Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D3.4

Strain(s) with the ability for biosurfactant production and suitability for large scale production and possible application to the environment

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1 About this deliverable

The contribution of University of Ulster (**UU)** is to evaluate strains from a collection of specialized microorganisms available at Ulster, for the production of biosurfactants that will be able to accelerate oil dispersion and emulsification and consequently, hydrocarbon bioavailability to microbial degradation. Until now, more than 10 strains were tested and the physicochemical properties of the produced biosurfactants were evaluated.

The screening activity carried out in WP4 by **UNIBO** during the first 12 months of the project allowed to select, among 25 isolates from desert sand and inland saline system in the south of Tunisia already adapted to high salinity, low nutrient availability and/or low water activity conditions, the strain *Bacillus subtilis* R39 as the most promising biosurfactant producer (see Deliverable D4.1). A screening of different carbon sources evidenced that growth of strain R39 and production of surface active molecules was higher and faster on glucose rather than on crude glycerol, soybean, maize and peanuts oils (see Deliverable D3.1).

The report at hand summarises the work performed by partner Actygea towards an up-scaling of the production of rhamnolipids and sophorolipids aiming at :

- Sufficient supply of material for micro- and mesocosm experimentation
- Determining the technical and economic feasibility of the production of the above biosurfactants in view of an *in*-field application.

2 Introduction

At **UU** three strains of *Pseudomonas* (*aeruginosa* PAO1, ST5 and DS10), four species of *Burkholderia* (*thailandensis, glumae, kururiensis* and *plantarii*), a *Bacillus* strain and also two yeast strains *Candida bombicola* and *Pseudozyma aphidis* were examined. These strains are producing mainly low molecular weight biosurfactants (rhamnolipids, sophorolipids and mannosylerythritol lipids) except for *Bacillus* that is producing higher molecular weight lipoproteins biosurfactants called surfactin.

The main activities of UNIBO as presented in section 5 were:

- to identify the best nitrogen source and C/N ratio for the cultivation of the isolate *Bacillus subtilis* R39 and
- to identify the optimal cultivation conditions of the same isolate in fermenter for the production of surface active molecules.

In addition, marine isolates were screened for the capability to produce surface active molecules, in order to further select biosurfactant producers (see DL 3.1 – month 24).

The evaluation of biosurfactants and bioemulsifiers of microbial origin suitable for the large scale production was carried out by **ACTY** based on the following principles:

- products already on the market for cleaning and environmental applications
- environmentally friendly (biodegradable, low toxicity)
- economically feasible due to the low cost requested and large volumes required.

Based on the above principles, rhamnolipids were selected for their effectiveness at low concentrations and for the relative ease of production. Furthermore, commercial applications for rhamnolipids were already described in recent literature with high rates of success in scaling up processes (Randhawa and Rahman, 2014; Irfan-Maqsood and Seddiq, 2014; Samsu *et al*., 2014). The

second category of biosurfactants was sophorolipids for their action of sinking agents for oil and for their ease of production and purification (Silva *et al*., 2014**;** Weber, *et al*., 2012).

3 Experimental set and analytical methods

3.1 Evaluation of various strains for biosurfactant production

3.1.1 Biosurfactant production

In Ulster University, all strains were maintained at −80°C in glycerol stocks. Fermentations were performed in either shake flasks (200 rpm) or bioreactors (1-L DASGIP and 5-L BIOSTAT fermenters). Biosurfactants were produced in mineral salt medium (MSM) with composition (in g/L) $NaNO_3$, 2.0; Na_2HPO_4, 0.9; KH_2PO_4, 0.7; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001; trace element solution, 1.0 ml. The trace element solution was prepared as follows (g/L): $ZnSO_4 \cdot 7H_2O$, 0.70; $CuSO_4 \cdot 5H_2O$, 0.50; $MnSO_4 \cdot H_2O$, 0.50; H_3BO_3, 0.26; $MoNa_2O_4 \cdot 2H_2O$, 0.060. It was supplemented with glycerol, glucose or soybean oil as carbon source. Experiments with various carbon substrates are in progress.

3.1.2 Biosurfactant analysis

During the fermentations performed at Ulster, bacterial growth was monitored by measuring optical density OD (600 nm) and the biosurfactant synthesis by measuring the reduction of the surface tension du Noüy ring method with a Krüss tensiometer (Helvac, et al., 2004). After the completion of the fermentation process microbial cells were removed and solvent extractions were carried out in order to obtain the crude extract of biosurfactants (Smyth et al. 2010), which was gravimetrically determined. For sample purification solid phase extraction (SPE) cartridges were used before the HPLC-MS analysis. Using this technique excess carbon sources, produced fatty acids or other impurities that are co-extracted with the biosurfactants can be removed. The measurement of the Emulsification Index (EI_{24}) was performed with kerosene as solvent.

