Screening of lipopeptide-producing strains of Bacillus sp. Using a new automated and sensitive fluorescence detection method

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Biotech Method

Screening of lipopeptide producing strains of *Bacillus* sp. using a new automated and sensitive fluorescence detection method[†]

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Abbreviations: CPC, cetylpyridinium chloride; **FL**, fluorescein, **BTB**, bromothymol blue; **RP-UPLC**, reversed phase ultra-performance liquid chromatography; **MALDI-ToF**, Matrix Assisted Laser Desorption Ionisation - Time of Flight. **MIC:** Minimum Inhibitory Concentration

Abstract

Lipopeptides, such as surfactins are important biosurfactants produced by *Bacillus* sp. that find applications in many areas (environment, medicine and food industries). Giving their importance, the use of simple detection methods will facilitate screening and quantification. In the present work, we described a completely automated workflow for the screening of lipopeptide producing strains and its quantification. Firstly, isolated colonies from environmental samples were automatically picked and inoculated in 96 wells growth plate. After overnight incubation, surfactin produced in the broth was quantified, using a new sensitive fluorescent method. The method uses fluorescein (FL), which is an anionic dye at neutral to alkaline pH and forms a stable complex with the cationic surfactant cetylpiridinium chloride (CPC), quenching fluorescence. Upon addition of surfactin or other lipopeptides, fluorescein is released from the CPC-FL complex and quantified. The robustness of this method was assessed by comparing the quantification results to those conventionally measured by RP-UPLC and the results of strain screening were confirmed by MALDI-ToF analysis. We report for the first time the successful application of this analytical method for high-throughput screening of novel lipopeptide-producing strains.

Highlights

Previous work based on colorimetric detection of surfactin uses the dye-complex CPC-BTB. In the present work, bromothymol blue was replaced by fluorescein, forming a stable complex at low concentration. By using automation, it was possible to design a complete workflow that is useful for quantification and high-throughput screening of novel surfactin-producing strains.

1   Introduction

Biosurfactants are important secondary metabolites produced by certain bacterial strains. The importance of these compounds are their applications in environmental remediation [1], their antimicrobial activities [2,3] and synergistic action with other secondary metabolites [4]. One of the most studied and active biosurfactants are surfactins, an heptapeptide with strong surface activity [5,6]. Two other compounds of the family of surfactins, namely pumilacidin and lichenysin have a structure very similar to surfactin. Other important lipopeptides produced by *Bacillu*s sp. are iturins (iturin A, mycosubtilin and bacillomycin) and fengycins (fengycin A and B, plipastatin), which have antifungal activities. The structural features of surfactins include the presence of a beta-hydroxy fatty acid (C_{12}-C_{16}) and the aminoacids L-glutamate and L-aspartate, which contribute with negative charges for this anionic biosurfactant [7,8]. The interest of the scientific community for these biosurfactant molecules, such as lipopeptides and particularly for surfactin has been increasing steadily since the last 10 years. For example, the number of scientific articles referring to surfactins over this period is 975 and 3450 more generally on lipopeptides, i.e. almost one scientific article per day (query on Scopus database the October 23rd, 2018). The growing interest in these molecules leads to an increased work of the scientific community for the optimization of their production either by genetic and metabolic engineering [8,9] or process engineering [10] but the limits of these approaches are being achieved. In addition, the society is increasingly in demand for natural products that do not originate from genetically modified organisms. In this context, search for new strain producing these molecules with interesting production capacities or which could be naturally optimized becomes an important issue. Quantification of surfactants is difficult to carry out by high-throughput methods [11]. The work of Chen et al. 2007 describes however, a method that is based on the effect of surfactant on the solution's meniscus and its consequence on the visualization of a grid that is placed under a microtiter plate [12].

The quantification of surfactin (or other lipopeptides from *Bacillus* sp.) usually is carried out by liquid chromatography (HPLC). However, it requires a clean-up step by solid phase extraction [8]. Other methods such as surface tension measurement [13], oil spreading [14], blood hemolysis [15] or drop collapsing [16] test have often been used [13]. Recently Yang et al. [17] presented a colorimetric procedure. The method uses the anionic dye bromotimol blue (BTB) as indicator and the cationic surfactant CPC as mediator. However, the dye complex is not stable. It only last a few hours before precipitating and losing color. In addition, the limit of detection seems to be around 0.1 g/L [17].

