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CMCase produced by a novel *Bacillus subtilis* strain Fatma/1 degraded bacterial biofilms of some nosocomial pathogens *in vitro*

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Abstract

Nosocomial bacteria's ability to produce biofilms helps them survive in a variety of environments, such as hospitals, wounds, and medical devices. Cellulases can destroy cellulose, one of the major structural components of biofilms, representing an important part of the bacterial biofilm matrix.

Bacterial strains were obtained from diabetic foot hospital patients and tested for their ability to produce biofilms *in vitro.* Isolates were identified as *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* using a standard set of biochemical assays commonly used at hospital laboratory. Biofilm degradation by CMCase enzyme was evaluated through *in vitro* tube method, microscopic observation and crystal violet assay.

CMCase had a high effectiveness in eliminating P. aeruginosa biofilms and a modest capacity to remove biofilms of other strains used in this study. Light microscopy demonstrated fully disseminated cells of *P. aeruginosa* biofilm exposed to CMCase. Besides, Using CMCase dramatically eliminated 87.5% of the carbohydrate content of its biofilm matrix.

Keywords: CMCase; Bacterial biofilm; *Pseudomonas aeruginosa*; *Bacillus subtilis*; Nosocomial infections.

1. Introduction

Biofilms are microbial aggregates in which cells are encased in a self-produced extracellular polymeric substance (EPS) attached to a surface [1]. After bacteria colonize a biotic surface or a medical implant, it instantly switches from motile to adherent or biofilm mode of growth [2].

The polysaccharide matrix is composed of up to 97% water, soluble components such as polysaccharides, proteins and extracellular DNA [3] along with insoluble components such as lipids, cellulose [4], pili, fimbriae, and flagella [5]. According to Makabenta *et al*. [6], the extracellular polymeric compounds form a protective shield against the passage of antimicrobials, or other destructive compounds produced by the host. Biofilms also protect bacteria from extreme environmental conditions, including ultraviolet light, pH fluctuations, salinity, nutrient depletion and desiccation [7].

Biofilms account for up to 80% of all bacterial human infectious diseases, such as endocarditis, wound infections, meningitis, and infections related to indwelling devices [8]. Biofilms can cause chronic wound infections, particularly when a prosthetic device is implanted [9]. Biofilms can potentially lead to an aggressive infection, assisting resistance to antibiotics and posing a threat of toxicity to tissues because of the topical agents used in treatment [10]. According

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to Elgharably *et al*. [11], Biofilm formation by a specific organism is a critical factor in the invasion and stability of infections, as it insulates pathogens from phagocytes and creates a physical shield which promotes antibiotic resistance [12]. Being confined within a biofilm protective substance, pathogens can hide their antigens, weaken antibiotic efficacy, and attenuate the protective immunity, stimulating persistence infections such as endocarditis, chronic renal stones, and cystic fibrosis while sacrificing free movement throughout the body [13,14].

A wide range of microbes release cellulases while living on cellulolytic materials [15]. Cellulase-producing bacteria and fungi were isolated from multiple sources such as agricultural wastes, soil, sediments, seawater, animal excreta and so on [16]. Cellulases degrade cellulose, one of the major components of biofilms, representing an important part of the biofilm matrix [3,17]. Few studies have applied bacterial cellulose degrading enzymes mostly produced by *Bacillus* species as biofilm degrading formulas to clean medical implants [18], prosthetic devices [19] and curing hospital acquired infections [20]. Such biofilm removal approaches, are critical alternates to antibiotics in the fight against major bacterial infections [21]. Effective microbial treatment demands the development of new biofilm degrading approaches.

In the current study, CMCase enzyme isolated form a novel strain of *Bacillus subtilis* was evaluated at different concentrations for its ability to degrade biofilms of some hospital-acquired pathogens via tube method, microscopic observation, crystal violet assay and measuring the biofilm's carbohydrate content.

