Chapter 1

Introduction and Review of literature

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The world wide requirement for the treatment of organic and inorganic pollutants from the effluents of domestic and industrial waste water has become prerequisite in this era (Pawelec *et al.,* 2011; Peng *et al.,* 2011). The increased levels of such pollutants contribute to undesired levels of turbidity, colour, high BOD and COD to the discharged water (Verma *et al.,* 2012). Hence it becomes an absolute requirement for the treatment of polluted discharged water before its release into water bodies like lake, rivers or ocean. Techniques such as industrial filtration, centrifugation, electro-coagulation, treatment with activated carbon have been considered as a method of choice by most of the industries (Wei *et al.,* 2018). However, with an increasing concern of the amount of waste water to be discharged the currently available methods becomes more and more costly. Hence there arises a need for an alternative cost-effective method for the significant management of industrial effluents. Bioflocculation is one of the ways in which this task is accomplished (Kurniawan *et al*., 2020; Liu *et al*., 2021).

The flocculants used at an industrial scale can either be organic or inorganic. The inorganic chemical flocculants that are widely used for the industrial purpose are alum (aluminum sulphate), aluminum chloride, polyaluminum chloride, ferrous sulphate, ferric chloride, magnesium chloride and calcium chloride. These flocculants function by the phenomenon of surface charge neutralization of the suspended particles. It must be noted that the charge neutralization of suspended particles depends on the amount of salts added. Optimum flocculation is observed when the zeta potential of the particles becomes zero (isoelectric point). Excessive use of metal coagulants can cause reversal of zeta potential that finally results in re-dispersal of suspended particles (Kleimann *et al*., 2005). There are several disadvantages of inorganic chemical flocculant, that includes their requirement in large amount for efficient flocculation, needs specific pH to induce flocculation, and toxicity to humans and other animals (Flaten T., 2001; Polizzi *et al.,* 2002; Ward *et al*., 2006; Banks *et al*., 2006). The organic chemical flocculants involved in direct flocculation are high molecular weight, linear or branched, anionic / cationic or amphoteric, water soluble

polymers synthesized products of petroleum industry (Suopajarvi *et al*., 2013) eg. polyacrylamide is one of the common flocculant synthesized by polymerization of monomers of acrylamide. Other such flocculants includes polyacrylic acid, polyamine and polydiallyl dimethyl ammonium chloride (Singh *et al*., 2000). Majority of chemically synthesized polymers cannot be degreded in the environment and have been distinguished as potentially unsafe for their large-scale applications (Brostow *et al*., 2009). The monomeric forms of chemical flocculants such as acrylamide, ethyleneimine, epichlorohydrin and dimethylamine are extremely toxic and can act as a potent carcinogen. The release of such toxic monomers into the environment can have adverse effect on living organisms by entering the food chain (Sharma *et al*., 2006).

1.1 Amyloid form of proteins and neurological disorders

Proteins are considered as a building blocks of life and are known to have two main secondary structures. i) The α helix and ii) the β sheet. The α helix is a coiled structure whereas the β sheet is a structure in which the sheets are stacked over one another. The stacking in β sheet may either be in parallel or antiparallel fashion. The stability of these structures depends on the external conditions such as pH, temperature and ionic strength. Any changes in these parameters can lead to conformational changes resulting in protein misfolding and aggregates.

Amyloids are filamentous protein structures that share a structural motif, the cross-beta structure. The interest in amyloids is caused by their relation to a vast group of human and animal diseases called amyloidoses. Some of these diseases caused by prions, a specific type of amyloids, are transmissible. Besides mammals, prion amyloids are described in lower eukaryotes, where they underlie non-chromosomal genetic determinants In case of mammals protein misfolding can have detrimental effects giving rise to neurological degenerative disorders like Alzheimer's, Huntington's, and Parkinson disease. These misfolded and aggregated proteins termed amyloid proteins are the aggregates of β sheet proteins that form thin, long fibrils. The β sheets are stabilized by systematic hydrogen bonding which imparts

its unique characteristics such as high stability in presence of physical and chemical denaturants. The dimension of typical amyloid fibril varies from 5 - 10nm in thickness and several micrometers in length (Salinas *et al.,* 2020). The disease associated amyloids are formed because of protein misfolding. The misfolded proteins then aggregate to form fibrils that are deposited as neurological plaques eventually leading to a condition known as amyloidosis. Many proteins can metamorphose into amyloids on account of mutations and misfolding into β sheets that aggregate to form fibers. The amyloids formed due to protein misfolding are physiologically harmful and are corroborated to be lethal in the long run.

1.1 Amyloid form of protein produced by microorganisms

Contrary to amyloids accumulated in diseases, bacteria and fungi make amyloid proteins that possess specific functions. The amyloids made by the bacteria and fungi are often termed as functional amyloids as they have a unique function associated with them (Salinas *et al.,* 2020; Picken *et al*., 2020). In contrast to the disease associated amyloids the functional amyloids are one of the eminent cell surface proteins in the prokaryotic world (Gebbink *et al.,* 2005; Otzen *et al.,* 2019; Evans *et al.,* 2018). The functional bacterial amyloid often abbreviated as FuBa, was first discoverd in *E.coli*. Microbial functional amyloids are extremely diverse and are involved in structural scaffolding and biofilm formation, adhesion, surface-tension modulation, regulation of the cell cycle and gametogenesis, toxicity, host–pathogen interactions, and symbiosis. Due the diversity in structure and regulation, microbial functional amyloid systems have functional diversity (Levkovich *et al*., 2020).

1.2 Amyloid producers in natural habitat

The functional amyloid has been widely distributed in fungal and bacterial populations. Studies of bacteria associated with diverse natural habitat such as fresh water lakes, drinking water reservoir and waste water treatment plants have shown existence of amyloids. Most of the biofilms formed under such natural habitat have dominance of amyloid producing bacteria. Assessment of bacteria producing amyloids have shown that they constitute 5 - 40%

of all the bacteria present in the biofilms. These bacteria have been documented to be catalogued into several phyla such as Proteobacteria, Bacteriodetes, Chloroflexi and Actinobacteria (Jordal *et al.,* 2009; Balistreri *et al*., 2020). The distribution of amyloid producers in various natural environments is widespread. Figure. 1.1 gives schematic representation of FuBa from different bacteria.

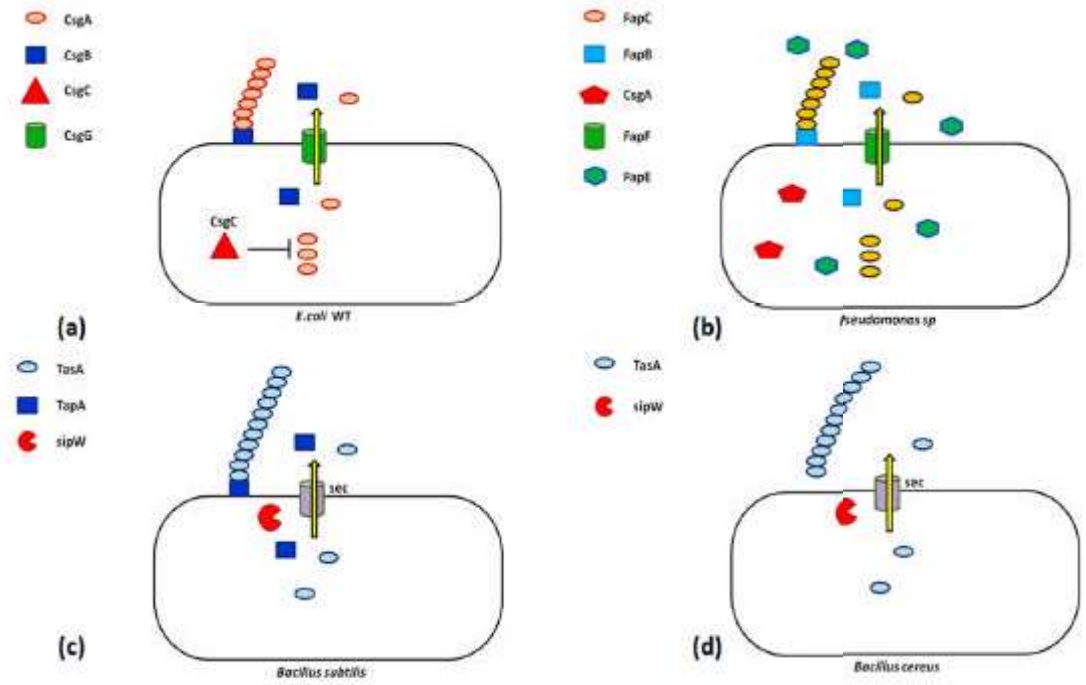

Figure 1. 1 Presence of functional bacterial amyloid in various bacteria a) *E. coli*. b) *Pseudomonas* sp. c) *Bacillus subtilis*. d) *Bacillus cereus*.

1.2.1 Curli amyloid

The amyloid present in *E. coli* was termed as curli (Figure. 1.1a). The curli amyloid has been discovered in pathogenic as well as non pathogenic *E. coli* (Dueholm *et al.,* 2012). This amyloid is one of the chief extracellular protein components 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).

