Chapter 2

Screening of amyloid producing bacteria from activated sludge and characterization of its bioflocculant.

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2.1 Functional bacterial amyloids (FuBa)

The microorganisms assemble cell surface amyloids to accomplish specific task such as cell aggregation, attachment on biotic and abiotic surfaces, biofilm formation, interaction with other species etc. Unlike pathogenic amyloids made by the mammals the microbial amyloids help the individual cell to perform specific task hence it is called as functional bacterial amyloid (FuBa). The presence of anti-parallel beta sheet structure makes these proteins robust in nature, with an ability to withstand harsh physical and chemical denaturants. Keeping these qualities in mind the amyloid producing bacteria were isolated from the flocs of activated sludge and screened with an aim to select a bioflocculant producer. Further, the amyloid bioflocculant obtained from the selected isolate henceforth referred to as bioflocculant CR4 was characterized.

2.2 Materials and Methods

2.2.1 Isolation of amyloid producing bacteria

Isolation of amyloid producers was done from the flocs of the activated sludge from the process tank. The activated sludge samples were collected from various sewage treatment plants of Vadodara (Gajrawadi sewage treatment plant, Aatladra sewage treatment plant [22.18N, 73.12E]) and Surat (Bheshan sewage treatment plant [21.12N, 72.12E]. The flocs were mixed in saline, serially diluted and then spread on 0.005% Congo red containing Luria Bertani agar plates, incubated at 37^{o}C for 24 to 48hrs. Colonies showing red color were selected for further studies (Hammar *et al*., 2002; Reichhardt *et al.,* 2015; Larsen *et al.,* 2007).

2.2.2 Staining of amyloid producing isolates by thioflavin T fluorescence

The isolates were grown in Luria broth and incubated at 37^{o}C for 24 to 48hrs. Stock solution of amyloid specific dye Thioflavin T having concentration 32mg/ml was prepared and diluted 1:2 with distilled water to make working solution. 2 ml of culture grown in Luria broth was centrifuged at 8500g for 15mins. The pellet obtained was washed twice with distilled water

to remove traces of culture media. The resulting biomass in the pellet was suspended in equal volume of distilled water and diluted 10 times (1:10 Dilution). A loop full of sample was taken on glass slide, heat fixed and stained with working Thioflavin T solution. The samples were incubated at room temperature for 5 min under dark (to avoid photo bleaching of the fluorescence dye). Excess of dye was removed by washing the slide with distilled water. The slides were air dried and observed under fluorescence microscope (Olympus Microscope BX43), with UV excitation and green emission at 540nm (Berg *et al*., 2010; Picken *et al.,* 2015).

2.2.3 Measurement of the flocculation activity

The isolates that showed green fluorescence with thioflavin T staining were selected for flocculation assay. All the positive isolates were cultivated individually in Luria broth at 37^{o}C for 24 hrs. For the measurement of flocculation activity, 0.5g amount of kaolin clay was suspended in 100 ml distilled water and mixed well to make uniform suspension to get final concentration of 0.5%. Culture broth of each isolate was taken in separate tubes and cell density of each isolate was adjusted to 0.4 OD (600nm). 1ml of the suspension was added to the kaolin and stirred for 1min with vortex mixer. The tubes were kept still for 10 min to allow the floccules to settle. 2ml of the upper phase was collected in separate micro-centrifuge tube and the optical density was measured at 595nm. The control experiment was performed by using distilled water instead of bacterial suspension. Estimation of % flocculation activity was determined by following equation (Wang *et al.,* 2007).

$$\% \text{ Flocculation} = 100 \times (OD_{550nm} \text{ Control} - OD_{550nm} \text{test}) / (OD_{550nm} \text{ Control}) \text{ ---------- (1)}$$

2.2.4 Identification of the selected isolate

The isolate showing maximum flocculation activity was identified by biochemical tests and 16S rRNA gene sequencing. The genomic DNA of the selected isolate was isolated (Sambrook and Russell, 2001) and subjected to PCR amplification of 16S rRNA gene using universal primers. 27F (AGAGTTTGATCMTGGCTCAG) and 1541R (AGGAGGTGATCCAGCCGCAG). The PCR conditions used for the amplification were as

follows: - Initial denaturing at 94ºC followed by 25 cycles of denaturing at 94ºC for 30 seconds, Annealing at 58ºC for 30 seconds, amplification at 72ºC for 1 min and final amplification at 72ºC for 5 minutes. The amplified PCR product was separated using 0.8% agarose containing 0.5µg/ml ethidium bromide. The PCR amplicon obtained was sent for Sanger sequencing at Scigenom Labs Pvt. Ltd., Kochi India. The nucleotide sequence obtained after sequencing was analysed by NCBI-nucleotide BLAST, identified and uploaded to NCBI-GenBank.

2.2.5 Characterization of isolate CR4

The characterization of amyloid in isolate CR4 was done by several techniques described as follows. The isolate CR4 was inoculated in LB broth and incubated at 37ºC for 16hrs at 160RPM agitation. 1ml of culture was collected and centrifuged at 8500g for 15min. The biomass in the pellet was washed several times with distilled water and resuspended in 1ml distilled water. Bioamss thus obtained used for characterization.

2.2.5.1 Congo red staining

A loopfull of culture was taken from the resuspended cells and used to make smear on clean slide. The smear was heat fixed and stained with 0.005% Congo red for 15mins. Excess of stain was removed and the slide was gently washed several times with distilled water. The cells were observed under flourescence microscope with green excitation and red emission.

2.2.5.2 Thioflavin T staining and Confocal microscopy

A loopfull of culture was taken from the resuspended cells and used to make smear on clean slide. The smear was heat fixed and stained with Thioflavin T 32mg/ml (Diluted 1:2) for 15mins. Excess of stain was removed and the slide was gently washed several times with distilled water. The cells were analyzed by Confocal and DIC microscope.

2.2.5.3 SEM analysis of *B. cereus* CR4

The cells were harvested by centrifugation and fixed with 2.5% glutaraldehyde followed by dehydration with series of increasing concentrations of ethanol ie. 10, 25, 50, 70, 80 and

100%. The cells were sputtered with platinum and observed under scanning electron microscope (JEOL JSM-7600F FEG-SEM at SAIF facility IIT Mumbai.)

2.2.6 Purification and characterization of amyloid produced by isolate CR4

The purification of amyloid protein was done as described by Sitaras *et al.,* 2011. The culture was grown in LB broth at 37^{o}C for 24hrs. 40ml of the culture broth was taken in centrifuge tube and the biomass was harvested by centrifugation at 8500g for 15 minutes. The pellet was washed twice with sterile distilled water to remove traces of growth media. The biomass in the pellet was suspended in 5ml 10mM Tris buffer (pH-7.5) followed by treatment with the extraction buffer [125 mM Tris glycine (pH-7.0), 4% SDS, 20% glycerol]. This mixture was heated for 10 minutes in boiling water bath, cooled and centrifuged at 10000g for 20 minutes. For purification of the protein of interest SDS-Agarose gel (2%) was prepared and circular wells were bored in the gel using 8mm cork borer. The samples were mixed with bromophenol blue and loaded in each well and electrophoresis was carried out at 75V for 40 min. The protein material retained in the wells was collected and used for further confirmatory tests (Sitaras *et al.,* 2011; Collinson *et al.,* 1993).

2.2.7 Flocculation activity of the amyloid protein obtained from isolate CR4

The flocculation activity of the purified amyloid protein was assayed as described earlier in section 2.2.3. In order to determine the effect of pH and bioflocculant concentration the flocculation activity was carried out with different concentrations of purified amyloid at different pH and the flocculation activity was determined. For this the purified bioflocculant from the wells of SDS-Agarose was centrifuged at 16000g for 30min and suspended in buffers having pH, 2, 3, 5, 7, 9, 11, 12 respectively (pH 2 and 3: citrate buffer, pH 4 and 5: acetate buffer ; pH 6 and 7 Phosphate buffer; pH 8 and 9 Tris buffer; pH 11 and 12 glycine NaOH buffer) and treated at different temperatures ranging from 4 to 100^{o}C for 30 min. Each set was again centrifuged at 16000g to recover the insoluble aggregates, resuspended in phosphate buffer pH 7 and used to study flocculation activity with 0.5% kaolin. Hence forth in the thesis the amyloid protein is referred to as bioflocculant CR4.

2.2.8 Characterization of the amyloid nature of the protein obtained from isolate CR4

2.2.8.1 Congo red birefringence assay

The purification of surface protein extracted as described by Sitaras *et al*., (2011) was done by SDS agarose gel electrophoresis. The material recovered from the wells of SDS agarose gel was collected and tested for presence of amyloid by birefringence test. The sample was taken on glass slide and fixed with formaldehyde. The slide was flooded with alkaline Congo red solution (made in 70% ethanol, saturated NaCl, Saturated Congo red and 2% NaOH) and incubated for 15min. Excessive stain was removed by washing the slide with distilled water. The slide was air dried and observed under polarized light with polarizer and analyzer crossed at 90^{o} in Olympus CX43 microscope (Puchtler *et al.,* 1962).