2. Material and methods

2.1. Carboxymethyl cellulase producing strain and enzyme production

Carboxymethyl cellulase producing strain used in this study was identified as *Bacillus subtilis* and submitted to GenBank with the accession number LC535007.1**.**

The strain was grown on fresh liquid medium (containing: (g/l): Carboxymethylcellulose (CMC), 18; yeast extract,9; K_2HPO_4,2; peptone, 2; $MgSO_4 \cdot 7H_2O$, 0.05; $MnCl_2.4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.05; and pH adjusted to 7.0) at 180 rpm and 37˚C for 24 hrs. To expel vegetative cells and excess media, the inoculated medium was centrifuged at 14,000 × g for 15 minutes. The pellet was discarded, and the remaining liquid was recovered and used as a crude CMCase.

2.2. Enzyme assay

The activity of CMCase was determined by employing CMC (1% w/v) as a substrate. CMC was solubilized in 0.05 M Na-citrate buffer (pH 5.0) by swirling continuously for 1 hour at 60°C, followed by sedimentation at 10,000 g for 10 minutes to achieve a homogeneous liquid. Then,100 µL of crude enzyme specimen was mixed with 900 µL of the substrate and maintained for 10 minutes at 50°C. The reaction was halted via supplementing the mixture with 1.5 mL of dinitrosalicylic acid (DNS reagent) which was then boiled for 5 minutes. The amount of reducing sugars was estimated at 540 nm against sample blank. One unit (1U) of CMCase activity was described as the quantity of the enzyme needed to create 1µmol of reducing sugar per minute at test conditions [22].

2.3. Isolation and identification of biofilm- forming strains

Swab samples obtained from diabetic foot hospital patients were used to collect pathogenic microorganisms. MacConkey agar was used to culture Gram negative bacterial pathogens whereas Gram positive strains were grown on Mannitol salt agar. Pure strain cultures were maintained in 20% glycerol and stored at -18°C. A standard set of biochemical assays commonly used at hospital laboratory were used to identify different clinical isolates.

2.4. Biofilm development by different clinical isolates

Aliquots of 200 µL of brain heart infusion broth (BHI) were delivered into microtubes (1.5 mL) under aseptic conditions. Each pathogenic bacterial strain (1×10^6 CFU/mL) was cultured in 10 µL of broth media and then transferred individually into the microtubes and aerobically grown at 37°C for 24 hrs. Tubes were then examined for their ability to develop biofilms.

2.5. Tube method for identification of biofilm producing bacteria

For identification of biofilm producer microorganisms, the method of Christensen *et al*. [23] was used with little modification. Isolates are inoculated into tubes of BHI liquid media and incubated at 37°C for 24 hrs. The tubes were supplied with CMCase enzyme (12 U/mL) and allowed to sit for an hour. Tubes with no CMCase were used as control. The biofilm formed on the tube walls were dyed with safranine and left for 1 hour. The dye was then removed by double rinsing the tubes using phosphate-buffered saline (PBS). Tubes were dried in inverted position, and biofilm degradation capacity of CMCase was assessed depending on color intensity of the ring formed. The test was performed in triplicate.

2.6. Crystal violet assay of different species biofilms treated with CMCase

The efficacy of the CMCase in the degrading the biofilms of different strains was investigated using the technique of Trivedi *et al*. [24] on polystyrene plates. CMCase at different activities (4, 8 and 12 U/mL) was supplied to overnight bacterial culture (10^6 CFU/mL) of each pathogenic strain grown in brain heart infusion broth. After incubation for 24 hrs at 37° C, the polystyrene plates were washed with deionized water to discard dispersed cells and medium. Plates were allowed to dry before adding crystal violet.

The wells were dyed using 400 µl of 0.1% (w/v) CV solution for 10 min. After that, CV was disposed, and the wells were double washed with saline. The wells were left to air dry prior to applying 1 ml of 95% ethanol to dissolve CV stain. The color intensities of the biofilms were then determined by reading OD_{600} in RayBiotech microplate reader. The test was performed in triplicate.

