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Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium

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Abstract:

The antifouling potential of commercial hydrolases, four proteases, seven glycosidases and one lipase was evaluated on the adhesion of marine Pseudoalteromonas sp. D41. The experimental method, adapted to screen antifouling agents, was based on bacterial adhesion in natural sterile sea water in a microtiter plate and on total biomass quantification by the fluorescent dye DAPI (4[prime]6-diamidino-2-phenylindole). Savinase (subtilisin) was the most effective hydrolase in both the prevention of bacterial adhesion and the removal of adhered bacteria. However, some enzymatic preparations tested such as Amano protease were not only ineffective but also increased the number of adhered bacterial cells. Enumeration using epifluorescence microscopy of CTC (5-cyano-2,3-ditolyl tetrazolium chloride) and DAPI stained adhered D41 cells confirmed these observations. Overall, these results demonstrated that hydrolases could either prevent adhesion and remove adhered bacterial cells effectively, or conversely increase bacterial adhesion, depending on enzymatic concentrations and the type of enzymes tested.

Keywords: adhesion; enzymes; *Pseudoalteromonas*; antifouling; marine biofilm

1. Introduction

Biofilm is a bacterial community which adheres onto biotic and abiotic surfaces and is embedded in a polymeric matrix composed mainly of polysaccharides, proteins, nucleic acids and mineral ions (Flemming & Wingender, 2001; Sutherland, 2001; Allison, 2003; Branda *et al*., 2005). Biofilm develops in many fields: health (nosocomial infection), industry (food industry, pulp and paper industry, textile industry, wastewater treatment…), equipment in natural water (vessels, pipelines, harbour facilities, aquaculture equipment, marine sensors, cooling water systems…). In this publication, we will focus on bacterial adhesion developed at solid / liquid interfaces involving sea water i.e. marine biofilm.
In fact, marine biofilms have an influence on marine macroorganisms adhesion leading to fouling (Holmström *et al*., 1992; Wieczorek & Todd, 1998; Qian *et al*., 2003). The latter induces critical material biodamages and economic problems. To fight against fouling, one strategy is to control biofilm development, one of the first steps of fouling adhesion, by physical or chemical technology. Chemical techniques use biocide products such as metal compounds (tin, copper), oxidant compounds (chloride, bromide, ozone) or synthetic non oxidant compounds (algicide, bactericide, fungicide usually used in agriculture). It has been shown that antifouling paints with organostannic agents are very toxic for marine flora and fauna (Alzieu, 1996 and 1998). Legislation on the use of biocides used is becoming more and more restrictive thanks to European regulations (Directive 1998/8/EC) which control their use, especially by banishing organostannic compounds in antifouling paints. Therefore, it is essential today to look for environmentally friendly antibiofilm agents.
The biofilm matrix is mainly composed of water (97%) and extracellular polymeric substances (EPS) made up of polysaccharides, but also proteins and nucleic acids, lipids, mineral ions and various cellular debris (Sutherland, 2001). It fulfills different functions such as adhesive foundation, structural integrity, bacteria protection and intercellular communication (Allison, 2003; Branda *et al*., 2005). When focusing on hydrolysing organic compounds involved in marine biofilm, hydrolases in commercial enzymatic preparations seem to be good antibiofilm agent candidates due to their (i) availability, some are already produced at an industrial scale; (ii) biodegradability, unlike to the long persistence of organometallic compounds; (iii) weak toxicity, contrary to oxidase enzymes. They may inhibit the adhesion by degrading involved biofilm matrix molecules instead of killing marine micro and macroorganisms.
In this study, the adhesion of a natural pioneer marine biofilm-forming bacteria was tested in the presence of hydrolases like proteases, glycosidases and one lipase in natural sterile sea water. The inhibition activity on microbial adhesion was measured in terms of the prevention of bacterial adhesion and of the detachment of already adhered bacteria using a new test in polystyrene microplates adapted to screen antifouling agents in marine conditions (Leroy *et al.*, 2007). Some enzymes partly inhibited *Pseudoalteromonas sp.* D41 adhesion but some others increased it.

2. Material and methods

All the chemicals and reagents were of analytical grade from Sigma-Aldrich.

Enzymes and enzymatic activities

Commercial enzymatic preparations, manufactured for a range of industrial applications, were supplied by different providers. Four proteases, seven glycosidases and one lipase were tested. Table I summerizes all the characteristics of the enzymes we selected to test against bacterial adhesion. Heat denaturated controls were tested on bacterial adhesion only for papaïne, -amylase, Lipolase and Glucanex. They were obtained by heating enzymatic preparations at 100°C for 20 min. and supernatants were recovered by centrifugation at 10 000 g for 10 min before testing on bacterial adhesion. In order to compare the enzymatic effects between each other and in marine conditions, we quantified each enzymatic preparation using the appropriate assay and substrate in sterile natural sea water at 20°C and at pH 8.15.
[Insert Table I about here]

Protease activity was measured with azocasein (Tomarelli *et al*., 1949). Subtilisin was incubated at different concentrations, from 0.5 to 10 g l-1, in natural sea water (previously filtered through a 0.22 µm membrane), at 25°C with 2% (w/v) azocasein in phosphate buffer of pH 8.15 at different incubation times from 3 to 12 min. The enzymatic activity was stopped by adding 10% trichloroacetic acid. The absorbance of enzymatically hydrolyzed casein was determined at 450 nm.

One unit of protease activity (UP) was defined as the amount of enzyme required to produce an absorbance unit change per minute at 25°C at pH 8.15.

Glycosidase activities were determined by measuring the release of reducing sugars according to the Somogyi-Nelson method using a glucose standard (Somogyi, 1960; Spiro, 1966). Glycosidases were incubated at different concentrations, from 0.005 to 10 g l^{-1} depending on the enzymatic preparation, in natural sea water (previously filtered through a 0.22 µm membrane), at 25°C with 12.5 g l^{-1} appropriate substrate at different incubation times from 3 to 12 min. The enzymatic activity was stopped by heating samples for 5 min at 100°C before measuring the amount of released reducing sugars. For glucanase, xylanase, pectinase, amylase and cellulase, the substrates used were : β-glucan, xylan, pectin, soluble starch and carboxymethyl cellulose with middle viscosity respectively. One unit of glycosidase activity (UG, UX, UPe, UA, UC) was defined as the amount of enzyme capable of forming one reducing sugar glucose equivalent per minute at 25°C at pH 8.15.

