ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 1, pp. 59–64. © Pleiades Publishing, Ltd., 2015.

EXPERIMENTAL ARTICLES

Enzymatic Degradation of Bacterial Biofilms Using *Aspergillus clavatus* MTCC 1323[1]

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Received June 25, 2014

Abstract—Fungal strain, *Aspergillus clavatus* MTCC1323 under solid state fermentation was found to produce enzymes (protease, amylase and pectinase) having potential of degrading the biofilms of *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*. Maximum specific enzyme activities were found to be 10.0, 8.0, and 10.086 U/mg for protease, amylase and pectinase, respectively, after 7 days of incubation at 27°C. Biofilms' degradation was analyzed through FTIR technique. Various proteins and carbohydrates were involved in the formation of biofilms as their concentrations were reduced after enzyme mixture treatment. The degradation of the biofilms was analyzed by viability assay using flow cytometry and fluorescence microscopy. Maximum biofilm degradation was found against *P. aeruginosa* and *B. subtilis* biofilms and showed 82 and 75% biofilm reduction, respectively, in terms of dry cell weight. Flow cytometry viability assay results indicated that the enzyme mixture of *A. clavatus* was capable of degrading the bacterial biofilms.

Keywords: biofilm, *Aspergillus clavatus*, pectinase, amylase, protease

DOI: 10.1134/S0026261715010130

Bacterial biofilms are potentially hazardous for natural, medical, food and industrial aspects related with critical public health safety. More significantly, it has been found that this sessile biofilm environment provides diverse species of bacteria to develop selective resistance to commonly administered antibiotics and protection from host immune responses during chronic infection of humans and animals. In the laboratory, several bacteria have been shown to form biofilms on a variety of surfaces including plant and animal food sources to cause disease upon consumption.

The biofilm matrix contains polysaccharides, glycoproteins and proteins [1]. Bacterial extracellular polysaccharides are composed of homo- and heteropolysaccharides of glucose, mannose, galactose, fructose, pyruvate, and mannuronic acid or glucuronic acid based complexes [2]. Different types of chemical bonds between the polysaccharides give rise to a multitude of polysaccharides including levans, polymannans, dextrans, cellulose, amylopectin, glycogen and alginate. In nutrient limited ecosystems, such as, in the aquatic environment, bacteria have a marked tendency to attach with surfaces and initiate the formation of a biofilm [3], causing problems such as, an increased frictional resistance to fluids in water conduits and on ship hulls (fouling) [4], decreased heat transfer from heat exchangers, corrosion of metallic substrata, and contamination in the food and biotechnology industries. Also, biofilms are severe problem for medical sciences industry, mostly they cause dental plaque, contaminate endoscopes and contact lenses, prosthetic device colonization, and biofilm formation on medical implants [5].

Bacterial cells after coming in contact with inert surfaces, firstly they exert through weak forces by their external structures (i.e., flagella, fimbriae and/or capsular components) and get bound to the substratum and afterwards they secrete sticky extracellular polymeric substances (EPS) to form a matrix gel that embeds several layers of cells after maturation of substances (EPS) to form a matrix gel that embeds several layers of cells after maturation of biofilm. In addition to proteins and nucleic acids, the bacterial biofilm matrix contains polysaccharides, lipids, mineral ions and cell debris [6]. Earlier reports showed that polysaccharides are possibly responsible for bacterial adhesion and biofilm formation on static surfaces [7]. However, the precise composition of biofilm polysaccharides is still insufficiently known, but available data on planktonic EPS and biofilm EPS suggests that some of their monomers are identical to those present in plant cell-wall material [6]. Some bacterial

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exopolysaccharides have been reported to be acceptable substrates for enzyme mixtures from non-bacterial sources [8].

These undesirable biofilms could be effectively controlled by understanding the type and nature of the contaminating residual materials (i.e., carbohydrates, fats, proteins, mineral salts etc.) and the microorganisms can be removed from the surfaces [9]. Recent studies have shown great interest in enzymatic degradation of bacterial biofilms. Enzymes are highly selective and capable of disrupting the structural stability of the biofilm EPS matrix [9]. Various studies have been done in the past pertaining to the enzymatic degradation of polysaccharides and proteins for biofilm detachment, as these are the two important components of the EPS. Some fungi have been reported for their capability to degrade bacterial biofilms by secreting various enzymes [9]. Due to the significant structural role of proteins and polysaccharides in the EPS matrix, a combination of various proteases and polysaccharases might be successful in biofilm removal/detachment [10]. Hence, keeping the aforesaid facts in view regarding the bacterial biofilm removal, the present study was performed to evaluate the applicability of the enzymes produced by *Aspergillus clavatus* MTCC 1323 for the degradation of bacterial biofilms.