2.7. Microscopic examination of biofilms of different strains

Each strain's biofilm was cultivated at 37°C on glass pieces (1 cm^3) fixed on microtiter plate containing culture medium. After 24 hrs of growth, CMCase was applied. Glass pieces containing intact or degraded biofilms were then mounted on glass slides and stained using Gram staining method. Control specimens containing biofilms without treatment were also stained and examined. Light microscopy was used to examine stained glass pieces which were then photographed using mobile camera.

2.8. Bactericidal effect of CMCase on different planktonic cells

The impact of CMCase at varied activities (4,8 and12 U/mL) on the growth or different bacterial strains used in this study was evaluated via Kirby-Bauer test on plates containing Mueller Hinton agar.

2.9. Determination of the biofilm's carbohydrate content

The formaldehyde-NaOH technique of [25] was used to extract the exo-polymeric matrix of biofilms of different control strains or specimens treated with CMCase (12 U/mL). Each well was supplied with 10% formaldehyde solution and the plate was maintained under shaking conditions for 1 hr at 4˚C. The reagent was then disposed and 1 mL of 1M NaOH solution was applied. The plate was maintained at shaker adjusted at 4°C for 2 hrs. Each treatment's solution was recovered from wells and filtered using a 0.2µm filter.

The product was then dialyzed against double dis.H_2O. The pellet was used to quantify the amount of total carbohydrate present in EPSs of treated and untreated bacterial biofilms using the phenol-sulfuric technique of Masuko *et al.* [26].

3. Results and discussion

3.1. Identification of bacterial pathogens

Isolates were identified as *Staphylococcus aureus* (Sa), *Acinetobacter baumannii* (Ap), *Klebsiella pneumoniae* (Kp), *Pseudomonas aeruginosa* (Pa), and *Escherichia coli* (Ec). E. *coli* strain was identified as lactose fermenting, indole positive, and oxidase negative rods., while K. *pneumoniae* were indole negative, ornithine positive, urea and citrate positive rod. P. *aeruginosa* were identified as non-lactose fermenter, gram negative, oxidase positive rods. Non-lactose fermenter, gram negative, oxidase negative cocci were identified as A. *baumannii*. S. *aureus* appeared as golden yellow colonies on Mannitol salt agar, coagulase, and catalase positive cocci.

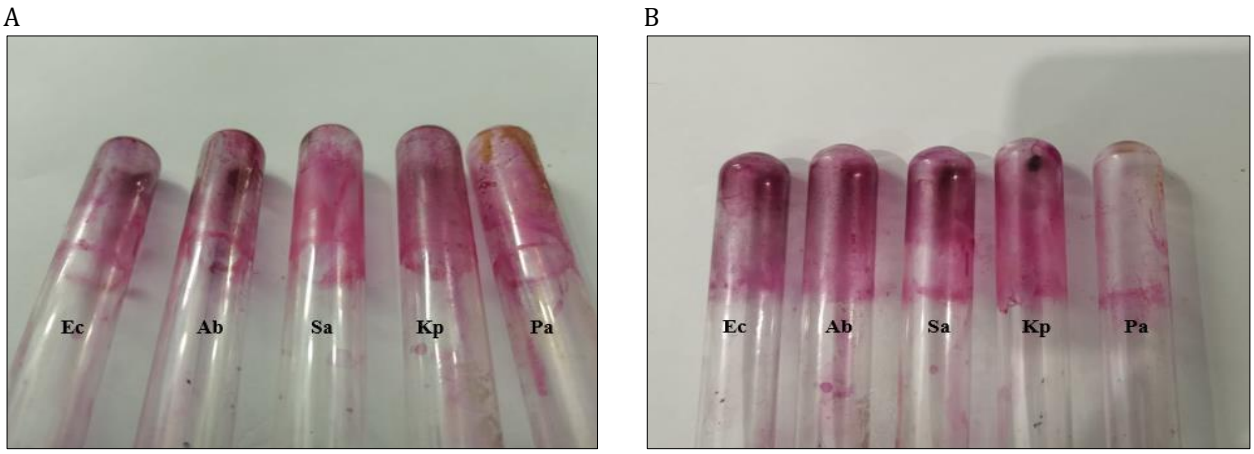

Figure 1 Assessment of biofilm degradation capacity of CMCase depending on pigment intensity of the formed safranin ring (A) control of different biofilms of *P. aeruginosa* (Pa), *K. pneumoniae* (Kp), *S. aureus* (Sa), *A. baumannii* (Ap) and *E. coli* (Ec) (B) biofilms of *P. aeruginosa* (Pa), *K. pneumoniae* (Kp), *S. aureus* (Sa), *A. baumannii* (Ap) and *E. coli* (Ec) treated with CMCase

