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Cepacian degrading *Cytobacillus* sp. strain Dbc1 with anti-biofilm activity potentiating antibiotic efficacy against *Burkholderia cepacia* biofilm development

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Abstract

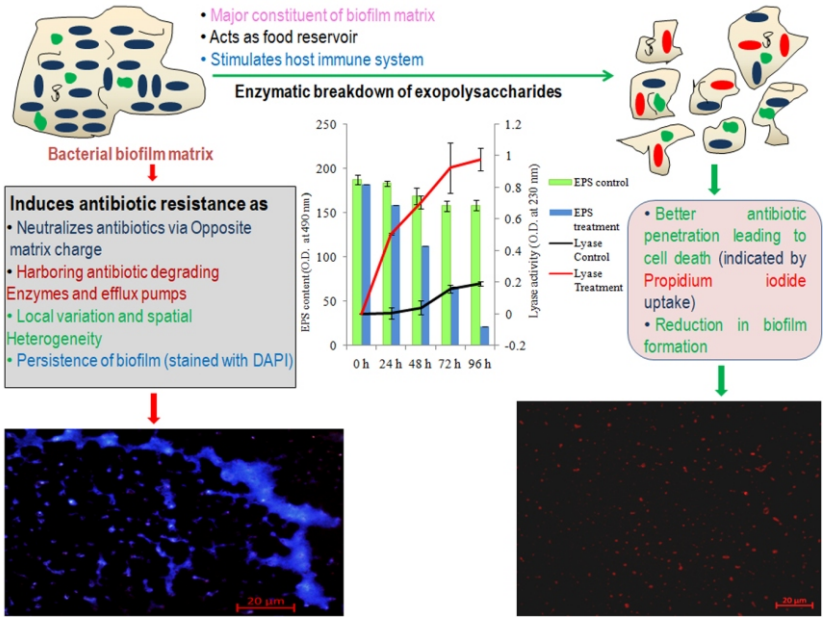
Aim: Inhibition of biofilm formation in *Burkholderia cepacia* through enzymatic degradation of predominant exopolysaccharide (cepacian) of *B. cepacia.*

Methodology: Cepacian was extracted from *B. cepacia* followed by isolation and identification of potent cepacian utilizing bacteria based on planktonic growth using cepacian as sole carbon source. Effective lyase activity (responsible for cepacian breakdown) and anti-biofilm activity of potent isolate against *B. cepacia* was determined. Effect of biofilm disintegration on antibiotic penetration into biofilm was detected. Lastly, inhibition of biofilm formation by crude lyase preparation of the isolate inside an external medical device was detected.

Results: *Cytobacillus* sp. strain Dbc1 was the most potent cepacian degrading bacteria which showed significant lyase activity and reduced total biomass of both newly formed and pre-formed biofilm of *B. cepacia.* Cepacian degradation potentiated chloramphenicol penetration within pre-formed biofilm leading to cell mortality in *B. cepacia*. *Cytobacillus* sp. strain Dbc1significantly reduced total biomass of established biofilm inside nasal oxygen catheter.

Interpretation: It can be concluded that *Cytobacillus* sp. strain Dbc1 significantly controlled mature *B. cepacia* biofilm through potent cepacian degrading and anti-biofilm activity along with better antibiotic targeting into established biofilm. Strain Dbc1 can be used to inhibit medical device- associated biofilms *in vitro.*

Key words: Antibiotic susceptibility, Biofilm inhibition, *Burkholderia cepacia*, Cepacian breakdown, Multi-drug resistance



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Introduction

Exopolysaccharide, being one of the major components of the biofilm matrix, plays a crucial role in bacterial adaptation to different environmental niches (Ferreira *et al.,* 2011). Exopolysaccharides and biofilms of pathogenic bacteria are among the major virulence factors associated with pathogenicity as EPS matrix serves several functions leading to the emergence of Multidrug Resistance (MDR) among them. Matrix charge (Walters *et al.,* 2003), antibiotic degrading enzymes, antibiotic specific efflux pumps within EPS matrix induce drug resistance (Gillis *et al.,* 2005). In most of the pathogenic bacteria, multi-layered biofilms show local variation in terms of diffusion of nutrients, secondary metabolites, oxygen, pH, anti-bacterial agents etc., which on other hand create heterogeneous micro-colonies within biofilms. Local variation also leads to difference in metabolic activity and gene expression among biofilm embedded cells (Jefferson, 2004) resulting in altered responses against antibacterial agents and antibiotics (Kırmusaoğlu, 2016). Thus, biofilm matrices of pathogenic bacteria put the therapeutic treatment in a challenge.

B. cepacia is an opportunistic, nosocomial pathogen which often mediates chronic infection and mainly exists as mature biofilm within Cystic Fibrosis airways (Singh *et al.,* 2000). Therefore, biofilm matrix and exopolysaccharides play an important role in disease persistence. Seven exopolysaccharides have been reported to be produced in the genus *Burkholderia* (Ferreira *et al.,* 2011) although cepacian is the predominant EPS in most of the Bcc strains (Cuzzi *et al.,* 2012). Cepacian consists of repeating units of branched hepta-saccharide including D-Glucose, D-rhamnose, D- mannose, D-galactose and D-glucuronic acid in a ratio of 1:1:1:3:1 and is highly acylated as well (Cerantola *et al.,* 1999; Cescutti *et al.,* 2000). Sub-inhibitory concentration of antimicrobial agents and antibiotics induce biofilm formation in pathogenic bacteria (Baker *et al.,* 2016). Cepacian confers protection against desiccation, metal ion stress (Ferreira *et al.,* 2010) and oxidative stress (Cuzzi *et al.,* 2012).

