https://dergipark.org.tr/en/pub/turkager 2025, 6(1): 59-73 DOI: 10.46592/turkager.1680203

TURKAGER
TURKISH JOURNAL OF AGRICULTURAL ENGINEERING RESEARCH
https://dergipark.org.tr/tr/pub/turkager
e-ISSN: 2717 - 8420

Inhibition of Quorum Sensing and Biofilm Development by Walnut Rhizosphere Bacteria

Belgin ERDEM[a*] İlkay AÇIKGÖZ ERKAYA[b], Dilek YALÇIN[c]

[a]*Department of Medical Services and Techniques, Health Services Vocational College, University of Kırşehir Ahi Evran, Kırşehir, TÜRKİYE*

[b]*Department of Environmental Engineering, Faculty of Architecture and Engineering, University of Kırşehir Ahi Evran, Kırşehir, TÜRKİYE*

[c]*Department of Food Quality Control and Analysis Program, Kahramankazan Vocational School, University of Başkent University, Ankara, TÜRKİYE*

ARTICLE INFO: **Research Article**
Corresponding Author: Belgin ERDEM, E-mail: *berdem@ahievran.edu.tr*
Received: 20 April 2025 / **Accepted:** 25 June 2025 / **Published:** 30 June 2025

Cite this article: Erdem, B., Erkaya, A.A., & Yalçın, D. (2025). Inhibition of Quorum Sensing and Biofilm Development by Walnut Rhizosphere Bacteria. Turkish Journal of Agricultural Engineering Research, 6(1), 59-73. *https://doi.org/10.46592/turkager.1680203*

ABSTRACT

Antibiotic resistance is a significant public health threat, and bacterial biofilms may play a role in the development of resistance to antibiotics. Natural biological agents hold promise for solving this problem due to their antibiofilm properties. In this study, extracts of walnut rhizosphere soil bacteria were used to investigate their anti-quorum sensing and antibiofilm characteristics. Four isolates (BT13, BT31, BT39 and BT40) from the walnut rizosphere demonstrated anti-quorum sensing action against Chromobacterium violaceum ATCC 12472 at dosages of 20 mg mL^{-1}. The crude extracts of BT13 (E. mundtii) showed the highest zone diameter, while crude extracts from BT33 did not. The crude extract from the BT39 (E. faecium) isolate, S. aureus (88%), and L. monocytogenes (82%) have the best biofilm inhibition activity. The study identified two walnut rhizosphere bacteria (BT13 and BT39) that exhibited promising antibiofilm and anti-quorum sensing properties, which could serve as natural biological controls against antibiotic-resistant bacteria. In addition, these bacteria may protect both plant and public health by acting as effective antibiotics against resistant pathogenic organisms.

Keywords: *Antibiofilm activity, Antiquorum sensing, Walnut rhizosphere, Enterococcus faecium Enterococcus mundtii*

INTRODUCTION

Antimicrobial resistance (AMR) is a significant global health concern. During the COVID-19 pandemic, the widespread and often inappropriate use of antibiotics has raised serious concerns regarding the acceleration of AMR (Ghosh *et al.*, 2021). Recent studies suggest the need for new natural biological antimicrobial agents to

combat the rapidly developing AMR. Diseases caused by pathogenic microorganism, especially in immunocompromised individuals, are significantly influenced by biofilm and quorum sensing (Khan *et al.*, 2021). Numerous illnesses linked to healthcare are caused by bacteria, which also result in longer hospital stays, higher medical expenses, and the development of biofilms on medical equipment (Vestby *et al.*, 2020; Dadi *et al.*, 2021). Despite the use of large amounts of antibiotics, these therapies are not always successful. Therefore, new methods to combat AMR and treatments that can change and eradicate biofilms and quorum sensing are needed. Recent research has shown promising outcomes in natural substances that can suppress quorum sensing and biofilm formation (Hughes and Webber, 2017).

Plant extracts from *Berginia ciliata, Clematis grata,* and *Clematis viticella* have shown anti-biofilm efficacy against *Pseudomonas aeruginosa* PAO1 (Alam *et al.*, 2020). Natural antibiofilms can show anti-quorum sensing and antibiofilm characteristics through naturally occurring bacterial secondary metabolites, and they can also suppress bacterial biofilm formation and quorum sensing activities. Secondary metabolite-producing soil microorganisms have garnered more attention in recent years (Alam *et al.*, 2020).

Examining the antibiofilm and antiquorum sensing characteristics of bacteria from natural settings like soil and water is crucial for developing novel compounds that combat biofilms and resistant infections (Asfour, 2018). Bacterial biofilms, a collection of microorganisms, are resistant to antibacterial treatments and tolerant of adverse conditions. They are responsible for a wide spectrum of chronic diseases, and antibiotic resistance in bacteria induced by biofilms makes diseases more difficult to cure (Donlan, 2002). New approaches, such as anti-quorum sensing detection (QS), are needed to counteract microbial resistance by blocking virulence factors (Roy *et al.*, 2018). QS inhibitors are used to treat chronic resistant bacterial infections and play a vital role in reducing resistance (Miller and Bassler, 2001). Anti-QS controls virulence factor production, bioluminescence emission, binding, motility, competence, biofilm formation, and sporulation, among other physiological processes (Cegelski *et al.*, 2008; Bhardwaj *et al.*, 2013).

Gram-negative bacteria regulate their virulence through a quorum sensing (QS) system, and Quorum Quenching (QQ) bacteria are a sustainable biocontrol method (Ma *et al.*, 2013). However, new medicines are needed to inhibit harmful bacteria that form biofilms and disrupt quorum sensing (Basavaraju *et al.*, 2016).

Research is needed to synthesize novel antimicrobial agents with anti-QS action, such as soil, water, and plant-derived microorganisms. The incidence and diversity of Quorum Quenching bacteria found in walnut rhizosphere soil were studied (Rajput and Bithel 2022; Sabu *et al.*, 2017). Although many studies have been conducted on the anti-quorum sensing and antibiofilm activities of leaf surface (phyllosphere) bacteria of medicinal plants and other plants, the bacteria found in the rhizosphere soil structure of the commercially important walnut tree have not been examined. Therefore, it is crucial to identify new control agents for biological protection against economically significant walnut plant diseases.

