Optimizing the production of the biosurfactant lichenysin and its application in biofilm control

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Running title: Lichenisyn and biofilm control

Abstract

Aims: Apply Response Surface Methodology (RSM) to develop and optimize an economical medium for lichenysin production, which is a surfactant produced by *Bacillus licheniformis* and evaluate the application of lichenysin in the prevention and disruption of pathogenic microorganism biofilm that creates health problems in the food industry and hospitals.

Results: An economical medium containing molasses was optimized to enhance lichenysin production by response surface methodology (RSM). A production of 3.2 g l^{-1} of lichenysin was achieved with an optimum medium containing 107.82 g l^{-1} of molasses, 6.47 g l^{-1} of $NaNO_3$ and 9.7 g l^{-1} of K_2HPO_4/KH_2PO_4, in which molasses and phosphate salts had a significant effect on biosurfactant production. Lichenysin was effectively applied in a surface pre-treatment to avoid microbial biofilm development of MRSA (68.73 %) and *Candida albicans* (74.35 %), with ED_{50} values of 8.3 and 17.2 µg ml^{-1}, respectively. It was also very efficient in a surface post-treatment to remove biofilms of MRSA (55.74 %) and *Yersinia enterecolitica* (51.51 %), with an ED_{50} of 2.79 and 4.09 µg ml^{-1}, respectively.

Conclusions: Lichenysin was found to have notable anti-adhesion activity, being able to prevent and eliminate the biofilm formation by pathogenic strains associated with foodborne illness. This new medium resulted in a four-fold increase in production compared with the non-optimized medium.

Significance and Impact of Study: Molasses can be regarded as a useful resource for biotechnological applications, such as the production of lichenysin. The use of agro-industrial substrates has an important role in the sustainable and competitive development of several industrial sectors, as well as in industrial residues management. Additionally, lichenysin is particularly effective in preventing biofilm formation by strains problematic for the food industry and in the hospital environment. Lichenysin also efficiently disrupts biofilm.

Keywords: *Bacillus licheniformis*, lichenysin, biosurfactant, molasses, production optimization, response surface methodology, adhesion, biofilms.

Introduction

Microorganisms have the ability to adhere to and grow on surfaces (e.g. stainless steel, polypropylene, rubber, wood) and develop ecosystems called biofilms. This may be a cause of contamination in different industries, since wet surfaces can provide a solid substrate for bacterial growth and persistence (Bridier *et al.* 2014). For example, pathogenic microorganisms such as *Escherichia coli*, *Shigella* spp. and *Staphylococcus aureus* have been detected in biofilm development in the dairy and egg processing industries (Sharma and Anand 2002; Shi and Zhu 2009). Biofilms may cause biodeterioration of materials and generation of diseases with serious clinical consequences. *Listeria monocytogenes* affects safety in the food industry and may cause a disease called listeriosis with a considerable mortality rate in sensitive groups (De Araujo *et al.* 2011). Hospitals are another problematic environment, where patients are at risk from *Candida albicans,* recognized as a major agent of hospital-acquired infection, and its emergence as an important nosocomial pathogen is related to its capacity to form biofilms on medical equipment (e.g. catheters) (Douglas 2003).

Pathogen implantation on industrial and medical equipment or products has been generally controlled by cleaning and disinfection procedures (Jahid and Ha 2012), but microorganisms possess a certain degree of resistance to the chemical-based products used (Srey *et al.* 2013). Therefore, new approaches to control biofilm formation have been introduced. Bridier *et al.* (2014) reviewed potential green strategies that include essential oils, bacteriophages, enzymes, biocides and biosurfactants (BS) to avoid biofilm formation. BS may be considered as environmentally friendly cleaning

products, because of their natural origin and relatively easy preparation and usability (Cameotra and Makkar 2004).

Due to their potential application in processes in food and biomedical industries, or in the environment, BS have attracted much attention in the scientific community and have been the focus of recent biotechnology research. BS are able to modify bacterial surface hydrophobicity and, consequently, microbial adhesion to solid surfaces. Their effect depends on the initial bacterial hydrophobicity as well on the BS type and concentration (Ahimou *et al.* 2000).

The BS surfactin, lichenysin, iturin and fengycin are lipopeptides that exhibit powerful biological effects due to their exceptional surface activity (Ongena and Jacques 2008). In a previous study, our group reported that *Bacillus licheniformis* AL 1.1 produces a mixture of lichenysin homologous with a molecular weight between 1006 and 1034 m/z. Its peptidic part is composed of glutamine as the N-terminal, and two leucines, valine, aspartic acid, leucine and isoleucine as the C-terminal. The lipid moiety contains a mixture of *β-hydroxy* fatty acids ranging in size from C_{14} - C_{16} (Coronel-León *et al.* 2015).

A strategy proposed to improve the BS production process is the use of agro-industrial wastes, which reduces the initial costs of raw materials (Mukherjee *et al.* 2006). Various agro-industrial substrates, such as waste frying oils (Haba *et al.* 2000), peanut oil cake (Thavasi *et al.* 2007), molasses (Saimmai *et al.* 2011), whey (Joshi *et al.* 2008a), okara with sugarcane bagasse (Slivinski *et al.* 2012) and rice straw (Zhu *et al.* 2013a), have been studied for BS production at the laboratory scale. Another important issue is the optimization of the culture medium. Response surface methodology (RSM) is one of the most efficient optimization strategies; based on a set of mathematical and statistical techniques, its main objective is to determine the optimal operating conditions for a system. Thus, by the modeling and analysis of a problem in which a response of interest is determined by several variables, the response variable can be optimized (Rodríguez-Carmona *et al.* 2011).

This work has two aims: firstly, to use RSM to develop and optimize an economical medium for lichenysin production based on an agro-industrial substrate, and secondly, to evaluate the application of lichenysin in the prevention and disruption of pathogenic microorganism biofilm that creates health problems in the food industry and hospitals.

Materials and methods

Microorganisms

Bacillus licheniformis AL1.1 was isolated from sediment samples from Deception Island of the Antarctic continent (Llarch *et al.* 1997). The strain was subcultured fortnightly on tryptone soy agar plates (TSA, Pronadisa, Barcelona, Spain), incubated for 24 h at 30 ºC and kept at 4 ºC. The strain was preserved frozen in cryovials (EAS laboratories, France) at -80 ºC in the culture collection of the Microbiology Unit, University of Barcelona, Spain.

Optimization of culture medium

Agro-industrial substrates for lichenysin production.

For the initial studies: molasses, cassava wastewater, cassava starch and whey were evaluated as a carbon source. The mineral medium (MM) used in the screening phase contained (g l^{-1}): carbon source, 20; $NaNO_3$, 4; KH_2PO_4/Na_2HPO_4, 9.7 (1:1); $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.21; $CaCl_2$ $7x10^{-6}$; trace element solution, 0.05 ml^{-1}; final pH 7. The mineral components and carbon source were autoclaved separately (121 ºC for 20 min).