MATERIALS AND METHODS

Microorganisms and culture conditions. Fungal strain *A. clavatus* MTCC 1323 was maintained on potato dextrose agar, containing potato infusion (4 g/L), D(+) dextrose (20 g/L), agar (15 g/L), and incubated at 28°C until adequate sporulation. Fungal spores were filtered and stored under condition of 10% glycerol at –20°C.

Culture medium containing (10%) lemon peel (10% wt/vol) along with $MgSO_4 \cdot 7H_2O$ (0.05%), NH_4NO_3 (0.1%), $(NH_4)H_2PO_4$ (0.1%) was used for fungal growth. One millilitre spore suspension (10^3 spores/mL) was added to 250 mL flask containing 20 mL of above on daily basis production medium and incubated for 7 days at 28°C under static condition. Aliquot samples were withdrawn on daily basis and fungal cells were separated by centrifuging the sample at 10000 rpm for 20 min. The fungal growth was measured by pellet weight (obtained by drying it at 40°C for 48 h) and the supernatant was used for the enzyme assay. All experiments were performed in triplicate.

Enzyme Assays

The biochemical tests for extracellular enzyme(s) production were carried out in triplicate using the following assays:

Amylase assay. The amylase activity was determined according to the dinitrosalicylic acid (DNS) method using 1% soluble starch as substrate [11]. The reaction mixture was comprised of 1 mL 1% starch solution (dissolved in 20 mM phosphate buffer of pH 6.0), 1 mL 20 mM phosphate buffer (pH 6.0) and 1 mL culture supernatant, and incubated at 40°C in water bath for 30 min. Three mL DNS reagent was added to stop the enzymatic reaction and finally samples were kept in boiling water bath for 15 min. Absorbance (optical density; OD) was measured $OD_{540\ nm}$ against a blank after cooling the samples at room temperature. One unit of the amylase activity was defined as the amount of enzyme required to produce 1 μmol of reducing equivalents per min from soluble starch under the assay conditions.

Protease assay. The protease enzyme assay was performed using a modified Kunitz caseinolytic assay as described by Janssen et al. [12]. Half mL of enzyme source was added to 2.0 mL of 0.5% casein in 50 mM phosphate buffer (pH 7.4) and incubated at 37°C. The reaction was terminated after 30 min by adding 3.0 mL of 5% trichloroacetic acid (TCA). The solution was kept for additional 30 min at room temperature and centrifuged at 10000 rpm and the absorbance of the supernatant was measured at $OD_{660\ nm}$. One unit of the protease activity was defined as the amount of enzyme that liberates 1 μg of tyrosine from the substrate (casein) per min under assay conditions.

Pectinase assay. The pectinase activity was assayed by the colorimetric method of Miller (1959) using glucose as standard. Briefly, 0.5 mL of cell free supernatant was incubated with 0.5 mL of pectin in 0.1 M acetate buffer (pH 6.0) at 40°C for 10 min under static condition. Afterwards, 1 mL of DNS reagent was added to the mixture and it was boiled for 5 min at 90°C. The reaction was stopped by adding 1 mL of Rochelle's salt and the mixture was diluted by adding 2 mL of deionized water and the absorbance was measured at $OD_{595\ nm}$. One unit of the pectinase activity was defined as the amount of enzyme which liberates 1 μmole glucose per min.

Biofilm Formation

Mono species biofilms of *Penicillium aeruginosa* MTCC 741, *Penicillium aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 251, *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 441 and *Bacillus subtilis* ATCC 6633 were developed on glass wool by inoculating 100 mL nutrient broth with 100 μL inoculum of the 24 h old culture suspensions of the above mentioned bacteria. After incubating at 27°C for 48 h under static conditions, the glass wools were withdrawn and dipped into saline solution (0.9%, w/v) to discard the loosely attached cells.

Biofilm Analysis

Flasks containing the bacterial biofilms were vortexed for 5 min to detach the loosely bound biofilm cells. Microbial contents were homogenized for 30 s

with homogenizer and centrifuged at 10000 rpm for 5 min at 4°C. The microbial pellets were dissolved in 1 mL phosphate buffer (pH 7) and used for the analysis [7].