2.2.8.2 Congo red spectral shift assay

The stock solution of 0.05% of Congo red was prepared in distilled water and diluted to 0.005% to make working solution. The 100µg of purified protein was mixed with 900µl of Congo red (0.005%) and incubated at room temperature for 10mins. The absorption spectra of the mixture was monitored from 400nm to 560nm. A solution of Congo red (0.005%) without addition of protein was used to measure the control spectra (Klunk *et al.,* 1989).

2.2.8.3 SDS-PAGE analysis

The protein recovered from the wells of SDS-Agarose was analysed on 10% SDS-PAGE. The resolving SDS-PAGE gel was prepared as follows. 3.3ml of 30% acrylamide-bisacrylamide solution was taken in glass beaker followed by addition of 2.5ml Tris-Cl buffer (pH 8.5). This was followed by addition of 4.85 ml distilled water, 50µl of 10% SDS and 50µl 10% ammonium persulphate. The polymerization reaction was initiated by addition of 5µl of TEMED. The mixture was mixed well and poured in between the glass plates followed by layering of methanol. Once the resolving gel was polymerized comb was placed on the top of the plates and stacking gel was prepared. For the preparation of stacking gel 0.33 ml of 30% acrylamide-bisacrylamide solution was taken in glass beaker followed by addition of 0.63 ml Tris-Cl buffer (pH 6.5). This was followed by addition of 1.5ml of water, 20µl SDS

and 20µl APS. The polymerization reaction was initiated by addition of 2µl of TEMED. The mixture was mixed well and poured on the top of resolving gel. The stacking gel was allowed to polymerize for 15mins and used for analysis. Before loading the gel the samples were mixed with the running buffer and boiled for few minutes. Once cooled the samples were loaded in the gel. Culture broth containing total proteins was used as a control. The electrophoresis was carried out at 75Volts for 4hrs. Once the electrophoresis was complete the gel was fixed in fixative (Methanol: acetic acid: water 50:40:10 ratio) for 2hrs followed by staining with silver nitrate staining. Briefly the gel was fixed in fixative (methanol: acetic acid: water, 50:40:10) for 30 min. The gel was then sensitized and treated with 10mM silver nitrate. The reaction was stopped by treating the gel with dilute sodium carbonate.

2.2.8.4 FTIR analysis

The purified protein from the wells of agarose was mixed with KBr pellets and the FTIR spectrum was analyzsed by Perkin Elmer RX1 Infrared spectrophotometer in the frequency range from 400 cm^{-1} to 4000 cm^{-1} (Markande and Nerurkar, 2016).

2.2.8.5 Ammoniacal silver staining

One of the classical methods used for visualization of neurofibrillary tangles and amyloid plaques in brains of Alzheimer patients is Bielschowsky method. This method uses ammoniacal silver solution for the visualization of plaques. The thickness of amyloid fibrils is around 10nm which is equivalent to the thickness of flagella. Based on this fact, bacterial flagella staining method for the visualization of amyloid on bacterial cells was used (West., 1977).

2.2.8.6 TEM analysis

The purified protein collected in the wells of SDS agarose gel (section 2.2.6) was placed in copper grid and stained with 1% uranyl acetate. The sample so prepared were studied under transmission electron microscope (Phillips CM200, at SAIF facility IIT Mumbai)

2.2.8.7 CD spectra analysis

The purified protein was dissolved in distilled water and used for performing CD spectroscopy. The spectrum was recorded in the range from 195 – 250nm wavelength (JASCO J810 at IIT Kharagpur)

2.2.9 Zeta potential analysis of flocs of kaolin particles produced by purified amyloid bioflocculant CR4

Zeta potential analysis was outsourced to SVNIT, India. The kaolin suspension was taken in a Zeta potential analyzer cell (cuvette) and electrodes were introduced. The movement of particles in electric filed was measured by analyzing light scattering. Based on the movement of particles the net charge on each particle was estimated.

2.2.10 Protein sequencing

The protein recovered from the wells of SDS agarose gel was treated with 1% formic acid for 15mins and separated by SDS-PAGE. The protein band corresponding 32KD was excised and purified by electrodialysis. The protein was lyophysized and sent for protein sequencing by LC MS/MS analysis at CCAMP (Centre for Cellular and Molecular Platforms) Bangalore, India.

2.2.11 Time course of bioflocculant production

The culture was grown in Luria broth at 37^{o}C for 24hrs. The optical density of grown cells was adjusted to 0.4OD at 600nm. 0.5ml of culture was then inoculated in 50ml Luria broth (1% inoculum) and incubated at 37^{o}C. The growth of bacteria was monitored by taking OD at 600nm and flocculation activity with biomass and supernatant was checked at various time intervals.

2.2.12 Quantification of amyloid by SDS agarose gel electrophoresis

For the quantification of amyloid during time course production SDS agarose gel electrophoresis was performed. SDS agarose was prepared with the composition: - 2% SDS, 1.5% agarose and TAE Buffer. After the gel was solidified circular wells were bored in the gel using cup borer. Samples for electrophoresis were prepared as described earlier. The

samples were loaded into wells along with 2% molten agarose and bromophenol blue as tracking dye. BSA (1µg, 2µg, 5µg, 10µg) was used as control. Once the agarose in the well was solidified the electrophoresis was carried out at 100V for 40 minutes. After electrophoresis the gel was fixed in fixative (methanol: acetic acid: water in ratio 50:40:10) for 3 hrs. The staining of the gel was performed with commassie brilliant blue for 12hrs. Destaining of the gel was performed using methanol: acetic acid: water (50:40:10) until all the background was destained. The Quantification of protein in the gel was done using BSA as standard using Image J Software (Sitaras *et al.,* 2010).

2.3 Results and Discussion

2.3.1 Isolation of amyloid producing bacteria

The presence of amyloid producing bacteria have been intensively documented to be present in the flocs of activated sludge process. Within the flocs of activated sludge, the amyloid producers play an important role in cell aggregation, cell adhesion and biofilm formation (Larsen *et al.,* 2007). Based on these observations the isolation of amyloid producing bacteria was carried out from the flocs of activated sludge collected from various sewage treatment plants of Gujarat. Isolation was accomplished on Congo red agar plates as a growth media. The amyloid is a state of protein that is typified by the presence of β sheets stacked over each other. The phenyl rings of the Congo red dye specifically bind the β sheets of amyloid ensuing in its detection. This excessive binding of the dye with the protein makes bacterial colonies appear red in colour while non amyloid producing bacteria shows white colonies (Figure. 2.1).

The use of Congo red dye as a indicative agent for the presence of amyloid have been used since several decades for diagnosis of amyloid plaques in degenerative diseases. The Congo red moelcule is a linear molecule which has bi-phenyl ring, two amino group and a sulphate group. The amino group and sulphate group can have a charge depending on the pH while the bi-phenyl group proffers hydrophobic nature to the dye. The hydrophobic region

specifically reacts with the anti parallel beta sheets of the amyloid giving rise to staining of amyloid fibrils.

Several authors (Chapman *et al*., 2003, Gebbink *et al*., 2005, Larsen *et al*., 2007) have exploited the use of this dye to identify amyloid producing bacteria and fungi based on their colony colour. The table 2.1 illustrates the colony morphology of the 18 isolates giving red colonies on Congo red agar obtained from the flocs of activated sludge.

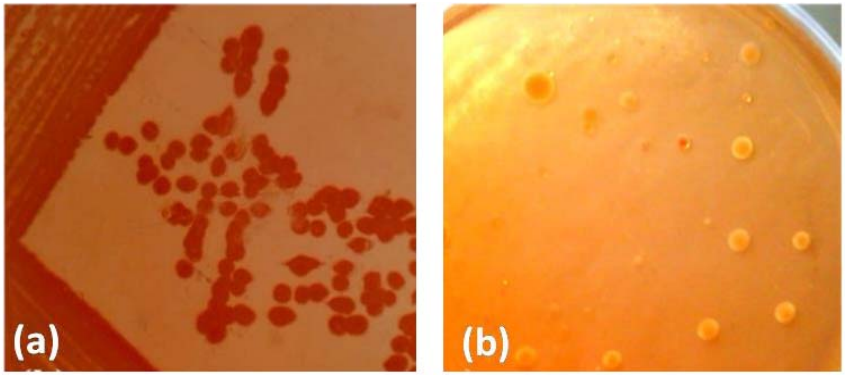


Figure 2. 1 Colony characteristics of amyloid producing bacteria on Congo red agar. a) amyloid producing bacteria gives red colonies. b) Non amyloid producing bacteria demonstrates white coloured colonies.

The detection of amyloid producing isolates based on red-coloured colonies on Congo red agar is one of the facile methods of detection. This is because the side chains of the dye can have non specific ionic interaction or hydrogen bonding with the biomolecules present of the surface of the cells. Hence each isolate obtained were subjected to another round of tests to corroborate the presence of amyloids.