The lipase activity was determined by measuring the increase in absorbance at 400 nm produced by the release of *p*-nitrophenol during the hydrolysis of *p*-nitrophenyl palmitate ($C_{16:\ 0}$) (Kilcawley *et al*., 2002). Absorbance was measured every minute for 10 min at 25°C. To initialize the reaction, lipase solution in natural sea water (previously filtered through a 0.22 µm membrane) was added to 50 mM substrate solution in acetonitrile. One unit of lipase activity (UL) was defined as the amount of lipase required to produce an absorbance unit change per minute at 25 °C at pH 8.15.

Biofilm forming bacterium

The D41 strain was isolated from a natural marine biofilm on Teflon coupons immersed for 24 h in the bay of Brest (France) (Rubio, 2002). For long-term storage, pure culture was maintained on Difco Marine Broth (MB) 2216E (Fischer Scientific Labosi, Elancourt, France) with 15% (v/v) glycerol at –80°C. A comparative 16S rRNA gene sequences analysis indicated that the D41 isolate belonged to the genus Pseudoalteromonas sp. (Rubio, 2002).
The bacterial suspension was prepared from *Pseudoalteromonas sp.* D41 grown overnight on Difco Marine Agar (MA) 2216E (Fischer Scientific Labosi, Elancourt, France) at 25°C, scraped and suspended in 10 ml of natural sea water sterilized by filtration through a 0.22 µm, to reach an OD_{600} of 2 (2 x 10^9 cfu ml^{-1}).

Bacterial adhesion tests and enzymatic screening

Pseudoalteromonas sp. D41 adhesion in black polystyrene microtiter plates (Microwell F96 FluoroNunc, Bioblock, Illkirch, France), in sterile sea water, at 20°C was performed as described by Leroy *et al.* (2007).
The enzymes were tested in two following ways.
Regarding the inhibition of bacterial adhesion : the prevention test, 50 µl of the enzymatic preparation at the desired concentration was placed in the wells one hour before the bacterial suspension (200 µl per well) and incubated for 3 and 24 h at 20°C.
Regarding the detachment of already adhered bacteria: the detachment test, the enzymes were added 3 hours after the D41 bacterial suspension and incubated for only 1 h at 20°C.
In all cases, three washings in 36 g l-1 NaCl were then performed before fixation for 1.5 h at 4°C with 200 µl of 36 g l-1 sterile NaCl containing 2.5% formaldehyde and DAPI staining (4 µg ml-1) for 20 min. Again three washings were performed and then, DAPI was solubilized into ethanol 95% for 15 min (200 µl). Fluorescence was measured at 350 nm excitation and 510 nm emission wavelengths using a Genios Plus microplate fluorescence reader (TECAN, Lyon, France). For each condition, the enzymatic preparations were tested four times and heat denaturated controls only twice. They were all tested at different concentrations using serial two fold dilutions inside microplate wells.
A blank with sterile sea water as well as a control with bacterial suspension without enzymes were included in each column of the experimental microplate. The bacterial adhesion reduction percentage (RP) was calculated from the fluorescence of the blank (FB; without bacteria), fluorescence of the control (FC; bacteria without enzyme) and fluorescence of the sample (FS; bacteria with enzyme) (Equation (1)). The concentrations necessary to reach a reduction percentage of 50% adhesion (C50) were calculated from a double-logarithmic plot of RP against enzymatic concentrations. Standard deviation was calculated from the four C50 calculated for each experiment and p values were calculated with the t-test (Excel software).

$$RP = \{[(F_C-F_B) – (F_S-F_B)] / (F_C-F_B)\} \times 100 \quad (1)$$

For CTC staining with Amano protease, the experiment was undertaken according to the prevention test described above. Amano protease was added one hour before D41 suspension, incubated for 3

and 24 h at 20°C, the adhered bacteria were washed three times with 36 g l^{-1} sterile NaCl solution, stained with CTC (5 mM) for 2 h, washed three times with 36 g l^{-1} sterile NaCl solution, fixed for 1.5 h at 4°C with 200 µl of 36 g l^{-1} sterile NaCl containing 2.5% formaldehyde and finally, CTC reduced into formazan (CTF) via the respiratory electron transport chain of bacteria, CTF was then solubilized into ethanol 95% for 15 min. Absorbance was then measured at 450 nm using the Genios Plus microplate fluorescence reader (TECAN, Lyon, France).
Regarding the enumeration of total and viable cultivable adhered bacteria in polystyrene microplates, the 24 h adhesion prevention test with Amano protease was used as described above: 50 µl of Amano protease or sterile natural sea water was added one hour before bacterial suspension. The bacteria were then incubated for 24 h at 20°C; the wells were washed three times with 36 g l^{-1} sterile NaCl solution. The adhered bacteria were scraped into 250 µl with 36 g l^{-1} sterile NaCl solution four times. A part of the scraped adhered bacteria was diluted with adequate serial ten fold dilutions and 100 µl of the last three dilutions were spread out on marine agar, incubated from 24 to 48 h at 25°C and the colony forming units were then enumerated. A part of the scraped adhered bacteria was incubated with formaldehyde solution (2.5% final concentration) for 1.5 h and with triton X-100 solution (0.05% final concentration) for 10 min before sonicating the solution for 10 min at 47 kHz. DAPI was then added to the final concentration of 4 µg ml^{-1} for 20 min. The DAPI stained bacteria solution was then filtered onto a 0.22 µm sterile black polycarbonate filter (45 mm diameter, Isopore GTBP Millipore, Fischer Scientific Labosi, Elancourt, France). The filters were rinsed with sterile water and mounted onto glass slides. The slides were observed under 100 magnification on epifluorescence microscopy using an Olympus BH201 under WU filter (Olympus, Rungis, France). Approximately 1250 cells were counted per filter from the 25 different fields. This experiment was carried out in four wells of one microplate.