Here, we present a complete automated workflow for screening of lipopeptides (such as surfactin) producing strains (Fig. 1). This workflow is divided in three steps: a first step for automated colony picking from agar medium, a second step for incubating the strains on microtiter plates containing production medium and a third step for quantifying the lipopeptides produced using fluorescent methods, thus inspired on the work described by Yang et al. [17]. The method developed and optimized here uses the anionic fluorescent dye FL as an indicator dye that was mixed with CPC as mediator, resulting in a more stable complex, with increased sensitivity. In addition, it produces better curves, as there is a gain in linearity, besides producing an automated method that could be used for high-throughput screening of lipopeptide production and especially for surfactins. This workflow has been successfully tested to screen novel isolated strains of *Bacillus sp.* and to quantify the production of surfactins. The method was validated by ultra-performance liquid chromatography (UPLC) and a coupled MALDI-ToF analysis confirmed the species of the novel isolated strains and the type of lipopeptides produced.

2 Materials and Methods

2.1 CPC-FL method optimization

Three steps of optimization were used to design this new quantification method. All experiments were performed in Greiner® black 96-flat-well polystyrene plates, incubated at 37 ± 1 °C and stirred for 30 s before measurement.

Initially, the excitation and emission wavelength of FL were optimized using a multimode microplate reader (SpectraMax i3, Tecan). The focalization height was also optimized and found to be ideal at 0.5 mm above the bottom of the well. FL is a fluorescent dye with absorption and emission in the visible range, respectively around 494 and 512 nm [18]. The results obtained here have shown that the fluorescein-displacing reaction can be optimally monitored using an excitation wavelength of λ_{ex} = 482 ± 9 nm and a detection at λ_{em} = 528 ± 15 nm. PMT gain was set to the highest value, with 6 flashes per read.

Secondly, relative fluorescence units (RFU) detected were correlated with FL concentration using a calibration range from 0 to 0.01 mM in 0.1 M sodium phosphate buffer pH 8.0. Results are presented in Fig. 2a. The optimal CPC/FL ratio and CPC concentration were determined using a 0.045 to 0.360 mM of CPC with 0.009 mM of FL, in presence of 0.7 to 45 mg/L of surfactin final concentration in the reaction mix. Results are presented in Fig. 2b.

Finally, the calibration curves of the different lipopeptides (surfactin, mycosubtilin and fengycin) were set by addition of 10 μL of a 7 to 450 mg/L lipopeptide solution in water, to 90 μL of 0.1 mM CPC and 0.01 mM FL solution in 0.1 M sodium phosphate buffer pH 8.0. Results are presented in Fig. 2c.

Lipopeptides used for method optimization were supplied from Lipofabrik SAS (Villeneuve d'Ascq, France). The anionic fluorescent dye fluorescein (FL) (Uranin AP) and cetylpyridinium chloride (CPC) were purchased from Sigma Aldrich (Saint Louis, USA).

Experiments were carried out 3 times and with a technical triplicate. The mean values and standard deviation are presented.

2.2   Determination of the MIC of CPC

The minimum inhibitory concentration (MIC) of CPC alone and the mixture of CPC-FL were determined in microtiter plate by cultivating the strain *Bacillus subtilis* ATCC21332 [ATCC Collection] with different concentration of CPC. The wells of a 96 well plate, containing 100 μL of LB medium with different concentration of CPC (from 0 to 0.2 mM) were inoculated with 10 μL of pre-culture to obtain an initial optical density of 0.1. After 24 hours of incubation at 37°C under 200 rpm orbital shaking, the growth in each well was measured by optical density at 605 nm (SpectraMax i3, Molecular Devices). Experiment was carried out in triplicate.

2.3   Screening of environmental strains using the CPC-FL method

Environmental samples were collected from a farm soil in the Haut de France region (France) and then freezed at -20°C before analysis. The samples were first diluted in sterile distilled water and heated at 80°C for 20 minutes, in order to select only sporulating strains. The samples were then streaked on 250*250 mm YPG agar plates containing 100 μg/mL amphotericin B and incubated at 37°C for 24 hours. Then, 10 isolated colonies were randomly selected regarding their different morphology, and robotically transplanted using the colony picker (QPix 460, Molecular Devices) into a deep-well plate (Bioblock) of 2.2 mL well capacity (Simport, Beloeil, Canada). The wellscontained 1 mL of modified Landy's medium that was adjusted to pH 7.0 using phosphate buffer as previously described [9] or 1 mL of LB medium. Landy medium, a chemically defined medium, is one of the most used medium for lipopeptide production. Cultures were then set at 37°C during 24 hours under 200 rpm orbital shaking. Two reference strains of *B. subtilis* were also used, respectively as negative control, strain 168 a

non-lipopeptides producer [*Bacillus* Stock Center Collection] [15] and positive control strain ATCC21332 a natural producer of surfactin and fengycin [ATCC collection].

