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Research article

Antifouling activities of proteinase K and α-amylase enzymes: Laboratory bioassays and *in silico* analysis

Sathianeson Satheesh *, Lafi Al Solami

Department of Marine Biology, Faculty of Marine Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

The application of enzymes as antifoulants is one of the environment-friendly strategies in biofouling management. In this study, antifouling activities of commercially available proteinase K and α-amylase enzymes were evaluated using barnacle larva and biofilm-forming bacteria as test organisms. The enzymes were also tested against barnacle cement protein through *in silico* analysis. The results showed that both enzymes inhibited the attachment of bacteria and settlement of barnacle larvae on the test surface. The lowest minimum inhibitory concentration of 0.312 mg ml^{-1} was exhibited by proteinase K against biofilm-forming bacteria. The calculated LC_{50} values for proteinase K and α-amylase against the barnacle nauplii were 91.8 and 230.96 mg ml^{-1} respectively. While α-amylase showed higher antibiofilm activity, proteinase K exhibited higher anti-larval settlement activity. Similarly, *in silico* analysis of the enzymes revealed promising anti-settlement activity, as the enzymes showed good binding scores with barnacle cement protein. Overall, the results suggested that the enzymes proteinase K and α-amylase could be used in antifouling coatings to reduce the settlement of biofouling on artificial materials in the marine environment.

1. Introduction

Biofouling growth on artificial substrates submerged in marine waters is a significant problem throughout the world with considerable economic significance. Though the use of chemical compounds has proved to be efficient in controlling biofouling growth, environmental concerns warrant the restricted applications of inorganic and organic compounds in antifouling coatings [1]. In aquaculture facilities, the toxic biocides used for biofouling management may affect the cultured organisms [2]. The research interest in searching for alternative eco-friendly antifoulants has grown in the recent past, particularly after the ban on tributyltin-based antifouling paints [3,4]. Natural products from terrestrial and aquatic sources have been suggested as efficient antifoulants [5–7]. Moreover, enzymes from microorganisms are also reported to possess strong antibiofilm activity against many bacterial strains [8,9].

Antifouling coatings consisting of enzymes as active ingredients are considered environmentally friendly [10] and may possess strong inhibitory activities against biofouling organisms [11,12]. In a previous study, Pettitt et al. [13] evaluated the activity of some commercial enzymes (including proteases and α-amylase) on the settlement of fouling organisms. Many other studies (e.g. Refs. [12, 14,15], (also tested the inhibitory activities of enzymes against different fouling organisms. However, *in silico* analysis of the interaction between enzymes and barnacle cement protein has not been studied in detail. Molecular docking is one of the cost-effective methods to determine the binding affinity of chemical compounds with potential targets [16]. Molecular docking methods are

* Corresponding author.
E-mail addresses: ssathianeson@kau.edu.sa, satheesh_s2005@yahoo.co.in (S. Satheesh).

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considered an important tool in the early stages of drug discovery [17]. Also, virtual screening tools are recently gaining attention in antifouling compound screening studies [18,19].

The basic principle involved in the application of enzymes for antifouling purposes is to cleave the adhesives produced by the fouling organisms and thereby inhibit the attachment [12]. These adhesives mainly consist of polysaccharides and proteins along with some other components [12,20,21]. Most of the fouling organisms such as barnacles and bivalves produce sticky materials for firm attachment on surfaces [22]. Barnacle cement is a proteinaceous substance that mainly consists of protein and a small amount of carbohydrates and lipids [23,24]. The compounds or agents that modify the biochemistry or degrade the barnacle cement may prevent the settlement on surfaces. In addition to barnacles, the initial biofilm communities, mainly bacteria settling on the substrates, play a key role in the settlement of macrofouling organisms [25,26]. For antifouling screening studies, biofilm-forming bacteria are commonly used as targets to understand the antibiofilm activity of the compounds [27,28]. While many enzymes are tested against bacteria for antibiofilm applications, studies on the antifouling activity of proteinase K are lacking. Among the proteases, proteinase K is highly stable in various environmental conditions [29]. Hence, this enzyme was selected from the proteases to test the antifouling activity. The major objectives of the present study were to (1) test the antifouling efficiency of proteinase K enzyme and (2) compare the antifouling performance between proteinase K and α-amylase enzymes. A study of this type will be useful for understanding the settlement-inhibiting activities of enzymes and designing enzyme-based biofouling control measures.

2. Materials and methods

2.1. *In silico analysis for the selection of enzyme from the protease group*

An initial *in silico* analysis was carried out to select the most suitable protease enzyme that cleaves the biofilm-associated protein (BAP) and barnacle cement protein. The BAP (UniProt ID: Q79LN3) and barnacle cement protein (UniProt ID: Q9GRC4) were loaded onto the PeptideCutter server (https://web.expasy.org/peptide_cutter/). PeptideCutter is an online server that predicts the potential cleavage sites of protease enzymes in a chosen protein sequence. All the available protease enzymes in the server were used against BAP and barnacle cement protein sequences. UniProt ID of the BAP and barnacle cement were uploaded in the server and cleavage protein function was performed. Based on the *in silico* analysis, proteinase K (serine protease) enzyme was used in this study. In addition, α-amylase enzyme was used for testing the antifouling activity. Previous studies reported the efficiency of α-amylases in the degradation of extracellular polymeric substance (EPS) matrix formed in biofilms [30].

2.2. *Enzymes*

The commercially available proteinase K (from *Tritirachium album*, P2308, Sigma-Aldrich) and α-amylase (from *Bacillus licheniformis*, A4551, Sigma-Aldrich) enzymes were used for laboratory assays. Protease (Subtilisin A, product code P5380, Sigma-Aldrich) from *Bacillus licheniformis* was used as positive control.

