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ORIGINAL ARTICLE

## Effect of proteases against biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis*

P.-H. Elchinger<sup>1,2,3</sup>, C. Delattre<sup>3</sup>, S. Faure<sup>1,2</sup>, O. Roy<sup>1,2</sup>, S. Badel<sup>4</sup>, T. Bernardi<sup>4</sup>, C. Taillefumier<sup>1,2</sup> and P. Michaud<sup>3</sup>

<sup>1</sup> Institut de Chimie de Clermont-Ferrand, Clermont Université, Université Blaise Pascal, Clermont-Ferrand, France

<sup>2</sup> CNRS, UMR 6296, ICCF, Aubière, France

<sup>3</sup> Institut Pascal UMR CNRS 6602, Polytech Clermont-Ferrand, Clermont Université, Université Blaise Pascal, Aubière Cedex, France

<sup>4</sup> BioFilm Control, rue Emile Duclaux, Biopôle Clermont Limagne, Saint-Beauzire, France

**Significance and Impact of the Study:** Three proteases were tested against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms in standard conditions. The Flavourzyme containing a mix of *Aspergillus oryzae* endo- and exoproteases demonstrated significant efficacy against *Staph. epidermidis* biofilm formation. These results could prove valuable in the effort to develop simple anti-biofilm methods.

### Keywords

anti-biofilm, biofilms, fouling, protease, *Staphylococcus aureus*, *Staphylococcus epidermidis*.

### Correspondence

Cedric Delattre, Polytech Clermont-Ferrand, Institut Pascal UMR CNRS 6602, Clermont Université, Université Blaise Pascal, 24 avenue des Landais, BP 206, 63174 Aubière Cedex, France.

E-mail: cedric.delattre@univ-bpclermont.fr

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### Abstract

Biofilms play a key role in bacterial resistance against antibacterial agents—an issue that causes multiple problems in medical fields, particularly with *Staphylococcus* biofilms that colonize medical indwelling devices. The literature reports several anti-biofilm strategies that have been applied in medicine. Disrupting the biofilm formation process creates new sites open to colonization by treatment-generated planktonic bacteria, so efforts have turned to focus on strategies to prevent and control the initial *Staphylococci* adhesion. Here, we investigated the preventive activities of three commercial proteases (Flavourzyme, Neutrase and Alcalase) against biofilm formation by two *Staphylococcus* strains. Some proteolytic extracts revealed interesting results with *Staphylococcus epidermidis* and *Staphylococcus aureus aureus* biofilms.

### Introduction

The simple model of biofilm infection proposed by Costerton in 1999; has since been adjusted to reach a more complex model for multi-species biofilms (Wolcott *et al.* 2013). These models explain the mechanisms that shield biofilms against anti-bacterial treatments and thus the shift in research efforts towards biofilm regulation strategies (Chen *et al.* 2013).

In general, the biofilm installation process involves four steps (Arciola *et al.* 2012). The first one is the initial attachment of cells to the material surface. This step includes hydrophobic interactions involving bacterial proteins such as autolysin and adhesin (Foster 1995; Heilmann *et al.* 1997; Hirschhausen *et al.* 2010). It leads to the irreversible attachment of cells. The bacteria accumulate in multiple layers mediated by the microbial surface components

recognizing adhesive matrix molecules (MSCRAMM) composed of proteins (Patti *et al.* 1994; Speziale *et al.* 2009) in the second step, that maturation of the biofilm starts in the third step, after production of exopolysaccharides, proteins, and extracellular DNAs (eDNAs) and the detachment of bacteria cells from the biofilm into a planktonic state occurs in the last step.

