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Bacterial Biofilm Degradation Using Extracellular Enzymes Produced by *Penicillium janthinellum* EU2D-21 under Submerged Fermentation

Anil Kumar Nagraj, Digambar Gokhale*

NCIM Resource Center, CSIR-National Chemical Laboratory, Pune, Maharashtra, India
Email: dv.gokhale@ncl.res.in

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Abstract

Bacterial biofilms are the bacterial aggregates that are embedded in the self-produced matrix of extracellular polymeric substances (EPS) that cause persistent bacterial infections posing significant medical challenges. They are recalcitrant to antibiotics and host defenses which make the treatments difficult and costly. *Penicillium janthinellum* mutant EU2D-21 was found to produce extracellular enzyme complex (amylase, cellulase, protease) under submerged fermentation. Maximum specific enzyme activities were found to be 3.04 IU/mg, 2.61 IU/mg and 3.39 IU/mg for alpha-amylase, cellulase and protease respectively, after 8 days of incubation at 30˚C. We evaluated the enzyme complex for its ability to target and degrade the biofilms of different bacteria. We found that it degraded biofilms of *Escherichia coli* (85.5%), *Salmonella enterica* (79.72%), *Pseudomonas aeruginosa* (88.76%) *and Staphyloccus aureus* (87.42%) within 1 h of incubation at 50˚C. The scanning electron microscopy (SEM), quantitation of biofilm removal assay and Crystal violet assay demonstrated that the enzyme complex detached the biofilm exo-polysaccharide matrix and bacteria from the cell surface. These results illustrate the feasibility and benefits of using this enzyme complex as anti-biofilm therapeutics to eradicate biofilms. This can also be used as a promising strategy to improve treatment of multidrug resistant bacterial infections.

Keywords

Penicillium janthinellum, Cellulase, Biofilm Degradation, Bacterial Biofilms

1. Introduction

Biofilms are complex structures associated with aggregates of microbes em-

bedded in self-produced extracellular polymeric substances (EPS). EPS is composed of exo-polysaccharides, proteins and extracellular DNA (eDNA). Formation of biofilms provides significant advantage to microbial cells because it mediates adherence to host cells [1] [2] and assists in the resistance to antimicrobial agents [3]. The exopolysaccharide component of the biofilm acts as a barrier against phagocytosis by host immune cells [4]. It is estimated that most of the human bacterial infections (60% - 80%) are due to biofilms [5] and hence there is an urgent need to develop novel and effective treatments that target and disrupt biofilms. Biofilms are resistant to antimicrobial agents due to the impaired penetration and hence it is necessary to develop prophylactic treatments that either inhibit biofilm formation [6] [7] or disrupt the biofilms [8]. *Pseudomonas aeruginosa* is ubiquitous opportunistic pathogen causing pulmonary infections in immune-compromised patients who suffer from chronic lung diseases. Mortality is associated with *P. aeruginosa* is high [9] and it is increasing with the emergence of multidrug resistance strains. Recent studies have shown great interest in the use of enzymes in targeting and disrupting the bacterial biofilms proving their potential in treatment and eradication of chronic bacterial diseases. *Staphylococcus aureus* is a leading cause of infections in human population. It is very difficult to treat the wounds infected with *S. aureus* due to its multidrug resistance and natural tendency of biofilm formation. Watters *et al.* [10] tested the efficacy of the enzymes like α-amylase, bromelain, lysostaphin against methicillin resistant strains of *S. aureus* biofilms and found that all of them decreased the biomass significantly within 24 h. The fungal strain, *Aspergillus clavatus* MTCC 1323 was found to produce enzyme complex (protease, amylase, pectinase) which degrades the biofilms of *P. aeruginosa*, *B. subtilis* and *S. aureus* [11]. It is reported that glycoside hydrolases proved to be potential therapeutic agents exhibiting activity against diverse microorganisms by degrading biofilms [12]. Blanchette and Wenke [13] have very nicely highlighted the current and upcoming therapies for prevention and disruption of bacterial biofilms in their review article.

We therefore sought to look for the enzyme complexes capable of targeting and disrupting the biofilms of pathogenic bacteria. *Penicillium janthinellum* mutant EU2D-21, was known to produce cellulase enzyme complex [14]. We wanted to evaluate this enzyme complex for bacterial biofilm degradation and see whether it can be potential therapeutic agent for degrading bacterial biofilms. We demonstrate that the addition of low concentrations of the enzyme complex can disrupt bacterial biofilms in the laboratory strains of *Pseudomonas aeruginosa, Escherichia coli, Salmonella enterica* and *Staphylococcus aureus* in vitro at very low concentrations. Our results suggest that the enzyme complex produced by *P. janthinellum* have the potential to effectively degrade bacterial biofilm and can act as an anti-biofilm therapeutics which can be used for treatment and eradication of chronic bacterial infections.