Besides having role in animal tissue incursion, the curli functional amyloid have also been found to be present in profuse environmental biofilms. In case of legumes, the root nodules constitute *Rhizobiales* sp that expresses curli for cell aggregation (Smith *et al.,* 2017).

The gene that codes for curly amyloid has been designated as csgA. The gene csgA is translated as a monomeric protein and is excreted out of the cell by a membrane bound CsgG protein. The CsgG identifies the singal sequnece 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 (Yan et al., 2020). 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 pathogesis (Miller *et al*., 2020). After their discovery in *E.coli* amyloid were also discovered in other members of the family *Enterobacteriaceae.*

1.2.2 TasA in *Bacillus* sp.

Bacillus sp. 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 conditions the *Bacillus* sp. form spores that are resistant to various external environmental stress. Studies of *Bacillus* sp. spores 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. TasA has been extensively studied in model strain *Bacillus subtilis* (Figure. 1.1c). The TasA amyloid is encoded by tapA-sipW-tasA operon (Branda *et al*., 2006; Romero *et al*., 2010). Upon its secretion in the extracellular environment the TasA monomers aggregates to form fibrillar structure. The fibers remain attached with the cell wall via accessory protein called TapA (Figure. 1.1c) (Beauregard *et al.,* 2013). In absence of TapA, the TasA fibers are released into the extracellular environment (Figure. 1.1d). The presence of TasA also provides hydrophobic nature to the cell surface, leading to autoaggregation of cells at air liquid interface and formation of pellicle. The pellicle formed by *Bacillus subtilis* are one of the highly rigid and sturdy pellicles formed by the bacteria (Asally *et al.,* 2012; Azulay *et al.,*

2020). The role of TasA is prominent in the environments where the bacterial cells experiences oxygen and nutrient gradients. In presence of TasA and *Bacillus* surface layer protein BslA the bacterial cells aggregate at the air liquid interface to form a thick floating biofilm termed as a pellicle. In contrast the strains of *Bacillus subtilis* devoid of TasA fails to form pellicle. The TasA amyloid fibers also protects the spores of *Bacillus subtilis* in adverse conditions and also help in spore dispersal. Besides biofilm formation and cell aggregation the TasA has major role in colonization of plant root tissues and protect the plant root against various invading pathogens (Nagorska *et al.,* 2007; Steinberg *et al.,* 2020). The TasA protein purified from *Bacillus subtilis* binds amyloid specific dye thioflavinT and Congo red, demonstrates characteristic green birefringence under cross polarized light and shows highly fibrous mesh under electron microscope (Chai *et al.,* 2013).

The amyloid produced by *B. cereus* is depicted in figure 1.1d. The amyloid TasA produced by *B. cereus* is similar to TasA amyloid produced by *B. subtilis*. However, this functional amyloid system is devoid of accessory protein TapA. The absence of TapA amyloid on the cell surface of *B. cereus* causes TasA amyloid fibers to be released free in the extracellular environment. The absence of TapA in *B. cereus* also results in formation of weak biofilms as compared to thick and sturdy biofilms of *B. subtilis.*

1.2.3 Tafi amyloid

Tafi amyloids are associated with members of *Salmonella* sp. Just like curli tafi amyloids have an important role in biofilm formation, invasion of host tissue for colonizing small intestine and pathogenesis. Similar to the curli amyloid of *E. coli* the tafi amyloid also binds host proteins like laminin and fibronectin. Interaction of amyloid protein with fibronectin allows them to be easiliy internalized with the aid of integrins. Once internalized the bacteria initiates pathogenesis (Li *et al.,* 2018).

Several proteins present in the human body have tendency to bind with the microbial amyloids which results in spread in infection and inflamation. This binding is usually governed by the cross-beta structure of the amyloid fibril and biochemical interactions such

as hydrogen bonding, hydrophobic interaction and ionic interaction (Kosolapova *et al.,* 2020). The mammalian protein such as tPA (tissue plasminogen activator), fibronectin and factor XII have been extensively explored for their interaction with the bacterial amyloids such as curli and tafi (Kranenburg *et al*., 2002). A specific domain termed as fibronectin binding domain, present in these proteins interacts with the parallel beta sheets of the amyloid protein (Hammar *et al.,* 1995; Loferer *et al.,* 1997). In contrast the mutant strains of *E.coli* and *Salmonella* having defective or no amyloid, fails to cause tissue invasion. The functional bacterial amyloids utilize both the proteins for escaping neutrophill phagocytosis leading to enhanced virulence. Hence the phenomonon of interaction of bacterial amyloids with the host proteins plays a major role in virulence as well as activating the host immune response. Also, during the course of evolution most of the virulent bacterial strains have lost ability of synthesize amyloids, in order to escape the host immune response.

1.2.4 Chaplin amyloid

Just like curli amyloids the chaplins are synthesized in the form of monomers and aggregated into fibrils on the cell surface. *Streptomyces coelicolor* contains eight forms of chaplins, out of which five are mature forms called long chaplins and are 55 amino acids in length (Claessen *et al.,* 2004, 2003). The remaining are called short chaplins that share the chaplin domain and are anchored within the cell wall. The rest of the monomers aggregate on the domain and form fibrils. The self assembly of chaplin monomers on the cell surface gives characteristic hydropohobic nature to the cell surface. The cell surface hydrophobicity allows the cells to attach on hydrophobic surfaces and escape the surface tension of water. The hydropohobic nature also prevents aggregation of spores allowing their dispersal in presence of water. These spores are widely distributed in moist and damp environment. Upon introduction in human body the spores induce inflamatory response and skin infections. However, the role of chaplins in the progression of infection is still not clear.

1.2.5 Harpins

Harpins are amyloids produced by plant pathogen *Xanthomonas* sp. Harpins are glycine rich proteins that may either exist in small oligomeric or long fibrillar form. Unlike mature fibril

which have long slender nature, the oligomers demonstrate spherical morphology when studied under electron microscope. The oligomeric form plays an important role in induction of hypersensitive reaction in rice plants. The hypersensitive reaction weakens the plant tissue making it susceptible to bacterial infection. The fibrous amyloid form plays an important role in colonization of plant tissues and formation of biofilm on plant surface (Yuan *et al.,* 2020).

1.2.6 Chorion

Chorion is a functional amyloid present in the outer egg shell of silk moth. The extraordinary sturdy nature of amyloid makes chorion resistant to various physical and chemical stress. This protects the embryo from external stress like high temperature, physical pressure, moisture and from various viruses and bacteria (Siniukova *et al.,* 2020).

1.2.7 Mycobacterium tuberculosis pili (MTP)

Human pathogens such as *Mycobacterium tuberculosis* is responsible for millions of deaths world wide. The pathogen possesses cell surface pili that has characteristic similar to tafi amyloid of *Salmonella* sp. This amyloid is termed as MTP (Alteri *et al.,* 2007). The cell surface pili of *Mycobacterium* remains stable in SDS loading buffer, heat and denaturants like urea (Levesque *et al.,* 2001; Collinson *et al.,* 2001). MTP has been demonstrated to bind host proteins like lamimin and mediate its pathogenesis. However, the contrasting feature of MTP is that it does not bind host proteins like type IV collagen and fibronectin. Strains of *Mycobacterium* lacking MTP show about 60% decline in their virulence, which suggests the role of MTP amyloid in virulence. The study of MTP by transmission electron microscopy demonstrates fibrils morphologically similar to curli and tafi amyloid (Barnhart *et al.,* 2006, Alteri *et al.,* 2007). However, at molecular level there is no sequence homology between MTP and amyloids of other bacteria showing similar phenotype. This observation suggests that even though the amyloids share similar phenotypic nature, the primary amino acid sequence might be completely diverse. Another contrasting feature of MTP is that the genes coding for this functional amyloid are not organized in clusters of operon but are scattered throughout the genome. Hence MTP is recognized as one of the unique functional amyloid presents in the bacterial world (Miller *et al.,* 2021).

1.2.8 Hydrophobins

These are multifunctional proteins made by diverse species of fungi. Just like bacterial amyloids they are synthehized in the form of monomers and are assembled on the cell surface. However, the contrasting feature of these proteins is that unlike bacterial amyloid that are loosely associated with the cell surface, the hydrophobin amyloid are tightly associated with the cell surface. The tight association of hydrophobin amyloids provides unique features to the cell surface which includes ability to attach on hydrophobic abiotic or biotic surfaces, reduction of surface tension of water, escape from water surface and spore dispersal. Besides being tightly associated with the cell surface, the monomeric forms of hydrophobins can assemble at the air liquid interface and act as a surfactant that ultimately decreases surface tension of water. The biosurfactant nature of amyloid at the air liquid interface allows the fungal hyphae to easily escape water and also allow the spore dispersal. Certain plant pathogenic fungi such as *Magnaporthe grisea* contains cell surface amyloid which are classified as class I hydrophobin MPG1. This protein allows attachment of spores on hydrphobic surfaces and develop an infective structure called appressorium. The appressorium has very high turgor pressure that allows penetration of the fungal hyphae into the plant tissue. The presence of cell surface amyloid allows the appressorium to withstand high turgor pressure and aid in plant invasion (Talbot 2003, Elliot 2003) (Fig.1.2). The strains of *Magnaporthe* devoid of amyloid fails to infect plant tissue. Human and animal fungal pathogens such as *Aspergillus fumigatus* has hydrophobin amyloid termed as RodA (Thau *et al.,* 1994, Paris *et al.,* 2003). The monomers of RodA assemble to form a rod like structure that acts as a functional amyloid. The RodA fibrils are present on the conidia and aids in its virulence. Besides virulence the RodA coated on conidia allows the fungi to escape neutrophils and macrophages (Parta *et al.,* 1994).

