Many microorganisms can produce acetoin under carbohydrate-rich conditions, which is an essential physiological role. Most of the bacteria use the acetoin fermentation as a protection against the acidic condition. In 1898, Daniel Voges and Proskauer observed the red color formation by bacterial culture media after treatment with potassium hydroxide. Later it was found that, in the presence of strong base the acetylmethylcarbinol was oxidized to diacetyl, which then reacts with guanidine compounds and leads to red color formation. The acetoin formation process helps the bacterium to avoid the acidic condition and in the regulation of NAD/NADH ratio. The produced acetoin acts as a carbon source.

In human pathogens like *V. cholerae,* acetoin formation plays a vital role in survival under carbohydrate-rich conditions (Yoon and Mekalanos 2006). The long-standing mystery in the history of cholera epidemiology was the replacement of classical *V. cholerae* by El Tor biotype. In 2009, Yoon and Mekalonas showed that the classical and El Tor O1 biotype of *V. cholerae* behaves differentially under glucose-rich condition. The classical biotype produces various acids especially lactic acid and pyruvate, which acidify the culture media and classical biotype loses its viability. On the other hand, the El Tor biotype while growing under carbohydrate-rich media, produces a neutral compound like acetoin 2, 3-butaindione. As a result, the growth of El Tor biotype is enhanced in glucose-rich media.

We started our study to address the facts that the Classical and El Tor biotypes of *V. cholerae* belong to O1 serogroup. They are genetically very close and having same quorum sensing mechanism involving identical proteins which are regulating same pathways. Even so, they behave differentially in carbohydrate-rich media.

In the first part of this study, we screened the El Tor hybrid, non-O1, and non-O139 *V. cholerae* strains for acetoin phenotype. We screened 34 different strains of *V. cholerae*, and found O395, Y4254, 02602 Hong Kong, I11509, VOC 723, 03802 Hong Kong B180, NM06-001, 02102 Vietnam, and 2322 strains to be acetoin negative. We also analyzed the growth pattern of acetoin negative *V. cholerae* in 1% glucose-rich media, and observed that all acetoin negative strains have growth defect while growing in glucose-rich media. There are two types of acetoin negative strains. a) due to mutations in acetoin operon genes, b) due to defect in regulatory mechanism. In order to know the nature of acetoin negative strains, we transformed the functional $AlsR_{N16961}$ in acetoin negative strain. The externally supplied AlsR shall activate the acetoin operon, and if strains have no mutation in acetoin operon genes, then they will produce acetoin, which can be observed through VP test. After transformation

of functional $AlsR_{N16961}$, we found that strains O395, VOC723, 2322 and Y4254 were able to produce acetoin, indicating that they have functional acetoin operon and their acetoin negative phenotype may be due to defect in regulatory mechanism. After analysis of AlsR of O395, VOC723, 2322 and Y4254, we found that AlsR of O395 have A69T and R113C amino acid mutation, and AlsR of 2322 have a four amino acid stretch deletion from R199 to G202. To further investigate the importance of mutation in AlsR of O395 we made A69T and R113C amino acid replacement in $AlsR_{N16961}$ and checked for the functionality of AlsR by acetoin formation. The VP test showed that The $AlsR_{O395}$ is active and defect for its acetoin phenotype is upstream to Acetoin operon. The ClustalW analysis showed that $AlsR_{2322}$ has four amino acid stretch deletion. To find out the importance of these four amino acids in the activity of AlsR we made sequential amino acid deletion in $AlsR_{N16961}$. The study of sequential single amino acid deletion of REVG stretch from R199 to G202 shows that after the deletion of each amino acid from REVG stretch, the AlsR loses its functionality, indicating that the length of REVG stretch is essential in the functionality of AlsR. In continuation with this study to assert the importance of each single amino acid in the REVG stretch, we did the sequential alanine replacement in REVG stretch of $AlsR_{N16961}$ which reveals that the R199 and V201 are essential for the functionality of AlsR. From this mutational study we concluded that the AlsR of O395 is functional and AlsR of 2322 is unable to activate acetoin operon.

