IJPSR (2018), Volume 9, Issue 6 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 11 September, 2017; received in revised form, 15 November, 2017; accepted, 30 December, 2017; published 01 June, 2018

BROAD SPECTRUM ANTIBIOFILM AND ANTIQUORUM SENSING POTENTIAL OF CRUDE SECONDARY METABOLITE EXTRACTS OF MICROBIAL ISOLATES OBTAINED FROM POLLUTED WALDHUNI RIVER

S. P. Lokegaonkar * and B. M. Nabar

Department of Microbiology, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar - 421003 Maharashtra, India.

Keywords:

Biofilm,
Quorum sensing,
Exopolysaccharide, eDNA,
Secondary Metabolites

Correspondence to Author:
Mr. Swapnil Pradip Lokegaonkar

Research Scholar,
Department of Microbiology,
Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar - 421003, Maharashtra, India.

E-mail: neel025@gmail.com

ABSTRACT: Bacterial biofilms due to antibacterial resistance are causing serious health issues with respect to infection control, its prognosis and treatment of disease. The current scenario demands need of new and alternative antibacterial compounds for treatment of such infections. The current study highlights the use of polluted river, Waldhuni, as a source of potent microorganisms with antibiofilm properties. 186 microorganisms were isolated from Waldhuni River, and tested for quorum sensing inhibitory activity (QSI) against *C. violaceum* MTCC 2656. Seven isolates exhibited QSI activity, out of which 1 isolate PB2 was selected for further study based on its high QSI potential. PB2 displayed broad spectrum antibiofilm activity against eight strains of biofilm forming pathogens. The effective minimum biofilm eradication concentration (MBEC) of crude extract of PB2 secondary metabolite (SME) was found to be 500ppm. The extract reduced exo-polysaccharide (EPS) by 94.16%, viability by 90% and biofilm density by 96% in 6h treatment. It also rendered to inhibit extracellular DNA of *Streptococcus mutans* MTCC497, *Enterococcus faecalis* MTCC 439 and *Pseudomonas aeruginosa* MTCC 2453. PB2 isolate was identified to be *Pseudomonas aeruginosa*. Thus, *Pseudomonas aeruginosa* PB2 with antiquorum sensing and broad spectrum biofilm inhibitory activity was isolated from highly polluted Waldhuni river.

INTRODUCTION: Biofilm is a complex matrix made up of microbial consortium where cells strongly adhere to each other. It has been estimated that approximately 70% to 80% of microorganisms form a biofilm and almost 80% of the microbial infections that occur in a human body are biofilm derived [1].

QUICK RESPONSE CODE

DOI:
10.13040/IJPSR.0975-8232.9(6).2378-86

Article can be accessed online on:
www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(6).2378-86

Biofilm mediated infections are responsible for a number of clinical challenges, which mainly include diseases involving unculturable pathogens, chronic inflammation, delayed wound healing, rapid antibiotic resistance, and systemic infections. Biofilm forming pathogenic bacteria exhibit an innate ability to resist disinfectants and host defenses. Due to ability to form a biofilm and persist, microorganisms are significantly less susceptible to antibiotics and host defenses than their planktonic forms [2]. Most common bacteria forming a biofilm are *Staphylococcus aureus, Staphylococcus epidermidis, Vibrio cholerae, Enterococci spp., Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [3 - 7].

Staphylococci, are a leading cause of nosocomial infections worldwide, is the etiologic agent of a wide range of diseases, ranging from relatively benign skin infections to potentially fatal systemic disorders [3]. Pathogens such as *Pseudomonas aeruginosa, Streptococcus mutans, Klebsiella pneumoniae* have rendered the ability to form biofilm on indwelling medicinal devices with increasing rates of persistence [8 - 9]. Emerging cases of *V. cholerae* colonizing intestine to form a biofilm leading to a development of fatal disease have been reported recently [10 - 11].

Conversely microorganisms are also known for production of various antimicrobial compounds. Actinomycetes, *Bacillus* species, *Pseudomonas* species are commonly known microorganisms which produce antimicrobial and antifungal compounds [12]. Soil and water are rich resources which offer abundant supply of such potentially novel microorganisms. In spite of decades of research, 99% of the diverse bacterial species from soil and water bodies are yet unexplored [13].

In the current study soil and water samples were collected from highly polluted Waldhuni river. Waldhuni river flows through four major cities of Thane District, Maharashtra state. It is polluted mainly due to industrial and domestic waste disposal. Such polluted environments tend to exhibit presence of highly stress tolerant microorganisms significant to human. These microorganisms can be tested for their biotechnological potential, such as bactericidal properties.

Present study intends to isolate the microorganisms from Waldhuni river and to study their antibiofilm potential against selected standard strains of pathogens.

