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### Hindering biofilm formation with zosteric acid

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## Hindering biofilm formation with zosteric acid

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The antifoulant, zosteric acid, was synthesized using a non-patented process. Zosteric acid at 500 mg l<sup>-1</sup> caused a reduction of bacterial (*Escherichia coli*, *Bacillus cereus*) and fungal (*Aspergillus niger*, *Penicillium citrinum*) coverage by 90% and 57%, respectively. Calculated models allowed its antifouling activity to be predicted at different concentrations. Zosteric acid counteracted the effects of some colonization-promoting factors. Bacterial and fungal wettability was not affected, but the agent increased bacterial motility by 40%. A capillary accumulation test showed that zosteric acid did not act as a chemoeffector for *E. coli*, but stimulated a chemotactic response. Along with enhanced swimming migration of *E. coli* in the presence of zosteric acid, staining showed an increased production of flagella. Reverse transcriptase-PCR revealed an increased transcriptional level of the *fliC* gene and isolation and quantification of flagellar proteins demonstrated a higher flagellin amount. Biofilm experiments confirmed that zosteric acid caused a significant decrease in biomass (–92%) and thickness (–54%).

**Keywords:** adhesion; biofouling; zosteric acid; modeling

### Introduction

Conventional biocidal practices often prove inadequate with respect to adherent microorganisms associated within biofilms (Costerton 2007). By adopting the sessile mode of life, microorganisms improve their resistance to antimicrobial agents up to several orders of magnitude (Shirliff et al. 2002). In addition, increasingly restrictive regulations limiting the use of substances hazardous to human health and the environment have resulted in several biocides being banned. As a consequence, new approaches to defeat deleterious biofilms have become imperative, and the best strategy is to anticipate biofilm formation. An innovative trend is to mimic antifouling (AF) strategies of higher organisms (Qian et al. 2010). For example, furanones produced by the alga *Delisea pulchra* perturbed biofilm processes interfering with bacterial cell-to-cell communication without affecting the growth rate (Hentzer et al. 2003). Ursolic acid from the tree *Diospyros dendo* inhibited *E. coli* biofilm formation 6 to 10-fold at the non-toxic concentration of 10 mg l<sup>-1</sup> (Ren et al. 2005). *N*-vanillylnonanamide, an analogue of hot pepper capsaicin, reduced the adhesion of cells of a *Bacillus* sp. on glass slides by 48% at a concentration of 205 μM (Villa et al. 2009).

The eelgrass *Zostera marina* possesses features that protect against microbial colonization, including the production of the secondary metabolite *p*-(sulphooxy)cinnamic acid (zosteric acid), which displays AF activities against marine bacteria, algae, barnacles and tubeworms at non-toxic concentrations (Todd et al. 1993; Callow and Callow 1998; Barrios et al. 2005; Jagani et al. 2009). Zosteric acid is a sulfated phenolic acid bearing a cinnamic acid moiety. Phenolic acids are known for their non-toxicity, and are used in nutritional and cosmetic formulations. Cinnamic acid may be used in foodstuffs according to the Food and Drug Administration (FDA 21 CFR 172.515 and 21 CFR 182.60), the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2000) and the Council of Europe (European Union 2000) and is also widely employed in the medical field (Yeh et al. 2009).

Xu et al. (2005b) evaluated the toxicity of zosteric acid to *Pseudomonas putida* and aquatic bacteria using the Microtox assay and static toxicity assessment. Moreover, zosteric acid showed no measurable LD<sub>50</sub> for larval fish, possessed an acute toxicity profile similar to table sugar and had a half-life of a few days in seawater (Flemming 2005). Xu et al. (2005a) observed a significant reduction (92.5%) in marine bacterial biofilm coverage with 50 mg l<sup>-1</sup> zosteric acid.

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Zosteric acid also reduced spore adhesion of the two phytopathogenic fungi *Magnaporthe grisea* and *Colletotrichum lindemuthianum* on abiotic surfaces as well as on plant leaves (Stanley et al. 2002).

In 1999, Alexandratos was granted a US patent for the synthesis and purification of zosteric acid (US Patent 5990336). However, the protocol did not produce large amounts of the potential antifoulant. Low-yields represent an important obstacle to the successful development of an AF product, with regards to its availability on the market. In addition, although the published literature supported the potential of zosteric acid as an AF compound, its efficacy in a complex scenario using a mathematical model has not been explored. In addition, none of the previous studies have reported a mode of action for zosteric acid. The goal of the present work was to enhance the zosteric acid literature by reporting for the first time a non-patented method of synthesizing a large amount of zosteric acid and to demonstrate its efficacy in complex scenarios against fungi and bacteria using a model-building strategy. Its mode of action against bacteria will also be demonstrated.

## Materials and methods

### Synthesis of *p*-(sulphooxy) cinnamic acid sodium salt

*Trans*-4-hydroxycinnamic acid (0.1 mol, 16.46 g) was dissolved in anhydrous *N,N*-dimethylformamide, DMF (30 ml). Sulfur trioxide pyridine complex (Py · SO<sub>3</sub>, 0.16 mol, 25.68 g) was added and the resulting solution stirred at 50°C for 2 h. After cooling to room temperature, 30% NaOH was added dropwise to pH 7. The precipitate was filtered and the resulting solution extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 20 ml). The aqueous phase was partially evaporated and methanol was subsequently added dropwise causing precipitation of a white solid that was removed by filtration. The solution was evaporated to dryness, which produced 28.35 g of the title compound, yield 95%. <sup>1</sup>H NMR (D<sub>2</sub>O), δ, 6.53 (d, 1 H, J = 16.0 Hz), 7.37 (d, 1 H, J = 8.4 Hz), 7.44 (d, 1 H, J = 16.0 Hz), 7.68 (d, 1 H, J = 8.4 Hz). <sup>13</sup>C NMR (D<sub>2</sub>O) δ, 121.7 (CH), 121.8 (CH), 124.0 (CH), 129.1 (CH), 129.2 (CH), 133.1 (C), 140.1 (CH), 152.0 (C), 175.4 (C). HPLC: Ascentis™ RP-amide (15 cm × 4.6 mm, 5 μm) H<sub>2</sub>O-CH<sub>3</sub>CN (0.1% trifluoroacetic acid) 70–30, 1 ml min<sup>-1</sup>, λ 298 nm; t<sub>R</sub> 2.9 min.

### Microbial strains and growth media

The microbial strains used were *Bacillus cereus*-group strain (UNIMI collection), *Escherichia coli* ATCC 25404, *Aspergillus niger* ATCC 9642 and *Penicillium citrinum* (UNIMI collection). These strains were selected as they form biofilms. Plate Count Broth,

PCB (Difco) and Potato Dextrose Broth, PDB (Merck) were used to culture the bacteria and fungi, respectively. Fungi were also grown on Potato Dextrose Agar, PDA (Merck) to obtain conidia.

### Cellular growth with zosteric acid

Bacteria were grown in untreated polystyrene 96-well plates (Thermo Fisher Scientific) at 30°C for 72 h in PCB medium. Cell enumeration was calculated from OD<sub>600</sub> using a Bio-Rad 680 microplate reader. Maximum bacterial growth rates with and without 500 mg l<sup>-1</sup> zosteric acid were calculated using the plot 'logarithm of the cellular density vs time'. Fungi were grown in 100 ml PDB with 0, 1, and 25 g l<sup>-1</sup> zosteric acid. Spore suspensions (10<sup>4</sup> spores ml<sup>-1</sup>) were incubated at 25°C for 10 days. Fungal biomass was assessed gravimetrically.

The ability of bacteria and fungi to grow on zosteric acid as their sole carbon and energy source was tested using mineral media (Maniatis et al. 1982; ASTM G21-96 2002) supplemented with the antifoulant at the same concentrations as for the growth test. The positive control was represented by the mineral medium supplemented with either 500 mg l<sup>-1</sup> of glucose or 25 g l<sup>-1</sup> of saccharose (Merck, Italy) for bacteria and fungi, respectively. Microbial growth was followed by determination of absorbance (OD<sub>600</sub>) whereas fungal growth was monitored gravimetrically by cell dry weight determination as previously described. All the experiments were repeated three times.