The dry weight of enzyme treated (for 1 h incubation after addition of 1 mL enzyme/mg biomass) and non-treated biofilm was measured after drying the biofilm in electronic oven at 60°C for 3 h.

Carbohydrate and protein concentrations of the enzyme treated and untreated biofilm samples were analysed employing Anthrone assay and Lowry assay method, respectively. The reductions in the protein and carbohydrate contents were further analysed by Fourier Transform Infrared (FTIR) spectroscopy. For the FTIR analysis, 48 h old biofilm was treated with enzyme solution (1 mL) for 1 h and biofilm without treated with enzyme was kept as control. The samples were analysed using (0.2 mL) chloroform as a solvent.

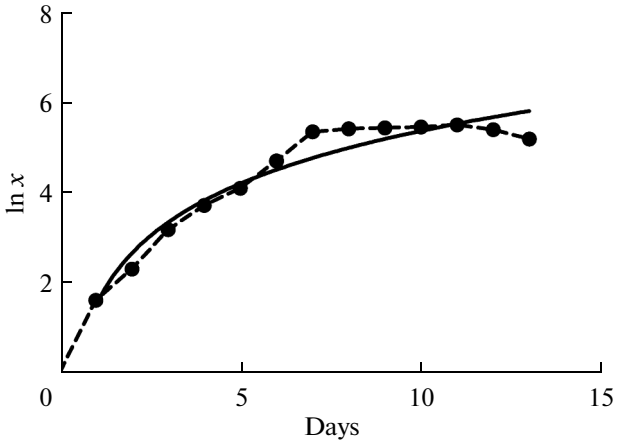

Fig. 1. Growth (in semilog) of *Aspergillus clavatus* cultured on lemon peel.

Biofilm Degradation

Microtiter plate method. The microtiter assay (a type of quantitative determination method) was performed according to the protocol given by Pitts et al. [13] with minor modifications. 200 μL of the standardized bacterial suspension was added to wells of polystyrene microtiter plate and incubated at 27°C with continuous shaking at 100 rpm for 48 h. Biofilm formation was monitored periodically by visual inspection. Following the incubation, the supernatant was discarded and 200 μL of enzyme supernatant was added to each well except the control well. The microtiter plates were incubated for 1 h at 27°C and washed twice with distilled water. The cells were fixed with 200 μL of 95% ethanol for 15 min and allowed to dry. Afterwards, 200 μL of crystal violet solution was added for 30 min and wells were washed three times each with sterile distilled water and 30% glacial acetic acid (200 μL), respectively. The microtiter plates were read at $OD_{595\ nm}$ using ELISA plate reader (SpectraMax). The microtiter plate method was performed using different volumes of enzymes (i.e., 50, 100, 200, 250 and 300 μL) and enzymes produced after different time incubations (i.e., 3, 5 and 7 days). The percentage biofilm degradation was calculated using the following formula:

$$\text{Percentage reduction} = [(C - T)/C \times 100], \%,$$

C: Average absorbance per well for control wells (biofilm, without treatment),

T: Average absorbance per well for treated wells (biofilm with treatment).

Fluorescence Microscopy

Biofilm was withdrawn from glass wool and dipped into 0.9% NaCl (w/v) for 1 min, and finally it was used as standard biofilm sample. In order to evaluate the biofilm degradation, the biofilm samples were treated with 1 mL fungal culture supernatant (enzyme) and incubated at 27°C for 1 h under static condition. Biofilm sample without enzyme treatment was considered as control and incubated under similar condition. The incubated biofilm samples were dried at 45°C and fixed on glass slide for 30 min using formaldehyde solution. The slide was stained with crystal violet solution for 2 min and scanned under fluorescence microscope at 959 nm wavelength.

Statistical Analysis

All data was represented as mean (±SD, $n = 3$) of three and one way ANOVA was performed followed by Dunnets test. The statistical significance level was maintained as p-value < 0.05.