MATERIALS AND METHODS:

Test cultures and growth conditions: Eight standard strains of pathogens were selected for the study. The selected bacterial strains were *S. mutans* MTCC 497, *S. aureus* MTCC 3160, *S. epidermidis* MTCC 3165, *E. faecalis* MTCC 439, *K. pneumoniae* MTCC 3384, *V. cholerae* MTCC 3906, *E.coli* MTCC 687 and *P. aeruginosa* MTCC 2453. For the quorum sensing inhibitory activity violacein producing *C. violaceum* MTCC 2656 was used. All strains were maintained in Brain heart infusion (BHI) broth medium at 37 °C, while *C. violaceum* MTCC 2656 was incubated at 30 °C. For all assays test culture suspension was prepared by using overnight growth with optical density adjusted to 0.1($OD_{610\ nm}$).

Collection of Soil and Water Samples: Soil and water samples were collected from 18 locations throughout the Waldhuni river. Based upon the earlier reports, locations with elevated levels of COD were selected in this study. Total 36 samples comprising 18 soil and 18 water samples, were collected from the river. The details of locations are listed in **Table 1**.

TABLE 1: DETAILS OF THE SAMPLING LOCATION

S. no.	Locations	Longitude	Latitude
1	Kakole Village	73.18280697	19.16968844
2	Phansipada	73.18302155	19.1820919
3	Additional Ambernath	73.19533825	19.18051112
4	Ambernath	73.18066120	19.19287323
5	Shiv Mandir Area	73.17692757	19.19846627
6	Kailash Colony	73.17319393	19.20328911
7	Kansai Bridge	73.17430973	19.20953021
8	Ambernath MIDC	73.17449212	19.21647438
9	Ambernath railway station	73.18484545	19.21087992
10	Saibaba mandir	73.18514585	19.21081103
11	Ulhasnagar railway station	73.16290498	19.21879508
12	SMT CHM college	73.16051245	19.21974232
13	Hiraghat	73.15734208	19.22705156
14	Kalyan Division	73.14806700	19.23666289
15	Shahad	73.15225124	19.24392235
16	Godrej Park	73.15396786	19.26446927
17	Mohan bridge	73.15224051	19.24254962
18	Kalyan Creek	73.15690756	19.25754921

Isolation of Microorganisms: All samples were subjected to enrichment for isolation of Bacteria, Fungi and Actinomycetes. 1 gm of soil sample and 10ml of water sample was inoculated in Luria Bertani (LB) medium, Potato dextrose broth (PD) and Actinomyces broth (supplemented with 80µg/ml of cylcoheximide and 80µg/ml of nalidixic acid). After enrichment period, broth was inoculated on to LBA, PDA and Starch casein agar (SCA). Colonies thus obtained were preserved and were utilized to study their quorum sensing inhibitory activity.

Screening for Quorum Sensing Inhibitory (QSI) Activity: The QSI activity of isolates were determined by using method described by Packiavathy *et al*., with minor modifications [14]. Isolates were inoculated into respective broth media, incubated for 2-3 weeks and centrifuged at 15000 rpm, for 20 min to obtain cell free

supernatant (CFS). CFS was subjected to the qualitative analysis to find out their QSI potentials against *C. violaceum* MTCC 2656. The violet coloured pigment violacein is produced by *C. violaceum* MTCC 2656 as a response to its QS signal molecule N-hexanoyl-L-homoserine lactone (HHL). 10 µl of overnight culture suspension of *C. violaceum* MTCC 2656 (adjusted to 0.1 OD at 610nm) was added into wells of sterile microtiter plates (MTP) containing 180 µl of LB broth and incubated along with 10 µl of individual CFS. The plates were incubated overnight at 37°C and observed for the reduction in violacein pigment production. Only the CFS which exhibited significant QSI activity against *C. violaceum* MTCC 2656 alone was further considered for subsequent experiments in this study.

The percentage of violacein inhibition was calculated by following the formula:

Percentage of violacein inhibition = (Control OD_{580nm} − Test OD_{580nm}/Control OD_{580nm}) ×100

Preparation of Secondary Metabolite Extracts (SME): The secondary metabolite extracts (SME) were prepared from the isolates which QSI activity[15-16]. Isolates were inoculated to the respective Broth media and then filtered, centrifuged at 15000 rpm, for 20 min to obtain cell free supernatant (CFS). Equal volume of ethyl acetate was added to CFS and kept under shaker conditions for 1 hour. In a separating funnel secondary metabolite was obtained as a layer between organic and aqueous layers. The layer of secondary metabolite was collected in a petridish and dried to evaporation. The dried secondary metabolite was dissolved in dimethyl sulfoxide (DMSO) and used to study the Antibiofilm properties.