### Design of experiments (DoE)

Variables studied that influenced biofilm formation were: the type of microorganism, the type of surface (hydrophobic surface, medium-binding microtiter plates and hydrophilic surfaces, polar high-binding microtiter plates), the zosteric acid concentration (10 and 500 or 1000 mg l<sup>-1</sup> for bacteria and fungi, respectively), the contact time (30 min and 24 h), the temperature (10°C and 40°C), the pH (6 and 8) and agitation at 250 rpm (+ or -). The experimental set up considered the zosteric acid concentrations, and pH and temperature levels that did not inhibit cell growth. The number of surface-bound cells was quantified by fluorometric measurements and a standard curve of cell number vs fluorescence intensity was determined for each microorganism. For statistical analysis, the variables were coded according to the following equation:

$$x_i = 2 \times (\text{real value} - \text{central value}) / \Delta \quad (1)$$

where Δ represents the difference between the maximum and the minimum value. In the case of skewed

data, a square root or logarithmic transformation was performed on the response prior to any calculation.

A Fractional Factorial Design (FFD) resolution IV was applied first. Since in this design two-factor interactions are confounded by each other, the design was extended to a Full Fractional Design. Finally, star and center samples were added to estimate quadratic terms and fully characterize the process by a response surface. As the experimental domain could not be enlarged, a Face Centered Composite Design (FCC) was performed. Experiments in triplicate were carried out in randomized run order. The relationships between the independent variables and the response were evaluated by multiple linear regression (MLR) taking into account the main, interaction and quadratic effects. The experimental data points (number of trials, range and levels of the variables) used for the calculations are given in Table 1.

Factors and interactions having  $P > 0.05$  were excluded. The significance of the regression was tested by comparing the effect or variability caused by the regression model to the overall error ( $\alpha = 0.05$ ). The goodness of fit of the polynomial models was evaluated by the coefficient of determination  $R^2$ , and the Lack of Fit (LOF) test ( $\alpha = 0.05$ ) for the model from ANOVA table. A LOF  $> 0.05$  indicates possible contributions in the variables-response relationship not accounted for by the model. The adjusted coefficient of determination,  $R^2_{\text{adj}}$ , was also calculated, taking into account the degrees of freedom.  $R^2_{\text{adj}}$  gives an indication if a new coefficient improves the model or might lead to overfitting. The modeling was performed using Unscrambler 9.8 (CAMO) and Microsoft Excel Worksheet.

The experimental data points used to confirm the mathematical models are shown in Table 2.

### Biofilm assay

Overnight bacterial cultures were centrifuged, then diluted to the desired concentration using phosphate-buffered saline (PBS, Sigma-Aldrich). Mature conidia were harvested from 10-day PDA cultures by flooding the surface with PBS with 0.05% Tween 20 (Sigma-Aldrich) and scraping with a sterile handle. Conidial suspensions were filtered through glass wool, centrifuged, and resuspended in PBS to the desired concentration. Two hundred  $\mu\text{l}$  of mineral medium containing  $10^6$  cells were placed in the following 96-well polystyrene-based microtiter plates: (i) hydrophobic medium-binding plates and (ii) hydrophilic and polar high-binding plates, and subjected to different experimental conditions (see the DoE section). Planktonic cells were washed three times with 200  $\mu\text{l}$  of PBS. The adhered cells were stained using either

4',6-diamidino-2-phenylindole or Fluorescent Brightener 28 (Sigma-Aldrich) for bacteria and fungi, respectively. Fluorescence intensity was measured using the fluorometer VICTOR™ X Multilabel Plate Readers (Perkin Elmer) (excitation 433 nm, emission 335 nm). A standard curve of fluorescence intensity vs cell number was determined for each microorganism. A negative control without zosteric acid was included in all the combinations. Three replicates of each combination were tested.

### Contact angle measurement

Bacterial wettability before and after zosteric acid treatment was evaluated by measuring the contact angle of water drops on lawns deposited on membrane filters with a Dataphysics OCA-20 contact angle with a CCD video camera (DataPhysics Instruments GmbH). One hundred fifty ml of a 12-h cell suspension ( $10^8$  cells  $\text{ml}^{-1}$ ) were centrifuged, washed three times with PBS and resuspended to 30 ml. The suspension was filtered using 0.2  $\mu\text{m}$  nitrile filters (Millipore) and the filters air-dried for 1 h to reach a plateau. Two measurements were carried out for each microorganism, six measurements for each filter.

### Bacterial motility assays

Swimming and swarming motility plates were prepared as reported elsewhere (Gómez-Gómez et al. 2007). Plates contained 0, 10, 100, 200, 300, 400, and 500  $\text{mg l}^{-1}$  zosteric acid and 5  $\mu\text{l}$  of bacterial PBS suspensions were inoculated in the plate centre. Swim plates were incubated at 30°C for 24 h and swarm plates at 30°C for 72 h. All the experiments were conducted in duplicate.

### Chemotaxis

Zosteric acid, as a chemorepellent or chemoattractant for *E. coli*, was investigated following Adler's capillary chemotaxis assay (Adler 1973). Cells in PCB in exponential growth were resuspended in chemotaxis buffer after centrifuging at 300 g for 5 min. The capillaries were then filled with 10 mM L-aspartate (positive control), 10 M glycerol (negative control), the chemotaxis buffer alone or 500  $\text{mg l}^{-1}$  zosteric acid. After incubation for 1h, cells in the capillary were assessed by plate counts. The following chemotactic responses were evaluated after 30 and 60 min: (i) 10 mM L-aspartate in the capillary and bacteria grown in PCB; (ii) 10 mM L-aspartate in the capillary and bacteria grown in PCB with 500  $\text{mg l}^{-1}$  zosteric acid; (iii) 10 mM L-aspartate and 500  $\text{mg l}^{-1}$  zosteric acid in the capillary and bacteria grown in PCB; (iv) 10 mM

Table 1. Experimental matrix design and the corresponding response for the FCC.