RESULTS AND DISCUSSION

Microorganism and enzyme production. The microbial growth and enzyme production capacity of *A. clavatus* MTCC 1323 was studied under solid state fermentation (SSF) conditions using lemon peel as a substrate. The growth pattern of *A. clavatus* MTCC 1323 has been given in Fig. 1. It is evident from the Fig. 1 that the growth of *A. clavatus* was highest after the 5th day of incubation and progressively reached to a plateau phase after the 7th day. From the growth curve, the specific growth rate and the doubling time of *A. clavatus* MTCC 1323 were 0.1625 per h and 4 h, respectively. Seven days incubated culture broth having specific enzyme activities of 8.0, 10.0, and 10.086 U/mg for amylase, protease, and pectinase, respectively, was found to be most suitable for biofilm degradation.

Microbial biofilm analysis and viability test by flow cytometry. The results of dry weights of mono species microbial biofilms treated and untreated with enzyme have been summarized in Table 1. After enzymatic treatment of the biofilms, the weight of the biofilm was

Biofilm analysis in terms of DCW, protein and carbohydrate contents along with SD

Microbes used for biofilm formation	Without enzyme treatment			With enzyme treatment		
	DCW, g/L	Protein, mg/mL	Carbohydrate, mg/mL	DCW, g/L	Protein, mg/mL	Carbohydrate, mg/mL
B. subtilis MTCC 441	2.41 ± 0.010	1.13 ± 0.070	0.48 ± 0.056	1.31 ± 0.042	0.76 ± 0.042	0.31 ± 0.035
B. subtilis ATCC 6633	2.09 ± 0.026	1.11 ± 0.028	0.37 ± 0.049	1.70 ± 0.035	0.83 ± 0.014	0.24 ± 0.028
P. aeruginosa MTCC 741	2.25 ± 0.030	1.15 ± 0.035	0.80 ± 0.035	1.20 ± 0.070	0.60 ± 0.035	0.42 ± 0.049
P. aeruginosa MTCC 2453	2.62 ± 0.055	1.15 ± 0.071	0.37 ± 0.049	1.74 ± 0.028	0.80 ± 0.035	0.20 ± 0.035
S. aureus MTCC 96	2.54 ± 0.031	1.18 ± 0.071	0.45 ± 0.035	1.65 ± 0.070	1.10 ± 0.035	0.35 ± 0.035
S. aureus MTCC 251	2.20 ± 0.025	1.23 ± 0.056	0.70 ± 0.035	1.85 ± 0.035	1.10 ± 0.035	0.57 ± 0.049

found to be decreased at varying degrees and the enzyme mixture was reduced more than 40% DCW in case of *P. aeruginosa* MTCC 741 and *B. subtilis* MTCC 441.

The microscopic observations of the biofilms revealed that the EPS of the untreated biofilm was dense and looked like granular structure with clear marked microcolonies and water channels. However, the enzyme treated biofilms were lacking such structures (Fig. 2). With increase in volume of enzyme mixture (i.e., from 100–300 μL) of *A. clavatus* MTCC 1323, the biofilm degradation ability of the strain was increased. The enzyme mixture was most effective against the biofilm formed by *P. aeruginosa* MTCC 741 and least effective against the biofilm formed by *S. aureus* MTCC 251 as observed by biofilm treatment with 300 μL enzyme mixture solution. The biofilm degradation results were further verified by analysing the protein and carbohydrate contents of the treated and untreated biofilms. Maximum (>45%) and minimum (10–20%) decrease in protein and carbohydrate contents were observed for *P. aeruginosa* MTCC 741 and *S. aureus* MTCC 251, respectively (table).

The microtiter assay was performed to determine the enzymes' efficiency for the degradation of microbial biofilms and extracellular polymeric substances excreted during biofilm formation. The biofim degradation efficiency of enzymes increased with increase in enzyme concentration (50–300 μL). The maximum biofilm reduction was observed for *B. subtilis* MTCC 441 and *P. aeruginosa* MTCC 741 and minimum biofilm reduction was found for *S. aureus* MTCC 251 and *P. aeruginosa* MTCC 2453. Further studies regarding incubation period for active enzyme concentration revealed that seven day incubated (grown) culture retained maximum degradation efficiency for various bacterial biofilms (Fig. 3).