Antibiofilm Properties:

Biofilm Control Assay: A microtiter plate based biofilm assay was carried out to identify the minimum biofilm eradication concentration (MBEC) of secondary metabolite extracts (SME) [17]. Test cultures (20µl) were inoculated in 180µl BHI broth supplemented with 2% glucose. After 48h incubation at 37 °C, medium was removed aseptically. SME were added to well with final concentration 5000, 500, 50, 5 & 0.5 ppm, with 2% Glucose containing BHI medium, and incubated for 6h. Medium was then aseptically removed, and each well was washed with sterile phosphate buffered saline (PBS) (pH 7.0). After three washings, each well was stained with 200 µl of 0.2% crystal violet aqueous solution for 45 minutes. Wells were washed five times gently with sterile distilled water. 200 µl of 95% ethanol to was added to the wells. 100 µl from each well was transferred to a new microtiter plate OD was measured at 595 nm. The control for each test culture was also maintained (*i.e.* untreated 48h old biofilm). The percentage reduction in biofilm was calculated by using following formula:

Percent reduction in biofilm = (Control $OD_{595\ nm}$ − Test $OD_{595\ nm}$)/Control $OD_{595\ nm}$ x 100

Reduction in Exopolysaccharide Content (EPS): Exopolysaccharide content was estimated by total carbohydrate assay [18]. Mature 48h old biofilm of each test culture was developed on 1 × 1 cm PVC slide. These biofilms were treated separately with MBEC of secondary metabolite for 3h.

After incubation slides were transferred to the glass tubes. 1ml of 5% phenol and 5ml of H_2SO_4 containing 1% hydrazine sulphate was added to each of the tube and incubated for 1 h in dark. Absorbance was measured at 490 nm. The control of biofilm for each test culture was maintained separately.

The percentage reduction in EPS content of biofilm was calculated by using following formula:

Percent reduction in EPS = (Control $OD_{490\ nm}$ −Test $OD_{490\ nm}$)/Control $OD_{490\ nm}$ x 100

Quantify Biofilm Density Reduction: To quantify the effect of SME on biofilm density, method described by Lokegaonkar and Nabar was used [17]. The biofilm (48h) of test cultures were grown on 1x1 cm PVC slides. These slides were exposed to MBEC of secondary metabolite extracts and incubated for 3h. After incubation the biofilm was scraped off from the slide and suspended in the 5 ml of phosphate buffer (pH 7.6). The 0.1ml of fluoresce in diacetate (FDA) in acetone, was added to 3 ml of the suspension. The mixture was incubated for 1 h at 37ºC.

Reaction was stopped using 3ml Acetone and absorbance was read at 490nm. 0.3ml of the scraped suspension was used to determine the bacterial titer by spread plate technique on LB agar.

The results were compared with the untreated biofilm of each test culture respectively. The biofilm density was calculated by following formula:

Surface density of biofilm =
(Bacterial concentration x Buffer Volume)/ Area of slide

Effect on Viability: The biofilm was developed in a microtiter plate by inoculating 20µl of test culture in 180µl of 2% Glucose containing BHI medium. Plates were incubated for 48h, and then medium was decanted. The biofilm was washed with sterile PBS (pH 7.2) to remove debris. Biofilm is exposed to MBEC of extract for 3h. After the treatment biofilm washed to remove residual SME. The biofilm was then disrupted and suspended in a sterile saline. The load of viable cells was determined by serial dilution technique using spread plate method. The positive control of untreated biofilm of test pathogens were also maintained. Reduction in viability was evaluated by comparing the load of bacterial viable cells of SME treated biofilm with control biofilm.

Reduction in Viability = (Control CFU/ml – Treated CFU/ml)/Control CFU/ml x 100

Effect on Extracellular DNA: DNAse activity of crude SME was estimated by using sub MBEC levels SME. In a micro centrifuge tube 200µl of test pathogens was inoculated in 1800µl 2% glucose containing BHI medium along with sub MBEC concentration. Tubes were incubated for 24h, at 37 °C. After incubation tubes were centrifuged at 5000 rpm for 10 minutes. Supernatants were subjected to agarose gel electrophoresis to check the presence of DNA. Electrophoresis was carried out with 1% Agarose gel in Tris Borate buffer with ethidium bromide as a visualizing agent. Agarose gel is observed under UV transilluminator to check the presence of DNA band. Absence of DNA in supernatant indicated the DNAse activity of crude extracts. The test pathogens without SME was kept as a control for each test culture.

Identification of Potential Microorganisms: Isolate with significant QSI activity, and antibiofilm properties was identified on the basis of colony characters, Gram's staining and biochemical analysis.