2.3. *Antibacterial activity*

The antibacterial activity of the enzymes was evaluated against biofilm bacteria isolated from submerged surfaces in the Red Sea. The biofilm bacteria are *Psychrobacter* sp. IMB8 (NCBI GenBank: ON003956), *Pseudoalteromonas issachenkonii* IMB12 (NCBI GenBank: ON003959) and *Pseudoalteromonas shioyasakiensis* IMB13 (NCBI GenBank: ON003960). These biofilm bacterial strains were isolated from the marine biofilm developed on artificial substrates submerged in the Red Sea [31]. The biofilm formation of these strains was confirmed previously by the microtitre plate method [31]. The agar well diffusion method was used for antibacterial assay. The biofilm-forming bacteria were cultured overnight in Zobell marine broth at 30 °C in an incubator shaker. Overnight cultures of the target biofilm strains were adjusted to a 0.5 McFarland standard (1.0×10^8 CFU/mL) using a spectrophotometer at OD_{600}. The suspensions were uniformly spread on freshly prepared Zobell marine agar (ZMA) plates using a sterile cotton swab. Sterilized cork borers were used to make 6-mm wells in the plates. α-Amylase was adjusted to 100 mg ml^{-1} in phosphate-buffered saline (PBS) while proteinase K was adjusted to 10 mg ml^{-1}. A 100 μL aliquot of each enzyme was added to the wells and allowed to incubate in the refrigerator at 4 °C for 1 h to ensure dispersion. The plates were incubated at 30 °C for 24 h and observed for zones of inhibition. The zones of inhibition were measured with a ruler to determine the size of the inhibition. The antibacterial activity was categorized as weak, moderate, or strong based on the size.

The minimum inhibitory concentration (MIC) of the enzymes was evaluated by micro broth dilution assay and confirmed with the addition of resazurin dye according to the Clinical and Laboratory Standards Institute (CLSI) method. The assay was employed in a 96-well microtitre plate. In each well, 100 μL of Zobell marine broth (ZMB) growth media was added, followed by 100 μL of the enzyme in the first column. Dilution was performed by transferring 100 μL to the next well, resulting in decreasing concentrations until the sixth column. For α-amylase, a concentration of 50–1.5625 mg ml^{-1} was used while for proteinase K the concentration was diluted six times from 5 to 0.15625 mg ml^{-1}. Next, 100 μL of the tested bacterial strain at a concentration of 1×10^8 CFU mL^{-1} was added. Two controls were used: a negative control with only medium and a positive control with medium and bacterial inoculum but without the enzyme extract. The plates were kept in an incubator at 30 °C for 24 h under static conditions. After incubation, a comparison of inhibition in the wells with the controls was made visually. Subsequently, the resazurin assay was performed by adding 30 μL of the resazurin solution to each well and incubating at 30 °C for 3 h. Colour changes were observed, with a change from pink to blue indicating the absence of growth, while a change from blue to pink indicated bacterial growth due to the reduction of resazurin. The MIC reading was

recorded as the lowest enzyme concentration that inhibited bacterial growth, preventing a change in colour from blue to pink. A change in pink colour in the growth control well indicated proper growth of the isolate, while the absence of contamination was confirmed by no colour change in the sterile control wells.

2.4. Biofilm inhibition assay

The antibiofilm activity of the enzymes was determined using a microtitre biofilm formation inhibition assay. The 96-well microtitre plate (round bottom) was used for this assay. The effect of the enzymes was assessed against the biofilm formation ability using a spectrophotometric assay. In each well of the microtitre plate, 100 μL of a bacterial suspension (prepared according to the McFarland standard) was added, followed by the addition of 100 μL of the enzymes. The control samples consisted of a culture with a culture only without any treatment and media only. The plates were then incubated at 28 °C for 48 h. After the incubation period, the biofilm inhibition was estimated by measuring the absorbance of the microtitre plates at OD_{600} using a microplate reader (Synergy, Biotek). The biofilm inhibition assay was carried out in duplicate, and the absorbance values recorded at OD_{600} were calculated using the equation given below:

Percentage of biofilm inhibition = [(**OD Control** − **OD** Sample)/**OD control**] × 100.

2.5. Culture of barnacle larvae

The adults of barnacle *Amphibalanus amphitrite* (=*Balanus amphitrite*) were collected from the Obhur Creek (21°41'46.52″ N and 39°00'14.07″E), central Red Sea, Saudi Arabia. The barnacle specimens were identified based on morphological characters as described previously [32]. The collected adult barnacles were cultured in the laboratory according to the method described previously by Salama, Satheesh and Balqadi [33]. In brief, the *A. amphitrite* individuals were kept in a glass aquarium with aeration in the laboratory. The nauplii released by the adults were transferred to 5-L glass tanks using a hand net. The tanks were kept in a walk-in type environmental chamber at 28 °C temperature under 12 h light: 12 h dark conditions. The nauplii were fed a mixed algal diet consisting of *Chaetoceros* and *Tetraselmis*. The nauplii were checked every day under a dissecting microscope (Lecia S6E) to confirm the larval stages and reared up to cyprid larva (settlement stage). Stage III nauplii were used for the toxicity assay and cyprid larvae were kept for anti-larval settlement assay.

2.6. Barnacle larval settlement assay

The cyprid larvae of barnacle *A. amphitrite* were used to test the larval settlement-inhibiting activity of enzymes. Petri dishes (polystyrene, 50 × 9 mm) were used as the test substrates for the settlement assay. The dishes were filled with 10 ml of sterile-filtered seawater (Millipore, 0.22 μm) and 25 cyprid larvae were introduced in each Petri dish. To this, the enzymes were added. The settlement assay was carried out using the three concentrations of the enzymes (5, 10 and 25 mg ml^{-1}) after calculating the LC_{50} values. The dishes that were maintained without the addition of enzymes served as the negative control. Subtilisin A at 25 mg ml^{-1} and copper sulphate (2 μg ml^{-1}) were used as positive controls for the larval settlement assays. Subtilisin A from the serine protease group was selected as positive control based on its proven antifouling activity [34–36]. The Petri dishes were kept at 28 °C in the dark. The settlement of larvae on the dish was checked under the stereomicroscope after 15, 24 and 48 h of treatment. The settlement assay was conducted in replicates (n = 3) and the mean values were taken for further analysis.