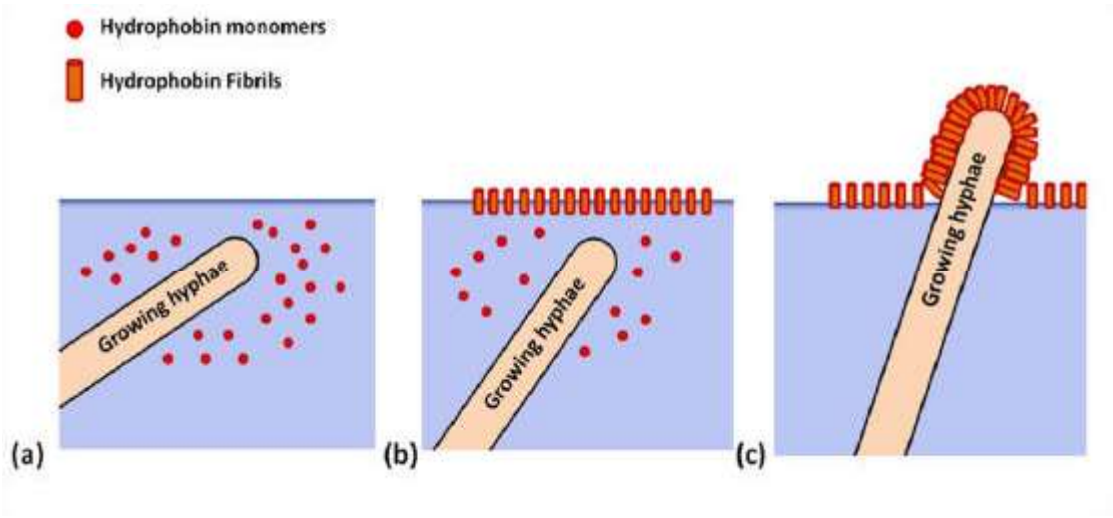

Figure 1. 2 Role of functional amyloid hydrophobin in escape of fungal hyphae from water a) Soluble monomers of hydrophobin secreted by the fungal hyphae. b) Aggregation of hydrophobin monomers at the air liquid interface and polymerization into amyloid fibrils. c) Functional amyloid fibrils at the air liquid interface reduces the surface tension of water and allows the escape of the fungal hyphae.

1.3 Methods to study amyloid

There are several techniques available for the confirmation of amyloid form of protein. Most of these techniques have evolved from the classical diagnostic methods used for detection of amyloids in brain biopsies of Alzheimer's patients. The same techniques have been employed for the detection and confirmation of amyloids in bacteria as well as fungi. These techniques include Congo red birefringence assay, Congo red red shift assay, thioflavin T fluorescence, resistance to SDS, TEM, SEM, FTIR and CD spectra. Nilsson *et al*. (2004) have developed criteria for classification of protein to be amyloid. This method is based on assigning numerical points to various tests done for detecting amyloid. The points given for each test are as follows. 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. The protien must atleast score 4 points to be confirmed as an amyloid protein.

1.3.1 Congo red 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 coloured colonies when grown on media containing Congo red. Screening colonies based on red coloured phenotype is one of the effortless assays to discern amyloid production. In addition, staining purified protein with Congo red demonstrates apple green birefringence under cross polarized light (Glenner *et al.,* 1972; Klunk *et al.,* 1989). The phenomenon of birefringence transpires when the planarly arranged phenyl ring of Congo red interacts with the β sheets of amyloid protein. This interaction causes torsional strain on the planarly arranged phenyl ring that changes the optical properties of the dye. This change in optical property 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.

1.3.2 Thioflavin T staining

Thioflavin T is a benzathiozole fluorescent dye documented with high specificity in binding with amyloid protein (Westermark *et al.,* 1999). Just like Congo red Thioflavin T has planarly arranged phenyl rings that binds β sheets of the amyloid protein. This binding results in increase in the fluorescence emission of the dye. Hence the bacteria that produces amyloid protein can be identified as bright green fluorescent cells under fluorescence / confocal microscope. Though the fibrils cannot be optically resolved under microscope, the aggregated fibrils do display fluorescence when viewed under the microscope.

1.3.3 Resistance to SDS (Sodium dodecyl sulphate)

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. The purified amyloid protein can be then subjected to

several assays like electron microscopy, FTIR and CD spectra analysis for further confirmation.

1.3.4 Electron microscopy

Both SEM and TEM are versatile methods used for the direct detection of amyloids. SEM provides crucial information regarding tightly bound amyloids on bacterial surface while TEM provies valuable information about the loosely bound amyloids on cell surface. Reports suggests that amyloids appear to be aggregated fibrous structures which are several micrometers in length and few nanometers in diameter.

1.3.5 FTIR

Fourier transformed infrared spectroscopy (FTIR) is a technique employed for the detection of chemical functional groups in biological molecules. Besides functional groups FTIR also provides valuable information regarding the secondary structure of proteins. The secondary structure like α helix shows absorption maxima at 1652 cm^{-1} while β sheet shows absorption maxima at 1630 cm^{-1}. The amyloid protein having typical β sheet can be easily identified on the basis of FTIR spectrum.

1.3.6 CD spectra

Besides FTIR circular dichroism is an ancillary technique for detection of β sheets in proteins. A protein with typical beta sheet structure will demonstrate negative absorption peak at 220nm thus making it a valuable tool for identification of protein secondary structure.

1.4 Biotechnological applications of amyloids

Owing to sturdy nature of amyloid fibrils they carry prodigious potential for several industrial applications. Studies have also shown potential of amyloid fibres to act as a supporting material for making nano wires (Bolisetty *et al*., 2012). The TasA amyloid produced by *Bacillus subtilis* have been recently exploited to prepare biocementing material with unique properties (Scheibel *et al*., 2003). Similarly studies herein tend to exploit the flocculation properties of bacterial amyloid.

1.5 Flocculation process

The phenomenon in which suspended particles aggregate to form large clusters is called flocculation. Two factors govern the settling of particles in suspension 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 tend to settle easily. Besides particle size, the particles in a suspension ususally 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. These binding favours 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 (Dotto *et al.,* 2019) (Figure. 1.3).

5.5.1 Chemical flocculants

The flocculants used for the clarification of suspended particles can either be organic or inorganic. Usually, metal ions are used as inorganic flocculants. They function by neutralizing the surface charge of suspended particles allowing them to come close to one another and result in their aggregation. This aggregation phenomenon is termed as coagulation. Unlike flocculation where the polymer binds the suspended particles to form aggregates, coagulation only involves neutralization of surface charge by metal ions that allow particles to come together and form aggregates (Pugazhendhi *et al.,* 2019). The aggregates formed by coagulants are less stable and are easily affected by external factors such as pH, temperature, turbulance and presence of other ions. Hence to obtain high floc density polymeric flocculants are often added along with coagulants to form large and stable flocs. Several inorganic compounds like polyaluminium chloride, calcium chloride, ferric chloride, aluminium sulphate acts by the mechanism of coagulation. It should be noted that

aggregation of particles by metal ions is governed by attraction between oppositely charged species. Hence only those particles with net negative charge can be aggregated using metal ions. Addition of coagulants to a particle suspension having net positive charge can abrogate coagulation process (Huang *et al.,* 2013; Blanco *et al.,* 2010).

Besides coagulants the aggregation of suspended particles can be achieved by using polymers that binds the particles and cause their aggregation. These polymers induce flocculation by the mechanism of electrostatic patching or bridge formation. The organic polymeric flocculants are the byproduct of petroleum industry. These polymers have high molecular weight and have long fibrous structure which may either be branched or unbranched. Polyacrylic acid, polyacryl amide, polyamine etc. are one of the commonly used organic flocculants for flocculation. Most of the chemical-based flocculants are toxic and hazardous for the environment. Hence in recent times organic flocculants extracted from biological sources have gained substantial importance (Zhang *et al.,* 2013; Ntsaluba *et al.,* 2011; Khan *et al.,* 2015).

1.5.2 Bioflocculants

The bioflocculants are high molecular weight compounds synthesized by living organisms. These biopolymers have multiple functional groups that can interact with the suspended particles resulting in their aggregation. The bioflocculants can be produced by plants as well as microorganisms. Depending on the pH the funcional groups of the biopolymer can have various electric charges, that facilitates electrostatic patching and bridge formation with the colloidal particles to induce flocculation. Unlike metal ions that can flocculate only negatively charged particles the biopolymers can aggregate any particles with net positive or negative charge. Besides this they have a unique advantage of being cheap, ecofriendly and non-toxic to the environment. The bioflocculants being biodegradable in nature can have wide variety of applications (Saha *et al.,* 2020; Ang and Mohammad., 2020).