The medium used for lichenysin ($Lch_{AL1.1}$) production optimization was a basal medium containing: molasses, $NaNO_3$ and K_2HPO_4/KH_2PO_4 (1:1). They were supplied as indicated in the experimental design shown in Tables 1 and 2. The carbon source and the salt solutions were sterilized separately at 121 ºC for 20 min, cooled and aseptically reconstituted at room temperature prior to use. The final pH was adjusted to 7 using 0.1 mol l^{-1} NaOH or 0.1 mol l^{-1} HCl. Since nitrogen, phosphorus and carbon are essential nutrients in the medium that influence the growth and accumulation of $Lch_{AL1.1}$, we optimized the concentration of their sources using RSM.

Molasses were obtained from Molasses Tababuela, a local company in Santa Rosa city, province of El Oro, Ecuador. This substrate had the following composition, according to the supplier: total sugar 53.73%, sucrose 38%, organic nitrogen 1% and micro minerals (mg/100 g molasses): calcium 850, magnesium 700, iron 8 and sodium 23. All the chemicals used were of analytical grade. Panreac (Barcelona, Spain) supplied chemical products, all of which were of ACS quality, and ADSA (ADSA, Barcelona, Spain).

Inoculum preparation, flask shaking experiments and cultivation conditions

Five hundred milliliter baffled Erlenmeyer flasks containing 50 ml of the corresponding medium (MM or different medium from the experimental design) were inoculated with a 2% cell suspension in sterile saline from an overnight culture incubated at 30 ºC on TSA; the bacterial suspension was adjusted by turbidimetry (A_{540}=2.00) in a UVIKON 922 spectrophotometer (Shimadzu, Japan). Incubation was carried out in an orbital shaker at 150 rpm at 30 ºC for 72 h. All the experiments were carried out in triplicate

Experimental design

In this study, response surface methodology (RSM) was applied using a central composite rotatable design (CCRD) to study the behavior in the optimum region of Y_1 = lichenysin production (g l^{-1}), considering the follow components: (x_1) carbon source, (x_2) NO_3^- source and (x_3) PO_4^{3-}. A CCRD was formed by a factorial design 2^K, where k= 3 (k is the number of selected factors), which resulted in a CCRD with 23 experiments, each one evaluated at five different levels (-1.68, -1.0, +1, +1.68). Table 1 shows the rank and levels of the independent variables as the real values that were investigated in this study. Twenty-three experiments were performed in triplicate.

The response variable was described as a function of the independent variables (x_1, x_2, x_3). As describing the experimental data for the curvature of the system was really important in this study, a second-order polynomial model was used:

Equation 1

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \sum \beta_{ij} x_i x_j$$

Where Y = dependent or response variable, β_o = constant or independent term, x_i are coded levels or independent variables x_i (i = 1–3), β_i= coefficient of linear effects, β_{ii} = coefficient of quadratic effects, β_{ij} = coefficient for interaction effects (i y j = 1–3).

The Essential Regression (ER) program (a Microsoft Excel macro of free diffusion available in http://www.jowerner.homepage.t-online.de/download.htm) was used to obtain the regression models and conduct the analysis of significance. The ER program

includes a tool called Optimize that allows us to determine the maximum and minimum values of a function using the Solver macro. This tool was able to determine the optimal concentrations of molasses (X_1), NO_3^- (X_2) and PO_4^{3-} (X_3) within the concentration ranges used in the experiments.

Finally, the optimum conditions predicted were validated by results obtained under specific experimental conditions. Ten new experiments were conducted under conditions different from those used to obtain the regression equation. In this case all the experiments were selected so that they were uniformly distributed over all possible values of responses from high to low. For each experimental value, three mutually independent determinations were performed from three separate experiments, which were carried out with different inocula under the same working conditions as for the points included in the matrix. Additionally, the kinetics of production of $Lch_{AL1.1}$ were measured to understand the behaviour of AL 1.1.

Analytical methods

Lichenysin determination

Cell-free supernatant was subjected to acid precipitation using concentrated hydrochloric acid (HCl) until pH 2, and left overnight at 4 ºC. The crude lichenysin ($Lch_{AL1.1}$) was collected by centrifugation (11,000 x g, 4 °C for 20 min), and washed twice with acid distilled water (pH 2) to eliminate any impurities. The $Lch_{AL1.1,}$ dried in an oven at 40 °C until constant weight, was quantified by gravimetry (g l^{-1}). The purified lichenysin was recovered from the crude lichenysin after three extractions with an ethyl acetate-methanol mixture 8:1 (v/v). The organic phases were combined, passed over anhydrous sodium sulfate and concentrated in a rotary vacuum evaporator (Büchi, Switzerland) and weighed.

Biomass determination

Cell dry weight (CDW) was determined gravimetrically (g l^{-1}): a 10 ml aliquot of the 72 h culture was centrifuged at 11,000 x g in a Beckman Coulter centrifuge for 20 min at 4 °C, the pellet was suspended and washed three times with deionized water. Finally, the pellet was placed in a pre-weighed vial, and dried to a constant weight at 100 °C; all measurements were made in triplicate. Biomass is expressed as g l^{-1} of CDW.

Finally, $NaNO_3$ was determined using Quantofix Nitrate strips (Mackerel-Nagel, Düren, Germany), and total carbohydrates were determined by the phenol-sulfuric acid method, using glucose as the standard. (DuBois et al. 1956).

Anti-adhesion assay on polystyrene surface

The effect of surface pre-treatment and post-treatment with purified lichenysin on microbial adhesion was tested using a modified O'Toole (2011) method. Briefly, for surface pre-treatment, purified lichenysin was dissolved in PBS (pH 7.2). Next, the wells of a microtiter plate were filled with 200 µł of several purified lichenysin concentrations (4000 to 0 µg ml^{-1}), incubated for 6 h at room temperature (25ºC), and washed twice with PBS. Control wells contained only PBS or lichenysin solution.

For biofilm formation, *E. coli* O157:H7 CECT 4267, *Y. enterocolitica* ATCC 9610, *L. monocytogenes* ATCC 15313, methicillin-resistant *Staphylococcos aureus* (MRSA) ATCC 43300, and *Candida albicans* ATCC 10231 were cultured overnight in Muller Hinton Broth (MHB). A 1:100 dilution in the medium proposed by O'Toole (2011) (g/l): glucose, 2; casamino acids, 5; KH_2PO_4, 3; K_2HPO_4, 7; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.12, was used to fill microtiter plate wells (200 µL), which were incubated for 20 h at 37ºC (O'Toole, 2011). *Campylobacter jejuni* ATCC 33292 was grown in MHB supplemented with serum albumin (10%) under microaerophilic conditions.

Wells were washed tree times with distilled water, fixed for 15 min with methanol, and stained for 20 min with crystal violet (1%). After washing with water and drying, the stain in the wells was diluted with 200 µl of acetic acid (33%), and the absorbance was determined at 595 nm. Percentages of microbial adhesion inhibition were calculated using the formula: percentage adhesion of inhibition = $[1 - (A_c / A_O)] \times 100$, where A_c represents the absorbance of the well with lichenysin at concentration c and A_o the absorbance of the control well (absence of lichenysin). All the results were represented as the average of three independent experiments.

For the post-treatment with purified lichenysin, first the wells of a 96-well *polystyrene* microtiter plate were incubated for 20 h at 37 ºC with 200 µl of bacterial suspension prepared as mentioned above. After incubation, the unattached microbial

cells were removed by washing three times with distilled water. Next, 200 µl of 4000 to 2 µg ml^{-1} lichenysin were added to each well and incubated at 25°C for 6 h. The quantification was carried out as in the pre-treatment.

