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Anti-biofilm activity of biogenic selenium nanoparticles and selenium dioxide against clinical isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*

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ABSTRACT

The aim of the present study was to investigate the anti-biofilm activity of biologically synthesized selenium nanoparticles (Se NPs) against the biofilm produced by clinically isolated bacterial strains compared to that of selenium dioxide. Thirty strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* were isolated from various specimens of the patients hospitalized in different hospitals (Kerman, Iran). Quantification of the biofilm using microtiter plate assay method introduced 30% of *S. aureus*, 13% of *P. aeruginosa* and 17% of *P. mirabilis* isolates as severely adherent strains. Transmission electron micrograph (TEM) of the purified Se NPs (produced by *Bacillus* sp. MSh-1) showed individual and spherical nano-structure in the size range of 80–220 nm. Obtained results of the biofilm formation revealed that selenium nanoparticles inhibited the biofilm of *S. aureus*, *P. aeruginosa*, and *P. mirabilis* by 42%, 34.3%, and 53.4%, respectively, compared to that of the non-treated samples. Effect of temperature and pH on the biofilm formation in the presence of Se NPs and SeO_2 was also evaluated.

Introduction

Biofilm is currently defined as structured bacterial communities enclosed in a self-produced extracellular polymeric substance (EPS) such as exopolysaccharide, extracellular DNA (eDNA), and proteins adhered to abiotic or biological surfaces [1,2]. Bacterial cells in biofilm are better protected, less subject to mutation, become more resistant to antibiotics, and represent lower metabolic activity [3]. There are several reports on relation between biofilm and antibiotic resistance which made this problem rarely resolved [4,5]. In this regard, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* are among the leading nosocomial pathogens capable of producing severe biofilm-related infections such as colonization on central venous catheters (CVCs), lower respiratory tract infections (due to contaminated ventilators), and catheter-related ascending urinary tract infections [6,7]. Furthermore, biofilm formation is important, because this mode of growth is associated with the chronic nature of the subsequent infections, and colonizing bacteria can resist against phagocytosis and evade the body's defense system [3]. Biofilm-associated infections have affected millions of people in both developed and developing countries and consequently caused death in their victims [4,8]. So, investigations on biofilm inhibitory activity of even natural or synthetic compounds have received more attention in recent decades [2].

Nanotechnology concerns the arrangement of materials at the atomic stage to achieve nanoscale materials with unique physicochemical and biological characteristics [9,10]. The ability of nanostructures for the inhibition or disruption of microbial-derived biofilm has been recently reported. For example, Naik and Kowshik [11] investigated the effect of sol–gel coatings of AgCl–TiO_2 nanoparticles for the inhibition of biofilm formed by *Escherichia coli*,

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S. epidermidis, and *P. aeruginosa*. Anti-biofilm activity of chemically synthesized ZnO and CuO nanoparticles was reported by Tabrez Khan et al. [12]. Kalishwaralal et al. (2010) [13] studied the inhibition effect of silver nanoparticles on the biofilm formation of *P. aeruginosa* and *S. epidermidis*.

Selenium (Se) is a micronutrient metalloid incorporated (in the form of selenomethionine, selenocysteine) in the structure of several enzymes such as glutathione peroxidases (GPx), iodothyronine deiodinases, and thioredoxin reductase (TrxR), which are involved in antioxidant defense, detoxification, and metabolism, respectively [14,15]. Excellent biological properties of selenium nanoparticles (Se NPs) such as antibacterial, antiviral, and antioxidant activity together with their lower toxicity have introduced it as an interesting subject in the field of nanotechnology [16,17]. Behind the physicochemical techniques applied for the synthesis of Se NPs [18,19], biological methods, (synthesis of nanostructures using some bacterial and fungal strains as well as several plant extracts) [20,21] supply novel, clean, non-toxic, and eco-friendly method for the production of Se NPs. To the best of our knowledge and according to a survey of the literature, there is no report on the anti-biofilm effect of the biogenic Se NPs. In the present study, the biogenic Se NPs was purified from the whole cell lysate of *Bacillus* sp. MSh-1 and their effects on the biofilm formation by three bacterial strains (*P. mirabilis*, *S. aureus*, and *P. aeruginosa*) isolated from clinical specimens compared to selenium dioxide were also studied.

Materials and methods

Chemicals

Selenium dioxide (SeO_2), nutrient broth, *n*-octyl alcohol, sodium dodecyl sulfate (SDS) and Tris-base were purchased from Merck Chemicals (Darmstadt, Germany). All other chemicals and solvents were of analytical grade.