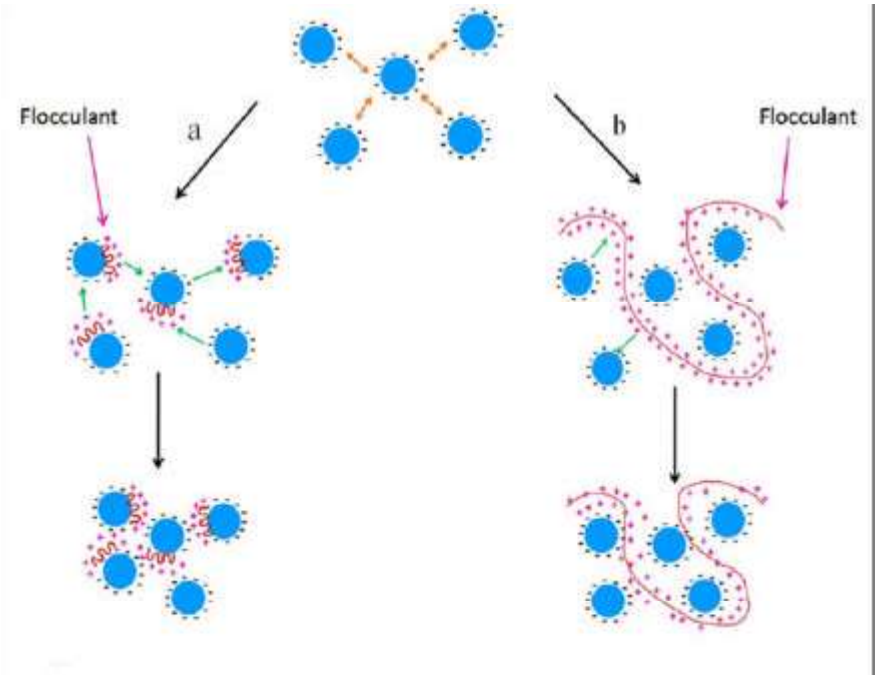

Figure 1. 3 Mechanism of flocculation by a) Electrostatic patching and b) Bridge formation.

1.5.3 Plant based bioflocculants

Plants are rich in biopolymers such as starch, mucilages, natural gums, chitosan and cellulose. These polymers can either by homopolysaccharides or heteropolysaccharides made up of several carbohydrate moieties like glucose, galactose, maltose, fructose, xylose and uronic acids (Jani *et al.,* 2009; Mirhosseini and Amid, 2012). Presence of these moieties give them a sticky nature and can bind several suspended particles by electrostatic and ionic interactions resulting in their flocculation. The naturally existing gum and mucilages in plants have been widely exploited in the treatment of various industrial effluents (Gupta and Ako, 2005). Sodium carboxy methyl cellulose a chemical derivative of naturally existing cellulose have been used for the treatment of drinking water (Khiari *et al.,* 2010). The extraction of plant based polymers is done by solvent extraction method. Recent studies show that the naturally

extracted plant-based polymers can also be chemically modified to add more branches. Such chemical modification of natural polymer is termed as grafting. The modification by grafting provides large surface area for attachment of particles for flocculation (Ghosh *et al.,* 2010; Pal *et al.,* 2012).

1.5.4 Microbial bioflocculants

Apart from plants the bacteria and fungi present in the nature can also produce variety of polymers having bioflocculant properties. The phenomenon of microbial bioflocculation was first discovered a century ago by Louis Pasteur in the year 1899, when he was studying yeast cells (Pasteur L, 1976; Wilhelmi, 1944). He documented that the yeast used for the fermentation of wine produced an adhesive like substance that caused cell aggregation. Bloch and other investigators in the year 1918 discovered the role of floc forming *Zoogloea ramigera* in the aggregates of activated sludge (Blöch M 1918). They also documented a strong correlation betwen the EPS production by bacteria and bioflocculation. Further investigations on bioflocculants revealed more detailed insight into the chemical nature of biofloccualnts (Ajao *et al.,* 2021). These biopolymers are usually made up of polysaccharides, extracellular polymeric substances (EPS), proteins, glycoproteins, lipids and glycolipids (Wang *et al.,* 2015; Tenney *et al.,* 1973). Depending on the pH and presence of ions, the EPS can possess net positive or net negative charge that allows its interaction with the charged particles in the suspension. Due to the presence of electric charge on the suspended particles the suspension remains stable due to electrostatic repulsion. The repulsion between the colloidal particles prevents aggregation and settling of particles. However, in presence of EPS the electric charge on the particles gets neutralized which leads to their aggregation and flocculation (Wan *et al.,* 2013; Kratochvil *et al.,* 1998).

The persence of EPS around bacterial and fungal cells bequeaths them with property of cell aggregation, biofilm formation and bioflocculation. The composition of EPS also differs between the species. The EPS is customarily composed of polysaccharides, proteins, lipids, glycoproteins and glycolipids which proffers them unique adherent nature. When dispersed in aqueous environment they form matrix around suspended particles and entraps them to

form large aggregates and floccules. Besides EPS few bacteria and diatoms can also make transport exopolymeric particles (TEP). Transport exopolymeric particles are the products of EPS that have experienced abiotic stress such as shearing forces. Just like EPS bioflocculants the TEP also interact with suspended particles by electrostatic patch or bridge formation. In the marine environment TEP plays an important role in flocculation and aggregation of several microalgae and diatoms. Several bacteria possessing flocculation activity have been isolated for their application in waste water treatment, dewatering of sludge, removal of dyes and heavy metals etc.

1.5.5 Bioflocculants from various bacteria

Among *Bacillus* species *B. lichiniformis* has been widely explored for application of its bioflocculant. The flocculant produced by *B. lichiniformis* was found to be composed of 91% polysaccharides and 8% protein and was produced from starch and ammonium chloride as a carbon and nitrogen source respectively (Li *et al*., 2009).

Besides polysaccharides and proteins some strains of *B. lichiniformis* are known to produce poly-gamma glutamic acid which demonstrated excellent flocculation activity with kaolin clay. The flocculation activity could be increased in presence of coagulants such as divalent metal ions. However, presence of trivalent metal ions demonstrated decrease in flocculation activity. In case if the zeta potential of suspended particles had high magnitude, then addition of divalent metal ions such as Ca^{+2} provided aid as a coagulant and decreased the zeta potential to an extent that prevented electrostatic repulsion between the particles. This made the flocculant most effective in causing aggregation and floc formation (Salim *et al.,* 2011). This flocculant produced by *B. lichiniformis* has demonstrated significant removal of COD and turbidity from waste water, as compared to conventional chemical-based flocculants. The glycoprotein biopolymer acted by the mechanism of electrostatic charge neutralization of the colloidal particles leading to decrease in zeta potential and formation of dense flocs (Li *et al*., 2009). The poly-gamma-glutamate produced by *B. lichiniformis* had also demonstrated efficient and cost-effective harvesting of microalgae *Nanno chloropsissalina* by flocculation in bench scale as well as pilot plant bioreactor. This application has a potential to reduce the

use of toxic aluminium salts that are used for large scale harvest of microalgae. In addition, the bioflocculant produced by *B. lichiniformis* was 2-3 times more effective as compared to the aluminium salt (Bharathiraja *et al.,* 2015; Farooq *et al.,* 2015).

Besides *Bacillus lichiniformis* other species such as *Bacillus megaterium* are known to produce high molecular weight EPS made up of repeating units of glucose, galactose and rahmnose along with other constituents like uronic acid, amino sugars and acetyl groups. Due to the presence of long polymer with side chains the EPS of *Bacillus megaterium* exhibited flocculation activity by electrostatic bridge formation. High yield of polymeric bioflocculant has been produced by use of simple carbon and nitrogen source like sucrose and ammonium chloride (Yuan *et al.,* 2011).

Another *Bacillus* species *Bacillus mojavensis* produced a biopolymer with 98% polysaccharide and 2% protein and demonstrated flocculation activity in presence of cations such as Ca^{+2}. The polymer has been documented to possess stability in extreme acidic pH and high temperature upto 75^{o}C (Elkady *et al*., 2011). In case of *Agrobacterium* sp. a highly pure polymeric bioflocculant has been obtained by employing alcohol precipitation and ion exchange chromatography. The flocculant was composed of polysaccharide made of repeating units of glucose and galactose. Low molecular weight of polymer and presence of simple sugars makes the bioflocculant extremely water soluble with high flocculation activity (Lee *et al.,* 2015). *Pseudomonas aeruginosa* if provided with appropriate carbon and nitrogen source demonstrated significant flocculation of activated sludge in presence of cations such as Ca^{+2}, Zn^{+2}, Mg^{+2}, Cu^{+}, Na^{+} and K^{+}. On the other hand, trivalent metal ions such as Al^{+} and Fe^{+3} acted as inhibitor of flocculation activity. The bioflocculant was pH stable and could tolerate temperature upto 100^{o}C for 60 minutes with 20% loss in flocculation activity. The biochemical analysis of the bioflocculant demonstrated presence of 27% protein and 70% carbohydrates that includes amino sugars, neutral sugars and presence of uronic acids (Gomaa *et al*., 2012; Gao *et al.,* 2017). Other studies indicated that *Pseudomonas sp* TJ-F1 showed significant improvement in flocculation of activated sludge when used with divalent

Ca^{+2} ions. The flocculation activity and time to filter (TTF) were better than organic flocculant such as polyacrylamide (Zhang *et al.,* 2010).