2.7. Toxicity of enzymes against barnacle larva

The stock solutions of each enzyme were prepared in PBS at the concentration of 100 mg ml^{-1}. The nauplii were taken in six-well polystyrene plates (25 individuals in each well) which contained filtered seawater. The enzymes (proteinase K and α-amylase) were added to the wells (in replicate, $n = 3$) in four different concentrations (5, 10, 25 and 50 mg ml^{-1}) to evaluate the toxicity against the nauplii. The wells without enzyme treatment were considered as negative controls. The plates were kept under dark conditions at 28 °C. The number of dead larvae in each well was counted after 15, 24 and 48 h under a stereomicroscope (Leica S6E). From the mortality data, the LC_{50} value for the enzyme treatment against barnacle nauplii was calculated using probit analysis [37]. The mean percentage of mortality in each concentration was used for the probit analysis. The LC_{50} values were calculated using the online excel tool available at https://probitanalysis.wordpress.com/.

2.8. In silico analysis

2.8.1. Collection and preparation of enzyme and protein structures for molecular docking

The *in silico* antifouling activity analysis of the enzymes proteinase K and α-amylase was carried out against the target barnacle cement protein. The structure of cement protein (cp-20k) from the barnacle *Megabalanus rosa* was downloaded from Alphafold (https://alphafold.ebi.ac.uk/) protein structure database (protein ID: AF-Q9GRC4-F1). The structure of proteinase K enzyme (PDB ID: 2PKC) of the fungus *Parengyodontium album* was downloaded from the protein data bank (https://www.rcsb.org/). The structure of the

α-amylase enzyme (UniProt ID: Q6GWE2) of *Bacillus licheniformis* was downloaded from UniProt (https://www.uniprot.org/). The structure of reference enzyme subtilisin (protein ID: AF-A7XPN6-F1) was downloaded from Alphafold. The PDB files of cement protein and enzymes were uploaded to BDSV (Biovia Discovery Studio Visualizer). In BDSV, the water molecules and hetatms associated with the enzymes and protein were removed. Following this, polar hydrogen was added to the enzymes and protein structures and the files were saved in PDB format and used for energy minimization before molecular docking. The energy minimization of protein and enzyme molecule structures was carried out using Chiron (https://dokhlab.med.psu.edu/chiron/login.php) web server [38].

2.8.2. Molecular docking of enzymes with barnacle protein structure

The enzyme structures were docked with target barnacle cement protein using the HDOCK online server [39] available at http://hdock.phys.hust.edu.cn/. The template-based docking function was used for docking and the residues of binding sites were not specified. The HDOCK server predicts the binding complexes between proteins based on global docking. Hence, it is not necessary to input binding site details. Templates used for the docking of each enzyme with barnacle cement protein is presented in Table 1. The default docking parameters and algorithms available in HDOCK were used for docking. The best docking model was selected based on the rank assigned by HDOCK and used for further analysis.

2.8.3. Visualization of enzyme–protein interaction

The docked enzyme–protein complex files were submitted to the PDBSUM (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum) online server for the analysis of the interaction between the enzymes and barnacle cement protein. Particularly, the bonded and non-bonded interactions between the amino acid (AA) residues of the enzymes and proteins were analysed to understand the effect of enzyme treatment on barnacle cement proteins.

2.8.4. Analysis of cement protein–enzyme complex interfaces

The binding affinity of the protein–enzyme complex was determined using PRODIGY (https://wenmr.science.uu.nl/prodigy/) web server [40]. The binding free energy of protein–enzyme complexes was predicted by the MM/GBSA method [41] using Hawkdock server (http://cadd.zju.edu.cn/hawkdock/). Further, PPCheck (http://caps.ncbs.res.in/ppcheck/index.html) web server [42] was used for predicting the hotspot residues (the residues that are energetically more important in the interface).

2.9. Statistical analysis

The data obtained from barnacle larval toxicity and settlement assay were analysed using two-way analysis of variance (ANOVA). Enzyme concentration and observation time were used as factors for two-way ANOVA. Alternatively, three-way ANOVA was carried out for the settlement assay data to check the variation between the anti-settlement activities of the two enzymes. Concentration, observation period and enzyme type were used as factors for three-way ANOVA. The homogeneity of the data was tested using Levene's 'C' test. Further, the post hoc Tukey test was conducted to analyse the difference between treatment groups (control and different enzyme concentrations) in the case of significant variations in ANOVA. The statistical analysis was conducted using the STATISTICA (ver.13) program. For the statistical tests, $p < 0.05$ was considered significant.

3. Results

3.1. In silico analysis for the selection of enzyme from protease group

The preliminary *in silico* analysis using PeptideCutter online server showed strong activity of proteinase K. Among the available proteases and chemicals in the server, proteinase K performed the maximum number of cleavages (1080 against BAP; 69 cleavages against barnacle cement protein). The results are presented in Tables S1 and S2.

3.2. Antibacterial activity of enzymes

The zone of inhibition and MICs of both proteinase K and α-amylase enzymes against the biofilm-forming bacteria are presented in

Table 1
Templates used by HDOCK server for the molecular docking of cement protein and enzymes.

	PDB ID	Chain ID	Align_length	Coverage	Seq_ID (%)
Cement protein-proteinase K complex					
Receptor	3WLW	A	153	0.757	25.0
Ligand	3WLW	C	123	0.441	24.2
Cement protein-alpha-amylase complex					
Ligand	1VJS	A	483	0.943	99.8
Cement protein-subtilisin complex					
Ligand	4GI3	A	275	0.726	99.3

Table 2. Proteinase K exhibited a maximum inhibition zone of 22 mm against the bacterium *P. issachenkonii* IMB12. The maximum zone of inhibition of 18 mm was observed against the same bacterium in α-amylase treatment. Proteinase K showed a very low MIC against the strain *Psychrobacter* sp. IMB8 (Table 2). While the MIC of proteinase K was low against all the tested bacterial strains (0.312–2.5 mg ml^{-1}), higher values were observed for the MIC of α-amylase (6.25–25 mg ml^{-1}).

