Chapter 4

Studies on amyloid production and biofilm formation of *B. cereus* CR4 and other *Bacillus* spp.

Chapter 4. Studies on amyloid production and biofilm formation of *B. cereus* CR4 and other *Bacillus* spp.

The bacterial biofilm is a multicellular bacterial community attached on a physical or biological surface. The attachment of bacterial cells on biotic or abiotic surface and the multicellular nature is governed by formation of extracellular polymeric substances (EPS) made by individual bacterial cells. Recent studies have demonstrated that besides EPS the functional amyloids present on the cell surface of bacteria also plays a significant role in giving strength and rigidness to the biofilm structure. The process of biofilm formation and detachment is governed by several environmental factors, nutrients, metal ions, cell signaling molecules and presence of cofactors on the surface of bacterial cells. In case of common soil dwelling bacterium *B. subtilis* carbon source such as glycerol and metal ions such as manganese have significant role in promotion of biofilms by stimulating formation of cell surface TasA amyloid fibrils (Shemesh *et al*., 2013). A smilar study in notorious food poisoning strain of *B. cereus* have demonstrated the role of manganese in stimulating formation of biofilms in invitro conditions (Gingichashvili *et al*., 2017). Besides glycerol and manganese, investigation of biofilms associated with reactors and pipes of dairy industry have demonstrated abudance of *B. cereus* biofilms. Lactose which is main component of effluents of dairy industry is known to promote biofilms of *B. cereus* in dairy vessels and pipelines. The significant contribution of lactobse in promoting *B. cerues* biofilms is due to upregulation of EPS and TasA amyloid genes (Duanis *et al*., 2016). Therefore, the effect of nutrients on amyloid production by *B. cereus* CR4 was studied.

Besides nutrients the presence of external stress has also demonstrated in regulating biofilm formation. In vivo studies with *E. coli* have demonstrated an increase in its virulence in presence of alcohol. This increase in virulence inturn causes *E. coli* to promote dense obnoxious biofilms. Detailed studies on effect of ethanol on *E. coli* have demonstrated over expression of curli amyloid genes that results in strong biofilms. Besides Ethanol, chemical stress agents such as DMSO have also demonstrated upregulation of curli genes leading to

development of biofilms (Lim *et al.,*2012). Hence in this perspective it became interesting to study the effect of several stress agents on amyloid production by *B. cereus* CR4.

The *Bacillus* sp. are known to form thick floating biofilm termed as pellicle under static condtions. The TasA amyloid produced by *Bacillus subtilis* is one of the major components that provides unique strength and hydrophobic nature to the pellicle. Studies were conducted with various *Bacillus* species including *B. cereus* CR4 with respect to cell surface hydrophobicity and form (pellicle or submerged) of biofilm to understand the significance of TasA presence in *Bacillus* spp.

Bacterial biofilms being notorious agents in various food industry, medical devices, pipelines etc. needs special attention for its control and eradiction. For any bacteria the biofilm stage is a unique stage where cells aggregate and behaves as a multicellular community. However, with time as the biofilm age, the lack of nutrients and accumulation of toxic waste products forces bacterial cells to return to its free planktonic stage of existence. However, the biofilms that have functional amyloid fibers as its adhering constituent are rigid and robust. Such biofilms are difficult to disintegrate by physical and chemical agents. An example of such strong and rigid biofilm is the floating pellicle formed by *B. subtilis* at air liquid interface (Romero *et al.,* 2010). The pellicle and biofilm of *B.subtilis* remains sturdy as long as TasA amyloid fibers are adhered on the cell surface. This adhering is supported by an accessory protein TapA that remains on the cell surface and anchors TasA fibers as depicted in Figure 1.1c. If the interaction between TasA and TapA is broken then the TasA amyloid fibers becomes free from the cell surface. This ultimately results in breakage of pellicle and biofilm structure. The TasA amyloid fibrils along with extracellular polymeric substances that adheres the individual bacterial cells gives the pellicle its unique strength. Under nutrient limiting conditions when it becomes necessary for biofilm cells to return to their planktonic stage the *B. subtilis* cells produces D-amino acids (D-Tyrosine, D-leucine, D-tryptophan, D-methionine) that causes detachment of TasA–TapA complex releasing the extracellular amyloid fibrils from the cell surface. This release of amyloid fibrils causes disintegration of biofilm with ease. Invitro studies suggests that the mixture of D amino acids are effective in

detaching the biofilm in nM concentration (Kolodkin *et al*., 2010; Warraich *et al*., 2020). The role of D amino acid has been widely studied in *B. subtilis,* making it interesting to study in other *Bacillus* species here. In above perspective, this chapter discusses the results of following studies.

I) Infleunce of various factors like nutrientsthat include lactose, glycerol and $MnCl_2$ and stress agents like ethanol, DMSO, SDS and NaCl on amyloid production has been studied on *Bacillus* cereus CR4.

II) Studies on biofilm form (pellicle or submerged) and cell surface hydrophobicity of different *Bacillus* spp.

III) Influence of D-amino acid on biofilm detachment of *Bacillus* spp.

4.1 Materials and Methods

4.1.1 Quantification of biofilm

The quantification of biofilm was done as described by Srinandan *et al.,* (2010). The biofilm assay was performed using 96 well microtitre well plate. The wells of microtitre plate were inoculated with the culture of *B. cereus* CR4 (10% inoculum, 0.4OD at 600nm). The plates were incubated at 37^{o}C for 24hrs with 160RPM agitation. The quantification of biofilm was done by staining the biofilm with 1% crystal violet for 10mins followed by destaining with 70% ethanol. The optical density was measured at 595nm in the crystal violet stain eluted in 70% ethanol.

4.1.2 Quantification of Amyloids

For the quantification of cell bound amyloids 50ml of TY broth (1% Tryptone and 0.15% Yeast extract) was prepared with different concentration of nutrients (Section 4.2.3) and inoculated with 10% *B. cereus* CR4 inoculum of 0.4 OD600nm. The flasks were incubated at 37^{o}C for 24hrs. The biomass was harvested by centrifugation at 8500g for 15mins and used for quantification of amyloids as described in chapter 2 section 2.2.11

4.1.3 Effect of Lactose, Manganese and Glycerol

4.1.3.1 Effect of Lactose, $MnCl_2$ and glycerol

TY broth with varying concentration of lactose (0.1%, 0.5%, 1%, 1.5%, 2%), $MnCl_2$ (20Mm, 40mM, 60mM, 80mM, 100mM) and glycerol (0.1%, 0.2%, 0.3%, 0.4% ,0.5%) was prepared individually and the biofilm assay was performed using 96 well microtitre well plate (Section 4.2.1.). The quantification of amyloids was done as described in section 4.2.2.

4.1.4 Effect of stress agents on growth, amyloid and flocculation activity.

The flasks containing TY broth was with varying concentration of SDS (0.005%, 0.01% and 0.05%), ethanol (0.05%, 0.5% and 1.5%), DMSO (0.05%, 0.5% and 1.5%) and NaCl (2%, 3% and 4%) was prepared and inoculated with the culture of *B. cereus* CR4 (10% inoculum). The TY broth without any additive was kept as a control. The flasks were incubated at 37^{o}C for 24hrs with 160RPM agitation. After growth the biomass was used for flocculation assay and quantification of amyloids.

4.1.5 *Bacillus* spp. used in the studies.

B. oleronius, B. clausii, B. badius, B. atrophaeus 78, *B. amyloliquefaciens* 98*, B. licheniformis* TT42, *B. licheniformis* K125 *B. mojavensis* JF2, *B. mojavensis* TT33, *B. subtilis* MW10*, B. subtilis* 168*, B. sphaericus, B. thuringiensis* HD73*, B. thuringiensis* HD1*, B. cereus* CR4*, B. megaterium, B. firmus, B. cereus* 14579T

4.1.6 Observation of biofilm form

Different *Bacillus* strains were checked for their ability to form pellicle or submerged biofilm. Each strain was grown in test tubes with 5ml Luria broth, at 37^{o}C for 48hrs under stationary conditions. The tubes were observed for the presence of pellicle or submerged biofilm.