Biosynthesis and purification of the Se NPs

Bacterial strain of *Bacillus* sp. MSh-1 which was previously isolated from the Caspian Sea (located in the northern part of Iran) and identified by 16S rDNA gene analysis technique was applied for the biosynthesis of Se NPs based on the method described by Shakibaie et al. [21]. In brief, 100 mL sterile nutrient broth (NB) medium containing SeO_2 (final concentration of 1.26 mM) was inoculated with 1 mL of the fresh inoculums (OD_{600}, 0.1) of *Bacillus* sp. MSh-1 and incubated in a shaker incubator (150 rpm) at 30 °C for 14 h. Thereafter, the bacterial cells were harvested by centrifugation (4000 × *g* for 10 min) followed by washing the obtained biomass with the sterile NaCl solution (0.9%) for three times. The bacterial pellets were then disrupted by grinding the frozen cells in liquid nitrogen using a mortar and pestle. The resulting slurry was consequently ultrasonicated at 100 W for 5 min using ultrasonic processor (Sonics Vibra Cell VC-505/220, Newtown, USA) over three 15 s periods, with an interval of 45 s between the periods. The sonicated extract was then washed for three times by the sequential centrifugation (14,000 × *g*, 5 min) with a 1.5 M Tris–HCl buffer (pH 8.3) containing SDS (1%) and deionized water, respectively. Subsequently, Se NPs were extracted and purified by organic–aqueous partitioning system (*n*-octanol–water), as previously described [21]. Surface morphology of the prepared biogenic Se NPs was examined by transmission electron microscope (Zeiss Supra 55 VP TEM, operated at 100 kV) equipped with an EDX (energy dispersive X-ray) microanalyzer. The related size distribution pattern of biologically synthesized Se NPs was plotted by manually counting of 400 individual particles from different TEM images.

Isolation and identification of biofilm-producing bacteria

Clinical specimens of wounds, urine, cerebrospinal fluid (CSF), and blood as well as lung and nasal secretion were collected from the patients hospitalized in different hospital wards like burn, ICU, pediatric, and surgery from August 2012 to March 2013 in Kerman, Iran. All the collected samples were aseptically transported to the nutrient broth within 45 min of sample collection. Each sample was then diluted (10^{-2}) using sterile normal saline solution (0.9%) and streaked onto MacConkey and sheep blood agar medium followed by incubation at 37 °C for 24 h and identification of isolated microorganisms based on the standard procedures [22]. The identified isolates were then mixed with 2 mL sterile Trypticase Soy Broth (TSB) containing glycerol (15%) and preserved at −70 °C.

Antibiotic susceptibility testing and determining the MIC for Se NPs

Susceptibility of each isolate to the antibiotics of methicillin, tetracycline, amikacin, gentamicin, ciprofloxacin, ceftazidime, vancomycin, erythromycin, chloramphenicol, amoxicillin + clavulanic acid, and imipenem was evaluated using disk diffusion method based on the protocol described by Clinical and Laboratory Standards Institute (CLSI 2006) [23]. All the applied antibiotic disks were purchased from Oxoid Inc. (Mumbai, India). Reference strains *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were included as controls. Zone of inhibition surrounding each disk was measured and labeled as resistance, intermediate, and sensitive according to CLSI protocol. In order to determine the minimum inhibitory concentration (MIC) of biogenic Se NPs (concentration range of 0–100 $\mu g\,mL^{-1}$) on the isolated strains, the agar dilution method was applied according to the method by Zare et al. [24].

Biofilm formation assay

The biofilm formed by the above isolates was quantified by microtiter method as described previously [25] with some modification. Briefly, one loopful from each isolated colony was inoculated into a sterile TSB medium (2 mL) containing glucose (1% W/V) to optimize biofilm production. Optical density (OD_{650}) was then adjusted to 0.13 to reach 0.5 McFarland standard (1.5×10^8 CFU mL^{-1}) followed by further dilution of the prepared bacterial suspension to reach $\sim 10^6$ CFU mL^{-1} and addition of 100 μL of each prepared inoculum to 96-well flat bottom tissue culture microplate. Similarly, 100 μL of the TSB medium without any bacterium (negative control) was added to the related well and the microtiter plate was then incubated at 37 °C under static condition. To evaluate the quality of the method, standard strain of *E. coli* (DH5α) was used as control for no biofilm microorganism. After 24 h incubation at 37 °C, non-adherent cell suspensions were aseptically aspirated, washed, and replaced with 10 μL of sterile phosphate buffered solution (pH 7.2) to remove any remaining suspended cells. In order to fix the biofilm, 150 μL of methanol was added to each well and kept at room temperature (25 °C) for 20 min. Methanol was then removed and replaced with 200 μL of crystal violet solution (1% W/V). The wells containing biofilm matrix were slowly washed with sterile deionized water and kept at room temperature until drying. Thereafter, 200 μL of glacial acetic acid (33% V/V) was added to each well and the optical density of each well was measured at 570 nm using Synergy 2 multi-mode microplate reader (BioTek, USA). The isolates were then classified into strongly adherent, moderately adherent, weakly adherent, and non-adherent strains based on the formula given by Stepanovic et al. [25]. All the mentioned experiments were performed in

triplicate and the most potent biofilm-producer isolates were selected for further investigations.