4. Tube method

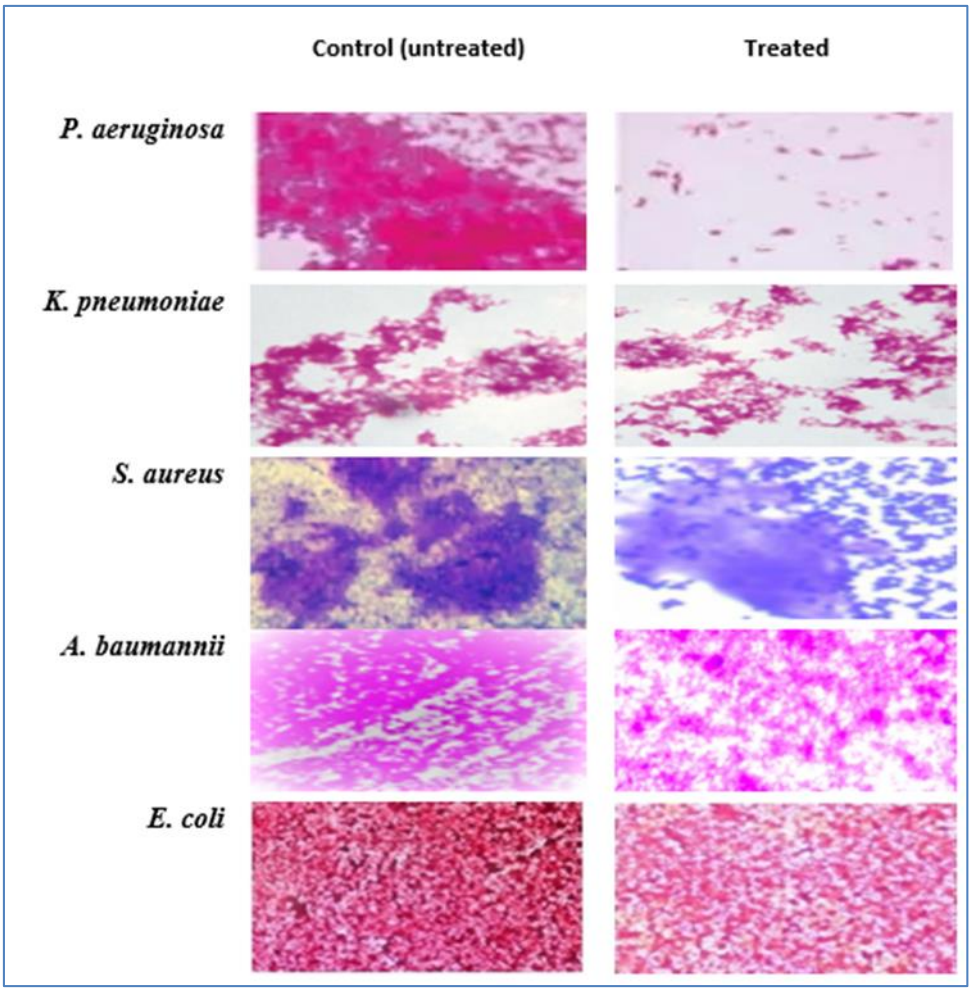

Figure 2 Light microscopy images (40×) showing biofilm dispersal of various pathogenic bacteria before and after 24 hrs of enzymatic treatment using CMCase