Table 2. 1 Colony morphology of isolates obtained from activated sludge

Sr No	Isolates	Colony morphology	Gram's nature
1	**M1**	Raised, convex, opaque	Gm +ve rods
2	**M2**	Raised, convex, opaque, slimy	Gm +ve rods
3	**M3**	Raised, convex, translucent	Gm +ve rods
4	**M4**	Raised, flat, translucent	Gm +ve rods
5	**M5**	Raised, convex, opaque	Gm +ve rods
6	**M6**	Raised, flat, opaque	Gm +ve rods
7	**M7**	Raised, convex, opaque, slimy	Gm +ve rods
8	**M8**	Raised, flat, translucent	Gm -ve short rods
9	**M9**	Raised, convex, translucent	Gm -ve short rods
10	**M10**	Raised, convex, translucent	Gm -ve short rods
11	**M11**	Raised, convex, translucent	Gm -ve short rods
12	**M12**	Raised, convex, translucent, slimy	Gm -ve short rods
13	**M13**	Raised, convex, opaque, slimy	Gm +ve long rods
14	**M14**	Raised, convex, translucent	Gm -ve short rods
15	**M15**	Raised, convex, opaque, slimy	Gm +ve long rods
16	**CR4**	Raised, flat, opaque	Gm +ve long rods
17	**CR6**	Raised, convex, translucent	Gm -ve short rods
18	**CR11**	Raised, convex, opaque	Gm -ve short rods

The presence of amyloid production by the isolates was further validated by thioflavin T staining. Just like Congo red, thioflavin T is an amyloid specific fluorescent dye that gives bright green fluorescence upon binding with amyloid protein (Picken *et al.,* 2015; Biancalana *et al.,* 2010; Sulatskaya *et al.,* 2010; LeVine *et al.,* 1999). Consequently, the isolates obtained from the flocs of activated sludge process were further screened by thioflavin T fluorescence assay. In presence of thioflavin T the amyloid positive bacteria give bright green fluorescence when illuminated under fluorescence microscope. *Solibacillus silvestris* AM1 known to produced amyloid protein was used as a positive control as it is known to make its own

amyloid, whereas *E. coli* curli mutant was used as a negative control. No fluorescence was detected with *E. coli* curli mutant (Figure. 2.2). The isolates that gave fluorescence with thioflavin T were subcultured and selected for further studies.

2.3.2 Flocculation activity of amyloid producing bacteria

Amyloid forming bacteria have been documented to exist in several diverse environments like fresh water lakes, drinking water pipes, flocs of activated sludge etc. (Larsen *et al*., 2007). Having widespread prevalence in nature the amyloids have shown to play an important role in cell aggregation, cell adhesion and bio-film formation (Hu, L 2018; Ramsook *et al.,* 2010; Jordal *et al.,* 2009; Otoo, 2008).

The Flocs of activated sludge have shown variety of amyloid producing bacteria that belongs to phylogenetically distant species in the phyla *Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes*, and *Actinobacteria*. In addition, the amyloid protein, being highly stable protein structure, can be a promising material with variety of industrial applications.

Based on these facts it was tempting to speculate flocculation activity associated with bacterial amyloid as most of the reports have documented its role in cell aggregation. The flocculation activity of selected bacterial isolates was checked with 0.5% kaolin. It was observed that one of the isolates named CR4 showed maximum flocculation activity of 55% as compared to other isolates (Figure. 2.3) Owing to the fact that isolate CR4 demonstrated red coloured colonies and had flocculation activity, it was selected for further studies. Several tests were carried out to confirm the amyloid production in isolate CR4.

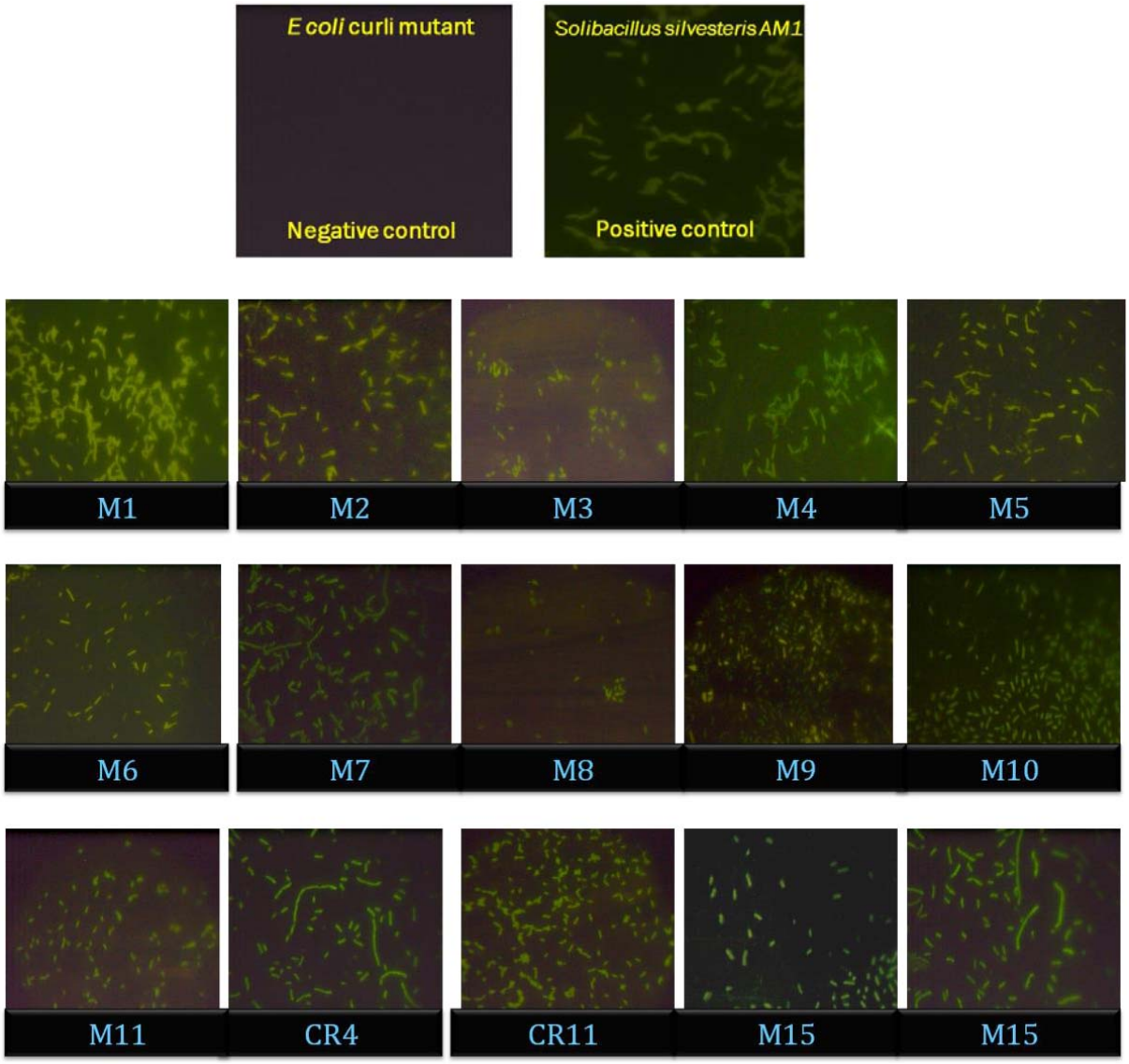


Figure 2. 2 Fluorescence micrographs of the 15 selected isolates stained with thioflavin T showing green fluorescence. (two of the isolates failed to give fluorescence)

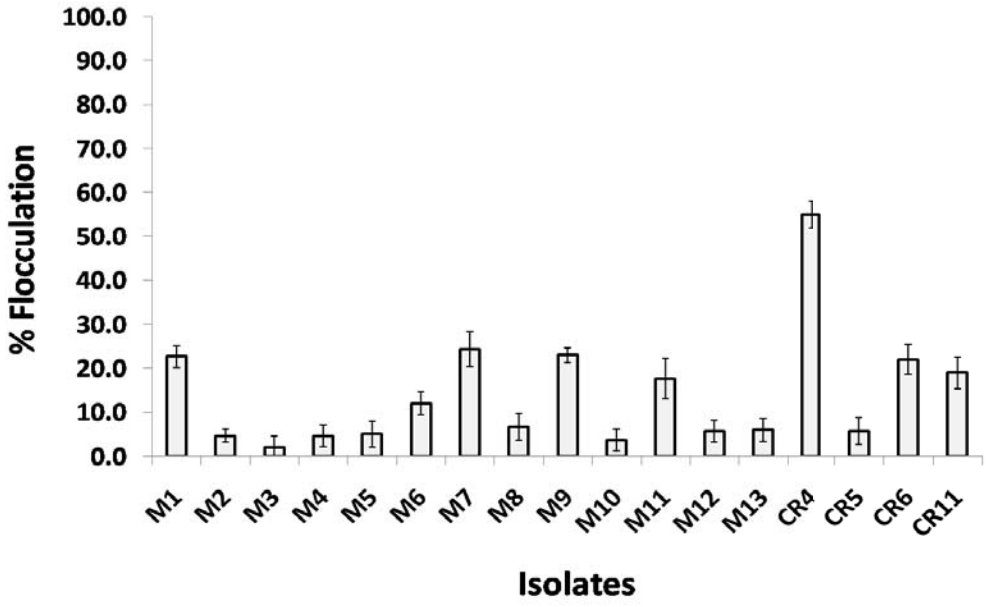


Figure 2. 3 Flocculation activity associated with the 17 selected isolates obtained from the activated sludge.

2.3.4 Identification of isolate CR4

The isolate was identified as *Bacillus cereus* on the basis of NCBI blast analysis, as depicted in table 2.2 and henceforth denoted as *B.cereus* CR4. And the identification was supported by the biochemical tests.