Experimental data were fitted to a log logistic model (y = d / 1 + exp (*b*log (x) - log (e))), with three parameters, where d is upper asymptote (maximal inhibition adhesion); b is the slope in the inflexion point of the curve and e is the effective dose 50 (ED_{50}).

3. Results

B. licheniformis AL 1.1 synthesizes lichenysin, a lipopeptide with high surface activity, capable of lowering the surface tension to 29.7 mN m^{-1}, with a cmc of 15 mg l^{-1}, and remaining stable under a variety of extreme environmental conditions (Tº, pH, NaCl) (Coronel-León *et al.* 2015). These properties suggest potential applications for lichenysin in food or sanitary industries, but to make it a competitive product and promote its use, its production needs to be economically viable. Strain AL1.1 achieved a good production of $Lch_{AL1.1}$ (Fig. 1) with molasses (0.73 g l^{-1}), considerably better than cassava starch (0.32 g l^{-1}), which might be an interesting alternative to explore in future studies. The other substrates (cassava wastewater and whey) were not suitable for $Lch_{AL1.1}$ production. For this reason, using RSM, we first optimized lichenysin production in a medium containing molasses, an agro-industrial substrate. The effect of the concentration of three variables (carbon source, nitrate and phosphates) on $Lch_{AL1.1}$ production was studied.

Optimization: ANOVA, regression and prediction equations

The CCRD results shown in Table 2 were fitted to two second-order polynomial equations with three independent variables $x_{i-j}; x_1 - x_3$. The following second-order polynomial regression model for $Lch_{AL1.1}$ production (*Y*) (Eq. 2) was obtained:

Equation 2

$$Y_1 = 3.14 - 0.78x_1^2 - 0.50x_3^2 - 0.45x_2^2 + 0.33x_3 + 0.13x_1 - 0.17x_1x_3 - 0.16x_1x_2$$

To check the statistical significance of the second-order model equations, an F-test (ANOVA) was used (Table 3), with no significant lack of fit of the regression models. The result of Fisher's F test for the regression model was highly significant ($p<0.05$). The R^2 calculated for the $Lch_{AL1.1}$ polynomial model was 0.973, indicating that 97% of the variability in the responses could be explained by the second-order polynomial prediction equations given above (Eq. 2). Consequently, the ANOVA results demonstrate the suitability of the model.

RSM allowed us to perform a mathematical analysis of the model shown in Equation 2, which consists of seven terms plus the intercept. The term with the most influence on the production of $Lch_{AL1.1}$ was x_3, corresponding to the PO_4^{3-} concentration, with a regression coefficient β_3= 0.331, followed by the term x_1, corresponding to the molasses concentration, for which β_1= 0.136. Interestingly, nitrate concentration had very little influence within the ranges studied. This is reflected in the fact that x_2 does not appear in the regression equation. With respect to the quadratic terms of molasses, NO_3^- and PO_4^{3-}, they were adjusted to a curve line, with the difference in the values of the slope, so that x_1^2 is in the first position of the regression equation and has a value β_{11}=-0.786, followed by β_{33} =-0.507. Finally, although x_2 has no significant effect on the production of lichenysin, its quadratic term x_2^2 appears in third position in the regression equation with a slope value lower than that of the quadratic terms corresponding to the concentration of molasses and phosphates.

According to the coefficients of the interaction terms, the effect of molasses concentration on production depends greatly on the phosphate concentration and to a lesser extent on nitrate concentration; the terms x_1x_3 and x_1x_2 appear, respectively, in sixth and seventh place in the regression equation with similar regression coefficients (β_{13}=-0.176 and β_{12}=-0.166).

Based on the regression equation (Eq. 2), the optimum conditions for $Lch_{AL1.1}$ production in coded unit were x_1 = 0.0515 for the molasses, x_2 = -0.009 for nitrate, and x_3 = 0.318 for phosphate, with their natural values: 107.82 g molasses l^{-1}, 6.47 g NO_3^- l^{-1}, and 9.7 g PO_4^{3-} l^{-1}. The predicted maximum production of $Lch_{AL1.1}$ corresponding to these values was 3.19 g l^{-1} after 72 h of growth, fourfold higher than that (0.73 g l^{-1}) obtained with the initial non-optimized medium.

To obtain additional information, surface plots based on the models were obtained as a function of two variables at a time (varied within the experimental ranges), holding the third variable constant at its optimum level. Figure 2 shows the effect of each of the variables on $Lch_{AL1.1}$ production when set at their optimal levels. At the optimum concentration of molasses x_1= 0.0515 (Figure 2a), it is clear that maximum $Lch_{AL1.1}$ production (3.19 g l^{-1}) was favored when both nitrate concentration (x_2=-0.009) and phosphate concentration (x_3= 0.318) were near their central values. In Figures 2b and 2c, the optimal nitrate and phosphate concentrations are kept constant and in both cases production of $Lch_{AL1.1}$ was favored at central values of both molasses concentration (x_1=0.0515) and phosphate concentration (x_3= 0.318), leading to the maximum value of 3.19 g l-1 of $Lch_{AL1.1.}$ The polynomial regression model (Eq. 2) indicates significant interactions ($x_1\ x_2, x_1\ x_3$), but the response surface graphs do not show these as clearly. The optimum values for $Lch_{AL1.1}$ production are close to the central point, as shown in the response surface (Figure 2); this indicates that the ranges selected are appropriate for the study.

To validate if the polynomial equation correctly describes the response function, a tool in the program Essential Regression (ER), which provides theoretical values of responses with a confidence interval of 95%, was applied. Figure 3 plots the theoretical and experimental values for the production of $Lch_{AL1.1}$ (Y_1). The degree of fit achieved between experimental and theoretical values $Y_{i\ theoretical} = Y_{i\ experimental}$ had a correlation coefficient of R^2= 0.9939. These slopes are relatively close to unity, confirming that the regression models obtained in this work were adequate to predict $Lch_{AL1.1}$ production, depending on the concentrations of the three tested components of the culture medium in the experimental range studied. We can see that the theoretical value for $Lch_{AL1.1}$ production was 3.19 g l^{-1}, compared to the experimental value of 3.20 g l^{-1}, giving an error of 0.35%. It can thus be assumed that the models adequately predicted the maximum production values.

Kinetics of production of $Lch_{AL1.1}$ by B. licheniformis in the optimal medium (72 h culture)

In Figure 4 it can be seen that strain AL 1.1 presented a log phase lasting from 12 to 36 h of cultivation, when there was an increase in the production of $Lch_{AL1.1}$ in relation to the cell growth. Surface tension reached the lowest value of 30 mN m^{-1} after 48 h of incubation, with a remarkable accumulation of $Lch_{AL1.1}$ in the culture medium (2.5 g/l). The production of $Lch_{AL1.1}$ was maintained until 72 h (stationary phase).

Effect of pre-treatment (polystyrene surface) with purified lichenysin to avoid microbial adhesion

The activity of purified lichenysin against microorganisms was defined using two parameters: effectiveness and efficiency. Effectiveness was defined as the percentage of adhesion inhibition induced by $Lch_{AL1.1}$. Efficiency was measured with the effective dose 50 (ED_{50}) or the BS concentration that reduces the microbial adhesion by half compared to the control.