Statistics: Each assay was performed in triplicate and the values were expressed as the means ± SD. One-way analysis of variance (ANOVA) was performed using SPSS version 17.

RESULTS AND DISCUSSION:

Isolation of Microorganisms: As per the recent study done by Nabar *et al*., Waldhuni river is highly polluted with respect to its physical, chemical as well as biological aspects [19]. Though COD value was reported 28931mg/L, high MPN levels indicate persistence of microorganism in such stressed locations [19]. Thus the locations were selected on the basis of COD and MPN values. From 36 collected samples total 186 isolates were obtained. Among the isolated microbes bacterial population was found to be highest, *i.e*. 127 (124 mesophiles, 3 thermophiles) while fungi as well as actinomycetes were relatively moderate *i.e.* 22 (mesophilic) and 37 (18 mesophilic, 19 thermophilic) respectively.

Presence of "Psychrophiles" was not evident among all sampling sites. The thermophilic microbes were obtained from the above selected polluted sites. All the isolates were screened for their ability to reduce violacein production by *Chromobacterium violaceum* MTCC 2656.

Quorum Sensing Inhibitory Activity Assay: The mechanism of QS regulation is established target for developing alternative biocontrol strategies [20]. O' Loughlin *et al*., reported to show that the anti-QS approach is a promising bio control method against *P. aeruginosa* infections [21]. Recent studies indicate that the soil and plant bacterial isolates produce degradation enzymes rendering efficient QSI activity [19, 22 - 24]. In current study all isolates were screened for violacein inhibition. As the experiment was conducted in dose independent manner, relative potential of each isolate to inhibit violacein was tested. 7 isolates were found able to reduce violacein pigmentation significantly, which include one fungal isolate, 2 Bacterial isolate and 4 Actinomycetes.

Maximum reduction in violacein, with highest significance was showed by bacterial isolate PB2. Hence this isolate was selected for further studies. **Fig. 1** shows the percentage reduction in violacein synthesis in presence of CFS. **Table 2** represents statistical analysis of mean percent reduction in violacein pigmentation.

In the present study QSI (*i.e.* violacein inhibition) activity is considered as indicator of quality antibiofilm properties. Hence further study emphasizes on biofilm eradication, EPS reduction and other biofilm parameters. The secondary metabolites were extracted using ethyl acetate precipitation method and studied for its antibiofilm potential.

Detection of MBEC of PB2 Crude Secondary Metabolite Extract: The percentage reduction in biofilm formation was checked by performing microtiter plate based quantitative assay. MBEC of PB 2 bacterial isolate was found to be 500ppm for selected Gram negative standard strains of pathogens. The effective MBEC for *Staphylococci* was found to be 500ppm, while for *S. mutans* and *E. faecalis* MBEC of crude SME was more than 500ppm. **Table 3** represent the statistical analysis of MBEC determination assay of PB2.

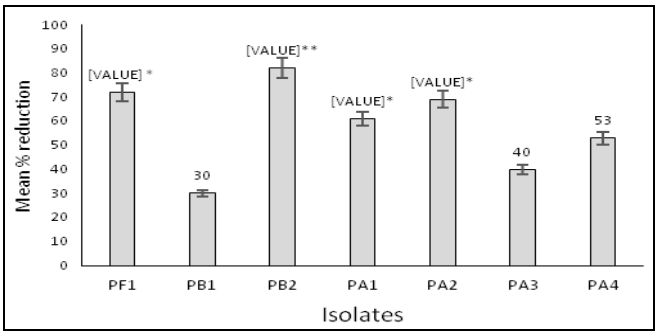

FIG. 1: PERCENTAGE REDUCTION IN VIOLACEIN

TABLE 2: DESCRIPTIVE ANALYSIS OF REDUCTION IN VIOLACEIN

Isolate	Mean Percentage Reduction	Std. Error	Std. Deviation	Variance
PF1	72*	3.28	5.68	32.33
PB1	30	3.75	6.50	42.33
PB2	82**	1.45	2.51	6.333
PA1	61*	2.02	3.51	12.33
PA2	69*	1.73	3.00	9.00
PA3	40	1.85	3.21	10.33
PA4	53	1.73	3.00	9.00

Results are represented as the percentage reduction in Violacein pigmentation. Mean values of triplicate individual experiments and Standard Deviations are shown. **Indicates statistical significance ($p<0.01$), * indicates statistical significance ($p<0.05$)