Biofilm inhibition assay for biogenic Se NPs and SeO_2

In order to evaluate the anti-biofilm effect of Se NPs and SeO_2, the most potent biofilm producer isolates of *P. mirabilis*, *S. aureus* and *P. aeruginosa* were separately seeded into 96-well microplates as previously described (section "Biofilm formation assay"). Thereafter, Se NPs and SeO_2 were added to the desired wells to reach the sub-MIC concentration of 0–16 μg mL^{-1}. After 24 h incubation at 37 °C, fixation and colorization of the formed biofilm were carried out by methanol and crystal violet, respectively, and the absorbencies were recorded at 570 nm as mentioned above. Simultaneously, CFU mL^{-1} of each well treated with Se NPs (concentration range of 0–16 μg mL^{-1}) was also determined. All the experiments were carried out in triplicate and mean of the obtained results was reported.

Effect of temperature and pH on biofilm inhibition

Effect of temperature on the biofilm formation in the presence of Se NPs and SeO_2 was studied as follow. After preparing the preculture of each biofilm-producing isolate in TSB medium (~10^6 CFU mL^{-1}), 100 μL of the bacterial suspension was added to 96-well polystyrene microplates; then, Se NPs and SeO_2 solutions were inserted into each well to reach the final concentration of 16 μg mL^{-1}. The microplates were then incubated at three different temperatures (25 °C, 37 °C, and 42 °C) for 24 h under static condition and the amount of biofilm formation was determined as previously described.

In order to evaluate the influence of pH on biofilm formation in the presence of Se NPs and SeO_2, the same experiments were designed by preparing of TSB medium with different initial pH (5, 7, and 9) followed by the addition of biofilm producing strain (100 μL from a preculture suspension, ~10^6 CFU mL^{-1}) and insertion of Se NPs or SeO_2 solutions into each well to reach the final concentration of 16 μg mL^{-1}. The prepared microplates were then incubated at 37 °C and the amount of biofilm formation was quantified as said above. All the experiments were repeated three times and mean of the obtained results was reported.

Statistical analyses

Each value was expressed as mean ± SD. SPSS software 15 for Windows (SPSS Inc., Chicago) was used for statistical analysis. Differences between the groups were determined using one-way analysis of variance (ANOVA) and *p*-values of less than 0.05 were considered to be significant.

Results and discussion

Biosynthesis and characterization of Se NPs

Se NPs was successfully synthesized using *Bacillus* sp. MSh-1 which was simply evident from significant color change in cultivation medium from colorless to insoluble orange–red elemental selenium (Se^0) (data not shown) and purified by liquid–liquid extraction method. Based on the TEM micrograph of the purified Se NPs (Fig. 1a), well dispersed nanostructures with spherical shape and diameter range of 80–220 nm were synthesized by *Bacillus* sp. MSh-1. EDX microanalysis of the purified NPs exhibited Se absorption peaks consisting of SeLα, SeKα and SeKβ at 1.37, 11.22 and 12.49 keV, respectively (Fig. 1b). Furthermore, elemental composition analysis indicated the presence of strong signals from the Se atoms with the weight percent equal to 100 without signals of other elements. Thus, the *n*-octanol/water partitioning system could be successfully applied to remove different soluble or insoluble impurities of biologically synthesized Se NPs. Size distribution pattern (Fig. 2) measured by manual counting of 400 individual particles from different TEM images revealed that the NPs with the size of 125 nm to 150 nm were the most frequent particles (Fig. 2).