Epifluorescence microscopic counts of total adhered bacteria

Lab-Tek™ wells with a glass surface (Lab-Tek™ NUNC, Fischer Scientific Labosi, Elancourt, France) were used and 360 µl of sterile natural sea water or 360 µl of Amano protease was added per well and incubated for 1 h at 20°C without shaking. 540 µl of bacterial suspension was then inoculated. The covered Lab-Tek™ glass slides were incubated for 24 h at 20°C with orbital shaking (300 rpm). The non adhered bacteria were eliminated by three successive hand washings : the Lab-Tek™ wells were manually reversed, 900 µl of 36 g l^{-1} sterile NaCl solution was added and Lab-Tek™ were shacken. The adhered bacteria were fixed for 1.5 h at 4°C with 900 µl of 36 g l^{-1} sterile NaCl solution with 2.5% formaldehyde and then desalted by successive baths in decreasing salinity solutions. The adhered bacteria were incubated with 900 µl of the DAPI solution (4 µgml^{-1}) for 20 min in the dark. The excess stain was removed by three hand washings then the Lab-Tek™ wells were removed and the glass surface was dried out. The slides were observed under 100 magnification on epifluorescence microscopy using an Olympus BH201 under UV filter (Olympus, Rungis, France).

3. Results

Effects of commercial enzymes on *Pseudoalteromonas sp*. D41 adhesion in sea water microplate tests

We tested the potential capability of several enzymes to inhibit the development of Pseudoalteromonas sp. D41 adhesion on the polystyrene microplate surface. The enzymes were tested for the prevention of bacterial adhesion and for the detachment of already adhered bacteria as described before.
[Insert Figure 1 to 3 and Table II about here]
The reduction percentages in relation to enzymatic activities are shown in Figures 1 to 3 depending on each enzymatic activity. As previously described, the enzymatic activity was evaluated using appropriate substrates. The influence of enzymatic activities depends on the type of protocol tested. Glucanases and proteases seemed to be effective, unlike the xylanases, amylase, cellulase and lipase. Savinase was the most effective protease, rapidly reaching the highest reduction percentage at the lowest proteolytic activity used in the well, whatever the preventive or curative protocol used (see Figure 1, row 1). The C50 values are given in Table II. To compare each C50 between all enzymatic preparations, the C50 were calculated in mg ml^{-1}. The results confirmed that Savinase was the most effective enzyme since it needed the lowest concentration to reach 50% inhibition. Savinase was more effective at 3h prevention compared to the detachment of adhered bacteria (result statistically

significant at 98 %, p = 0.02). Moreover, less Savinase was needed to prevent bacterial adhesion in the 24 h test compared to 3 h, as indicated by C50. This result was only observed for Savinase. This showed that the effectiveness of Savinase, in the prevention of bacterial adhesion, increased with the incubation time.
Only Glucanex, Lipolase, -amylase and papaïne could be tested after heating. All enzymatic preparations in Table 1 could not be heat denaturated , some (generally the liquid ones) became solid, thus testing was impossible. For Glucanex, α-amylase and Lipolase, heat denaturated enzymes presented a partial inhibition of bacterial adhesion whatever the protocol tested and approximately the same pattern was observed for the non denaturated enzymes. Nevertheless, a lower bacterial inhibition could be observed for heat denaturated Glucanex and Lipolase in the 3 h prevention test compared to non heat denaturated Glucanex and Lipolase when the highest concentrations were used. Only heat denaturated papaïne showed less or no bacterial inhibition. No heat-denatured enzyme preparations showed any residual enzymatic activity (data not shown).
Amano protease was partially able to prevent D41 adhesion for 3 h but it increased the final bacterial cell amount if D41 bacteria were allowed to settle for 24 h (Figure 1, row 4). In fact, reduction percentages after a 24 hour D41 adhesion were negative showing that the total adhered bacteria amount was greater with Amano protease than without. This was also the case for Shearzyme (Figure 2, row 4), Celluclast (Figure 3, row 1), Pectinex (Figure 3, row 3) and α-amylase (Figure 3, row 2). Umamizyme (Figure 1, row 2), Finizym (Figure 2, row 1) and Glucanex (Figure 2, row 2) also promoted bacterial adhesion in the 24 h prevention test at low concentrations and inhibited it at higher concentrations. This effect was also observed with Pectinex (Figure 3, row 3), α-amylase (Figure 3, row 2), Celluclast (Figure 3, row 1), Glucanex (Figure 2, row 2) and Shearzyme (Figure 2, row 4) in the detachment test. Finizym inhibited bacterial adhesion in the prevention test and increased it in the detachment protocol of already adhered bacteria (Figure 2, row 1).

To conclude, in some conditions of enzyme concentrations and depending upon the protocol used (prevention or detachment of bacterial adhesion) some enzymatic activities were capable of increasing bacterial adhesion.

Amano protease effect on *Pseudoalteromonas sp*. D41 adhesion

[Insert Figure 4 to 6 about here]

The effectiveness of Amano protease in the prevention of D41 adhesion was studied for adhesions during periods of 3 h and 24 h (Figure 4). In the case of a 3 h adhesion, Amano protease inhibited the number of adhered bacteria for both total and respiring cells in relation to its concentration (Figure 4). In the second case (24 h adhesion), the more concentrated the Amano protease, the greater the total adhered bacteria amount was since the calculated biofilm reduction percentages were negative. Adhesion of respiring bacteria were still inhibited at an average rate of about 25%. Figure 5 confirmed this latter observation by the enumeration of total and also viable and cultivable adhered bacteria in the well after scraping. DAPI stained and cultivable viable bacteria enumerationsled to an increment of 99.6% of the total bacteria amount, and an inhibition of 75.1% of the cultivable viable adhered bacteria amount. Surprisingly, the total amount of cells counted using epifluorescence microscopy was lower than the number counted by plating in the control treatment. In fact, DAPI stained cells were sonicated before enumeration, suggesting that sonication could partly lead to the lysis of cells, as previously observed (Garabetian *et al*., 1999; Fykse *et al*., 2003). This phenomenon did not interfere with the observations of total DAPI cells increment and cultivable viable cell inhibition in the presence of Amano Protease since DAPI stained cells enumeration in controls was compared with DAPI stained cells enumeration with Amano protease, and in the same way for cultivable viable cell enumeration.

The pattern of surface coverage by *Pseudoalteromonas sp.* D41 in the presence of Amano protease was studied on glass slides after DAPI staining of total bacteria (Figure 6). The adhesion was allowed to set for 24 hours. The coverage was relatively uniform and homogeneous without Amano protease (Figure 6, A). When, Amano protease was added, clusters of adhered cells were formed (Figure 6, B).