Cepacian can interfere with the host immune system by inhibiting neutrophil chemotaxis, phagocytosis and reactive oxygen species (ROS) scavenging activity (Ferreira *et al.,* 2011). Further, cepacian acetylation has also been reported to provide toughness and protection against ROS-mediated damages (Whitefield *et al.,* 2015). Thus, EPS protect bacteria in biofilms from several stresses like desiccation, metallic cations, ultraviolet radiation, host immune defenses and antimicrobial agents. Conventional cleaning procedures as well as antibiotic treatments are insufficient to eradicate biofilm infections. In this context, enzymatic breakdown of biofilm matrix polysaccharide can disintegrate the biofilm of bacteria particularly pathogenic bacteria which, in turn, releases inhabitant cells and its constituents making them more prone to antimicrobial attack. Biofilm disintegration can also reduce the occurrence of chronic infections, which are really difficult to eradicate (Fleming and Rumbaugh, 2017). In this study, *Cytobacillus* sp. Dbc1 was isolated from rhizospheric soil, collected from local rice field of Bardhaman, West Bengal that can utilize cepacian the sole carbon source when grown on a minimal medium and can disintegrate B. cepacia biofilm potentiating antibiotic targeting within thick biofilm matrix. Crude lyase enzyme of *Cytobacillus* sp. Dbc1 also inhibited mature biofilm development inside external medical device.

Materials and Methods

Bacterial strains, culture conditions and chemicals used: *Burkholderia cepacia* (MCC 2275) was used as a test organism and regularly cultured at 28°C in R2A medium. Chloramphenicol was purchased from HIMEDIA, (India). Ethyl alcohol 95% was used for extraction of Cepacian exopolysaccharide from *B. cepacia.* MTT (3-[4, 5-dimethylthiazol-2-yl]- 2,5 diphenyltetrazolium bromide), DAPI (4', 6-diamidino-2-phenylindole) and Propidium Iodide were purchased from HIMEDIA (India).

Extraction and characterization of Cepacian exopolysaccharide: *B. cepacia* was grown on Davis Mingolis minimal medium where Carbon:Nitrogen was10:1 for 72 hrs with gentle shaking. Cepacian was extracted following the method of Suryawanshi *et al.,* 2019) with slight modification. Briefly, after incubation *B. cepacia* culture was centrifuged at 8000 rpm for 20 min. Supernatant was mixed with absolute alcohol in 1:3 ratio and kept for 24 hrs at 4°C. Thereafter, the mixture was further centrifuged at 8000 g for 20 min. Finally, the precipitation was allowed to dry at 50°C and stored for further analysis. Total carbohydrate content of cepacian was estimated following the acid hydrolysis method (Dubois *et al.,* 1956) and sugar concentration was determined from the standard curve using D-glucose as standard. Experiments were done in triplicate and mean values were taken.

Isolation of Cepacian degrading bacteria: Soil sample was collected from the rhizosphere of *Oryza sativa* L. in Crop Research and Seed Multiplication Farm, Burdwan University (23.252405 N, 87.843423 E). Soil sample was suspended in sterile distilled water and homogenized for 1 hr in a shaker incubator. Then serial dilution was made up to 10^{-5} and plated over Davis Mingioli minimal agar using 3% cepacian as sole carbon source, incubated for 7 days at 28°C. Pure culture of two isolated bacteria was obtained after four-way streaking and repeated sub-culturing. Gram staining and antibiotic sensitivity profiling of two isolates were done.

Screening of potent isolate: Both isolate Dbc1 and Dbc2 were grown in Davis Mingoli minimal broth medium supplemented with 3% cepacian and incubated at 28ºC for 96 hrs. Planktonic growth was recorded at 600 nm at a regular interval of 6 hrs to determine the growth pattern and cepacian utilization of two isolates. Antibiotic susceptibility of isolate Dbc1 and Dbc2 was performed against a number of standard antibiotic stripes (HIMEDIA, India) on Mueller Hinton agar plates followed by incubation at 28ºC.

Biochemical characterization of potent isolate and optimization of culture condition: For biochemical

characterization of potent isolate, some routine tests like indole test, Methyl Red and Voges-Praskauer (MR-VP) test, catalase test, starch hydrolysis test, nitrate reduction test, citrate utilization test were performed. following standard protocols (Powers and Latt, 1977; Evans and Klesius, 2004; Buxton, 2011). For cardinal temperature detection, isolate Dbc1 was inoculated in the presence of cepacian and incubated at 0ºC, 10ºC, 20ºC, 30ºC, 40ºC , 50ºC and 60 ºC for 72 hrs. For optimum salinity and pH determination, isolate Dbc1 was grown on Davis Mingoli minimal broth using 3% cepacian with different NaCl concentration (0.0M-2.0M) and different pH (pH4-pH11) and incubated at 28ºC for 72 hrs. Bacterial growth was measured at 600 nm at regular interval of 24 hrs using a UV-VIS spectrophotometer (SHIMADZU UV-1900).

Phylogenetic identification of potent isolate: Genomic DNA of potent isolate was extracted by CTAB-NaCl method (Ausubel *et al.,*1994) followed by amplification of 16S rRNA gene of the isolate using universal primers [8F-5' AGAGTTTGATCCTG GCTCAG3' and 1492R 5'TACGGTTACCTTGTTACGACTT3'] (Weisburg *et al.,* 1991). The sequencing was done in Barcode Bioscience, Bangalore, India and raw data was comparatively analyzed by NCBI Blast. Phylogenetic tree was constructed by Neighbour Joining method using Mega7 software. The evolutionary history was determined following Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree obtained from 1000 replicates was considered for detecting evolutionary history of the taxa analyzed (Felsentein, 1985). Branches corresponding to the partitions reproduced in less than 50% bootstrap replicates were rejected. The sequence was deposited to the NCBI database.