2. Materials and Methods

2.1. Fungal Microbial Strain and Enzymes Production

P. janthinellum mutant EU2D-21 was isolated in our laboratory [14] and was deposited in NCIM Resource Center, CSIR-National Chemical Laboratory, Pune, India with the accession no NCIM 1368. The strain was grown on Potato Dextrose Agar (PDA) slopes incubated at 30˚C for 7 days and sub-cultured once in every 3 months. Bacterial strains *Escherichia coli* NCIM 2674*, Salmonella enterica* NCIM 2257*, Pseudomonas aerogenosa* NCIM 2200 *and Staphylococcus aureus* NCIM 5021 were obtained from NCIM Resource Center, CSIR-National Chemical Laboratory, Pune, India. Basal fermentation medium consisted of 0.2% KH_2PO_4, 0.03% $CaCl_2$, 0.03% urea, 0.14% $(NH_4)_2SO_4$, 0.01% yeast extract, 0.025% bacto-peptone, 0.03% $MgSO_4.7H_2O$, 0.0005% $FeSO_4.7H_2O$, 0.00016% $MnSO_4{\cdot}7H_2O$, 0.00014% $ZnSO_4{\cdot}7H_2O$, 0.0002% $CoCl_2$ and 0.1% Tween 80. The pH of the fermentation medium was adjusted to 5.2 prior to sterilization. Submerged fermentation (SmF) was carried out in 250 ml Erlenmeyer flask with 70 ml of fermentation medium containing cellulose powder (1%) and wheat bran (2.5%). The flasks were inoculated with spores (approximately 10^7) from 15-days old culture grown on PDA slant and incubated at 30˚C with shaking at 180 rpm. The samples were removed after 8 days of incubation and centrifuged at 7500 rpm for 10 min at 10˚C. The supernatant was analyzed for cellulase, amylase, and protease enzymes. Protein was estimated according to Lowry method with bovine serum albumin as standard [15].

2.2. Enzyme Assays

Cellulase (FPase) was determined by DNS method as reported earlier [14]. The assay mixture of 2 ml contained 1.9 ml citrate buffer (50 mM, pH 4.5) and Whatman filter paper no. 1 (50 mg, 2 - 6 cm) and 0.1 ml of suitably diluted supernatant. The reaction was initiated by the addition of enzyme and the assay mixture was incubated at 50˚C for 60 min. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose produced per min under the assay conditions. The α-amylase activity was determined by the method of Bernfield [16]. The reaction mixture of 1 ml consisting of 0.5 ml of suitable diluted supernatant and 0.5 ml of 1% soluble starch in 50 mM citrate buffer, pH 4.5 was incubated at 50˚C for 10 min. The reducing sugars released were estimated as glucose equivalents by DNS method. One unit of enzyme activity was defined as 1 umole of sugar released per min per ml of culture filtrate. Protease activity was determined as reported earlier using Bovine Serum Albumin (BSA) as substrate [17]. The reaction mixture of 2 ml contained 10 mg of BSA in 50 mM sodium citrate buffer, pH 4.5 and suitably diluted culture filtrate containing the enzyme. The reaction was incubated for 60 min at 50˚C and the reaction was terminated by adding 3 ml of 5% tri-chloroacetic acid. The acid soluble material was estimated at 280 nm after removing the precipitate by filtration through Whatman No. 1 filter paper. One unit of activity was de-

fined as the amount of enzyme which catalyzed the release of 1 µmol of tyrosine per min per ml of the culture filtrate.