The ability of CMCase to degrade the biofilm of different strains was assessed by tube method as illustrated in Figure 1. The least color intensity was obtained after treating the biofilm of *Pseudomonas aeruginosa* with CMCase. Treating the biofilm of *Klebsiella pneumoniae* (Kp) with CMCase also resulted in a mean reduction in color intensity of the stain which means that CMCase efficiently degraded the previously formed biofilm. Reduction in color intensity was also achieved with other strains but to a lesser degree. The best result was obtained with *Pseudomonas aeruginosa* followed by *Klebsiella pneumoniae* (Kp), *Staphylococcus aureus* (Sa), *Acinetobacter baumannii* (Ap) and *Escherichia coli* (Ec), respectively. Thus, CMCase revealed high potency in eliminating *P. aeruginosa* biofilms and medium capacity in degrading the EPS of other strains used in this study.

4.1. Examination of biofilms via light microscopy

The anti-biofilm effect of CMCase was assayed through light microscopic images displayed at Figure 2. Glass pieces containing the biofilm either control or enzymatic treated were stained by Gram stain and placed on slides that were checked for biofilm formation or dissemination using light microscopy.

Specimens of *Klebsiella pneumoniae* (Kp), *Staphylococcus aureus* (Sa), *Acinetobacter baumannii* (Ap) and *Escherichia coli* (Ec) biofilms treated with CMCase exhibited relatively moderate dissemination of the biofilm substance whereas specimens of *P. aeruginosa* biofilms treated with CMCase exhibited completely disseminated cells as shown in Figure 2. CMCase alone revealed promising anti-biofilm effect against the biofilms of *P. aeruginosa.* Other enzymes or formulas may be required to assist CMCase for complete dispersion of the biofilms of *Klebsiella pneumoniae* (Kp), *Staphylococcus aureus* (Sa), *Acinetobacter baumannii* (Ap) and *Escherichia coli* (Ec).

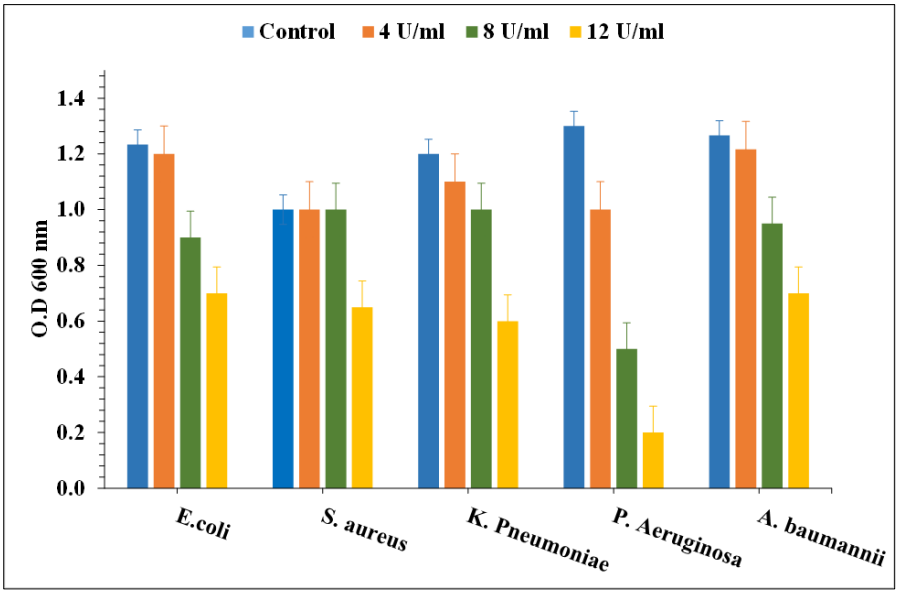

Figure 3 Crystal violet assay showing biofilm dispersal of various pathogenic bacteria after 24 hrs of CMCase treatment

4.2. Crystal violet assay of different species biofilms treated with CMCase

CMCase's potential to degrade biofilms produced by bacterial pathogens in BHI was evaluated. P. aeruginosa biofilms were considerably diminished when exposed to varied doses of CMCase enzyme with 85% of the biofilm being degraded using CMCase at 12U/mL, however *S. aureus* biofilms were more tolerant to CMCase at concentrations of (4 and 8 U/mL) showing a notable decrease (35%) in biofilm at 12 U/mL CMCase (Figure 3).