Besides the removal of suspended particulate matter, colloids, BOD, COD and heavy metals from waste water the bioflocculants have also been employed for the removal of pathogenic organisms by the phenomenon of cell aggregation and flocculation. The EPS produced by *Klebsiella pneumoniae* demonstrated flocculation of pathogenic organisms like *amoeba cysts* present in the water (Zhao *et al.,* 2013).

This bioflocculant composed of high molecular weight polymer made up of 85% carbohydrate and 6% protein was shown to be produced by *Klebsiella pneumonia*. The carbohydrate component was composed of sugars such as rahmnose, galactose and mannose. The side chains of the polymer had charged functional group that contributed to its flocculation activity.

Study of floccules generated by the high molecular weight polymer under electron microscope demonstrated the mechanism of bridge formation with kaolin particles while zeta potential measurements revealed significant charge neutralization during mechanism of floc formation. This bioflocculant was superior in terms of its pH tolerance, temperature stability and its ability to flocculate in absence of divalent cations. These qualities made such flocculants highly suitable for the treatment of biomedical waste water, industrial waste water and domestic sewage (Yin *et al.,* 2014).

Efforts have been done for the production of bioflocculant at low cost and the development of consortia for efficient flocculation (Mu *et al*., 2021). In one of such study the bioflocculant made by co-cultivation of *Staphyloccus* and *Pseudomonas* species demonstrated efficient flocculation activity. The production of bioflocculant could be easily carried out using brewery water as a sole source of carbon fortified with trace amounts of yeast extract and ammonium sulfate. This bioflocculant has been exploited for the treatment of pharmaceutical waste water, waste water from food industry and effluent containing textile dyes (Zhang *et al.,* 2007).

Klebsiella mobilis isolated form dairy effluents made polysaccharide based bioflocculant which was devoid of amino acids. The bioflocculant could be easily produced using simple carbon and nitrogen sources. Besides demonstrating flocculation with kaolin the bioflocculant had proven to be excellent for the treatment of industrial dyes such as Cibacron yellow and Violet HFRL (Sanayei *et al.,* 2009, Sanayei *et al.,* 2010).

Bioflocculant produced by *Klebsiella* sp. showed 96% flocculation of kaolin at very low dose of 34mg/l. When compared to chemical flocculants such as polyacrylamide and aluminium salts the results were significantly similar (Yang *et al,* 2012). *Sphingomonas paucimobilis* isolated from drainage waste water demonstrated 98% flocculation activity with kaolin. When employed for the treatment of textile waste water it demonstrated efficient removal of water-soluble dyes without significant change in the BOD and COD values. The bioflocculants produced by different species, their optimal media conditions, requirements of coagulant aid, chemical composition of bioflocculant and their unique characteristics is depicted in table 1.1.

1.5.6 Role of Nutrients in bioflocculant production

Several factors affect bioflocculant production by the microorganisms. These include temperature, pH, ionic strength, carbon source and nitrogen source. Generally, the optimum pH and temperature for bioflocculant production could be screened by one factor at a time (OFAT) method. Once optimum pH and temperature are determined the role of different nutrients like carbon source, nitrogen source and salts could be screened by statistical designs like Plackett Burman analysis. The most common nutrients used for bioflocculant production includes glucose, ammonium sulfate and urea (Table. 1.1). Optimization is then carried out by using response surface methodology as described in the next section. Once the screening of nutrients is done by Plackett Burman analysis, Box Behnken design and Central composite design that are included in RSM are used for optimization.

Table 1. 1 Bioflocculants produced by different species and their unique properties.

Organism	Growth Media	Coagulant aid	Chemical composition	Functional groups in flocculation	Ability to withstand stress	References
***Bacillus* sp. A-5A**	Glucose and ammonium nitrate, pH 6	Ca^{+2}	Uronic acid, neutral sugar, and protein	methoxy, hydroxyl, and amino groups	Thermal stable bioflocculant that retains 65% activity after heating at 100 °C for 25 min	Ugbenyenn *et al*., (2014)
***Halomonas* sp. and *Micrococcus* sp. Consortia**	Glucose, ammonium sulfate, and urea	Ca^{+2} Mn^{+2}, Al^{+3}	Uronic acid, protein, and sugar	methoxy, hydroxyl, and amino groups	Thermal stable bioflocculant that retains 70% activity at 80 °C for 30 min	Okaiyeto *et al.,* (2013)
Bacillus licheniformis	Minimal media	Ca^{+2}	Proteoglycan, neutral sugar, amino-sugar carbohydrate, and protein	Carboxyl, hydroxyl, and amino groups	Stable below 80 °C and pH range 3–8	Xiong *et al.,* (2010)
***Pseudoaltero monas* sp.**	Minimal media	Ca^{+2} and Fe^{+2}		Acetyl groups	Work in saline conditions with 35% NaCl, work at low temperature below 15 °C	Li *et al.,* (2008)
***Methylobacter ium* sp.**	Minimal media	Ca^{+2}		Carboxyl and hydroxyl groups	Thermostable, retains 70% activity at 100 °C for 30 min	Luvuyo *et al.,* (2013)
***Pseudomonas* sp.**	Minimal media		Carbohydrates, proteins, and glycol-proteins	Carboxyl, hydroxyl, and methoxyl groups	Thermally stable between 5 and 100 °C	Zaki *et al.,* (2011)

1.6 Optimization of media for bioflocculant production by using Design of experiments (DOE)

1.6.1 Plackett Burman analysis

One of the well-known experimental designs for the selection of factors giving significant response is the Plackett Burman design. This design was introduced by Robin Plackett and JP Burman in 1946. Plackett Burmann analysis is one of the widely used statistical methods for the assortment of nutrient sources with profound effect on production of coveted bio molecule as well as growth. The method involves measurement of desired response by ascribing high and low concentrations of selected nutrients often indicated as +1 or -1. The +1 denotes maximum concentration of the nutrient used while -1 denotes minimum concentration used. A table is generated in the multiples of 4. These tables are often generated by a software for example Design Expert (StatEase inc. USA). Once experimental runs are complete the value of each response is used to calculate the variance due to each factor. This variance is used to compute statistical F test which is the ratio of variance due to each factor by the variance due to dummy. The critical values of F test are obtained using F table. Any ratio above the critical value will show significant effect in the response. The significant variables are then selected for optimization by response surface methodology (RSM) (Stanbury *et al.,* 2013)

1.6.2 Response surface methodology

Commonly used RSM methods Box Behnken design and Central composite design are based on the use of regression equation for point prediction. The experiments are carried out at various levels and the response is measured due to each selected factor. This response is then used to compute a mathematical equation using a software like Design Expert. The mathematical equation is usually a regression equation which may either be linear, quadratic or cubic. The response calculated by the equation is then compared with the actual response measured experimentally. This comparison is done by studying the graph of actual vs predicted and ANOVA table for the particular equation. Once the equation truly predicts the actual experimental response it is then used to generate contour plots for prediction of optimal

response. Optimization involves the use of various factors at different concentration and monitoring of the response. As exemplified in (Figure. 1.4) each red dot instantiates the experimental run at the respective concentration for factor A and factor B. The experiments can be carried out for each point and the response is recorded. The experimental response generated for each respective point gives idea about the concentration of factor A and factor B required to yield optimal response (Stanbury *et al.,* 2013).

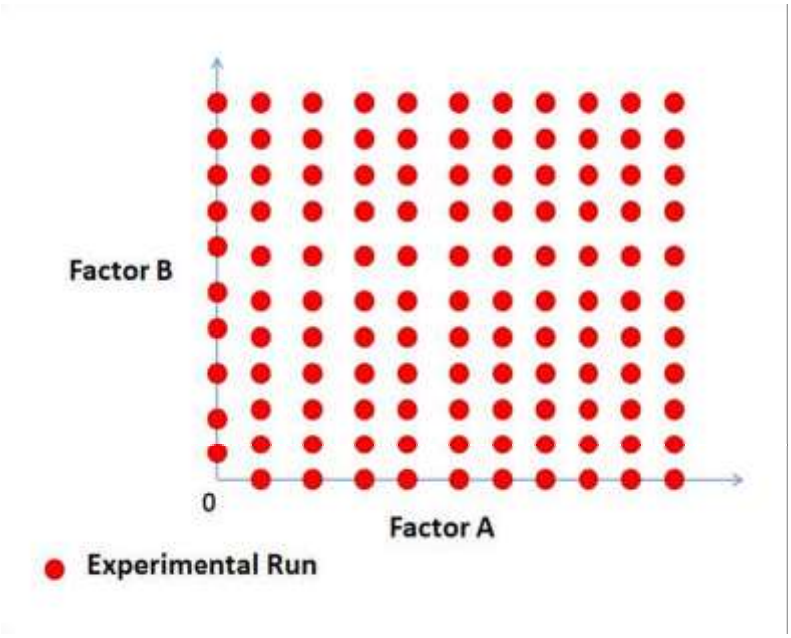

Figure 1. 4 Optimization of two factors, A and B by OFAT analysis. Red dot indicates total number of experimental runs required for optimization.

