Summary

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- **Isolation of amyloid producing bacteria from flocs of activated sludge.**
- Screening for amyloid producing bacteria was done on Luria agar supplemented with Congo red as an indicator. As red coloration to the bacterial colonies may be imparted by the surface amyloid protein, 17 isolates demonstrating red colored colonies on Congo red agar presumably due to amyloid prtein were screened for their flocculation activity. Isolate CR4 from among the 17 isolates one demonstrated maximum 55% flocculation activity as compared to other isolates and was selected for further studies.
- The isolate CR4 was classified under group 18 of Bergeys manual as of Gram-positive long rods that were motile and formed endospores.
- Using biochemical tests based on Bergys mannual of determinative bacteriology 9th ed. and 16S rRNA gene sequencing isolate CR4 was identified as *Bacillus cereus* and *designated* as *B.cereus* CR4 in the entire studies.
- **Studies on time course bioflocculant production by *B.cereus* CR4**
- Time course production in LB broth studies demonstrated that *B. cereus* CR4 demonstrated possessed 58% cell bound bioflocculation activity at the end of exponential growth phase at 16hrs. The flocculation activity demonstrated by the cell free supernatant peaked to 52% in stationary phase at 28hrs.
- **Confirmation of amyloid nature of the bioflocculant produced by *B.cereus* CR4.**
- The presence of amyloid protein in *B. cereus* CR4 was confirmed by several confirmatory tests that were performed with the *B.cereus* CR4 cells as well as purified protein. Confirmation of amyloid production by isolate *Bacillus cereus* CR4 was done as follows:
- Presence of redcoloured colonies on Congo red agar.
- Cells showing red fluorescence when stained with Congo red.
- **Amyloid specific Thioflavin T staining**: Cells showing bright green fluorescence due to cell surface amyloids in fluorescence microscopy and clumps of cells of *B. cereus* CR4

embedded in aggregates showing green fluorescence due to amyloid production in conflocal microscopy.

- **Scanning electron microscopy. S**tudies of *B. cereus* CR4 cells with SEM shows the presence of fibrillar structure around the cell, along with cell bound EPS.
- **Flagella staining.** Using classical flagella staining method the presence of amyloid fibrils around the cells was directly visualized a dense fibrillar aggregates festoon around the cells observed under bright field microscope.
- **Confirmation of amyloid characteristics if purified surface protein from *B cereus* CR4**
- **SDS Agarose gel electrophoresis.** Amyloid nature of the purified protein was established as SDS resistant protein aggregates that failed to enter the wells of SDS agarose during electrophoresis.
- **Thioflavin T staining.** The purified protein demonstrated green fluorescence under UV transilluminator after staining with Thioflavin T.
- **Congo red birefringence assay.** The purified protein stained with Congo red demonstrated apple green birefringence under polarized light microscopy.
- **Transmission Electron microscopy.** Transmission Electron microscopy of purified protein demonstrated presence of fibrillar structures corresponding to presence of amyloid fibrils.
- **FTIR spectroscopy.** Fourier transformed Infrared Spectroscopy of the purified protein demonstrated peaks in Amide I region of the spectrum. The analysis of amide I region of the spectrum showed the peak corresponding to wave-number 1632 cm^{-1} that confirmed the presence of β sheet in the protein that is characteristic of amyloid form of proteins.
- **CD spectra.** Analysis of CD spectra of the purified protein demonstrated a negative peak at 220nm that showed the presence of β sheet in the protein.
- **Congo red – Red shift assay.** The absorption maxima of Congo red mixed with purified amyloid protein was found at 510nm whereas free Congo red demonstrated its absorption

maxima at 470nm. Spectral shift from 470nm to 510nm is typically given by amyloid form of protein thus confirming the amyloid nature of bioflocculant CR4 protein.