Although the economic value of the Walnut plant is high, research on its rhizosphere soil has not yet been done sufficiently. However, a lot of research has concentrated on the anti-quorum sensing and anti-biofilm properties of rhizosphere

bacteria, traditional medicinal plants, and other plants. Therefore, finding new biocontrol agents is crucial to preventing diseases that could harm the economically significant walnut plant. This study examined how pathogenic bacteria's quorum sensing and biofilm formation were affected by *E. mundiit and E. faecium* bacteria isolated from walnut soil.

Our objective was to assess these bacterial extracts' suitability for creating novel antimicrobial agents to treat illnesses in people, animals, and plants. Quorum sensing is essential for stopping the development of biofilms and pathogen resistance to antibiotics. Knowing these characteristics could lead to new strategies for the environmentally friendly management of infectious diseases and the control of harmful bacteria. In this direction, researchers are creating novel biocontrol methods that will transform infection control and microbial ecology.

The current study aims to assess the potential applications of bacteria isolated from walnut rhizosphere as novel bactericidal medications for the treatment of illnesses in people, animals, and plants.

The antibiofilm and antiquorum sensing properties of the bacterial isolates were evaluated. These features are essential for preventing dangerous germs to developing resistance to drugs. Therefore, more study is needed to find novel alternatives to antibiotics that will avoid resistant bacteria and ensure the sustainability of renewable therapies.

MATERIALS and METHODS

Collecting soil samples

Rhizosphere soil samples were collected from different walnut growing area of Kırşehir-Kaman (latitude N 39° 22′16″38.51″, longitude E 33° 42′46" 36.16″), at the root depth of young walnut trees. Sampling was carried out under aseptic conditions in the period between August and September 2021.

Bacteria isolated from the rhizosphere of walnuts

In the study, 1 gram of rhizosphere soil was diluted with 10 ml of water to isolate bacteria from the soil sample. The diluted soil samples were plated on nutrient agar plates and incubated at 28°C for 24 to 48 hours. After incubation, colonies with different morphological characteristics were selected and moved onto agar using the streak plate method. Pure isolates were stocked and preserved for identification and analysis (Chikere and Udochukwu, 2014).

The MALDI-TOF-MS automated microbiology system was used to identify the isolates. The presence of these bacteria in the soil was confirmed by the study's identification of 5 bacteria from two species using the MALDI-TOF MS method.

These genera and species are given in Table 1. The anti-quorum sensing experimentation was performed with the violacein producing *Chromobacterium violaceum* ATCC12472. Except for *C. violaceum* ATCC12472, which was incubated at 28°C, all medicinal strains were kept in Tripticase soy agar (TSA) and Tripticase soy broth (TSB) medium at 37°C.

S. aureus ATCC 29213, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. aerogenes* ATCC 51342, *L. monocytogenes* ATCC 7644, and *B. cereus* 709 Rome were

the pathogenic bacteria used in the anti-biofilm. The agar was then incubated overnight at 37°C overnight.

Identifying strains using MALDI-TOF-MS

MALDI TOF - MS was used to identify the species of strains isolated from walnut rhizosphere soil (Bruker Daltonics, Autoex Speed). This was achieved by identifying mass signals from the most prevalent and conserved genus-, species-, or subgroup-specific ribosomal protein fractions. MALDI-TOF MS log (score) values between 2.000 and 2.299 were considered as stable genus and likely species identifications, whereas values between 2.3 and 3.000 were interpreted as extremely plausible species-level identifications (Alatoom *et al.*, 2011).

The formic acid procedure, as used by Bizzini *et al.* (2010) was used to extract ribosomal protein, and samples were prepared for MS analysis. The samples were moved to the steel target plate using a sterile wood applicator from a single bacterial colony (Ground Steel Target, Bruker Daltonics). The transplanted bacteria were covered with a saturated HCCA matrix (a solution of cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid; CAS Number 28166-41-8). The material is left to dry at room temperature in order to allow for co-crystallization (Bizzini *et al.*, 2010) (Table 1).

Table 1. *Genus and Species Identified by MALDI-TOF MS Method of bacteria isolated from walnut rhizosphere.*

Bacteria code	Organism		MALDI- Biotyper Score Value
	Genus	Species	
BT13	*Enterococcus sp.*	*E. mundtii*	2.443
BT31	*Enterococcus* sp.	*E. faecium*	2.297
BT33	*Enterococcus* sp.	*E. faecium*	2.432
BT39	*Enterococcus* sp.	*E. faecium*	2.366
BT40	*Enterococcus* sp.	*E. faecium*	2.38

Production of crude extract for anti-quorum sensing and anti-biofilm studies

By placing a colony in 5 ml of TSB, 5 rhizosphere isolates were created. For fresh activation, 1 ml of each activated bacteria was added to 100 ml of TSB. After 48-72 hours of incubation at 30°C and 100 rpm in the orbital shaker, the cell-free supernatant from the 100 ml bacteria culture was collected, and after centrifugation at 13800 rpm for 30 minutes, it was diluted with an equivalent volume of ethyl acetate. After that, the crude extract is evaporated in an oven vacuum over night. In order to achieve these final concentrations, 1% dimethyl sulfoxide (DMSO) was added, and the mixture was kept at -20°C. The final concentrations were 5, 10, and 20 mg mL^{-1} stock (w v^{-1}) (Kanagasabhapthy *et al.*, 2009; Younis *et al.*, 2015).

Testing quorum sensing inhibition activity in the crude bacterial extract

Anti-quorum sensing behavior of bacterial extracts against *C. violaceum* was tested using the agar gel diffusion method. On TSA, active *C. violaceum* was spread using a clean cotton swab. Then, 75 µL of extracts at concentrations of 5, 10 and 20 mg mL^{-1} were injected into the opened wells. DMSO, incubated under the same conditions (30°C for 24 hours), was used as a control during the experiment.

It was possible to see anti-quorum sensing activity through a foggy halo field against the background of violeceum pigment. A total of three times this test was run (Abudoleh and Mahasneh, 2017). The plates were then kept at 30°C for a further 24 hours. A translucent condition indicates growth inhibition, while a murky halo (purple hue removal exclusively) is indicating QSI activity (Nithya *et al.*, 2010b).