2.3. Biofilm Treatment with Enzyme, Crystal Violet (CV) Assay and Scanning Electron Microscopy

To test the efficacy of enzyme preparation, 1.5 ml (Total protein 10 mg, 5 mg, 2.5 mg) of filter sterilized crude enzyme in 50 mM sodium citrate buffer, pH 4.5 was added to each well and kept at 50˚C for 1 hr in Heidolph Titramax-1000 incubator with shaking 450 rpm. Wells containing citrate buffer without enzyme were used as control. After incubation for 1 h, the degradation was visualized by CV assay. Crystal violet (CV) assay was performed as described by Trivedi *et al.* [18]. The washed biofilms were stained with 0.1% CV for 10 min and washed again with saline. The CV dye was eluted with 95% of ethanol and quantified by measuring OD_{600} with a microplate reader (Spectramaxplus 384 plate reader, Molecular Devices Inc.). Blank samples with LB in absence bacteria were included in all CV assays. The OD_{600} readings of the blanks were subtracted from the OD_{600} readings of the test samples to account for any non-specific binding of CV in the wells. A modified SEM method was used for analysis of biofilms of all four organisms [19]. All test samples were fixed in 2% glutaraldehyde at 4˚C for 2 h, washed with 100 mM PBS two times and then dehydrated in a gradient alcohol concentration (30%, 50%, 80%, and 90%) in water for 10 min at 4˚C. The samples were further dehydrated with 100% ethanol at 4˚C for 10 min. The specimen was left in absolute alcohol and mounted onto an aluminum stub with carbon tape, sputter-coated with gold before examination.

2.4. Effect of pH and Temperature on Biofilm Removal

The optimum temperature of biofilm removal was determined by treating the biofilms with enzyme mixture containing 5 mg of total protein in 50 mM citrate buffer, pH 4.5. The biofilms along with the enzymes were incubated at different temperatures ranging from 40˚C to 50˚C for 1 h in shaking plate incubator (450 rpm). The biofilms incubated in citrate buffer with no enzyme were considered as control. The effect of different pH on biofilm removal was determined by treating the biofilms in buffers at various pH (3.0 - 6.0) containing enzyme and incubating them at 50˚C in shaking plate incubator (450 rpm) with taking appropriate control. The removal of the biofilms was determined using CV assay as described earlier.

2.5. Estimation of Carbohydrate and Protein Content of Biofilm

For comparison between control vs enzyme treated biofilms, the EPS was extracted by modified formaldehyde-NaOH method [20]. Briefly, each well was added 1 ml of 10% formaldehyde solution and kept at 4˚C for 1 h. The formaldehyde solution was removed gently and added 1 ml of 1M NaOH solution prepared in distilled water and kept at 4˚C for 2 h under shaking. The suspension of

each well was collected and filtered by 0.22 micron filter. The filtrate was dialyzed (3 kDa membrane) against double distilled water. Total carbohydrate content of biofilms were determined by phenol-sulfuric method using glucose as standard [21] and the protein content was determined by Lowry Method using BSA as standard.

3. Results

3.1. Determination of Enzyme Activity

P. janthinellum EU2D-21 produced high amounts of extracellular cellulase (FPase), CMCase and β-glucosidase under submerged fermentation conditions. In addition to the cellulase, *P. janthinellum* EU2D-21 was found to produce amylase and protease enzymes under submerged fermentation. The specific activities of 2.8, 3.0 and 3.4 IU/mg were for cellulase, amylase and protease respectively (**Table 1**). This enzyme preparation was further evaluated for degradation of biofilms produced by *Escherichia coli* NCIM 2674, *Salmonella enterica* NCIM 2257, *Pseudomonas aerogenosa* NCIM 2200 *and Staphylococcus aureus* NCIM 5021.

3.2. Evaluation of Enzyme Efficacy for Biofilm Degradation

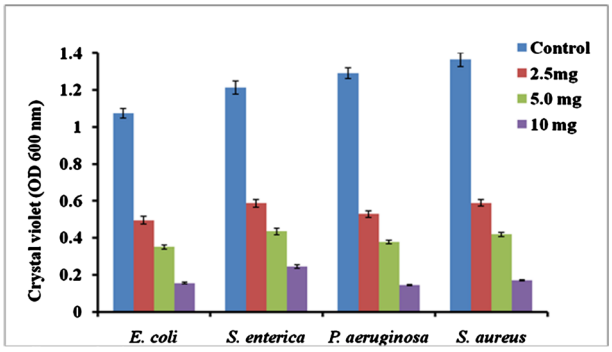
The crude extracellular enzyme preparation produced by fungal strain *P. janthinellum* EU2D-21 was used for the bacterial biofilm degradation. The CV assay was used to determine the activity of enzymes on biofilms. Crude extracellular enzyme (containing total 10 mg of protein) showed the highest percentage removal of biofilms produced by *Escherichia coli* (85.5%), *Salmonella enterica* (79.72%), *Pseudomonas aeruginosa* (88.76%) *and Staphyloccus aureus* (87.42%) in 1 hour at 50˚C (**Figure 1(a)**). The removal of the biofilms by the enzyme was also analyzed visibly using CV assay (**Figure 1(b)**). SEM analysis confirmed that crude extract of *P. janthinellum* could effectively degrade the biofilms formed by all tested bacterial strains. The SEM observations of the biofilms revealed that the untreated biofilms were dense and looked like organized structure with clear marked microcolonies. However, the enzyme treated biofilms were lacking such structures (**Figure 2**) showing the consistency with the CV assay observations.