Detection of cepacian utilization and enzyme activity in potent isolate: To detect cepacian degradation and lyase activity in the cell free supernatant, potent isolate was grown on modified Davis Mingoli minimal medium in the presence of 3% cepacian previously described. On a regular interval (0 hr, 24 hrs, 48 hrs, 72 hrs and 96 hrs), the available EPS content present in the culture filtrate of isolate Dbc1 was estimated by the acid hydrolysis method described previously. In another experiment, cell free lysate of Dbc1 was diluted six times with sterile distilled water and optical density was recorded at 230 nm (Cescutti et al., 2006). An uninoculated medium was used as a control.

Detection of anti-biofilm activity

Inhibition of biofilm formation: The potential of the potent isolate to inhibit biofilm formation in *B. cepacia* was also determined by crystal violet staining method (O'Toole *et al.,* 1999). In brief, isolate Dbc1 was grown in modified Davis Minimal broth supplemented with 3% cepacian. R2A broth was inoculated with fresh culture of *B. cepacia*, with/without of cell free lysate of isolate Dbc1 and incubated at 28ºC for 72 hrs at static condition. After incubation, the planktonic growth was read at 600 nm, while the attached cells were gently washed twice with sterile physiological saline and stained with 0.1% crystal violet, incubated for 15 min. Thereafter, 30% Acetic acid was used to solubilize the stain and the absorbance was recorded at 550 nm.

Eradication of mature biofilm

Crystal violet staining method: Disintegration of biofilm of *B. cepacia* by the potent isolate was determined by static micro- titer plate assay (O'Toole *et al.,* 1999). *B. cepacia* Biofilm was grown in micro-well plate at 28ºC at static condition for 48 hrs. Thereafter, 200 µl of Dbc1 culture filtrate was added to the pre-formed biofilm, mixed thoroughly and the *B. cepacia* biofilm without any culture filtrate served as control. Then biofilm biomass was estimated by crystal violet staining.

Colony Forming Unit (CFU) method: Viable cell number of *B. cepacia* biofilm was evaluated by CFU counting. Briefly, *B. cepacia* biofilm was allowed to form in micro-well plate following the previously described method. Planktonic cells were discarded. After the incubation period, biofilm entrenched cells attached to the well bottom were washed gently with sterile physiological saline water and suspended by repeated pipetting, diluted six times in phosphate buffered saline. Cell suspension was uniformly spread over R2A agar plates and incubated for 24 hrs at 28°C followed by recording the CFU.

Effect on biofilm-matrix components of *Burkholderia cepacia*: As described previously, isolate Dbc1 was grown in modified Davis Minimal broth in the presence of cepacian. After incubation, cell free culture filtrate of isolate Dbc1 was mixed thoroughly to the pre- formed biofilm and further incubated for another two days. Thereafter, cepacian was extracted from *B. cepacia* biofilm by the method of Maes *et al.* (2019). Briefly after incubation, *B. cepacia* planktonic cells and culture medium were discarded gently and each well was washed with sterile physiological saline twice. The biofilm adhered to the well was scraped gently using a sterile micro-tip and suspended in saline water for the extraction of biofilm-bound EPS. NaCl solution was added to the suspension and adjusted to a final concentration of 1.5M NaCl followed by vigorous vortexing and centrifugation at 5000 g for 10 min. Finally, the supernatant was used for further chemical analysis. Carbohydrate and protein content of EPS were estimated by phenol-sulfuric acid method (Dubois *et al.,* 1956) and Bradford method (Bradford 1976) respectively. Bovine Serum Albumin was used as standard.

Effect on antibiotic penetration within *B. cepacia* biofilm MTT Assay: For this experiment, a modified method was followed (Saggu *et al.,* 2019). At first, *in vitro* sensitivity of *B. cepacia* against a number of conventional antibiotics was tested and Chloramphenicol was selected on the basis of zone of inhibition. Minimum Inhibitory Concentration (MIC) of chloramphenicol was detected against *B. cepacia* by broth micro-dilution method. Then isolate Dbc1 was grown in Davis minimal medium containing cepacian. After incubation, broth culture of isolate Dbc1 was centrifuged at 10,000 g for 5 min. R2A broth medium was inoculated with 200µl of fresh culture of *B. cepacia* in each well and incubated for 48 hrs in static condition at 28ºC. After biofilm

formation, biofilm was treated with either culture filtrate of Dbc1 (200 µl) or chloramphenicol (5×MIC) or combination of chloramphenicol (5×MIC) and culture filtrate of isolate Dbc1 (200 µl) and left for further incubation overnight. Cultures without any treatment served as controls. Next day, the planktonic cells and culture medium were discarded. Wells were washed with physiological saline to eliminate non-adherent cells without disturbing the biofilm. Biofilm embedded cells were suspended in physiological saline water after scraping the well bottom via repeated pipetting to loosen the bacterial aggregates and further subjected to MTT assay to determine cell viability in *B. cepacia* biofilm.

Fluorescence microscopic study: Live/dead staining followed by fluorescent microscopy was also done to observe antibiotic accessibility of chloramphenicol into biofilm. Isolate Dbc1 was grown in 3% of cepacian. Initially, *B. cepacia* biofilm was allowed to grow on sterile cover slips placed inside 12 micro-well plate for 48 hrs. Next, cell free lysate of isolate Dbc1 was added to the micro-well plate containing pre-formed biofilm of *B. cepacia* grown (48 hrs) on the sterile cover slip (Blue Star, India) and incubated at 28ºC overnight. Control set was devoid of any treatment. After incubation, all the cover slips were rinsed twice with physiological saline to remove non-adherent cells. The cover slips were then stained with 1µl of DAPI and propidium iodide for 1 min separately taken from 1mg/ml stock solutions. Excess stains were removed by washing with physiological saline. Microscopic images were recorded with a fluorescent microscope (Zeiss, Axiolab 5).