Violaceum quantitative evaluation

The spectrum of violacein activity by *C. violaceum* (CV12472) in the presence of isolates was investigated by using the Blosser and Gray (2000) method of extracting the violaceum and measuring it. In nutrient broth, bacterial isolates were diluted two-fold, and 50 µl of grown culture (1.7x 10^7CFU / ml) was inoculated and incubated at 28°C until full pigmentation was observed in the blank (untreated culture). To begin, 200 µl of treated and untreated cultures are placed in an Eppendorf tube and lysed by adding 200 µl of 10% SDS, vortexing for 5 seconds, and incubating at room temperature for 5 minutes. Decrease in pigment production in the presence of bacteria isolate measured as percent inhibition = OD of control- OD of treated /OD of control x100 (Khan *et al*, 2009).

Quantification of anti-biofilm activity

For this test, pathogenic bacteria were cultured in Trypticase Soy Broth (TSB) at 37°C for 24 h. 100 µL of crude extract and 100 µL of bacterial cultures were deposited into 96-well polystyrene microtiter plates, and the microplates were then incubated at 37°C for 24 h. Planktonic cells were then discarded. Double distilled water was used to wash the adherent cells and allowed to air dry. After the biofilms were stained with 200 µL of 0.4% (w/v) crystal violet solution, the wells were rinsed twice with clean water. After the wells were air dried, the crystal violet was dissolved in 200 µL of ethanol. The optical density at 595 nm was measured using a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA). Bacterial cultures without extract were used as controls and BHIB medium was preferred for comparison. There were three runs of this experiment (Theodora *et al.*, 2019).

$$\textit{Percentage biofilm inhibition} = \frac{(\textit{Control OD595} - \textit{Treated OD595})}{(\textit{Control OD595})} \times 100\%$$

Scanning electron microscopy visualization of biofilm inhibition

BT-13 (*E. mundtii*) and BT-39 (*E. feacium*), *S. aureus,* and *E. coli* biofilms were examined by using scanning electron microscopy (SEM). First, biofilms on a glass coverslip were fixed with 2.5% glutaraldehyde for 30 min at 37°C. Coverslips were fixed, followed by three PBS rinses and 15-minute intervals of drying with a graded ethanol solution (30%). The samples were then freeze-dried after reintroducing ethanol using isoamyl acetate. Ultimately, utilizing a scanning electron microscope SEM (FEI Model Quanta FEG 450) study, coverslips were coated with gold. Scanning electron microscopy was used to describe the size, form, and morphology of bacterial antibiofilms. The Yozgat Bozok University Science and Technology Application and Research Center in Yozgat performed SEM (Turkey) (Relucenti *et al.*, 2021).

Finding pathogenic components in the bacterial isolates

It was also established that the bacterial isolates have extracellular enzyme activity for the enzymes lipase, proteinase, hemolysin, and amylase. Using tributyrin plate halo test, lipolytic activity was quantified. This procedure employed 1% tributyrin (v/v) as an enzyme substrate (Kim *et al.*, 2001). On agar plates with skim milk, protease activity is being monitored (Yu *et al.*, 2009). On 1.2% agar plates with an addition of 10% (v/v) sterile skimmed milk, protease generation and proteolytic activity were discovered.

A cleaning zone formed around the colonies as a result of proteolytic strains. On agar-based 5% sheep erythrocytes, hemolytic activity was investigated. Around the colonies, there was a distinct, colorless zone that was a sign of hemolytic activity. A specific substrate was supplied to the solid medium and discovered utilizing a diffusion approach involving developing colonies in order to manufacture extracellular enzymes. At 4 and 28°C, the isolated bacteria were inoculated, and the assays were carried out twice. The open areas surrounding the colonies were quantified in mm as the difference between the halo and the colony's diameter and were thought to be a sign of enzymatic activity (Wang *et al.*, 2007).

RESULTS and DISCUSSION

Anti-quorum sensing and anti-biofilm activity were screened.

The anti-qouroum sensing and anti-biofilm activities of 5 bacteria isolated from 30 rhizosphere soil samples were investigated.

Antiquorum sensing activity detection

Agar well was performed by diffusion method for anti-QS scanning using *C. violaceum* CV12472 strain. The screened 5 soil bacteria at a concentration of 20 mg mL^{-1}, with a pigment inhibition zone of 13.2 and 11.4 mm against CV12472 strain, significant inhibition in pigment production of isolates was detected, respectively (Table 2). Inhibition of pigment production was also detected in bacterial isolates, with a pigment inhibition zone ranging from 11-13 mm against CV12472. No effect on pigment inhibition by bacterial isolates was observed at the tested concentrations of 5 and 10 mg mL^{-1}. Violecein extraction was carried out in cultures of CV12472 treated with soil isolates to prevent the production of violecein.

Table 2. *In disc diffusion assays and pigment inhibition assays, strains have anti-quorum sensing activity.*

Anti-quorum sensing activity									
Strain No	**Origin of isolates**	**Bacteria**	**Inhibition zone (mm)**			**Concentrations (mg mL^{-1})**			
			Concentrations (mg mL^{-1})			**Violacein inhibition (%)**			
			5	**10**	**20**	**2**	**4**	**8**	**16**
BT13	Rhizosphere soil	*E. mundtii*	0	0	13.2	63.3	53.9	23.8	17.8
BT31	Rhizosphere soil	*E. faecium*	0	0	12.3	60.2	45.8	42.0	33.4
BT33	Rhizosphere soil	*E. faecium*	0	0	0	68.3	56.2	58.1	34.5
BT39	Rhizosphere soil	*E. faecium*	0	0	13.1	71.2	61.9	52.8	42.0
BT40	Rhizosphere soil	*E. faecium*	0	0	11.4	72.3	66.7	62.5	56.2

A number of bacteria were isolated as a result of the screening technique utilized in this investigation, ten of which were capable of generating antibacterial substances, and seven of which had both QSI and bacterial growth inhibitory activity against *C. violaceum* ATCC 12472. In order to determine whether Isolates 5 (BT13, BT31, BT33, BT39, and BT40) may produce anti-quorum sensing, additional testing was conducted on them.