3.3. Effect of pH and Temperature on Biofilm Reduction

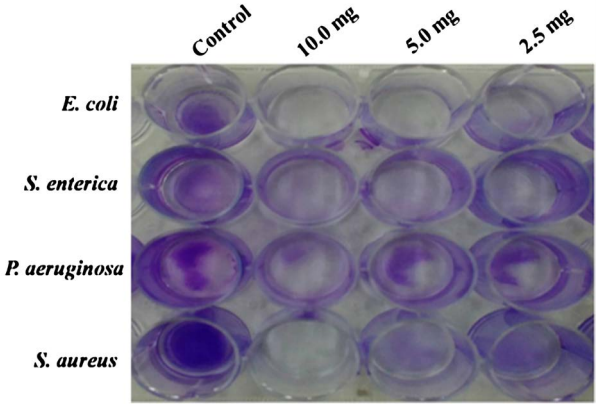
Maximum biofilms reduction was observed maximum at pH 4.5 at 50˚C with a significant loss (>30%) of biofilm degradation ability at pH 3.0 for each tested

Table 1. Enzyme activities of crude broth from *P. janthinellum* EU2D-21.

Enzyme	Specific Activity (IU/mg)
Celllulase	2.85
Alpha-amylase	3.08
Protease	3.46



(a)



(b)

Figure 1. (a) Quantitative biofilm reduction using different concentration of enzyme range from 2.5 - 10 mg produced by *Penicillium janthinellum* EU2D-21; (b) Wells of crystal violet staining with control biofims of each bacterial strain followed by enzyme treated at differnet total enzyme (total potein ranging from 2.5 - 10 mg).

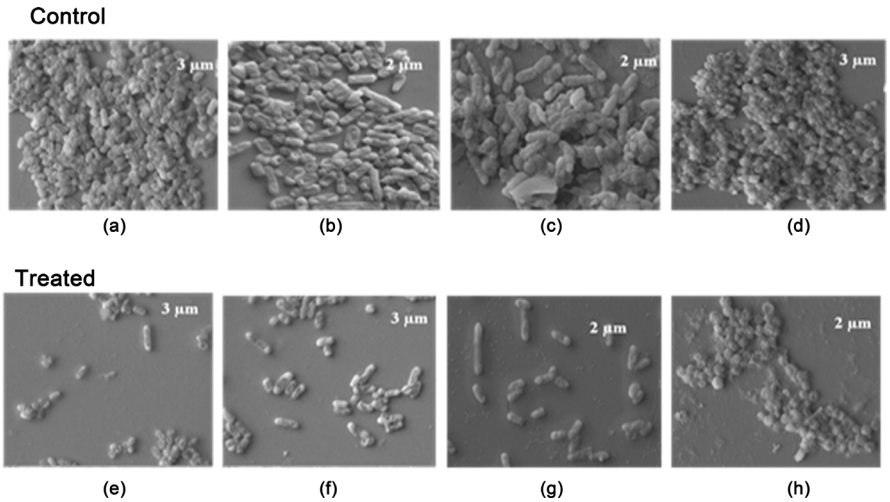


Figure 2. Visualization of enzyme effect on bacterial biofilms. (a)-(d): 1% CV staining of *Escherichia coli* (*NCIM* 2674), *Salmonella enterica* (*NCIM* 2257), *pseudomonas aeruginosa* (*NCIM* 2200), *Salmonella enterica NCIM* 2257 respectively without enzymatic treatment (Control) (e)-(h) 1% CV staining of all above respective biofilms with 5 mg of crude enzyme treatment in 50 mM citrate buffer pH 4.5 for 1 h. The magnification is 100x with oil immersion lense.

bacterial biofilms. However, at pH 6.0, enzyme showed around less than 15% of biofilm reduction for all tested bacterial biofilms (Figure 3). Maximum biofilms reduction was obtained at 50˚C with decrease in biofilm degradation at temperatures 40˚C as shown in the figure (Figure 4(a)). The effect of temperature on biofilm