4.1.7 Measurement of cell surface hydrophobicity

The measurement of cell surface hydrophobicity was done by MATH (Microbial adhesion to hydrocarbons) assay (Tecon *et al.,* 2010). Each *Bacillus* strain was grown in Luria broth

for 24hrs at 37^{o}C and 160RPM agitation. The cell biomass was collected by centrifugation at 8500g for 15mins and suspended in equal volume of distilled water. The cell density was adjusted to 0.4 OD at 600nm. For the assay 3ml of culture suspension was mixed with 150µl of Hexane with vortex. The mixture was allowed to stand for 10min and the optical density of aqueous phase was measured at 600nm. The % hydrophobicity was measured as per following equation.

$$\% \text{ Hydrophobicity} = 100 \times \frac{\text{Initial OD (600nm)} - \text{Final OD (600nm)}}{\text{Initial OD (600nm)}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

4.2 Results and Discussion

Infleunce of various factors like lactose, glycerol and $MnCl_2$ and stress agents like ethanol, DMSO, SDS and NaCl on amyloid production by on *Bacillus cereus* CR4.

4.2.1 Effect of Lactose, $MnCl_2$ and Glycerol

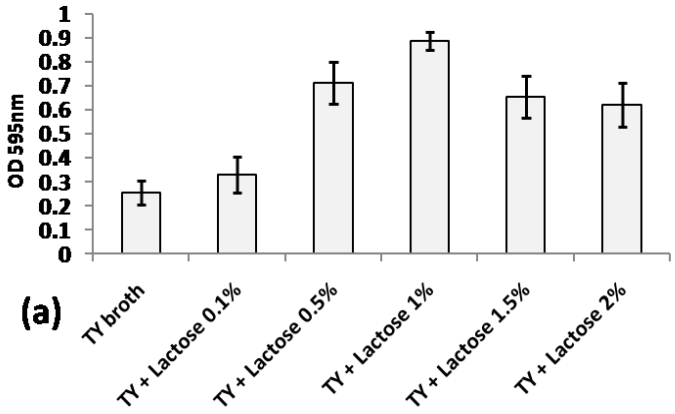
The effluents of dairy industry contain nutrients that assists growth of several bacterial species among which *Bacillus* strains have a dominant role in forming biofilms and pellicle. The biofilms of several *Bacillus* species are supported by the presence of cell surface TasA amyloid proteins, that results in development of rigid biofilms. In case of *B. subtilis* the TasA aids in formation of strong floating pellicle at air-liquid interface. The nutrients of dairy effluents such as lactose have been demonstrated to upregulate production of TasA amyloid, resulting in cell aggregation and formation of thick biofilms (Ostrow *et al.,* 2019). Similarly, metals ions such as manganese and carbon sources such as glycerol have a significant role in promoting biofilm and pellicle formation. Both glycerol and manganese stimulate KinD histidine kinase that inturn causes expression of EPS, Spo0A and TasA amyloid genes. This expression results in formation of dense biofilm containing TasA fibers and EPS of *Bacillus* (Shemesh *et al.,*2013).

In the present study, when the effect of lactose was tested on the biofilm formation by *B. cereus* CR4 it was observed that the biofilm formation increases by three times in presence

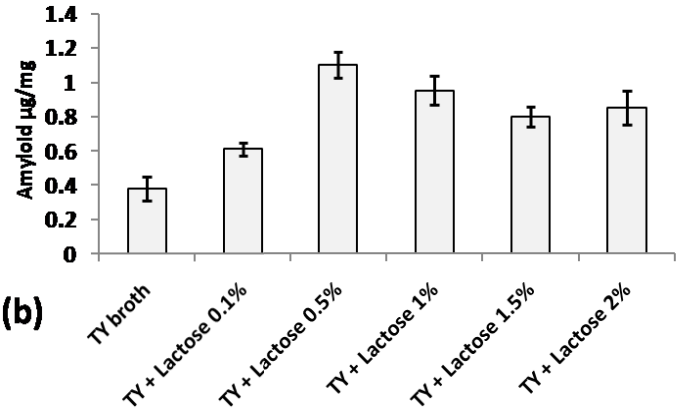
of 0.5% lactose. While higher concentration of lactose from 1% to 2% showed no significant difference (Figure. 4.1a). Quantification of cell surface amyloids revealed that, the presence of 0.1% lactose did show an increase in amyloid from 0.38µ/mg to 0.61µ/mg when compared with the control (Figure. 4.1b). However, the same experiment did not show any significant increment in the biofilm. Hence it can be concluded that presence of trace amount of lactose (0.1%) is capable of increasing cell surface amyloids but not biofilm. However, on increasing the concentration of lactose to 0.5% and above there was substantial increase in both cell surface amyloids as well as increase in the biofilm. The biofilm formation in presence of lactose was correlated with the cell surface amyloids at concentrations higher than 0.5%. The role of lactose in promoting increase in cell surface amyloids and biofilm can be understood by the fact that lactose has been reported to upregulate tasA and eps operons. This upregulation is dependent on autoinducer AI2. Dairy sugars like lactose induces quorum sensing in *B. subtilis* by production of AI2 that in turn upregulates tasA and eps genes resulting in formation of obnoxious biofilms (Duanis-Assaf *et al.,* 2016).

When the effect of manganese ions was checked, the manganese concentration upto 40mM did not show significant increase in the amyloid formation as well as biofilm formation. On increasing the concentration of manganese upto 60mM and above it, demonstrated increase in the biofilm formation and cell surface amyloids by two-fold (Figure. 4.1 c, d). On the other hand the presence of glycerol was insignificant in increasing the biofilm formation and cell surface amyloids (Figure. 4.1 e, f).

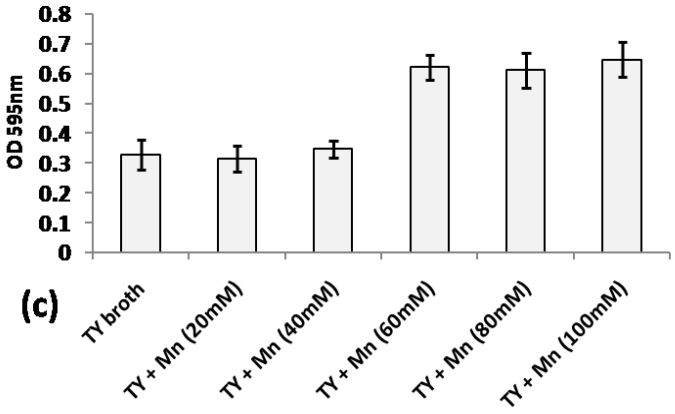
On the basis of above results it can be concluded that dairy sugar lactose played a significant role in increasing the flocculation activity and cell surface amyloids.


OD 595nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TY broth
TY + Lactose 0.1%
TY + Lactose 0.5%
TY + Lactose 1%
TY + Lactose 1.5%
TY + Lactose 2%

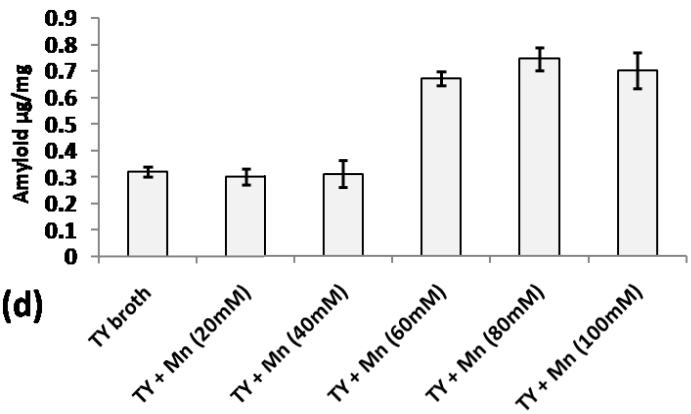
(a)


Amylold µg/mg
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TY broth
TY + Lactose 0.1%
TY + Lactose 0.5%
TY + Lactose 1%
TY + Lactose 1.5%
TY + Lactose 2%

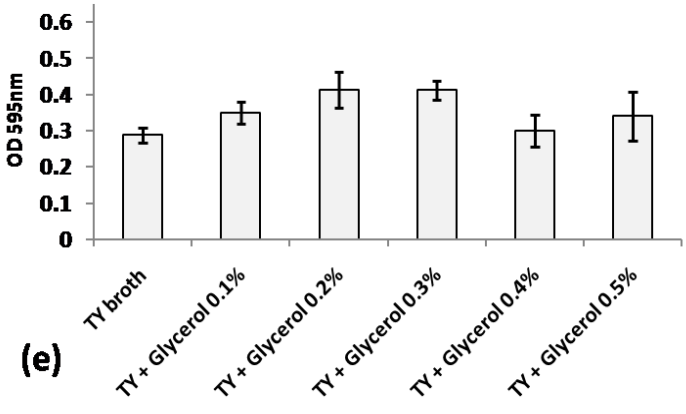
(b)