Biochemical tests demonstrated that the isolate belongs to group 18 (Gram positive sporulating rods) of Bergeys manual of determinative bacteriology 9th edition. The isolate was gram positive, rod shaped spore forming bacteria that was motile, showed positive reaction for amylase production, casein utilization, citrate utilization, catalase test and Voges Proskauer test. The isolate demonstrated negative results for gelatin hydrolysis, indole test, oxidase test, arabinose utilization and lactose utilization.

Table 2. 2 NCBI Blast Analysis of 16S rRNA sequence of isolate CR4

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus strain CR4 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2833	2833	100%	0.0	100.00%	1534	MG281958.1
Bacillus anthracis strain FDAARGOS_695 chromosome	Bacillus anthracis	2743	30075	100%	0.0	99.09%	5135792	CP054816.1
Bacillus anthracis strain FDAARGOS_702 chromosome	Bacillus anthracis	2743	30075	100%	0.0	99.09%	5272559	CP054800.1
Bacillus anthracis strain FDAARGOS_703 chromosome	Bacillus anthracis	2743	30075	100%	0.0	99.09%	5261520	CP054797.1
Bacillus thuringiensis strain FDAARGOS_791 chromosome, complete...	Bacillus thuringiensis	2743	38333	100%	0.0	99.09%	5281841	CP054568.1
Bacillus thuringiensis strain FDAARGOS_792 chromosome, complete...	Bacillus thuringiensis	2743	38296	100%	0.0	99.09%	5251676	CP053938.1
Bacillus thuringiensis strain FDAARGOS_794 chromosome, complete...	Bacillus thuringiensis	2743	35560	100%	0.0	99.09%	5214223	CP053934.1
Bacillus cereus strain FDAARGOS_797 chromosome, complete geno...	Bacillus cereus	2743	38346	100%	0.0	99.09%	5413450	CP053931.1
Bacillus cereus strain FDAARGOS_780 chromosome, complete geno...	Bacillus cereus	2743	35577	100%	0.0	99.09%	5271040	CP053997.1
Bacillus cereus strain FDAARGOS_781 chromosome, complete geno...	Bacillus cereus	2743	35577	100%	0.0	99.09%	5271029	CP053991.1
Bacillus thuringiensis strain FDAARGOS_795 chromosome, complete...	Bacillus thuringiensis	2743	35586	100%	0.0	99.09%	5228070	CP053980.1
Bacillus cereus strain FDAARGOS_802 chromosome, complete geno...	Bacillus cereus	2743	38329	100%	0.0	99.09%	5342923	CP053965.1
Bacillus thuringiensis strain FDAARGOS_793 chromosome, complete...	Bacillus thuringiensis	2743	38333	100%	0.0	99.09%	5256259	CP053981.1

2.3.5 Confirmation of amyloid production

The red-coloured colonies obtained on Congo red agar suggests the production of amyloid protein by the isolates which prognosticates the presence of amyloid. However, Congo red being a charged molecule at neutral pH can non-specifically bind to other biological molecules like exopolysaccharides present on the cell surface (Sivanathan *et al.,* 2013). Hence it is mandatory to perform several confirmatory tests to rectify the amyloid production by the isolate.

In an effort to detect amyloid production by *B.cereus* CR4 and associated flocculation activity with it, two approaches were employed. i) Detection of amyloids on cell surface of *B.cereus* CR4 and ii) Confirmation of amyloid nature of purified bioflocculant CR4.

Detection of amyloids on cell surface of *B.cereus* CR4.

In the first approach, for the demonstration of cell bound amyloid protein the cells of *B.cereus* CR4 were stained with Thioflavin T and observed under fluorescence microscope.

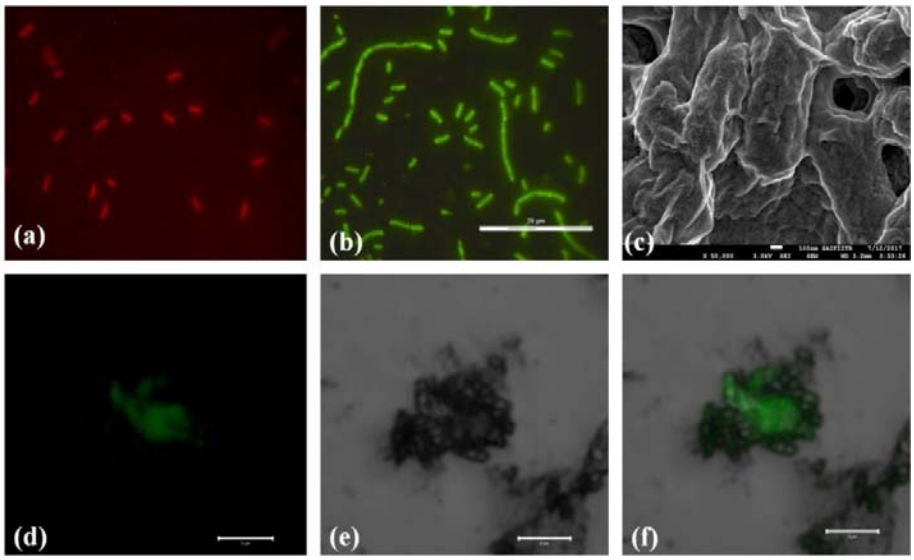


Figure 2. 4 Confirmation of amyloid in *B.cereus* CR4 by various techniques.Confirmation of amyloid production in isolate *B. cereus* CR4 by various techniques. a) Congored Fluorescence. b)Thioflavin T fluorescence. c) Scanning electron microscopy. d) Confocal microscopy. e) DIC microscopy of the confocal image. f) Merged image.

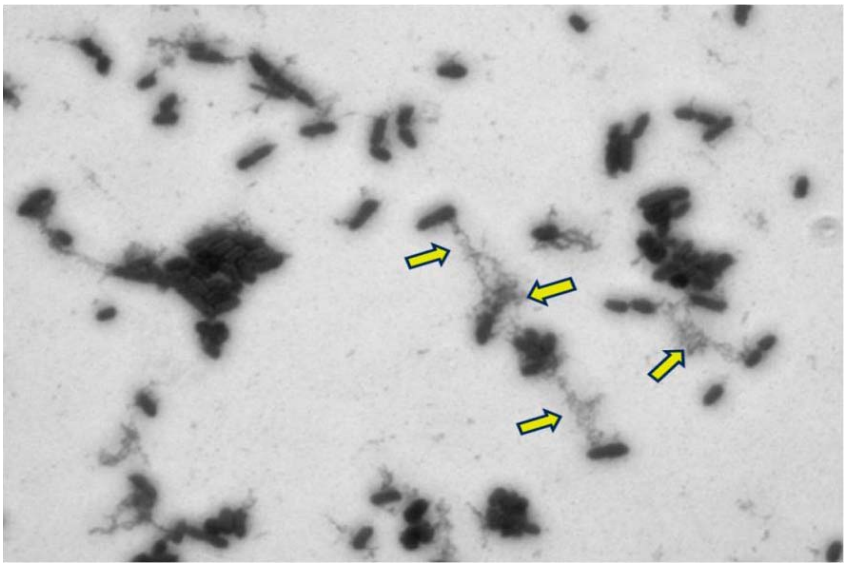


Figure 2. 5 Use of flagella staining method to observe amyloids on cell surface using bright field microscopy. The arrow indicates amyloid fibrils present on the cell surface.

Thioflavin T is considered as one of the most sensitive dyes that binds amyloids (Berg *et al.,* 2010; Jordal *et al*., 2009) and have been recognized as gold standard for selectively staining and identifying amyloid fibrils (Biancalana *et al*., 2010; Hanczyc *et al*., 2021). The dye exhibits green fluorescence upon binding with β sheets of amyloids.

Azo dyes like Congo red have also been used for the detection of amyloid proteins (Larsen *et al.,* 2007). The cells of *B.cereus* CR4 stained with Congo red showed rapidly bleaching bright red fluorescence indicating the presence of amyloid protein on its cell surface (Figure. 2.4a). The green fluorescence of Thioflavin T upon binding to amyloid have been previously established as a diagnostic tool for detecting amyloids. The cells of *B.cereus* CR4 stained with Thioflavin T showed bright green fluorescence under fluorescence microscope showing presence of amyloid protein on its cell surface (Figure. 2.4b). Larsen *et al.,* (2007) too has documented the presence of amyloid producing bacteria in microcolonies from natural biofilms existing in the environment. His studies emphasis similar observations with Thioflavin T staining giving strong green fluorescence while Congo red giving red fluorescence which bleached after some time. Further confirmation of cell surface bound

amyloid was obtained from scanning electron microscopic analysis. SEM has been one of the widely used technique for observation of the amyloids around the bacterial cells (Zheng *et al.,* 2015, Romero *et al.,* 2010).

SEM analysis showed presence of extrapolymeric substance coating the cell surface in *B.cereus* CR4 (Figure. 2.4c). Figure. 2.4d shows cell aggregates with green fluorescence under confocal microscope. The pseudo 3D image of cell aggregates was observed in differential interference contrast microscope, as shown in figure 2.4e. Figure. 2.4f shows the merged images from (d) and (e) implying that the extrapolymeric substance contained in the aggregates of *B.cereus* CR4 contained amyloid.