Role of enzymes in the degradation of biofilms. The cellular composition of gram-positive bacteria contain the cytoplasm, the cytoplasmic membrane, and the cell wall, whereas, gram-negative bacteria also contain the outer membrane. The bacterial cell wall has a shape-giving function and protects the cells from osmotic disruption. It is made up of the peptidoglycan, whose primary structure consists basically of disaccharide and pentapeptide subunits with some specific features, like, the presence of alternating D- and L-

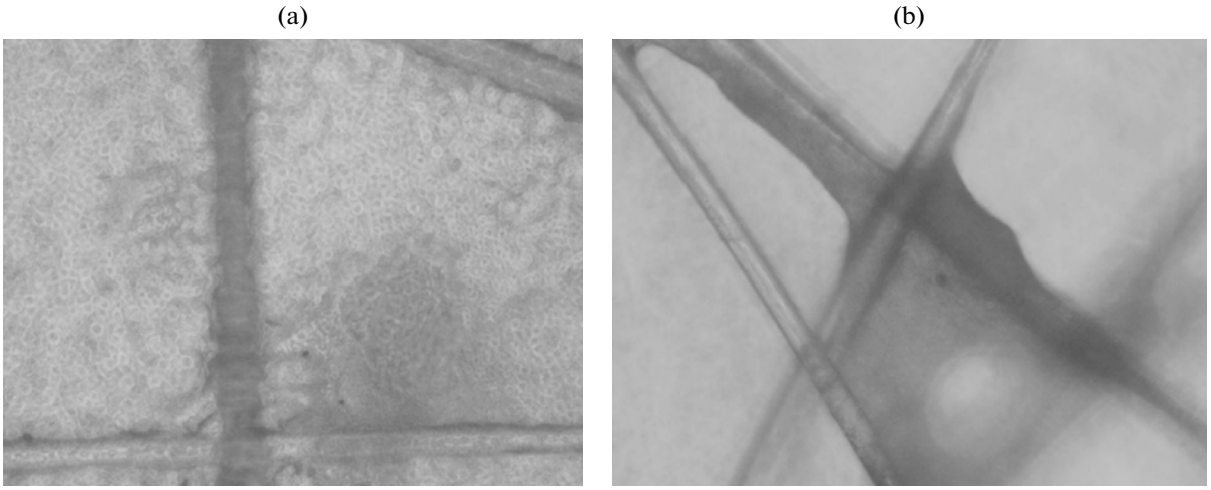

Fig. 2. Microscopic images of *P. aeruginosa* MTCC 741 biofilm-Control (a) and enzyme treated (b)].

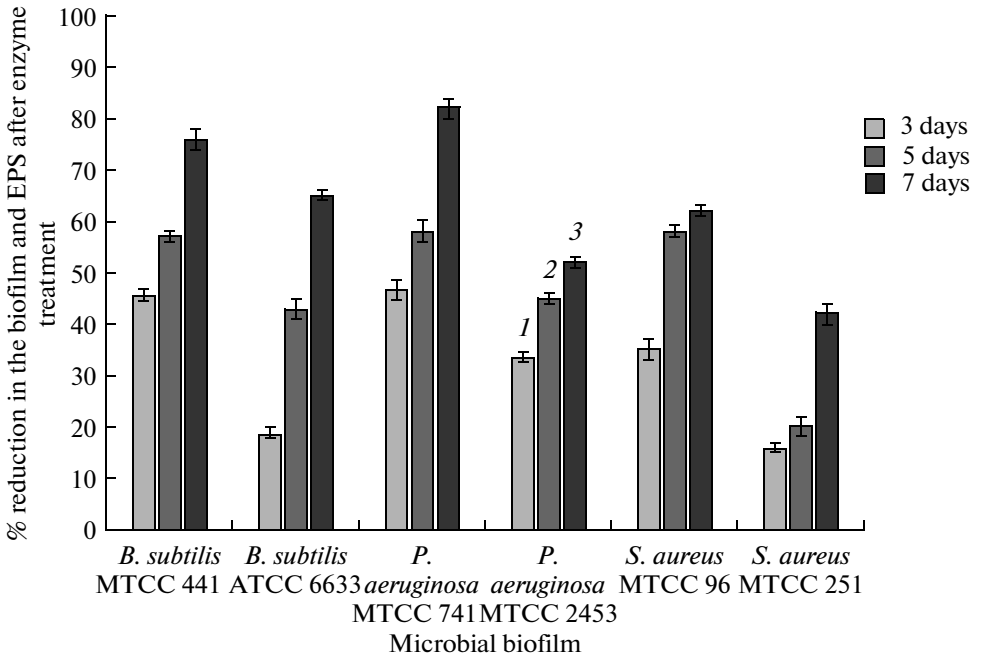

Fig. 3. Effect of incubation period of *A. clavatus* on EPS for biofilm degradation determined by Micro titer Plate method. (*1*)—3 days; (*2*)—5 days; (*3*)—7 days.