Antibiotics therapeutic efficacy is linked to their bactericidal effect, (Lobritz *et al.* 2015) but selective pressure can lead to antibiotic resistance in bacteria (Maeda *et al.* 2012). Conventional therapies are losing effectiveness, and the incidence of antibiotic-resistant pathogenic bacteria is growing uncontrollably (Saga and Yamaguchi 2009). The use of antipathogenic materials to decrease bacterial virulence has become a paradigm for infection control, allowing for no selective pressure on antibiotic resistance development. This approach opens new scientific avenues for developing new therapeutic drugs and addressing the growing issue of antibiotic resistance (Maeda *et al.*, 2012).

Anti-pathogenic materials have emerged as a recent paradigm for bacterial infection control, aiming to decrease virulence rather than destroy the pathogen. This approach does not selectively pressure antibiotic resistance in bacteria and opens up new research avenues for developing new therapeutic drugs, as it does not selectively pressure the growth of antibiotic resistance in bacteria.

Quorun sensing inhibition activity of the bacterial extracts

This study isolated ten bacteria capable of producing antibacterial materials and seven with QSI and bacterial growth inhibitory activity against *C. violaceum* ATCC 12472. Isolates 5 (BT13, BT31, BT33, BT39, and BT40) were tested for anti-quorum generation. The soil from land and beaches was used as a reservoir for isolating microorganisms (Weng *et al.*, 2012). Twelve potentially QSI-active isolates were successfully isolated from the 500 isolates. The oil extract of *Syzygium aromaticum* also showed antibacterial and QSI activity (Khan *et al.*, 2009).

Theodora *et al.* (2019) isolated 11 phyllosphere bacteria with anti-quorum sensing activity. Another study used anti-quroum sensing to separate an actinomycetes strain from soil exposed to Gamma radiation (Lokegaonkar and Nabar, 2017). *B. subtilis* strain R-18 bacterial cell-free culture supernatant was tested against

C. violaceum and *S. marcescens*, demonstrating its quorum sensing inhibitory ability (Devi *et al.*, 2018).

The study found that 4 out of 5 rhizosphere isolates can be used as anti-quorum sensing agents due to their ability to thrive in a demanding environment with changing physical conditions and restricted nutrient availability (Hunter *et al.*, 2010). The results showed that extracts at concentrations of 5 mg mL^{-1} and 10 mg mL^{-1} had no activity, while activity was being observed at concentrations of 20 mg mL^{-1}. The study also observed inhibition of violet pigments in AHL rhizosphere from *C. violaceum* due to the degradation of metabolites generated by bacteria (Stauff and Bassler, 2011). The bacteria producer and extract concentration also impacted quorum quenching activity (Abudoleh and Mahasneh, 2017) which regulates the gene expression of biofilm-forming cells (Biradar and Devi, 2018; Roy *et al.*, 2018).

Future sequencing of rhizosphere bacteria's metabolites could help identify their quorum-quenching agents. Four extracts from walnut rhizosphere showed significant anti-quorum sensing activity against *C. violaceum* CV12472 and anti-biofilm properties against pathogenic bacteria without affecting growth. These extracts showed various results based on biofilm activity quantification in inhibition steps (Table 2). Disrupting autoinducer synthesis, cell-to-cell exchange, autoinducer reception and transduction, and autoinducer degradation can disrupt pathogenic bacteria's quorum sensing mechanism, leading to biofilm inhibition (Grandclement *et al.* 2016; Zhou *et al.*, 2017). Biofilm destruction activity may be caused by enzymes or small molecules that hydrolyze biofilm compounds (You *et al.*, 2007). Pathogenic bacteria biofilms have different EPS compositions (Gunn *et al.*, 2016), and specific enzymes may degrade various EPS compounds (Fleming and Rumbaugh, 2017).

Violacein quantification

The violacein inhibition percentage was 72.3 and 56.2% when *C. violaceum* ATCC 12472 was administered with separate concentrations of extract of BT40 at concentrations of 0.5 and 0.0625 mg mL^{-1} of the bacterial QSI compounds, respectively. This finding is consistent with the findings of Choo *et al.* (2006) who discovered that vanilla extract decreased violacein production by up to 98 %, and Packiavathy *et al.* (2012) who discovered that *Cuminum cyminum* inhibited violacein production by 90%. The extract of *Rosmarinus officinalis* leaves inhibited the synthesis of violacein by 40%, according to Vattem *et al.* (2007).

These findings clearly showed that bacterial QSI compounds can be isolated from natural habitats. Rather than suppressing the pathogen, QS inhibition is an alternative method of pathogen control that involves manipulating gene expression. Bacterial QSI agents would certainly aid in the battle against newly emerged resistant pathogenic bacteria. The area is still in its early stages, and further screening and testing procedures are needed.

Quantification of anti-biofilm agents

The anti-biofilm activity experiment revealed that crude extracts varied in their inhibitory efficacy against all pathogenic bacteria tested, with the greatest results

coming from *S. aureus* and *E. coli* and the worst results coming from *L. monocytogenes* and B. *cereus* 709 Roma (Table 3). Pathogens *P. aeruginosa, E. aerogenes and B. cereus* 709 Roma did not show antibiofilm activity. Crude extracts obtained from BT39 (*E. faecium*) isolate, *S. aureus* (88%) and *L. monocytogenes* (82%) had the best biofilm inhibitory activity.