OD 595nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TY broth
TY + Mn (20mM)
TY + Mn (40mM)
TY + Mn (60mM)
TY + Mn (80mM)
TY + Mn (100mM)

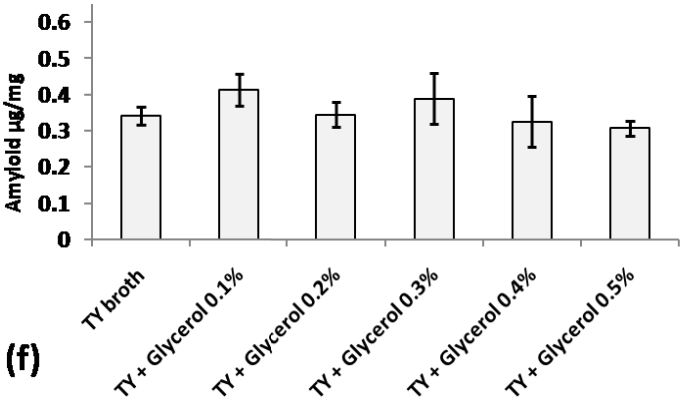
(c)


Amylold µg/mg
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TY broth
TY + Mn (20mM)
TY + Mn (40mM)
TY + Mn (60mM)
TY + Mn (80mM)
TY + Mn (100mM)

(d)


OD 595nm
0.6
0.5
0.4
0.3
0.2
0.1
0
TY broth
TY + Glycerol 0.1%
TY + Glycerol 0.2%
TY + Glycerol 0.3%
TY + Glycerol 0.4%
TY + Glycerol 0.5%

(e)


Amylold µg/mg
0.6
0.5
0.4
0.3
0.2
0.1
0
TY broth
TY + Glycerol 0.1%
TY + Glycerol 0.2%
TY + Glycerol 0.3%
TY + Glycerol 0.4%
TY + Glycerol 0.5%

(f)

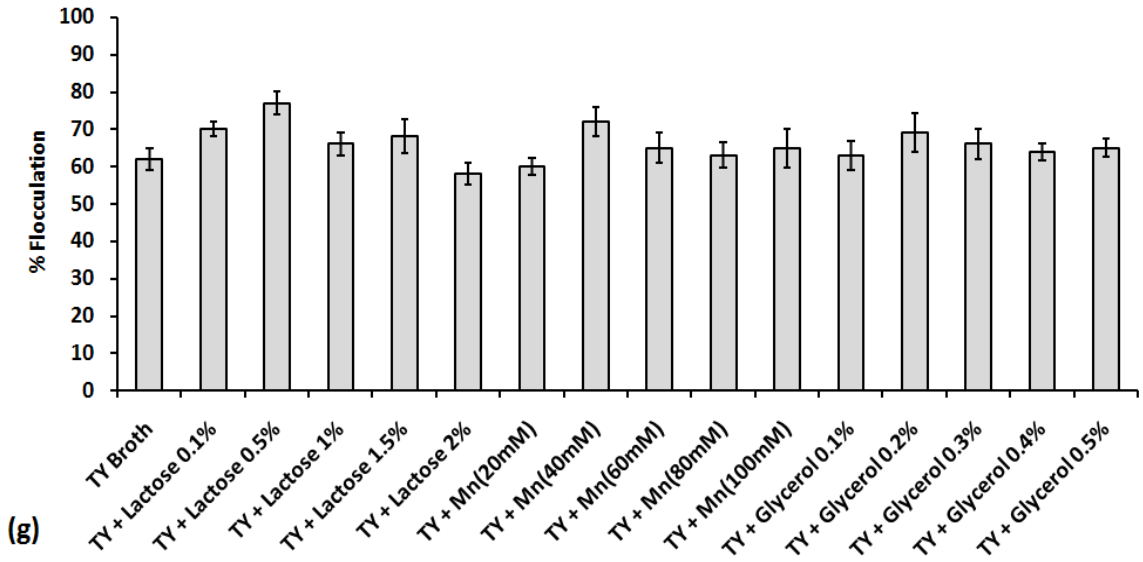


Figure 4. 1 Effect of lactose, manganese and glycerol on biofilm formation and amyloid production by *B.cereus* CR4. a) Effect of lactose on biofilm formation. b) Effect of lactose on amyloid production. c) Effect of manganese on biofilm formation. d) Effect of manganese on amyloid production. e) Effect of glycerol on biofilm formation. f) Effect of glycerol on amyloid production g) Effect of nutrients on % flocculation

This observation can have significance from the viewpoint of the industrial production of the Bioflocculant CR4. Particularly so since the results discussed here have correlated with the study of nutrients required for significant increase in flocculation activity, by Plackett Burman analysis and response surface analysis in chapter 3 where lactose was found to be significant for the bioflocculant production. biofilm formation by 1.5-fold as compared to the control, while at other concentrations taken the results were insignificant. The amyloid production by the cells treated with 0.05% SDS was maximum (Figure. 4.2c), whereas the biofilm formation was insignificant when compared with the control. This difference can be explained by the fact that SDS being a surfactant may act as biofilm inhibitor at higher concentrations.

When the effect of SDS stress was tested on amyloid and bioflocculant production it was observed that the presence of 0.05% SDS produced 76.2% flocculation activity with minimal cell density of 0.2 OD (600nm). At the same time the maximum amyloid production of 3.1 µg/mg of biomass was observed (Figure. 4.2b). The % flocculation remained constant at about 76 % with cell density 0.4 OD and it gradually decreased upto 60.4% at increased cell density of 1.0 OD (Figure. 4.2a). Comparatively the control experiment without SDS showed a gradual increase in flocculation from 43.1% to 80.6% at cell density of 0.8OD. Figure. 4.2a further demonstrates that with an increase in SDS concentration from 0.005% to 0.5% there was gradual increase in the flocculation activity by 20% at cell densities from 0.2 to 0.6. This decrease is due to the fact that phenomenon of flocculation occurs by electrostatic patching or bridging mechanism and excessive addition of bioflocculant saturates the suspended particles with bioflocculant cells with uniform electric charge that results in repulsion and gradual decrease in flocculation activity.

4.2.2 Effect of ethanol stress

Studies have demonstrated that there is overexpression of curli amyloid genes that results in strong biofilms due to influence of ethanol on *E. coli* (Lim *et al*., 2012). Studies on effect of ethanol on amyloid and bioflocculant production demonstrated that 0.5% ethanol showed maximum flocculation activity of 84% with cell density of 0.4OD (Figure. 4.3a). The amount of amyloid produced per mg of biomass was 4.9 µg (Figure. 4.3b). Further increase in cell density upto 1.0 OD decreased the % flocculation by 20.5%. In comparison the control showed maximum flocculation activity of 83.2% at cell density 0.8 OD which was double as compared to the cells treated with 0.5% ethanol and cell density 0.4 OD (Figure. 4.3a). Further analysis of results showed that an increase in cell density from 0.2 to 0.4OD resulted in increase in flocculation activity. The % flocculation gradually showed a decrease with an increase in cell density above 0.6 OD which was expected due to excessive presence of cells causing inhibition in flocculation. Treatment with 1.5% ethanol showed minimum flocculation activity of 40.8% and 57.9% at respective cell densities of 0.2 and 1 OD. Overall decrease in flocculation in presence of 1.5% ethanol might be due to the inhibitory effect of

ethanol on the cells. In case of ethanol the trend between biofilm formation and amyloid production was similar. Treatment with 0.5% ethanol shows amyloid formation of 4.3µg/mg biomass and maximum biofilm formation. As compared to the control the biofilm formation was accounted to be two-fold higher (Figure. 4.3c).

