Conclusion

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Major studies on functional bacterial amyloids have always been with respect to their physiological aspect such as cell aggregation, pellicle formation and surface tension reduction. Till now there has been very less attention to the unique properties of amyloid proteins that makes them potential candidate for various industrial applications. These unique properties include, resistance to physical and chemical denaturants, resistant to detergents and high mechanical strength comparable to that of steel. These unique properties make amyloid protein different as compared to other biological proteins. Based on these interesting features it was decided to explore the amyloid proteins produced by bacteria and exploit their unique features for specific application.

Since large number of amyloids producing bacteria were reported to be present in the flocs of activated sludge, we isolated amyloid producing bacteria from the activated sludge of domestic waste water treatment plants. The screening was performed on Congo red agar as this dye has demonstrated its unique interaction with the amyloid fibrils that makes the colony of isolates appear dark red in color. Besides red color phenotype the interaction between amyloid fibrils and Congo red dye was checked by in vitro assays with the purified bioflocculant CR4. Upon staining with Congo red the protein aggregates demonstrated apple green birefringence under cross polarized light microscopy. The phenomenon of apple green birefringence has been considered as one of the gold standards for detecting amyloid protein in biopsies of brain tissue collected from patients with neurological disorders. The specificity of this assay lies in the unique interaction between planar Congo red molecules with anti-parallel beta sheets of amyloid protein. This interaction results in torsion twist in the molecule giving rise to apple green birefringence under cross polarized light. Besides birefringence another phenomenon exhibited by Congo red molecules bound to amyloid fibrils is the red shift in its absorption maxima as compared to free Congo red dye. Besides Congo red, amyloid specific dyes such as Thioflavin T have been shown to exhibit enhanced fluorescence upon binding with amyloid fibrils. Staining of *B. cereus* CR4 cells with

Thioflavin T demonstrated bright green fluorescent cells under fluorescence and confocal microscope.

The unique properties of amyloid protein lie in the fact that it possesses the presence of anti-parallel beta sheet structure. Hence it becomes inevitable to detect the presence of anti-parallel beta sheet in *B. cereus* CR4 protein before categorizing it as an amyloid protein. The detection of beta sheets in *B. cereus* CR4 protein was done by analysis of CD spectra and FTIR spectra as both the spectroscopic methods have been considered as one of the most sensitive methods for the detection of protein secondary structures such as alpha helix and beta sheets. Both CD and FTIR spectra demonstrated peaks that corresponds to the presence of antiparallel beta sheets in *B. cereus* CR4 protein. Besides these assays further corroboration for the presence of amyloids was done by direct visualization of the presence of amyloid fibrils. One of the benchmark techniques used for the direct visualization of amyloid fibrils on cell surface of *B. cereus* CR4 was flagella staining method. This classical method relies on thickening of the flagellar structures by tannic acid followed by staining with hot ammonical silver solution and makes nano sized structures thick enough to be visualized under bright field microscope. The cells of *B. cereus* CR4 demonstrated the presence of fibrillar aggregates around the cell surface. Such aggregates were also observed by visualizing purified *B. cereus* CR4 protein under Transmission electron microscope. Hence after performing series of assays, it was concluded that *B. cereus* CR4 possess its own functional amyloid. For the identification of this amyloid protein, the purified protein was outsourced for sequencing by LC MS-MS method. The protein sequencing results showed the presence of TasA functional amyloid which is common amyloid present in *Bacillus* genus. The TasA amyloid have also been studied significantly in *B. cereus* and have demonstrated to play an important role in cell aggregation and biofilm formation, especially in the pipelines and vessels of dairy industry. Several studies have shown the role of TasA amyloid in various physiological processes such as cell aggregation, biofilm formation, impartment of cell surface hydrophobicity and pellicle formation. The purified amyloid protein from *B. cereus* CR4 was confirmed to possess bioflocculant activity. Hence based on

this fact we further explored the application of *B. cereus* CR4 amyloid bioflocculant or bioflocculant CR4.