Purified lichenysin presented anti-adhesive activity against all tested microorganisms. When a polystyrene surface was treated with $Lch_{AL1.1}$, a clear decrease of bacterial and yeast adhesion was observed (Figure 1, supplementary material). As shown in Table 4a, $Lch_{AL1.1}$ presented a concentration-dependent anti-adhesive effect. At the studied concentrations, the highest anti-adhesive effect was observed against *C. albicans* and MRSA (74.45 % and 68.73 %, respectively). Intermediate inhibition was obtained for *E. coli* O157:H7 and *L. monocytogenes* (49.9% and 47.8%, respectively), while the effect on *Y. enterocolytica* and *C. jejuni* was low (39.9 and 36.7 %).

Experimental anti-adhesion data were adjusted to a logistic model, indicating process saturation with a rapid response when BS concentration increased (Figure 5). The calculated effective dose with 50 % adhesion inhibition (ED_{50}) was 17.2 µg ml^{-1} for *C. albicans*, 8.3 µg ml^{-1} for MRSA, 16.1 µg ml^{-1} for *Y. enterocolitica* and 188.5 µg ml^{-1} for *C. jejuni*. These results indicate that low concentrations of lichenysin are very active (high efficiency) in preventing adhesion, especially of MRSA, *Y. enterocolytica* and *C. albicans*. Results obtained with *E. coli* O157:H7 and *L. monocytogenes* do not fit with the logistic equation, which is reflected in an absence of process saturation.

Disruptive effect of purified lichenysin on microbial adhesion (post-treatment)

Another approach is to remove the attached microorganisms from the surface after biofilm formation. Experimental data for the post-treatment are shown in Table 4b. The maximum disruption produced by $Lch_{AL1.1}$ was 55.74 % for MRSA, 51.51% for *Y. enterocolitica*, 45.9 % for *L. monocytogenes*, 42.83 % for *E. coli* O157:H7 and 40.70 % for *C. jejuni*. As in the first part of the study, the adhesion results were adjusted to a logistic equation (Figure 5b), showing a rapid reduction in adhesion when the $Lch_{AL1.1}$ concentration was increased, also indicating process saturation. According to the logistic model, the ED_{50} indicated a high efficiency, since the values obtained were very low: 2.8

µg ml^{-1} for MRSA, 4.1 µg ml^{-1} for *Y. enterocolitica*, 24.5 µg ml^{-1} for *E. coli*, 30.7 µg $ml^{-1}$for *C. jejuni* and 76.9 µg ml^{-1} for *L. monocytogenes* (Figure 5b). The lowest elimination effectiveness was obtained against *C. albicans* (37.97%). This process was different in that process saturation was not detected, as biofilm disruption did not present saturation at high concentrations, and $Lch_{AL1.1}$ showed its lowest desorption effectiveness.

When comparing both processes, it is clear that the action of $Lch_{AL1.1}$ was more effective in the pre-treatment against four of the six tested organisms, the exceptions being *Y. enterocolitica* and *L. monocytogenes*. In terms of efficiency, the lowest ED_{50} values were obtained in the post-treatment; however, inhibition values were lower than those achieved in the pre-treatment. The most marked difference in the BS effect occurred with *C. albicans*, since it gave the highest value of inhibition in the pre-treatment, while in the post-treatment the action of $Lch_{AL1.1}$ was very low.

Discussion

With the aim of increasing the lifetime of the raw materials and reducing the costs of BS production, substrates such as potato peel have been used to produce lipopeptides from *Bacillus subtilis* (Das and Mukherjee 2007), rice straw for the production of surfactin by *Bacillus amyloliquefaciens* XZ-173 (Zhu *et al.* 2013), and cassava wastewater for the production of surfactin by *Bacillus subtilis* (Nitschke and Pastore 2006). Molasses, the subproduct of sugarcane extraction, is mainly destined for animal feed, but its low price and nutritional compounds (e.g. nitrogen, vitamins) give it a broader industrial interest (Sarka *et al.* 2012). A key benefit of molasses is associated with its complex composition, as it includes a variety of mineral salts. In this work, when molasses was used as the substrate, only nitrate and phosphate salts needed to be added to the medium to obtain bacterial growth and BS production.

To the best of our knowledge, this study is the first comprehensive analysis of the use of molasses as a substrate to increase lichenysin production, applying response surface methodology to determine the optimal conditions for the main components of the culture medium. Molasses is a highly complex substrate that can be compared with other agro-industrial substrates evaluated using regression models in the literature. For example, surfactin production by *B. amyloliquefaciens* grown on rice straw gave a regression coefficient (R^2) of 0.98 (Zhu *et al.* 2013), or when pure carbon substrates such as glucose were used for the production of surfactin by *B. subtilis* DSM 3256, the R^2 of

the regression model was 0.93 (Sen and Swaminathan 2004). In the particular case of lichenysin, when produced by *B. licheniformis* with glucose, the optimal conditions determined by RSM gave an R^2 value of 0.918 (Joshi *et al.* 2008b), lower than that obtained in our work, which confirms the appropriate level of prediction of our proposed model.

The optimal concentration of molasses for the production of lipopeptides from *Bacillus licheniformis* TR7 and *Bacillus subtilis* SA9 was previously reported to be around 40 g l^{-1} (4% w/v) (Saimmai *et al.* 2011). Other researchers used about 70 g l^{-1} (7% w/v) of molasses for lipopeptide production by *B. subtilis* and *Bacillus* HS3 20B, indicating that production was inhibited when the concentration exceeded 90 g molasses l^{-1} (9% w/v) (Joshi *et al.* 2008a). In contrast, in our study, the optimal concentration of molasses was 107.82 g l^{-1} (10% w/v), three times that reported by other authors. This variation might be due to the composition of molasses, which can vary depending on the conditions under which the sugarcane is cultivated.

It is noteworthy that the effect of the nitrogen source ($NaNO_3$) on the production of $Lch_{AL1.1}$ was not significant (Eq. 2) within the ranges studied, even though the product is a lipopeptide, suggesting that the nitrogen content of the raw material is enough to support amino acid synthesis. However, Figure 2b shows that increasing or decreasing this variable led to small changes in the production of $Lch_{AL1.1}$. These results are consistent with those reported for *B. subtilis* in media poor in nitrogen, in which biosurfactant production was inhibited (Das and Mukherjee, 2007). Finally, phosphates mainly had a buffering effect, maintaining a pH suitable for *B. licheniformis* AL1.1 to produce $Lch_{AL1.1}$. This explains the importance of this variable for the regression model (Eq. 2), since it did not affect microbial growth. Similar behavior was reported by Qiu *et al.* (2014) indicating that Na_2HPO_4/KH_2PO_4 act as a buffering system and osmotic balance for lichenysin production by mutant *B. licheniformis* WX02-Psrflch.

As mentioned in Coronel-León *et al.* (2015), when AL 1.1 used glucose as a carbon source, the maximum production was reached at 24 h and was growth-linked accumulation. In the current study, three stages in production were observed, which might be due to the complex nature of molasses. The kinetics of accumulation of $Lch_{AL1.1}$ by *B. licheniformis* AL 1.1 presents a characteristic profile of a metabolite partially associated with bacterial growth. It is clear that the accumulation of $Lch_{AL1.1}$ began during the exponential phase and continued after the growth ceased. In accordance with our results, Nitschke and Pastore (2006) described the biosynthesis of a surfactant from

cassava wastewater, whose production began in the exponential phase and continued during the stationary phase.