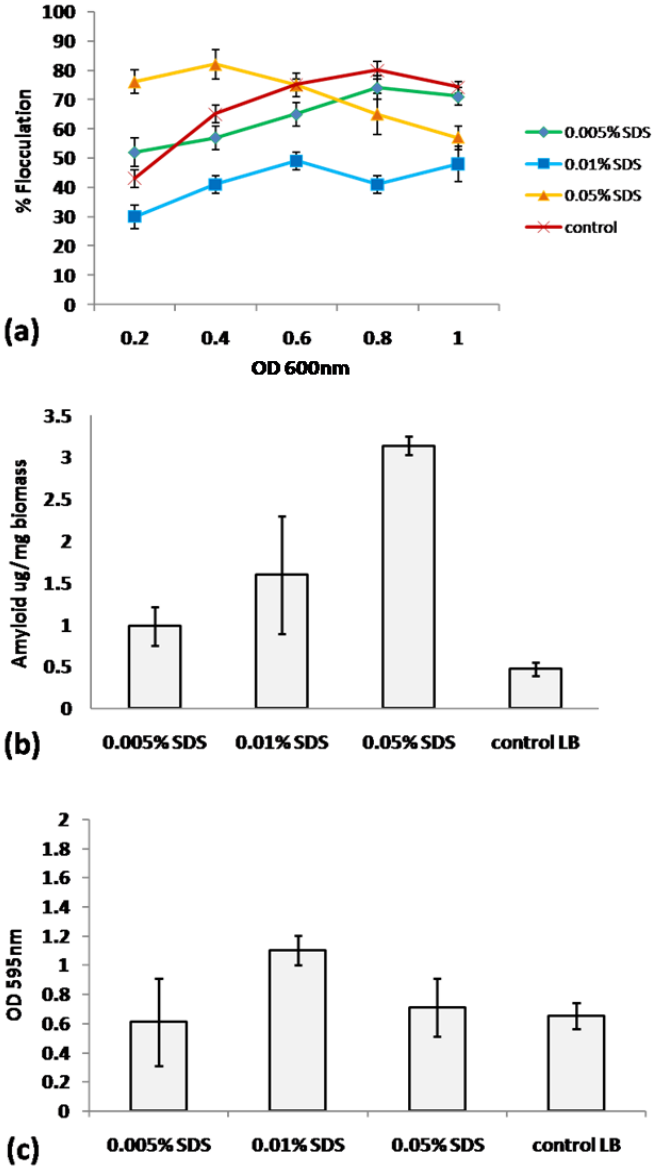


Figure 4. 2 Effect of SDS stress on a) bioflocculation activity b) amyloid production and c) biofilm formation of *B.cereus* CR4

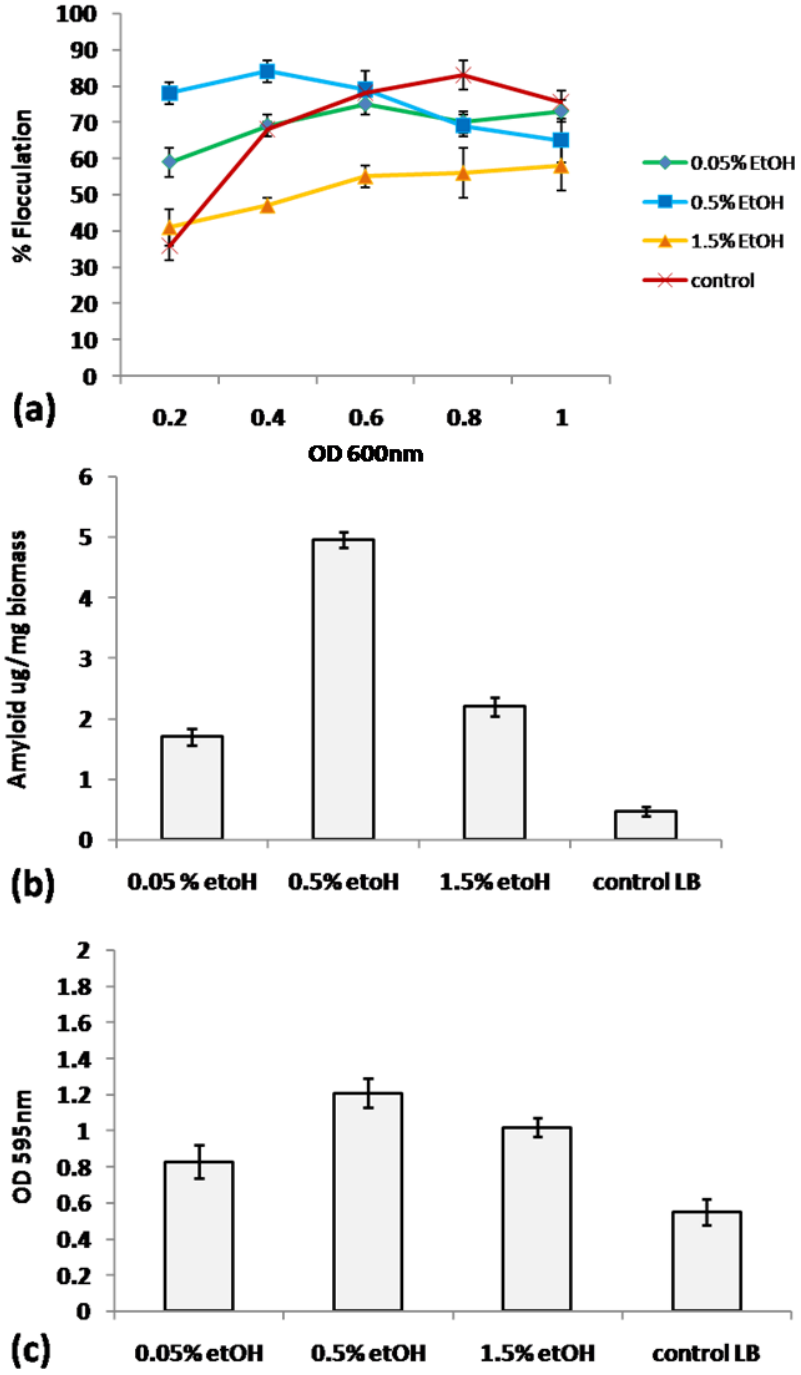


Figure 4. 3 Effect of ethanol stress on a) bioflocculation activity b) amyloid production and c) biofilm formationby *B. cereus* CR4

4.2.3 Effect of DMSO stress

Like ethanol, chemical stress agents such as DMSO have also demonstrated upregulation of curli genes leading to development of biofilms (Lim *et al.,* 2012). The presence of DMSO as a stress agent showed a very significant increase in % flocculation activity. The flocculation activity demonstrated by 0.2 OD cell density was 84.5 % which was several folds high as compared to the control with similar cell density (Figure. 4.4a). Here, the amount of amyloid produced was 4.3µg/mg biomass which was maximum as compared to other stress agents (Figure. 4.4b). The % flocculation activity associated with the control cells reached 84.7% at cell density of 0.8OD which was 4 times more as compared to the cells treated with 1.5% DMSO during growth. The presence of DMSO at lower concentration 0.05% and 0.5% also demonstrated gradual linear increase in the flocculation activity. Presence of 1.5% DMSO was superior as compared with other treatments in giving maximum flocculation activity with minimal cell density and maximum amyloid production. When the biofilm studies were performed with DMSO, it was observed that with an increase in DMSO stress from 0.05% to 1.5% there was a linear increase in the biofilm formation (Figure. 4.4c).

Solvents like ethanol and DMSO can be lethal for the bacteria cells at higher concentration. However, at low concentration, these solvents often act as stress agents. Both ethanol and DMSO have demonstrated typical agglutination phenotype where the cells showed self aggregation and settling after treatment during its growth. Studies have demonstrated that *E. coli* strains with deletion of amyloid genes have failed to show cell aggregation and biofilm formation (Lim *et al.,* 2012). The curli amyloid produced by *E.coli* is one of the well studied functional bacterial amyloid since decades. Curli amyloid have a unique role in biofilm formation on biotic and abiotic surfaces of *E. coli* that cause food borne outbreaks and pathogenesis. Ethanol and other stress agents like DMSO have been reported to upregulate biofilm formation, cell aggregation and pathogenesis in *E. coli*. This increase in virulence and biofilm formation have been directly linked with overexpression of curli amyloid produced by *E. coli*. DMSO at concentration of 4% v/v and ethanol at concentration 2% v/v

have been found by Lim *et al*., (2012) to upregulate curli amyloid expression by 13-fold and 8-fold respectively.