Table 3. *Quantification of anti-biofilm action against pathogenic bacteria*

Pathogens	Activity	BT13	BT31	BT33	BT39	BT40
		E. mundtii	*E. faecium*	*E. faecium*	*E. faecium*	*E. faecium*
S. aureus ATCC 29213	Inhibition	18	0	3	88	3
E. coli ATCC 25922	Inhibition	85	15	15	60	16
P. aeruginosa ATCC 27853	Inhibition	0	0	0	0	0
E. aerogenes ATCC 51342	Inhibition	0	0	0	0	0
L. monocytogenes ATCC 7644	Inhibition	0	0	0	82	0
B. cereus709 Roma	Inhibition	0	0	0	0	0

Evaluation of anti-biofilm activity

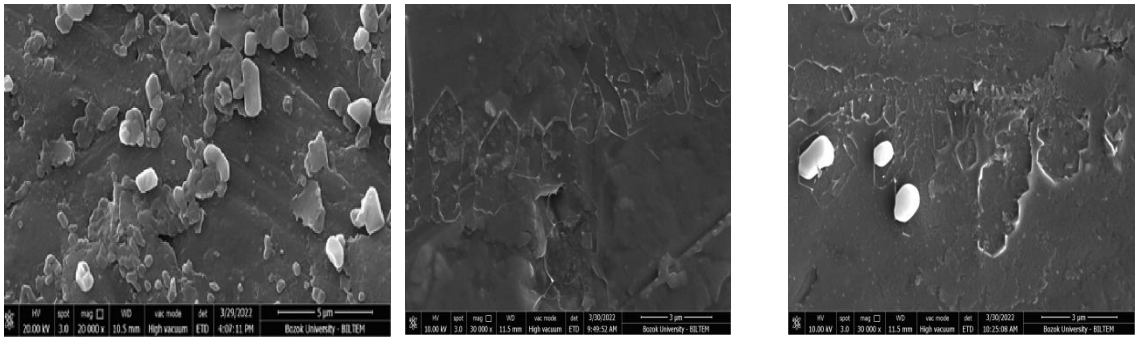
The study found that BT39 isolate extract significantly inhibited biofilm formation without affecting the growth of two standard pathogenic bacteria, *S. aureus* and *L. monocytogenes*. These isolates have a wide spectrum of anti-biofilm function in inhibition ways, and crude extracts of rhizosphere isolates have shown promise as quorum quenching and anti-biofilm agents against some biofilm-forming pathogenic bacteria studied (Table 3). Our results are consistent with previous findings that some marine Bacillus species inhibit *P. aeruginosa* biofilm (Nithya *et al.*, 2010a).

Bacteria prefer biofilm life due to increased resistance to antibacterial agents, which prevents successful treatment of biofilm-associated infections (Høiby *et al.*, 2010). Quorum sensing and biofilm formation are essential for bacteria in social life, and preventing biofilm formation in quorum sensing is crucial for disease treatment (Nadell *et al.*, 2008). Recently, commercially available QQ compounds have been shown to increase the susceptibility of bacterial biofilm to antibiotics, making them suitable for effective treatment of biofilm-associated infections (Brackman *et al.*, 2011).

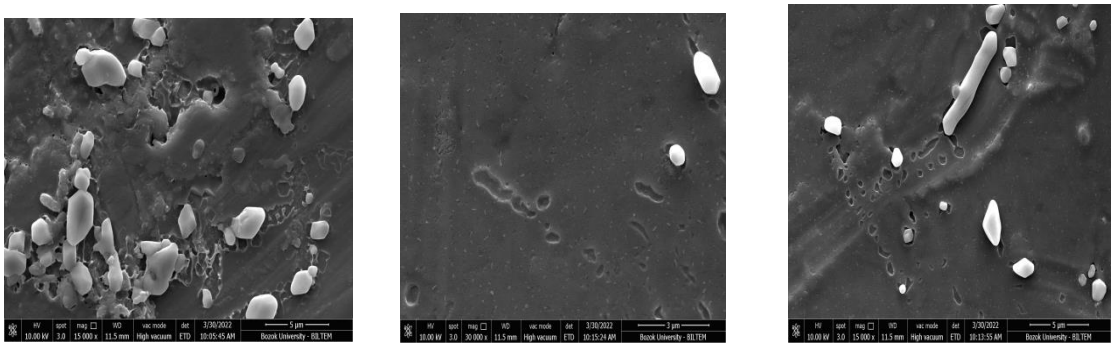
Antibiofilm Activity Evaluation Using Scanning Electron *Microscopy*

The biofilms made up of *E. mundtii* (BT-13), *S. aureus,* and *E. coli* were examined by using and scanning electron microscopy (SEM). First, biofilms on a glass coverslip were fixed with 2.5% glutaraldehyde for 30 min at 37°C. Coverslips were fixed, followed by three PBS rinses and 15-minute intervals of drying with a graded ethanol solution (30%). The samples were then freeze-dried after reintroducing ethanol using isoamyl acetate. Ultimately, utilizing a scanning electron microscope

SEM (FEI Model Quanta FEG 450) study, coverslips were coated with gold. Scanning electron microscopy was used to describe the size, form, and morphology of bacterial antibiofilms (SEM). The Yozgat Bozok University Science and Technology Application and Research Center in Yozgat performed SEM (Turkey).



A) *S. aureus* (Control) B) *E. mundtii* (BT-13) C) *E. faecium* (BT-39)



A) *E. coli* (Control) B) *E. mundtii* (BT-13) C) *E. faecium* (BT-39)

Figure 1. *SEM pictures showing the variations in cell morphology in the biofilm structures of BT-13 and BT-39 bacterial extracts as well as control cells S. aureus and E. coli. (The arrows show that although control cells are intact and tightly encased in the EPS matrix, they are injured and removed from the EPS matrix).*

Evaluation of biofilm inhibition by SEM

SEM was used to detect the biofilm inhibitory activity of bacteria isolated from the walnut rhizosphere against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. Visualization using SEM provided additional confirmation of this conclusion (Figure 1). The integrity of the cell walls is compromised, and the multilayer biofilm growth's thickness is reduced. The test bacterial strains also failed to preserve their usual morphology and did not cluster when *P. aeruginosa* was present. The threatened cell walls are to blame for this.

The production of EPS by bacteria, which helps them attach to different surfaces and play a vital role in the growth of microcolonies that result in the creation of biofilms, is another significant feature that has been researched (Leme *et al.* 2006). BT13 and BT39 extracts inhibit a QS system that regulates the release of extracellular DNA needed by the pathogen *E. coli* to form biofilms. These extracts soften the biofilm and make it more susceptible to antibiotics. BT13 and BT39 extracts caused cell death by forming pores in the cell wall of gram-positive bacteria, including pathogen *S. aureus.*

Detection of Virulent Factors of the bacterial isolates

A study was done on the bacterial biofilm supernatants' capacity for hemolysis, lipolysis, and proteolysis. The pathogenicity potential of ten different bacteria was evaluated (Table 4). The size of colonies and haloes was used to gauge enzyme activity. Hemolysis, lipase, and proteinase are used to break down and disperse biofilms. On 5% sheep blood agar plates, biofilm supernatants showed the small-scale and hemolytic activity of hemolytic halos.