In medical settings, biofilms produced by *Staphylococcus aureus* and *Staphylococcus epidermidis* strains are responsible for a number of nosocomial infections and infections on indwelling medical devices (Otto 2008; Gil *et al.* 2013). Biological disruptor agents have already been used against *Staphylococcus* biofilms, such as DNase I which targets eDNAs or Dispersin B which targets exopolysaccharides (Hall-Stoodley *et al.* 2008; Kaplan 2009). In the work of Augustin and Ali-vehmas (2004), they study the effect of commercial enzyme (including some

protease) against *Pseudomonas aeruginosa*, *Staph. thermophilus*, *Listeria* and *E. coli* biofilms and proved the interest of protease extracts. For more details, the recent review of Cordeiro and Werner (2012) collated numerous application of enzymes in antifouling application and highlighted the importance of biofilm control.

The aim of this work was to prove that some proteases are particularly good anti-biofilm agents that exclusively target the proteins produced by *Staphylococcus* strains early in biofilm formation. The objective is to propose new surface treatments using specific proteases that act early in bacterial attachment before the biofilm is allowed to mature. Flavourzyme, from *Aspergillus oryzae*, is an aminopeptidase that acts via exolytic and endolytic mechanisms (Del-Mar *et al.* 2013). Neutrase, from *Bacillus amyloliquefaciens*, exhibits endoprotease activity in neutral conditions, and its anti-biofilm activities have already been tested against a large panel of bacterial biofilms (*Pseudomonas*, *Acinetobacter*, *Serratia*; Aldridge *et al.* 1994; Marion and Sanchez 2004). Alcalase, from *Bacillus licheniformis*, is a serine endopeptidase essentially composed of subtilisin A that was recently used in the paper industry to remove multi-species biofilms (Marcato-Romain *et al.* 2012).