amino acids etc. [14]. In Gram negative bacteria a layer of lipoprotein covalently bound to the peptidoglycan is also present. This outer membrane is the major permeability barrier that protects these cells against bile salts, degradation by digesting enzymes [15]. Beside this, several antimicrobial agents fail to penetrate the biofilms mainly due to the presence of extracellular polymeric substances (EPS) which act as a barrier and protect the bacterial cells. Due to the heterogeneous nature of EPS, a mixture of enzymes might be necessary for efficient degradation of bacterial biofilms. Previous reports have shown that enzymes' mixture can degrade the EPS of the bacterial biofilms [16, 17]. Enzymes degrade the biofilms directly by destroying the physical integrity of the extracellular polymeric substances through weakening the proteins, carbohydrate and lipid components of the extracellular polymeric substances [18].

In the present study, biofilms formed by various bacteria (treated and untreated with enzyme mixture) along with EPS were analyzed through FTIR, where functional groups were found in excited state and vibrated at particular frequencies, and showed the characteristics of different biomolecules (i.e., proteins, carbohydrates and other biomolecules). The region between 4000 and 3100 cm^{-1} was dominated by the bands resulting from –OH (3400 cm^{-1}) and N–H stretching modes (amide A 3300 cm^{-1} and amide B 3030 cm^{-1}). The region between 3100 and 2800 cm^{-1} exhibited the C–H stretching vibrations of $-CH_3$ and CH_2 functional groups. The region between 1800 and 1500 cm^{-1} was dominated by the amide I and amide II bands, which were most intensive bands in the spectra of nearly all bacterial test samples. Complex absorption profiles were observed between 1300 and 1500 cm^{-1} aroused predominantly from CH_2 and CH_3 bending modes of lipids and proteins. A characteristic, but weak, feature was often observed around 1400 cm^{-1}, which may be attributed to the symmetric stretching vibrations of –COO– functional groups of amino acid side chains or free fatty acids [15]. The C–O stretching of polysaccharides had characteristic band in fingerprint region 800–1100 cm^{-1} [19].

In the untreated samples, the bands of OH stretching and CCO for carbohydrate moiety (3500 and 760 cm^{-1}, respectively), NH and CO stretching for amide moiety (3200 and 1639 cm^{-1}, respectively) were clearly distinct, whereas, those bands were found degraded in the treated samples. The results suggested that both proteolytic and carbohydrate-degrading activities were efficient for biofilm removal. Orgaz et al., 2006 [20] reported that the combination of both the enzymes, which occurs naturally in the cultures of *Penicillium* and *Aspergillus* growing on sodium alginate, have biofilm removal capability. Based upon the experiments performed in this study, the enzyme mixture was found to be effective to degrade more than 40% of the biofilm formed by *B. subtilis* MTCC 441 and *P. aeruginosa* MTCC 741. The carbohydrate and protein contents of the biofilm along with EPS were analyzed for various bacterial strains (table). O'Toole and Kolter, 1998 [21] reported that the protein-rich appendages play an important role in the initial steps of bacterial cell adhesion to surfaces. Presence of pro-

teinases in higher ratio facilitates the cell detachment and interruption of biofilm formation. Pectinase doesn't degrade the bacterial biofilm, it deacetylates the polysaccharide present in the biofilm matrix, makes the film more softer and possibly more porous, thus allows protease and amylase to penetrate and degrade the protein and polysaccharide present in the biofilm [20]. In the present study, attempts have been made to to assess the enzymatic degradation of biofilms produced by various bacteria. We found that *A. clavatus* MTCC 1323 was capable of producing extracellular enzymes (proteases, amylases and pectinases) that can efficiently degrade biofilms produced by cultures of *Pseudomonas, Bacillus* and *Staphylococcus*. The present study also warrants for further research work for analyzing the biofilm degradation ability of an individual enzyme (proteases, amylases, and pectinases) along with its mechanistic characterization.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support received from the Council of Scientific and Industrial Research (CSIR) and Department of Science and Technology (DST), India. Authors also like to thank SAIF Division, CSIR–CDRI, Lucknow, UP, India for providing help in flow cytometry and florescent microscopy studies.

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