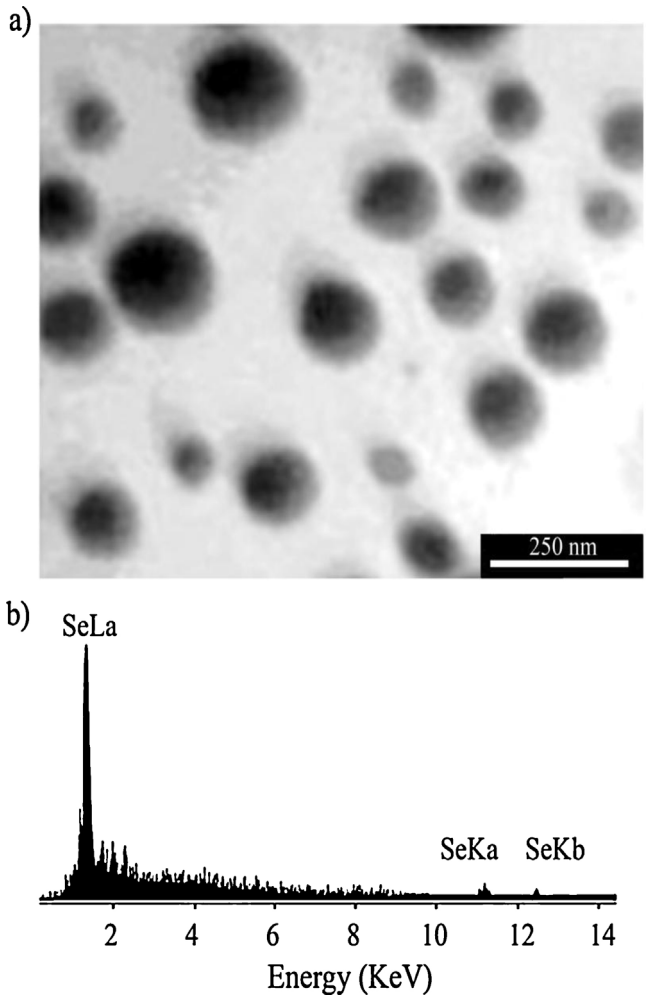

Fig. 1. (a) Transmission electron microscopy (TEM) and (b) energy dispersive X-ray (EDX) images of Se NPs synthesized by *Bacillus* sp. MSh-1 and purified using *n*-octyl alcohol/water extraction system.

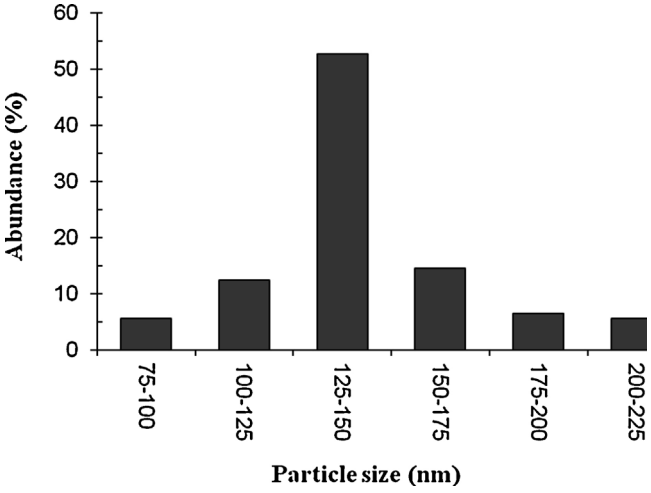

Fig. 2. Particle size distribution histogram of the biogenic Se NPs obtained by manual counting of 400 individual particles from different TEM images.

Recent studies have revealed excellent optical and physical characteristics of Se NPs (as an indirect elemental semiconductor) such as high photoconductivity (*ca.* 8×10^4 S cm^{-1}), saturable absorption, good photoelectrical, nonlinear optical properties, anisotropy of thermo-conductivity, and thermoelectric response [26,27]. Furthermore, biological activities of Se NPs like induction of apoptosis in cancer cell line [28] and protective effect on Cisplatin-induced spermatotoxicity [29] as well as antioxidant [14], antibacterial, and antileishmanial [16] properties have been also reported. So, development of cost-effective methods for the synthesis of Se NPs has been considered by many investigations, among which application of biological resources such as bacterial, fungal, and plant extracts for the production of Se NPs have gained great interest during two last decades [14,30]. The obtained results of the present work introduced the marine strain of *Bacillus* sp. MSh-1 as an efficient bacterial strain able to reduce selenate to Se^0. The halotolerant bacterial strain, *Bacillus megaterium*, which could tolerate 7% NaCl, was isolated from Bhitarkanika mangrove soil [31]. This Gram-positive bacterial strain efficiently reduced selenite (up to 0.25 mM) to Se NPs after 40 h of incubation. Application of the culture supernatant of *Aspergillus terreus* toward SeO_2 (final concentration of 100 μg mL^{-1}) led to the formation of Se NPs with the average size of 47 nm [32]. The study carried out by Ramamurthy et al. [33] introduced fenugreek seed extract as a herbal source for producing Se NPs (with the particle size of 50–150 nm) after 72 h incubation.