Inhibition of biofilm formation on medical device *in vitro*: Standard nasal oxygen catheter (2 m long) purchased from a surgical shop was cut into small pieces (4 cm long) aseptically. Each segment was inoculated with R2A medium and overnight grown *B. cepacia* and both ends of each segment were sealed with sterile non-absorbent cotton, incubated at 28ºC for 48 hrs. After incubation, pre-formed biofilm on the inner side of nasal oxygen catheter segment was treated with cell free lysate of isolate Dbc1 (200 µl) and allowed to incubate. Cultures, not subjected to any treatment were set as control. Biofilm disruption was assessed for 3 days at a regular interval of 24 hrs following previously described crystal violet staining method (O'Toole *et al.,* 1999) In brief, culture medium was discarded from inside of each segment, non-adherent cells were removed with the help of physiological saline and 0.1% crystal violet was used to stain the attached biofilm on the inner surface of each nasal oxygen catheter segment. After 30 min, crystal violet was discarded and segments were washed twice. Acetic acid (30%) was further used to solubilize the stain inside. After 15 min absorbance was read at 550 nm.

Statistical analyses: All the experiments were carried out in triplicate. Mean values of triplicate data were taken and statistical errors were calculated using Microsoft Excel.

Results and Discussion

B. cepacia is a potent human pathogen, resistant against a number of antibiotics and can escape host's innate immune responses by forming biofilm. Moreover, cells of *B. cepacia* within biofilm play an advantageous role in establishing prolonged infection due to their enhanced drug resistance by varied unique mechanisms different from their planktonic counterparts. It is reported that the antibiotic resistance increases 10-1000 times in

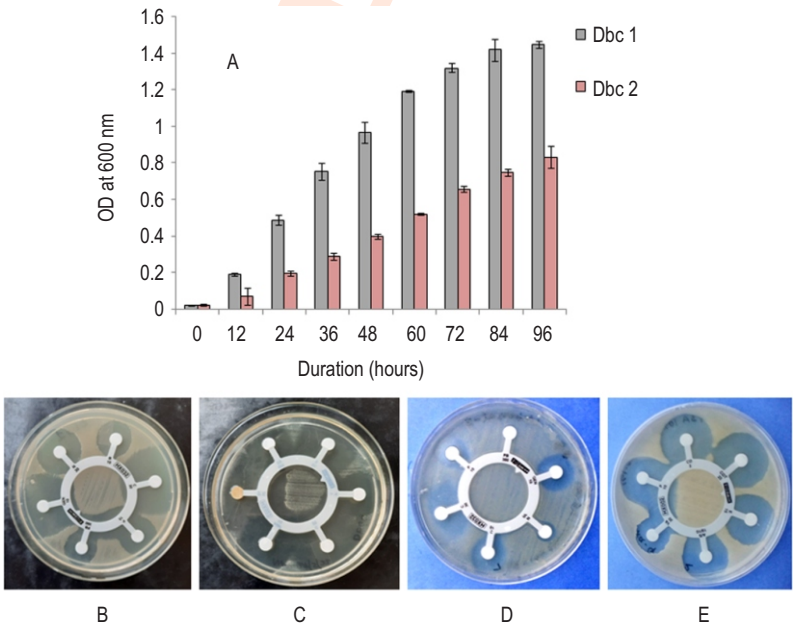

Fig. 1: Planktonic growth of Isolate Dbc1 and Dbc2 over time using cepacian as sole carbon source.

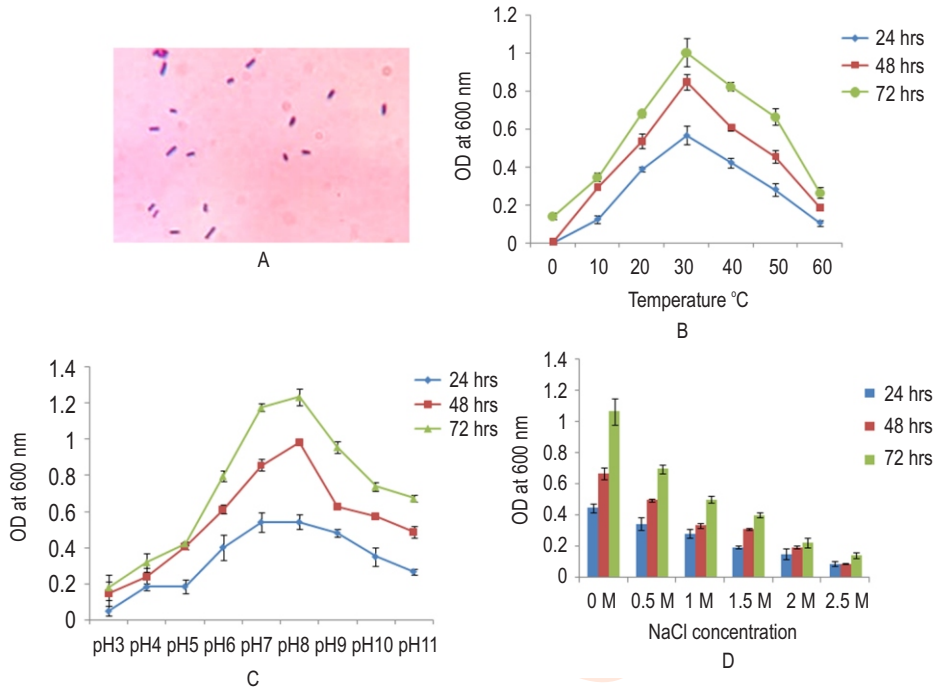

Fig. 2: (A) Microscopic image of isolate Dbc1. Graphical representation showing optimized growth condition of Isolate Dbc1 in terms of (B) cardinal temperature, (C) optimum pH and (D) optimum salinity.