- The purified surface protein from *B. cereus* CR4 demonstrated flocculation activity which confirmed that bioflocculant produced by *B.cereus* CR4 has amyloid nature. The bioflocculant termed bioflocculant CR4 in the entire studies retained 60 % of its flocculation activity between pH 3 to 9 upto temperature 90ºC.
- **Identification of bioflocculant CR4 protein.**
- Analysis of purified amyloid bioflocculant CR4 by SDS-PAGE revealed presence of 32KD protein.
- The purified CR4 bioflocculant upon LC-MS/MS analysis showed presence of TasA protein that is amyloid protein commonly found in *Bacillus* species. In *B. cereus* too the TasA amyloid have shown to play an important role in cell aggregation and biofilm formation.
- **Mechanism of flocculation by CR4 bioflocculant.**
- Zeta potential analysis demonstrated that kaolin particles in absence of bioflocculant possess -20mV electric charge that shifts to 0mV upon addition of bioflocculant CR4. This change in zeta potential values indicates the role of charge neutralization by bioflocculant CR4. This suggested that the negative surface charge on kaolin particles is neutralized by CR4 bioflocculant by electrostatic patching mechanism.
- **Media optimization for bioflocculant production**.
- *B.cereus* CR4 demonstrated optimal bioflocculant production at pH 7 and temperature 37ºC.
- Screening of Different nutrient sources that significantly contributed in the bioflocculant production by Plackett Burman analysis revealed the role of Lactose, Yeast extract and Tryptone in supporting bioflocculant production.
- Optimization of media for maximum bioflocculant production by Central composite design revealed optimal medium containing 0.25% lactose, 0.5% yeast extract and 0.7%

tryptone that demonstrated 89.2% flocculation activity of bioflocculant CR4 at pH 7 and temperature 37^{o}C.

- **Optimization of flocculation conditions.**
- Studies on flocculation of kaolin with purified bioflocculant CR4 demonstrated 61% flocculation activity at acidic pH 5 and 60µg/ml of amyloid concentration.
- Optimization of flocculation conditions for kaolin using Central composite design resulted in enhanced 90% flocculation activity at pH 3 when the amyloid concentration was 72mg/l. respectively.
- **Media optimization for growth of microalgae *Scenedesmus* sp.**
- Screening of nutrients that significantly supported growth of *Scenedesmus* sp. revealed the role of $NaNO_3$ and $ZnSO_4$ as a growth supporting nutrients.
- The growth of *Scenedesmus* sp. optimized by Central composite design demonstrated 3.46g/l of biomass production in presence of 125mg% of $NaNO_3$ and 2.55mg% of $ZnSO_4$ respectively.
- **Optimization of flocculation of *Scenedesmus* sp. by *B. cereus* CR4**
- Screening of metal ions as coagulants for flocculation of *Scenedesmus* sp. with *B. cereus* CR4 demonstrated maximum % flocculation of 58.5% in presence of Fe^{+3} followed by 22% flocculation activity in presence of Ca^{+2} ions.
- Optimization of flocculation conditions for the recovery of *Scenedesmus* sp. biomass by flocculation with *B. cereus* CR4 revealed maximum flocculation of 82.3% at pH, biomass and Fe^{+3} concentration adjusted to 4.0, 0.400 (OD 750nm) and 182 mg% respectively.
- Bright field microscopy of flocculated *Scenedesmus* biomass with *B. cereus* CR4 revealed the presence of *B. cereus* CR4 cells as a bridging agent between *Scenedesmus* cells.
- Studies on factors affecting cell viability of *Scenedesmus* sp. after flocculation with *B. cereus* CR4 revealed that 99% of the cells remains viable at pH 7.
- The cell viability remains at 92.8% at pH 6 and 0.55mg% $FeCl_3$ whereas the cell viability dropped to 8% when pH was dropped to 2 at 0.55mg% $FeCl_3$ concentration.