The growth was first measured by optical density at 605 nm (SpectraMax i3, Molecular Devices) and strains were reisolated on separate LB agar plates for further analysis by MALDI-ToF. Then, 96 well-plates were centrifuged (Alegra X-30R, Beckman Coulter) for 30 min at 4 °C and 2700 g. A sample of 200 μL was kept at 4°C before RP-UPLC analysis. A sample of 10μL was transferred to a Greiner® black 96-flat-well plate. Sample preparation steps were performed on a Biomek FXp liquid handling station from Beckman Coulter. The fluorescent lipopeptide detection was automated on a multimode microplate reader (SpectraMax i3, Molecular Devices). The reaction mixture was composed of 10 μL of sample solution, and 90 μL of 0.1 mM CPC and 0.01 mM FL in 0.1 M sodium phosphate buffer pH 8.0. Calibration curves of each lipopeptide were prepared using 0.7 to 45 mg/L FL in modified Landy's and LB medium, which were previously incubated at 37 °C for 24 h at 200 rpm orbital shaking. Fluorescence measurements were performed after 30 s of orbital shaking at 30 °C, using λ_{ex} = 482 ± 9 nm as excitation wavelength and λ_{em} = 528 ± 15 nm for detection as described previously. Experiments were carried out 3 times and with a technical triplicate. The mean values and standard deviation are presented.

2.4 Lipopeptides quantification using RP-UPLC

Lipopeptides (i.e. iturins, fengycins and surfactins) were quantified using an ultra-performance liquid chromatography (UPLC) ACQUITY H-Class system from WATERS (Saint-Quentin En Yvelines, France) and 2.1X50 mm BEH C18 Column. Lipopeptides were analysed using gradient with the initial solvent acetonitrile/water/trifluoroacetic acid 30:70:0.1 (vol/vol/vol) and reach 45:55:0.1 (vol/vol/vol) in 15 minutes to elute iturins. Then, a linear gradient to reach 55:45:0.1 (vol/vol/vol) in 10 min was applied. Under these conditions, fengycins are eluted. Following this step, another gradient of 5 minutes was performed to reach 80:20:0.1 (vol/vol/vol). Then surfactins were eluted isocratically during 5 min. Pure iturins A, fengycins and surfactins from Sigma-Aldrich were used as

references. Retention time and second derivatives of UV-visible spectrum (Diode Array Waters PDA, Empower Software) of each peak were used to identify and quantify the eluted molecules.

2.5   Strain identification by MALDI-TOF-MS

Isolated bacterial strains from LB agar plate were loaded three times onto ground steel MALDI target according to the manufacturer's instructions (Bruker Daltonics, Bremen, Germany). Briefly, 3 bacterial colonies were taken off from agar plates with a 10 µL pipet tip, smeared onto the target and dried at room temperature. The on-target deposits were overlaid with 1 µL of 70% formic acid solution, dried at room temperature, overlaid again with 1 µL of matrix solution [10 mg/mL of HCCA dissolved in ACN/water/TFA (50/47.5/2.5; v/v/v)] and dried again [19,20]. Samples were automatically analyzed using MALDI-TOF mass-spectrometer (AutoflexSpeed from Bruker Daltonics) running Flexcontrol 3.4 software as described recently [19].

Mass spectra were processed using the BioTyper software (version 3.0; Bruker Daltonics) running with the BioTyper database version DB 5989, containing 5989 reference MALDI-TOF MS profiles (5298 of bacteria, 626 of yeasts and 65 of filamentous fungi). Matching between experimental MALDI-TOF MS profiles obtained from bacteria isolates and the reference MALDI-TOF MS profiles is expressed by BioTyper according to a Log (Score) and an associated colour code (green, yellow and red) as decribed recently [19].

2.6 Characterization of the lipopeptides production in novel isolates strains by MALDI-TOF-MS

On the same deposits (smears) produced previously for strain identification, the MS signals were acquired in reflectron mode in the 700–3,500 Da m/z range by summing 8000 laser shot spectra, according to the manufacturer's automatic method RP_700-3500.par (voltage values of ion sources #1 and #2 set as 19 and 16.70 keV, respectively; voltage values of reflectron #1 and #2 set as 21 and 9.5 keV, respectively; lens tension =

6.9 keV; pulsed extraction = 120 nsec; laser intensity between 30 and 50%). Mass spectra were visualized using FlexAnalysis software (version 3.4; Bruker Daltonics).

3   Results and discussion

In this work, we designed, optimized and assessed a fully automated workflow for screening bacterial lipopeptide (especially surfactin) production and quantification on microtiter plates using CPC-FL. The method is based on the release of FL upon interaction with anionic lipopeptides.