These results show that and hemolysin are produced in biofilms and then transferred to the extracellular environment. Larger diameter haloes of proteolysis and lipolysis were noticed in the identical supernatants of tributyrin agar and skim milk agar (Table 3).

Table 4. *Virulent factors of the bacterial isolates measured of diatemer and the colony halo in mm at 4 and 28°C.*

		Temperature (°C)							
		Diameter of virulent factors halo (mm)							
		Lipase		Protease		Hemolysin		Amylase	
Isolates	**Identified species**	4°C	28°C	4°C	28°C	4°C	28°C	4°C	28 °C
BT13	*E. mundtii*	-	-	10.0	15.2	-	-	-	-
BT31	*E. faecium*	-	-	12.2	30.0	-	-	-	-
BT33	*E. faecium*	-	-	12.1	25.0	-	-	-	-
BT39	*E. faecium*	-	-	15.3	25.0	-	-	-	-
BT40	*E. faecium*	-	-	18.2	30.2	-	-	-	-

-No clean zone surrounding the colony + Clear zone surrounding the colony

At 4 and 28°C, *E. faecium* (BT31, BT33, BT39, and BT40) had high protease activity (12.2-30.2 mm), but no lipase, hemolysin, or amylase activity. At 28°C (15.2 mm) and 4°C, *E. mundtii* (BT13) had high protease activity (10.0 mm). The results of present study indicate the difference in the expression of protease by *E. mundtii* (BT13) and *E. faecium* (BT31, BT33, BT39 and BT40) isolates. The results show that rhizosfer isolates in this study displayed proteolytic activity (Table 4).

Detection of Virulent Factors of the bacterial isolates

In this work, bacteria isolated from walnut rhizosphere soil were tested for the presence of virulence factors such lipase, protease, hemolysin, and amylase in specific medium (Abdollahzadeh *et al.*, 2020).

The strains of *E. faecium* (BT31, BT33, BT39, and BT40) and *E. mundtii* (BT13) did not generate lipase, hemolysin, or amylase. However, the clear translucent halo that developed around the colony on the protease-selective media demonstrated that every isolate produced protease.

Despite the fact which this research did not witness the formation of lipase, hemolysin, or amylase enzymes, it has been demonstrated that soil habitats like the rhizosphere of walnuts include bacteria that produce these types of enzymes, which

are employed in a variety of uses in industry (Saha *et al.*, 2019). For instance, it has been noted that bacterial amylolytic activities increases within waste soils that include garbage that is abundant in amylase than in garden soils. Food, feed, textile, paper, and biofuel are just a few of the industries that heavily rely on enzymes like pectinase, which break down materials that contain pectin (Abdollahzadeh *et al.*, 2020). Furthermore, bacteria found in the walnut rhizosphere contribute significantly to the cycling of plant nutrients and promote plant health. Four bacteria from the walnut rhizosphere were identified and isolated for this investigation. Gram-negative bacteria taken from comparable soil settings had been shown in earlier research to be useful in biological control against insects in walnut trees, despite the fact that these isolates were Gram-positive. The synthesis, optimisation, and biotechnological potential of the enzymes derived from these isolates require more research.

CONCLUSION

Enterococcus bacteria found in walnut rhizosphere soil can be evaluated in agricultural and medical applications due to their ability to sense pathogenic quorum and prevent biofilm formation. By exploiting these natural antagonistic properties, researchers can develop innovative approaches to combat antibiotic resistance and reduce chronic infections. Future research should focus on understanding the specific molecular interactions and environmental factors that facilitate these beneficial effects and develop solutions against antibiotic-resistant bacteria that pose a threat to public health. These beneficial bacteria can inhibit biofilm formation, reduce virulence, and increase host resistance by producing signaling molecules or enzymes. This sustainable and environmentally friendly approach to antibiotic resistance and infection management requires further research to identify specific bacterial strains or metabolites for therapeutic applications.

ACKNOWLEDGEMENT

Authors are grateful for financial support from Kırşehir Ahi Evran University Scientific Research Unit (Project No: SAG.A4.21.002)

DECLARATION OF COMPETING INTEREST

The authors declare no competing interests.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Belgin ERDEM: Investigation, methodology, conceptualization, validation, writing - original draft and visualization
İlkay AÇIKGÖZ ERKAYA: Formal analysis, data curation, validation and review.
Dilek YALÇIN: Methodology, validation, formal analysis and editing.

ETHICS COMMITTEE DECISION

This article does not require any Ethical Committee Decision.

REFERENCES

Abudoleh SM and Mahasneh AM (2017). Anti-quorum sensing activity of substances isolated from wild berry associated bacteria. *Avicenna Journal of Medical Biotechnology, 9(1): 23-30.*

Abdollahzadeh R, Pazhang M, Najavand S, Fallahzadeh-Mamaghani V, Amani-Ghadim AR (2020). Screening of pectinase-producing bacteria from farmlands and optimization of enzyme production from selected strain by RSM. *Folia Microbiol, 65: 705-719.* https://doi.org/10.1007/s12223-020-00776-7

Alam K, Farraj DAA, Mah-E-Fatima S, Yameen MA, Elshikh MS, Alkufeidy RM, Mustafa AEMA, Bhasme P, Alshammari MK, Alkubaisi NA, Abbasi AM and Naqvi TA (2020). Anti-biofilm activity of plant derived extracts against infectious pathogen-*Pseudomonas aeruginosa* PAO1. *Journal of Infection and Public Health, 13(11): 1734-1741.*

Alatoom AA, Cunningham SA, Ihde S M, Mandrekar J and Patel R (2011). Comparison of direct colony method versus extraction method for ıdentification of gram-positive cocci by use of bruker biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology, 49(8): 2868-2873.* https://doi.org/10.1128/JCM.00506-11

Asfour HZ (2018). Anti-Quorum sensing natural compounds. *Journal of Microscopy and Ultrastructure, 6(1): 1-10.*