Trial	E. coli												B. cereus						A. niger and P. citrinum					
	No. adhered cells				No. adhered cells				No. adhered cells				No. adhered cells		No. adhered cells		No. adhered cells		No. adhered cells					
	A	B	C	D	HI	Hb	A	B	C	HI	Hb	A	B	C	A	B	A	B	Hb <sub>A. niger</sub>	Hb <sub>P. citrinum</sub>				
1	0	0	-1	0	4.65 × 10 <sup>5</sup>	± 6%	1.71 × 10 <sup>5</sup>	± 6%	0	9.61 × 10 <sup>5</sup>	± 11%	7.08 × 10 <sup>5</sup>	± 18%	-1	1	6.95 × 10 <sup>3</sup>	± 3%	8.41 × 10 <sup>4</sup>	± 7%					
2	-1	1	-1	1	5.81 × 10 <sup>5</sup>	± 14%	8.51 × 10 <sup>5</sup>	± 14%	0	2.48 × 10 <sup>5</sup>	± 6%	1.48 × 10 <sup>5</sup>	± 1%	1	1	5.40 × 10 <sup>3</sup>	± 2%	3.95 × 10 <sup>4</sup>	± 6%					
3	0	1	0	0	9.21 × 10 <sup>5</sup>	± 24%	3.35 × 10 <sup>5</sup>	± 11%	-1	1	5.24 × 10 <sup>6</sup>	± 1%	8.58 × 10 <sup>6</sup>	± 6%	1	0	5.56 × 10 <sup>3</sup>	± 2%	3.78 × 10 <sup>4</sup>	± 1%				
4	-1	-1	1	1	1.79 × 10 <sup>6</sup>	± 10%	3.12 × 10 <sup>5</sup>	± 8%	-1	-1	1.54 × 10 <sup>5</sup>	± 9%	1.88 × 10 <sup>5</sup>	± 5%	0	0	6.24 × 10 <sup>3</sup>	± 2%	5.25 × 10 <sup>4</sup>	± 0.2%				
5	1	1	-1	-1	5.01 × 10 <sup>5</sup>	± 4%	8.74 × 10 <sup>4</sup>	± 4%	1	1	1.36 × 10 <sup>6</sup>	± 3%	7.53 × 10 <sup>5</sup>	± 4%	0	1	6.14 × 10 <sup>3</sup>	± 2%	5.75 × 10 <sup>4</sup>	± 7%				
6	1	1	1	1	1.74 × 10 <sup>6</sup>	± 10%	2.03 × 10 <sup>6</sup>	± 1%	0	0	5.69 × 10 <sup>5</sup>	± 13%	4.48 × 10 <sup>5</sup>	± 8%	-1	-1	7.05 × 10 <sup>3</sup>	± 0.2%	5.53 × 10 <sup>4</sup>	± 3%				
7	1	1	1	-1	3.98 × 10 <sup>6</sup>	± 10%	2.53 × 10 <sup>5</sup>	± 8%	0	-1	1.71 × 10 <sup>5</sup>	± 8%	1.53 × 10 <sup>5</sup>	± 6%	-1	0	6.99 × 10 <sup>3</sup>	± 5%	6.86 × 10 <sup>4</sup>	± 3%				
8	-1	-1	-1	1	6.10 × 10 <sup>6</sup>	± 20%	2.81 × 10 <sup>5</sup>	± 19%	0	1	1.36 × 10 <sup>6</sup>	± 5%	5.86 × 10 <sup>5</sup>	± 3%	1	-1	5.75 × 10 <sup>3</sup>	± 3%	3.61 × 10 <sup>4</sup>	± 3%				
9	-1	-1	-1	-1	3.87 × 10 <sup>5</sup>	± 35%	3.86 × 10 <sup>5</sup>	± 6%	-1	-1	3.22 × 10 <sup>5</sup>	± 2%	3.27 × 10 <sup>5</sup>	± 5%	0	0	6.09 × 10 <sup>3</sup>	± 2%	5.26 × 10 <sup>4</sup>	± 2%				
10	1	-1	1	1	6.70 × 10 <sup>5</sup>	± 5%	7.29 × 10 <sup>5</sup>	± 4%	1	-1	1	388 × 10 <sup>5</sup>	± 12%	2.13 × 10 <sup>4</sup>	± 6%	0	-1	6.38 × 10 <sup>3</sup>	± 1%	4.50 × 10 <sup>4</sup>	± 2%			
11	1	0	0	0	5.64 × 10 <sup>5</sup>	± 17%	1.67 × 10 <sup>5</sup>	± 16%	1	0	3.57 × 10 <sup>5</sup>	± 7%	1.27 × 10 <sup>5</sup>	± 1%										
12	1	-1	-1	-1	2.24 × 10 <sup>5</sup>	± 3%	7.57 × 10 <sup>4</sup>	± 5%	-1	-1	1	4.80 × 10 <sup>5</sup>	± 15%	7.29 × 10 <sup>5</sup>	± 13%									
13	-1	1	1	-1	5.07 × 10 <sup>6</sup>	± 1%	1.65 × 10 <sup>6</sup>	± 1%	0	1	0	1.17 × 10 <sup>6</sup>	± 12%	6.07 × 10 <sup>5</sup>	± 4%									
14	-1	0	0	0	9.86 × 10 <sup>5</sup>	± 20%	5.51 × 10 <sup>5</sup>	± 7%	1	-1	-1	1.50 × 10 <sup>5</sup>	± 15%	1.85 × 10 <sup>5</sup>	± 11%									
15	0	-1	0	0	5.88 × 10 <sup>5</sup>	± 3%	2.07 × 10 <sup>5</sup>	± 4%	1	1	-1	1.42 × 10 <sup>5</sup>	± 13%	7.83 × 10 <sup>4</sup>	± 28%									
16	1	-1	-1	1	3.77 × 10 <sup>5</sup>	± 1%	1.13 × 10 <sup>5</sup>	± 6%	0	0	5.89 × 10 <sup>5</sup>	± 9%	4.48 × 10 <sup>5</sup>	± 6%										
17	0	0	0	0	7.92 × 10 <sup>5</sup>	± 7%	2.25 × 10 <sup>5</sup>	± 11%	0	0	6.25 × 10 <sup>5</sup>	± 4%	3.09 × 10 <sup>5</sup>	± 8%										
18	-1	1	-1	-1	5.68 × 10 <sup>5</sup>	± 29%	7.21 × 10 <sup>5</sup>	± 1%	0	0	5.50 × 10 <sup>5</sup>	± 34%	2.91 × 10 <sup>5</sup>	± 6%										
19	1	-1	1	-1	1.29 × 10 <sup>6</sup>	± 26%	1.80 × 10 <sup>5</sup>	± 4%	0	0	4.88 × 10 <sup>5</sup>	± 8%	4.43 × 10 <sup>5</sup>	± 21%										
20	0	0	0	1	8.44 × 10 <sup>5</sup>	± 20%	3.68 × 10 <sup>5</sup>	± 20%	0	0														
21	1	1	-1	1	5.20 × 10 <sup>4</sup>	± 19%	1.61 × 10 <sup>5</sup>	± 5%																
22	-1	1	1	1	2.96 × 10 <sup>6</sup>	± 2%	8.13 × 10 <sup>5</sup>	± 5%																
23	-1	-1	1	-1	1.15 × 10 <sup>6</sup>	± 1%	4.13 × 10 <sup>5</sup>	± 15%																
24	0	0	0	-1	6.24 × 10 <sup>5</sup>	± 8%	1.96 × 10 <sup>5</sup>	± 4%																
25	0	0	1	0	1.57 × 10 <sup>6</sup>	± 12%	5.09 × 10 <sup>5</sup>	± 1%																
26	0	0	0	0	7.17 × 10 <sup>5</sup>	± 6%	2.56 × 10 <sup>5</sup>	± 16%																

A, zosteric acid concentration: -1 = 10 mg l<sup>-1</sup>; 0 = 255 mg l<sup>-1</sup>; 1 = 500 mg l<sup>-1</sup>; B, temperature: -1 = 10°C; 0 = 25°C; 1 = 40°C; C, time: -1 = 0.5 h; 0 = 12.25 h; 24 h; D, pH: -1 = 6; 0 = 7; 1 = 8; HI, hydrophilic surface; Hb, hydrophobic surface.

Table 2. Comparison of predicted and experimental values of the response (number of adhered cells).

Microorganism	Parameters	Predicated value ( $\pm$ RMSEP%)	Experimental value ( $\pm$ SD%)	Deviation (%)
<i>E. coli</i> Hb surface (1/response)	A: 450 mg l <sup>-1</sup> B: 37°C C: 2.6 h D: 6.2	$9.36 \times 10^{-6} \pm 7\%$	$9.05 \times 10^{-6} \pm 9\%$	-3.3
<i>E. coli</i> HI surface (1/response)	A: 450 mg l <sup>-1</sup> B: 13°C C: 2.67 h D: 6.2	$3.41 \times 10^{-6} \pm 21\%$	$3.55 \times 10^{-6} \pm 7\%$	4.1
<i>B. cereus</i> Hb surface (In response)	A: 450 mg l <sup>-1</sup> B: 13°C C: 21.65 h	$10.95 \pm 2.0\%$	$10.89 \pm 0.71\%$	-0.56
<i>B. cereus</i> HI surface (In response)	A: 450 mg l <sup>-1</sup> B: 13°C C: 2.67 h	$12.05 \pm 0.98\%$	$12.12 \pm 1.40\%$	0.54
<i>A. niger</i> Hb surface	A: 900 mg l <sup>-1</sup> B: 37°C	$5.59 \times 10^3 \pm 1\%$	$5.55 \times 10^3 \pm 2\%$	-0.64
<i>P. citrinum</i> Hb surface (sqrt response)	A: 900 mg l <sup>-1</sup> B: 13°C	$196.10 \pm 0.58\%$	$195.47 \pm 0.71\%$	-0.32

Validation experiments were carried out within the experimental domain that ensured the minimum response. Parameters: A, zosteric acid concentration; B, temperature; C, time; D, pH; HI, hydrophilic surface; Hb, hydrophobic surface. The predicted values reported the root mean squared error of prediction (RMSEP) for measuring the predictive ability of the model. The experimental values reported the mean of three replicates and the SD. Deviation was calculated as follows: [(experimental value-predicted value)  $\times$  100]/ predicted value.