4. Discussion

Hydrolases had an effect on the adhesion of *Pseudoalteromonas sp.* D41 in polystyrene microplates when tested at 20°C in natural sea water. Some significantly increased adhesion whereas others

inhibited it. Proteases especially Savinase seemed to be the most effective enzyme in preventing *Pseudoalteromonas sp*. D41 adhesion onto a polystyrene surface in marine conditions. Amylase, pectinase, cellulase and xylanase were not effective in either the prevention or detachment of *Pseudoalteromonas sp.* D41 whereas β-glucanases like Finizym and Glucanex showed limited inhibition of bacterial adhesion, especially in the prevention test.

Some bacterial inhibition observed for α-amylase or Lipolase could not be attributed to a specific enzymatic effect as heat denaturated enzymes displayed the same pattern of bacterial inhibition. This observation suggests that additives or denaturated enzymes in enzymatic preparations could be involved in the bacterial inhibition effect. This phenomenon has already been observed by Petitt *et al*. (2004) as regards marine macroorganism spores and larvae. Additives or denaturated enzymes could be adsorbed onto the surface modifying its chemical properties and leading to different bacterial adhesion. Further studies with enzymes after being re-purified from commercial preparations should be undertaken. However, the effectiveness of enzymes such as Glucanex and papaïn, in the 3h prevention test, seems to be due to specific enzymatic effects.

The antibiofilm effectiveness of hydrolases could be assigned to the hydrolysis of a substrate involved in bacterial adhesion like EPS, Quorum Sensing (QS) molecules or adhesins. In fact, mutanase and dextranase, effective against mutan and dextran EPS, have been shown to inhibit the formation of dental biofilms by *Streptococcus mutans* and *S. sobrinicus* (Wiater *et al*., 2004). Dispersin B catalyses the hydrolysis of β-1,6-N-acetyl-D-glucosamine adhesin (Ramasubbu *et al*., 2005) and inhibits *Escherichia coli* and *Staphylococcus epidermis* biofilms (Kaplan *et al*., 2004; Itoh *et al*., 2005). More recently, the susceptibility of staphylococcal biofilms to enzymatic treatments has been shown to depend on their chemical composition. Staphylococcal biofilms producing poly-N-acetylglucosamine were sensitive to Dispersin B while staphylococcal biofilms producing proteins were sensitive to proteases (Chaignon *et al*., 2007). Target substrates could also be molecules implicated in QS pathway like N-acyl homoserine lactone (AHL) involved in a biofilm composed of Gram negative bacteria. Degradation by AHL lactonase (Wang *et al*., 2004) inactivates QS (Roche *et al*., 2004) and attenuates the virulence of the pathogenic bacteria *Erwinia carotovora* (Dong *et al,* 2000). In our study, Savinase effectiveness could be related to the physicochemical feature of the external layer of *Pseudoalteromonas sp*. D41 cells which was shown to be rich in proteins by Fourier Transform Infra-Red spectroscopy (FTIR), X-ray Photoelectron Spectroscopy (XPS) and Time-Of-Flight Secondary-Ion Mass Spectrometry (ToF-SIMS) (Pradier *et al*., 2005). Moreover, the EPS of *Pseudoalteromonas sp.* D41 after fermentation and during adhesion onto glass slides showed a high rate of proteins (Leroy, 2006). Thus, it could be hypothesized that the Savinase target could be proteins involved in D41 adhesion. On the other hand, Finizym and Glucanex have also shown their effectiveness in preventing adhesion suggesting the involvement of β-glucans in the first adhesion step of *Pseudoalteromonas sp.* D41. Epifluorescence microscopy of a 24 h D41 adhesion onto glass slides confirmed these observations showing a good fluorescence using the β-glucan stain calcofluor white (Leroy, 2006).

Surprisingly, some enzymes were able to increase the amount of adhered bacteria. In the presence of Amano protease, *Pseudoalteromonas sp*. D41 adhesion was prevented if it was allowed to adhere for 3 h. But after 24 h, the adhesion was greater when Amano protease was used. Since Amano protease is not naturally fluorescent and has no affinity to DAPI (data not shown), the observed fluorescence after DAPI staining in the presence of Amano protease is really due to an increase in bacteria amount. Biofilm accumulation has already been observed when an insufficient amount of biocide was applied (Grant & Bott, 2005). This increase in adhered bacteria could be related to sedimentation, the accumulation of planktonic bacteria, or to cell growth within the biofilm whereas Amano protease in addition to its enzymatic activity could be a substrate supporting bacteria growth. Dead cells in clusters may also be a nutrient source, allowing other cells to develop. Moreover, the enumeration of viable and cultivable bacteria and the epifluorescence microscopic counts of DAPI stained adhered bacteria confirmed that total adhered bacteria are in greater number when Amano protease is used. The number of respiring adhered bacteria (CTC stain) and viable cultivable adhered cells were not shown to increase in relation to the number of total adhered cells (DAPI stain). The cells may have entered into a dormant state, non respiring and non growing known as the “viable but non culturable(VBNC)” state and already described in the literature (Oliver, 2005). This could be a way for bacteria to protect themselves from a non suitable environment. To confirm this hypothesis, it could be interesting to test the capacity of these bacteria to return to a respiring metabolism and cultivable state. *Pseudoalteromonas sp*. D41 adhered in clusters in the presence of Amano protease. This phenomenon has already been observed in the presence of antimicrobial agents (Mah & O’Toole, 2001; Stewart & Costerton, 2001). Aggregate formation may affect Amano protease diffusion, and this could be a way for bacterial cells to resist the action of protease. Moreover, the presence of Amano protease may condition the polystyrene surface, modifying its chemical properties resulting in an