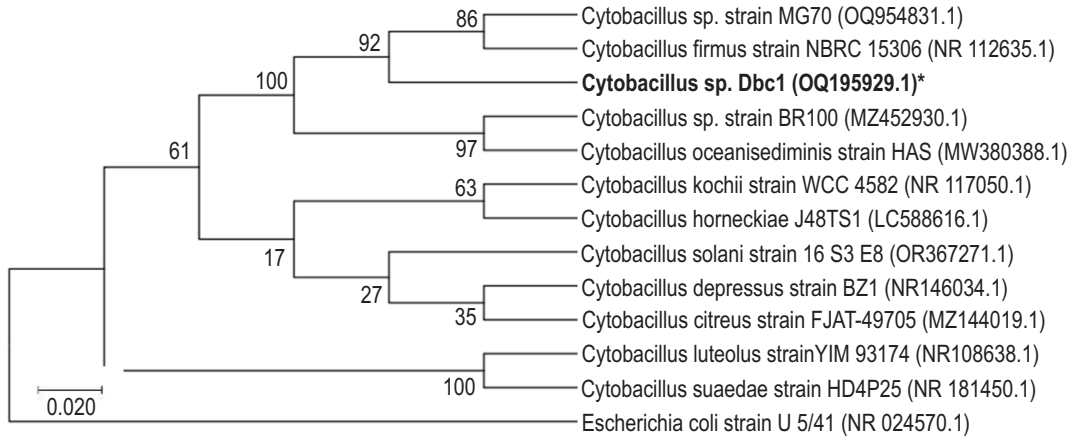

Fig. 3: Phylogenetic tree of *Cytobacillus* sp. strain Dbc1 showing its relationship with closest Blast hits generated by the Neighbour Joining method using Mega7 software.

biofilm embedded cells than their planktonic counterparts (Kisil *et al.,* 2020). Exopolysaccharides build the scaffold of a biofilm matrix which, in turn, plays an important role in biofilm maturation. In addition, these exopolysaccharides hold eDNA, proteins, enzymes and capsular polysaccharides (Sutherland, 2001; Matsukawa and Greenberg, 2004) which serve specific roles in biofilm development as well as antibiotic resistance (Li *et al.,* 2021). Apart from that, biofilm matrix also represents spatial heterogeneity in terms of nutrients, oxygen level, metabolites and pH (Li *et al.,* 2021) which otherwise creates several micro-environments having differences in terms of genetic expression, cellular metabolisms, micro-colony formation, antibiotic sensitivity within biofilm embedded cells. Therefore, dissociation of biofilm matrix may aid higher drug penetration and drug

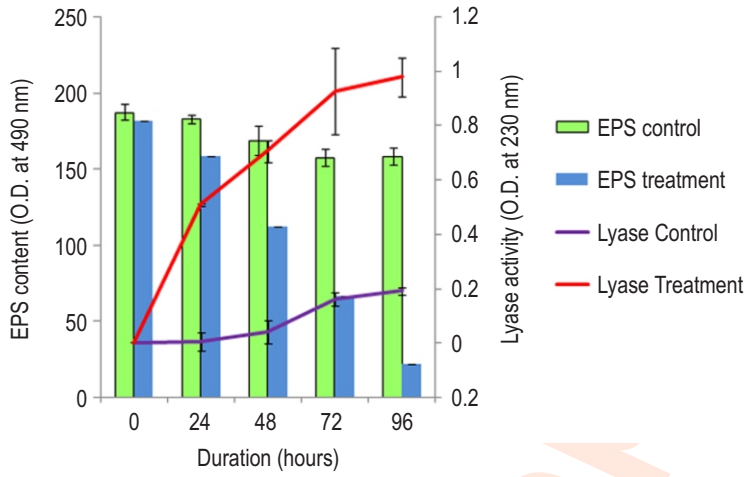

Fig. 4: Graphical representation showing cepacian utilization and crude lyase enzyme activity of *Cytobacillus* sp. Dbc1

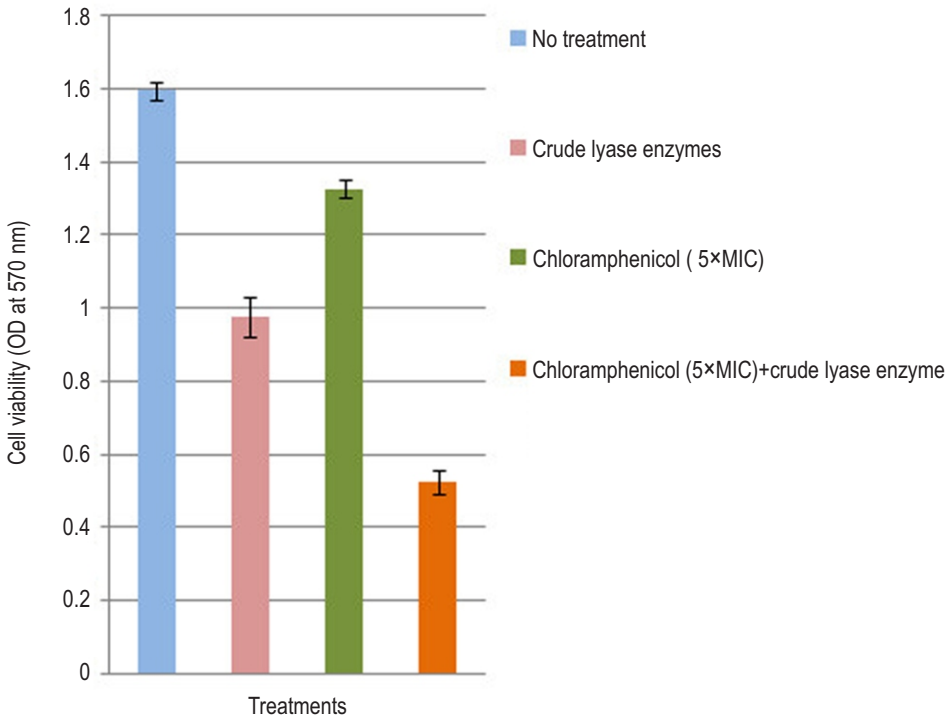

Fig. 5: Increase in antibiotic susceptibility of *B. cepacia* biofilm due to Cepacian degradation by crude Lyase activity of *Cytobacillus* sp. Dbc1.