Biofilm-producing bacterial strains

More than 30 species of *S. aureus*, *P. aeruginosa* and *P. mirabilis* were isolated from various specimens of the patients hospitalized in different hospital units. The most potent biofilm-producing bacterial strains were selected based on the biofilm formation assay. Furthermore, the antibiotic resistance pattern of each isolate were determined (Table 1). For *S. aureus*, 30% and 70% of the isolates found to be severely and moderately adherent, respectively. In the case of *P. aeruginosa*, the obtained results revealed that 13% of isolates was severely attached, 70% was moderately attached, and 17% was weakly attached. For *P. mirabilis*, 17%, 40% and 36% of isolates exhibited to be severely adherent, moderately adherent, and weakly adherent, respectively, and 7% of the isolates did not develop any biofilm. The obtained results of MIC determination for the isolated strains in the presence of Se NPs and SeO_2 revealed that all the applied clinical isolates were resistant to Se NPs and SeO_2 up to 100 μg mL^{-1}. So, the sub-MIC concentration of Se NPs and SeO_2 was used in order to evaluate the antibiofilm activity of biogenic selenium nanoparticles. Determination of CFU mL^{-1} of each isolate against the sub-MIC concentration of Se NPs (0–16 μg mL^{-1}, Fig. 3) demonstrated a mild decrease in CFU mL^{-1} of all the isolates by increasing the concentration of Se NPs, concentration indicating that Se NPs was moderately non-toxic for all the applied bacterial strains in the applied Se NPs concentration range.

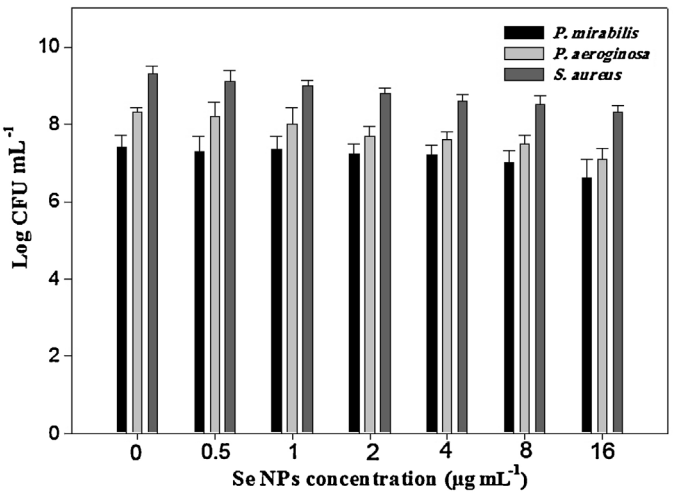

Fig. 3. Effect of Se NPs on growth of three biofilm-producing bacterial strains. Each well received 10^5 CFU mL^{-1} of each isolate and cultures were incubated at 37 °C for 24 h and the produced CFU mL^{-1} was then determined.

According to the report of National Institutes of Health about 65% of all infections involve with biofilm formation among, which the Gram-negative bacterium of *P. aeruginosa* and the Gram-positive staphylococci, *S. aureus* are the most common ones [34]. Both physiological and phonotypical characteristics of sessile (biofilm-associated) cells have been found to be different from those of non-adhered planktonic cells [2]. Behind health problems produced by development of biofilm producer, such strains can also have a detrimental effect in industrial systems [2]. So, isolation and identification of biofilm-producing strains and their biofilm structure as well as fighting with biofilm-producing microbial strains (which are often resistant to the available antibiotics) have been targeted by many investigations [35] since the first report of biofilm description in 1978 [36]. The obtained results of the present study revealed presence of resistant bacterial strains able to produce biofilm in the samples collected from hospitalized patients. In the study performed by Ansari et al. [37], it was found that out of 40 isolates of *E. coli* and 6 of *Klebsiella* spp. (which could produce extended

Table 1
Antibiotic susceptibility pattern of thirty *P. aeruginosa, S. aureus and P. mirabilis* isolates to commonly used antibiotics.[a]

Antibiotic	*P. mirabilis*			*S. aureus*			*P. aeruginosa*		
	R	S	I	R	S	I	R	S	I
CIP	31.81	46.59	21.95	33.3	0	66.6	16.65	73.26	9.99
TE	ND[*]	ND	ND	66.6	0	33.3	86.58	0	13.32
GM	40.04	39.7	18.18	33.3	0	66.6	49.95	0	49.95
AN	46.59	40.9	12.50	23.3	10%	66.6	19.98	16.65	63.27
CAZ	35.22	46.59	18.18	40	36.6	23.3	19.98	63.27	16.65
AMC	90	0	10	86.6	13.3	0	100	0	0
MET	ND	ND	ND	26.7	0	73.3	ND	ND	ND
VAN	ND	ND	ND	0	16.66	83.3	ND	ND	ND
E	ND	ND	ND	36.6	0	63.3	100	0	0
C	22.72	53.4	23.36	0	0	100	83.25	0	16.65
IMP	0	0	100	ND	ND	ND	19.98	66.6	13.32

MET, methicillin; TE, tetracycline; AN, amikacin; GM, gentamicin; CP, ciprofloxacin; CAZ, ceftazidime; VAN, Vancomycin; E, erythromycin; C, chloramphenicol; AMC, amoxicillin + clavulanic acid; IMP, imipenem; R, resistance; S, sensitive; I, intermediate. Figures indicate percentage of antibiotic resistance, sensitive or intermediate in bacterial population.