Biofilms of K. *pneumoniae,* A. *baumannii* and E. *coli* were clearly degraded using different concentrations of CMCase with 12 U/mL exhibiting the highest degradation rate of 50, 44.7 and 43.24% respectively as revealed in Figure 3.

4.3. Antimicrobial action of CMCase on vegetative cells

CMCase enzyme revealed no antibacterial effect on tested pathogens. No inhibition zones surrounding any disc filled with CMCase at different activities. Trizna *et al.* [27] observed the same results during his work on P. *aeruginosa*.

4.4. Determination of carbohydrate concentrations in the biofilm EPS

The overall amount of carbohydrate in extracted EPs was determined pre and post hydrolytic treatments with CMCase. The carbohydrate concentration in the EPS matrix of biofilms were significantly reduced after treatment with CMCase as seen in Table 1. This demonstrates the importance of the CMCase enzyme in eradication of biofilms of different strains.

Table 1 The net carbohydrate constituent of biofilms from different isolates were compared pre and post enzymatic treatment with CMCase

Microorganism	**Pre- enzymatic treatment**	**Post- enzymatic treatment**
	Carbohydrate(µg)	Carbohydrate(µg)
E. coli (Ec)	82.4±0.09	69.8±0.45
S. aureus (Sa)	127.8±0.46	67.4±0.09
K. Pneumoniae (Kp)	93±0.65	54.8±0.5
P. aeruginosa (Pa)	87.47±0.4	10.9±0.07
A. baumannii (Ap)	57.3±0.4	38.65±0.4

The CMCase enzyme significantly eliminated 87.5 % of the carbohydrate content of *P. aeruginosa* 's biofilm structure although eradicated about 47.26% of *S. aureus*'s biofilm matrix. About 41 % of the carbohydrate content of the EPS of *K. Pneumoniae (Kp)* was eliminated. However, 67.45% and 84.7 % of the carbohydrate matrix of *A. baumannii (Ap)* and *E. coli (Ec)* respectively remained after the treatment. This suggests the high content of cellulose and related substances present in the biofilm of *P. aeruginosa.*

The nature and constituents of the biofilm matrix determines what appropriate biofilm decomposing substances should be used [28]. Depending on the main components of the biofilm, certain enzyme or a combination of enzymes would be effective [29].

It has been claimed that releasing cells from the biofilm's insulating layer makes them highly vulnerable to antibiotics and the defensive reaction of the host [30]. Numerous studies have documented the efficient destruction of extracellular domains by the cellulases [18,31]. Biological enzymes are generally produced and employed by microbes for the degradation and dissemination of biofilms, therefore their utilization is a natural process [30].

P. aeruginosa generates at least three polysaccharides (alginate, Pel, and Psl) that impact the integrity of its biofilm structure [32]. Pel is an exopolysaccharide made up of 1-4 glycosidic bonds connecting N-acetylgalactosamine and N-acetylglucosamine [33]. CMCase could be useful as an antibiofilm agent since it can cleave internal -(1–4) or -(1–3) bonds of the polysaccharide chains of the biofilm.

Pseudomonas aeruginosa is a well-known human pathogen that causes a variety of illnesses, especially in immunodeficient patients as it releases multiple virulence factors [32]. Furthermore, *P. aeruginosa*'s exceptional ability to develop biofilms in a variety of conditions makes antibiotic treatments ineffective thus, promoting chronic infectious illnesses [34]. Since enzyme-based detergents have been used safely for many years, cellulase can be used safely on human skin without any risk [35]. It follows from the above that CMCase provides a promising agent for complete dispersion or removal of *P. aeruginosa*'s biofilm.