L-aspartate and 500 mg l<sup>-1</sup> zosteric acid in the capillary and bacteria grown in PCB with 500 mg l<sup>-1</sup> zosteric acid. All the experiments were conducted in duplicate.

#### Light microscope detection of flagella

*E. coli* flagella were detected using the procedure reported by Heimbrook et al. (1989). Bacterial cells were grown on a swim plate with and without 500 mg l<sup>-1</sup> zosteric acid for 24 h as described above. Drops of motile cells were prepared by touching the colony margin with a loopful of water. Drops of motile cells were placed on a slide, which was covered with a coverslip and two drops of staining solution were applied to the edge of the coverslip. After 10 min at room temperature, the stained bacterial flagella were observed by phase-contrast microscopy using a Leica DM 4000 B microscope at a magnification of 1000  $\times$  under oil immersion. Digital images were acquired using a CoolSNAP CF digital camera (Photometrics Roper Scientific, Germany) and elaborated using ImageJ ver. 1.34s software (Rasband 1997–2007, downloaded from <http://rsbweb.nih.gov/ij/>).

#### Semi-quantitative reverse transcriptase (RT)-PCR expression analysis

*E. coli* cultures were grown for 6 h at 30°C in PCB with and without 500 mg l<sup>-1</sup> zosteric acid. Total RNA was extracted using RNAprotect Bacteria Reagent mini kit

(Qiagen Inc, Italy) and cleaned by passing through RNeasy mini kit with on-column DNase I treatment (Qiagen Inc, Italy) as per the manufacturer's instructions. RNA concentrations were calculated by measuring absorbance at 260 nm using a SmartSpec<sup>TM</sup> 3000 spectrophotometer (BioRad, Italy). cDNA was synthesized from 500 ng RNA using a RevertAid H Minus First Strand cDNA Synthesis Kit as recommended (Fermentas, Italy). The secondary PCR reaction was performed in 25  $\mu$ l of a mixture containing 0.5  $\mu$ l of cDNA, 1X PCR buffer, 1.8 mM of MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.5  $\mu$ M of each primer and 0.6 of Taq polymerase (Promega, Italy). PCR amplification was carried out in a TProfessional Basic Gradient thermocycler (Biometra, Italy) using the following program: initial denaturation at 94°C for 4 min; 15 cycles of 94°C for 45 s, annealing at 62°C for 45 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. The primers used in this study were KF1 (5'-GCACAAGTCATTAATACCAACAGCCTC-3') and KR2 (5'-CCCTGCAGCAGACAGAACCTGCTGC-3') based on the DNA sequence of the *fliC* gene of *E. coli* K12 (Amhaz et al. 2004). The PCR reaction was also performed on cDNA samples prepared without reverse transcriptase (RT) to confirm that there was no genomic DNA contamination of the RNA preparation. Amplification products were evaluated by gel electrophoresis. The resulting PCR products were identified by sequencing to further confirm the expression of *fliC* gene (Primm, Milan). The sequences were analyzed using the BLASTN software ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)).

### Isolation and quantification of flagellar proteins

*E. coli* cultures were grown overnight at 30°C in PCB with 0, 100, 200, 300, 400, and 500 mg l<sup>-1</sup> zosteric acid. Cells were counted using Thoma-Zeiss chamber to obtain flagellar proteins from the same amount of biomass. Cells were harvested by centrifugation at 5600 g for 15 min, 4°C, and resuspended in 2 ml of PBS. Suspensions were vortexed for 10 min at 2000 rpm. Flagella were isolated by removing whole cells by two centrifugations (3500 g, 4°C, 15 min). Then, the supernatant was centrifuged at 130,000 g, 4°C for 90 min. Pellets containing flagellin subunits were resuspended in 250 µl of ultrapure water.

Flagella preparations were qualitatively analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gels and stained with Coomassie blue according to Leammli's method (1970). The molecular weights of the protein bands were determined by comparison to molecular mass standards. The total protein concentration in the flagellin extract was determined using the Bradford assay (1976) and assumed to be flagellin. All the experiments were conducted in duplicate.

### Colony biofilm, staining, cryosectioning and microscopy

*E. coli* biofilms were prepared as described by Anderl et al. (2000) using an LB plate. A Japanese paper moistened with phosphate buffer solution (PBS, Sigma Aldrich) or with 500 mg l<sup>-1</sup> zosteric acid was placed between the agar culture medium and the black membrane filters for the control and the treated samples respectively and replaced every 6 or 12 h. After 48 h, the membrane-supported *E. coli* biofilms were removed, suspended in PBS, serially diluted and plated onto LB agar by the drop plating method. All the experiments were conducted in duplicate.

Forty-eight h-old membrane-supported biofilm was stained with 10 µg ml<sup>-1</sup> 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich srl, Italy) and Alexa fluor<sup>®</sup> 488-labelled Concanavalin A (ConA, Invitrogen, Italy) as per the manufacturer's instructions to visualize biomass and extracellular polymer substances (EPS) respectively. Stained biofilms on polycarbonate membrane filters were covered carefully with a layer of Killik (Bio Optica, Italy) and placed on dry ice until completely frozen. Frozen samples were sectioned at -19°C using a Leitz 1720 digital cryostat (Leica, Italy). The 10-µm thick cryosections were mounted on a poly-L-lysine coated slides (VWR International srl, Italy), examined by fluorescence microscopy using a Leica DM 4000 B microscope at a magnification of 400 × as previously described. The biofilm thickness was measured for each image at three different

locations randomly selected along the profile. These measurements were used to calculate the average thickness and the associated standard deviation (SD).

### Statistical analysis

Analysis of variance (ANOVA) via MATLAB software (Version 7.0, The MathWorks Inc, Natick, USA) was applied to evaluate statistically significant differences among samples. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by *P* values <0.05.

## Results

### Synthesis of *p*-(sulphooxy)cinnamic acid sodium salt

Zosteric acid was generated by treating *trans*-4-hydroxycinnamic acid with the sulfur trioxide pyridine complex, a solid, easy to handle compound, in *N,N*-dimethylformamide (DMF) as solvent (Figure 1). After 2 h at 50°C, *trans*-4-hydroxycinnamic acid was completely converted to zosteric acid. The latter was isolated as the sodium salt by adding 30% NaOH to pH 7, followed by extraction with dichloromethane to remove pyridine and DMF. The resulting clear aqueous solution was concentrated and methanol added. The precipitate thus formed was filtered off and the filtrate evaporated to dryness to give a quantitative yield of sodium zosterate as a white solid containing <5% of sodium sulfate. <sup>1</sup>H NMR of sodium zosterate showed a downfield shift of aromatic protons adjacent to the strong electron withdrawing sulphooxy group with respect to the same protons of *trans*-4-hydroxycinnamic acid.