2.3.5.1 Ammoniacal silver staining

Ammoniacal silver staining is a common method used for staining and visualization of bacterial flagella under bright field microscope (West *et al*., 1977). The flagella can be several micrometers long and about 10nm thick which is same as that of the bacterial amyloid. Hence based on the propinquity in the dimensions of flagella and amyloid fibrils, the protocol for flagella staining was used to stain and visualize amyloid fibrils. As envisaged the bacterial cell surface showed long slender fibrous structure (Figure. 2.5) which was similar in appearance to the reported TEM images of curli amyloid of *E. coli.*

Confirmation of amyloid nature of purified bioflocculant CR4.

The second category of tests were initiated with purification of the amyloid protein. SDS agarose gel electrophoresis was performed with the bioflocculant CR4 as per Sitaras *et al.,* (2011). This method attempts to exploit the unique properties of amyloid to withstand denaturing agents like heat and presence of anionic detergents like SDS. Apart from physical and chemical denaturants the fibrous nature of amyloid proteins restricts them to enter 2% agarose gels. This allows effortless purification of the amyloid protein directly from the wells of SDS agarose gels. The amyloid protein perpetuated in the sampling well can also be visualized by loading the wells with sample and molten agarose followed by staining the gel

with Comassive brilliant blue**.** The purified protein also demonstrated fluorescence with amyloid specific dye thioflavin T, when observed in UV transilluminator (Figure. 2.6). BSA protein used as a control did not demonstrate any fluorescence while the protein recovered from the wells of SDS agarose demonstrated bright green fluorescence.

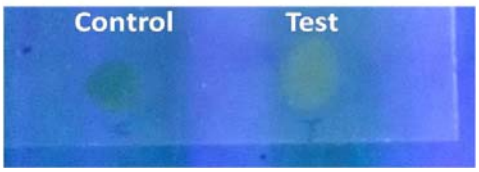


Figure 2. 6 Thioflavin T fluorescence of purified protein observed on UV transilluminator. Control:- BSA, Test:- Purified protein.

Further analysis of the gel suggests that other proteins advanced in the gel during electrophoresis, where as the fibrous amyloid protein remains trapped in the wells of agarose (Figure. 2.7a). The pure protein CR4 recovered from the wells of the gel was subjected to several assays for amyloid confirmation. These assays include Congo red birefringence assay, Congo red spectral shift assay, Transmission electron microscopy, FTIR and CD spectra.

SDS-PAGE is one of the common methods used for the analysis of proteins. As amyloids are stable protein aggregates having fibrous structure, they fail to enter polyacrylamide gels (Figure. 2.7b). Hence the fibrous protein aggregates must be monomerized before analysis by SDS PAGE. One of the common methods used for monomerization of amyloid protein is depolymerization by formic acid and subsequent analysis of the monomers by SDS PAGE. Once the protein was purified from the wells of SDS agarose it was treated with 1% formic acid and analyzed by SDS PAGE. After the electrophoresis was complete the gel was stained with Comassie brillaint blue. The analysis of the gel showed a single band corresponding to molecular weight of 32KD (Figure. 2.7c). The observed molecular weight points to the presence of amyloid protein like TasA which has been widely cited in *Bacillus* species and a molecular weight of 32KD.

2.3.5.2 Congo red birefringence assay.

It has been well established that the amyloid protein interacts with the aromatic bi-phenyl ring of the Congo red and shows characteristic apple green birefringence under cross polarized light (Westermark 1999; Glenner 1972). Hence it was of our interest to study the behaviour of *B.cereus* CR4 amyloid protein stained with Congo red under polarized light. The recovered protein from the wells of SDS-agarose was stained with alkaline Congo red made in saturated NaCl. The presence of alkaline conditions prevents non specific hydrogen bonding of Congo red with the protein whereas presence of saturated salt prevents non specific ionic bonding. This allows the binding of dye specifically to the hydrophobic region of the stacked β sheets of the protein. This unambiguous interaction causes torsional strain in the planar arranged bi-phenyl group which ensues two phenomena. i) The birefringence of polarized light and ii) Red shift in the absorption maxima of the dye. The dye bound with the amyloid fibrils converts the linearly polarized light into elliptically polarized light. This conversion allows the elliptically polarized light to cross the second polarizer making amyloids sample to glow under the microscope. Furthermore, the absorption maxima of the Congo red molecules bound with the fibrils is red shifted, which allows emission of green wavelength (Girych *et al.,* 2016; Prusiner *et al.,* 1983). The elliptically polarized green light crosses the analyzer while the linearly polarized light fails to cross the filter. Hence the amyloid positive sample appears green under polarized light microscope.

The *B.cereus* CR4 amyloid protein recovered from the wells of SDS agarose demonstrated apple green birefringence when studiedunder polarized light microscopy (Figure. 2.7d). As the birefrinigence is a distinct phenomenon that occurs due to specific interaction of planar aromatic rings of Congo red with the cross β sheets of amyloid protein, this test has been appraised as one of the confirmatory tests for the presence of amyloid protein (Puchtler *et al*., 1962; Howie *et al*., 2009). The birefringence assay has been cited widely for the detection and confirmation of amyloid protein in the biopsies of brain tissue for the diagnosis of neurodegenerative disorders.

2.3.5.3 Congo red spectral shift assay

Red shift is a phenomenon in which the absorption maxima of a dye shifts to higher wavelengths. Free Congo red shows absorption maxima at 470nm. Upon binding with amyloid fibrils, the dye has been reported to show hyperchromacity at 510nm (Klunk *et al*., 1999). To test this unique phenomenon the absorption spectra of free Congo red and in presence of purified protein was monitored at different wavelengths (Figure. 2.7e). The absorption spectra of the Congo red with purified protein showed a shift from 470 to 510nm which indicates the amyloid nature of purified protein. As expounded earlier this phenomenon occurs due to the twisting of the planar hydrophobic biphenyl group of Congo red molecule. The birefringence and the red shift phenomenon is considered as one of the defining tests for the corroboration of amyloid proteins (Klunk *et al*., 1989; Klunk *et al*., 1999).

2.3.5.4 Direct visualization of amyloid under Transmission electron microscope

Further substantiation for the presence of amyloid was supported by direct visualization of the purified protein in transmission electron microscope. Transmission electron microscopy has been considered as one of the superlative techniques for direct visualization of amyloid proteins (Gras *et al.,* 2011).

Unlike other tests that are based on chemical or physical phenomenon, this technique is effortless and provides straight forward attestation for the existence of amyloid. In order to examine the presence of amyloid in the material collected from the wells of agarose, the samples were stained with uranyl acetate and studied under TEM at SAIF facility in Indian Institute of Technology, Mumbai. The TEM micrographs depict long slender fibrillar structures several micrometers long and about 10nm thick (Figure. 2.7f). The presence of fibrils in TEM micrograph provides strong evidence for the occurrence of amyloid in purified bioflocculant.

2.3.5.5 Fourier transformed infrared spectroscopy (FTIR)

Fourier transformed infrared spectroscopy is one of the conclusive techniques for detecting presence of various molecular bonds, α helices, β sheets and random coil structure in proteins. As amyloid proteins have been reported to have presence of β sheets stabilized by hydrogen bonds, the use of FTIR can be efficacious for confirming the presence of β in the sample (Shivu *et al.,* 2013; Zou *et al.,* 2014).

Analysis of purified *B.cereus* CR4 amyloid protein by FTIR showed the presence of peak in the amide 1 region (Figure. 2.7g). The peak corresponding to wavenumber 1632 cm^{-1}confirmed the presence of β sheet in the CR4 protein. Most of the documented studies on amyloid proteins have portrayed similar peaks in the amide I region of the FTIR spectrum (Halverson *et al.,* 1991).

2.3.5.6 Circular dichroism (CD) spectra

Besides FTIR, Circular dichroism is widely used accessory technique for study of protein structure like the beta sheet rich amyloids (Terzi *et al.,* 1995). CD spectrum of the *B.cereus* CR4 amyloid protein gave a characteristic negative peak at 220nm (Figure. 2.7h) synonymous to the previously documented CD spectra of β sheet rich bacterial amyloids like curli, chaplin, tafi etc. (Cherny *et al.,* 2005; Dueholm *et al.,* 2010).

2.3.5.7 Criteria for classification of protein as amyloid

There are several reported tests for detection and confirmation of amyloid protein. Nilsson *et al*., (2004) have established several criteria for the confirmation of amyloid protein. These criteria involves assigning numerical points to various tests used for amyloid detection. This requires minimum 4 points for the protein to be categorized as an amyloid. The *B.cereus* CR4 amyloid protein 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.