[a] Muller-Hinton agar was used for susceptibility testing. Inoculum diluted to obtain 1×10^8 CFU mL^{-1}.

[*] ND = not determined.

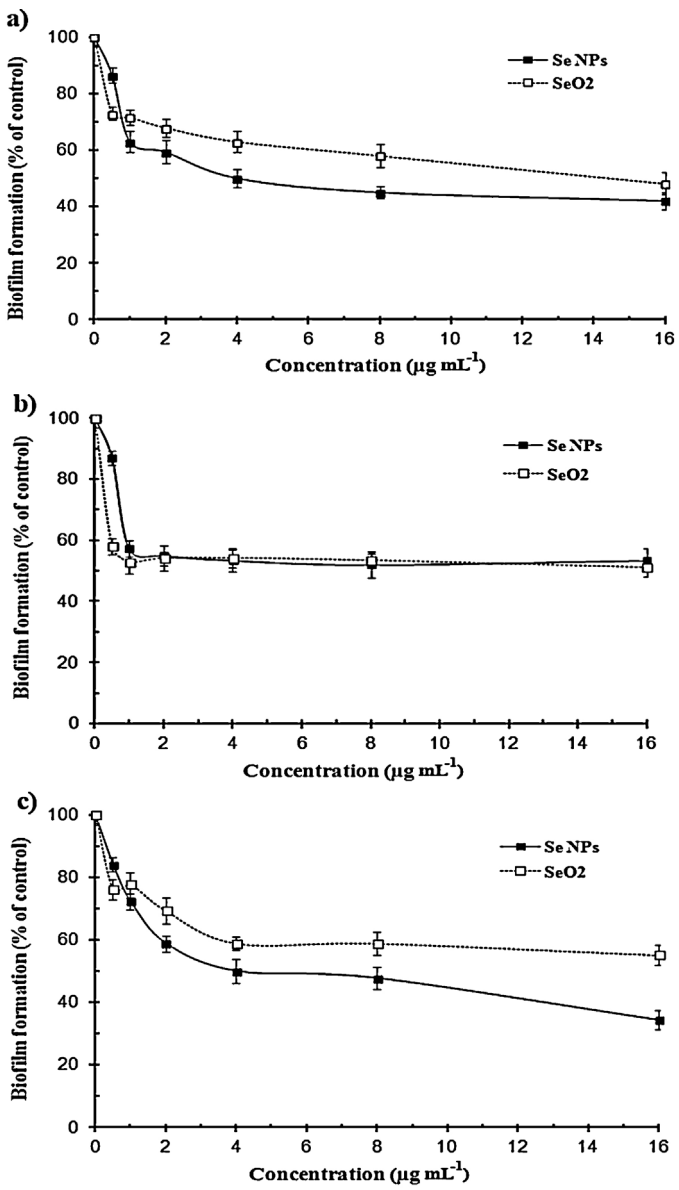

Fig. 4. Biofilm formation of (a) *S. aureus*, (b) *P. mirabilis*, and (c) *P. aeruginosa* in the presence of different concentration (0–16 μg mL^{-1}) of selenium dioxide (SeO_2) and biogenic Se NPs. Data are expressed as the mean ± SD ($n = 3$). Wells containing biofilm-forming bacteria in the absence of Se NPs or SeO_2 were designed as control.

spectrum β-lactamases) tested for biofilm formation using Congo red agar (CRA) method, 26 (65%) isolates of *E. coli* and 4 (66.67%) isolates of *Klebsiella* spp. produced black colonies. Two biofilm producer strains (identified as *P. aeruginosa* and *S. epidermidis*) were isolated from the contact lenses in the study conducted by Kalishwaralal et al. [13].