FL is a very useful dye that finds many applications in analytical chemistry [21]. The spectroscopic properties of FL are based on its high fluorescence emission at 520 nm for the deprotonated species [22]. In this work, we have explored the properties given by the complex formation between FL and CPC, which quenches fluorescence. The quenching phenomenon is potentially given by electrostatic interactions between the cationic surfactant and deprotonated FL at pH 8.0. At 25°C, the critical micellar concentration (CMC) of CPC in water is approximately 1 mM [23]. The working concentration of CPC used in the experiment was (0.09 mM), which is approximately 10 times below the CMC of CPC. Therefore, we expect that the interaction between CPC and FL is given by charge interactions occurring at the molecular level. However, the ion-pair formed between CPC and FL may decrease the solubility of CPC, by charge neutralization. Thus, it may induce surfactant self-aggregation. A similar pattern was observed for the interaction between CPC and another anionic dye (phenol red)[24]. For surfactin, the lowest concentrations of the analytical curve are close to its reported CMC (0.0075 mM)[25]. Therefore, it is possible that the interaction of surfactin with CPC involves adsorption of CPC on surfactin micelles. The binding of CPC to surfactin micelles is certainly stronger when compared to its binding to FL. The CPC-surfactin complex involves both electrostatic and hydrophobic interactions, possibly forming mixed micelles. Also, it must be considered that surfactin has two negative charges in the molecule, while fluorescein has only one negative charge

at pH 8.0 [22]. Because of surfactin binding, free FL is released, and fluorescence is detected.

Several calibration curves were prepared to find the best parameters for the CPC-FL method to evaluate surfactin production, during the growth of *Bacillus* sp. Relative fluorescence units (RFU), which were detected in correlation with FL concentration, show good linearity, up to 0.01 mM (r2 = 0.9976) (Fig. 2a) and an excellent LOD (Limit Of Detection) of 1.5 x 10-4 mM, which was determined according to IUPAC specifications. Considering linearity as an important parameter, the optimal concentration of CPC required to obtain the calibration curve was calculated. A 10-fold factor between CPC and FL concentration was found ideal, with a final concentration of 0.09 mM in CPC and 0.009 mM in FL, as shown on Fig. 2b. It is important to note that the parent method using BTB-CPC as the reporting dye complex has a problem of stability. In our experiments, the dye complex tends to precipitate. Spectrophotometric analysis showed a reduction of absorbance of 50% in 40 hours due to precipitation, while the FL-CPC dye remained stable for longer period, especially at the established concentration above (data not shown). From this, the optimized method consists in the use of 0.009 mM FL, as indicator dye, mixed with 0.09 mM CPC in 0.09 M sodium phosphate buffer pH 8.0.

Using the above described conditions, the lipopeptides calibration curves in water (Fig. 2c), in modified Landy's medium (Fig. 2d) and in LB medium (Fig. 2e) were determined. A very low LOD (0.015 g/L) could be calculated for the calibration curve in water. The LOD for the modified Landy´s medium curve showed even better results (0.008 g/L) for surfactin, according to IUPAC specifications. In both cases, a perfect linearity was found up to 450 mg/L of surfactin (r^2 = 0.9912 and 0.9988 respectively). In LB, however, the LOD was found higher, and the linearity not as good as in the previous conditions, with respectively 0.044 g/L and r^2 = 0.9838. This is correlated to a much lower slope for the calibration curve in LB than in water and Landy, that might come from the very high quantities of peptides present in LB and that may interfere with the test. These samples were also analyzed by RP-UPLC and similar quantification results were obtained (data not

shown). Better linearity was obtained using this CPC-FL method in comparison to CPC-BTB method (r^2 = 0.9793) [17]. For fengycin, the LOD found in water, Landy and LB media were of 0.041 g/L, 0.058 g/L and 0.051 g/L, respectively. Linearity coefficient were also acceptable with respectively r^2 = 0.9913, r^2 = 0.9985 and r^2 = 0.9747 in the three media. Here again, the linearity was lower in the case of LB, showing the negative impact of this broth on the assay response. Finally, for mycosubtilin, LOD were of 0.096 g/L and 0.110 g/L for water and Landy respectively, with correlation coefficients of r^2 = 0.9967 and r^2 = 0.9952. No LOD could be calculated from the mycosubtilin calibration curve in LB as no linear correlation was observed between the concentrations. Interaction of iturinic compound with CPC is significantly less good because they are uncharged. Nevertheless, in a chemically defined medium, it can certainly interact via a hydrophobic interaction and can be detected. More globally, this shows again the good correlation existing between the slope and the LOD value.