Basavaraju M, Sisnity VS, Palaparthy R and Addanki PK (2016). Quorum quenching: Signal jamming in dental plaque biofilms. *Journal of Dental Sciences, 11(4): 349-352.*

Bhardwaj AK, Vinothkumar K and Rajpara N (2013). Bacterial quorum sensing inhibitors: Attractive alternatives for control of infectious pathogens showing multiple drug resistance. *Recent Patents on Anti-Infective Drug Discovery*, *8(1): 68-83.*

Biradar B and Devi P (2011). Quorum sensing in plaque biofilms: challenges and future prospects. *Journal of Contemporary Dental Practice*, *12(6): 479-485.* https://doi.org/10.5005/jp-journals-10024-1080

Bizzini A, Durussel C, Bille J, Greub G and Prod'hom G (2010). Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. *Journal of Clinical Microbiology, 48(5): 1549-1554.* https://doi.org/10.1128/JCM.01794-09

Brackman G, Cos P, Maes L, Nelis H and Coenye T (2011). Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics in vitro and in vivo. *Antimicrobial Agents and Chemotherapy*, *55 (6): 2655-61.* https://doi.org/10.1128/AAC.00045-11

Cegelski L, Marshall GR, Eldridge GR and Hultgren SJ (2008). The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol, 6 (1): 17-27.* https://doi.org/10.1038/nrmicro1818.

Chikere CB and Udochukwu U (2014) Effect of growth media and incubation time on the culturability of soil bacteria. *IOSR Journal of Pharmacy and Biological Sciences, 9(2):6-9.* https://doi.org/10.9790/3008-09210609

Choo JH, Rukayadi Y and Hwang JK (2006). Inhibition of bacterial quorum sensing by vanilla extract. *Letters in Applied Microbiology*, *42(6): 637-641.* https://doi.org/10.1111/j.1472-765X.2006.01928.x

Dadi NCT, Radochová B, Vargová J and Bujdáková H (2021). Impact of Healthcare-Associated Infections Connected to Medical Devices-An Update. *Microorganisms, 9 (11): 2332.* https://doi.org/10.3390/microorganisms9112332

Devi KR, Srinivasan S and Ravi AV (2018). Inhibition of quorum sensing-mediated virulence in *Serratia marcescens* by *Bacillus subtilis* R-18. *Microbial Pathogenesis, 120: 166-175.* https://doi.org/10.1016/j.micpath.2018.04.023

Donlan, RM (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases, 8(9): 881-890.* https://doi.org/10.3201/eid0809.020063

Fleming D and Rumbaugh KP (2017). Approaches to dispersing medical biofilms. *Microorganisms*, *5(2): 15.* https://doi.org/10.3390/microorganisms5020015

Grandclement C, Tannieres M, Morera S, Dessaux Y and Faure D (2016). Quorum quenching: role in nature and applied developments. *FEMS Microbiology Reviews, 40(1): 86–116.* https://doi.org/10.1093/femsre/fuv038

Gunn JS, Bakaletz LO and Wozniak DJ (2016). What's on the outside matters: the role of the extracellular polymeric substance of Gram-negative biofilms in evading host immunity and as a target for therapeutic intervention. *Journal of Biological Chemistry, 291(24): 12538-12546.* https://doi.org/10.1074/jbc.R115.707547

Ghosh S, Bornman C and Zafer MM (2021). Antimicrobial resistance threats in the emerging COVID-19 pandemic: Where do we stand? *Journal of Infection and Public Health, 14(5): 555-560.* https://doi.org/10.1016/j.jiph.2021.02.011

Høiby N, Bjarnsholt T, Givskov M, Molin S and Ciofu O (2010.) Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents, 35 (4): 222–332.* https://doi.org/10.1016/j.ijantimicag.2009.12.011

Hughes G and Webber MA (2017). Novel approaches to the treatment of bacterial biofilm infections. *British Journal of Pharmacology, 174(14): 2237-2246.* https://doi.org/10.1111/bph.13706

Hunter PJ, Hand P, Pink D, Whipps JM and Bending GD (2010). Both leaf properties and microbe–microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* species) phyllosphere. *Applied and Environmental Microbiology, 76(24): 8117–8125.* https://doi.org/10.1128/AEM.01321-10

Kanagasabhapthy M, Yamazaki G, Ishida A, Sasaki H and Nagata S (2009). Presence of quorum-sensing inhibitors-like compounds from bacteria isolated from the brown alga *Colpomenia sinusoa. Letters in Applied Microbiology, 49(5): 573-579.* https://doi.org/10.1111/j.1472-765x.2009.02712.x

Khan MS, Zahin M, Hasan S, Husain F, and Ahmad I (2009). Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. *Letters in Applied Microbiology, 49(3): 354-360.* https://doi.org/10.1111/j.1472-765X.2009.02666.x

Khan J, Tarar SM, Gul I, Nawaz U and Arshad M (2021). Challenges of antibiotic resistance biofilms and potential combating strategies: a review. *3 Biotech, 11(4): 169.* https://doi.org/10.1007/s13205-021-02707-w

Kim EK, Jang, WH, Ko JH, Kang JS, Noh, MJ and Yoo OJ (2001). Lipase and its modulator from Pseudomonas sp. strain KFCC 10818: Proline-to-glutamine substitution at position 112 induces formation of enzymatically active lipase in the absence of the modulator. Journal of Bacteriology, *183(20): 5937-41.* https://doi.org/10.1128/JB.183.20.5937-5941.2001

Leme AFP, Koo H, Bellato CM, Bedi G and Cury JA (2006). The role of sucrose in cariogenic dental biofilm formation-new. *Insight. Journal of Dental Research, 85(10): 878-887.* https://doi.org/10.1177/154405910608501002

Lobritz MA, Belenky P, Porter CB, Gutierrez A, Yang JH, Schwarz EG, Dwyer DJ, Khalil AS and Collins JJ (2015). Antibiotic efficacy is linked to bacterial cellular respiration. *Proceedings of the National Academy of Sciences of the United States of America, 112*,(27): *8173-8180.* https://doi.org/10.1073/pnas.1509743112