adhesion limiting contact. However, cluster accumulation may form an anaerobic environment or an altered osmotic environment inside the biofilm resulting in higher mortality.
The potential of enzymatic preparations against biofilm formation associated to pathogen bacteria has already been shown (Boyd & Chakrabarty, 1994; Johansen *et al*., 1997; Kaplan *et al*., 2004; Itoh *et al*., 2005), as well as against dental biofilm (Hahn Berg *et al.*, 2001; Walker *et al*., 2003; Wiater *et al*., 2004), and industrial biofilm (Aldridge *et al*., 1998), but never against marine biofilm under marine conditions. We have shown here that commercial enzymatic preparations could inhibit marine *Pseudoalteromonas sp*. D41 adhesion in natural sterile sea water. Savinase was the most effective in our test conditions and this may be related to proteins directly involved during adhesion (data not shown) and a proteic external cell layer of *Pseudoalteromonas sp.* D41 (Pradier *et al*., 2005). Moreover, Alcalase, an enzymatic preparation based on subtilisin like Savinase, inhibits the adhesion of fouling macroorganisms (Pettitt *et al*, 2004). However, unlike the Pettitt *et al*. (2004) results, Savinase effectiveness seems to be stable, even better, when bacterial adhesion time in the prevention test increases from 3 hours to 24 hours. This result differs from those observed by Pettitt *et al*. (2004) using Alcalase on the adhesion of cypris larvae and spores of green algae or diatoms, suggesting that biochemical molecules involved in bacterial adhesion for 24 hours did not change significantly. Thus, it is evidently better to use subtilisin preparations like Savinase or Alcalase during the first stages of bacterial or macroorganism adhesion, by conditioning the surface after immobilizing this enzyme directly onto the surface or by incorporating it into an antifouling paint.
To conclude, hydrolases were shown to reduce and enhance bacterial adhesion, depending on the time of exposure, concentration and the nature of the enzymes. Despite these observations, one enzyme seems to be very effective in our marine test conditions: Savinase. In addition to previous antifouling studies (Pettitt *et al*., 2004) this result emphasizes the high antifouling potential of subtilisin.

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Tables

TABLE I Characteristics of commercial enzymes tested

Commercial name	Manufacturer	Microorganism source	EC Number	Name (Class)	Form	Optima		Application
						pH	Tp (°C)	
Amano Protease A	Unipex	*Aspergillus oryzae*	EC3.4.24.39	deuterolysine, (metalloendopeptidase)	Powder	7	50	Protein process
Papaïn	Sigma Aldrich	Papaya latex	EC3.4.22.2	papaïn (cysteine endopeptidase)	Powder	6-7	25	Varied (foods, pharmaceutical, textile)
Umamizyme	Unipex	*Aspergillus oryzae*	EC3.4.11.1	leucyl aminopeptidase (aminopeptidase)	Powder	8	45	Brewing
Savinase 16L type EX	Novozymes	*Bacillus sp.*	EC3.4.21.62	subtilisin (serine endopeptidase)	Liquid	>8,5	50	Textile
Celluclast 1,5L	Novozymes	*Trichoderma reesei*	EC3.2.1.4	cellulase	Liquid	4,5-6	50-65	Varied cellulose process
Pulpzyme HC	Novozymes	*Bacillus sp.*	EC3.2.1.8	endo-1,4-β-xylanase	Liquid	7	50	Pulp and paper
Glucanex 200G	Novozymes		EC3.2.1.58	glucan 1,3-β-glucosidase, β-glucanase + β-1,6-glucanase, protease, chitinase, cellulase	Powder			Oenology
Finizym 200L	Novozymes	*Aspergillus niger*	EC3.2.1.6	endo-1,3(4)-β-glucanase + cellulase, xylanase, pentosanase and arabanase	Liquid	5	60	Brewing
Shearzyme 500L	Novozymes	*Aspergillus oryzae*	EC3.2.1.8 EC3.2.1.6 EC3.2.1.4	endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, cellulase	Liquid	5	70	Wheat process
α-Amylase	Novozymes	*Bacillus sp*	EC3.2.1.1	α-amylase	Liquid			
Pectinex Ultra SPL	Novozymes	*Aspergillus aculeatus*	EC3.2.1.15	Polygalacturonase + hemicellulase, arabanase, β-glucanase et xylanase	Liquid	3,5	35	Fruit juice process
Lipolase 100L	Novozymes	*Aspergillus oryzae*	EC3.1.1.3	lipase (phosphoric monoester hydrolases)	Liquid			Detergent composition

TABLE II Concentration (mg ml^{-1}) to get a reduction of adhered bacteria of 50% calculated from a double-logarithmic plot of RP against enzymatic concentrations[a].

	3 h Prevention test	24 h Prevention test	3 h Detachment test
Savinase	4.6 ± 1.4	1.7 ± 0.5	6.2 ± 0.9
Umamizyme	10.5 ± 3.2	30.6 ± 1.3	60.3 ± 21.0
Papaïne*	12.8 ± 3.1	41.4 ± 10.7	184.8 ± 32.4
Amano protease	38.8 ± 10.0	ND[b]	79.2 ± 29.2
Finizym	40.8 ± 9.4	44.6 ± 0.2	ND[b]
Glucanex*	69.9 ± 4.9	85.5 ± 15.8	39.8 ± 4.9
Pulpzyme	175.1 ± 46.3	241.9 ± 16.6	178.0 ± 29.4
Shearzyme	183.7 ± 16.5	ND[b]	201.5 ± 19.2
Celluclast	221.9 ± 24.5	ND[b]	195.4 ± 21.3
α-amylase*	ND[b]	ND[b]	193.8 ± 9.0
Pectinex	698.8 ± 10.7	926.5 ± 118.3	372.0 ± 16.3
Lipolase*	81.9 ± 12.8	183.9 ± 42.8	ND[b]

* enzymes that were also tested heat denaturated

[a] Data are means ± SD (n = 4)

[b] Some C50 were not determined (ND) because 50% of reduction percentage of adhesion was not reached