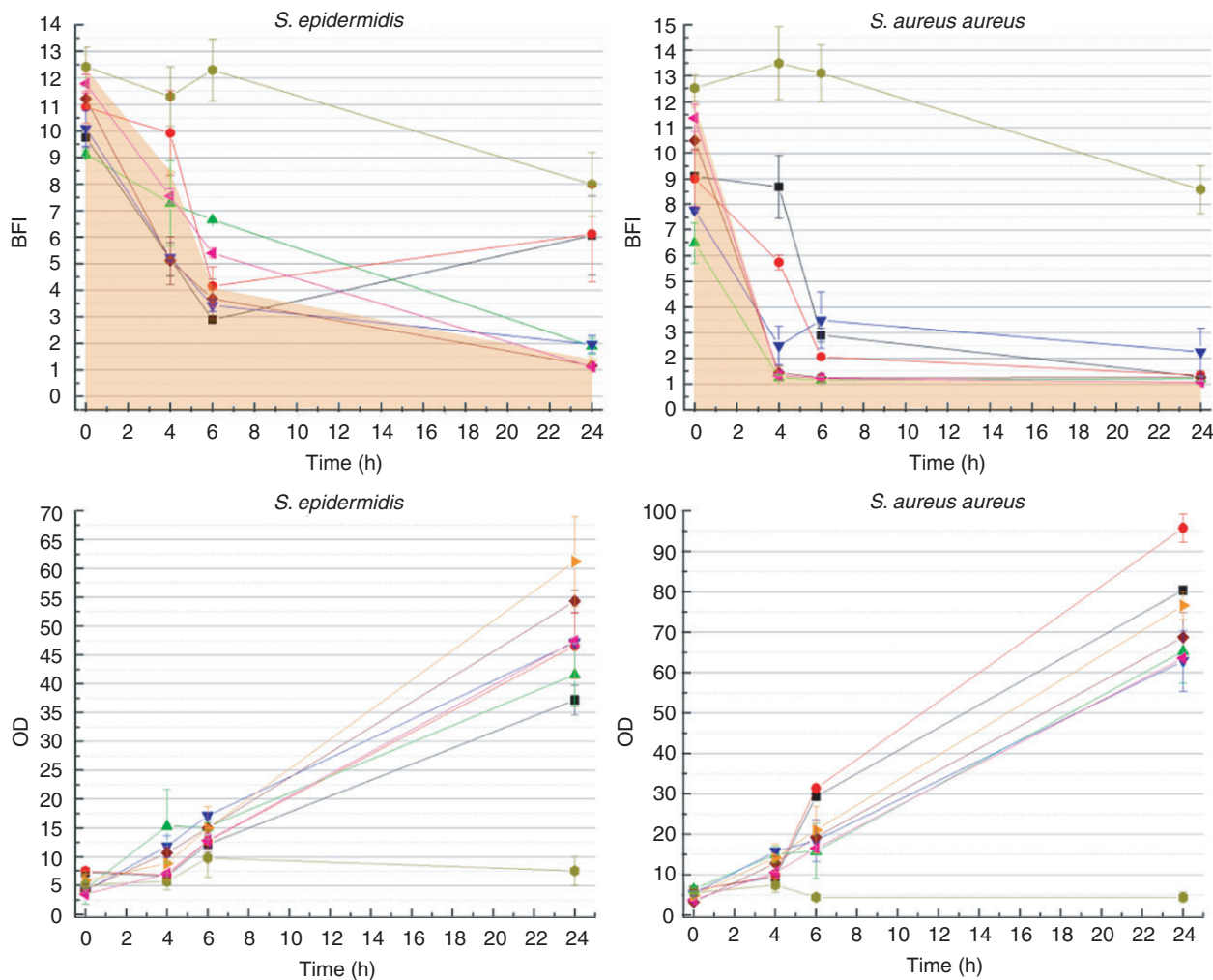
## Results and discussion

This *in vitro* study tested the efficacy of three proteases against biofilms of *Staph. epidermidis* and *Staph. aureus aureus*. Biofilm disruption has generally been assessed using colorimetric analysis such as crystal violet or safranin (Wu *et al.* 2013; Yoneda *et al.* 2013). The general procedure of colorimetric methods has been to remove the unbound cells not embedded in the biofilm and to stain the remaining cells. Staining intensity is proportional to biomass and thus correlated to amount of biofilm. Here, we investigated the BioFilm Ring Test (BRT) designed to evaluate the kinetics of biofilm formation by *Staph. epidermidis* and *Staph. aureus aureus* (Chavant *et al.* 2007; Badel *et al.* 2008, 2011a,b). BRT is a powerful analytical tool that enables quick and convenient screening of anti-biofilm agents based on the mobility of magnetic beads in the bacterial culture medium. This method has several advantages over colorimetric analysis as it can quantify biofilm without having to first remove or washing planktonic cells (Chavant *et al.* (2007).

The kinetics of biofilm formation by *Staph. epidermidis* and *Staph. aureus aureus* remained stable throughout incubation (Biofilm Index, BFI < 2), that is, through 24 and 4-h incubation, respectively. Based on these results, culture media inoculated with *Staphylococcus* strains (Brain Heart Infusion, BHI, pH 7.4) were supplemented with several concentrations of Neutrase, an endoproteolytic extract, and incubated at 37°C to evaluate their impact on biofilm for-