Nevertheless, to carry out experiments at each concentration of the selected factors (nutrients) may not be economically feasible. The method may also prove to be time consuming and may not yield reproducible results. For example, ten different concentrations of factor A and factor B will give 10 x 10 (100) combinations which may not be feasible at the laboratory scale. If there are three factors selected by the Placket Burmann design, then carrying out

experiments at ten different concentrations of each factor will result in 10 x 10 x 10 (1000) combinations which may be arduous task to perform at laboratory scale. Hence RSM methodology is a method of choice for optimization of factors where the Box Behnken design involves experiments which are done at high, low and central values (3 levels) while the Central composite design has 5 levels Viz. extreme high, high, central, low and extreme low (Figure. 1.5).

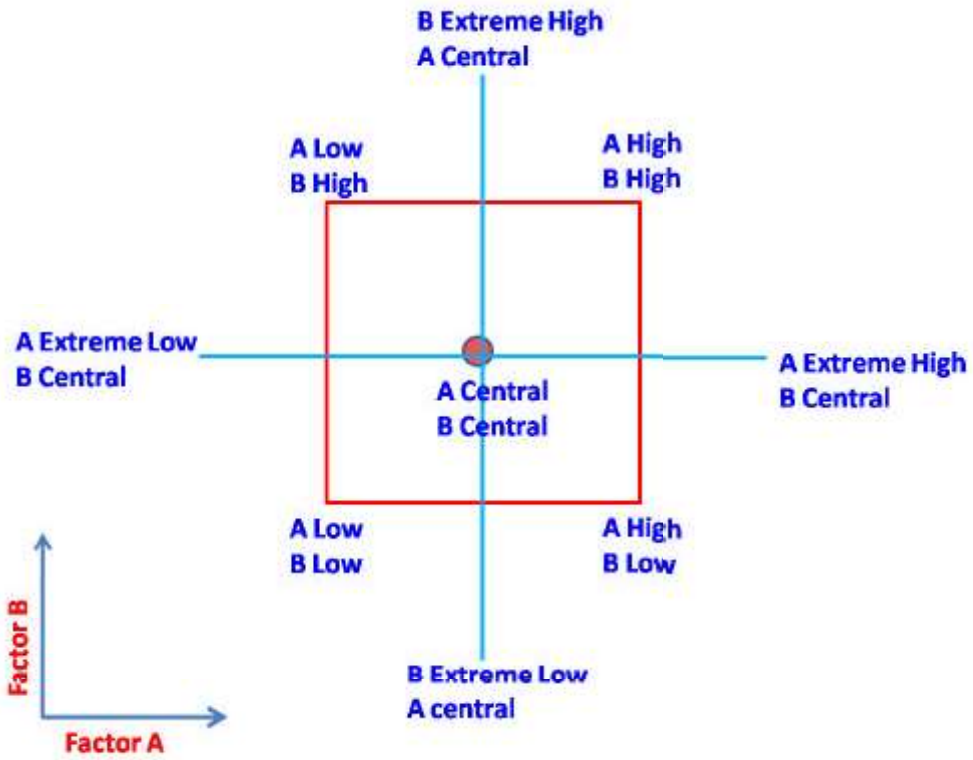

Figure 1. 5 Optimization using Central composite design. The vertical axis represents concentration of factor B while the horizontal axis represents concentration of factor A.

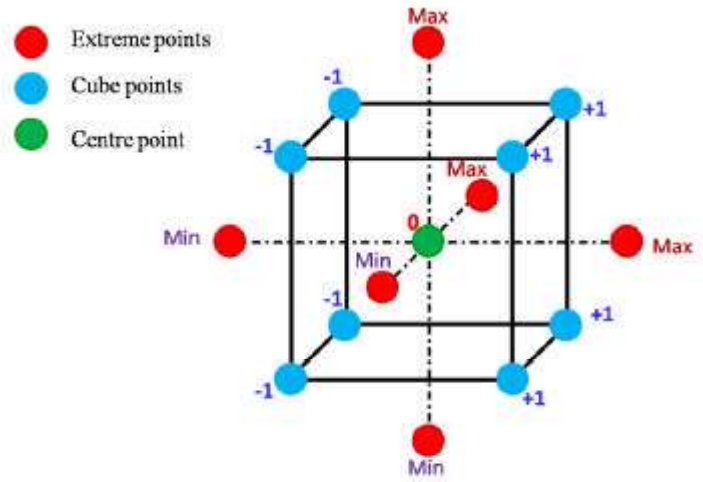

Figure 1. 6 Central composite design involving 3 factors for media optimization.

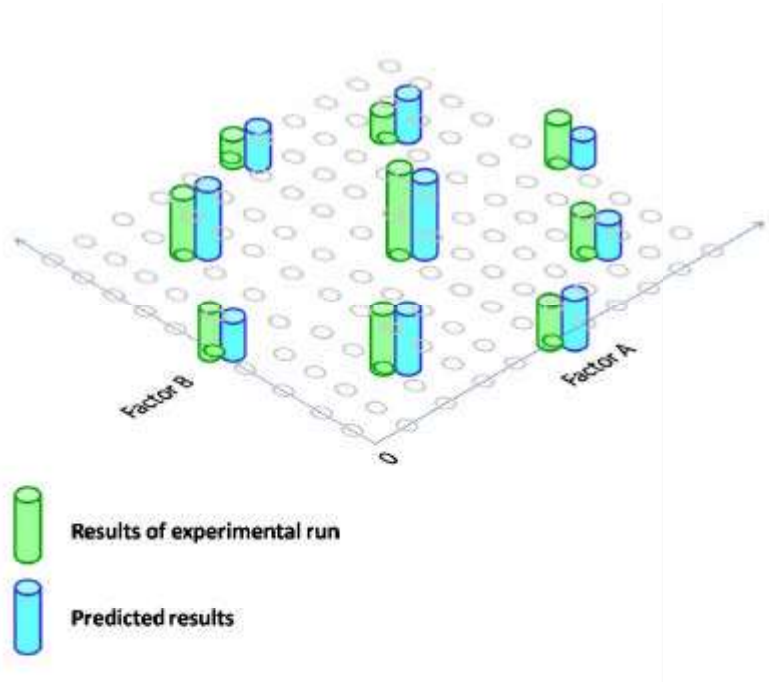

Figure 1. 7 Prediction of response in RSM using mathematical equation generated on the basis of experimental values (blue cylinder). The comparison between predicted and actual experimental results in demonstrated by keeping blue and green cylinders side by side.

Depending on the number of factors selected for studies the experimental runs are determined. Figure. 1.5 illustrates that when two factors (factor A and factor B) are selected the total number of experimental runs will be nine. These nine experimental runs include four experiments at extreme values, four experiments at high and low values and one experiment at central values for the selected factor. In case if the selected factors under study are three, then fifteen experiments will be carried out according to the cubic design as shown in figure 1.6. These fifteen experimental runs include six experiments at extreme high and low values, eight experiments at high and low values and one experiment at the central value. The experiments are carried out at each level and the results are entered in the software like Design Expert for further analysis.

The software generates mathematical equation for the prediction of response at points other than the experimental points. The mathematical equation is a regression equation that is usually quadratic or cubic equation which is in terms of the selected factors (nutrients). Once derived the equation is then used for prediction of the optimal response at the desired levels of selected factors (Figure. 1.7). Each experimental value is the compared with the predicted value and their resemblance is demonstrated in the ANOVA table.

The equation that calculates analogous response to the actual experimental values is selected and used for prediction of response at the remaining points. The response at each point is joined to create a contour plot which predicts the optimal response (Figure. 1.8). The predicted optimal response calculated by the mathematical equation is then corroborated experimentally. This tapers the total number of experimental runs, gives unambiguous results, mitigates the overall cost and denigrates experimental discrepancy. The CCD is most preferred as the experiments carried out at five levels minimizes error and generates punctilious equation for the accurate prediction of the response (Stanbury *et al.,* 2013).

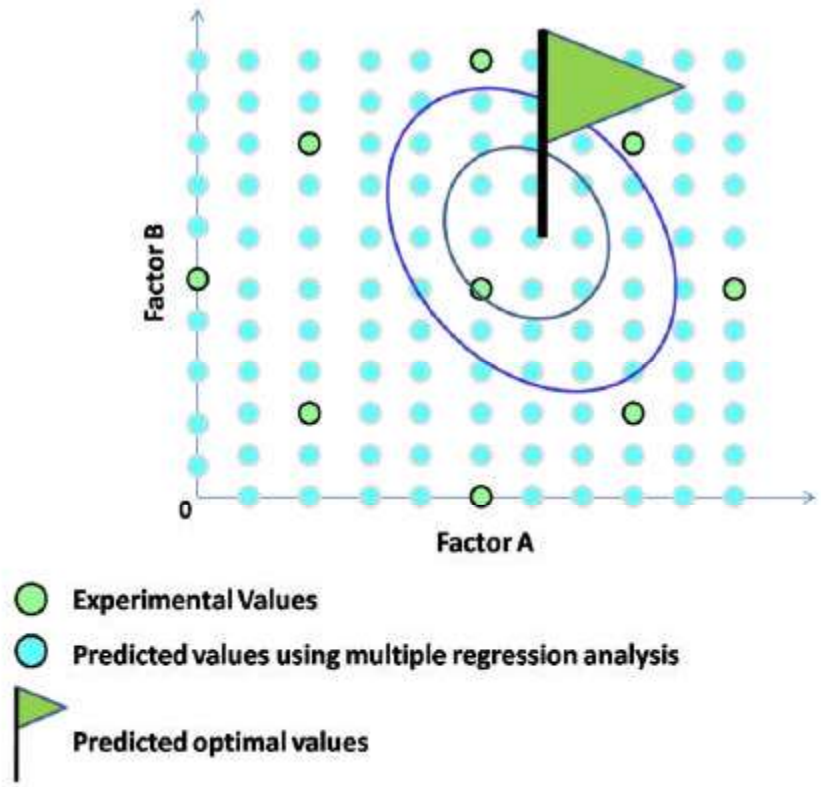

Figure 1. 8 Contour plots generated on the basis of predicted values. The flag represents the optimal value predicted using the contour plots.