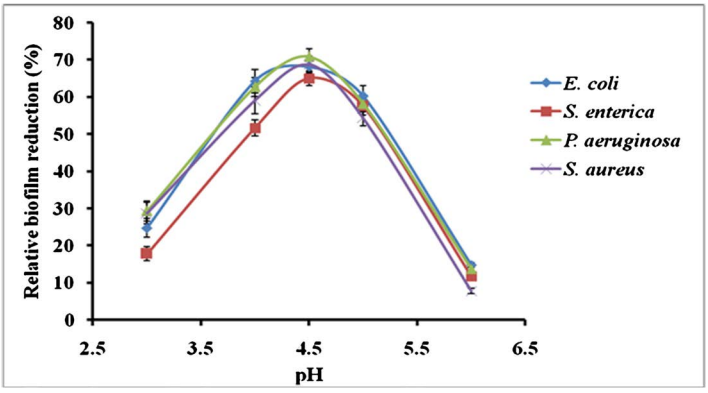
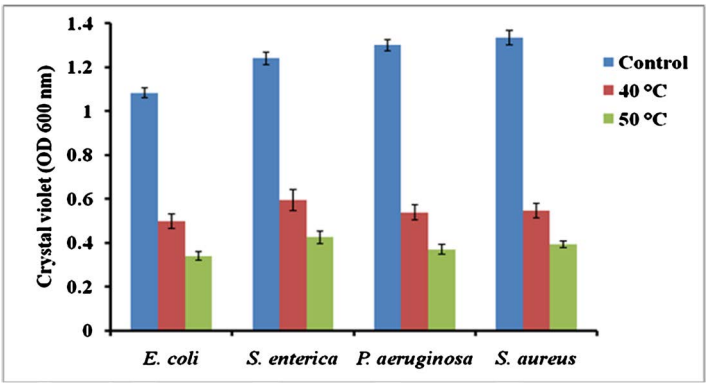
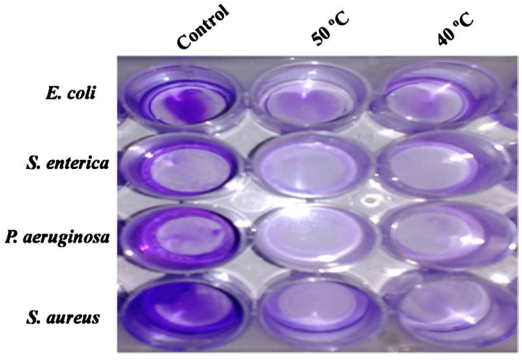


Figure 3. Effect of pH (3 - 6) on Biofilm reduction using 5 mg of enzyme and incubated at 50˚C for 1 h.



(a)



(b)

Figure 4. (a) Effect of temperature on Biofilm reduction using 5 mg of enzyme and incubated at different temperature for 1 h. The biofilms incubated in citrate buffer with no enzyme were considered as control; (b) Wells of crystal violet staining with control biofims of each bacterial strain followed by enzyme treated using 5 mg of total protein content at different temperature for 1 hr of incubation time. The biofilms incubated in citrate buffer with no enzyme were considered as control.

degradation was also evaluated using CV assay (Figure 4(b)) and it showed similar pattern.

3.4. Estimation of Carbohydrate and Protein from Biofilm

The carbohydrate and protein content of enzyme treated and untreated bacterial biofilms has been summarized in Table 2. After enzymatic treatment, the carbohydrate and protein content of the biofilms was found to be reduced which is indicative of actions of cellulase, amylase and protease enzymes present in the enzyme preparation.

4. Discussion

One of the prominent features of the biofilms is the exo-polysaccharide (EPS) matrix that embeds the associated bacteria. This matrix plays a major role in protection of associated bacteria due to its structural stability in addition to its role in nutrient delivery and genetic transfer. However, majority of the antimicrobial agents do not reach the embedded bacterial cells due to the presence of biofilms formed by the bacteria which act as a barrier and protect the bacterial cells. Therefore, the disruption of the biofilm matrix is essential for reducing the defenses of the embedded bacteria. The matrix (EPS) contains three components namely, carbohydrates, proteins and extracellular DNA (eDNA). Active degradation of matrix components is mediated by number of compounds. DNases have been used for degradation of eDNA in the biofilms leading to disruption of biofilm matrix [22] [23]. However, there are certain limitations in using DNases since they are effective only on nascent biofilms and not on matured biofilms. In addition, DNases are expensive and hence their use in biofilm disruption is limited. Though most of the compounds are unable to degrade matured biofilms, nitric oxide [24], *cis*-2 decenoic acid [25] and anti-biofilm peptide [26] showed both prevention and disruption of *P. aeruginosa* biofilms. However, these compounds required more than 24 h incubation for efficient degradation of *P. aeruginosa* established biofilms. Antimicrobial peptides such as glycoside or acid hydrolases produced by microbes are most effective in degradation of biofilm components leading to disruption of structural integrity of the matrix [12] [27]. A number of proteases, especially serine proteases, and amylases showed the