### Zosteric acid did not affect cell growth and was not a carbon and energy source

Before evaluating zosteric acid as a potential anti-foulant, its impact on the growth of the bacteria at 500 mg l<sup>-1</sup> was studied. The maximum growth rate of the *B. cereus* was 1.307 ± 0.058 (ln cells ml<sup>-1</sup>) h<sup>-1</sup> without zosteric acid, and 1.213 ± 0.138 (ln cells ml<sup>-1</sup>) h<sup>-1</sup> with zosteric acid. The maximum growth

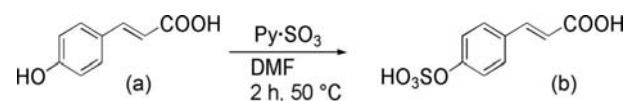


Figure 1. Synthesis of zosteric acid: (a) *trans*-4-hydroxycinnamic acid; (b) zosteric acid.

rate of *E. coli* without the zosteric acid was  $0.438 \pm 0.039$  (ln cells ml<sup>-1</sup>) h<sup>-1</sup>, and  $0.500 \pm 0.022$  (ln cells ml<sup>-1</sup>) h<sup>-1</sup> in the presence of zosteric acid. ANOVA showed no difference between the growth rates (*P. cereus*: 0.341; *P. E. coli*: 0.185); hence, zosteric acid did not inhibit growth.

Fungal growth in liquid culture was unaffected by zosteric acid as the dried mycelial weights were the same for both treated and untreated samples (*P. A. niger*: 0.102; *P. P. citrinum*: 0.463).

Although both bacterial species grew in the mineral medium supplemented with glucose, they did not exhibit growth on zosteric acid as the sole carbon and energy source. The minimum saccharose concentration that supported fungal growth was 25 g l<sup>-1</sup> (*A. niger*: 0.051 ± 0.001 mg<sub>dry weight</sub>; *P. citrinum*: 0.066 ± 0.001 mg<sub>dry weight</sub>). Low biomass was produced by 25 g l<sup>-1</sup> zosteric acid, the yield being about five-fold lower than for saccharose (*A. niger*: 0.013 ± 0.001 mg<sub>dry weight</sub>; *P. citrinum*: 0.012 ± 0.003 mg<sub>dry weight</sub>).

#### Zosteric acid affected cell adhesion

Bacterial and fungal cells stained with DAPI and Fluorescent Brightener 28, respectively, appeared uniformly labeled and revealed a linear relationship between cell number and fluorescent intensity in a range from 10<sup>2</sup> to 10<sup>7</sup> cells.

Biofilm assays were first investigated using the FFD approach. As the response was mainly affected by the type of microorganism and surface, insomuch as they masked the effects of any other experimental variables, the subsequent experimental designs were developed for each microorganism on each surface. The best explanatory equations that fitted the models and produced the response to surfaces that predicted the number of adhered cells were expressed as follows:

#### *B. cereus* hydrophobic surface Equation (2)

$$\begin{aligned} \text{Ln no. adhered cells} = & 12.69 + 0.606 \times \\ & \text{time} - 0.913 \times \text{zosteric acid concentration} + 0.713 \\ & \times \text{temperature} - 0.565 \times \text{zosteric acid concentration} \\ & \times \text{time} + 0.792 \times \text{time} \times \text{temperature} \end{aligned} \quad (2)$$

#### *B. cereus* hydrophilic surface Equation (3)

$$\begin{aligned} \text{Ln no. adhered cells} = & 13.24 + 0.921 \times \text{time} \\ & + 0.587 \times \text{temperature} - 0.314 \times \text{zosteric acid} \\ & \text{concentration} - 0.227 \times \text{time} \times \text{time} + 0.371 \\ & \times \text{time} \times \text{temperature} - 0.242 \times \text{zosteric acid} \\ & \text{concentration} \times \text{temperature} \end{aligned} \quad (3)$$

#### *E. coli* hydrophilic surface Equation (4)

$$\begin{aligned} 1/\text{no. adhered cells} = & 1.39 \times 10^{-6} \\ & - 7.6 \times 10^{-7} \times \text{time} + 3.72 \times 10^{-7} \\ & \times \text{zosteric acid concentration} + \\ & 3.26 \times 10^{-7} \times \text{time} \times \text{pH} - 3.0 \times 10^{-7} \\ & \times \text{temperature} - 2.8 \times 10^{-7} \times \text{zosteric acid} \\ & \text{concentration} \times \text{temperature} + 2.61 \times 10^{-7} \\ & \times \text{temperature} \times \text{pH} - 2.3 \times 10^{-7} \times \text{zosteric} \\ & \text{acid} \times \text{time} - 2.2 \times 10^{-7} \times \text{pH} \end{aligned} \quad (4)$$

#### *E. coli* hydrophobic surface Equation (5)

$$\begin{aligned} 1/\text{no. adhered cells} = & 4.11 \times 10^{-6} + 2.17 \times 10^{-6} \\ & \times \text{zosteric acid concentration} - 1.9 \times 10^{-6} \times \\ & \text{time} - 1.7 \times 10^{-6} \times \text{zosteric acid concentration} \times \\ & \text{time} - 9.7 \times 10^{-7} \times \text{pH} - 1.2 \times 10^{-6} \times \text{zosteric acid} \\ & \text{concentration} \times \text{pH} - 8.9 \times 10^{-7} \times \\ & \text{temperature} + 1.64 \times 10^{-7} \times \text{time} \times \text{pH} \end{aligned} \quad (5)$$

#### *P. citrinum* hydrophobic surface Equation (6)

$$\begin{aligned} \text{Sqrt no. adhered cells} = & 228.06 - 33.98 \times \\ & \text{zosteric acid concentration} + 15.20 \times \\ & \text{temperature} - 11.54 \times \text{zosteric acid} \\ & \text{concentration} \times \text{temperature} \end{aligned} \quad (6)$$

#### *A. niger* hydrophobic surface Equation (7)

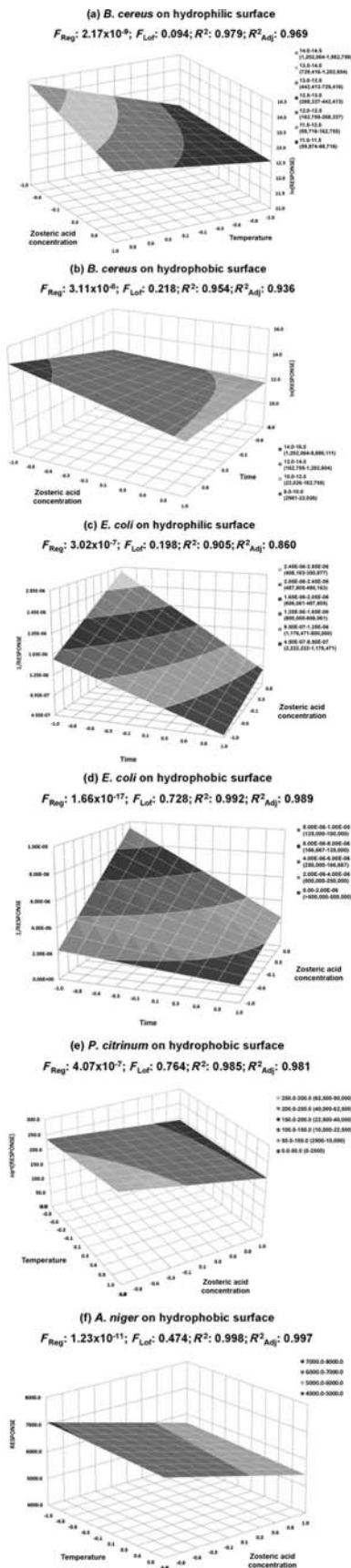
$$\begin{aligned} \text{No. adhered cells} = & 6254.6 - 714.17 \times \\ & \text{zosteric acid concentration} - 116 \times \text{temperature.} \end{aligned} \quad (7)$$

At a significance of 95%, only zosteric acid concentration and temperature influenced the fungal number of adhered cells. Both variables, together with contact time, influenced *B. cereus* adhesion. *E. coli*, whose behavior depends also on pH, showed the more complicated pattern. In all assays, zosteric acid inhibited cell adhesion while higher temperatures and longer exposure times promoted cell adhesion.

A significant reduction in bacterial coverage on both hydrophobic and hydrophilic surfaces was observed. In contrast, in all fungal experiments, zosteric acid reduced the number of attached conidia on the hydrophobic surface only.