1.7 Microalgae cultivation and its harvesting

Microalgae are one of the common microorganisms found in various water bodies like rivers, lakes and ocean. They grow by using sunlight along with dissolved nutrients like nitrates and phosphates present in the water body. In recent years with an urging need of fossil fuels due to increase in population have unlatched the demand of alternatives fuels like biodiesel and bioethanol (Foley *et al.,* 2011; Sivasankar *et al*., 2020). The microalgae, if cultivated in cost effective way can be an excellent source of biodiesel. Microalge have been documented to accumulate lipids like triglycerides that can be transesterified into biodiesel using methanol as a solvent. Besides this the microalgal biomass can be exploited to make bioplastic, bioethanol and methane which can have cogent uses in everyday life (Brennan *et al.,* 2010; Chisti *et al.,* 2007; Schaub 2009; Wijffels and Barbosa, 2010).

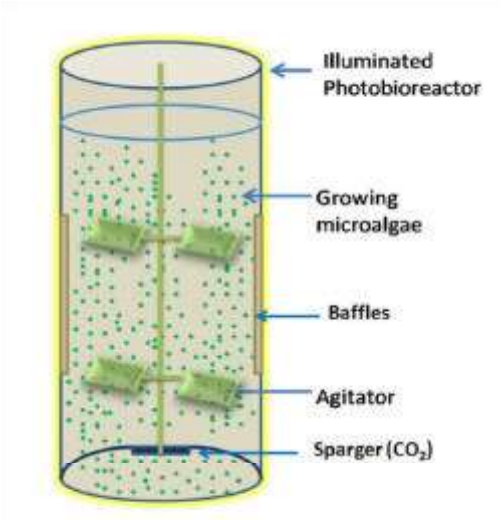

Figure 1. 9 Photobioreactor for large scale cultivation of microalgae.

Large scale cultivation of microalgae is routinely carried out in open ponds and photobioreactors. Open ponds, often termed as High-rate algal ponds (HRAP) are artificially contrived pond that provides necessary requirements for the growth of microalgae. These ponds are equipped with an impeller at the bottom that provides agitation for creating turbulence and nutrient mixing (Chiu *et al.,* 2009; Vandamme *et al.,* 2013). The atmospheric carbon dioxide diffuses in the pond water and acts as a carbon source. The HRAP ponds may also be equipped with CO_2 sparger to increase the yield of algal biomass. However, this attachment may not be economically feasible as most of the CO_2 bubbles ends up in the atmosphere (Mata *et al.,* 2010). Besides these the parameters such as pH and temperature cannot be controlled in this system.

Photobioreactors on the other hand are most efficient for large scale production of microalge (Figure. 1.9). When compared with open ponds the photobioreactors provides disparate precedence over open pond cultivation method. This ascendancy includes pH control,

temperature control, better bulk mixing, nominal evaporative loss and superior growth rate (Mata *et al.,* 2010). Air lift photobioreactors equipped with sparger at the bottom provides excellent aeration and mixing of nutrients in the reactor, resulting in high yield of biomass (Merchuk *et al.,* 2007; Azov *et al.,* 1982). Flat plate bioreactors are constructed with transparent rectangular plates. The illumination of light in flat plate reactor remains even without dark zones which results in excellent growth. In recent years Offshore Membrane Enclosure for Growing Algae (OMEGA) have been invented by NASA for the waste water treatment using microalgae. The OMEGA system uses specially designed plastic bags that are partially permeable to oxygen and carbon dioxide. The plastic bags containing microalge floats in the water body, where the microalgae utilize nutrients present in the waste water. The treated waste water leaves the system by osmosis and the biomass so generated can be later collected to generate biofuels (Trent *et al.,* 2010). However, one of the limitations of photobioreactor is the toxic effect of oxygen accumulated in the reactor due to photosynthesis.

Besides photobioreactor immobilizing micoalgae in polymeric gel such as alginate have demonstrated excellent results in waste water treatment. The immobilized microalgae have shown better accumulation of pigments and lipids as compared to algae grown in open water pond or photobioreactor (Carvalho *et al.,* 2006; De Bashan *et al.,* 2002). However immobilized microalge have been shown to be economically costly as compared to other methods of microalgae cultivation.

1.7.1 Microalgae for waste water treatment

One of the widely used methods for the waste water treatment entails activated sludge process that involves colossal aeration of the waste water to eradicate COD and BOD making water favorable for discharge in water bodies and agricultural fields. However the conventional biological wastewater treatment systems like activated sludge process, trickling water and stabilization ponds do not facilitate enhanced nutrient removal such as removal of nitrates and phosphates (Wang *et al.,* 2018; Chong *et al.,* 2012; Brostow *et al.,* 2009; Chevalier *et al.,* 2000). The consumption of water containing high levels of phosphates and nitrates have

shown to cause health hazards such as methemoglobinemia (Richard *et al*., 2014). Hence application of an appropriate technology for enhanced removal of nitrates and phosphates is desideratum in today's age. The three major methods employed for the removal of nitrates and phosphates includes a) Physical methods b) chemical methods and c) biological methods. The most popular physical methods include methods like electrodialysis, ion exchange and reverse osmosis etc. however their use is limited due to high recurring cost. Chemical methods of nitrate removal include the use of metals as a catalyst for nitrate reduction to ammonia. Mixture of such Pd/Cu, zero valent Iron (ZVI) have been used for catalytic nitrate reduction. However, the large amount of ammonia released as a by product is known to create secondary environmental problems. In contrast the use of biological methods of nitrate removal have received a considerable attention in the last decade as these methods are environmentally friendly and cost effective (Su *et al.,* 2019: Dias et al., 2021).

One of such biological methods involves the use of microalgae. The ability of microalgae, to use phosphates and nitrates from wastewater can be exploited for the waste water treatment (Christenson and Sims, 2011; Gupta *et al.,* 2005; Hoffmann *et al.,* 1998). Several ways have been employed for the cultivation of microalgae. These includes open ponds (Stirred and non stirred), closed systems that includes flat plate bioreactor, tubular bioreactor and plastic bag bioreactor (Larkum *et al.,* 2012; Grima *et al.,* 1999). Several studies conducted in the last decade have demonstrated that species of microalgae such as *Chlorella, Phormidium*, *Scenedesmus, Chlamydomonas, Arthrospira* and *Botryococcus* have been exploited in diversified ways for bioremediation of heavy metals, toxins, organic pollutants, removal of nitrates and phosphates from waste water. The growth of microalgae depends on the presence of carbon, nitrogen and phosphorous in the environment. The carbon to nitrogen to phosphate ratio of the domestic waste water is 20:8:1 while the ratio required for the growth of microalgae is 50:8:1. These values indicates that the nitrogen to phosphate ratio of the waste water and requirement of microalgae are similar. Microalgae can utilize additional carbon in the form of atmospheric carbon dioxide, which makes the waste water a concurring source for the growth of microalgal biomass. The biomass generated by cultivation of microalgae

can also be exploited economically. In recent years the demand of alternatives fuels like biodiesel and bioethanol has increased (Foley *et al.,* 2011; Pugazhendhi *et al*., 2020).

The challenging task while cultivating microalgae for any industrial purpose is its harvesting. The widely used industrial method for the harvesting of microalgal biomass is centrifugation. This method consumes excess of energy which adds upto 30% of cost in the treatment process (Uduman *et al.,* 2010; Pugazhendhi *et al*., 2020). Hence cost-effective harvesting of microalgal biomass is a major requirement for large scale manoeuvring of microalgae for the waste water treatment and other applications (Laraib *et al*., 2021). There are several methods employed industrially for the large-scale harvest of microalgae (Prajapati *et al.,* 2016; Delrue 2015; Pal *et al.,* 2012). The most common method involves use of chemicals such as alum that aggregates microalgal cells by the phenomenon of coagulation. However, the residuum of metal salts in microalgal biomass can act as an undesired contaminant, which may interfere in the extraction of lipids for biofuel production. Aside from metal ions organic polymers such as polyacrylamide have been extensively used for the microalgal harvest. However, the use of polyacrylamide at such large scale can have neurotoxic effects of acrylamide monomers (Ghosh *et al.,* 2010; Gonçalves *et al.,* 2015). Hence the flocculants derived from biological materials offers disparate ascendancy over toxic chemical flocculants for the harvest of microalgal biomass. Bioflocculation seems to be promising method to solve this problem. Bioflocculation is a phenomenon in which naturally existing biopolymers aggregates suspended particles into large stable clumps that settles nimbly (Renault *et al.,* 2009). Depending on their biochemical composition the biopolymers can either have positive or negative charge that interacts with particles of opposite charge and resulting in aggregation. The large polymeric bioflocculants can also act as bridge thereby bringing particles together resulting in flocculation. In case of small size biopolymers, they tend to bind surface of the particles carrying opposite charge. This binding facilitates binding of other particles on the polymer, thereby forming flocs that settles easily. The phenomenon is termed as electrostatic patching.