The bioflocculant producing *B. cereus* CR4 showed optimum flocculation activity at pH 7 and 37^{o}C temperature, which is common pH and temperature for the growth of several *Bacillus* spp. To obtain optimal bioflocculant production screening of media components was done by Plackett Burman analysis followed by optimization by central composite design. The Plackett Burmann studies showed the role of three nutrient factors viz. Lactose, Yeast extract and Tryptone that played significant role in production of bioflocculant CR4. Lactose is one of the major components of the dairy industry that has demonstrated its role in promoting obnoxious biofilms in the vessels and pipelines of the dairy industry. Majority of these biofilm are due to the presence of *Bacillus* spp. that possess TasA functional amyloid as its extracellular surface protein. Lactose is also known to upregulate EPS and TasA operon that enhances cell aggregation and biofilm formation. Further Yeast extract and Tryptone being rich nitrogen sources has significant contribution in exponential growth and production of amyloid bioflocculant. As a result, the bioflocculant production was found to be at its peak at the end of growth phase and it dropped gradually during the stationary phase. Thus the bioflocculant CR4 can be said to be a secondary metabolite. The TasA amyloid is a common amyloid protein present in *Bacillus* spp. In *B. subtilis* the TasA fibrils remains anchored on the cell surface with the aid of accessory protein TapA. In contrast *B. cereus* cells shows absence of TapA protein, causing TasA amyloid fibers to be released free in the extracellular environment. Hence the flocculation activity associated with the cell free supernatant increases during stationary phase of *B. cereus* CR4 growth. The presence and absence of TapA protein also had a significant correlation in biofilm development by various *Bacillus* spp. The biofilm development in several *Bacillus* spp. can either be of submerged type or floating type termed as the pellicle. In case of model *Bacillus* spp., *B. subtilis* the presence of extracellular TasA amyloid is known to make the cell surface hydrophobic, causing cells to float and aggregate at air liquid interface and form thick floating pellicle type biofilm. Studies with several *Bacillus* spp. demonstrated that there was a strong correlation between

cell surface hydrophobicity and formation of pellicle type biofilm. Most of the species possessed TasA amyloid and developed pellicle biofilm and demonstrated median % hydrophobicity of 55.7%. However, *B. cereus* CR4 demonstrated submerged form of biofilm with 28 % of cell surface hydrophobicity. The absence of accessory protein TapA in *B. cereus* spp. causes TasA amyloid fibers to be released free in the extracellular environment after they appear on the cell surface ultimately causing loss in cell surface hydrophobicity. Besides the role of nutrient sources as described above, chemical stress agents such as Ethanol, DMSO, SDS and NaCl demonstrated an increase in the cell surface amyloid production, biofilm formation and flocculation activity by several folds in *E. coli*. This shows that not only in *E. coli* but other amyloid producing species such as *B.cereus* CR4 also overexpresses its amyloid genes. The stimulation of amyloid production during stress demonstrates protective role of amyloid fibrils for the bacterial cells. This seems to be highly contrasting when compared with mammalian amyloids that proves to be extremely harmful to the individual. The increase in amyloid production by bacterial cells under stress allows easy aggregation of bacterial cells and formation of biofilms that in turn favours protection and survival of bacterial cells. Besides its physiological importance, the stimulation of excessive amyloid production by stress agents can also prove to be a promising strategy for cost effective industrial production of functional amyloid.

The unique ability of *B. cereus* CR4 to cause aggregation and flocculation was further explored in this study flocculation of microalgae *Scenedesmus.* In the current decade the study on use of microalgae for waste water treatment and production of biofuels have increased significantly. However, one of the major drawbacks that underrates microalgae for its use in biofuel production is the harvesting step. Hence *B. cereus* CR4 was exploited for the flocculation of *Scenedesmus* sp. after waste water treatment at bench scale and continuous scale reactor. As amyloid protein has unique ability to withstand physical and chemical stress the application of *B. cereus* CR4 for harvest of *Scenedesmus* biomass holds great industrial importance.

With an aim to identity the bioflocculant producing gene, CDAG (Curli dependent amyloid generator) system was used. This system uses *E. coli* curli mutant that gives white colored colonies on Congo red agar. Upon cloning of functional amyloid gene it demonstrates red colored colonies, making it easy to screen the presence of amyloid gene cloned in the plasmid. For the identification of amyloid gene in *B. cereus* CR4 the ORFs generated by PCR were ligated in pVS72 plasmid and transformed in *E.coli* curli mutant VS16. One of the colony was red colored that indicated presence of amyloid gene as an insert. The PCR amplification of the insert demonstrated the presence of 600bp PCR product that was sent for sequencing. The DNA sequencing of the PCR product revealed the presence of tasA gene. The bioflocculant possessing tasA gene was further amplified using tasA specific primers and cloned in *E. coli* curli mutant in CDAG system.

Future Directions

Thus, functional amyloid protein that is produced by several bacterial species in nature can be exploited for its unique and interesting properties. The unique ability of amyloid proteins to cause cell aggregation and biofilm formation in nature can be exploited as a potent bioflocculant. The TasA amyloid produced by isolate *B. cereus* CR4 can be used for bulk scale industrial harvest of microalgae following waste water treatment. This would not only reduce the cost of microalgal biomass harvest but would also provide an environmentally safe and ecofriendly strategy of treating waste water rich in phosphates and nitrates with economic benefits. The microalgal biomass harvested by bioflocculant is safe for fodder consumption and production of fuels such as biodiesel. Common nutrients such as milk sugar lactose which is rich in the effluents of dairy industry, physiological stress agents such as ethanol, DMSO and SDS can be employed for improving the yield and decreasing the cost of bioflocculant at industrial level. Besides industrial applications the use of D-amino acids to prevent amyloid biofilms provides an effective strategy of combating the problems caused by biofilms.