Figures

FIGURE 1

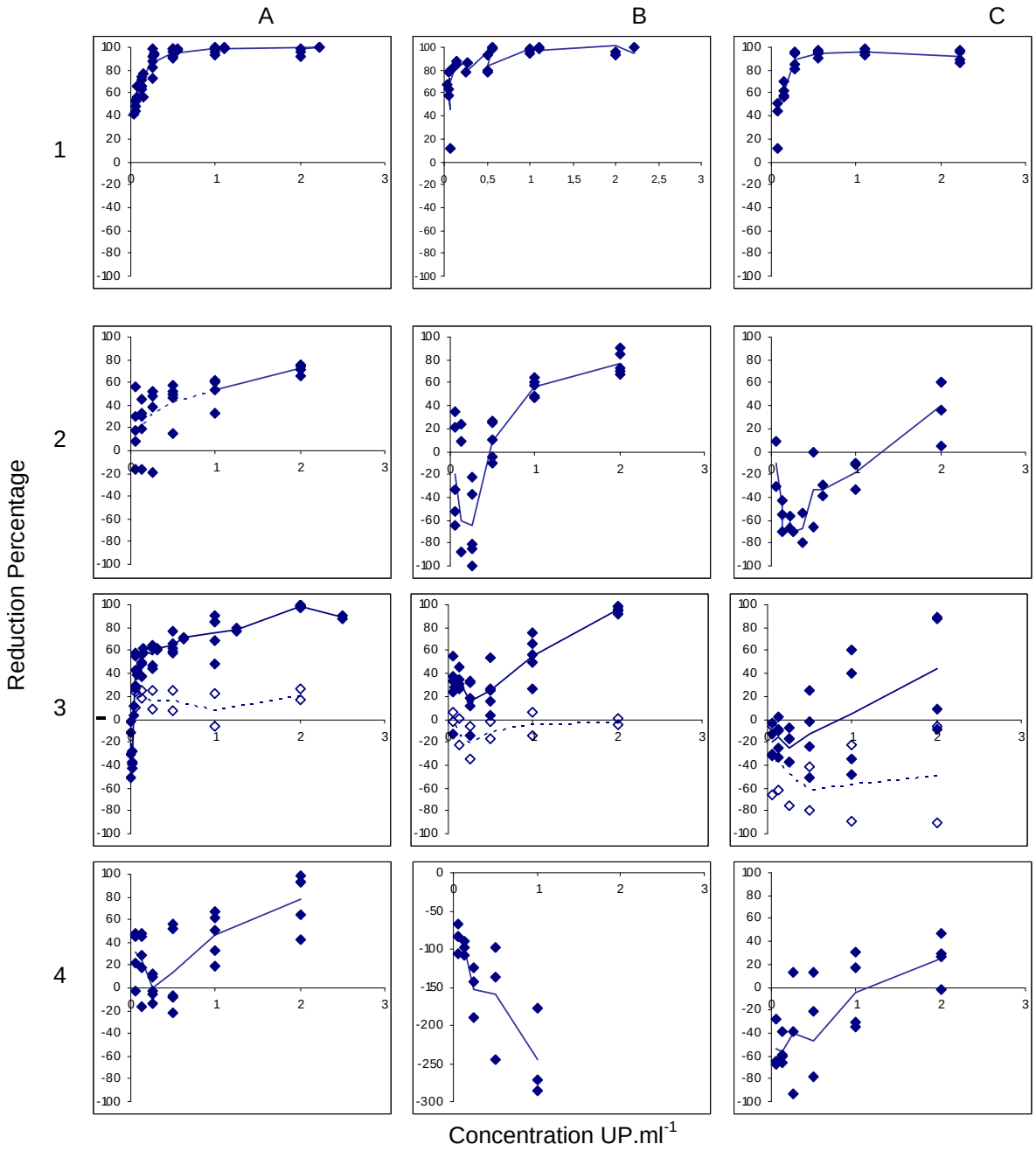

FIGURE 1 Reduction percentage of bacterial adhesion in microtiter plate according to enzyme concentration in the well of Savinase (row 1), Umamizyme (Row 2), papaïne (Row 3) and Amano protease (Row 4) with the three protocols tested: prevention of bacterial adhesion measured after 3 h (A), prevention of an adhesion after 24 h (B) and detachment of bacteria already adhered after 3 h (C). Heat denaturated papaïn tested was represented as ◊ (row 1). Full and dotted lines represent average of enzymes and heat denaturated enzymes tested respectively. All experimental values are shown; four experiments were done per enzyme concentration tested and two for heat denaturated enzyme. UP is used for protease activity unit.

