus*. Yeast spotting assay of VopR homologue in *V. alginolyticus* (VAVopR) revealed that VAVopR was also functionally active and showed cytoxicity in yeast cells, similar to VopR. Our spotting data clearly demonstrated the dispensability of first 100 amino acids at N-terminal region for VAVopR toxic activity and the cruciality of last 25 amino acids from C-terminus in VAVopR mediated lethality to yeast model system. Further, C-terminal truncation analysis of VAVopR revealed that even a deletion of last 10 residues from C-terminus resulted in loss of VAVopR yeast toxic activity. Biochemical subcellular fractionated membrane blot

analysis of yeast cell expressing VAVopR and its truncated constructs revealed that VAVopR localizes to plasma membrane like VopR, with the help of localization signal located in N-terminus of VAVopR. Chemical treatment of subcellular membrane fractions of yeast cells expressing VAVopR resulted that VAVopR majorly extracted either through detergent treatment or under denaturing condition but not released under high salt or high pH treatment.

Yeast cell model was employed to investigate the effect of two more *V. parahaemolyticus* effectors, VopL and VPA050. Domain deletion analysis of VopL revealed that all the three WH2 domains and PRM motifs were playing important role in VopL mediated yeast growth inhibition. Further, FACS and light microscopic analysis of yeast cells expressing VopL revealed that expression of VopL in yeast cells resulted in growth arrest phenotype with increased cell size. Similarly, yeast viability CFU count analysis revealed the cytotoxic nature of VPA0450. Further, alanine mutagenesis of conserved amino acid residues present in catalytic motifs suggested the importance of D261 residue in VPA0450 mediated yeast cytotoxicity. Yeast viability CFU count analysis of VP0450 alanine mutants indicated the partial arrest type phenomenon of yeast cells expressing VPA0450^{D261A}.

Collectively, the data presented within describe the identification and partial characterization of *V. parahaemolyticus* T3SS effectors using yeast as a model system. This model will be a valuable tool to expedite the identification of intracellular target of *V. parahaemolyticus* T3SS effectors and also useful to screen small molecule inhibitors against these effector

molecules. Hence, we can say that functional hints of effectors provided by employing yeast as a simplified single cell model system can provide valuable information. The work presented here further strengthening the possibility of exploring yeast for effectors studies in future.