In comparison to *E. coli* 0.5% ethanol and 1.5% DMSO treatment on *B. cereus* CR4 demonstrated 2.5-fold and 2-fold increase in production of cell surface amyloid respectively. The significant difference between *E. coli* and *B. cereus* CR4 can be due to the fact that curli amyloid fibers in *E.coli* tend to remain on cell surface anchored by accessory curliB proteinwhile in case of *B.cereus* the absence of cell surface anchoring protein TapA results in release of cell surface amyloid fibers in the extracellular environment.

4.2.4 Effect of NaCl stress

NaCl stress was studied since it would impart osmotic stress which would give interesting results. Also, it has not been studied hitherto. In case of NaCl, maximum % flocculation was seen with 0.4 OD cell density pretreated with 3% NaCl during growth (Figure. 4.5a). This was correlated with maximum amyloid production that accounted for 2.3µg/mg of biomass (Figure. 4.5b). The increase in NaCl concentration from 3% to 4% showed a decline in flocculation activity as well as growth. Presence of 2% NaCl showed a peak of flocculation activity at 0.6 OD cell density that accounted for 66.2% activity. In case of NaCl the biofilm formation in 2% NaCl and 3% NaCl was twice as compared to the control. The control and 4% NaCl showed similar biofilm formation with no significant difference (Figure. 4.5c). The amyloid and biofilm formation did not show strong corelation. This variation might be due to the fact that presence of metal ions has a significant impact on the stability and formation of biofilm. Ca^{+2} and Mg^{+2} ions have been demonstrated to contribute increase in biofilm formation and its stability in several organisms (Srinandan *et al*., 2010, Song and Leff, 2006). Thus, presence of Na^{+} ions might have its own effect in the biofilm formation and because of this effect the role of surface amyloids in biofilm formation may not be obvious.

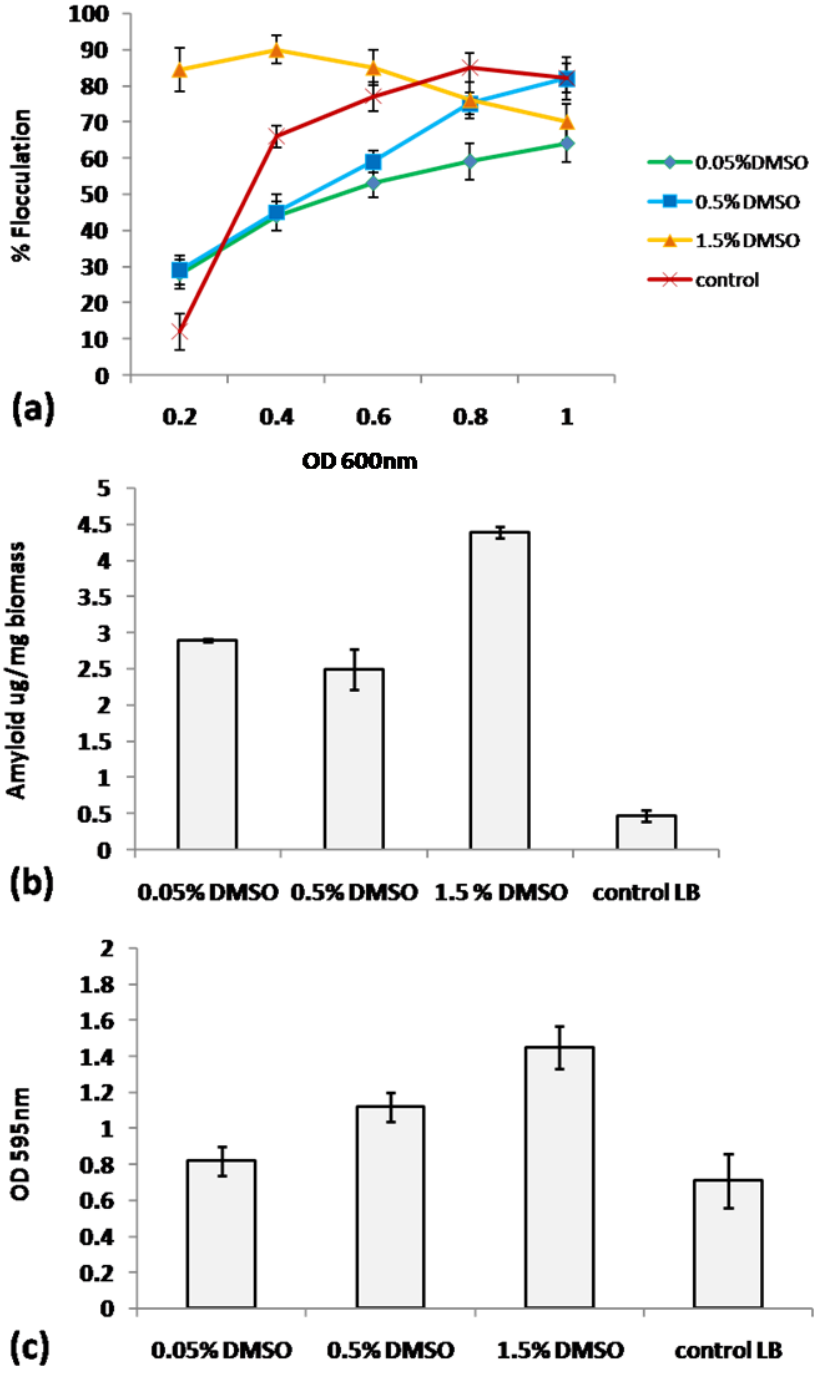


Figure 4. 4 Effect of DMSO stress on a) bioflocculation activity b) amyloid production and c) biofilm formation by *B.cereus* CR4

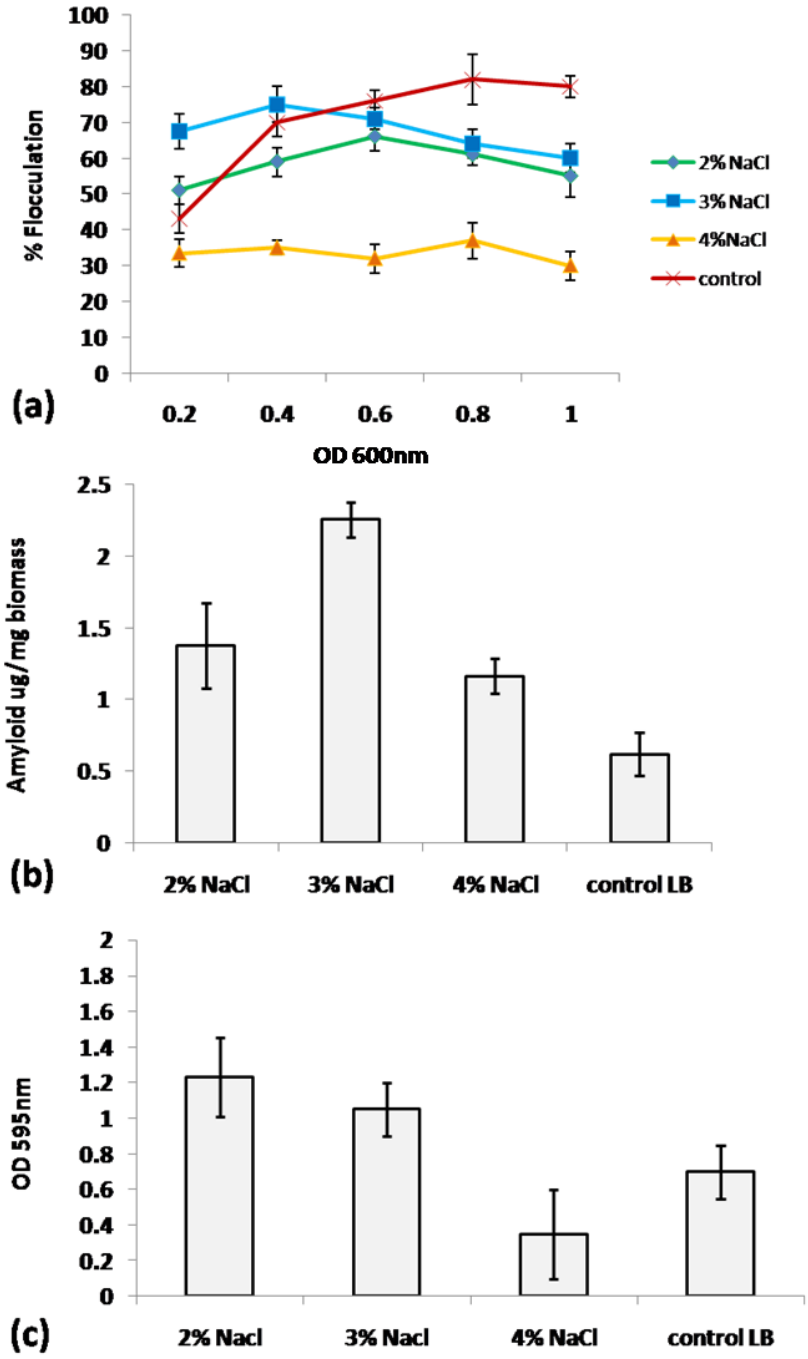


Figure 4. 5 Effect of NaCl stress on a) bioflocculation activity b) amyloid production and c) biofilm formation by *B.cereus* CR4

Studies on biofilm form and cell surface hydrophobicity of different *Bacillus* spp.