FIGURE 2

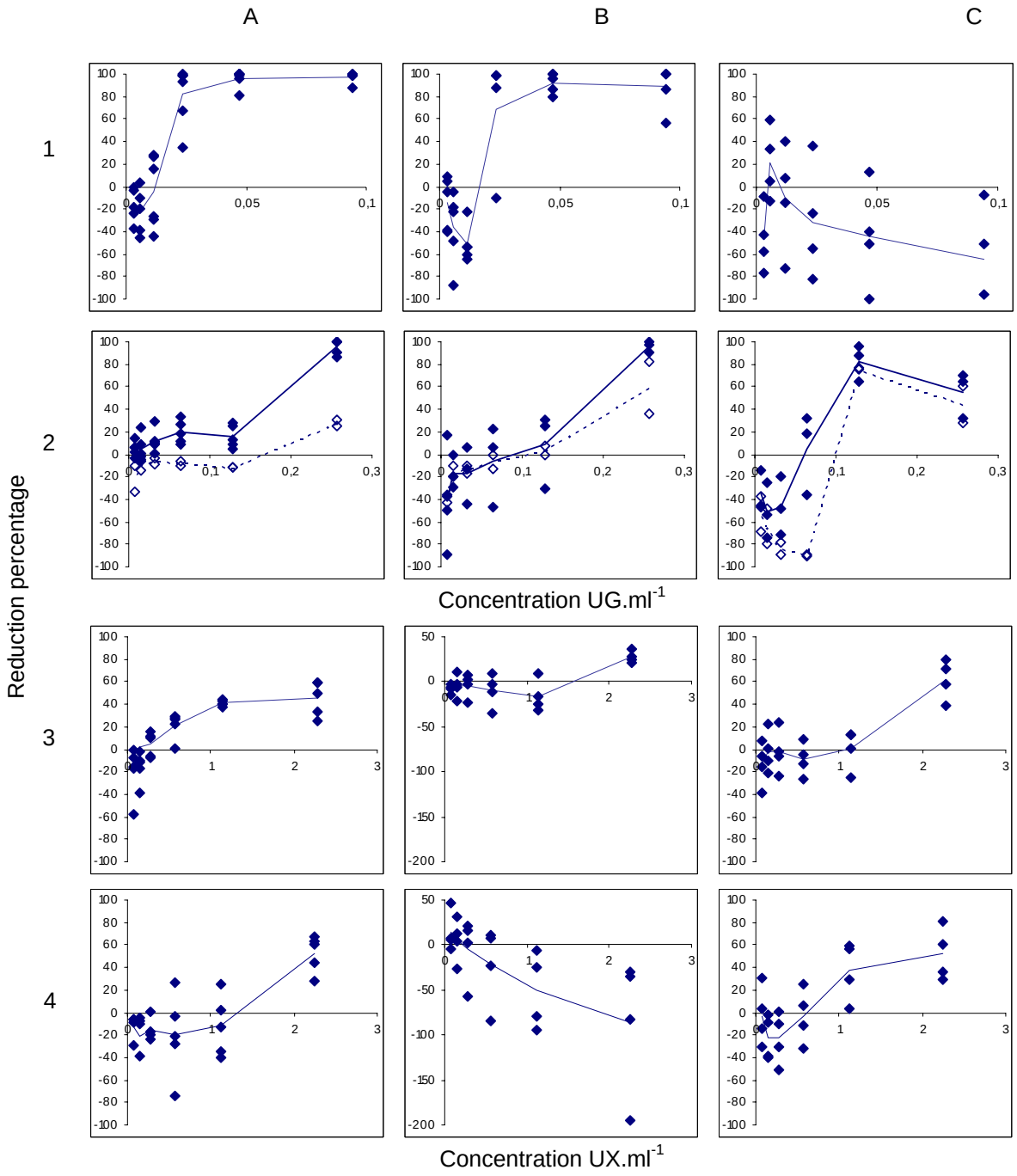

FIGURE 2 Reduction percentage of bacterial adhesion in microtiter plate according to enzyme concentration in the well of Finizym (row 1), Glucanex (Row 2), Pulpzyme (Row 3) and Shearzyme (Row 4) with the three protocols tested: prevention of bacterial adhesion measured after 3 h (A), prevention of an adhesion after 24 h (B) and detachment of bacteria already adhered after 3 h (C). Heat denaturated Glucanex tested was represented as ◊ (row 1). Full and dotted lines represent average of enzymes and heat denaturated enzymes tested respectively. All experimental values are shown; four experiments were done per enzyme concentration tested and two for heat denaturated enzyme. UG and UX are used respectively for glucanase and xylanase activities unit.

FIGURE 3

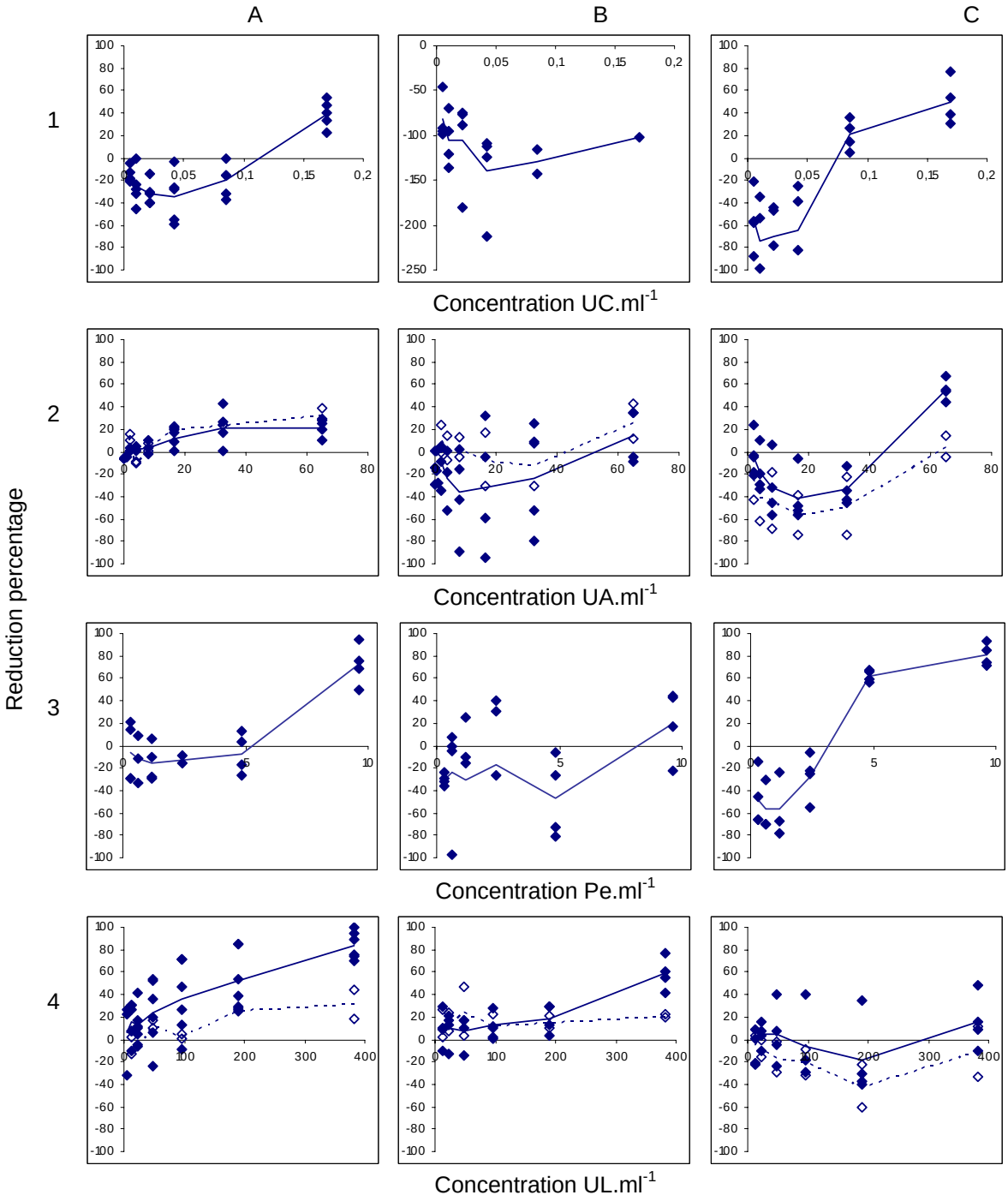

FIGURE 3 Reduction percentage of bacterial adhesion in microtiter plate according to enzyme concentration in the well of Celluclast (row 1), -amylase (Row 2), Pectinex (Row 3) and Lipolase (Row 4) with the three protocols tested: prevention of bacterial adhesion measured after 3 h (A), prevention of an adhesion after 24 h (B) and detachment of bacteria already adhered after 3 h (C). Heat denaturated α-amylase and Lipolase tested were represented as ◊ (row 1 and row 3 respectively). Full and dotted lines represent average of enzymes and heat denaturated enzymes tested respectively. All experimental values are shown; four experiments were done per enzyme concentration tested and two for heat denaturated enzyme. UA, UPe, UL and UC are used respectively for amylase, pectinase, lipase and cellulase activities unit.

