Synopsis of the Thesis
on

Functional and Biotechnological importance of amyloids of bacteria from Activated Sludge

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By

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Introduction

Functional bacterial amyloids (FuBa) are microbial cell surface bound filamentous proteins rich in β sheets arranged perpendicular to the fiber that accomplish specific functions like cell adhesion and biofilm formation (Gebbink *et al.* 2005). Unlike the disease associated amyloids that cause physiological dysfunctions in the mammalian body the functional amyloids help the microorganisms to accomplish a specific task. For example: in case of *E.coli*, *Salmonella* and other members of gram negative bacteria the amyloids play a predominant role in cell adhesion, biofilm formation and invasion of host tissues. In case of fungi they play an important role in creation of hydrophobic shell around the hyphae allowing fungi to escape water and in spores dispersal of spores.

Following are the widely studied amyloids in microorganisms.

Functional amyloid TasA in *Bacillus* spp.

Bacillus are gram positive bacteria that are found in wide range of natural habitat like soil, rhizosphere of plants waste water etc. Under stress and nutrient limiting conditionsthe *Bacillus* sp. form spores that are resistant to various external environmental stress. Studies of spores of *Bacillus* sp. under electron microscope have demonstrated the presence of fibrillar structures around the spores. These fibrillar proteins have been termed as TasA which is a functinal amyloid of *Bacillus* sp often termed as spore coat protein. The role of tasA is prominent in the environments where the bacterial cells experience oxygen and nutrient gradients. In presence of tasA and *Bacillus* surface layer protein BslA the bacterial cells aggregates at the air liquid interface to form a thick floating biofilm termedas a pellicle. It should be noted that tasA protein cannot attach itself to cell surface ofthe bacteria by itself it requires aid from an accessory protein termed as tapA which ispresent on the cell wall and attaches the fibrillar tasA on the cell surface. Recent studies on functional amyloids of *Bacillus* suggests that tapA is present in *Bacillus subtilis* and has a cruital role in aggregation of amyloid fibrils on the cell surface. However tapA seems to be absent in *Bacillus cereus* which shows release of fibres in the extracellular medium by the cells. Besides aggregation at air liquid interface, the hydrophobic nature of theseproteins also provides protection against harmful chemicals.

Curli amyloid

This amyloid is one of the chief extracellular protein component having divergent functions like biofilm formation, invasion of host tissues, induction of host inflammatory response and aids bacteria to persist harsh ambience of the host (Castonguay *et al.* 2006; Barnhart *et al.* 2006). The gene that codes for curly amyloid has been designated as CsgA. csgA is translated as a monomeric protein and is excreted outside the cell by a membrane bound CsgG protien. The CsgG identifies the singal sequence present in the CsgA and transports it in the extracellular environment. The cell surface of *E.coli* has CsgB protein that anchors CsgA on the cell surface. This anchoring makes the cell surface of *E.coli* decorated with fibrils of CsgA amyloid. It has been well established that curli amyloid binds diverse proteins such as laminin and fibronectin present in the host ultimately aiding *E.coli* in pathogenesis. After their discovery in *E.coli* they were further discovered in other members of the family Enterobacteriaceae.

Tafi amyloid

Tafi amyloids are associated with members of *Salmonella sp*. Just like curli tafi amyloidshave an important role in biofilm formation, invasion of host tissue for colonizing small intestine and pathogenesis. Just like curli amyloid of *E.coli* the tafi amyloid also bindshost proteins like laminin and fibronectin. Interaction of amyloid protein with fibronectin allows them to be easiliy internalized with the aid of integrins. Once internalized thebacteria initiates pathogenesis.

Chaplin amyloid

Chaplin amyloids are made by *Streptomyces sp*. Once translated they as exported outsidethe cell in the monomeric form. Chaplins being amphiphatic in nature congregate at airwater interface resulting in decrease in surface tension of water. This aids in growth ofhyphae outside the water and spore dispersal (Gebbink *et al.* 2005).

Hydrophobins amyloids

Fap amyloids are widely distributed in most of the fungi. Unlike bacterial amyloids that are loosely associated with the cell surface, the hydrophobins are tightly associated with the cell surface. The tight association of these proteins on the cell surface creates hydrophobic shell around the fungal hyphae. The hydrophobicity provided by these proteins helps the hyphae to escape water and facilitate dispersal of spores. Besides hydrophobicity they also help fungi to

attach inert surfaces, biofilm formation and pathogenesis.