mation and bacterial growth (Figure 1). No significant inhibition of *Staph. epidermidis* biofilm installation was detected from 0 to 6 h, whatever the Neutrase concentration. However, an anti-biofilm effect was observed at Neutrase concentrations of 50 and 10 mU ml<sup>-1</sup> at 24 h of incubation (BFI values were 6.1 with anti-biofilm agent vs 1.4 for the BHI control) once the *Staph. epidermidis* biofilm was installed. This anti-biofilm activity was attributed to the endoprotease activity of Neutrase against proteins from the *Staph. epidermidis* biofilm. This result is consistent with the reported anti-biofilm effects of lysostaphin, another endoprotease, that has been widely described and used against *Staphylococcus* biofilms in medical settings (Shah *et al.* 2004; Wall *et al.* 2005; Placencia *et al.* 2009; Pangule *et al.* 2010; Belyansky *et al.* 2011; Satishkumar *et al.* 2011). The effect on the strain *Staph. aureus aureus*, followed a different pattern, as seen in Figure 1 showing a 72% inhibition of *Staph. aureus aureus* biofilm formation at 4 h of incubation for a Neutrase concentration of 50 mU ml<sup>-1</sup> but a lower 47% inhibition at a Neutrase concentration of 10 mU ml<sup>-1</sup> (BFI of 8.7 for 50 mU ml<sup>-1</sup>, 5.7 for 10 mU ml<sup>-1</sup> and 1.4 for control) and a loss of effect once incubation time went beyond 6 h. This reactivity difference of Neutrase between the two biofilms may be due to a difference in the expression of protein involved in *Staphylococci* biofilms, such as BAP (biofilm-associated protein), Aap (Accumulation-associated proteins), Autolysins, MSCRAMMs, Sas (*Staph. aureus* surface protein) or Ses (*Staph. epidermidis* surface protein), which is variable in different strains, or may be due to a different accessibility of this protein to the Neutrase (You *et al.* 2014). OD measurements carried out to evaluate the putative anti-bacterial effect of Neutrase led to the conclusion that the tested protease (Neutrase) had no anti-bacterial effect against *Staph. epidermidis* and *Staph. aureus aureus* (Figure 1) at any of the concentrations tested.

The same experiment was conducted with Alcalase used as a putative anti-biofilm compound (Figure 2). This endoproteolytic extract contains subtilisin, a serine endopeptidase with a broad spectrum of activity (Leroy *et al.* 2008a). Subtilisins are known to be regularly used by bacteria in biofilm regulation (Longhi *et al.* 2008; Thallinger *et al.* 2013) due to their specific activity against adhesins (Leroy *et al.* 2008b). However, Alcalase showed no anti-biofilm effect against *Staph. aureus aureus* biofilms and only mild inhibition of *Staph. epidermidis* biofilms (BFI of 6.6 for 7 mU ml<sup>-1</sup> and 4.1 for controls after 6 h). This lack of effect could be explained by the fact that the standard physical-chemical culture parameters (pH and temperature) and natural acidification of the culture medium (BHI) were nonoptimal for Alcalase activity (optimal conditions for Alcalase are pH 8 and 50–60°C). A slight increase of bacterial growth was observed (Figure 2) in the