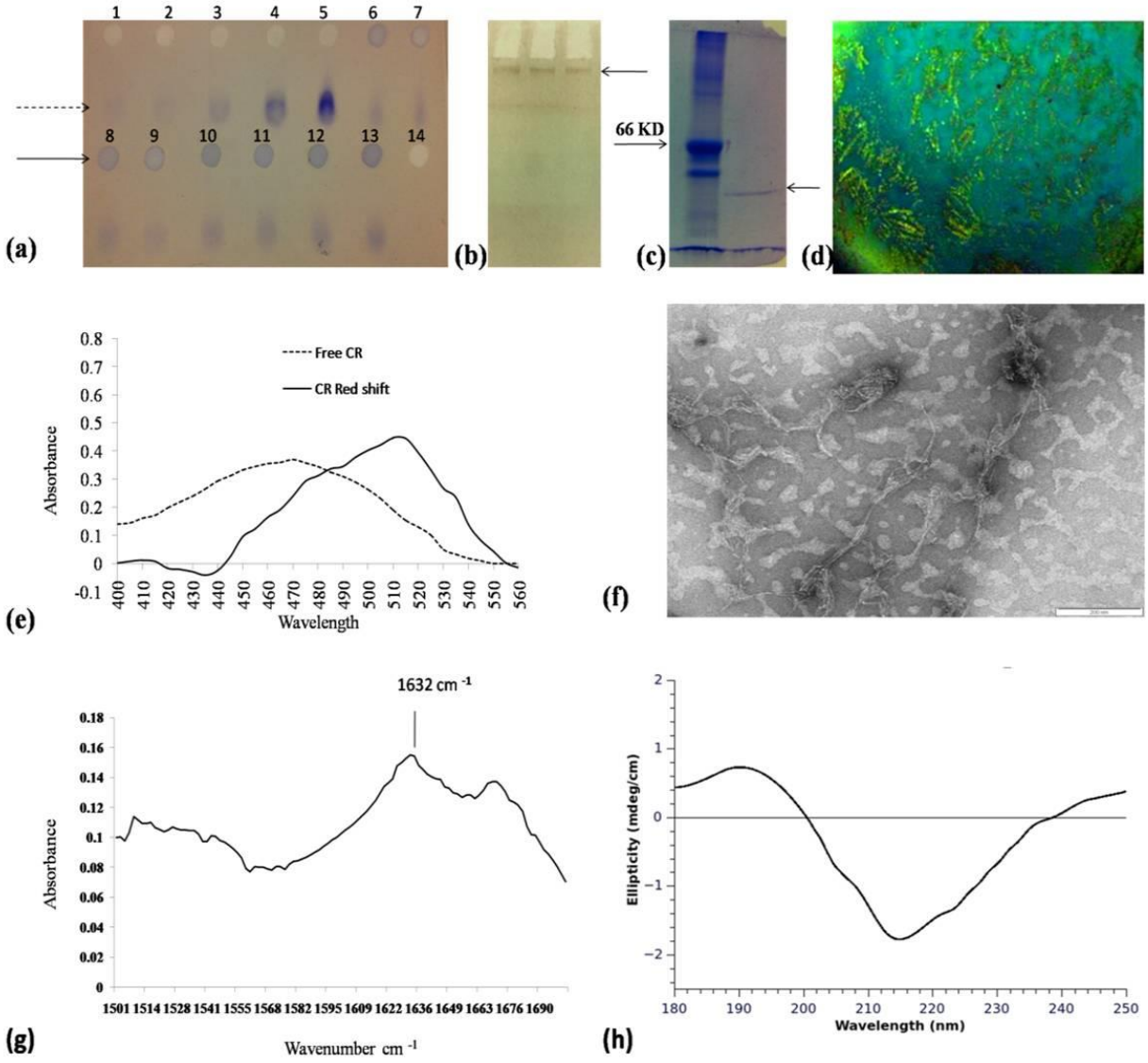


Figure 2. 7 Purification, quantification and confirmation of amyloid in *Bacillus cereus* CR4. a) SDS agarose gel electrophoresis for the quantification of amyloids. b) SDS-PAGE of purified protein. c) SDS-PAGE of purified protein after formic acid treatment. d) Congo red birefringence assay. e) Congo red shift assay. f) Transmission electron microscopy of purified protein. g) FTIR of purified protein. h) CD spectra of purified amyloid protein.

2.3.6 Zeta potential analysis

Zeta potential analysis is a method that reveals net electric charge present on the particles in suspension. The particles with net positive or negative charge tends to repel each other and make the suspension stable. On the contrary particles with net zero charge easily aggregates and settle down as there is no repulsive force to keep them in suspended form (Meraz *et al.,* 2016; Lopez *et al.,* 2014; Yu *et al.,* 1996).

Several factors govern the presence of net electric charge on the surface of particles. These includes pH, presence of ions, charged polymers and other charged particles. Charge neutralization often leads to aggregation of the suspended particles and easy settling.

In order to study the mechanism of flocculation by bioflocculant produced by *B. cereus* CR4 the zeta potential of kaolin particles was measured in presence and absence of bioflocculant. The zeta potential analysis reveals that the kaolin particles have net electric potential of -20mV that changes to 0mV in presence of bioflocculant. The shift in electric potential from -20mV to 0mV reveals that the bioflocculant has a net positive charge and neutralizes the kaolin particles having net negative charge (Figure. 2.8). This charge neutralization results in aggregation of particles by electrostatic patching and bridge formation, ultimately forming flocs and aggregates. This mechanism is described in Chapter 1 section 1.5

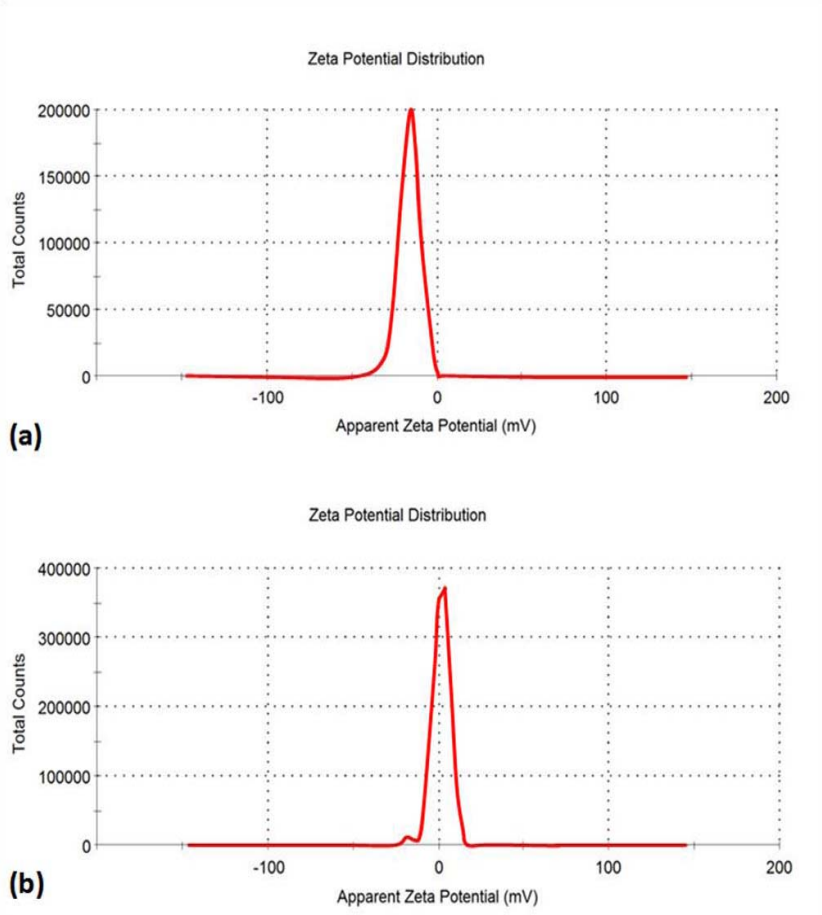


Figure 2. 8 a) zeta potential analysis of kaolin particles in absence of bioflocculant. b) zeta potential analysis of kaoliln particles in presence of purified bioflocculant.

2.3.7 Quantification of *B.cereus* CR4 amyloid bioflocculant CR4

In order to observe the time couse production of the bioflocculant its quantification is required. For the quantification of amyloids SDS agarose gel electrophoresis was used (Sitaras *et al.,* 2011). This system consists of 4% SDS agarose gel for analysing the samples. The unknown samples were mixed with molten agarose and loaded in the wells of agarose gel. To make the standard curve BSA of different concentration was loaded in the gel along with molten agarose. Once the run was complete the gel was stained with Comassie brilliant

blue and analyzed by Image J software after destaining. The amyloid protein being fibrous in nature fails to enter the agarose gel and is retained in the gel. Hence the blue colour of the well indicates trapped amyloid fibrils (Figure. 2.9 red boxes). In contrast small proteins such as BSA easily tends to migrate in the gel leaving the wells empty (Figure. 2.9 Blue box). The intensity of blue colour was estimated by Image J software. The intensity of BSA was used to plot the standard curve (Figure. 2.10) and utilizing this information the concentration of unknown amyloid protein was estimated.

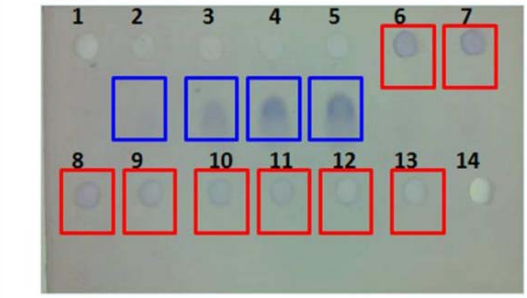


Figure 2. 9 SDS Agarose gel electrophoresis for quantification of amyloids. Blue boxes show the migrated standard protein bovine serum albumin while red boxes show the retained amyloid bioflocculant CR4 protein.