Several physiochemical tests have been employed for the detection and confirmation of amyloids. These methods include formation of red coloured colonies on Congored agar, green fluorescence with thioflavin T, green birefringence of Congo red under polarized light, Scanning and Transmission electron microscopy, CD spectra and FTIR spectra.

Applications of amyloids

The study and application of amyloids can have important industrial applications as these protein structures are sturdy and ecofriendly. Recent studies have shown that the unique properties of amyloids can be exploited for various industrial use such as bioremediation of heavy metals for contaminated water (Bolisetty *et al.* 2016), immobilization of enzymes (Sarah *et al.* 2010) and for making nano wires (Scheibel *et al.* 2003). One of the earlier study from our group shows potential application of amyloid protein produced by *Solibacillus silvesteris* as bioemulsifier (Markande *et al.*. 2016). Based on this observation we were interested in exploring the bioflocculation properties of bacterial amyloids as they have a major role in cell aggregation and biofilm formation.

Biofloculants

The phenomenon in which suspended particles aggregates to form large clusters is called flocculation. It is governed by two factors i) size of particles and ii) electric charge on particles. The first factor is governed by the Stokes law of viscosity. According to Stokes law, smaller the size of particle, more is the viscous drag and hence longer it takes to settle. Whereas large particles tends to settle easily. Besides particle size, the particles in a suspension usually have an electric charge which causes them to repel each other. This repulsion further avoids the particles to come closer and form aggregates. The addition of flocculant to the suspended particles allows formation of large clusters ultimately causing them to settle. The aggregation of particles by flocculant is consummated by two mechanisms. i) Electrostatic patching and ii) bridge formation. Electrostatic patching occurs by the binding of oppositely charged flocculant on the particles. This binding favors binding of other particles on the flocculant, ultimately resulting in aggregation. Bridge formation involves binding of large polymeric flocculant on the particles and bringing them closer to form large flocs. Bioflocculants produced by variety of microorganisms are high molecular weight compounds that aggregate solid particles, colloidal particles and bacterial cells

causing flocculation. Bioflocculants produced by most of the *Bacillus* sp. are reported to be composed of neutral sugar, amino sugar and uronic acid (Xiong*et al.*.2010, Wu*et al.*. 2007). Analysis of the flocs of activated sludge have shown the abundance of amyloid producing bacteria that belongs to phylogenetically distant species in the phyla *Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes,* and *Actinobacteria*. Amyloids have highly stable structures that are resistant to thermal and chemical denaturants as well as pH (Larsen *et al.*. 2007).

Methods of studying properties of amyloid proteins

Congored binding

The ability of amyloid protein to bind Congo red dye is one of the classical assays for detection of amyloid. Pure cultures of bacteria and fungi producing amyloids have demonstrated red colored colonies when grown on media containing Congo red. (Glenner *et al.* 1972; Klunk *et al.* 1989). Binding of Congo red with amyloid also changes the optical property that includes the phenomenon of birefringence of cross polarized light and red shift in the absorption maxima of Congo red from 480nm to 510nm. The birefrincence assay is one of the extensively used technique for the diagnosis of disease associated amyloids.

Thioflavin T staining

Barring Congo red, Thioflavin T is a benzathiozole fluorescent dye documented with high specificity in binding with amyloid protein (Westermark *et al.*1999). Hence the bacteria that produces amyloid protein can be identified as bright green fluorescence under fluorescence / confocal microscope.

Resistance to SDS

The unique and interesting properties of amyloids includes resistance to denaturants like heat and detergents like SDS. Besides harsh treatment the fibrous nature of the amyloid protien allows them to be separated from other proteins by conventional agarose gel electrophoresis. The non amyloid protein tends to migrate in the gel while the fibrous amyloid protein remains in the wells and can be easily purified.

Electron Microscopy

Both SEM and TEM are versatile methods used for the direct detection of amyloids. SEM provides curial information regarding tightly bound amyloids on bacterial surface while TEM provides valuable information about the loosely bound amyloids on cell surface.