3.3. Antibiofilm activity of enzymes

Microtitre plate showing the biofilm growth in control and enzyme treated wells is presented in Fig. 1. The microtitre plate assay results indicated a higher reduction in biofilm in the wells treated with α-amylase enzyme (Fig. 2). Proteinase K showed strong antibiofilm activity against the bacterial strain *P. shioyasakiensis*. Further, proteinase K treatment did not show biofilm inhibitory activity against the strain *P. issachenkonii*.

3.4. Barnacle larval settlement inhibitory activity

The settlement-inhibiting activity of the enzyme used in this study is given in Fig. 3. About 89.33 % of cyprid settlement was observed in the control dishes (without enzyme treatment) during the experimental period. However, the cyprids treated with enzymes showed a reduction in settlement. Cyprids treated with 25 mg ml^{-1} of proteinase K recorded 18.66 % settlement after 48 h of observation. At higher concentrations of α-amylase (25 mg ml^{-1}) treatment, 32 % of cyprids settled on the test surfaces. The positive controls subtilisin (25 mg ml^{-1}) and copper sulphate (2 μg ml^{-1}) treatment showed 29 % and 21 % settlement respectively (Table 3). Two-way ANOVA of settlement data indicated a significant ($p < 0.001$) variation between different concentrations of enzyme and the observation period (Table 4). Further, the post hoc Tukey test confirmed that the settlement percentage of larvae varied significantly between control and treatment concentrations (Table S3). The observed settlement inhibitory activity was significantly (Table S4) high in proteinase K treatment.

3.5. Toxicity of enzymes against barnacle larva

The toxicity of proteinase K and α-amylase against the barnacle larvae is presented in Fig. 4. An increase in mortality was observed with higher concentrations of enzymes. In control wells, a maximum of 4 % nauplii mortality was observed after 48 h (Fig. 4). The nauplii treated with 50 mg ml^{-1} of proteinase K and α-amylase recorded a mortality percentage of 50.66 and 29.33 respectively after 48 h. ANOVA results indicated a significant ($p < 0.001$) variation in the mortality of barnacle larvae in relation to the concentration of enzymes and observation period (Table 4). Further, the post hoc Tukey test indicated significant variation in mortality between control and all treatment concentrations in proteinase K (Table S3). For α-amylase-treated larvae, a significant variation in mortality was observed between control and all treatment concentrations except 5 mg ml^{-1} (Table S3). The calculated 48-h LC_{50} values for proteinase K and α-amylase against the barnacle nauplii were 91.8 and 230.96 mg ml^{-1} respectively (Fig. S3).

3.6. Molecular docking of cement protein and enzymes

The best docking model obtained from HDOCK server is presented in Fig. 5. Docking of cement protein with proteinase K revealed a HDOCK score of −238.58 and a confidence score of 0.854 (Table 5). The cement protein- α-amylase docking complex showed a HDOCK score of −234.24 and a confidence score of 0.843. The docking of reference enzyme subtilisin with cement protein showed a HDOCK score of −244.88 and the confidence score was 0.869 (Table 5). The docking model obtained for the cement protein–subtilisin complex is presented in Supplementary Figs. S2 and S4.

3.7. Visualization of enzyme–protein interaction

The PDBsum data revealed the presence of salt bridges, hydrogen bonds and non-bonded interactions between the enzymes and barnacle cement protein (Fig. 6, Fig. S4). Overall, 14 AA residues from barnacle cement protein and 20 residues from proteinase K were involved in the interaction (Fig. 6). Also, 17 residues from cement protein and 21 residues from α-amylase enzyme were involved in the interaction (Fig. 6). According to the data, the enzyme proteinase K made eight bonding interactions (three salt bridges and five hydrogen bonds) with the target barnacle cement protein and 154 non-bonded interactions (Table 6). The cement protein–α-amylase complex revealed 3 salt bridges, 5 hydrogen bonds and 165 non-bonded interactions (Table 6). In the complex predicted between the

Table 2
Zone of inhibition and Minimum inhibitory concentration (MIC) and antibacterial activity of enzymes against biofilm bacteria. The antibacterial activity (zone of inhibition) was measured by disc diffusion method. MIC was determined using broth dilution method.

	Zone of inhibition (mm)		MIC (mg ml^{-1})	
Organism	Alpha-amylase	Proteinase K	Alpha-amylase	Proteinase K
Psychrobacter sp. IMB8	10	13	6.25	0.312
Pseudoalteromonas issachenkonii IMB12	18	22	25	1.25
Pseudoalteromonas shioyasakiensis IMB13	12	14	12.5	2.5

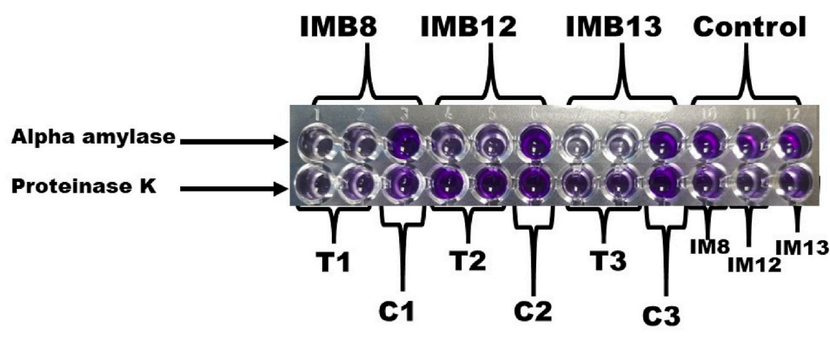

Fig. 1. Microtiter plate assay showing the antibiofilm activity of proteinase-k and alpha-amylase enzymes. IMB8, IMB12 and IMB 13 are biofilm-forming bacteria used as target for antibiofilm assay. T1, T2 and T3 are treatment wells; C1, C2 and C3 are control wells.