The consumption of molasses was measured as residual glucose, and its assimilation was clearly associated with the growth of strain AL 1.1 and production of $Lch_{AL1.1}$, confirming the importance of this parameter, as described in the analysis of the response surface. As shown in Figure 4, the detectable nitrate was consumed after 30 h of cultivation. In spite of this, both the bacterial growth and production of $Lch_{AL1.1}$ continued, and increased considerably. In the case of growth, this process could be the result of utilization of nitrogen sources stored during the initial growth. Another possibility would be the contribution of nitrogen nutrients present in molasses, which may play an important role in achieving the maximum values of production (3.2 g l^{-1}). Throughout the growth of AL 1.1, the pH value (6-7) did not change significantly, confirming the importance of phosphate as a buffer agent. In recent work, Qiu *et al.* (2014) reported that *B. licheniformis* WX02-Psrflch used lichenysin as a substrate for the "second growth" after the glucose was consumed. In our study, this effect was avoided, since a decrease in the production of $Lch_{AL1.1}$ was not observed, suggesting a continuous use of the culture feeding systems.

When *B. licheniformis* AL1.1 was grown under optimal conditions, the production of $Lch_{AL1.1}$increased from 0.73 g l^{-1} to 3.2 g l^{-1}, representing a fourfold increase. The 3.2 g l^{-1} of $Lch_{AL1.1}$ produced by *B. licheniformis* AL 1.1 is higher than the 1.1 g l^{-1} of lichenysin reported in *B. licheniformis* R2 when grown on a far more complex mineral medium, with glucose as the carbon substrate (Joshi *et al.* 2008b). *B. subtilis* LB5 grown on cassava wastewater yielded 3 g l^{-1} of crude surfactant (Nitschke and Pastore, 2006). In the case of *B. subtilis* B20, 2.29 g l^{-1} of crude BS was obtained from molasses (Al-Bahry *et al.* 2013), and using a similar substrate, 3.26 and 3.56 g l^{-1} was produced by *B. licheniformis* TR7 and *B. subtilis* SA9, respectively ((Saimmai et al. 2011). A mutant *Bacillus licheniformis* WX02-Psrflch improved the lichenysin production using glucose as the carbon source, achieving a value of 2.14 g/L (Qiu *et al.* 2014).

Microbial adhesion to surfaces is a challenge for the food industry and hospitals. The addition of a BS to a surface modifies its hydrophobicity, interfering in the microbial adhesion and desorption process, and can be used as a strategy to delay the start of biofilm formation (Gudiña *et al.* 2010). The effect of $Lch_{AL1.1}$ pre-treatment and post-treatment of polystyrene surfaces against biofilm formation by six microorganisms (*E.coli*

O157:H7, *Y. enterocolitica*, *L. monocytogenes*, MRSA, *C. albicans* and *C. jejuni*) of particular concern in the food industry and hospital environment was studied.

Bacterial adhesion depends on surface type and bacterial charge, and the third component, the BS (lichenysin), interferes in this relationship. Surfaces of most bacterial cells are negatively charged, to an extent that varies with growth environments (Shi and Zhu 2009). Lichenysin is a cyclic heptapeptide considered anionic due to aspartic acid residues that are negatively charged at pH 7.2 (zeta-potential =-37.6 mV). In addition, $Lch_{AL1.1}$ was effective above its critical micelle concentration (15 mg l^{-1}), which suggests that the polystyrene surface became covered by lichenysin micelles, an effect similar to that described for rhamnolipids and surfactin (Zezzi do Valle Gomes and Nitschke 2012). Therefore, the effect of inhibition could be the result of the forces of electrostatic repulsion between the negative charges of the microbial surface and the negative charge of the polystyrene surface (coated with lichenysin molecules), which translates into high percentages of elimination of microbial adhesion, mainly against *C. albicans* and MRSA. Whereas, the $Lch_{AL1.1}$ activity in the post-treatment could be a consequence of BS penetration and absorption at the interface between the solid surface and the attached biofilm-forming bacteria, thus reducing the interfacial tension and favouring bacterial detachment. McLandsborough *et al.* (2006) describe that the attractive interactions between the microbial surface and solid surface can be reduced in the presence of BS, which would facilitate the removal of biofilm.

For *E. coli*, *L. monocytognes*, *Y. enterocolitica* and *C. jejuni*, the values of adhesion inhibition were less than 50% (pre-treatment). The common denominator in these bacteria is the presence of flagella, suggesting that the presence of these appendages might favour the adhesion of these bacteria. Van Houdt and Michiels (2010) indicated that flagella can affect adhesion and biofilm formation by different mechanisms depending on the type of bacteria. In other work, Shi and Zhu (2009) also mention the importance of hydrophobic surfaces as the flagellum, exopolysaccharides to reduce the forces of repulsion between the two surfaces, thereby favouring microbial adhesion. As mentioned, $Lch_{AL1.1}$ was not very effective in removing the biofilm formed by *C. albicans* (37.97%). Douglas (2003) indicates that a distinctive feature of *C. albicans* biofilms is the presence of a mixture of several morphological forms. Moreover, the initial adhesion of this yeast occurs after 3 to 6 hours of incubation, so under the experimental conditions of our work (20 h of incubation), adhesion of *C. albicans* would have been very strong,

thus limiting the BS action. Other factors that could explain the low activity of $Lch_{AL1.1}$ against *C. albicans* are the negative charge neutralization due to the existence of cell wall changes in *C. albicans* during growth in response to the operating conditions (temperature and incubation time), or by the excretion of acid metabolites, which produce a decline in the forces of repulsion without modifying the microbial adhesion (Shakerifard *et al.* 2009).

The data presented here are consistent with other reports of biosurfactant anti-adhesion activity. In this work, lower BS concentrations were used than by Gudiña et al. (2010), who demonstrated high anti-adhesion of *Lactobacillus paracasei* crude BS against *S. aureus* (72.0 %), *S. epidermidis* (62.1 %), and *S. agalactiae* (60 %), and low activity against *P. aeruginosa* (16.5 %) and *E. coli* (11.5 %) at a concentration of 25 mg mL^{-1}. The adhesion of pathogenic bacteria (*E.coli* CFT073 and *S. aureus* ATCC 29213) to polystyrene surfaces was inhibited by a specific anti-adhesion activity of two lipopeptides produced by *B. subtilis* and *B. licheniformis*, which selectively inhibited biofilm formation (Rivardo et al. 2009). The adhesion of *L. monocytogenes* to microtitre plates (polystyrene) was reduced by 84% when the surface was treated with surfactin (1 mg mL^{-1}) and 82% when treated with purified ramnolipids (7.5 mg mL^{-1}) (De Araujo et al. 2011), at a surfactant concentration twice as high as that of lichenysin in the current work. The antiadhesive action of pseudofactin II against *C. albicans* SC 5314, *C. albicans* ATCC 20231, *Proteus mirabilis* ATCC 21100 and *E. coli* ATCC 10536 was reported, with 80-99% of reduction at a concentration of 0.5 mg ml^{-1}(Janek *et al.* 2012). Other authors reported that the action of surfactin (0.25 mg mL^{-1}) and rhamnolipids (1mg mL^{-1}) against *L. monocytogenes* reduced adhesion by 57.8 and 42%, respectively, and while surfactin was unable to prevent adhesion of *S. aureus*, rhamnolipids (1 mg ml^{-1}) reduced it by 67.8% (Zezzi do Valle Gomes and Nitschke 2012).