The method was then evaluated in the complete workflow described above and schematically shown in Fig. 1 for the screening of unknow environmental sample and using 2 different reference strains of *B. subtilis*. The time required to pick and analyze the production capacity of, for example, 96 strains (which corresponds to a 96-well plate) using this automated workflow, was 2 h (compared to at least 35 min of UPLC analysis per sample). To this period, 16 h of incubation is required on Petri dishes for bacterial growth, in addition to 24 h for surfactin production, after inoculation of single colonies on the liquid broth. However, these two incubation periods are inherent to all methods. At the end of the workflow, the new CPC-FL quantification method was compared to the usual method using RP-UPLC. The results are presented in Fig. 3. During the first step of screening 10 different colonies were selected and picked but only 5 of these grew on LB and modified Landy's medium. Analysis of culture supernatants by RP-UPLC indicated no fengycins or iturins production by any of the analyzed strains, including the producing strain ATCC 21332. The low cultivation time (24h) has not been suitable for the production of these lipopeptides, which are most often produced during the stationary

phase of growth [26]. RP-UPLC analysis of the 168 strain's fermentation broths (a non-producer of lipopeptides) reveals no surfactin production while CPC-FL method gives a response of 0.0098 ± 0.005 g/L in LB medium and 0.039 ± 0.011 g/L in modified Landy's medium. This result cannot come from the medium since the non-inoculated medium (negative control) used in this experiment produces almost no response. Nevertheless, these background noise values were used to normalize (by subtraction) the results obtained for the other strains screened.

The production results obtained with the reference strain ATCC 21332 show the effectiveness of the detection method described herein. It will be noted a good match of quantifications in the LB medium at around 0.015 g/L while a larger difference is observed in the Landy's medium between the two methods of quantification. Indeed surfactin production was measured at 0.38 g/L by RP-UPLC and at 0.44 g/L by the CPC-FL method. For the other five strains analyzed here, it can observe initially that two of these do not produce lipopeptides (strains A and E). For these two strains, the two quantification methods gave similar results, which are very close to the background noise obtained for the strain 168. This result is confirmed by the MALDI-ToF analysis, where no characteristic peak of the three families of lipopeptides (surfactins, iturins or fengycins) was detected (data not shown). For the other three strains, isolated from environmental sample, the production of surfactin was successfully quantified by both the CPC-FL and RP-UPLC to be in the range of 0.029 g/L to 0.053 g/L in the LB medium. The difference between the two methods of quantifications was not judged as statistically significant. These results were also confirmed for strain B in the modified Landy's medium. Nevertheless, for strains C and D, the quantification obtained by RP-UPLC after cultivation in the modified Landy's medium is very different from that obtained by the CPC-FL method. These results seem to reveal the production of other compounds which can interfere in the assay. One of the explanations would be an interference caused by primary metabolites resulting from the degradation of glucose that is present in Landy medium and not in the LB, such as acetate and lactate. These two compounds are very often

produced by *B. subtilis* under low oxygen transfer, as is the case of liquid growth on microtiter plates [8,27]. To verify this, measurements were made with different concentrations of acetate and lactate (between 0 and 0.5 g/L) using the CPC-FL complex. However, no fluorescein release was observed in the presence of these compounds (data not shown). Therefore, it is possible to infer that the difference between the UPLC results and the fluorescence-based quantification of surfactin, using a glucose based medium is due to production of additional primary or secondary metabolites by specific strains during cultivation. Interestingly, Yang et al., seem to have detected a similar pattern, when developing their method with CPC-BTB and using glucose based medium. This conclusion is based on the authors' specification that the optimal range for quantification, using the CPC-BTB method, is between 0.1 and 0.5 g/L [17]. This information suggests that other metabolites may give a corresponding false response at a concentration of 0.1 g/L. In addition to that, if longer incubation periods are to be analyzed, one must consider the contribution given by fengycins, which are additional anionic lipopeptide produced by *Bacillus* strains. In our studies, we found that this other biosurfactant give less sensitive response using the CPC-FL method, possibly because surfactin has 2 negative charges in comparison with fengycin. Finally, to validate our screening method further analysis was carried out by MALDI-ToF-MS. The analysis was performed directly on isolated colonies for species classification and also to determine their lipopeptide production pattern. For strains B, C and D, the characteristic peaks (m/z) of surfactin ([M + Na]$^+$ 1030, 1044, 1058, [M + K]$^+$ 1074) were found as shown for example for the strain B (Fig 4). Identification results also show that these strains belong to the species of *Bacillus subtilis*, *Brevibacillus borstelensis*, *Bacillus licheniformis* and *Bacillus oleronius* (data not shown). All these species belong to the *Bacillus* genus and are known to produce lipopeptides of the surfactins family such as surfactins, pumilacidins or lichenysins.