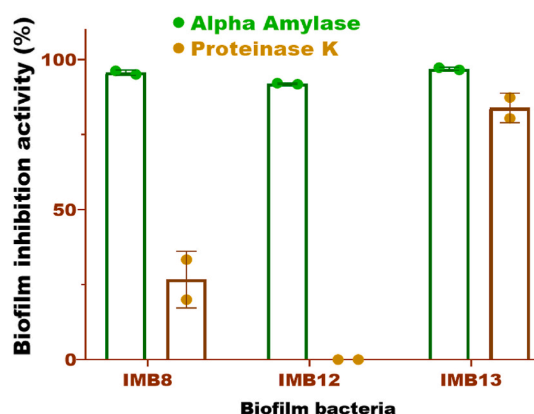

Fig. 2. Antibiofilm activity of enzymes against biofilm bacteria. The percentage of biofilm inhibition was calculated from the absorbance values of microtitre plate wells based on crystal violet staining of attached bacterial cells. Error bars indicate mean ± SE. Key: IMB8, *Psychrobacter* sp.; IMB12, *Pseudoalteromonas issachenkonii*; IMB13, *Pseudoalteromonas shioyasakiensis*.

reference enzyme subtilisin and barnacle cement protein, 21 residues of barnacle cement protein and 26 residues from subtilisin were involved in the interaction. The interaction of cement protein and subtilisin includes 2 salt bridges, 9 hydrogen bonds and 141 non-bonded interactions (Table 6). Overall, both proteinase K and α-amylase enzymes interacted with the target protein through hydrogen bonds. The number of predicted hydrogen bonds in the complexes formed by these two enzymes was lower than that in the complex formed by the reference enzyme subtilisin.

3.8. Analysis of cement protein–enzyme interface

The binding affinity of cement protein–proteinase K complex was found to be −13.1 kcal/mol. Also, a binding affinity of −14 kcal/mol was observed between cement protein and α-amylase interaction (Table 7). The interaction between cement protein and the subtilisin (reference enzyme) showed a binding affinity of −14.7 kcal/mol. The predicted binding free energies of cement protein–-proteinase K complex and cement protein–α-amylase complex were −42.57 and −12.86 kcal/mol respectively (Table 7). The total number of hotspot residues (energetically more important residues in the interface) were nine for barnacle cement protein–proteinase K interaction and cement protein–α-amylase interaction (Table S5).

4. Discussion

The results of the present study revealed antibacterial and antibiofilm activities of commercially available proteinase K and α-amylase enzymes. Proteinase K enzyme showed strong antibacterial activity against all the target biofilm-forming bacteria. However, the antibiofilm activity was very weak against the strain IMB8 and no activity against IMB12. α-Amylase showed higher biofilm inhibitory activity against the biofilm-forming bacteria. The higher biofilm inhibitory activity of α-amylase may be due to its degrading ability on EPSs produced by the bacteria during attachment. A previous study by Craigen et al. [43] indicated that treatment of biofilm bacteria with amylase enzyme inhibited biofilm formation as well as EPS production. Further, Divakaran et al. [44] reported that α-amylase was the most efficient enzyme for the inhibition of polysaccharides. Many studies suggested that enzymes that degrade the