Frequently, the efficiency in biofilm disruption in a post-treatment is lower than in a pre-treatment. Pseudofactin II (0.5 mg ml^{-1}) was able to remove the biofilm formed by *C. albicans* SC 5314, *C. albicans* ATCC 20231, *Proteus mirabilis* ATCC 21100 and *E. coli* ATCC 10536, with a performance ranging from 26 to 70% (Janek et al. 2012). In another study, surfactin (0.1 mg ml^{-1}) was able to eliminate the biofilm formed by *L. monocytogenes* (95.9 %), while ramnolipids (0.25 mg ml^{-1}) were less efficient (26.5 %). Rhamnolipids (0.25 mg ml^{-1}) and surfactin (0.1 mg ml^{-1}) eliminated the biofilm formed by *S. aureus* (58.5 and 63.7 %, respectively), but against *S. enteritidis*, surfactin (0.1 mg ml^{-}

^{1}) and rhamnolipids (0.25 mg ml^{-1}) were less efficient (35.3 and 30.9 %, respectively) (Zezzi do Valle Gomes and Nitschke, 2012).

Srey *et al.* (2013) mention that cells in biofilms are more resistant than planktonic bacteria to antimicrobial agents, because they have a barrier consisting of extracellular polymeric substances (EPS) that prevents or reduces contact. The application of antimicrobial agents to control biofilms generally results in a decline in effectiveness after repeated exposure to the microorganisms, which can generate resistant variants. Therefore, the surface activity and the weak antimicrobial property (data not shown) of lichenysin can be an advantage in treatments against biofilm generation by resistant strains.

Lichenysin could be an interesting alternative for controlling the growth of biofilms of MRSA, *E. coli* O157:H7, *Listeria monocytogenes*, *Y. enterocolitica* and *C. jejuni*, which have been described as foodborne pathogens (Shi and Zhu 2009). On the other hand, taking into account that *C. albicans* is recognized as an important pathogen in nosocomial infections (Boucherit-Atmani *et al.* 2011), the yeast response described in this work is of particular interest, especially when lichenysisn is used for the surface pre-treatment (Figure 1, supplementary material).

Acknowledgments

Financial support was received from the Spanish Ministerio de Economia y Competitividad, project CTQ2014-59632-R and the Generalitat de Catalunya Project 2014-SGR-534 and the Universitat de Barcelona. We thank Ecuador's Government for a SENESCYT-ESPOL grant. We thank Dr. G. Lorén from the Unity of Microbiology. Faculty of Pharmacy from University of Barcelona for useful discussion.

Conflict of Interest

The authors declare no conflict of interest.

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Figures caption

Figure 1. Effect of different carbon sources on the growth of *B. licheniformis* AL 1.1 and in the production of lipopeptides after 72 h culture at 30º C and 120 rpm. Surface tension supernatant (blacks bars), cell dry weight (white bars) and $Lch_{AL1.1}$ (grey bars).

Figure 2. Response surface graphs showing the effect of the molasses concentration (x_1), nitrates concentration (x_2) and phosphates concentration (x_3) at the optimum

conditions for $Lch_{AL1.1}$ production (*Y*) maximization by *B. licheniformis* AL 1.1. a) Influence of x_2 and x_3 on $Lch_{AL1.1}$ production keeping x1 at its optimum level (x_1= 0.05). b) Influence of x_1 and x_3 on $Lch_{AL1.1}$ production keeping x_2 at its optimum level (x_2= -0.009). c) Influence of x_1 and x_3 o $Lch_{AL1.1}$ production keeping x_3 at its optimum level (x_3= 0.31).

Figure 3. Predicted $Lch_{AL1.1}$ /experimental $Lch_{AL1.1}$ correlation graphic, validation of the regression model for *B. licheniformis* AL 1.1.

Figure 4. Time course of $Lch_{AL1.1}$ production in molasses medium optimum by *B.ysin licheniformis* AL 1.1. Surface tension (▪); residual glucose (x); Biomass (●); lychenisin (♦) residual sodium nitratre (Δ). Bars represent the standard deviation of three measurements.

Figure 5. Curve dose – response (adhesion inhibition) using logistic model (y = d / 1 + exp b(log (x)- log (e))). (a) Pre-treatment with purified lichenysin and (b) Post-treatment with purified lichenysin. *C. albicans* (x) ; *S. aureus* (•); *E. coli* (▪); *Y. enterocolitica* (♦); *L. monocytogenes* (▲); *C. jejunii* (▼).

Figure 1

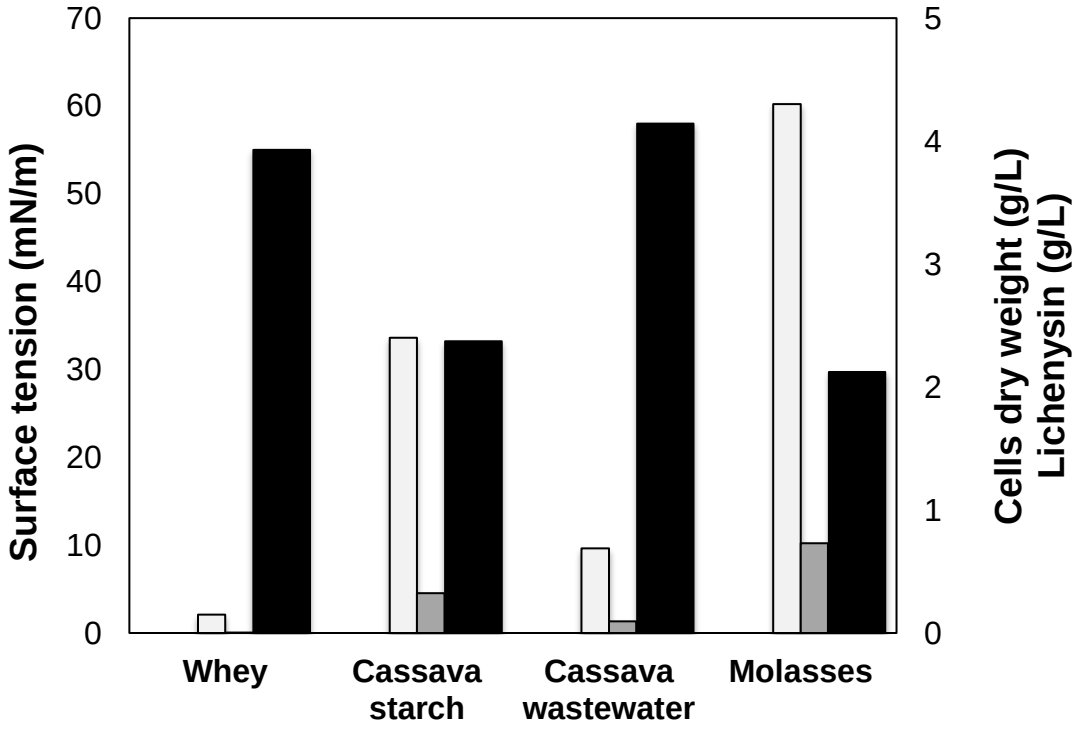
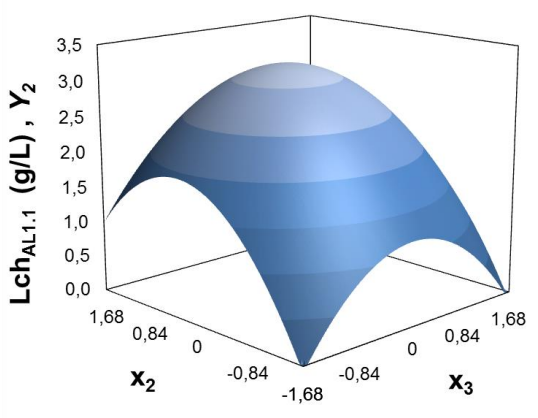
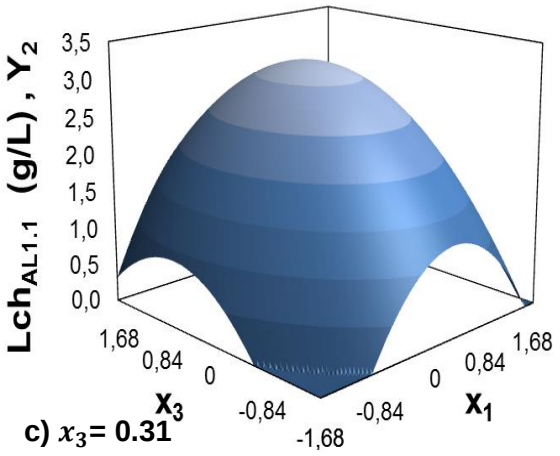