5. Conclusion

CMCase enzyme isolated from the novel bacterial strain showed a promising ability to degrade the biofilm produced by the nosocomial pathogen *Pseudomonas aeruginosa* via degradation of the cellulose which is a major content of its biofilm. CMCase also showed a great reduction in the biofilm`s carbohydrate content. CMCase can be used effectively in treating infections due to *P. aeruginosa* along with cleaning medical implants and prosthetic devices after in vivo studies. CMCase partially degraded the biofilms of *Escherichia coli*, *Klebsiella pneumoniae, Acinetobacter baumannii* and

Staphylococcus aureus. Thus, more studies are required to investigate the contents of their biofilms to use the suitable enzyme or a mixture of enzymes.

Compliance with ethical standards

Acknowledgments

Acknowledgments must be inserted here.

Disclosure of conflict of interest

The authors declare that they have no competing interests. Authors' contribution FMA carried out experiments and wrote the manuscript. RH assisted the scientific experiments, provided some instruments, as well as instructions for writing and editing. AMI proposed the research concept, designed the experiments, and provided necessary materials and instruments for experiments. All authors read and approved the manuscript.

References

[1] Karygianni L, Ren Z, Koo H, Thurnheer T. Biofilm matrixome: extracellular components in structured microbial communities. Trends in Microbiology. 1 Aug 2020; 28(8): 668-81.

[2] Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrobial Resistance & Infection Control. Dec 2019; 8(1): 1-0.

[3] Flemming HC, Wingender J. The biofilm matrix. Nature reviews microbiology. Sep 2010; 8(9): 623-33.

[4] Serra DO, Richter AM, Klauck G, Mika F, Hengge R. Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. MBio. 19 Mar 2013; 4(2): e00103-13.

[5] Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. FEMS microbiology reviews. Sep 2015; 39(5): 649-69.

[6] Makabenta JM, Nabawy A, Li CH, Schmidt-Malan S, Patel R, Rotello VM. Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. Nature Reviews Microbiology. Jan 2021; 19(1): 23-36.

[7] Yin W, Wang Y, Liu L, He J. Biofilms: the microbial "protective clothing" in extreme environments. International journal of molecular sciences. Jan 2019; 20(14): 3423.

[8] Khatoon Z, McTiernan CD, Suuronen EJ, Mah TF, Alarcon EI. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. Heliyon. 1 Dec 2018; 4(12): e01067.

[9] Akers KS, Mende K, Cheatle KA, Zera WC, Yu X, Beckius ML, Aggarwal D, Li P, Sanchez CJ, Wenke JC, Weintrob AC. Biofilms and persistent wound infections in United States military trauma patients: a case–control analysis. BMC infectious diseases. Dec 2014; 14(1): 1-1.

[10] Bianchi T, Wolcott RD, Peghetti A, Leaper D, Cutting K, Polignano R, Rosa Rita Z, Moscatelli A, Greco A, Romanelli M, Pancani S. Recommendations for the management of biofilm: a consensus document. Journal of wound care. 2 Jun 2016; 25(6): 305-17.

[11] Elgharably H, Mann E, Awad H, Ganesh K, Ghatak PD, Gordillo G, Sai-Sudhakar CB, Roy S, Wozniak DJ, Sen CK. First evidence of sternal wound biofilm following cardiac surgery. PloS one. 1 Aug 2013; 8(8): e70360.

[12] Goodman SD, Obergfell KP, Jurcisek JA, Novotny LA, Downey JS, Ayala EA, Tjokro N, Li B, Justice SS, Bakaletz LO. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. Mucosal immunology. Nov 2011; 4(6): 625-37.

[13] Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annual Reviews in Microbiology. Oct 2003; 57(1): 677-701.

[14] Edwards R, Harding KG. Bacteria and wound healing. Current opinion in infectious diseases. 1 Apr 2004; 17(2): 91-6.

[15] Thapa S, Mishra J, Arora N, Mishra P, Li H, O' Hair J, Bhatti S, Zhou S. Microbial cellulolytic enzymes: diversity and biotechnology with reference to lignocellulosic biomass degradation. Reviews in Environmental Science and Bio/Technology. Sep 2020; 19(3): 621-48.