4.2.5 Study of *Bacillus* spp. biofilms

Recent studies on biofilms have demonstrated that the bacterial cells have tendency to aggregate at the air-liquid interface and form a thick floating structure termed as the pellicle. Studies of pellicle with transmission and scanning electron microscope have demonstrated that the presence of amyloids plays an important role in stability and structure of pellicle. The amyloids present on the cell surface are known to provide hydrophobic environment for the cells which ultimately allows cells to accomplish specific function (Gebbink *et al*., 2005). These functions can include cell aggregation, attachment on hydrophobic surfaces, reduction in surface tension of water and escape of cells from the water (Gebbink *et al*., 2005). In a similar way the pellicle forming bacterial species have demonstrated the role of cell surface hydrophobicity in pellicle formation and its stability.

In contrast there are bacterial species that shows aggregation on inert solid surfaces and form biofilms that remain submerged in the liquid environment. The cell surface hydrophobicity is a major factor that governs the formation of pellicle or submerged biofilms. High cell surface hydrophobicity causes cells to move away from the aqueous environment and float on the air liquid interface. The aggregation of cells on the air-liquid interface ultimately results in pellicle. In recent years the amyloids of most of the bacteria and fungi have demonstrated to possess a hydrophobic nature. In case of *Bacillus subtilis* the hydrophobic amyloid TasA has a major role in cell aggregation and pellicle formation at the air-liquid interface. However, there is sparse information regarding the pellicle formation, type of biofilm formation (pellicle vs submerged) and role of amyloid in various other *Bacillus* species. To study this correlation different strains of *Bacillus* species were inoculated in Luria broth and incubated at 37ºC for 24hrs under stationary conditions. *Bacillus subtilis* was used as a control and it showed a dense thick pellicle formed at the air-liquid interface (Figure. 4.6a). Whereas *B. cereus* CR4 demonstrated the presence of submerged biofilm (Figure. 4.6b).

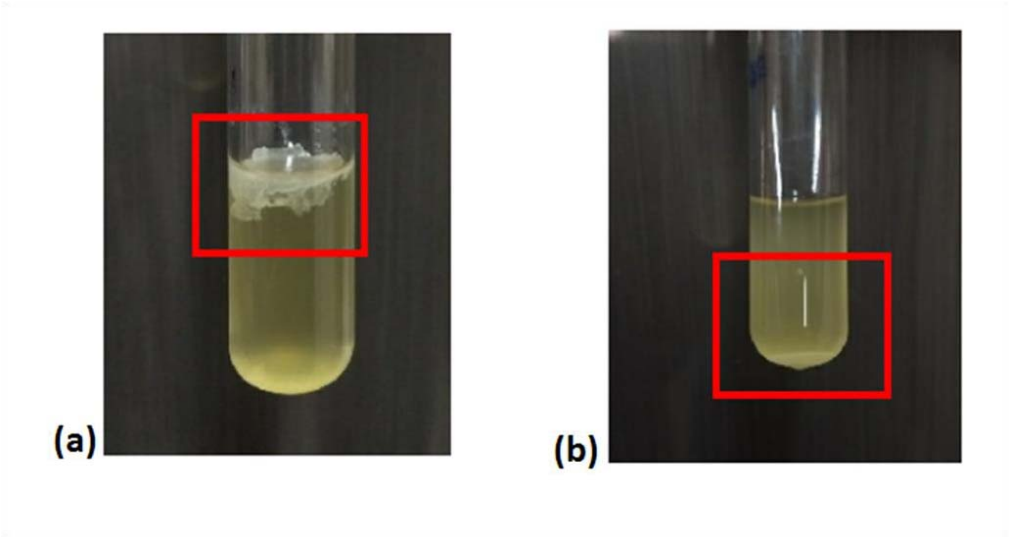


Figure 4. 6 Representative photographs of biofilms formed by different *Bacillus* species. a) Surface pellicle formation at air-liquid interface. b) Submerged biofilm formation at bottom of the tube. (a) *B. subtilis* (b) *B. cereus* CR4

The formation of pellicle or submerged biofilm by various other *Bacillus sp* is presented in the Table 4.1. It was observed that among the eighteen *Bacillus* species selected for study there were two distinct forms. In the first category ten species *B. oleronius, B. clausii, B. badius, B. atrophaeus* 78, *B. amyloliquefaciens* 98, *B. licheniformis* TT42, *B. licheniformis* K125 *B. mojavensis* JF2, *B. mojavensis* TT33, *B. subtilis* MW10, *B. subtilis* 168 demonstrated formation of floating surface pellicle form while in the second the remaining eight species *B.sphaericus, B. thuringiensis* HD73, *B. thuringiensis* HD1, *B.cereus* CR4, *B.megaterium, B. firmus, B.cereus* 14579T demonstrated formation of submerged biofilm form. Inorder to identify the presence and absence of tasA gene in different *Bacillus* species the tasA gene sequence of *B. subtilis* available on NCBI was used to carry out BLAST analysis with different *Bacillus* species. The presence and absence of tasA gene found out by bioinformatic analysis in different *Bacillus* species have been demonstrated in table 4.1

Table 4. 1 Biofilm form and distribution of tasA in various *Bacillus* sp

Species	Biofilm form	tasA
B. oleronius	Pellicle	Absent
B. clausii	Pellicle	Absent
B. badius	Pellicle	Absent
***B. atrophaeus* 78**	Pellicle	Present
***B. amyloliquefaciens* 98**	Pellicle	Present
B. sphaericus	Submerged	Absent
***B. licheniformis* TT42**	Pellicle	Absent
***B. licheniformis* K125**	Pellicle	Absent
***B. mojavensis* JF2**	Pellicle	Present
***B. thuringiensis* HD73**	Submerged	Absent
***B. thuringiensis* HD1**	Submerged	Absent
***B. mojavensis* TT33**	Pellicle	Present
***B. cereus* CR4**	Submerged	Present
B. megaterium	Submerged	Absent
B .firmus	Submerged	Absent
***B. cereus* 14579T**	Submerged	Present
***B. subtilis* MW10**	Pellicle	Present
***B. subtilis* 168**	Pellicle	Present

4.2.6 MATH Assay for estimation of cell surface hydrophobicity

Previous reports (Arnaouteli *et al.,* 2016) on pellicle formation by *Bacillus subtilis* suggests that cell surface hydrophobicity due to the presence of amyloid fibers has a major contribution in the development of such floating structures. Hence based on this rationale the cell surface hydrophobicity of each strain measured by MATH assay revealed that the *Bacillus* spp. could be categorised into two distinct groups. Sevent strains in first group showed cell surface hydrophobicity between 30 % and 40 % while eleven strains in second

group showed 50% and above hydrophobicity (Figure. 4.7). Furthermore, when the results of the MATH assay were matched with the groups of species forming pellicle and submerged biofilm there was a strong correlation between the cell surface hydrophobicity and ability of cells to form pellicle (Figure. 4.8). The cultures demonstrating average % hydrophobicity of 28% showed submerged biofilm formation while those demonstrating the average % hydrophobicity of 57.6% showed pellicle formation (Figure. 4.8a).