TABLE 3: DETERMINATION OF MINIMUM BIOFILM ERADICATION CONCENTRATION (MBEC) OF PB2 AGAINST STANDARD PATHOGENS

	Concentration of Secondary Metabolite (PPM)					
Pathogens	5000	500	50	5	0.5	p-Value
	Mean percentage reduction in biofilm formation					
E.coli	100±5	99±8	69±15	61±10	18±2	0.011*
P. aeruginosa	100±9	98±6	47±10	36±18	5±3	0.001*
K. pneumoniae	99±11	99±15	67±3	32±23	13±4	0.000*
V. cholarae	99±7	98±1	97±2	80±13	66±3	0.000*
S. aureus	100±2	98±21	68±51	47±25	49±14	0.208
S. epidermidis	99±1	98±18	65±55	57±25	48±12	0.121
S. mutans	96±6	60±13	62±14	16±7	1±1	0.019*
E. faecalis	90±17	69±9	56±6	57±24	19±9	0.048*

One way ANOVA was performed using SPSS 17., * indicates significance value less than 0.05, Confidence interval : 95%

Reduction in Exopolysaccharide (EPS): The EPS degradation ability of crude SME was studied by exposing a mature biofilm of test pathogens to PB2 extract. Total carbohydrate content of treated and untreated biofilm was compared to evaluate the reduction EPS. Hydrogen peroxide (H_2O_2) is also used as standard antibiofilm agent, to compare the test result. SME reduced EPS content of test pathogens upto 94.16%. While in case of *S. mutans* MTCC 497 76% of EPS of biofilm was degraded compared to control biofilm. In case of Gram negative standard strains of pathogens more than 81% EPS reduction is observed. Crude SME degraded EPS of Gram positive pathogen biofilms significantly. Hydrogen peroxide (H_2O_2) (100ppm) was more efficient against Gram positive pathogens with respect to EPS reduction of a biofilm. Results showed SME have wide range of EPS reduction, degrading EPS of Gram positive as well as Gram negative pathogen biofilms **Table 4**. From **Fig. 2** it is clear that broad spectrum effect on biofilm EPS was exhibited by crude extract of secondary metabolites.