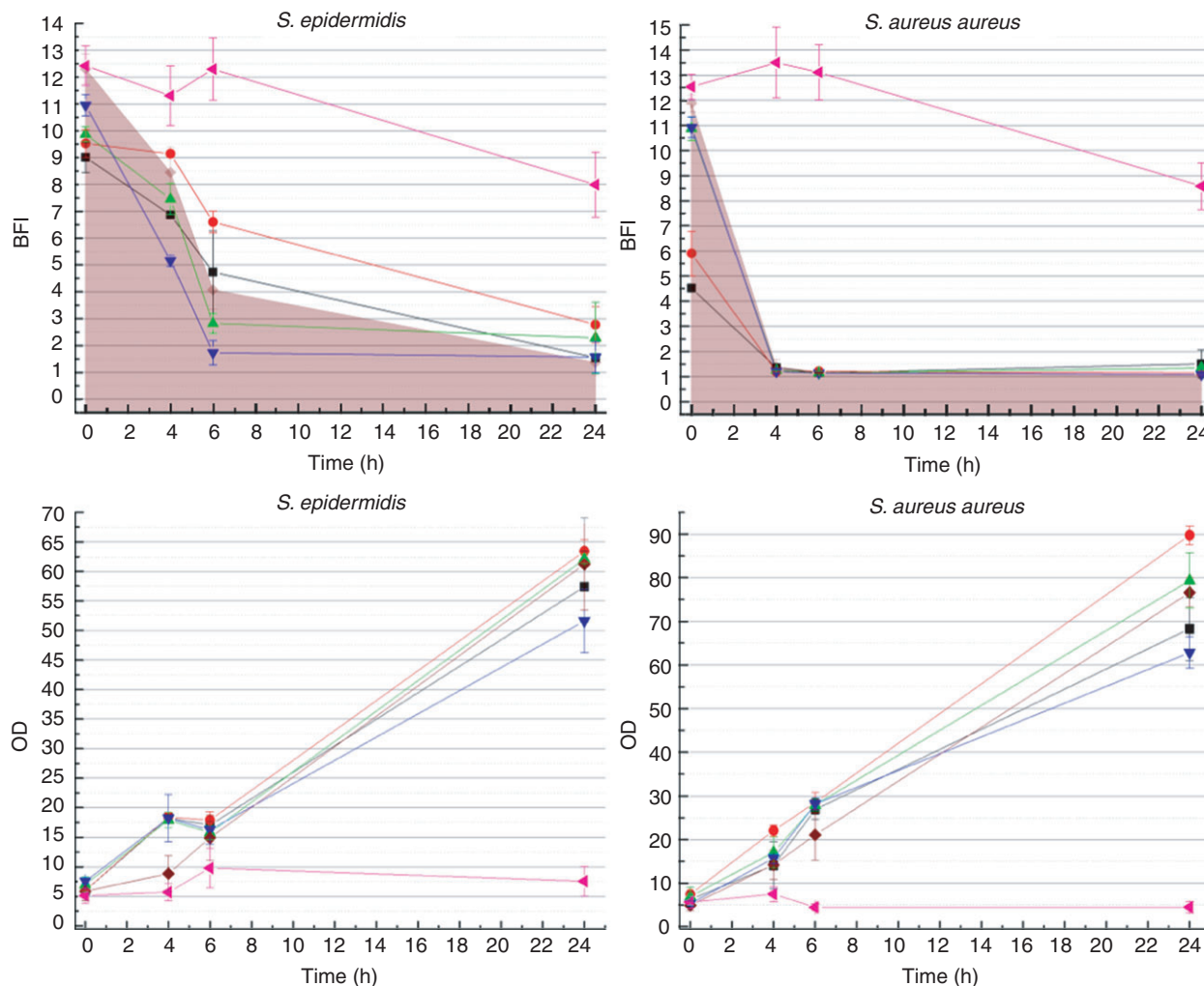
**Figure 1** Evolution of Biofilm Index and OD (600 nm) over 24 h for culture media of *Staphylococcus aureus aureus* and *Staphylococcus epidermidis* with Neutrase and controls (Neutrase at: ■ 50 mU ml<sup>-1</sup>; ● 10 mU ml<sup>-1</sup>, ▲ 5 mU ml<sup>-1</sup>, ▼ 2.5 mU ml<sup>-1</sup>, ◆ 0.5 mU ml<sup>-1</sup>, ◇ 0.05 mU ml<sup>-1</sup>; ▲ Strain control; ● Brain Heart Infusion (BHI) control).

presence of higher enzyme supplementation in the *Staph. aureus aureus* culture media, which was attributed to the presence of glycerol as an Alcalase enzyme stabilizer.

The third protease used in this study was Flavourzyme, an extract that combines exoprotease and endoprotease activities. Figure 3 charts the action of this enzymatic mixture on bacterial growth and biofilm formation. A significant anti-biofilm effect of Flavourzyme against *Staph. epidermidis* biofilm was visible at 6 h at 6 and 3 U ml<sup>-1</sup>, with a BFI of 9.0 for 6 U ml<sup>-1</sup>, 7.0 for 3 U ml<sup>-1</sup> and 4.1 for controls. This anti-biofilm efficacy was maintained at 24 h with a BFI of 7.8 for 6 U ml<sup>-1</sup>, 8.4 for 3 U ml<sup>-1</sup> and 1.4 for controls. The anti-biofilm action against biofilm formation by *Staph. epidermidis* was proportional to enzyme quantity, as there was no observable inhibition with the diluted Flavourzyme solution (0.6