The pellicle formation in *Bacillus* spp. is strongly dependent on the amyloid formation by the aggregated cells. All the *Bacillus* spp. under study showed strong correlation between the pellicle formation and presence of the tasA gene except *B. cereus* that showed submerged biofilm inspite of having tasA gene. This observation was supported by the fact that the amyloid fibers in *B. cereus* appears on the cell surface during exponential phase and gets released into the extracellular environment during stationary phase. The release of TasA fibrils in the extracellular environment is favored by the absence of cell wall anchoring protein TapA. In *Bacillus subtilis* the TapA protein present on the cell surface keeps TasA amyloid fibrils anchored on the cell surface. This allows the cells of *Bacillus subtilis* to form strong intracellular aggregates that floats at air-liquid interface due to its hydrophobic nature. In contrast *B. cereus* possess loosely bound amyloid fibers that are released free during the stationary phase. Hence the cells are less hydropohobic and forms submerged biofilm instead of surface pellicle.

The further analysis of the MATH assay resulted in segregating the *Bacillus* species into two groups. The group forming TasA and the one devoid of TasA (Figure. 4.10b). The box plot demonstrates that the median for % hydrophobicity in presence of TasA was 55.7% while the median in absence of TasA was 29.5%. The median value of 55.7% was close to the median value of the strains that exhibited formation of strong pellicle (57.3%). While the median value of TasA absence group 29.5% was close to median hydrophobicity value of strains that formed submerged biofilm (28%). Hence presence of TasA fibers showed significant contribution in providing hydrophobicity to the cells which in turn governs the formation of sturdy floating pellicle.

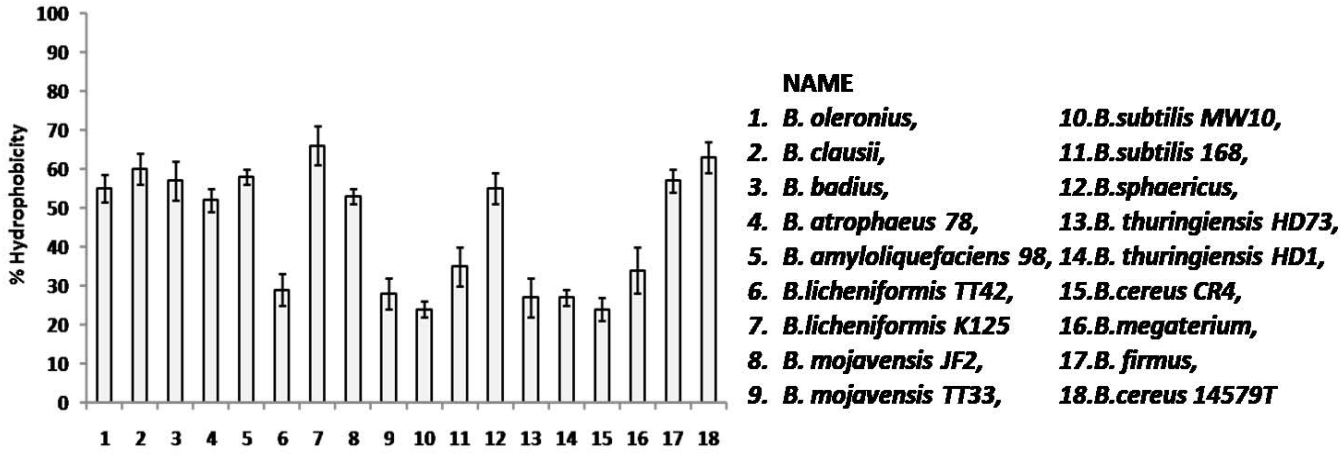


Figure 4. 7 MATH assay for quantification of % Hydrophobicity of different *Bacillus* spp.

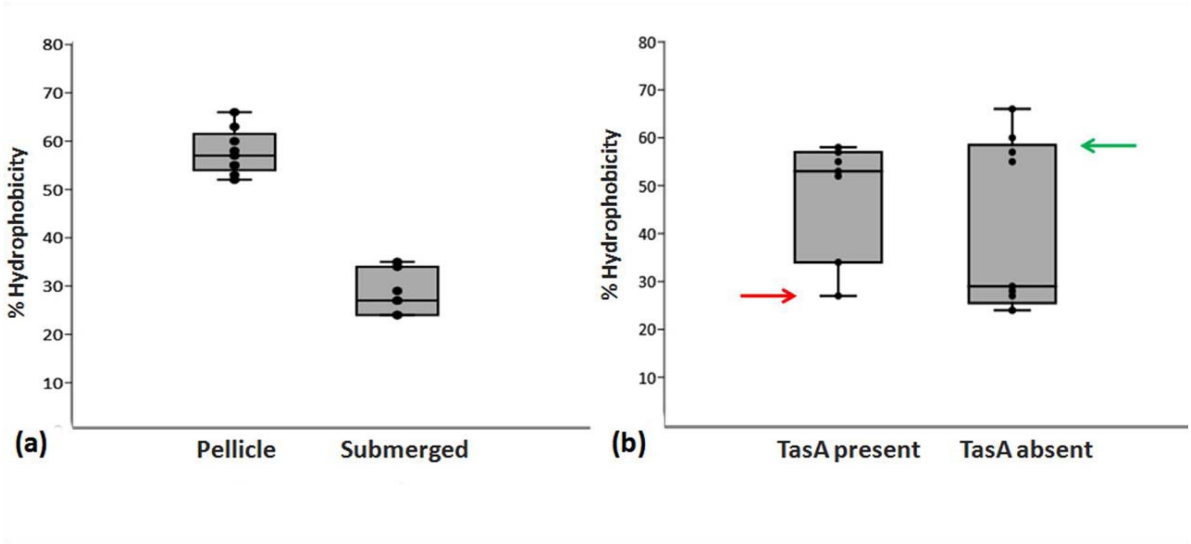


Figure 4. 8 Analysis of MATH assay results on the basis of a) biofilm form and b) tasA presence in different *Bacillus* species

However few species including *B. cereus* showed deviation in this behavior. In spite of having presence of TasA the % hydropohobicity of *B. cereus* was at the extreme low end of the range (Figure. 4.8b red arrow). A few strains like demonstrated in the box plot showed higher % hydrophobicity (Figure. 4.8b green arrow) as compared to the median of the same group. This can be due to the fact that besides amyloid fibers there are other factors that also contributes to the cell surface hydrophobicity of the cells.

Influence of D-amino acid on biofilm formation of *Bacillus* spp.

4.2.7 Effect of D amino acids on biofilm formation

The process of biofilm formation by the bacterial cells is a highly dynamic process. The formation of biofilm commences by binding of bacterial cells on the surface. The amyloid protein present on the cell surface plays a major role in such binding. Once the cells bind on the substratum the cell surface amyloids also aid in aggregation of other cells promoting growth and maturation of biofilm. With time the cells in the biofilm matrix experiences many changes. The sequential events that occur as the bacterial biofilm ages, includes limiting nutrients availability to the biofilm cells, accumulation of waste products, as well as death of cells. Hence it is advantageous for the biofilm-associated bacteria to return to the planktonic state. These unpropitious conditions induce many bacterial cells to produce D-amino acids. The D-amino acids in turn causes detachment of amyloid fibrils from the cells causing destabilization and disruption of biofilm.

The D amino acids have demonstrated significant disintegration of amyloid based bacterial biofilms in *Bacillus subtilis.* The protein complex which is strongly affected by the presence of the D amino acids is the TasA-TapA complex. The presence of D amino acids disturbs the TasA-TapA complex eventually leading to biofilm disruption. Based on this rationale, the effect of D-amino acids (DL methionine, DL tyrosine, DL leucine, DL tryptophan) at concentration of 10nM was studied on biofilm formation by different *Bacillus* species using 96 well microtitre plate. The mixture of same amino acid with L isomerc form was used as

an amino acid control. It was observed that *B. amyloliquefaciens, B. mojavensis* and *B. licheniformis* failed to show significant reduction in biofilm formation while other species demonstrated, significant reduction. The decrease in biofilm was also found significant in presence of L amino acid that was used as a control (Figure. 4.9, 4.10). This decrease might be due to the presence of racemases present in the cell, that converts the L form into the D one. A similar result was noted when DL tyrosine was tested for its ability to affect biofilm formation. *B. amyloliquefaciens,* and *Bacillus licheniformis* failed to show significant decrease in the biofilm formation.