targeting which, in turn, can minimize long-term medication and establishment of chronic infection. Moreover, biofilm disruption does not change any cellular function, so it neither imposes selective pressure on the bacterial growth nor promotes significant counteractive resistance mechanisms by them (Ghosh *et al.,* 2022). Though there are several reports on enzymatic degradation of biofilm matrix components of pathogenic bacteria such as exopolysaccharides (Baker *et al.,* 2016), proteins (Saggu *et al.,* 2019) etc., reports on cepacian EPS degradation of *B. cepacia* is scanty. In the present study, cepacian extracted from *B. cepacia* showed that the total carbohydrate content of cepacian was 556.3333 µg ml^{-1}. Two potent cepacian degrading sample bacteria namely Dbc1and Dbc2 were isolated from the rhizospheric soil samples collected from local paddy field. Both the isolates were Gram positive rods. Growth pattern of two isolates was evaluated in the presence of cepacian. Fig. 1A

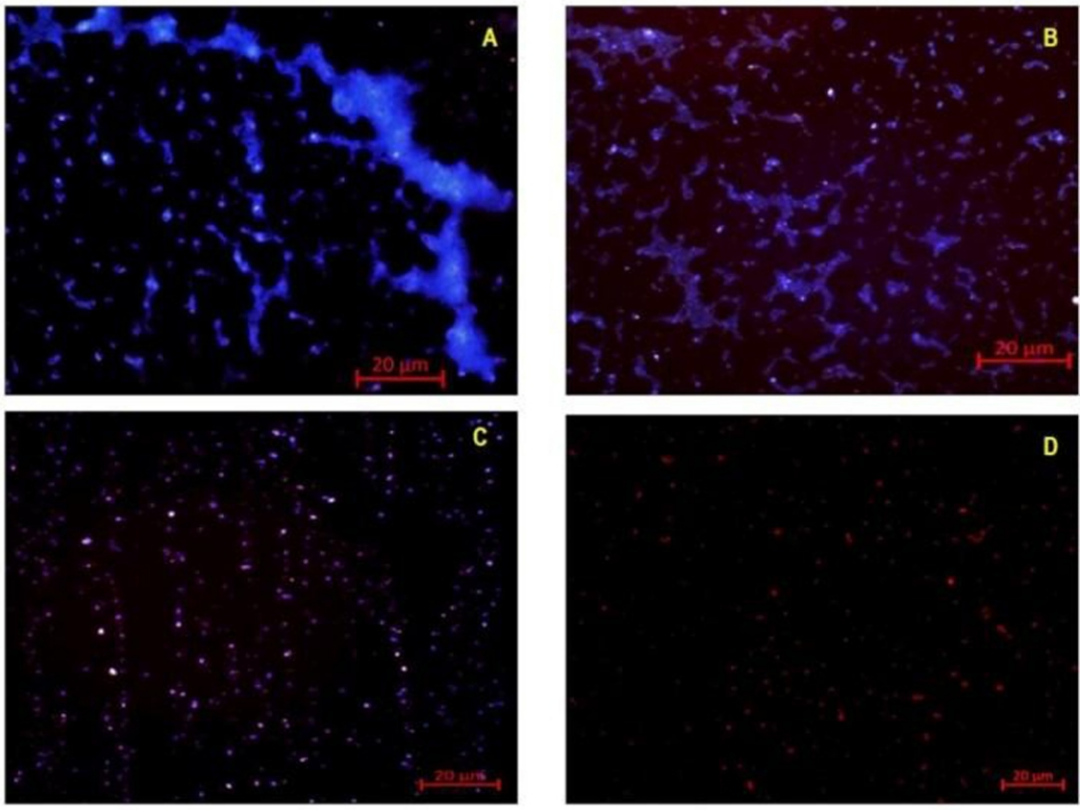

Fig. 6: Fluorescent microscopic image of *B. cepacia* biofilm stained with DAPI and propidium iodide (A) Control (B) treated with chloramphenicol [5×MIC] (C) treated with crude lyase preparation of *Cytobacillus* sp. Dbc1 and (D) treated with combination of chloramphenicol (5×MIC) and crude lyase of Dbc1.

showed faster planktonic growth due to better cepacian utilization by isolate Dbc1 over time than isolate Dbc2. Moreover, isolate Dbc1 was highly sensitive to all the antibiotics tested (Fig. 1B,1C), while isolate Dbc2 was resistant to Polymyxin B, Neomycin and showed less sensitivity to the remaining antibiotics tested (Fig. 1D, 1E and Table 1).