FTIR

Fourier transformed infrared spectroscopy is a technique employed for the detection of chemical functional groups in biological molecules. Besides functional groups FTIR alsoprovides valuable information regarding the secondary structure of proteins. The amyloid protein having typical sheet can beeasily identified on the basis of FTIR spectrum.

CD spectra

Besides FTIR circular dichorism is an ancillary technique for detection of β sheets in proteins. Once of the widely used classical amyloid confirmation assay is congored birefringence assay. This method is based on the binding of Congo red with the β sheets of amyloidprotein. This interaction of congo red with the protein allows amyloid producing bacteria to be identifed as red colour colonies on Congo red agar plates. Besides red colonies the purified protein stained with Congored demonstrates apple-green birefringence undercross polarized light.

Rationale:

In case of Enterobacteriaceae amyloids have an important role in cell adhesion and biofilm formation. In *Salmonella enterica* and *E coli* amyloids have been reported to have major role in biofilm formation and biofilm structure (Chapman *et al.* 2002, Barnhart *et al.*, 2006) In case of fungi like *C. albicans* and *Bacillus subtilis* amyloids have role in cell aggregation and biofilm formation. Knock out mutants of amyloid genes shows abrogated cell aggregation and biofilm formation (Gracia *et al.* 2011). Flocs of activated sludge have shown the abundance of amyloid producing bacteria that belongs to phylogenetically distant species in the phyla Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes, and Actinobacteria (Larsen *et al.*. 2008). In our laboratory we have earlier studied bioemulsifier property of amyloids from bacteria. In this context a bioflocculant having amyloid nature would be rugged and at the same time offer a suitable ecofriendly alternative to chemical flocculants. Hence based on these observations our aim was to investigate role of amyloids in flocculation and apply it to solve problems such as microalgae harvest and waste water treatment.

Objectives

1. Screening of amyloid producing bacteria from flocs of activated sludge process.
2. Biotechnological applications of the purified amyloid proteins
3. Study of physiological importance of amyloids in the selected isolates.
4. Cloning of amyloid producing genes form the selected isolate.

Work done.

The screening of amyloid producing bacteria was done from the flocs of activated sludge collected from various sewage treatment plants of Vadodara and Surat. The screening of amyloid producing bacteria was done on Congo red agar where the amyloid producing isolates showed red color colonies while non amyloid producers showed white colonies. The red color colonies so obtained were further confirmed by Thioflvain T staining and fluorescence microscopy. Each of the amyloid positive isolate were screened for flocculation activity. One of the isolate identified as *B.cereus* CR4 showed maximum flocculation activity and was selected for further studies

In an effort to confirm the amyloid production by *B. cereus* CR4 and assign the flocculation activity to it,two categories of the tests were performed. i) Demonstration of amyloids on cell surface of *B. cereus* CR4 and ii) Purification of amyloid protein and investigation of its flocculation activity.In the first category of tests, for the demonstration of cell bound amyloid protein the cells of *B. cereus* CR4 were stained with Thioflavin T and observed under fluorescence microscope. Green fluorescence with Thioflavin T upon binding with proteins like amyloids that are rich in beta pleated sheath and have been recognized as gold standard for selectively staining and identifying amyloid fibrils.Therefore green fluorescence of Thioflavin T upon binding to amyloid may be used as a diagnostic tool for the presence of amyloid structure Staining the cells of *B.cereus* CR4 with Thioflavin T showed bright green fluorescence under fluorescence microscope showing presence of amyloid protein on its cell surface.

The second category of tests were initiated with purification of the amyloid protein.SDS agarose gel electrophoresis performed with the bioflocculant CR4 as per Sitaras*et al.*.(2011)