The results supported the fitness of the polynomial models evaluated by ANOVA and calculations of regression coefficients. The lack of fit ( $\alpha > 0.05$ )





indicated that the selected models were adequate for describing the observed data. Regression coefficients and statistics for the fit are reported in Figure 2. A summary of the statistical results for each of the FCC final models is given in Table 1.

The effect of the main factors and interactions derived from FCC are summarized in Table 3. The response to surfaces shows the relation between the number of adhered cells and the influent variables (Figure 2). Validation experiments (Table 2) were carried out within the experimental domain that ensured the minimum response, which is in the optimum zone, according to the calculated equation for each model. Table 2 reports the predicted values with the model prediction error calculated as Root Mean Squared Error of Prediction (RMSEP) divided by the mean of the experimental responses. The results showed that the experimentally determined responses are not statistically different from the predicted ones, confirming the accuracy of the models.

#### *Zosteric acid did not affect cell-surface wettability*

Without zosteric acid, the water contact angles of bacterial lawns ranged between  $21.2^\circ \pm 5.3$  for *E. coli* and  $18.3^\circ \pm 2.8$  for *B. cereus*-group strain. With zosteric acid, the water contact angles of bacterial lawns ranged from  $25.8^\circ \pm 1.2$  (*E. coli*) to  $16.9^\circ \pm 1.9$  (*B. cereus*). Without zosteric acid, the mean contact angles were  $129.7^\circ \pm 6.5$  for *A. niger* and  $117.6^\circ \pm 2.2$  for *P. citrinum* conidia, whereas, in its presence, the mean contact angles were  $123.8^\circ \pm 2.1$  and  $120.3^\circ \pm 3.7$ . There were no significant differences in cell-surface wettability between treated and untreated samples ( $P_{E.coli}: 0.087$ ;  $P_{B.cereus}: 0.425$ ;  $P_{P.citrinum}: 0.330$ ;  $P_{A.niger}: 0.121$ ) suggesting that zosteric acid did not influence surface hydrophilicity.

#### *Induction of bacterial motility by zosteric acid*

The effect of zosteric acid on two flagella-driven motility types, swimming and swarming, was investigated. Zosteric acid did not affect swarming migration,

Figure 2. 3D surface plots showing the effect of zosteric acid on cell adhesion and its interaction with different variables. (a) *B. cereus* on hydrophilic surfaces; (b) *B. cereus* on hydrophobic surfaces; (c) *E. coli* on hydrophilic surfaces; (d) *E. coli* on hydrophobic surfaces; (e) *P. citrinum* on hydrophobic surfaces; (f) *A. niger* on hydrophobic surfaces. Ranges in the legend represent the numbers of adhered cells. Values used in the figure are mathematical transformation of these values. Levels of variables are shown in Table 1. The graphs report the regression coefficients and the statistic for the fit showing the adequacy of the models in describing the observed data.

Table 3. Linear and interaction effects of statistically significant variables on the number of adhered cells.

Variables	<i>B. cereus</i>		<i>E. coli</i>		<i>P. citrinum</i>		<i>A. niger</i>	
	Hb	HI	Hb	HI	Hb	HI	Hb	HI
Zosteric acid (A)	– (P: 3.1 × 10 <sup>-7</sup> )	– (P: 2.4 × 10 <sup>-6</sup> )	– (P: 5.1 × 10 <sup>-6</sup> )	– (P: 3.4 × 10 <sup>-4</sup> )	– (P: 4.9 × 10 <sup>-9</sup> )	– (P: 1.3 × 10 <sup>-7</sup> )	– (P: 1.3 × 10 <sup>-7</sup> )	– (P: 1.3 × 10 <sup>-7</sup> )
Time (B)	+ (P: 2.6 × 10 <sup>-5</sup> )	+ (P: 4.9 × 10 <sup>-10</sup> )	+ (P: 1.3 × 10 <sup>-6</sup> )	+ (P: 5.8 × 10 <sup>-8</sup> )	n.s.	n.s.	n.s.	n.s.
Temperature (C)	+ (P: 4.8 × 10 <sup>-6</sup> )	+ (P: 8.3 × 10 <sup>-8</sup> )	+ (P: 1.2 × 10 <sup>-3</sup> )	+ (P: 2.2 × 10 <sup>-3</sup> )	+ (P: 6.0 × 10 <sup>-7</sup> )	+ (P: 1.1 × 10 <sup>-2</sup> )	+ (P: 1.1 × 10 <sup>-2</sup> )	+ (P: 1.1 × 10 <sup>-2</sup> )
pH (D)	n.s.	n.s.	+ (P: 2.1 × 10 <sup>-3</sup> )	+ (P: 1.9 × 10 <sup>-2</sup> )	n.s.	n.s.	n.s.	n.s.
Interactions								
Zosteric acid × Time (A × B)	– (P: 1.5 × 10 <sup>-4</sup> )	n.s.	+ (P: 5.6 × 10 <sup>-4</sup> )	+ (P: 1.9 × 10 <sup>-2</sup> )	– (P: 1.0 × 10 <sup>-5</sup> )	n.s.	n.s.	n.s.
Zosteric acid × Temperature (A × C)	n.s.	– (P: 1.2 × 10 <sup>-3</sup> )	n.s.	+ (P: 5.7 × 10 <sup>-3</sup> )	n.s.	n.s.	n.s.	n.s.
Zosteric acid × pH (A × D)	n.s.	n.s.	+ (P: 4.9 × 10 <sup>-3</sup> )	n.s.	n.s.	n.s.	n.s.	n.s.
Time × Temperature (B × C)	+ (P: 5.2 × 10 <sup>-6</sup> )	+ (P: 3.1 × 10 <sup>-5</sup> )	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Time × pH (B × D)	n.s.	n.s.	n.s.	– (P: 1.9 × 10 <sup>-3</sup> )	n.s.	n.s.	n.s.	n.s.
Temperature × pH (C × D)	n.s.	n.s.	n.s.	– (P: 9.0 × 10 <sup>-3</sup> )	n.s.	n.s.	n.s.	n.s.
Time × Temperature × pH (B × C × D)	n.s.	–	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

–, inhibition of the adhesion process; +, promotion of the adhesion process; n.s., non significant; HI, hydrophilic surface; Hb, hydrophobic surface.

but it promoted swimming motility in a dose-dependent manner (Figure 3a). The magnitude of bacterial swimming on plates containing zosteric acid remained constant at concentrations ranging from 300 to 500 mg l<sup>-1</sup> (Figure 3b). The swimming movement was increased by 40%.

**Chemotaxis of *E. coli* was enhanced by zosteric acid**

Using the capillary accumulation assay, zosteric acid was found to be neither a chemorepellent nor a chemoattractant as it showed the same response as the chemotaxis buffer control (Figure 4a). A second set of

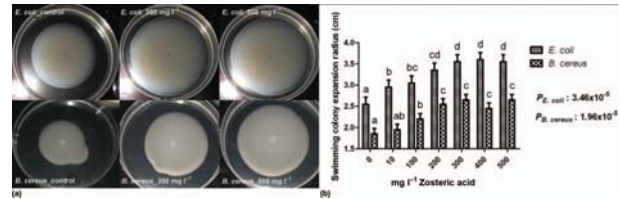


Figure 3. Swimming motility of bacteria induced by zosteric acid. (a) motility of *E. coli* (first row) and *B. cereus* (second row) on swimming plates without and with zosteric acid and incubated for 24 h at 30°C; (b) swimming colony expansion radius vs zosteric acid concentration. The graph provides the *P*-values obtained by ANOVA analysis (*P* < 0.05). Error bars display the HSD intervals using Tukey multiple comparison with a 95% confidence level (Tukey’s HSD, *P* < 0.05). According to *post hoc* analysis, means sharing the same letter are not significantly different from each other. The statistical analysis was performed separately for each microorganism.