Effect of Se NPs and SeO_2 on biofilm formation

Effect of Se NPs and SeO_2 on biofilm formation by *S. aureus*, *P. mirabilis*, and *P. aeruginosa* is shown in Fig. 4. In the case of *S. aureus*, the amount of biofilm formation was sharply decreased by increasing Se NPs (59.3 ± 2.1%) and SeO_2 (67.9 ± 1.9%) up to 2 μg mL^{-1} and reached 42 ± 1.8% and 48.1 ± 1.5%, respectively, at the concentration of 16 μg mL^{-1}, as presented in Fig. 4a. In the case of *P. mirabilis* treated with Se NPs and SeO_2 (0–16 μg mL^{-1}), the amount of biofilm formation was decreased to 53.4 ± 2.3% and 51.1 ± 2%, respectively, and remained constant at the concentration above 2 μg mL^{-1} (Fig. 4b). For *P. aeruginosa*, the amount of biofilm formation was dropped to 34.3 ± 1.4% and 55.1 ± 1.7% in the presence of Se NPs and SeO_2 (0–16 μg mL^{-1}), respectively (Fig. 4c).

Inability of antimicrobial agents for penetration into biofilm network (one of the most important reasons for the development of resistant microbial strains) could be overcome *via* the application of nanostructures exhibiting anti-biofilm activity [37,38]. Nowadays, nanoantibiotics have been constructed and evaluated for the inhibition of bacterial growth, even in planktonic or sessile forms [39]. Potential of silver nanoparticles (synthesized by chemical or biological methods) for the inhibition of biofilm formation has been determined by many investigations. For example, Ansari et al. [37] reported complete inhibition of biofilm formation of *E. coli* and *Klebsiella pneumoniae* in the presence of Ag NPs at the concentration as low as 50 μg mL^{-1}. The Same results were reported by Kalishwaralal et al. [13] who determined 95–98% reduction in the biofilm formation of *P. aeruginosa* and *S. epidermidis* biofilms after the addition of Ag NPs at the concentration of 100 nM.

Literature review revealed no reports on the antibiofilm activity of either chemically or biologically synthesized Se NPs. Results of the present work showed that the effect of biogenic Se NPs on biofilm formation by *P. mirabilis*, *S. aureus* and *P. aeruginosa* was not significantly higher than that of SeO_2 ($p > 0.05$) (Fig. 4). Although, the anti-biofilm activity of Se NPs (at the concentration above 1 μg mL^{-1}) on biofilm formation by *S. aureus* and *P. aeruginosa* was more than SeO_2, this reduction was not statistically significant ($p > 0.05$) (Fig. 4a and c). In addition, the anti-biofilm activity of Se NPs (>4 μg mL^{-1}) on biofilm formation by *S. aureus* and *P. aeruginosa* was higher than that of *P. mirabilis*; however, this effect was not significant ($p > 0.05$). On the other hand, the anti-biofilm activity of SeO_2 (>0.5 μg mL^{-1}) on biofilm formation by *P. mirabilis* was not significantly higher than those of *S. aureus* and *P. aeruginosa* ($p > 0.05$). Bearing in mind the lower toxicity of biogenic Se NPs (based on the investigations in both *in vivo* and *in vitro* models) compared to that of selenite or selenate [17], Se NPs or antimicrobials loaded on the surface of Se NPs might be the candidate as novel antibiofilm agents. However, further investigations are needed to evaluate the mentioned effect.

Effect of temperature and pH on biofilm inhibition

Effect of temperature on biofilm formation in the presence of Se NPs and SeO_2 (16 μg mL^{-1}) is presented in Fig. 5a. Results showed that, at 25 °C and 42 °C, the anti-biofilm activity of Se NPs on *P. mirabilis*, *S. aureus*, and *P. aeruginosa* was not significantly different from each other ($p > 0.05$). The anti-biofilm activity of Se NPs at 37 °C on *S. aureus* and *P. aeruginosa* was significantly higher than that of *P. mirabilis* ($p < 0.05$). For SeO_2, the results showed that the anti-biofilm activity for *P. mirabilis*, *S. aureus*, and *P. aeruginosa* was not significantly different from each other ($p > 0.05$); but, at 25 °C and 37 °C, the anti-biofilm activity for *S. aureus* and *P. aeruginosa* was significantly greater than *P. mirabilis* ($p < 0.05$). Furthermore, the biofilm-inhibitory effect of SeO_2 for *S. aureus* and *P. aeruginosa* was significantly higher than that of Se NPs at 25 °C and 37 °C ($p < 0.05$) and the biofilm-inhibitory effect of SeO_2 for *P. mirabilis* was not significantly higher than that of Se NPs at 25 °C and 37 °C ($p > 0.05$).