where the property of amyloid to withstand denaturing agents like SDS was exploited. Accordingly, the amyloid protein was retained in the sampling well while other proteins advanced in the gel during electrophoresis. The amyloid protein accumulated in the wells was visualized by staining the gel with Comassive brilliant blue and SDS PAGE. The pooled protein was purified by this method and subjected to several assays for amyloid confirmation.It has been well established that the amyloid protein interacts with the aromatic ring of Congo red and shows characteristic feature of i) birefringence under cross polarized light and ii) red shift in the absorption spectra.The purified protein, after staining with Congo red showed apple green birefringence when viewed under cross polarized light and red shift in absorption maxima from 470 nm due to free Congo red to 510 nm. The birefringence and the red shift phenomenon is considered as one of the definingtests for the confirmation of amyloid proteins (Klunk*et al.*. 1989).Further strong evidence for the presence of amyloid was supported by visualization of fibrils in transmission electron microscopy. This evidence was supported by FTIR and CD spectra, which are one of the versatile tools to confirm β sheet rich amyloid proteins (Calero *et al.*. 2012) The results were in accordance with the proposed criteria for the confirmation of amyloid protein as per Nilsson *et al.*.(2004) that requires minimum 4 points to be present in a protein to be categorized as amyloid form. The protein CR4 produced by *B.cereus* CR4 scored 7 points (presence of β secondary structure detected by FTIR/CD 2 points, Congo red binding 2 points, Thioflavin T binding 2 points and low solubility is SDS 1 point) which confirmed the amyloid nature of protein produced.

The pooled purified amyloid protein from SDS demonstrated flocculation activity.The pH and concentration of bioflocculant governs the aggregation phenomenon that leads to flocculation. pH determines the net charge on the bioflocculant which inturn determines the interaction of bioflocculant with the suspended kaolin particles. In case of purified amyloid protein *B.cereus*CR4 was 61% flocculation activity was observed at an acidic pH of 5 and 60 μgml^{-1} amyloid concentration.

To study the kinetics of bioflocculant production on the bacterial cell surface the flocculation activity associated with the biomass and supernatant was analyzed at different stages of growth. The flocculation activity demonstrated by the biomass increased during logarithmic growth phase and reached maximum towards its end, which indicates that the bioflocculant production was growth associated. The quantification of amyloid in biomass at different

growth stages was correlated with the flocculation activity associated with biomass. There was a gradual increase in flocculation activity in supernatant during logarithmic phase of growth of the culture that peaked at 16h corresponding to maximum flocculation activity. Indeed it has recently been shown in our laboratory that the cell bound amyloid produced by *Soli Bacillus silvestris,* gradually gets released in the extracellular environment, and shows aggregation during growth phase (Markande *et al.*. 2013). This gradual increase may also be due to the fact that the amyloids are produced on the cell surface and are constantly released into the extracellular environment as they are loosely associated with the cell surface.

The identification of the bioflocculant gene for amyloid protein produced by *B.cereus*CR4 was done by C-DAG system (curli-dependent amyloid generator). For the identification purpose as no prior information about the gene was available primers were designed so as to amplify most of the ORFs and ligate them in pVS72 vector in order to keep the gene in-frame. The curli-dependent amyloid generator (C-DAG) uses *E.coli* strain VS16 which has deleted curli operon and harbors a plasmid pVS72that overexpresses CsgG under IPTG induction. CsgG is a transmembrane protein that identifies signal sequence of curli amyloid and exports it outside the cell (Sivanathan *et al.*. 2013). The gene of interest is cloned in plasmid pVS72 that translates a fusion product of the gene of interest and the curli signal sequence which ensures its export outside the cell. After transformation the transformants were screened on TC agar medium containing arabinose and IPTG for induction.The transformant producing red colored colony and non transformant colorless colony was subjected to colony PCR with the primers flanking the gene of interest specific for plasmid and the product was sent for sequencing at SciGenom Labs, Kochi, India. The sequencing results show that the product was 89% similar to the *tas*A amyloid gene of *Bacillus subtilis*.

The flocculation ability of amyloid was further exploited in the harvesting of microalgae (Vandamme *et al.* 2013). *Scenedesmus* was grown in BG11 media, and flocculated with *B.cereus* CR4 in presence of Fe^{+3} as a coagulant. The contour plots representing flocculation of *Scenedesmus* at various concentrations of $FeCl_3$andpH.At pH below 4.5 and$FeCl_3$concentration 156mgl^{-1}, 86.87% flocculation activity was observed after addition of amyloid producing *B.cereus* CR4. Once the harvest of microalgae was standardized the flocculation ability of *B.cereus* CR4 was used for harvesting microalgae following waste water treatment.

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