4   Concluding remarks

A novel analytical method was described that is very efficient to screen new lipopeptides producing strains. The method allows the detection and quantification of lipopeptides, such as surfactins, from environmental isolates in a single run. This completely automated method can be used also to collect kinetic data on surfactin production, study novel medium compositions or select novel biosurfactant producers, without requiring an expensive and time-consuming procedure such as liquid chromatography. The CPC-FL method was based on a previously described procedure using the dye BTB and it was shown to be more sensitive and present better linear response, besides it was adapted to a completely automated system and validated by RP-UPLC analysis. A parallel analysis of the CPC-FL detection method and RP-UPLC revealed the potentialities of the described method for screening novel surfactin-producing strains. This was also corroborated using MALDI-ToF, which selects bacterial strains by their lipopeptide producing capacity. Nevertheless, special attention must be taken if medium highly enriched in a carbon source is used, which enables the cell to grow well and thus produce other molecules that can interfere in the assay. The use of a fluorescent reporting dye also opens opportunities for the CPC-FL method to be applied directly during the growth in micro-fermentation devices such as the Biolector® (from m2p-labs), which has inbuilt fluorescence detector using similar excitation and emission filters. To evaluate this, further studies will have to be conducted because CPC is toxic to microbial cells. Our studies also have shown that the MIC of CPC is approximately 0.1 mM.

Conflict of Interest   The authors declare that they have no conflict of interest.