U ml<sup>-1</sup>). The fact that bacterial growth was unaffected by the presence of Flavourzyme points to the conclusion that Flavourzyme acts specifically on the biofilm components of *Staph. epidermidis*. To the best of our knowledge, this proteolytic enzyme extract has never yet been used specifically against biofilms. Note that Flavourzyme was unable to disturb *Staph. aureus aureus* biofilm formation whatever the enzyme concentration used (Figure 3). This difference in efficacy of Flavourzyme against these two kinds of biofilms is similar to Neutrase assays and may be correlated to the nature of the strains and the accessibility of enzyme extracts to proteins biofilm.

This study was designed to evaluate the *in vitro* efficiency of three proteases against the formation of *Staph. aureus aureus* and *Staph. epidermidis* biofilms. The findings highlight that Flavourzyme has a specific



**Figure 2** Evolution of Biofilm Index and OD (600 nm) over 24 h for culture media of *Staphylococcus aureus aureus* and *Staphylococcus epidermidis* with Alcalase and controls (Alcalase at:  $\blacksquare$  14  $\mu\text{M ml}^{-1}$ ,  $\bullet$  7  $\mu\text{M ml}^{-1}$ ,  $\blacktriangle$  1.4  $\mu\text{M ml}^{-1}$ ,  $\blacktriangledown$  0.14  $\mu\text{M ml}^{-1}$ ;  $\blacklozenge$  Strain control;  $\blacktriangleleft$  Brain Heart Infusion (BHI) control).

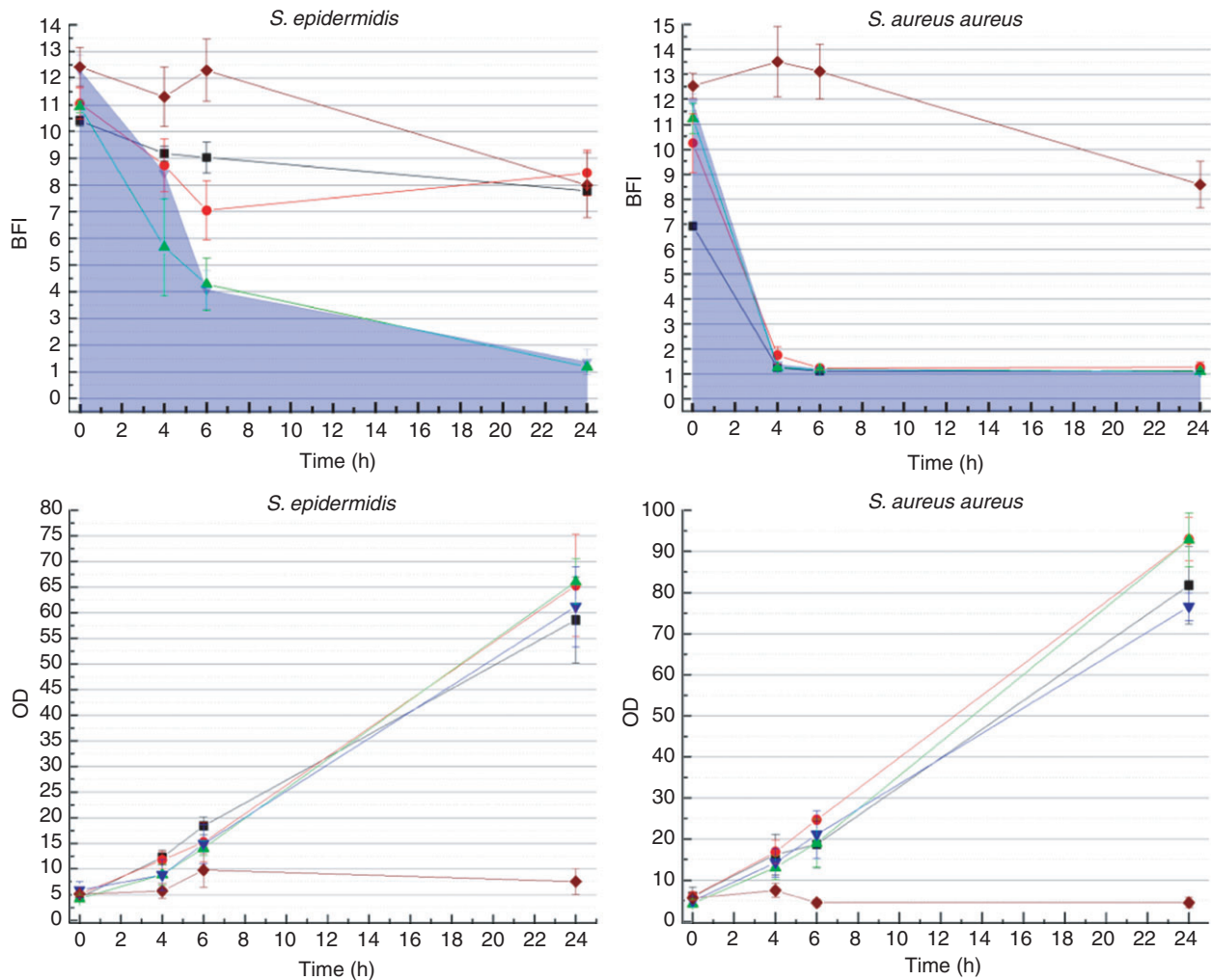
activity against *Staph. epidermidis* biofilm installation at concentrations of 3  $\text{U ml}^{-1}$  and over. This is the first report of Flavourzyme protease extract as an effective anti-biofilm agent. Moreover, OD measurements demonstrated that the anti-biofilm activity of Flavourzyme is not correlated with an anti-bacterial action. The Neutrase presented a significant *in vitro* anti-biofilm activity, increasing with incubation time, against biofilm of *Staph. epidermidis* and a slight inhibition effect of *Staph. aureus aureus* biofilm installation, whereas Alcalase presented not real effectiveness in these conditions. The two active enzyme extracts could be candidates as efficient anti-biofilm tools and consequently warrant further studies, with medical support, in dynamic reactors and *in vivo* assays, as described for example by Machado *et al.* (2012). In their work, they focused on ventilator-