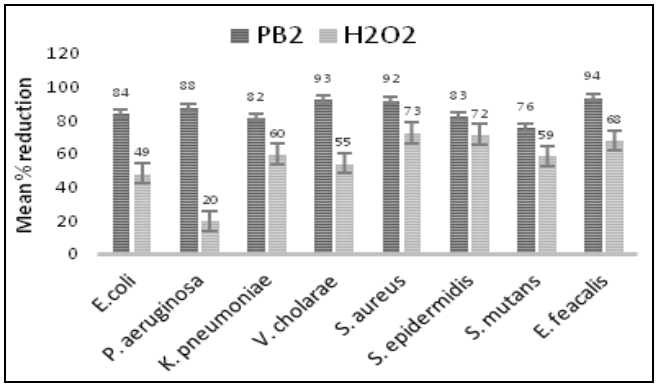

FIG. 2: EFFECT ON EPS CONTENT OF BIOFILM AFTER 6 h TREATMENT WITH PB2 SME AND 100ppm H_2O_2

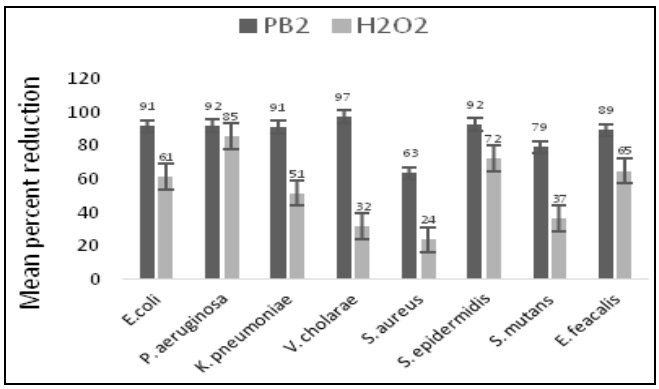

FIG. 3: EFFECT ON VIABLE LOAD OF BIOFILM AFTER 6 h TREATMENT WITH PB2 SME AND 100ppm H_2O_2

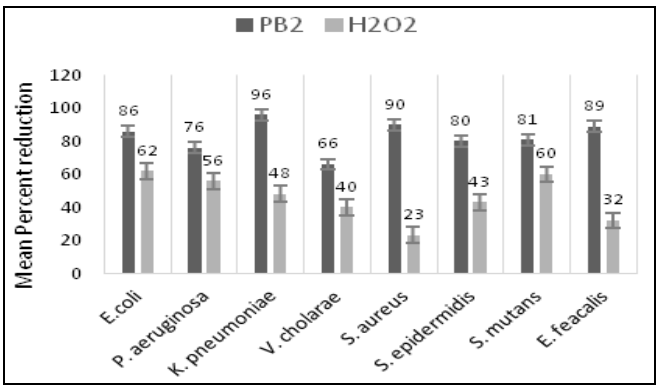

FIG. 4: EFFECT ON BIOFILM DENSITY AFTER 6 h TREATMENT WITH PB2 SME AND 100ppm H_2O_2

FIG. 5: EFFECT ON eDNA RELEASE WITH SUBMBEC CONCENTRATIONS OF PB2 SME

1.=*S. mutans* MTCC 497, 2.=*S. aureus* MTCC 3160, 3.=*S. epidermidis* MTCC 3165, 4.=*E. faecalis* MTCC 439, 5= *K. pneumoniae* MTCC 3384, 6=*V. cholarae* MTCC 3906, 7=*E.coli*, 8=*P. aeruginosa* MTCC 2453, 9= Control. Image was captured using digital camera, and processed with Microsoft paint to add number to each well

TABLE 4: EFFECT OF SECONDARY METABOLITES ON THE VIABLE LOAD, EPS CONTENT AND BIOFILM DENSITY OF STANDARD STRAINS OF PATHOGENS

	Viable count (10^{12} CFU/ml)			EPS (OD at 490nm)			Biofilm Density (%)		
	Positive Control	PB2	H_2O_2	Positive Control	PB2	H_2O_2	Positive Control	PB2	H_2O_2
E.coli		54±9	241±18	1.4± 0.6	0.22±0.1	0.72±0.3	100±10	14±5	38±4
P. aeruginosa		70±10	120± 8	1.0± 0.4	0.12±0.07	0.80±0.7	100±10	24±2	44±10
K. pneumoniae	909± 12	84±12	441± 29	1.1± 016	0.20±0.05	0.44±0.9	100±10	4±3	52±8
V. cholarae	440± 30	14±10	301± 40	1.1± 0.2	0.08±0.02	0.51±0.3	100±10	34±5	60±14
S. aureus	202± 25	74±8	624± 45	1.4± 0.1	0.11±0.03	0.38±0.2	100±10	10±8	77±8
S. epidermidis	510± 41	40±10	822± 5	1.1± 0.3	0.19±0.09	0.31±0.1	100±10	20±4	57±12
S. mutans	350± 19	74±17	222± 38	1.0± 0.2	0.24±0.01	0.41±0.1	100±10	19±6	40±8
E. feacalis	400± 22	44±10	141± 8	1.2± 0.3	0.07±0.02	0.38±0.2	100±10	11±8	68±11

Results are presented in Mean values with the standard deviation obtained with the help of SPSS 17. All experiments are performed in triplicates.

Effect on Viability: Reduction in viable load of bacteria in biofilm was studied by treating the 48h old biofilm for 6h with SME. The control biofilm of each test pathogen was considered as 100% viable mass in a biofilm. After 6h treatment significant reduction in the viability of bacterial population of biofilm was observed. 90% reduction was achieved after only 6h of exposure to the extract.

H_2O_2 was found to be more bactericidal towards Gram negative biofilm than that of Gram positive pathogens **(Fig. 3)**. PB2 proved to more consistent with respect its bactericidal properties.

Effect on Biofilm Density: The biofilm density is a measure of bacterial population per biofilm area. The efficient antibiofilm agent not only affects virulence properties, but also impacts on physiology and structure of biofilm, *i.e.* biofilm density. Result data is shown in **Table 4**. Highest reduction (*i.e.* 96%) in biofilm density was observed in case *K. pneumoniae* MTCC 3384 by PB2 SME in 6h treatment. Though EPS content and viability of *V. cholare* MTCC 3906 was reduced efficiently, PB2 reduced its biofilm density only by 66% **Fig. 4**. H_2O_2 reduced the biofilm density upto 66% in case Gram negative pathogens, and upto 60% in case of Gram positive pathogens.

Extracellular DNA: Bacteria in a biofilm stage release DNA, which enhances adhesion of cells to biotic and abiotic surfaces. This eDNA thus considered to be a potent virulence factor in transformation of planktonic stage to compact biofilm. Compounds having DNAse activity are proven to control the biofilm development. The effect of SME on eDNA release by pathogens have been analysed during the biofilm formation. Hence pathogens were incubated along with subMBEC levels of PB2 SME. After centrifugation, supernatants were electrophoresed on agarose gel and checked for presence of DNA **Fig. 5**. Well no. 9 shows the presence of control DNA. Well no 1, 4 and 8 does not show presence of DNA band.

Extracellular DNA was not obtained from *S. mutans* MTCC497, *E. faecalis* MTCC439, and *P. aeruginosa* MTCC 2453 in presence of SME of PB2. Recently many studies have reported the significance of eDNA*in Streptococcus mutans, S. aureus, P. aeruginosa, V. cholerae* [25 - 27]. Chiang et al elaborated eDNA mediated aminoglycoside resistance in a biofilm forming *Pseudomonas aeruginosa* [28].