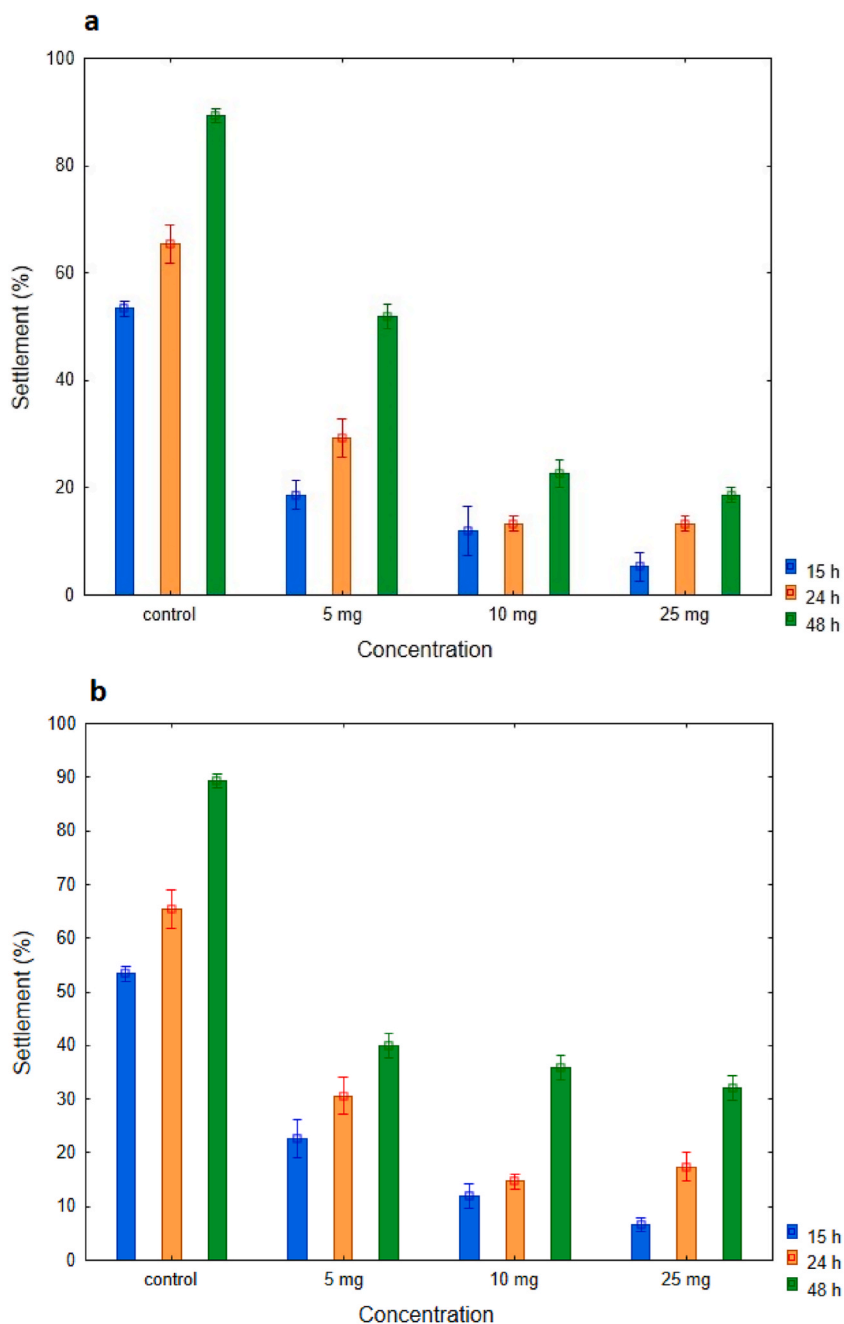

Fig. 3. Barnacle larval settlement-inhibiting activity of enzymes. Larval settlement inhibitory activity of (a) proteinase K and (b) α-amylase. The concentrations of enzymes are expressed in mg ml^{-1}. Error bar indicates SEM ($n = 3$).

Table 3
Barnacle larval settlement inhibition activity of positive controls subtilisin enzyme and copper sulphate. Values are percent (mean ± SD, n = 3) of settlement after treatment.

Time (h)	Subtilisin (percent of settlement)	Copper sulphate (Percent of settlement)
15	22.66 ± 2.08	13.33 ± 1.52
24	24.33 ± 2.3	18.66 ± 1.15
48	29 ± 2	21 ± 2

Table 4
Two-way ANOVA results for the toxicity and anti-settlement activity of enzymes (Proteinase K and α-amylase) against barnacle larvae. Enzyme concentration and observation time were used as factors.

	Mortality					Settlement				
		Proteinase K		α-amylase			Proteinase K		α-amylase	
Effect	df	F	P	F	P	df	F	P	F	P
Concentration	4	88.06	<0.001	32.25	<0.001	3	298.10	<0.001	273.26	<0.001
Time	2	31.30	<0.001	11.32	<0.001	2	82.52	<0.001	112.90	<0.001
Concentration*Time	8	2.12	0.064	1.71	0.135	6	6.95	<0.001	3.30	0.01
Error	30					24				

$P < 0.05$ is significant.

polysaccharide would efficiently remove the biofilms from the surfaces (for a review, see Ramakrishnan et al. [45]). Also, the anti-biofilm activity of the amylase enzyme was widely reported in the literature [9,46,47]. The observed results indicated that proteinase K and α-amylase may reduce the biofilm biomass on artificial substrates due to their bactericidal or biofilm inhibitory activity. A study conducted by Boles and Horswill [48] indicated that serine protease group enzymes play a key role in the removal of biofilms. The mode of action of proteinase K on biofilm disruption may be due to the breaking of biofilm matrix proteins [49]. As the crystal violet biofilm development assay used in this experiment only reveals the biomass of bacterial cells attached on the substrate [50], a further study is required to confirm the viability or number of cells that adhered on the wells of the microtitre plate.

Among the fouling organisms, barnacles are commonly used as target organisms for antifouling assays [51,52]. The results indicated the reduction in the settlement of cyprid larva of barnacles on experimental substrates due to enzyme treatment. Though proteinase K did not exhibit antibiofilm activity against some bacterial strains, it showed strong anti-larval settlement activity, significantly higher than α-amylase. This difference may be due to the mode of action of enzymes on bacteria and barnacle larvae. This indicates the necessity to include more organisms in antifouling screening studies. Proteinase K may inhibit the barnacle larval settlement by disrupting the 'footprints' or temporary adhesives released during surface exploration, which need further experimental studies.

The results of this study also indicated that both α-amylase and proteinase K affect the survival of barnacle nauplii at all the treatment concentrations. α-Amylase enzyme did not show significant mortality at 5 mg ml^{-1} concentration. Also, the LC_{50} value of proteinase K was lower than that of α-amylase. This indicates that α-amylase is less toxic to barnacle larvae. The α-amylase enzyme used in this study was isolated from *B. licheniformis*. Many food-grade enzymes are isolated from *B. licheniformis* and this bacterium is considered non-toxic to human and environmental health [53]. On the contrary, proteinase K is commonly used in molecular biology and other areas where the degradation of protein is needed [54].

The cypris larva of barnacles releases adhesive material (also known as barnacle cement) for surface exploration and permanent attachment [55]. The adhesive ('cyprid cement' or 'barnacle cement') released during permanent attachment on substrates is important for the firm settlement and further metamorphosis into adult barnacles [56]. Hence, the compounds that affect the barnacle cement protein may serve as potential antifouling agents. To validate this assumption, an *in silico* analysis was carried out to understand the interaction between enzymes (proteinase K and α-amylase) against the barnacle cement protein.

The results of the *in silico* analysis showed that the enzymes made strong interactions with the barnacle cement protein by hydrogen and non-hydrogen bonds. Such enzyme–cement protein interaction could interfere with the polymerization of cement protein. Dickinson, Vega, Wahl, Orihuela, Beyley, Rodriguez, Everett, Bonaventura and Rittschof [57] reported the prevention of barnacle cement polymerization by trypsin inhibitor. Therefore, the interaction of α-amylase and proteinase K with the cement protein may affect the adhesion of barnacles on the surfaces. The adhesive of barnacles consists of more than 10 proteins [58]. Among the identified proteins, those with molecular weights 20 and 19 kDa are the macromolecules that may play an important role in coupling effects on surfaces [59]. The tertiary structure of barnacle cement protein used for molecular docking in this study was 20k (MrCP20). According to Xu, Liu, Zhang and Xu [58], the main AA composition of 20k macromolecule includes cysteine (Cys), glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

In silico AA residue interaction analysis of docked cement protein–enzyme complex indicated hydrogen bonds between AAs of cement protein and enzymes (Fig. 5). Hydrogen bonds are important for many cellular functions and play an important role in protein–ligand binding and enzyme catalysis [60,61]. Hence, the presence of hydrogen bonds between the AA residues of cement protein and enzymes revealed the stability of the protein–protein complex predicted through the docking. The presence of salt bridges between protein and enzymes also indicates the stability of the complex predicted in this study. Salt bridges are believed to play a key role in protein–protein interaction, protein folding and stability of the protein–protein complexes [62]. Overall, the results of the molecular docking study indicated that the α-amylase and proteinase K enzymes interacted strongly with the cement protein of the barnacle, which may be one of the possible antifouling mechanisms of enzymes.