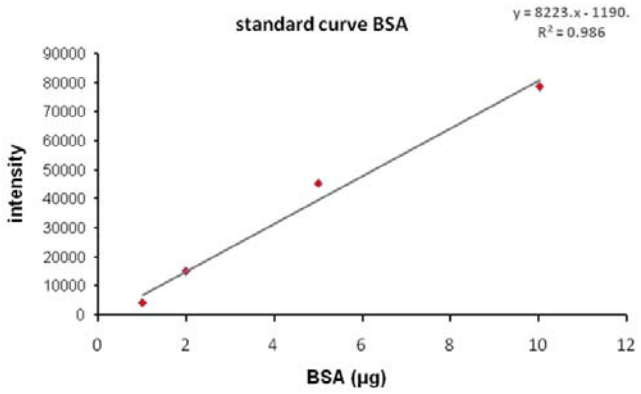


Figure 2. 10 BSA standard curve for quantification of amyloids by SDS-agarose gel electrophoresis.

2.3.8 Time course of bioflocculant production

To find whether the bioflocculant produced by *B.cereus* CR4 was associated with exponential or stationary phase: the growth, bioflocculant activity and amyloid production of isolate CR4 was studied at different time intervals. The flocculation activity was checked with the cells (biomass) and the supernatant. The % flocculation increases as the log phase progresses while the activity in the supernatant increases during the stationary phase (Figure. 2.11). This can be explained by the fact that amyloid protein of *B. cereus* CR4 are loosely associated with the cell surface. As the time proceeds the loosely bound amyloids gets detached from the cell surface and shows flocculation activity in the supernatant. This observation was similar to our previous observation with bioemulsifier producing *Solibacillus silvesteris* AM1 in our laboratory which showed maximum production of amyloid emulsifier during log phase and subsequent release of amyloid during stationary phase (Markande and Nerurkar, 2012).

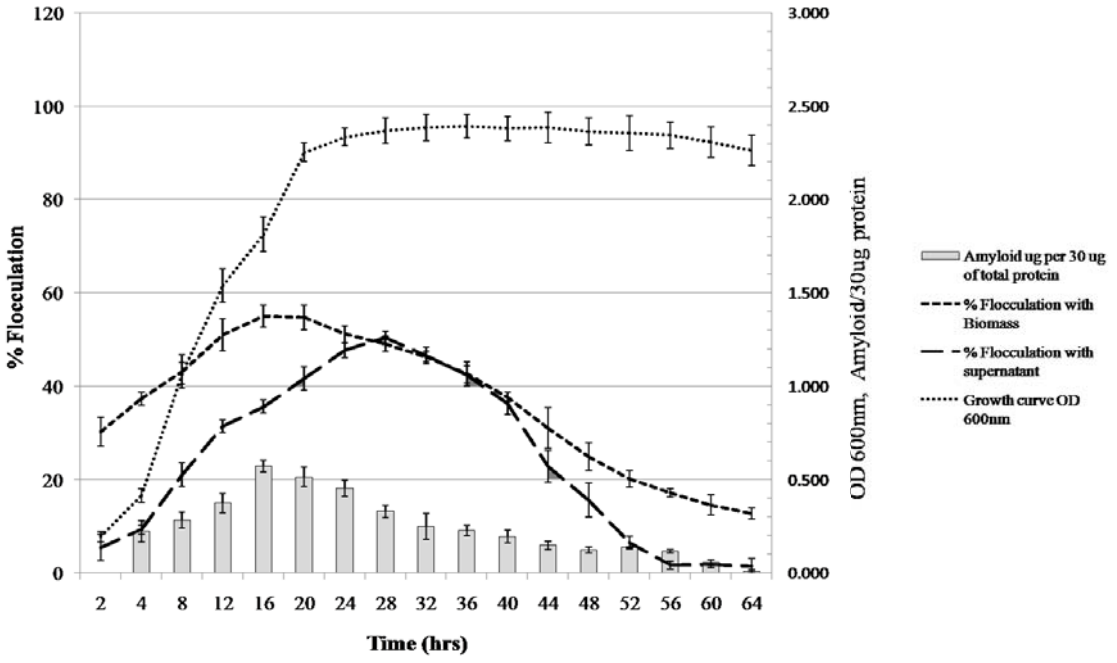


Figure 2. 11 Time course of bioflocculant production by isolate *Bacillus* CR4

2.3.9 pH and Temperature stability of bioflocculant

The unique properties of amyloid protein include its ability to withstand denaturants like heat and pH. These properties make amyloid protein as an interesting candidate for its applications. Interestingly bioflocculant CR4 showed large range flexibility in withstanding thermal and pH stress. There was no loss in flocculation activity even at 100°C at pH 7. The bioflocculant CR4 retained 60 % of its relative activity between pH 3 to 9 upto 90°C, indicating that its stability in wide pH range. The sturdy nature of purified amyloid bioflocculant shows that it can be used for an industrial purpose. (Figure. 2.12)

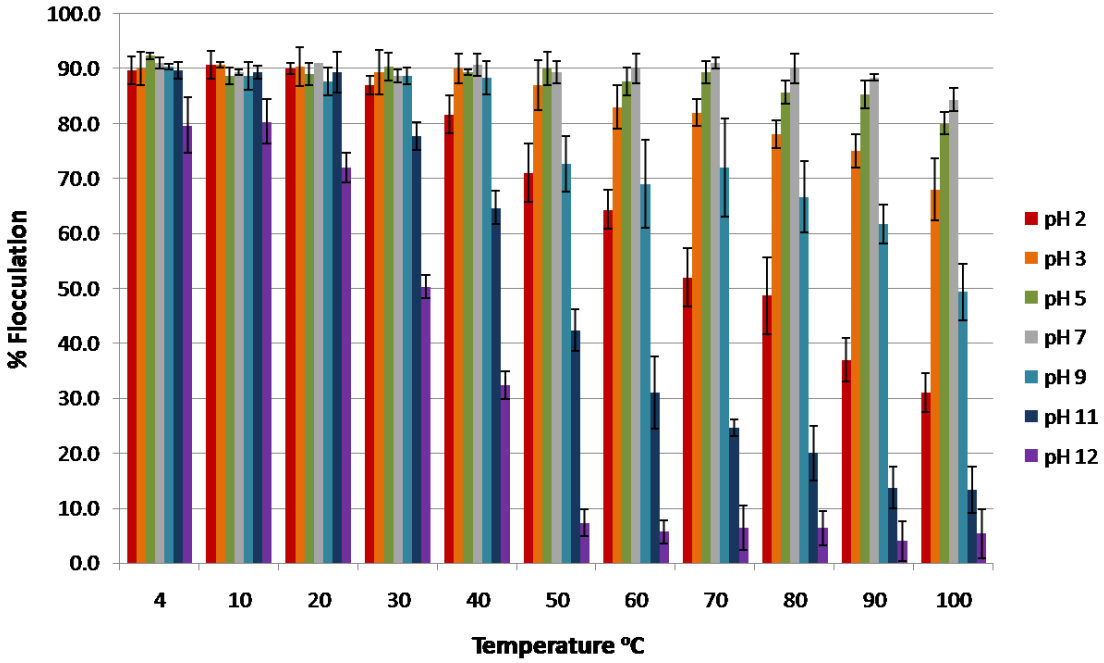
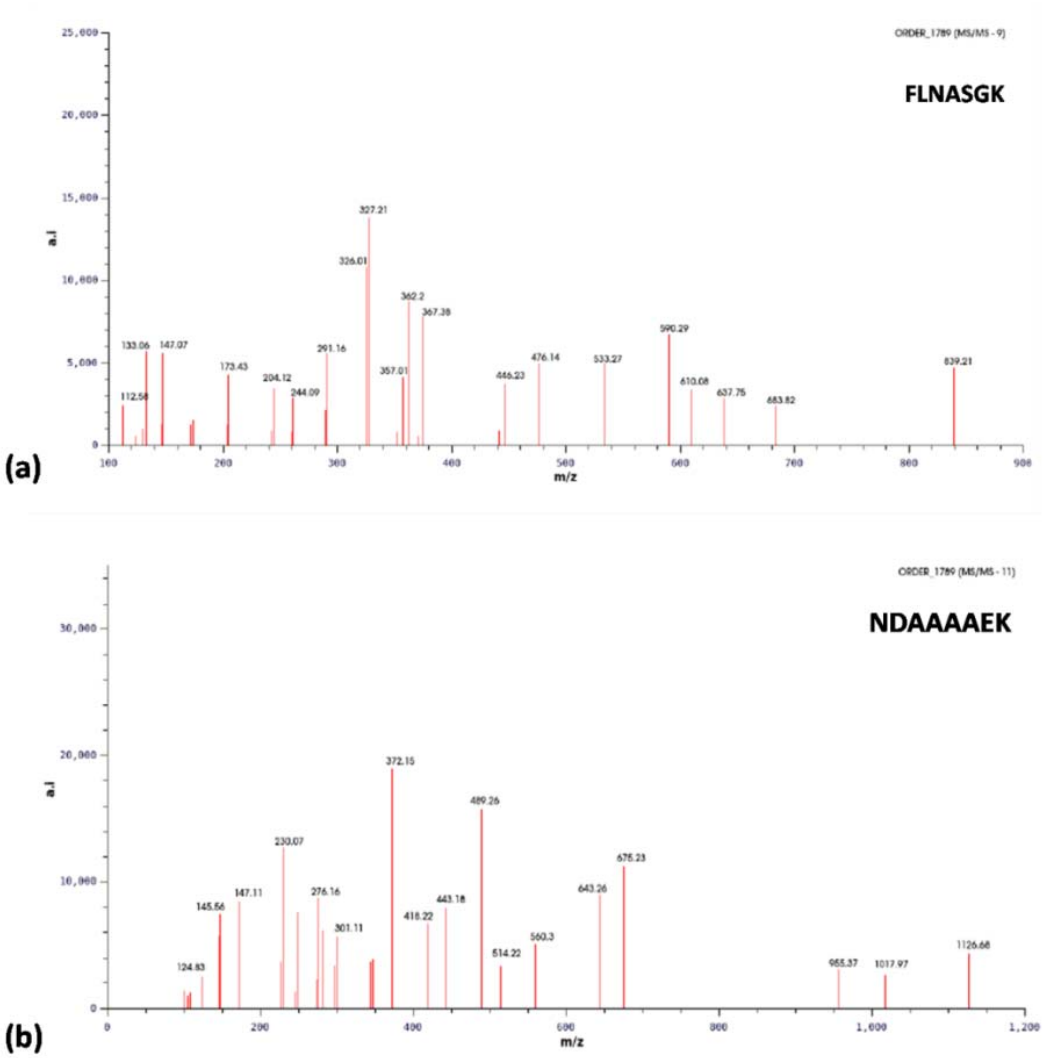