FIGURE 4

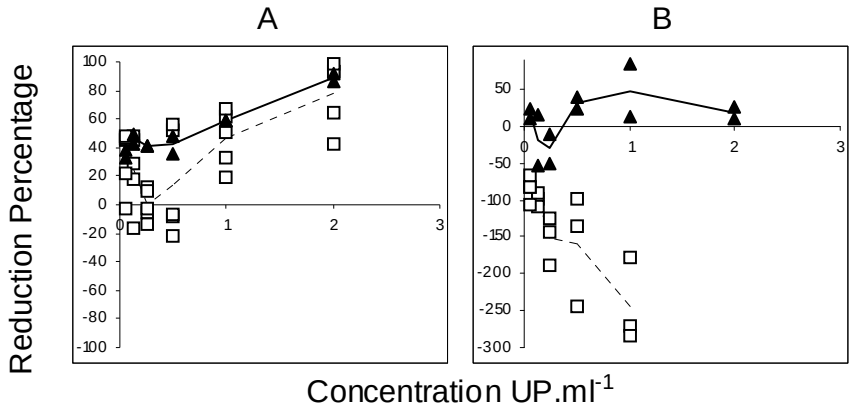

FIGURE 4 Reduction percentage of total adhered bacteria (DAPI stained) (□) and respiring adhered bacteria (CTC stained) (▲) according to Amano protease concentration in the well (UP ml^{-1}) in prevention of an adhesion allowed during 3 h (A) and prevention of the adhesion measured after 24 h (B).). Full and dotted lines represent average of DAPI and CTC stained adhered bacteria respectively. All experimental values are shown, four experiments were done for DAPI staining (□) and two experiments were tested for CTC staining (▲).

FIGURE 5

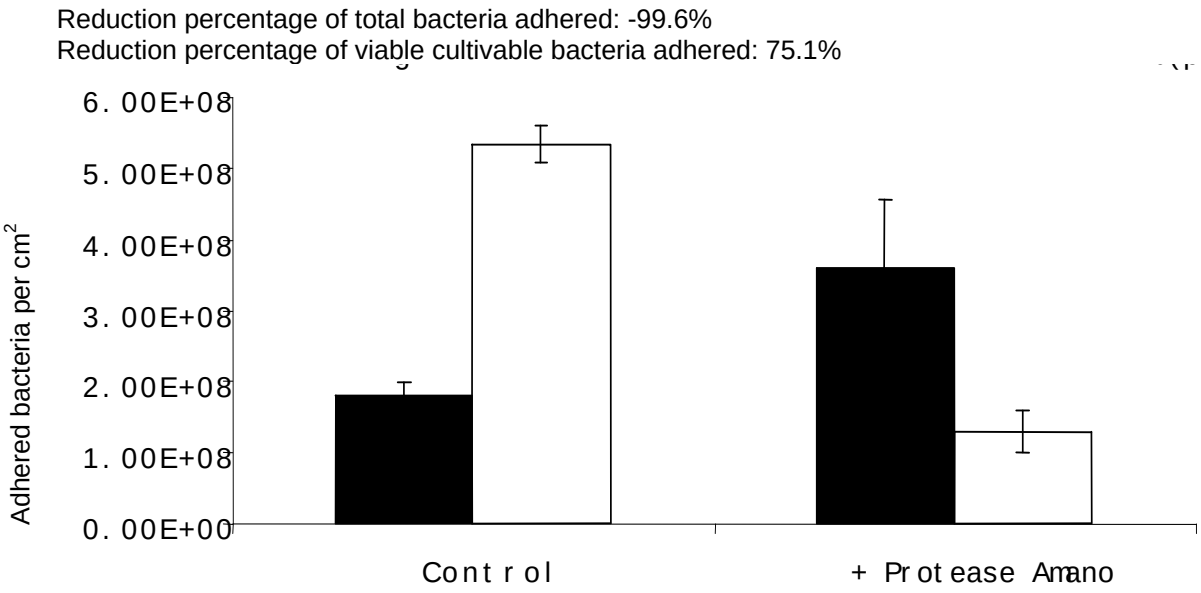

FIGURE 5 Effect of Amano protease in prevention of an D41 adhesion on polystyrene surface in microplate and measured after 24 h. Number of total (▉) and viable cultivable (▒) adhered bacteria per cm^2 are shown. Amano protease is pre-incubated in sea water in microplate at 1 UP ml^{-1} during 1 h, then *Pseudoalteromonas sp*. D41 is added per well and incubated 24 h at 20°C in sterile natural sea water. SD were calculated from four experiments.

FIGURE 6

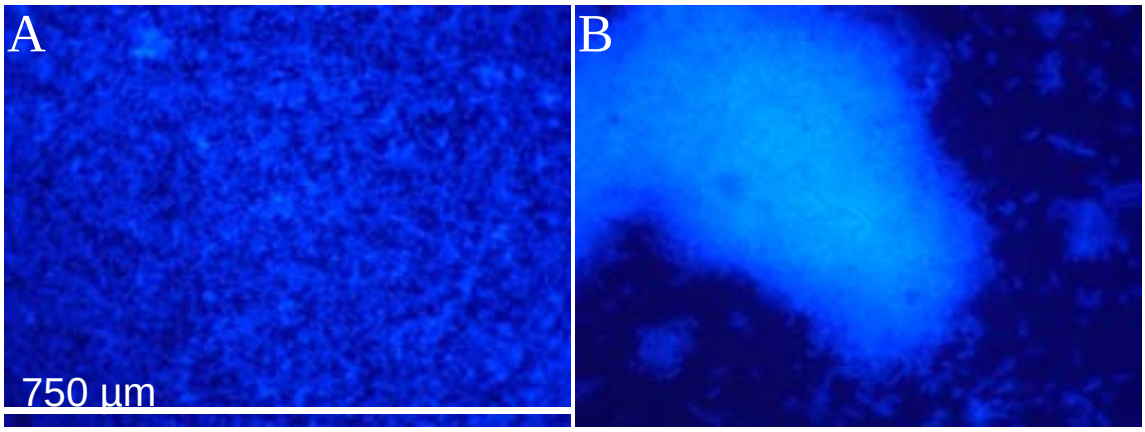

FIGURE 6 Epifluorescence microscopy analysis of DAPI stained Pseudoalteromonas sp. D41 adhered onto glass slide after 24 h in sea water at 20°C (A). Amano protease is pre-incubated in sea water in microplate at 1 UP ml-1 during 1 h, then Pseudoalteromonas sp. D41 is added per well and incubated 24 hours at 20°C in sterile natural sea water (B).