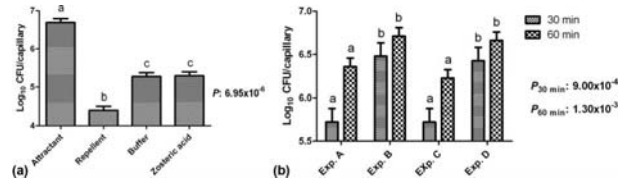


Figure 4. Capillary accumulation assays. (a) Chemotactic response of *E. coli* towards zosteric acid; (b) chemotactic response of *E. coli* to the chemoattractant aspartate in combination with zosteric acid. The experiments were performed as follows: (Exp. A) aspartate in the capillary and bacterial cells grown without zosteric acid; (Exp. B) aspartate in the capillary and bacterial cells grown with zosteric acid; (Exp. C) aspartate and zosteric acid in the capillary and bacterial cells grown without zosteric acid; (Exp. D) aspartate and zosteric acid in the capillary and bacterial cells grown with zosteric acid. The graph provides the *P*-values obtained by ANOVA analysis (*P* < 0.05). Error bars display the HSD intervals using Tukey multiple comparison with a 95% confidence level (Tukey’s HSD, *P* < 0.05). According to *post hoc* analysis, means sharing the same letter are not significantly different from each other. The statistical analysis was performed separately for each time.

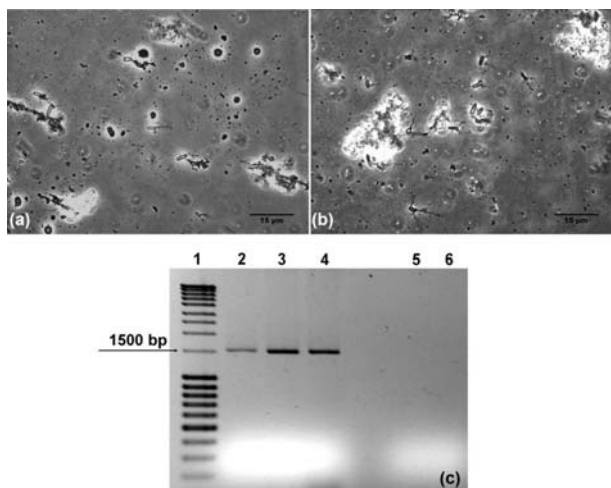


Figure 5. (a, b) Visualization of flagella in *E. coli* cells; (c) analysis of expression of *fliC* gene by RT-PCR in *E. coli* cells. Flagella were observed by phase-contrast microscopy after staining cells grown on swimming agar plates (a) without and (b) with 500 mg l<sup>-1</sup> zosteric acid. (c) Transcription level of *fliC* gene in *E. coli* cells grown in the absence (lane 2) or presence (lane 1) of 500 mg l<sup>-1</sup> zosteric acid detected by semi-quantitative RT-PCR. Lane 1, molecular size standard; lane 4, PCR positive control containing all the PCR reagents and genomic DNA template; lane 5, DNase control containing all the PCR reagents and RNA template; lane 6, PCR negative control containing all the PCR reagents and no template.

experiments was carried out to investigate the effect of zosteric acid on aspartate taxis. Figure 4b shows a greater *E. coli* chemotactic response toward the attractant aspartate in both the experiments B and D. The number of cells migrating into the capillary tube increased when bacteria were grown with 500 mg l<sup>-1</sup> zosteric acid prior to the assay, implying an effect on cell motility. However, the effect of zosteric acid did not increase proportionally to the exposure time, suggesting a saturation effect in the capillary at 60 min. After 60 min, zosteric acid enhanced the number of cells migrating into the capillary tube by 55%.

#### Zosteric acid affected the number of flagella

Phenotypic analysis of *E. coli* cells grown on swimming agar plates supplemented with 500 mg l<sup>-1</sup> zosteric acid revealed a substantial increase in the number of flagella in comparison to the control (Figure 5a). The altered ability of zosteric acid-treated cells to produce flagellar filaments was also confirmed at both transcriptional and proteomic level.

The sequences of the amplified RT-PCR products showed 100% homology with the *fliC* gene encoding flagellin. Semi-quantitative RT-PCR analysis showed that the transcription of *fliC* gene increased in the

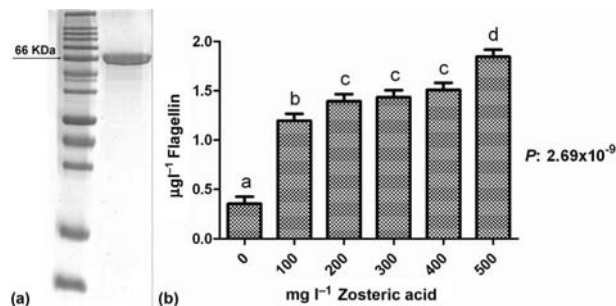


Figure 6. Effect of zosteric acid on the amount of flagellin in *E. coli*. (a) SDS-PAGE analysis of flagellin shows the adequacy of the extraction protocol (arrow indicates the band at 66 kDa); (b) results of protein concentration in the flagellin extract quantification by the Bradford method, assumed to be flagellin, at different zosteric acid concentrations. Statistical analysis shows that the amount of flagellin increased five-fold with 500 mg l<sup>-1</sup> zosteric acid. The graph provides the P-value obtained by ANOVA analysis ( $P < 0.05$ ). Error bars display the HSD interval using Tukey multiple comparison with a 95% confidence level (Tukey's HSD,  $P < 0.05$ ). According to post hoc analysis, means sharing the same letter are not significantly different from each other.

zosteric acid-treated samples compared to the control (Figure 5b).

The weight of the SDS-PAGE *E. coli* putative flagellin protein band was assessed by comparison to the molecular mass standard. Purified flagellin protein *FliC* from *E. coli* corresponded to the 65 kDa band as reported by several authors (Sharma et al. 2006; Khan et al. 2008). The flagellar preparation showed a single visible protein band with molecular mass approximately of 65 kDa, suggesting the adequacy of the extraction protocol (Figure 6a). The results from flagellin quantification by the Bradford method are reported in Figure 6b. The amount of flagellin increased with increasing zosteric acid concentration.

#### Zosteric acid affects biofilm formation

The fluorescent staining procedure demonstrated the presence of EPS and thus the formation of a biofilm. The same level of ConA binding was observed when bacterial biofilms were grown in the presence of zosteric acid suggesting that the anti-adhesion compound did not affect the EPS matrix. After 48 h of growth as a colony biofilm, a considerable decrease in biofilm formation was observed in the presence of zosteric acid. Treatment with zosteric acid produced biofilms containing  $4.66 \times 10^{10} \pm 7.92 \times 10^9$  CFU membrane<sup>-1</sup> (control  $5.79 \times 10^{11} \pm 5.80 \times 10^{10}$  CFU membrane<sup>-1</sup>), leading to a cell reduction of 92%. Cryosectioning of biofilm combined with microscopy observations revealed that *E. coli* biofilms

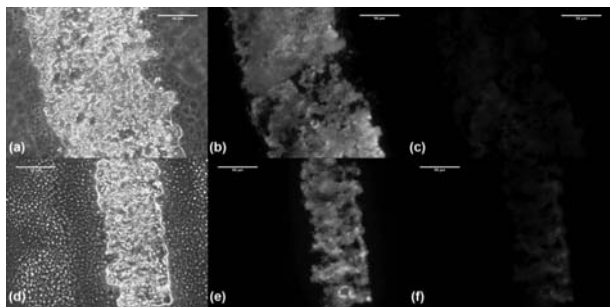


Figure 7. Images of cryosectioned *E. coli* biofilms grown without (first row) and with (second row) zosteric acid. The same area is shown in phase contrast view (a, d) and epifluorescent microscope field with DAPI filter cube (b, e) to visualize cells and Texas Red filter cube (c, f) to visualize biofilm matrix stained with Alexa fluor 488-labelled Concanavalin A.

formed in the presence of zosteric acid (thickness  $62 \pm 10 \mu\text{m}$ ) were significantly less thick than those formed without zosteric acid (thickness  $137 \pm 24 \mu\text{m}$ ). The yield reduction in biofilm thickness was by 54%. Zosteric acid-treated biofilms retained similar morphological patterns to those observed in the control (Figure 7).