Lokegaonkar SP and Nabar BM (2017). In vitro antibiofilm, antiquorum sensing activity of gamma tolerant streptomyces against gram negative pathogens. *International Journal of Pharmacology and Clinical Research, 9 (11): 665-670.*

Ma A, Lv D, Zhuang X and Zhuang G (2013). Quorum quenching in culturable phyllosphere bacteria from tobacco. *International Journal of Molecular Sciences, 14(7): 14607–14619.* https://doi.org/10.3390/ijms140714607

Maeda T, García-Contreras R, Pu M, Sheng L, Garcia LR, Tomás M and Wood TK (2012). Quorum quenching quandary: resistance to antivirulence compounds. *ISME Journal 6, 493-501.* https://doi.org/10.1038/ismej.2011.122

Miller MB and Bassler BL (2001). Quorum sensing in bacteria. *Annual Review of Microbiology, 55: 165-199.* https://doi.org/10.1146/annurev.micro.55.1.165

Nadell C, Xavier J, Levin S and Foster K (2008). The evolution of quorum sensing in bacterial biofilms. *PLOS Biology, 6 (1):171– 179.* https://doi.org/10.1371/journal.pbio.0060014

Nithya C, Begum MF and Pandian SK (2010a). Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. *Applied Microbiology and Biotechnology, 88(1): 341-358.* https://doi.org/10.1007/s00253-010-2777-y

Nithya C, Aravindraja C and Pandian SK (2010b). *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. *Research in Microbiology, 161(4): 293-304.* https://doi.org/10.1016/j.resmic.2010.03.002

Packiavathy IA, Agilandeswari P, Mustafa KS, Pandian SK and Ravi AV (2012). Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against Gram negative bacterial pathogens. *Food Research International*, *45(1): 85-92.* https://doi.org/10.1016/j.foodres.2011.10.022

Rajput M and Bithel N (2022). Phytochemical characterization and evaluation of antioxidant, antimicrobial, antibiofilm and anticancer activities of ethyl acetate seed extract of *Hydnocarpus laurifolia* (Dennst) Sleummer. *3 Biotech ,12(9): 215.* https://doi.org/10.1007/s13205-022-03267-3

Relucenti M, Familiari G, Donfrancesco O, Taurino M, Li X, Chen R, Artini M, Papa R and Selan L (2021). Microscopy methods for biofilm ımaging: Focus on SEM and VP-SEM pros and cons. *Biology* (Basel). *12;10 (1):51.* https://doi.org/1010.3390/biology10010051

Roy R, Tiwari M, Donelli G and Tiwari V (2018). Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. *Virulence, 9(1): 522–54.* https://doi.org/10.1080/21505594.2017.1313372

Sabu R, Soumya KR and Radhakrishnan EK (2017). Endophytic *Nocardiopsis* sp. *from Zingiber officinale* with both antiphytopathogenic mechanisms and antibiofilm activity against clinical isolates. *3 Biotech, 7(2):* 115. https://doi.org/10.1007/s13205-017-0735-4

Saga T and Yamaguchi K (2009). History of antimicrobial agents and resistant bacteria. *Japan Medical Association Journal, 52(2): 103-108.*

Saha ML, Islam KN, Akter T, Rahman IA, Islam T and Khan T (2019). Isolation and identification of amylolytic bacteria from garbage and garden soil. *Bangladesh Journal of Botany, 48 (3): 537-545.* https://doi.org/10.3329/bjb.v48i3.47915

Stauff DL and Bassler BL (2011). Quorum sensing in *Chromobacterium violaceum*: DNA recognition and gene regulation by the CviR receptor. *Journal of Bacteriology, 193 (15): 3871–3878.* https://doi.org/10.1128/JB.05125-11

Theodora NA, Dominika V and Waturangi DE (2019). Screening and quantification of anti-quorum sensing and antibiofilm activities of phyllosphere bacteria against biofilm forming bacteria *BMC Research Notes*, *12: 732.* https://doi.org/10.1186/s13104-019-4775-1

Vattem DA Mihalik K, Crixell SH and McLean RJ (2007). Dietary phytochemicals as quorum sensing inhibitors. *Fitoterapia* ,*78(4): 302-310.* https://doi.org/10.1016/j.fitote.2007.03.009

Vestby LK, Grønseth T, Simm R and Nesse LL (2020). Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics (Basel), 3(2): 59.* https://doi.org/10.3390/antibiotics9020059

Wang HY, Liu DM, Liu Y, Cheng CF, Ma QY, Huang Q and Zhang YZ (2007). Screening and mutagenesis of a novel Bacillus pumilus strain producing alkaline protease for dehairing. Letters in Applied Microbiology, *44 (20): 1-6.* https://doi.org/10.1128/JB.183.20.5937-5941.2001

Weng LX, Zhang YQ, Meng H, Yang YX, Quan ZX, Zhang YY and Wang LH (2012). Screening and isolating quorum sensing inhibitor from bacteria. *African Journal of Microbiology Research, 6(5): 927-936.* https://doi.org/10.5897/AJMR11.813

You JL, Xue XL, Cao LX, Lu X, Wang J, Zhang LX and Zhou SN (2007). Inhibition of vibrio biofilm formation by a marine actinomycete strain A66. *Applied Microbiology and Biotechnology, 76 (5): 1137-–1144.* https://doi.org/10.1007/s00253-007-1074-x

Younis KM, Usup G and Ahmad A (2015). Secondary metabolites produced by marine streptomyces as antibiofilm and anti-quorum sensing inhibitor of uropathogen *Proteus mirabilis*. *Environmental Science and Pollution Research, 23(5): 4756–4767.* https://doi.org/10.1007/s11356-015-5687-9.

Yu Y, Li H, Zeng Y and Chen B (2009). Extracellular enzymes of cold adapted bacteria from Arctic sea ice Canada Basin. Polar Biology, *32: 1539-1547.* https://doi.org/10.1007/s00300-009-0654-x

Zhou J, Bi S, Chen H, Chen T, Yang R, Li M, Fu Y, Jia A (2017). Anti-biofilm and antivirulence activities of metabolites from *Plectospaerella cucumerina* against *Pseudomonas aeruginosa*. *Frontiers in Microbiology, 3(8): 1-17.* https://doi.org/10.3389/fmicb.2017.00769