Figure 2

a) x_1=0.0515



b) x_2= -0.009



c) x_3= 0.31

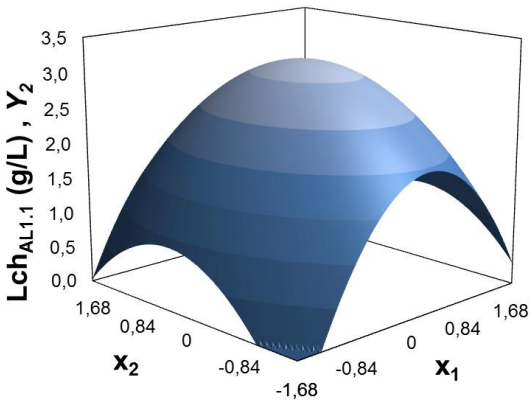


Figure 3

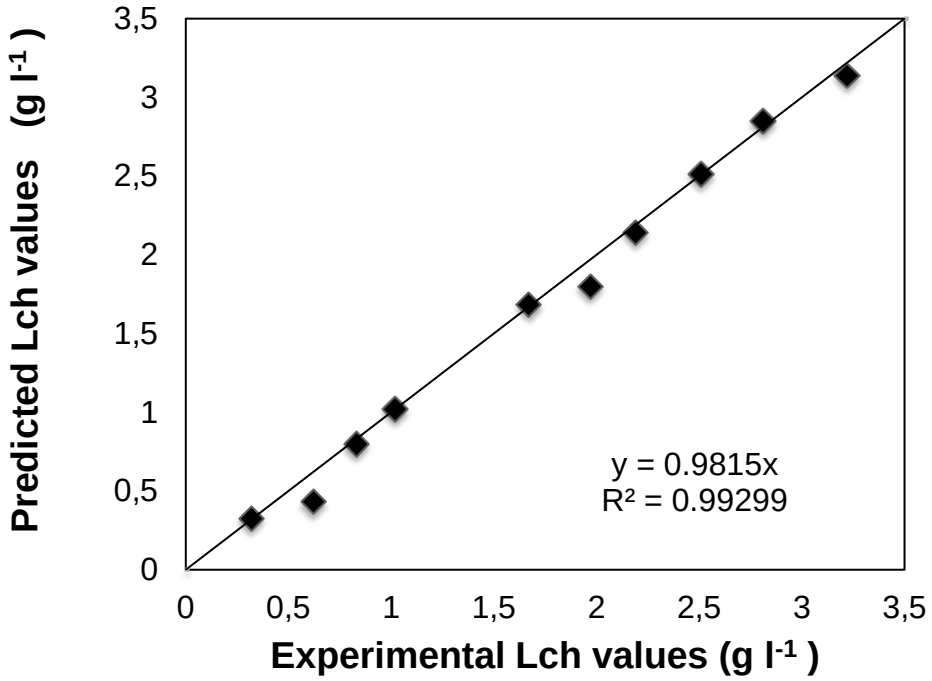


Figure 4

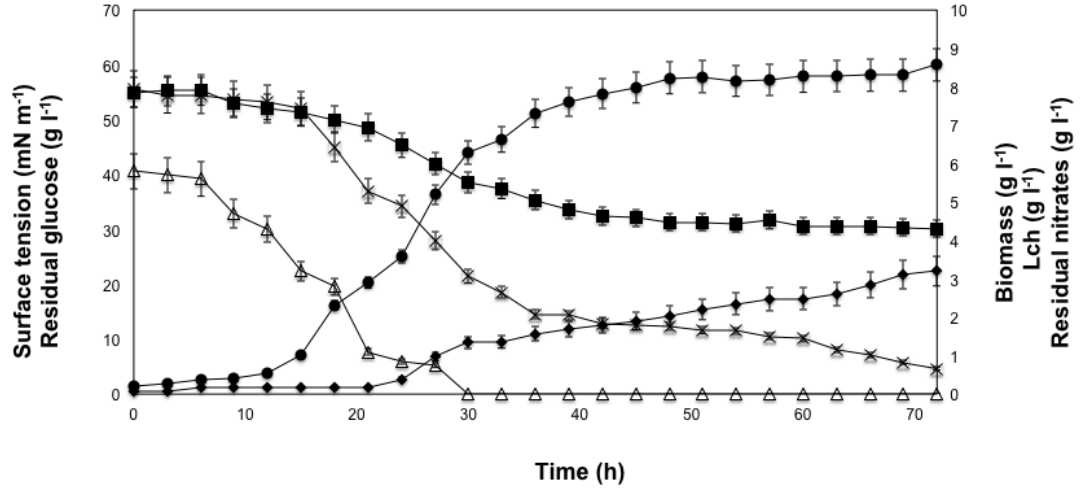
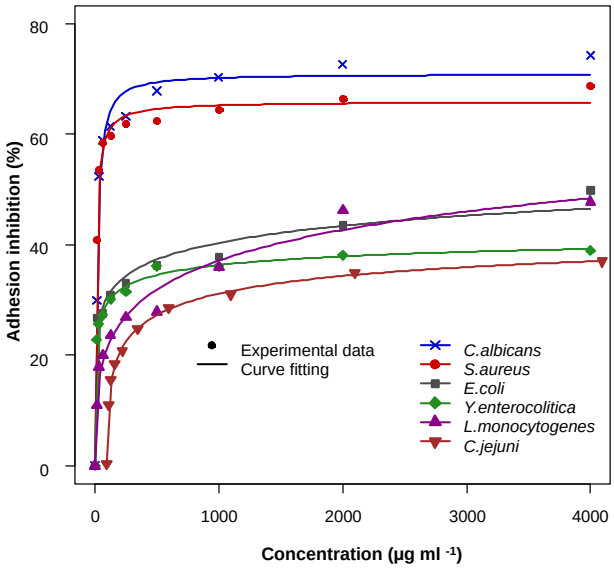