Though both enzymes showed excellent antibiofilm/anti-larval settlement activities, the performance of the enzymes against biofilm-forming bacteria and barnacle larvae differed. This observation confirmed that a combination of enzymes that break the extracellular matrix of biofilms and invertebrate larval adhesives may work better than the individual enzymes. Previously, the combined antifouling activity of enzymes was tested and found to be effective against fouling organisms [63]. Further, Jee, Kim, Sung and Kadam [64] observed an enhanced anti-biofilm activity of the protease and amylase enzyme combination. Tsiaprazi-Stamou et al. [65] also reported the synergistic action of amylase, protease and lipase enzymes on biofilm removal from different surfaces. Hence, it

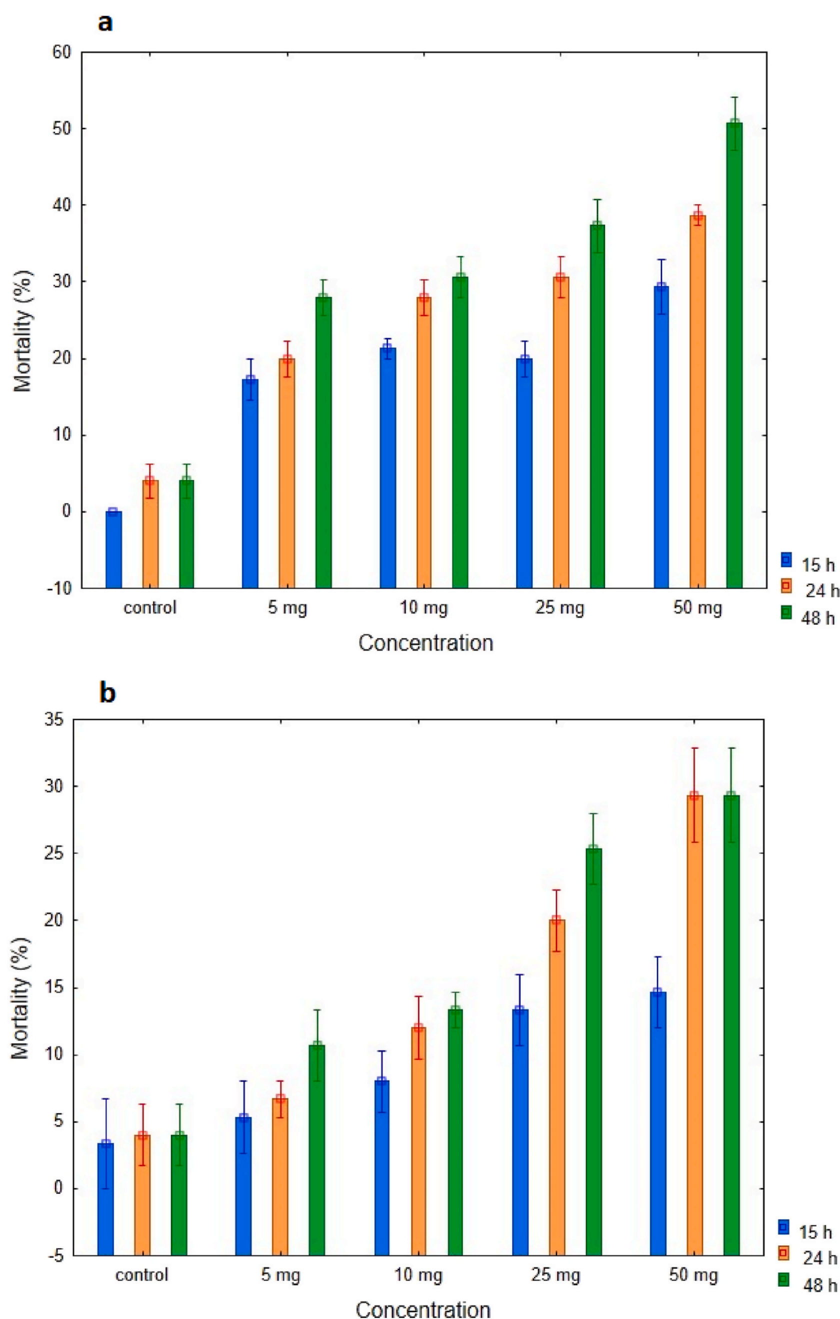

Fig. 4. Mortality of barnacle nauplii after treatment with enzymes. Toxicity of (a) proteinase K and (b) α-amylase against barnacle nauplii. The concentrations of enzymes are expressed in mg ml^{-1}. The percentage of mortality was based on a toxicity assay conducted using 25 barnacle nauplii for each treatment. Error bar indicates SEM ($n = 3$).

is necessary to study the synergistic antifouling activity of enzymes against biofouling organisms.