On the basis of these observations, Dbc1 was selected for further studies. Biochemical profile of isolate Dbc1 is presented in Table 2. Growth condition of isolate Dbc1 was optimized. Isolate Dbc1 showed the growth in a wide range of temperature from 10-50ºC. Cardinal temperature was detected at 30ºC (Fig. 2B). Isolate Dbc1 showed wide range of pH tolerance starting from pH 6 to 11 which indicates alkaliphilic nature, although optimum growth was observed at pH 8 (Fig. 2C). Isolate D23 showed tolerance up to 2M NaCl revealing the halo-tolerant nature (Fig. 2D). Another strain of *Cytobacillus* showed more or less similar result regarding optimum growth condition (Tarasov *et al*., 2023). On the basis of 16S rRNA gene sequence homology, isolate Dbc1 was identified as a member of the genus *Cytobacillus* (Fig. 3) The nucleotide sequence was deposited in the GenBank database, NCBI with the accession number OQ195929.1. A gradual increase in the planktonic growth of Dbc1 along with a sharp, corresponding decrease in available cepacian content present in the supernatant of Dbc1was observed when grown in 3% cepacian as sole carbon source. A significant increase in crude lyase activity of Dbc1 over time was recorded (Fig. 4). Potent lyase activity allowed continuous utilization of cepacian as sole carbon source by Dbc1. Similar result was reported by Cescutti *et al.* (2006) in *Bacillus* sp. where they showed a characteristic double bond in between C-4 and C-5 of Glucuronic acid residues in each repeating unit of cepacian due to lyase activity which can be measured by an increase in OD_{230}. As *Cytobacillus* sp. strain Dbc1 effectively utilized cepacian exopolysaccharide supplemented in basal medium due to their lyase activity, it was tested whether cell free lysate of *Cytobacillus* sp. strain Dbc1 could inhibit biofilm formation of *B. cepacia*. The addition of cell free supernatants (CFS) of strain Dbc1 to the established biofilm of *B. cepacia* reduced total biofilm biomass up to 62% without hampering their planktonic growth (Table 3). CFS of Dbc1 also interfered with viability of mature *B. cepacia* biofilm embedded cells revealed in terms of CFU. Although, CFS of strain Dbc1 inhibited biofilm formation of *B. cepacia* up to 45.52% (Table 3) in comparison to the control set. Several previous reports also showed that enzymatic degradation of biofilm matrix by glycoside hydrolase (Baker *et al.,* 2016), α- amylase of *Bacillus*

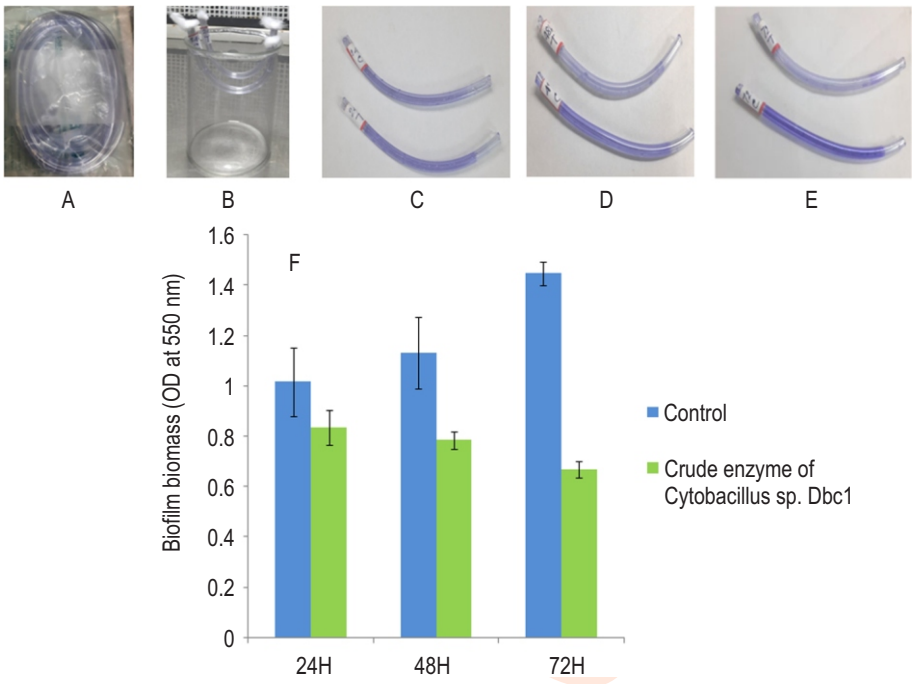

Fig. 7: Disruption of established biofilm of *B. cepacia* by crude lyase preparation of *Cytobacilllus* sp. Dbc1 inside external medical device (A) entire nasal oxygen catheter, (B) experimental setup, (C), (D) and (E) segmented catheter stained with crystal violet after 24, 48 and 72 hrs of *B. cepacia* biofilm growth respectively, (F) Graph showing reduction in biofilm biomass of *B. cepacia* by *Cytobacillus* sp. strain Dbc1 inside nasal oxygen catheter.

Table 1: Antibiotic susceptibility of isolate Dbc1 and Dbc2

Antibiotic strips	Antibiotics	Antibiotic sensitivity	
		Isolate Dbc1	Isolate Dbc2
Hexa Universal 1	Bacitracin (B)	++	++
	Chloramphenicol ©	+++	++
	Penicillin (P)	++	++
	Polymixin B(PB)	++	-
	Gentamicin (GEN)	+++	++
	Neomycin (N)	++	-
	Co-Trimaxazole (COT)	+++	++
Hexa G Plus 2	Penicillin (P)	++	++
	Clindamycin (CD)	++	++
	Erythromycin (E)	+++	++
	Vancomycin(VA)	+++	++
	Ampicillin/Sulbactum (A/S)	+++	++

amyloliquefaciens (Ivanova *et al.,* 2015) prevented both biofilm formation and disintegration of mature biofilm of *Pseudomonas aeruginosa* and mixed biofilm of *P. aeruginosa* and *Escherichia coli*. In a parallel experiment, it was observed that, treatment of crude lyase preparation reduced total carbohydrate content and protein content significantly in *B. cepacia* biofilm over time (Table 4). Enzymatic degradation of cepacian perhaps hampered mature biofilm structure of *B. cepacia* as some earlier reports suggest that proteins and enzymes in the biofilm matrix remain attached to the exopolysaccharides (Sutherland 2001; Matsukawa *et al.,* 2004). Therefore, dissociation of biofilm matrix can reduce the chance of maintenance of properly functionality of biofilm leading to less virulent downstream effects. Generally destabilization of biofilm or biofilm dispersion releases planktonic bacteria which can colonize and form biofilms again to new sites. Thus combination of anti-biofilm agents and antibiotic treatment can be able to manage the newly released planktonic bacteria making them susceptible to antimicrobial agents (Okshevsky *et al.,* 2015). Several reports