associated pneumoniae due to *Staph. aureus* and developed an interesting model to study lung infections. The effect of a combination of these enzymes (Flavourzyme or Neutrase) plus anti-bacterial agents should also be tested, as described for example by Izano *et al.* (2008) on *Staph. aureus* and *Staph. epidermidis* biofilms with the cationic detergent cetylpyridinium chloride.

## Materials and methods

### Materials

Bacteria were sourced from the Pasteur Institute Collection. *Staphylococcus aureus aureus* is referenced CIP 76 25, and *Staph. epidermidis* is referenced CIP 105 777. Bacteria were cultivated in BHI broth (Becton, Dickinson and



**Figure 3** Evolution of Biofilm Index and OD (600 nm) over 24 h for culture media of *Staphylococcus aureus aureus* and *Staphylococcus epidermidis* supplemented with Flavourzyme and controls (Flavourzyme at:  $\blacksquare$  6 U ml<sup>-1</sup>,  $\bullet$  3 U ml<sup>-1</sup>,  $\blacktriangle$  0.6 U ml<sup>-1</sup>;  $\blacktriangledown$  Strain control;  $\blacklozenge$  Brain Heart Infusion (BHI) control).

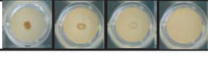
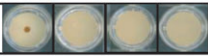

Company, Pont de Claix (Le), France). Flavourzyme 1000 L (1000 U g<sup>-1</sup>), Neutrase 0.8 L (0.8 U g<sup>-1</sup>) and Alcalase 2.4 L FG (2.4 U g<sup>-1</sup>) were purchased from Novozymes. Enzymes were dissolved and diluted in Milli-Q water (Millipore, Molsheim, France) to obtain concentrations of between 6 and 0.6 U ml<sup>-1</sup> for Flavourzyme, 50 and 0.05 mU ml<sup>-1</sup> for Neutrase and 14 and 0.14 mU ml<sup>-1</sup> for Alcalase, so as to maintain good in-well mobility of magnetic beads.

### The BioFilm Ring Test<sup>®</sup> technique

The BRT is a kit that includes microplates (12 polystyrene strips of eight wells, SBS format), magnetic beads in solution with a bead diameter of around 1 μm, a contrast agent (a nontoxic and inert opaque oil used for reading

steps), a dedicated bloc test (magnet support) and a plate reader (scanner). Results are obtained by optical analysis between a premagnetization image and a postmagnetization image (Figure 4). The protocol was similar to that reported elsewhere (Badel *et al.* 2011b).

In brief, 20 μl of each enzyme solution was distributed into each well. *Staphylococcus aureus aureus* and *Staph. epidermidis* suspensions were prepared from overnight cultures in BHI broth and then the A<sub>600 nm</sub> was adjusted to 1. Each suspension was diluted 250-fold to obtain final suspensions with a concentration of 10<sup>6</sup> CFU ml<sup>-1</sup>, magnetic beads were added at a rate of 10 μl ml<sup>-1</sup>, and 180 μl of the final suspension was distributed into the wells prefilled with enzymes. Enzyme controls were composed of 20 μl of enzymatic solution and 180 μl of BHI broth, with 10 μl ml<sup>-1</sup> of magnetic beads solution to verify their mobil-

<i>S. epidermidis</i> Control	Well images				
	BFI	12.3	8.4	4.1	1.3
<i>S. aureus aureus</i> Control	Well images				
	BFI	11.9	1.4	1.2	1.1
BHI Control	Well images				
	BFI	12.4	11.3	12.3	7.9
Time (h)		0	4	6	24

**Figure 4** Control wells after magnetization.

ity in presence of enzymes. Culture controls were composed of 200  $\mu\text{l}$  *Staph. aureus aureus* or *Staph. epidermidis* cultures with 10  $\mu\text{l}$   $\text{ml}^{-1}$  of magnetic beads solution. One microplate per read-time (0, 4, 6, 24 h) was filled and incubated at 37°C in a high-humidity chamber. A first scan of the plates with the plate reader gave a measure of reflectance, converted to  $A_{600\text{nm}}$  via an algorithm appropriate to Staphylococci genus.  $A_{600\text{nm}}$  measure is used to evaluate turbidity and thus culture growth in each well. Before the second reading, the contrast liquid was added in each well and then the plate was scanned to obtain an image (I0) with no spot visible. The well strip was placed on the bloc test for 1 min. to magnetize the beads. In the bloc, 96 magnets are placed opposite the bottom of each well. A second reading gave a second image (I1). (I0) and (I1) were compared using BIOFILM CONTROL Software, and an algorithm was used to estimate discrepancy between two images of the same well, giving a value termed BFI ranging from 0 to 16. A  $\text{BFI} \leq 2$  is representative of a stable and fully established biofilm.

The percentages of inhibition are calculated according to Eqn 1.

$$\%_{\text{inhibition}_x} = \frac{100(\text{BFI}_x - \text{BFI}_{\text{Strain control}})}{\text{BFI}_{\text{BHI control}} - \text{BFI}_{\text{Strain control}}} \quad (1)$$

This equation define the percentage of inhibition of a tested sample 'x', by setting for a chosen reaction time, the mobility of beads with the BHI control at 100% and the mobility of beads with the strain control at 0%.

All tests were performed in duplicate wells. The significance of differences between means was calculated using a Student's *t*-test. Threshold for significance was set at a *P*-value of < 0.05.

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## Conflict of Interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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