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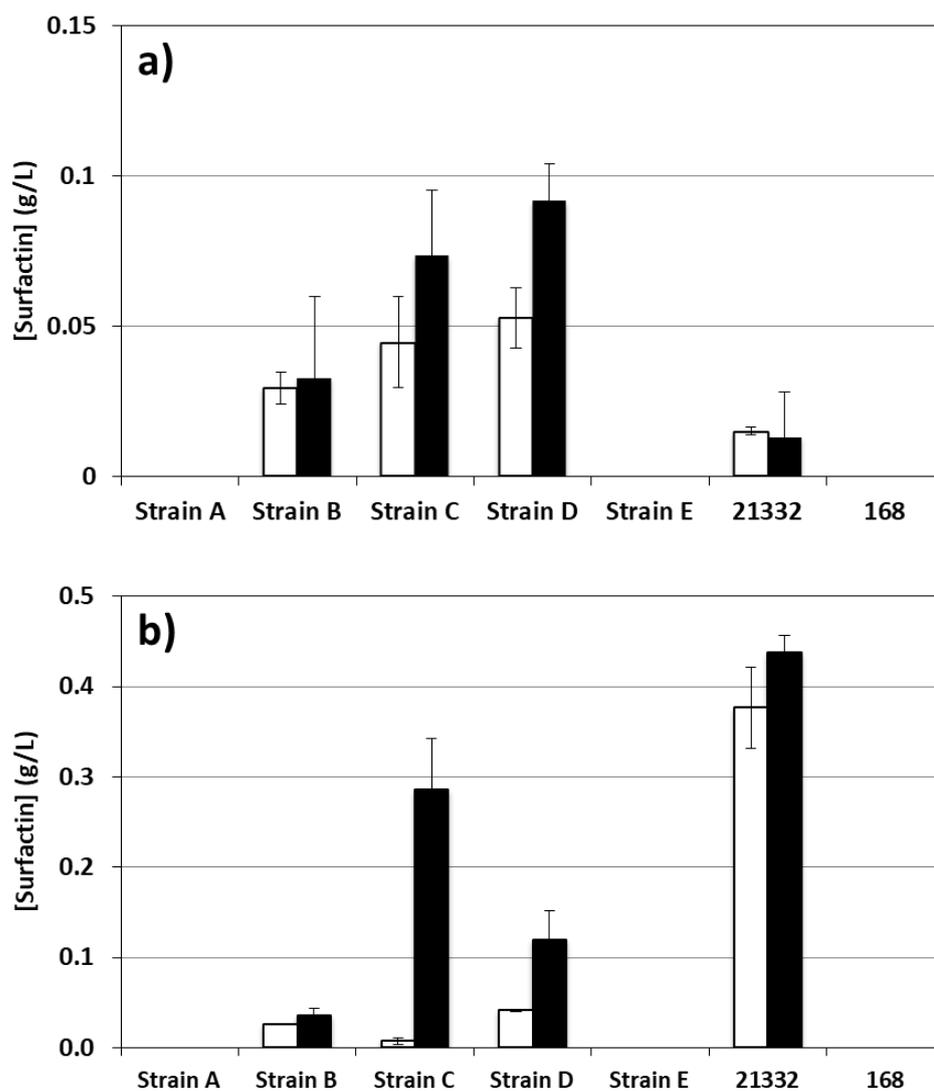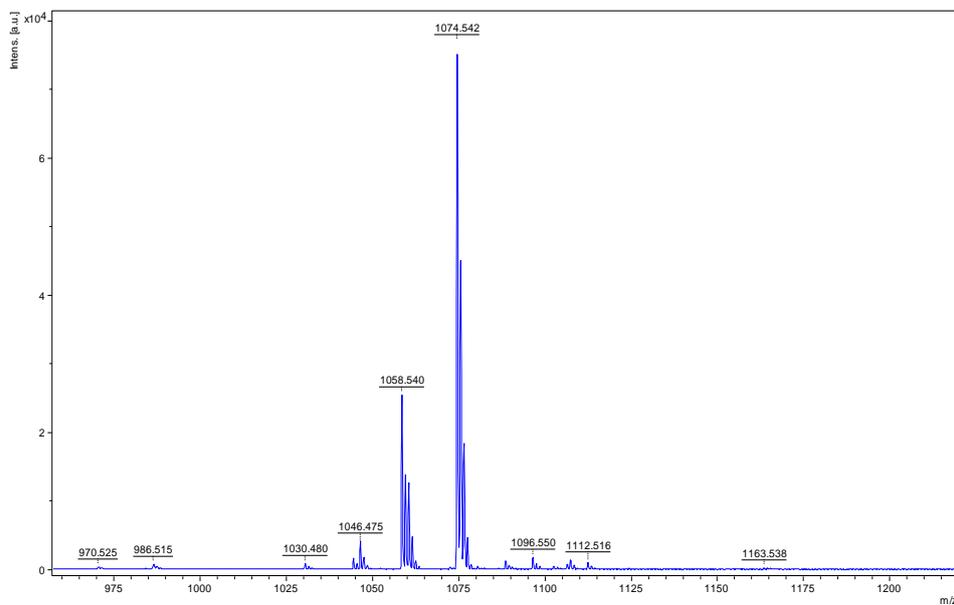
Figures
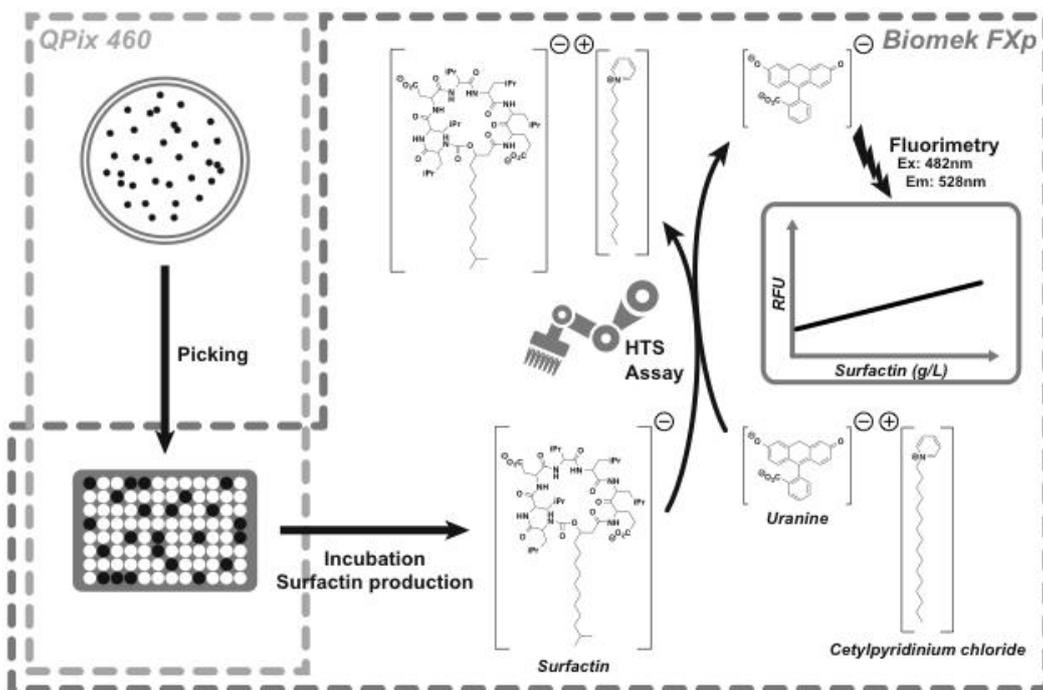
Figure 1. Complete workflow for high-throughput screening of strains producing anionic lipopeptides such as surfactin. The workflow is divided in three steps: firstly colony picking; followed by incubation on microtiter plates and quantification based on fluorescein displacement from the CPC-FL complex.
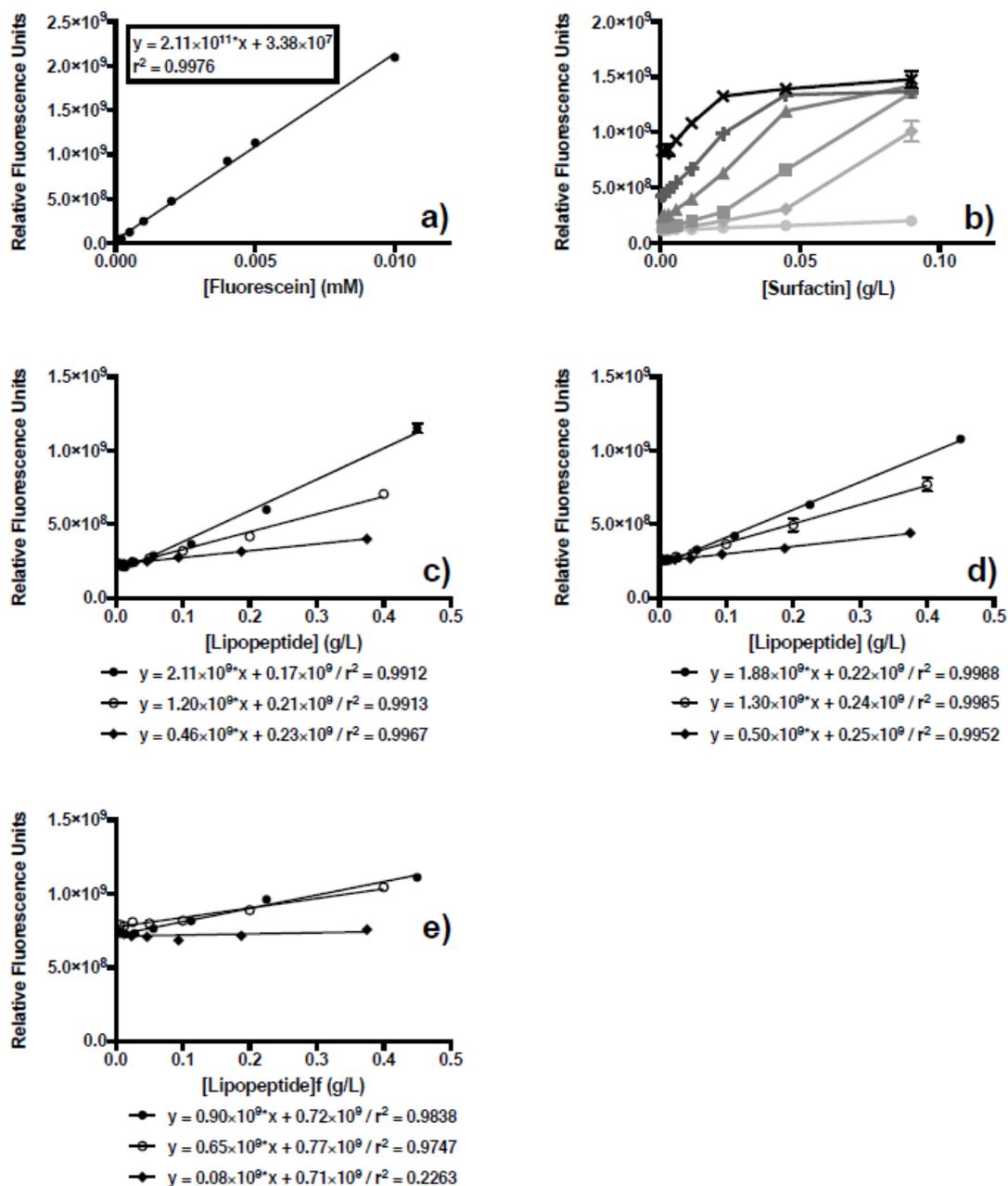
Figure 2. a) Fluorescein calibration curve. b) Relative fluorescence units measured in function of surfactin and CPC ($\times$ 0.045 mM; + 0.068 mM; ▲0.090 mM; ■ 0.135 mM; ◆ 0.180 mM; ● 0.360 mM) final concentrations, with 0.009 mM of FL. c) Lipopeptides calibration curves in water. d) Lipopeptides calibration curves in modified Landy's medium. e) Lipopeptides calibration curves in LB medium. Mean values and standard deviation are presented (n=9).

Figure 3. Quantification of surfactin produced by the different strains after 24 h of growth in (a) LB medium and (b) modified Landy's medium at 37°C and under agitation (200 rpm) using CPC-FL method (black bars) and compared to RP-UPLC method (white bars). Background noise values obtained in the case of the 168 strain's fermentation broth (0.0098 ± 0.005 g/L in LB medium and 0.039 ± 0.011 g/L in modified Landy's medium) were used to normalize (by subtraction) the results obtained for the other strains. Mean values and standard deviation are presented (n=9).

Figure 4. MALDI-ToF-MS analysis of the lipopeptides produced by the environmental strain B. The MS signals were acquired in reflectron mode in the 700–3,500 Da m/z range by summing 8000 laser shot spectra