Table 2: Biochemical characterization of isolate Dbc1

Biochemical test	Result
Indole test	+
MR test	+
VP test	-
Nitrate reduction test	+
Citrate utilization test	-
Starch hydrolysis test	+
Catalase test	+

Table 3: Anti-biofilm effectivity of *Cytobacillus* sp. Dbc1 against *B. cepacia*

Parameter		Control set	Treatment set
Bacterial growth (Od_{600})		1.1123±0.083439	1.034867±0.105511
Inhibition of formation of biofilm biomass (Od_{550})		1.2975±0.1567	0.7068±0.1048
Disintegration of mature biofilm	Total biomass	1.367867±0.1393	0.522333±0.112342
	CFU ml^{-1} (Log_{10})	6.42±0.41328	3.496667±0.355293

Table 4: Reduction of biofilm matrix components of *B. cepacia* biofilm by crude Lyase enzyme of *Cytobacillus* sp. Dbc1

Percentage of decrease in total carbohydrate content	69.5511+10.8237
Percentage of decrease in total protein content	40.42547+2.12765

showed that biofilms and exopolysaccharides enhance antibiotic resistance by serving as penetrating barriers to the conventional antibiotics (Van Acker *et al.,* 2014; Abede, 2020). Therefore, it can be predicted that disintegration of exopolysaccharides could weaken the biofilm matrix and make more susceptible to antibiotics. Initially, antibiotic sensitivity of *B. cepacia* was tested using a number of standard antibiotic discs (HIMEDIA, India), among them chloramphenicol was selected on the basis of diameter of zone of inhibition (data not shown). The MIC of chloramphenicol was µg ml^{-1} against *B. cepacia.* The effect of combination of antibiotic and CF of potent isolate was evaluated. Fig. 5 shows that high concentration of chloramphenicol (5×MIC) alone exerted less inhibitory effect on cell viability of *B. cepacia* biofilm cells. However, the combination of cell free lysate of *Cytobacillus* sp. strain Dbc1 and chloramphenicol resulted in almost 3-fold decrease in bacterial cell viability, however, crude lyase preparation of *Cytobacillus* sp. strain Dbc1 alone also showed significant reduction in cell viability.

This result positively indicates that lyase activity of *Cytobacillus* sp. disintegrated the biofilm matrix of *B. cepacia* and facilitated penetration and targeting of antibiotics into biofilm embedded cells. Fluorescent microscopic study using live/dead staining method also highlighted the result of MTT assay. Biofilms treated with either Dbc1CFS or Chloramphenicol (5×MIC) alone showed the presence of live cells at higher density which were stained blue by DAPI (Fig. 6B, 6C), but their combination hampered the biofilm entity immensely resulting in higher cell death and less biomass as well (Fig. 6D). Similar results have been reported earlier where kanamycin or colistin were less effective against biofilms of *Staphylococcus aureus* and *P. aeruginosa* when applied even in much higher concentration over their MICs. However, effectivity of colistin and kanamycin against biofilm cells increased considerably when they were applied in combination with glycoside hydrolase and metalloprotease, respectively (Baker *et al.,* 2016; Saggu *et al.,* 2019). Degradation of cepacian backbone in *B. cepacia* biofilm perhaps enhanced better antibiotic penetration and targeting resulted in higher mortality. *B. cepacia* frequently contaminates medical settings and accessories in hospitals (Jones *et al.,* 2001). Particularly indwelling medical device-associated biofilms have created serious problems in case of nosocomial infections (Li *et al.,* 2021) Therefore, new research is going on to combat formation of such medical device-associated biofilms. Coating of medical device surfaces with bioactive compounds (Costa *et al.,* 2017) or application of antimicrobials peptides (deBreji *et al.,* 2018), nanomaterials (Tran *et al.,* 2020) etc., are in practice to reduce the chance of *B. cepacia* infection from those contaminated devices.

In the present study, it was observed that crude lyase enzyme of *Cytobacillus* sp. Dbc1 successfully reduced biofilm biomass on the inner side of nasal oxygen catheter over a time period of 72 hrs when applied on established biofilm of *B. cepacia* (Fig. 7F) in contrast to the control set. Enzymatic degradation of biofilm matrix by α-amylase of *Bacillus amyloliquefaciens* also effectively reduced biofilm formation of *Staphylococcus aerues*, *Pseudomonas aeruginosa* and their mixed biofilm inside urinary catheters (Ivanova *et al*., 2015). It can be concluded that *Cytobacillus* sp. strain Dbc1 is a potent cepacian degrading candidate with significant anti-biofilm activity against *B. cepacia*. Strain Dbc1 also potentiated antibiotic effectivity against *B. cepacia* biofilm. Cytotoxicity and stability of crude lyase under physiological condition were not tested, at this juncture it can be effectively and safely used to reduce the chance of *B. cepacia* biofilm development in external medical devices like oxygen catheters. Lyase does not impose any selection pressure on the biofilm cells, chance of development of resistance against lyase is remote.

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