Identification of Isolate PB2: Isolate PB2 showed significant quorum sensing inhibitory activity and broad spectrum antibiofilm properties. It was identified on the basis of Bergey's manual of determinative bacteriology, colony characters, Grams characters and biochemical tests. **Table 5** represents the details of identification. Bacterial isolate PB2 was identified as *Pseudomonas aeruginosa*. It will be further subjected to molecular level identification tests such as 16S rRNA.

TABLE 5: IDENTIFICATION OF PB2 BASED ON BIOCHEMICAL TEST ANALYSIS

S. no.	Biochemical tests	Standard	Isolate PB2
1	Glucose	-	-
2	Sucrose	-	-
3	Lactose	-	-
4	Mannose	-	-
5	Xylose	-	-
6	Arabinose	-	-
7	Esculin	-	-
8	Gelatinase	+	+
9	Urea	-	-
10	Methyl Red Test	+/-	-
11	Voges–Proskauer	-	-
12	Indol	-	-
13	PPA	-	-
14	Starch hydrolysis	-	-
15	Citrate	+	+
16	Motility	+	+
17	Oxidase	+	+
18	Catalase	+	+
19	Pigmentation	v	V

Key: + = Positive test, - = Negative test, v= variable
Results of Standard *Pseudomonas aeruginosa* are derived from Bergey's manual determinative bacteriology $9^{th}Ed^{n}$.

CONCLUSION: Polluted conditions not only affect the water environment of a water body but also the soil ecosystem nearby it. Also pollutants are gradually absorbed in soil compared to water, making conditions less vulnerable for soil microbial flora and fauna to survive. Microorganisms adapt diverse metabolical mechanism while growing in such stressed environment.

Waldhuni river, heavily polluted with chemicals and a biological waste, lead microorganisms to

grow in stressed environment. The adapted microbial flora was isolated and tested for Quorum sensing inhibitory activity. *Pseudomonas aeruginosa* PB2, isolated from Waldhuni river exhibits significant QSI activity. Crude SME *P. aeruginosa* PB2, rendered broad spectrum antibiofilm properties against the selected standard strains of pathogens. Its high efficiency to degrade EPS, reduce biofilm density, bactericidal properties and denaturing eDNA proves it to be antibiofilm agent. Though purification of active component from SME is needed *P. aeruginosa* PB2 isolates highlights consideration of polluted environments as a potent source of microorganisms.

ACKNOWLEDGEMENT: Nil

CONFLICT OF INTEREST: The authors declare no conflict of interest.

REFERENCES:

1. Sun F, Qu F, Ling Y, Mao P, Xia P, Chen H and Zhou D: Biofilm-associated infections: antibiotic resistance and novel therapeutic strategies. Future Microbiology. 2013; 8:877-886.
2. Olsen I: Biofilm-specific antibiotic tolerance and resistance. European Journal of Clinical Microbiology and Infectious Diseases. 2015; 34:877-886.
3. Foster TJ, Geoghegan JA, Ganesh VK, Höök M: Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nature Reviews Microbiology. 2014; 12:49-62.
4. Gomes F, Teixeira P, Oliveira R: Mini-review: *Staphylococcus epidermidis* as the most frequent cause of nosocomial infections: old and new fighting strategies. Biofouling. 2014; 30:131-141.
5. Sugimoto S, Iwamoto T, Takada K, Okuda KI, Tajima A, Iwase T and Mizunoe Y: *Staphylococcus epidermidis* Esp degrades specific proteins associated with *Staphylococcus aureus* biofilm formation and host-pathogen interaction. Journal of Bacteriology. 2013; 195:1645-1655.
6. Holmberg A and Rasmussen M: Mature biofilms of *Enterococcus faecalis* and *Enterococcus faecium* are highly resistant to antibiotics. Diagnostic Microbiology and Infectious Disease. 2016; 84:19-21.
7. Taylor PK, Yeung AT and Hancock RE: Antibiotic resistance in *Pseudomonas aeruginosa* biofilms: towards the development of novel anti-biofilm therapies. Journal of Biotechnology. 2014; 191:121-130.
8. Krzyściak W, Jurczak A, Kościelniak D, Bystrowska B and Skalniak A: The virulence of *Streptococcus mutans* and the ability to form biofilms. European Journal of Clinical Microbiology and Infectious Diseases. 2014; 33:499-515.
9. Chatterjee S, Maiti PK, Dey R, Kundu AK and Dey RK: Biofilms on indwelling urologic devices: microbes and antimicrobial management prospect. Annals of Medical and Health Sciences Research. 2014; 4:100-104.
10. Augustine N, Goel AK, Sivakumar KC, Kumar RA and Thomas S: Resveratrol–a potential inhibitor of biofilm formation in *Vibrio cholerae*. Phytomedicine. 2014; 21:286-289.
11. Kalpana BJ, Aarthy S and Pandian SK: Antibiofilm activity of α-amylase from *Bacillus subtilis* S8-18 against biofilm forming human bacterial pathogens. Applied Biochemistry and Biotechnology 2012; 167:1778-1794.
12. Demain AL: Pharmaceutically active secondary metabolites of microorganisms. Applied Microbiology and Biotechnology 1999; 52:455-463.
13. Tiwari K and Gupta RK: Bioactive metabolites from rare actinomycetes. Studies in natural product chemistry. Elsevier, Amsterdam 2014, 419-509.
14. Packiavathy IA, Priya S, Pandian SK and Ravi AV: Inhibition of biofilm development of uropathogens by curcumin–an anti-quorum sensing agent from *Curcuma longa*. Food Chemistry. 2014; 148:453-460.
15. Usup G, Younis KM and Ahmad A: Secondary metabolites produced by marine *Streptomyces* as antibiofilm and quorum-sensing inhibitor of uropathogen *Proteus mirabilis*. Environmental Science and Pollution Research. 2016; 23:4756-4767.
16. Nabar BM and Lokegaonkar S: Larvicidal activity of microbial metabolites extracted from extremophiles against vector mosquitoes. International Journal of Mosquito Research. 2015; 2:61-65.
17. Lokegaonkar SP and Nabar BM: Inhibition of *Strptococcal* biofilms using *Morus alba* leaf extract. Journal of Microbial World 2010; 12:161-167.
18. Mary RN and Banu N: Screening of antibiofilm and anti-quorum sensing potential of *Vitex trifolia* in *Pseudomonas aeruginosa*. International Journal of Pharmacy and Pharmaceutical Sciences. 2015; 78:242-245.
19. Nabar BM, Lokegaonkar SP, Shepal KB, Lele HM, Krishna B and Panjwani D: Study of the seasonal variation of the physico-chemical and microbiological parameters of the Waldhuni river in thane district, India. Khoj, 2011; 91-97.
20. Brackman G and Coenye T: Quorum sensing inhibitors as anti-biofilm agents. Current Pharmaceutical Design. 2015; 21:5-11.
21. O'Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF and Bassler BL: A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. Proceedings of the National Academy of Sciences 2013; 110, 17981-17986.
22. Chow JY, Yang Y, Tay SB, Chua KL and Yew WS: Disruption of biofilm formation by the human pathogen *Acinetobacter baumannii* using engineered quorum-quenching lactonases. Antimicrobial Agents and Chemotherapy. 2014; 58:1802-5.
23. Kalia VC: In search of versatile organisms for quorum-sensing inhibitors: acyl homoserine lactones (AHL)-acylase and AHL-lactonase. FEMS Microbiology Letters. 2014; 359:143-143.
24. Vinoj G and Vaseeharan B, Thomas S, Spiers AJ and Shanthi S: Quorum-quenching activity of the AHL-Lactonase from *Bacillus licheniformis* DAHB1 inhibits *Vibrio* biofilm formation *in vitro* and reduces shrimp intestinal colonisation and mortality. Marine Biotechnology. 2014; 16:707-715.
25. Liao S, Klein MI, Heim KP, Fan Y, Bitoun JP, Ahn SJ, Burne RA, Koo H, Brady LJ and Wen ZT: *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released *via* membrane vesicles, and influenced by components of the protein secretion machinery. Journal of Bacteriology 2014; 196:2355-2366.

26. Okshevsky M and Meyer RL: The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. Critical Reviews in Microbiology, 2015; 41:1-11.
27. Pammi M, Liang R, Hicks J, Mistretta TA and Versalovic J: Biofilm extracellular DNA enhances mixed species biofilms of *Staphylococcus epidermidis* and *Candida albicans*. BMC Microbiology 2013; 3:1-12.
28. Chiang WC, Nilsson M, Jensen PØ, Høiby N, Nielsen TE, Givskov M and Tolker-Nielsen T: Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy 2013; 57: 2352-2361.

How to cite this article:
Lokegaonkar SP and Nabar BM: Broad spectrum antibiofilm and antiquorum sensing potential of crude secondary metabolite extracts of microbial isolates obtained from polluted Waldhuni River. Int J Pharm Sci Res 2018; 9(6): 2378-86. doi: 10.13040/IJPSR.0975-8232.9(6).2378-86.