The biofouling process in the sea starts with the formation of biofilms mainly by bacteria and diatoms, followed by the settlement of larval forms of invertebrates [66]. The formation of biofilm and biofouling growth is detrimental to marine structures and technical objects submerged in the sea [4]. The antifouling compounds are generally tested against biofilm organisms as well as macrofouling organisms under both laboratory and natural conditions [67]. In this study, three bacterial strains isolated from the microfouling community and barnacle larvae were used to test the antifouling efficiency of proteinase K and α-amylase enzymes. Barnacles were selected due to their significance in the biofouling community on artificial substrates throughout the world seas [68]. Though laboratory-based antifouling assays provide inhibitory activity and toxicity details of the compounds, field experiments are essential to understand the antifouling efficiency due to the involvement of various factors in the biofouling process. This study revealed that

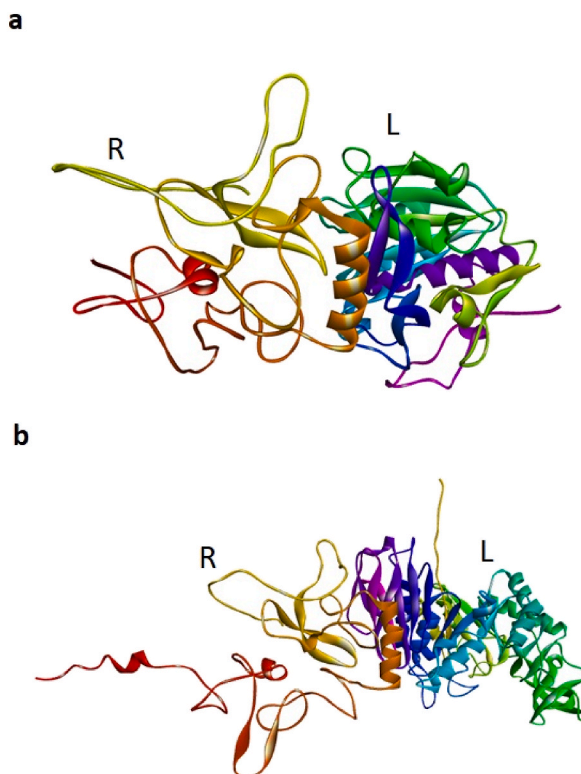

Fig. 5. Docking pose of barnacle cement protein with enzymes. Interaction between (a) cement protein and proteinase, and (b) cement protein and α-amylase. The docking pose view of the enzyme–protein complex was generated using BIOVIA discovery studio program. Key: R, receptor (cement protein); L, ligand (enzyme).

Table 5
Docking scores obtained for the barnacle cement protein-enzyme complex.

Parameters	Cement protein-proteinase K	Cement protein- α-amylase	Cement protein-subtilisin
Docking score	−238.58	−234.24	−244.88
Confidence score	0.854	0.843	0.869
Ligand RMSD (Å)	70.31	133.27	138.86

enzymes could be used to prevent or disrupt the biofilms as well as reduce the barnacle settlement. However, for successful implementation of enzymes for antifouling purposes, a proper additive material may be needed to maintain stability under harsh marine conditions.

5. Conclusion

The laboratory bioassays in this study indicated the antibacterial and antibiofilm activities of proteinase K enzyme. Further, proteinase K inhibited the settlement of barnacle larva. The anti-settlement activity of the proteinase K was significantly higher than α-amylase. *In silico* analysis revealed that both enzymes could interfere with the settlement process of barnacles by binding with the cement protein. Strong binding affinity values were obtained for the models predicted for the cement protein–enzyme complex. The stability of cement protein–enzyme complex models predicted through molecular docking was supported by the presence of hydrogen bonds and salt bridges between the AA residues of cement protein and enzymes. The observed results could be useful for the development of enzyme-based antifouling strategies after further field studies.

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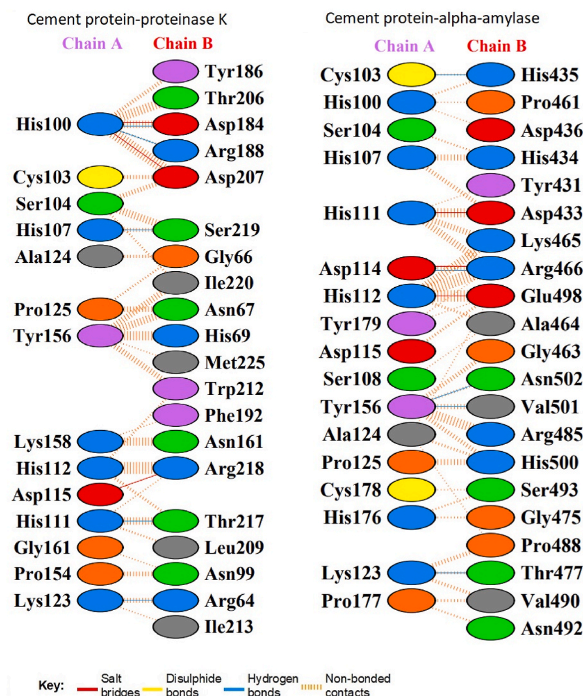

Fig. 6. Interactions between amino acid residues across the interface in the cement protein–enzyme complex predicted by the PDBSUM server. Chain A is from cement protein and B indicates corresponding enzymes as shown above the figure. Coloured lines indicate the interactions between the residues as shown in the key.

Table 6
Interface statistics of cement-protein complex predicted using PDBsum server (Chain A-cement protein, chain B- enzyme).

	Chain	No. of interface residues	Interface area (Å2)	Salt bridges	Disulphide bonds	Hydrogen bonds	Non-bonded contacts
Cement protein-proteinase K	A	14	969	3	–	5	154
	B	20	829				
Cement protein-α-amylase	A	17	1101	3	–	5	165
	B	21	970				
Cement protein-subtilisin	A	21	1402	2	–	9	141
	B	26	1264				

Table 7
Predicted binding affinity, binding free energy and Kd values of cement protein and enzyme complexes.

Protein-protein complex	Binding affinity (kcal mol^{-1})	Kd (M) at 25 °C	Binding free energy (kcal mol^{-1})
Cement protein-proteinase K	−13.1	2.3e-10	−42.57
Cement protein-alpha-amylase	−14.0	5.3e-11	−12.86
Cement protein-subtilisin	−14.7	1.5e-11	−32.26

Data availability statement

All data generated or analysed in this study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Sathianeson Satheesh: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lafi Al Solami:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31683.

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