[16] Gunavathy P, Boominathan M. Isolation and Molecular Characterization of Cellulose Producing Bacteria from Soil of Sacred Grove, Puducherry, India, International Journal of Applied Research Journal of Current Microbiology and Applied Sciences. May 2016; 4(12): 585-590.

[17] McCrate OA, Zhou X, Reichhardt C, Cegelski L. Sum of the parts: composition and architecture of the bacterial extracellular matrix. Journal of molecular biology. 15 Nov 2013; 425(22): 4286-94.

[18] Loiselle M, Anderson KW. The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. Biofouling. 1 Apr 2003; 19(2): 77-85.

[19] Rajasekharan SK, Ramesh S. Cellulase inhibits Burkholderia cepacia biofilms on diverse prosthetic materials. Pol J Microbiol. 1 Jan 2013; 62(3): 327-30.

[20] Huertas MG, Zarate L, Acosta IC, Posada L, Cruz DP, Lozano M, Zambrano MM. Klebsiella pneumoniae yfiRNB operon affects biofilm formation, polysaccharide production and drug susceptibility. Microbiology. 1 Dec 2014; 160(12): 2595-606.

[21] Rumbo C, Vallejo JA, Cabral MP, Martínez-Guitián M, Pérez A, Beceiro A, Bou G. Assessment of antivirulence activity of several d-amino acids against Acinetobacter baumannii and Pseudomonas aeruginosa. Journal of Antimicrobial Chemotherapy. 1 Dec 2016; 71(12): 3473-81.

[22] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry. 1 Mar 1959; 31(3): 426-428.

[23] Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. Journal of clinical microbiology. Dec 1985; 22(6): 996-1006.

[24] Trivedi A, Mavi PS, Bhatt D, Kumar A. Thiol reductive stress induces cellulose-anchored biofilm formation in Mycobacterium tuberculosis. Nature communications. 25 Apr 2016; 7(1): 1-5.

[25] Pan M, Zhu L, Chen L, Qiu Y, Wang J. Detection techniques for extracellular polymeric substances in biofilms: a review. BioResources. 4 Aug 2016; 11(3): 8092-115.

[26] Masuko T, Minami A, Iwasaki N, Majima T, Nishimura SI, Lee YC. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. Analytical biochemistry. 1 Apr 2005; 339(1): 69-72.

[27] Trizna E, Bogachev MI, Kayumov A. Degrading of the Pseudomonas aeruginosa biofilm by extracellular levanase SacC from *Bacillus subtilis*. BioNanoScience. Mar 2019; 9(1): 48-52.

[28] Chaignon P, Sadovskaya I, Ragunah C, Ramasubbu N, Kaplan JB, Jabbouri S. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Applied microbiology and biotechnology. May 2007; 75(1): 125-32.

[29] Johansen C, Falholt P, Gram L. Enzymatic removal and disinfection of bacterial biofilms. Applied and environmental microbiology. Sep 1997; 63(9): 3724-8.

[30] Rumbaugh KP, Sauer K. Biofilm dispersion. Nature Reviews Microbiology. Oct 2020; 18(10): 571-86.

[31] Fleming D, Chahin L, Rumbaugh K. Glycoside hydrolases degrade polymicrobial bacterial biofilms in wounds. Antimicrobial agents and chemotherapy. 1 Feb 2017; 61(2): e01998-16.

[32] Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. The formation of biofilms by Pseudomonas aeruginosa: a review of the natural and synthetic compounds interfering with control mechanisms. BioMed research international. Oct 2015.

[33] Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, Secor PR, Tseng BS, Scian M, Filloux A, Wozniak DJ. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix. Proceedings of the National Academy of Sciences. 8 Sep 2015; 112(36): 11353-8.

[34] Bjarnsholt T. The role of bacterial biofilms in chronic infections. Apmis. May 2013; 121: 1-58.

[35] Basketter DA, English JS, Wakelin SH, White IR. Enzymes, detergents and skin: facts and fantasies. British journal of dermatology. Jun 2008; 158(6): 1177-81.