1.8 Amyloids and biofilms

Biofilm can be defined as a bacterial community of same or different species, adhered together in an enclosed matrix. The matrix of biofilm is composed on various components secreated by the bacteria in the extracellular environment. These components include extracellular polymeric substances (EPS), proteins, eDNA, amyloid fibrils and cellulose. The components of the extracellular environment allow the attachment of bacterial cells on various biotic and abiotic surfaces. The process of biofilm formation starts with the attachment and aggregation of cells on the surface. This initial attachment is termed as the monolayer formation (Moorthy and Watnick, 2004). With time the monolayer advances into the formation of microcolonies which further develops into mature biofilm. When the conditions become unfavorable the mature biofilm disperses the bacterial cells into planktonic state by the breakdown of the complex matrix (Hall-Stoodley *et al.,* 2004). It must be noted that the matrix also generates gradients of nutrients, oxygen and various secreted metabolites such as cell communication or quorum sensing molecules. Biofilms have been considered as an intolerable nuisance in food industries. The nutrient rich environment of the food industries provides competent environment for the easy growth and spread of biofilm (Nikolaev and Plakunov, 2007; Srey *et al*., 2013; Coughlan *et al.,* 2016). Most of the biofilm forming species in food industries can act as food poisoning agents and human pathogens (Colagiorgi *et al*., 2017). The harmful strains can include *Pseudomonas*, *Geobacillus stearothermophilus* and *Vibrio* spp. The biofilm development by these species in various processing parts of industry such as tanks, pipelines, centrifuges and packaging tools has been considered as one of the major nuisances for the spoilage of food and puts human health to risk (Camargo *et al.,* 2017). *Bacillus* is a gram positive, facultative anaerobic or aerobic spore forming bacterium that can tolerate wide variety of environments and stress. The vegetative spores of *Bacillus* can survive harsh food processing such as pasteurization. *Bacillus* has been considered as a major nuisance in dairy industry. The non-hemolytic enterotoxin produced by *Bacillus cereus* has been responsible for causing abdominal pain, cramps and diarrhoea (López *et al*., 2015; Tschiedel *et al*., 2015; Soni *et al*., 2016). *Bacilllus*

cereus develops a submerged biofilm in the tanks of dairy vessels and being motile in nature the bacteria is capable of spreading rapidly in various parts connected with the vessel. Besides dairy industry *Bacillus cereus* also acts as a food poisoning nuisance in other industries such as fruit juice industry, red meat industry and poultry industry. The biofilm formed by *Bacillus cereus* consists of complex exopolysaccharieds and amyloid fibers that provides sturdiness to the biofilm matrix. The only effective known strategy for the removal of *Bacillus* biofilm is heat treatment involving high temperature short time exposure. However, this method has its limitations and cannot be applied in all parts of the industry. Hence there arises a demand for the removal of such biofilms by other methods.

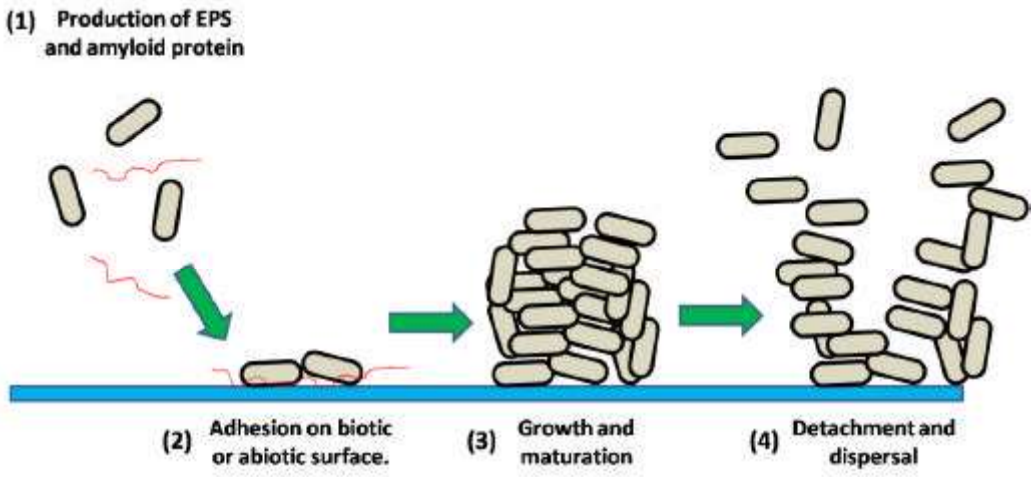

Figure 1. 10 Stages in biofilm formation and breakdown.

In case of other *Bacillus* species like *Bacillus subtilis* the biofilm formed is dense and very thick. The stationary cultures of *Bacillus subtilis* forms a thick biofilm called pellicle at the air liquid interface. The pellicle is composed of extracellular polymeric substances and functional amyloid fibers termed as TasA. The presence of TasA amyloid on the cell surface allows the cells to form dense aggregates that are highly stable and sturdy. The unique

property of amyloid fibers like resistance to denaturants, physical and chemical stress provides unique properties to the biofilm.

1.9 Biofilm disruption

The TasA fibrils remains attached with the cell wall with the aid of an accessory protein called TapA. When the conditions for biofilm sustenance become unfavorable the biofilm undergoes disintegration releasing the aggregated cells into their planktonic form. The *Bacillus subtilis* cells have a unique interesting mechanism that governs the collapse of dense sturdy biofilm to its planktonic stage. This mechanism is governed by the production of D-amino acids in late stationary phase that acts as a molecular signal for the breakdown of biofilm. The D-amino acids such as D-tryptophan, D-leucine and D-methionine have demonstrated disassembly of dense biofilms made by *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, the use of D-amino acids for biofilm disruption is limited to few bacterial species and need to be extended in others (Romero *et al*., 2010). The unique ability of D animo acids to cause biofilm disruption has also been patented in recent years. The amino aids have been found to be effective in the range of parts per billion. The D amino acid along with the use of biocide have been applied as a tool for eliminating biofilm. The most effective amino acid found to possess antibiofilm activity was D-tyrosine. The combined use of D tyrosine in the range of 100ppb to 100ppm and biocides such as tetrakis hydroxymethyl phosphonium sulfate (THPS) in the range of 10ppm to 1000ppm have been found to destroy the biofilm and planktonic cells respectively. The biocides such as THPS is a broad-spectrum biodegradable biocide. The effectiveness of THPS gets reduced when the cells are adhered together in a complex structure such as biofilm. Therefore, D amino acids in combination with THPS can be synergistically effective. The bacterial biofilm is a major source of trouble leading to problem of biofouling in several industries (Gu *et al*., 2015).

Rationale

Rationale

Functional amyloids of bacteria are the cell surface proteins produced by several microorganisms to execute specific functions. These diverse functions include cell aggregation, attachment on biotic and abiotic surfaces, biofilm formation, reduction in surface tension of water, spore dispersal, pellicle formation etc. As compared to other proteins amyloid proteins have unique properties that outcompetes other cellular proteins in many ways. These proteins are resistant to physical and chemical denaturants such as pH, temperature and presence of detergents like SDS. The presence of stacked antiparallel beta sheet in these proteins also provides a unique characteristic such as high tensile strength comparable to that of steel. Amyloid protein being an extracellular microbial product is strong, sturdy, robust and ecofriendly. These characteristics of amyloid protein makes it an interesting candidate for variety of industrial applications. These aspects of bacterial amyloids would give novel products. Studies on amyloid producing bacteria have demonstrated their role in cell aggregation and floc formation in several water bodies such as drinking water reservoir, waste water effluents and activated sludge. Several studies have demonstrated bioflocculant production by various bacterial species. The naturally existing functional amyloids of microbial origin possess several attributes that includes surface active and emulsification properties as well as ability of causing cell aggregation before biofilm formation. However, these functional amyloid proteins have not been exploited for its biotechnological applications especially as a flocculant. Studies on investigation of flocculant properties of the bacterial amyloids would have novelty. Based on these observations it was of interest to isolate and characterize amyloid producing bacteria possessing bioflocculant activity. Further studies were planned on the application of functional bacterial amyloid as a bioflocculant for the harvest of microalgal biomass in nutrient removal from waste water. Role of amyloid in biofilm formation, its detachment and cloning of bioflocculant gene were other aspects of interest that were studied.

Objectives

Objectives

1. Screening of amyloid bioflocculant producing bacteria from flocs of activated sludge and characterization of its bioflocculant.
2. Optimization of production of bioflocculant from the selected isolate and studies on its biotechnological application.
3. Studies on amyloid production from selected isolate and its biofilm formation.
4. Cloning of amyloid producing gene form the selected amyloid producing isolate.

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