- **Application of *Scenedesmus* sp. for nutrient removal from waste water and its harvest by floc forming *B.cereus* CR4.**
- Application of *Scenedesmus* sp. to remove nitrates and phosphate from synthetic waste water using batch and continuous reactor demonstrated that *Scenedesmus* sp. could remove 99% nitrate and 43.90% phosphate in batch reactor while 42.95% nitrate and 20.05% phosphate removal was observed at critical flow rate 4.2 ml/min in a continuous reactor.
- **Effects of Different Nutrients and stress agents on flocculation activity.**
- Stress agents such as SDS (0.05%), Ethanol (0.5%) DMSO (1.5%) and NaCl (2%) demonstrated flocculation activity of 82%, 84%, 89% and 66% respectively as compared to control (without stress agent) where it demonstrated 62% flocculation.
- The amyloid production in presence of SDS (0.05%), Ethanol (0.5%), DMSO (1.5%) and NaCl (2%) was 1.7, 5.1, 4.5 and 1.45 μg/mg of biomass respectively. These observations suggests that presence of stress agents increased amyloid production as well as flocculation activity, this is an important observation making it important with respect to industrial production of CR4 bioflocculant.
- **Biofilm studies**
- Biofilm stimulating agents such as Lactose and Manganese at respective concentration of 0.5%, 60mM demonstrated substantial increase in biofilm and cell surface amyloid production in *B. cereus* CR4.
- Besides nutrient factors effect of stress agents such as SDS (0.05%), ethanol (0.5%), DMSO (1.5%) and NaCl (2%) showed biofim formation that was four-fold higher as compared to TY broth as a control.
- Studies on cell surface hydrophobicity and biofilm form demonstrated that pellicle type biofim form has median % hydrophobicity of 57% while submerged biofilm has median % hydropohobicity of 27%. which shows that hydrophobicity had major role in governing pellicle vs surface biofilm formation.

- The *Bacillus* spp. that demonstrated presence of TasA amyloid showed median for % hydrophobicity of 55.70% while the *Bacillus* spp. in which TasA amyloid was absent showed median % hydrophobicity of 29.50%. This proves that the presence of TasA amyloid provides hydrophobic nature to the cell surface that in turn favors pellicle formation, while absence of TasA makes the cells less hydrophobic and promotes formation of surface biofilms.
- Except *B. amyloliquefaciens,* and *B. licheniformis* all *Bacillus* spp. under study demonstrated two-to-three-fold decrease in biofilm in presence of DL amino acids.
- Mixture of DL methionine, DL tyrosine, DL leucine, DL tryptophan at concentration as low as 10nm were effective in preventing the biofilm formed by most of the *Bacillus* spp.
- **Cloning of bioflocculanat gene.**
- The ORFs amplified from the genomic DNA demonstrated multiple bands when observed after agarose gel electrophoresis.
- The amplified ORFs were ligated in vector pVS72 and transformed in *E.coli* curli mutant VS16. Most of the colonies screened on Congo red agar after transformation demonstrated white color that shows absence of amyloid gene.
- One of the colonies that showed red color was used to perform colony PCR using plasmid specific flanking primers to amplify the cloned gene.
- Amplification of cloned gene demonstrated the presence of 600 base pair PCR product which was further sent for DNA sequencing.
- DNA sequencing analysis suggests that the gene responsible for producing amyloid bioflocculant in *B. cereus* CR4 was found to be tasA after sequencing.
- Thus an ecofriendly, biodegradable, environmentally safe and sturdy bioflocculant with amyloid nature was found from common bacterium *B. cereus CR4.* The amyloid bioflocculant was identified as TasA protein that has shown to play an important role in cell adhesion, aggregation and biofim formation in *B. cereus CR4* demonstrated optimum bioflocculant production in presence of lactose and stress agents like ethanol, DMSO and SDS. The ability of TasA to cause cell aggregation was explored for flocculation and

harvest of *Scenedesmus* sp. The bulk growth of *Scenedesmus* sp can be obtained by using waste water rich in phosphates and nitrates, at industrial scale. Once cultivated cost effective harvesting of *Scenedesmus* sp. can be achieved by the use of *B.cereus* CR4 biomss. Further, the use of D-amino acids at nano molar concentrations demonstrates an effective way for preventing formation of biofilms.