Among *Bacillus subtilis* and *B. cereus* the presence of D amino acids caused significant prevention of *Bacillus subtilis* biofilm whereas in *B. cereus* CR4, the biofilm breakdown was abrogated. The possible reason for abrogated biofilm disruption might be due to the absence of TapA which is an anchoring protein for TasA amyloid fibrils. Further experiments were done to check, whether the D-amino acids affected disassembly of their biofilms, in another *Bacillus* sp. Besides *B. cereus* and *B. subtilis* D amino acids detached biofilms of most of the *Bacillus* sp significantly. This observation could serve as removal of notorious biofilms associated with dairy industries and biomedical fields. Table 4.2 depicts the summary of the concentration of various factors studied in this chapter that significantly influenced the amyloid production, biofilm formation and flocculation. Nutrients like lactose and stress agents such as DMSO can be used to enhance the production of the amyloid bioflocculant, while the use of DL amino acids can be exploited for its anti biolfilm activity.

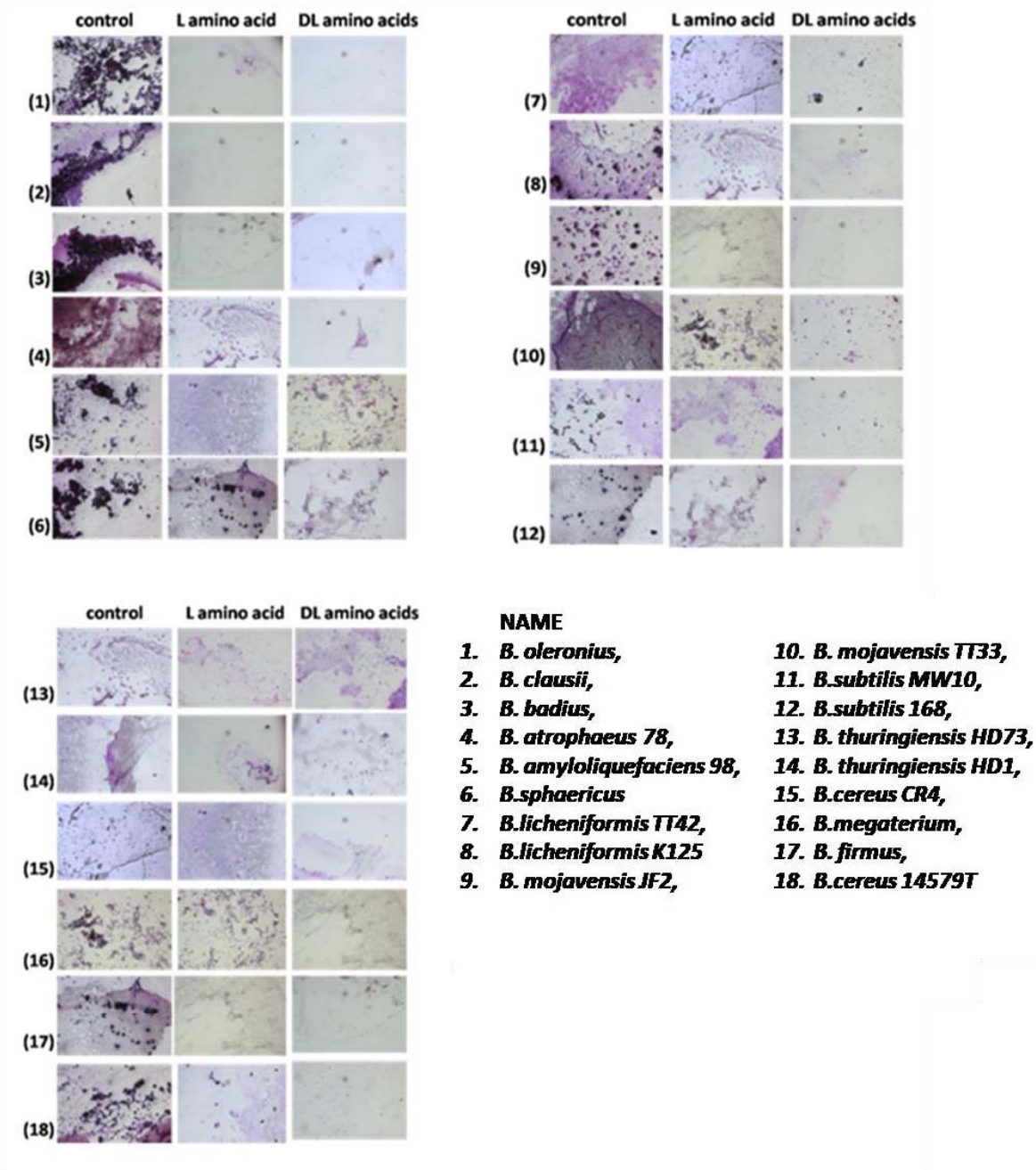


Figure 4. 9 Bright filed microscopy for analysing the effect of D-amino acids on biofilm formation by various *Bacillus* species.

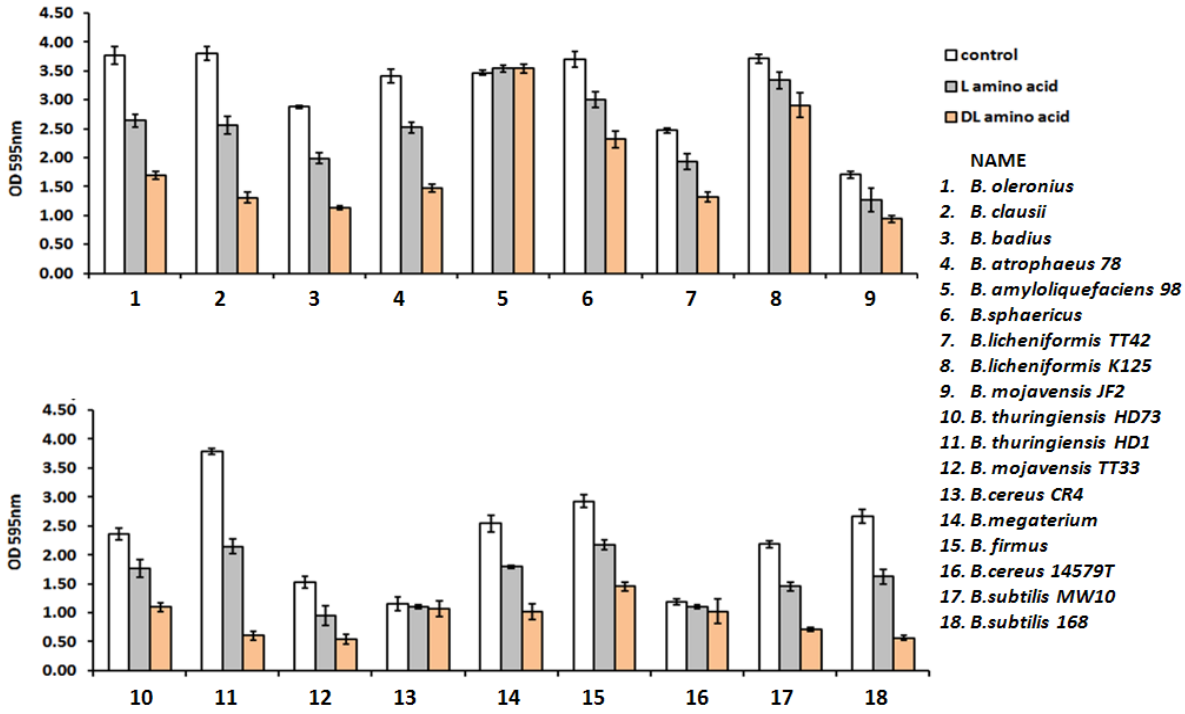


Figure 4. 10 Effect of D-amino acids on biofilm formation by various *Bacillus* species.

Table 4. 2 Summary of factors and their significant concentrations that enhanced amyloid production, biofilm formation and flocculation.

Stress / Nutrients	Amyloid (µg/mg)	Biofilm (OD 595nm)	% Flocculation
TY Broth (control)	0.38	0.26	62
0.5% Lactose	1.13	0.73	78
60mM $MnCl_2$	0.71	0.63	67
0.05% SDS	1.7	1.15	82
0.5% Ethanol	5.1	1.2	84
1.5% DMSO	4.5	1.45	89
2% NaCl	1.45	1.2	66

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