Table 2. Comparison of total carbohydrate and protein content of each biofilm without enzyme/enzyme treated biofilms.

Organism	Without Enzyme		Enzyme treated	
	Carbohydrate (µg)	Protein (µg)	Carbohydrate (µg)	Protein (µg)
E. coli (NCIM 2674)	88.65 ± 2.88	46.52 ± 1.1	10.88 ± 0.56	7.75 ± 0.52
S. enterica	67.16 ± 1.64	51.23 ± 0.98	11.62 ± 0.73	8.42 ± 0.34
P. aeruginosa	102.09 ± 2.32	40.23 ± 1.25	13.25 ± 0.82	6.24 ± 0.44
S. aureus	59.1 ± 1.54	35.11 ± 1.02	7.24 ± 0.48	5.57 ± 0.32

capability of degrading biofilms with varying degrees of success [28] [29]. In our study, the enzyme preparation produced by *P. janthinellum*, EU2D-21 consisted of cellulase, amylase and protease enzymes when grown in cellulose containing medium. It appears to be a potent disruptor/degrader of the biofilms produced by *E.coli, S. enterica, P. aeruginosa and S aureus*. Maximum biofilm degradation was found within 1 h against *E. coli* and *P. aeruginosa* showing 80% and 78% biofilm reduction respectively in terms of carbohydrate removal. *Aspergillus clavatus* MTCC 1323 produce enzyme preparation with protease, amylase and pectinase activities under solid state fermentation. This enzyme degraded the biofilms of *Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus* after 7 days of incubation [11]. Newly formulated enzyme cleaners, known as Deconex Prozyme Active consisted of mixture of four enzymes viz. protease, polysaccharidases, lipases and DNases. This enzyme formulation degraded 95% of the biofilms formed by clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* [30]. Deconex Prozyme Active was proved to be the best in the removal of biofilms of clinical isolates when compared to the commercially available enzymatic detergents. Our enzyme preparation pf *P. janthinellum* is comparable to commercial enzyme formulation (deconex Prozyme active) which can cleave around 90% biofilm produced by *Pseudomonas aeruginosa and Staphyloccus aureus*. Watters *et al.* [10] tested commercially available ά-amylase, bromelain, lysostaphin and papain against the biofilms of *S. aureus*. They found maximum biomass was decreased (98%) by ά-amylase, bromelain, and papain whereas lysostaphin reduced the biomass up to 75%. Biofilms formed by *Mycobacterium tuberculosis* is primarily composed of polysaccharides with cellulose as a key component [18]. Our *P. janthinellum* enzyme could be the potential candidate to degrade such cellulose containing biofilms to prevent *M. tuberculosis* infections.

5. Conclusion

Bacterial biofilms are single or multispecies microbial communities that are composed of cells embedded in self-produced extracellular polymeric substances (EPS). These biofilms protect the embedded bacteria from sheer stress, disinfectants, host immune defenses and antibiotics which lead to perpetual bacterial infections. The use of novel therapies to combat perpetual bacterial infections is a promising strategy to improve patient outcomes. The most commonly used enzymes for dispersal of biofilms are glycoside hydrolases such as DNase, amylases, proteases, and cellulases. Our study focused on evaluation of extracellular enzyme preparation of *Penicillium janthinellum* EU2D-21 for degradation of biofilms formed by *Escherichia coli, Stphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella enterica.* The enzyme of *P. janthinellum* EU2D-21 consisted of cellulase, protease and amylase which degraded the selected bacterial biofilms using within 1 h of incubation. The carbohydrate and protein content of treated biofilm were drastically reduced indicating the biofilm degrading abil-

ity of the enzyme. Future studies will be carried out on the degradation of biofilms formed by clinical bacterial cultures isolated from wound pathogens.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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