In the next step to understand the molecular basis responsible for the acetoin negative phenotype of strains having functional AlsR (O395, Y4254 and VOC 723 strains) we did protease assay to find out the HapR status. The non-functional HapR is unable to block AphA at high cell density and AphA in turn inhibit the Acetoin operon. The protease assay showed that the HapR of O395 is inactive and HapR of VOC723, 2322 and Y4254 is active.

In 2002, a study showed that the mutation in the HapR binding site of -77 of *aphA* promoter region inhibited the binding of HapR ((Rutherford, Van Kessel et al. 2011)(Skorupski K. 2002). To investigate the same or related situation we did sequence analysis of *aphA* promoter of all ten acetoin negative strains. The sequence analysis showed that only 2322 strain does not have the mutation at the HapR binding site and remaining nine acetoin negative strains have a mutation at -77 positions of *aphA* promoter at the HapR binding site. Because of this mutations, the AphA expression is unchecked in these acetoin negative strains at high cell density. To confirm this, we disrupted the AphA of VOC723 and AphA in O395 strain and analyzed their acetoin formation and growth behavior pattern in glucose-rich

media. After disruption of AphA, VOC723 and O395 strains were able to produce acetoin and their growth pattern is improved in glucose-rich media.

In conclusion, the O395, Y4254, VOC723 are acetoin negative due to the mutation in the HapR binding site at *aphA* promoter region. The length of REVG stretch is essential for AlsR activity, and the R199 and V201 are essential amino acid for the functionality of AlsR.

The subsequent five amino acid deletion study was done to find a crucial stretch of proposed HTH domain of AlsR. The results showed that these AlsR constructs (from AlsR 5 to 10 amino acid del to AlsR 40 to 45 amino acid del construct) loses its activity showing the importance of these five amino acid stretches.

The acetoin formation is influenced by various factors in *B. subtlis* ((Sharma and Noronha 2014)). To find out the factors influencing the acetoin formation in *V. cholere,* we have checked the influence of pH and various metabolite on acetoin formation. We found that the acetoin formation in *V. cholere* is induced under high glucose, acetate and at moderate low pH. In continuation with this study we did the docking study to find out the AlsR protein and acetate interaction. The protein-ligand complex in docking showing the S104, L229 and R230 amino acids are interacting with Acetate ligand.

The alarming increase in the antibiotic-resistant strains of pathogenic bacteria including *V. cholerae* had forced the researchers to find out new methods of treatment ((Kitaoka, Miyata et al. 2011); (Mandal, Dinoop et al. 2012)). As *V. cholera* are acid labile and pH maintenance of culture media is vital for its survival. The acidic condition in glucose-rich media can be created in two ways and which can be employed against *V. cholerae* 1) using bacteria that acidify the media in the presence of glucose and 2) using compounds that can block the acetoin formation in glucose-rich media. Keeping these facts in mind, we did a co-culture study of El Tor hybrids and *E .coli.* In our study we found the growth of El Tor hybrid strain is inhibited in the presence of *E. coli* in glucose-rich media. On the contrary, the growth of acetoin negative *V. cholerae* in the spent culture of *B. subtilis* is enhanced as compared to growth in glucose-rich media. Similarly, in co-culture experiment, the growth of acetoin negative *V. cholerae* O395 is maintained in glucose-rich media in the presence of acetoin positive *V. cholerae* N16961 strain. Here the acetoin positive N16961 *V. cholerae* protects the acetoin negative O395 from acid shock, but the growth rate of N16961 outcompete the O395(Pradhan, Baidya et al. 2010). To explore the compound that can block the acetoin

formation in glucose-rich media, we screened in house library with the help of Dr. Raman Parkesh. Unfortunately, in this study all the primary and secondary compound screened as positive were turn out cytotoxic. However, acetoin inhibition is the best target against *V. cholera* infection.

In the screening of acetoin formation of different vibrio belonging to vibrio genus from our lab collection, we found the *V. alginolyticus* is acetoin positive, but it is unable to maintain its growth in carbohydrate-rich media. The acetoin formed during the metabolism of carbohydrate could not neutralize the acid in *V. alginolyticus*.