Figure 2. 12 pH and temperature stability of bioflocculant obtained from *B.cereus* CR4

2.3.10 Identification of amyloid bioflocculant CR4

Identification of amyloid bioflocculant was carried out by purification of amyloid by SDS-agarose gel electrophoresis followed by treatment with formic acid to monomerize the

fibrillar structure. As described earlier the protein monomers demonstrated molecular weight of 32KD when analyzed by SDS–PAGE. The 32KD band was excised from gel and purified by electrodialysis. The protein retained during dialysis was lyophilized and sequenced by Edman degradation. The LC-MS/MS analysis of the protein showed several peptides with sequence similar to spore coat protein TasA of *Bacillus* sp. As shown in Figure. 2.13 and Table 2.3 the longer peptides number 9,11,17,18 & 21 (in bold font) reveal the identity of the bioflocculant CR4 as similar to TasA.



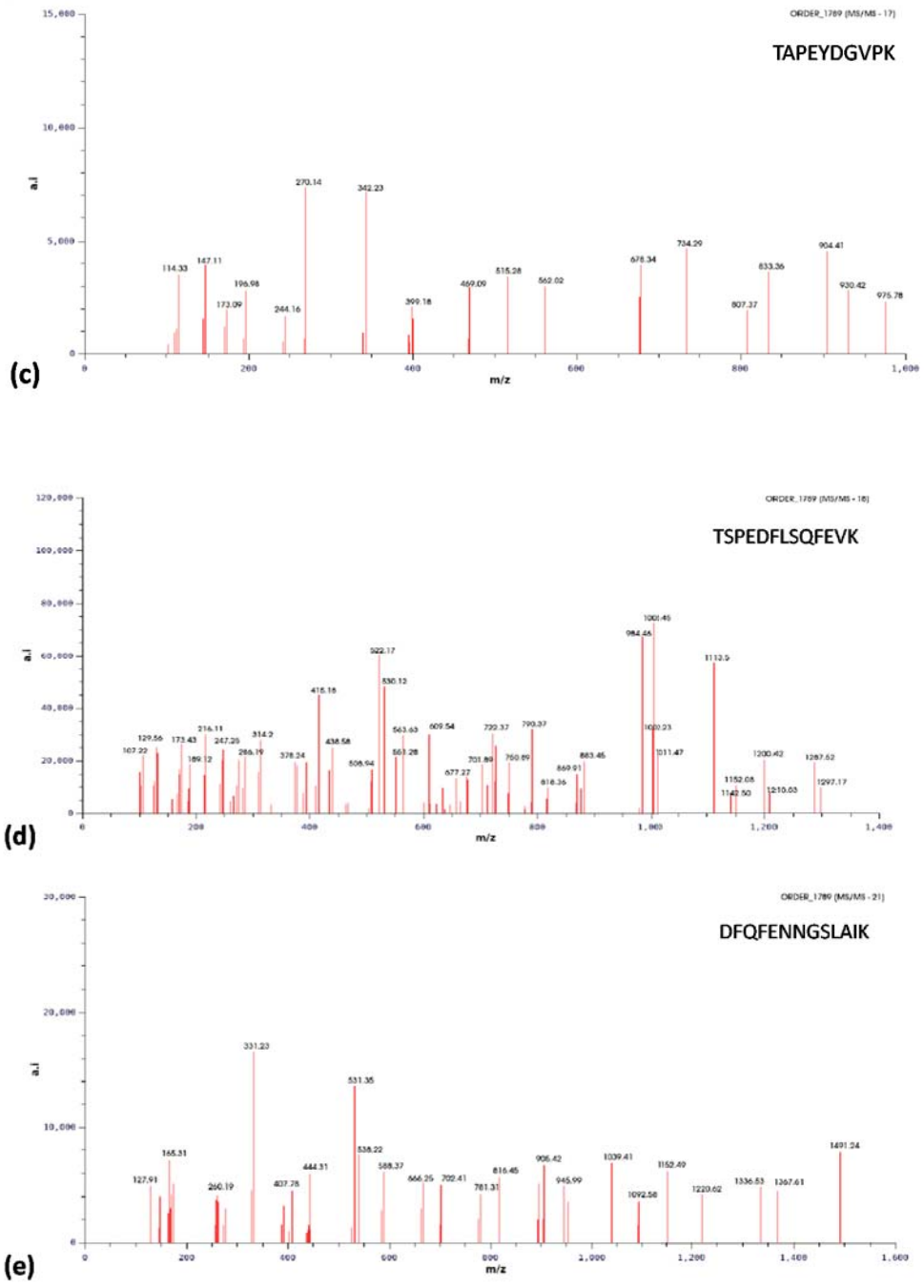


Figure 2. 13 LC-MS/MS analysis for the identification of purified amyloid protein bioflocculant CR4.

Table 2. 3 List of peptides detected by LC MS/MS analysis of purified CR4 protein.

Sr no	Peptide	Similarity
1	TSGK	ECM-binding protein homolog *Staphylococcus epidermidis* RP62A
2	GNMK LN GA	Centrosomal protein *Homo sapiens* - -
3	VLD GA	- -
4	PQWG	Immunoglobulin heavy chain junction region *Homo sapiens*
5	HLTC	-
6	HQPK	-
7	VNNQK YFDD FLGPK NGDK NGNND	Zonadhesin; Flags: Precursor *Homo sapiens* Verlamelin biosynthesis protein S *Lecanicillium* sp ATP-dependent helicase/nuclease subunit A *Lactobacillus casei BL23* Uncharacterized protein YeeJ *Escherichia coli* 157:H7
8	NNVADEFK WRMDSGF PVINK APTK	RNA helicase DHR1 *Saccharomyces cerevisiae* S288C BCLAF1 and THRAP3 family member 3 *Homo Sapiens* Chromodomain-helicase-DNA-binding protein 8 [*Homo Sapiens* -
9	**FLNASGK**	**Spore coat-associated protein *Bacillus* sp**
10	LTK	-
11	**NDAAAAEK**	**Major biofilm matrix component *Bacillus subtilis***
12	QLDK WSM	Extracellular matrix protein FRAS1 *Homo sapiens* -
13	APVLLNDK GSCNQK	Levansucrase *Pseudomonas savastanoi* Lysine-specific demethylase 5B *Homo sapiens*
14	PGDK	ATP-binding cassette sub-family A member 5 *Homo sapiens*
15	AVNDK	Cullin-associated NEDD8-dissociated protein 1 *Aspergillus nidulans*
16	GSTLK	Unnamed protein product *Homo sapiens*
17	**TAPEYDGVPK**	**MULTISPECIES: spore coat protein *Bacillus***
18	**TSPEDFLSQFEVK**	**MULTISPECIES: biofilm matrix protein TasA *Bacillus***
19	ENEK	-

	PVDK	-
20	ENEK	-
21	**DFQFENNGSLAIK**	**MULTISPECIES: biofilm matrix protein TasA *Bacillus***
22	ASK PVW	- -
23	NAGF	-
24	GGASSSVTCN	Uncharacterized protein TRIREDRAFT_102680 *Trichoderma reesei* QM6a
25	VLD PLNMM	- Hypothetical protein, partial *Streptomyces* sp E5N91

The significant outcome of the screening of various isolates from the flocs of activated sludge provided a special strain of *B. cereus* that produced extracellular amyloid protein which was found to possess flocculant activity. The bioflocculant produced by the specially selected isolate *B. cereus* CR4 was named bioflocculant CR4. Bioflocculant CR4 was found to be similar to TasA amyloid protein found in many *Bacillus* spp. It was characterized and further studied for its biotechnological applications, the results of which are discussed in succeeding chapters.

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