In general, most of mesophilic bacteria represent their highest attachment capacity at optimum cultivation temperature and the number of attached cells decreases by alteration from related optimum temperature [40]. However, depends on the applied strain in biofilm investigations the effect of temperature on biofilm formation found to be varied. For example, Nichols et al. [41] reported that production of exopolysaccharides in *Pseudoalteromonas* species maximally occurred at −2 °C and 10 °C compared

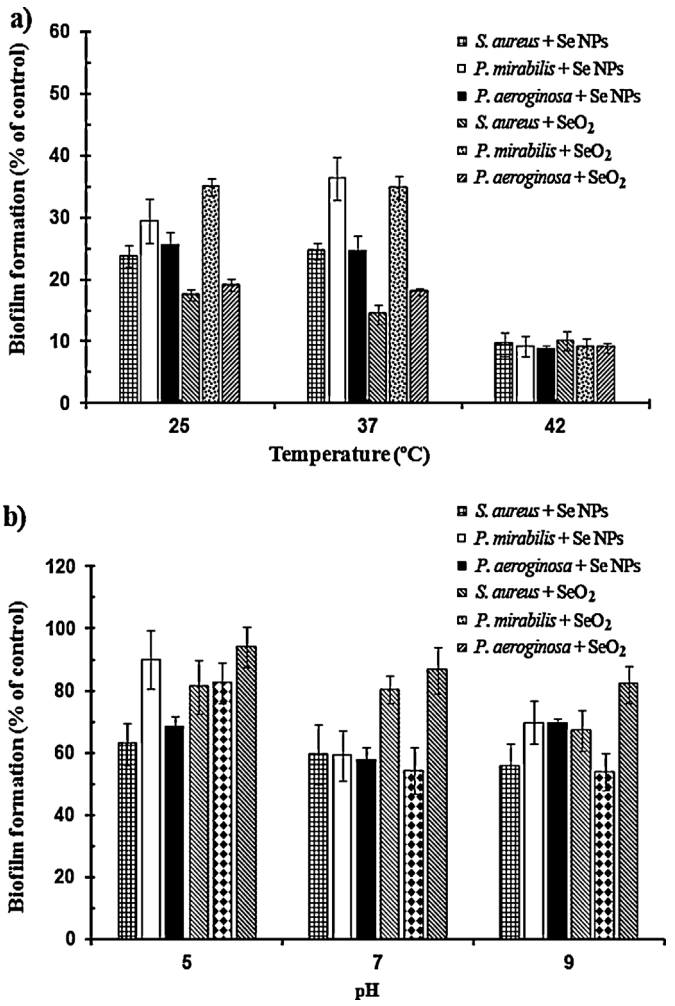

Fig. 5. Effect of (a) temperature and (b) pH on biofilm formation by three clinical isolates in the presence of biogenic Se NPs (16 μg mL^{-1}) and SeO_2 (16 μg mL^{-1}). Control is non treated wells containing biofilm-forming bacteria in absence of Se NPs or SeO_2.

to that of 20 °C. On the other hand, in the case of *Salmonella typhimurium* it was revealed that increasing of temperature up to 37 °C increased the rate of biofilm formation while the number of attached cells decreased at 42 °C [42]. In the case of *Listeria monocytogenes* (one of the most important foodborne pathogens) it was found that formation of the biofilm was temperature-independent [43].

Effect of pH on biofilm formation in the presence of Se NPs and SeO_2 (16 μg mL^{-1}) was studied (Fig. 5b). Results revealed that, at pH 7 and 9, the anti-biofilm activity of Se NPs on *P. mirabilis*, *S. aureus*, and *P. aeruginosa* was not significantly different from each other ($p > 0.05$). In contrast, at pH 5, the biogenic Se NPs for *S. aureus* and *P. aeruginosa* represented a significant increase in anti-biofilm activity compared to *P. mirabilis* ($p < 0.05$).

Literature review revealed the key role for pH of cultivation media on biofilm formation. In the study of Dat et al. [44] who investigated on the biofilm formation of *Bacillus licheniformis* NBRC 12195 and *Lactobacillus paracasei* subsp. *paracasei* NBRC 15889 on stainless steel, it was found that optical densities of biofilms formed in the pH-adjusted samples were significantly lower than those of pH-unadjusted samples. However, in the case of *Salmonella enteritidis* the biofilm formation was reported to be independent of the pH value [45].

Conclusion

To sum up, the obtained results of the present work introduced biologically synthesized Se NPs as an antibiofilm-forming agent against clinically isolated bacterial strains. It seems that different oxidation states of selenium (Se^0 and Se^{4+}) exhibits different effects on biofilm formation in the presence of each bacterial strain at different pH and temperature. However, the molecular mechanisms of biofilm-inhibitory effect for Se NPs and other Se compounds have not been completely understood and merits further studies.

Conflict of interest

The authors declare that they do not have any conflict of interest in this study.

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