Figure 5

a)


Adhesion inhibition (%)
0
20
40
60
80
0
1000
2000
3000
4000
Concentration (µg ml -1)
Experimental data
Curve fitting
C.albicans
S.aureus
E.coli
Y.enterocolitica
L.monocytogenes
C.jejuni

b)

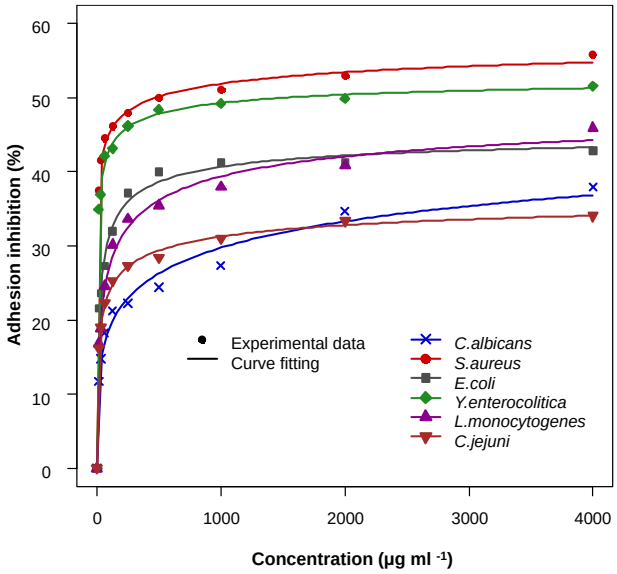

Adhesion inhibition (%)
0
10
20
30
40
50
60
0
1000
2000
3000
4000
Concentration (µg ml -1)
Experimental data
Curve fitting
C.albicans
S.aureus
E.coli
Y.enterocolitica
L.monocytogenes
C.jejuni

Table 1. Range and levels of independent variables in CCRD experiments with three factors at five levels each one

Independent Variable	Units	Symbols		Coded level of variables				
		Uncoded	Coded	-1.68	-1	0	1	+1.68
[Molasses]	g l^{-1}	X_1	$\boldsymbol{x_1}$	10	48.45	105	161.55	200
[NO^{3-}]	g l^{-1}	X_2	$\boldsymbol{x_2}$	1	3.23	6.5	9.77	12
[PO_4^{3-}]	g l^{-1}	X_3	$\boldsymbol{x_3}$	2	4.63	8.5	12.37	15

Table 2. CCRD matrix of three factors and observed response experimental data

Exp nº	Variable coded values			Variable naturals values			
	x_1	x_2	x_3	X_1 Molasses	X_2 NO^{3-}	X_3 PO_4^{3-}	Y $Lch_{AL1.1}$
				(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})
1	1	1	-1	161.55	9.77	4.63	1.17
2	-1	-1	-1	48.45	3.23	4.63	0.73
3	0	0	0	105	6.5	8.5	2.69
4	0	0	0	105	6.5	8,5	3,33
5	0	1.68	0	105	12	8,5	1,63
6	-1	1	-1	48.45	9.77	4.63	0,75
7	0	0	0	105	6.5	8.5	3.16
8	1	1	1	161.55	9.77	12.37	1.61
9	-1.68	0	0	10	6.5	8.5	0.62
10	0	0	0	105	6.5	8.5	3.15
11	-1	-1	1	48.45	3.23	12.37	1.55
12	0	-1.68	0	105	1	8.5	1.98
13	1.68	0	0	200	6.5	8.5	1.12
14	0	0	1.68	105	6.5	15	2.08
15	1	-1	-1	161.55	3.23	4.63	1.52
16	-1	1	1	48.45	9.77	12.37	2.19
17	0	0	-1.68	105	6.5	2	1.24

18	1	-1	1	161.55	3.23	12.37	1.93
19	0	0	0	105	6.5	8.5	3.23
20	0	0	0	105	6.5	8.5	3.28
21	0	0	0	105	6.5	8.5	3.14
22	0	0	0	105	6.5	8.5	3.16
23	0	0	0	105	6.5	8.5	3.16

Table 3. Analysis of Variance (ANOVA) for the significance of regression model for $Lch_{AL1.1}$ production (Y_2) by *Bacillus licheniformis* Al 1.1, with 7 terms and 23 experiments.

R=0.9868, R^2=0.973, Adjusted R^2 = 0.9614, Coefficient of variation =8.828

Source	**Sum of squares**	***df***	**Mean square**	***F_0***	**Probability P= P:(H_0:$F_0 \leq F_{crit}$)**
Regression model	19.17	7	2.739	79.30	1.0387E-10
Residual or error	0.518	15	0.03454		
LOF error	0.189	6	0.03157	0.8645	0.555
Pure error	0.329	9	0.03652		
Total	19.69	22			

Table 4. Microbial adhesion inhibition in the polystyrene surface (microtiter plate) by purified lichenysin. a) Pre-treatment and b) Post-treatment.

a)

Pre-treatment Adhesion inhibition (%)						
Concentration (µg ml $^{-1}$)	***C. albicans***	***S. aureus* MRSA**	***E. coli* O157:H7**	***Y. enterocolitica***	***L. monocytogenes***	***C. jejuni***
4000	74.35	68.73	49.86	38.91	47.77	36.70
2000	72.6	66.43	43.54	38.1	46.2	34.65
1000	70.23	64.48	37.84	36.1	35.96	30.75
500	67.87	62.34	36.25	36.1	27.9	28.24
250	63.22	61.95	33.02	31.48	26.84	24.49
125	61.5	59.74	30.89	30.19	23.66	20.56
63	58.93	58.53	27.59	27.28	20.00	18.15
31	52.33	53.6	26.77	25.62	17.92	15.22
16	29.96	40.93	26.75	22.8	10.94	10.75
0	0.00	0.00	0.00	0.00	0.00	0.00

b)

Post-treatment Adhesion inhibition (%)						
Concentration (µg ml $^{-1}$)	***C. albicans***	***S. aureus* MRSA**	***E. coli* O157:H7**	***Y. enterocolitica***	***L. monocytogenes***	***C. jejuni***
4000	37.97	55.74	42.83	51.51	45.9	40.70
2000	34.65	52.87	41.19	49.83	40.78	34.65
1000	27.39	50.96	41.19	49.16	37.91	30.75
500	24.42	49.88	39.96	48.32	35.45	28.24
250	22.26	47.97	37.09	46.14	33.61	24.49
125	21.2	46.17	31.97	43.12	30.12	20.56
63	18.28	44.5	27.25	42.11	24.59	18.15
31	14.78	41.51	23.57	36.91	18.85	15.22
16	11.78	37.51	21.57	34.91	16.85	10.75
0	0.00	0.00	0.00	0.00	0.00	0.00