## Discussion

In the past, zosteric acid was isolated from *Zostera marina* through extraction with methanol, followed by a cumbersome purification procedure yielding 66 mg of pure compound from 1700 g dry biomass (Zimmerman 1995, US Patent 5384176). Later, zosteric acid was prepared by treating *trans*-4-hydroxycinnamic acid with chlorosulfonic acid in pyridine (Alexandratos 1999, US Patent 5990336). Although this procedure enables a scaled-up synthesis of zosteric acid, thus avoiding the costly and time consuming extraction from a natural source, it has limitations. The reaction was carried out in pyridine in the presence of chlorosulfonic acid, a corrosive liquid that reacts violently with water. Moreover, only a 50% yield of the pure compound was achieved after a tedious purification involving elution through ionic exchange resins followed by water evaporation. The new procedure proposed here provides the required amount of zosteric acid and promises to be easily scaled up and eliminates harvesting of the natural source material.

In the study reported here, zosteric acid did not affect bacterial or fungal growth at the concentrations tested. In addition, it did not represent a carbon and energy source at concentrations  $\leq 1000 \text{ mg l}^{-1}$ . Interestingly, both fungal species were able to use zosteric acid as a nutrient at  $25 \text{ g l}^{-1}$ , a concentration much higher than those evaluated in other assays.

DoE was used to investigate the response to zosteric acid when changes in several environmental factors are considered at the same time, a situation that better simulates *in vivo* conditions. Indeed, since many parameters govern cell adhesion, the evaluation of their relative contribution and interaction is impossible using the classical approach of 'one variable at time'. The DoE strategy was also used to study interactions among factors and to identify the optimal conditions needed to reduce the number of adhered cells by carrying out the minimal number of experiments. It was also used to study variability of the system under investigation. In addition, the polynomial equations obtained can be used to predict the performance of zosteric acid in different environmental conditions within the chosen domain.

Although there are several papers suggesting that the colonization process is significantly affected by many parameters like the substratum, the microorganisms involved and environmental factors (Holder and Keyhani 2005; Boks et al. 2008), none of these authors used the mathematical tool DoE for process optimization. The mathematical models described here clearly showed that, although zosteric acid exhibited a species-specific behavior, it always decreased bacterial and fungal adhesion, thus displaying potential as a broad range antifoulant. In all the investigations performed, zosteric acid appeared to be more effective in deterring microbial adhesion on the hydrophobic rather than the hydrophilic surface. In particular, the molecule did not affect fungal spore adhesion on the hydrophilic surface. Preferences for hydrophobic surfaces have been extensively observed for spores of several plant pathogens including *Botrytis cinerea* and *Penicillium expansum* (Doss et al. 1993; Amiri et al. 2005). This is not surprising as the aerial surfaces of the plants are hydrophobic (Koch et al. 2009). The use of sublethal concentrations of zosteric acid proved to be one of the most important factors affecting the number of adhered cells of bacteria and fungi. The estimated equation showed that  $50 \text{ mg l}^{-1}$  of zosteric acid ensured a reduction of bacterial coverage on the hydrophobic surface by 35% and 49% for *B. cereus* and *E. coli*, respectively. The number of adhered cells decreased with an increase in zosteric acid concentration. Thus,  $300 \text{ mg l}^{-1}$  zosteric acid reduced cell adhesion on the hydrophobic surface by  $\sim 60\%$  and  $84\%$ , for *E. coli* and *B. cereus*, respectively. Interestingly,  $500 \text{ mg l}^{-1}$  of zosteric acid ensured a percentage reduction of bacterial adhesion of  $>90\%$ . In contrast, the effective concentration for fungi was  $1000 \text{ mg l}^{-1}$  which led to a maximum reduction of 57%. The fungal regression models predicted that  $50 \text{ mg l}^{-1}$  of zosteric acid would reduce spore

adhesion on hydrophobic surface by about 3%. The experimental results were in agreement with those of Xu et al. (2005a), who observed a reduction in bacterial biofilm coverage by 98.2% at 500 mg l<sup>-1</sup> of zosteric acid. Likewise, Stanley et al. (2002) showed that in the presence of 1000 mg l<sup>-1</sup> of zosteric acid, adhesion of *Colletotricum lindemuthianum* on leaf surfaces was reduced by 40%.

In this study, temperature alone was another important variable positively affecting cell adhesion in both the bacterial and fungal models. Toren et al. (1998) observed that elevated seawater temperatures caused coral bleaching, promoting the adhesion of *Vibrio* strain AK-1. The adhesion of *Pseudomonas putida* to cellulose fibers increased rapidly with an increase in temperature (Rochex et al. 2004), as well as the capability of *Listeria monocytogenes* to colonize austenitic stainless steel (Mai and Conner 2007). Although changes in temperature alone promoted surface colonization, in most cases its interaction with zosteric acid exerted a significant negative synergistic effect. The mathematical investigation revealed that zosteric acid successfully counteracted the positive effects of some environmental variables promoting surface colonization, such as temperature, time and pH, and that agitation did not significantly affect cell adhesion. Interestingly, Zilm and Rogers (2007) reported that a rise in pH increased the ability of *Fusobacterium nucleatum* to colonize and infect gingival epithelial cells.

As there were no obvious differences in the wettability of either the bacterial or the fungal cells with or without the antifoulant, other explanations of the mechanism of action of zosteric acid were investigated, specifically its role on the bacterial flagellar-driving movements. Zosteric acid stimulated cell movement in liquid medium in a dose-dependent manner, increasing swimming by 40%. In contrast, zosteric acid did not influence swarming motility, suggesting that it interferes with adhesion rather than blocking cell-to-cell communication. Kovacicova et al. (2005) observed that the substantial increased motility in *Vibrio cholerae* by overexpression of protein AcgA reduced its ability to form biofilm. In addition, Ren et al. (2005) showed that the non-toxic plant extract ursolic acid inhibited *E. coli* biofilm formation by up-regulating chemotaxis and motility genes. Zosteric acid was neither a chemoattractant nor a chemorepellent, suggesting that it probably did not act at the level of motor rotation for *E. coli*. The capillary assay with aspartate corroborated the results of the swim plate assay, showing a strong response to the chemoattractant when bacterial cells were grown with zosteric acid.

The hyper-motile phenotype observed in the zosteric acid-treated samples, prompted the question whether the antifoulant controlled the degree of flagellation in the swim cell-state. Using flagellar staining, an increased

number of flagella in *E. coli* cells grown in the presence of 500 mg l<sup>-1</sup> of zosteric acid was observed. Motility and production of flagellin are strongly linked (Smith et al. 2003; Karlsen et al. 2008). The increased transcriptional level of the motility gene *flhC* supported this finding, as well as the increased amount of flagellar proteins. Likewise, Fujinami et al. (2007) observed that a *Bacillus* species with a hyper-motile phenotype exhibited higher flagellin levels. These results suggest that sub-lethal concentrations of zosteric acid negatively impacted *E. coli* cell adhesion making the cells too motile for normal biofilm formation.

To further investigate the effect of zosteric acid on biofilm formation, the membrane-supporting biofilm reactor was used to form *E. coli* biofilm. This technique permitted cells to be forced to attach to a surface, a feature that allowed direct investigation of the effect of zosteric acid on the structural development and organization of the biofilm, whilst bypassing the effect on the adhesion phase. Although within 48 h a mature biofilm structure was observed in the presence of zosteric acid, biofilm development was retarded with a significant decrease in biomass and mean thickness. These findings support the results obtained from the mathematical models that revealed the ability of zosteric acid to slow down surface colonization. The increase in motility contributes to the understanding of the mechanism of action of zosteric acid. Zosteric acid is a promising broad spectrum antifoulant.

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