For the detection of the produced biosurfactants ESI-MS Quadrupole ion-trap analysis was carried out. All samples were dissolved in methanol at a concentration of ~0.5 g/L and injected directly in the system. The mass spectrum provides the molecular ions of the main congeners in its sample as a verification of their production. For a more detailed analysis HPLC-MS was carried out for rhamnolipids and sophorolipids. Characterization was carried out using the LCQ quadropole ion trap mass spectrometer (Finnigan MAT, San Jose, California, USA) utilizing ESI connected to a thermospectra HPLC system by a Luna C18 column (250 mm x 4.6 mm x 5 mm) for rhamnolipid analysis and Gemini C18 column (250 mm x 4.6 mm x 5 mm for sophorolipids. Water (mobile phase A) and acetonitrile (mobile phase B) were used at an initial concentration 70% A and 30% B which was gradually raised to 70 % mobile phase B after 40 minutes.

3.1.3 Optimisation conditions for biosurfactant production by Bacillus subtilis R39

3.1.3.1 Selection of the N source and C/N ratio for biosurfactant production with R39 strain

The growth and production of biosurfactants in UNIBO by *Bacillus* strains has been reported to be remarkably affected by the Carbon/Nitrogen (C/N) ratio (Fonseca *et al.*, 2007). Since *B. subtilis* R39 strain was previously grown only with sodium nitrate (SN) 2g/L as Nitrogen (N) source in the presence of glucose 10g/L as C source (corresponding to a Carbon/Nitrogen ratio of 13), experiments in flasks were conducted in order to assess the effect on growth and biosurfactant production of (i) the concentration of nitrogen source in terms of Carbon/Nitrogen (C/N) ratio, and (ii) the use of an

inorganic or of an organic nitrogen source. Lower C/N ratios were tested by employing sodium nitrate or urea as nitrogen source at increasing initial concentrations, while keeping the glucose concentration constant (Table 1). The medium employed (MSMg) was a minimum salt medium (pH 7.0) containing KH_2PO_4 (0.7 g/L), Na_2HPO_4 (0.9 g/L), $MgSO_4 \bullet 7H_2O$ (0.4 g/L), $CaCl_2 \bullet 2H_2O$ (0.1 g/L), NaCl (5 g/L), $FeSO_4 \bullet 7H_2O$ (4 mg/L), $MnSO_4 \bullet 1H_2O$ (2.3 mg/L), $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ (0.12 mg/L), glucose (10 g/L) as carbon source and different concentrations of $NaNO_3$ or urea as nitrogen source (Table 1). Incubations were at 30°C and 150rpm with monitoring of microbial growth (OD_{600}), glucose concentration (HPLC-RID), Emulsification Index (EI_{24}) with toluene as solvent and drop collapse activity (DC) on parafilm (scores from 0 to 3, where 0 corresponded to the drop collapse of distilled water and 3 of 0.5% SDS solution in distilled water).

Table 1 Conditions of cultivation of strain R39 in flask

Condition	N source	N concentration (g/L)	N source concentration (g/L)	C/N Ratio
SN13	$NaNO_3$	0.3	2.0	13
SN8	$NaNO_3$	0.5	3.1	8
SN3	$NaNO_3$	1.4	8.5	3
SN1	$NaNO_3$	3.0	18.5	1
Ur13	Urea	0.3	0.7	13
Ur8	Urea	0.5	1.1	8

3.1.3.2 Optimization of R39 cultivation and biosurfactant production under fermentation conditions

Batch cultivations of strain R39 were performed in UNIBO in a Biostat®B bioreactor (Sartorius) consisting of a 2L working volume jacketed borosilicate vessel equipped with two Rushton turbines (6 paddles each) for stirring, temperature, pH and dissolved oxygen controls. The media used were either the MSMg described above including $NaNO_3$ 2g/L as nitrogen source, or a medium with an identical composition except for the concentrations of phosphates which were incremented (KH_2PO_4 and $Na_2HPO_4 \bullet 7H_2O$ 9.32 g/L and 32.7 g/L, respectively), to increase the buffer capacity of the medium at pH 7 (MSMgP). Temperature was controlled to 30°C. One L medium was inoculated at 5% (v/v) with a culture grown in MSMg medium at 30°C, 150 rpm. Some preliminary tests were performed by growing the inoculum from a single colony in 50 mL of MSMg medium for 30h (one-step inoculum growth). The remaining tests were performed by growing the inoculum first from a single colony in 20 mL of MSMg medium for 40h, that was then transferred (5% v/v) in 50mL MSMg medium, grown until the OD_{600} was between 1.5 and 2.0 (i.e., for about 18-20h) and diluted in MSMg to a final OD_{600} of 1.7 before being used to inoculate the bioreactor (two-step inoculum growth).

Growth was monitored via the measurement of the optical density at 600 nm (OD_{600}) in a Varian Cary100 double beam spectrophotometer; glucose concentration in the culture supernatant was measured with an HPLC Infinity1260 system equipped with a refractive index detector and a Hi-Plex H column (Agilent Technologies), while the accumulation of surface active molecules in the supernatant was monitored via the Emulsification Index (EI_{24}) with toluene as solvent, the drop collapse test (DC) on parafilm and the Interfacial tension (IFT) with a contact angle DSA130 instrument equipped with Drop Shape analysis software in “pendant drop” mode. Distilled water and a 0.5% (w/v) SDS solution were used as negative and positive controls, respectively, in all tests.

3.2 Up-scale fermentations

Although several media and protocols for the production of biosurfactants have been described, neither the fermentation times nor the composition of the medium warrant an economic feasibility of industrial production. The first step of large-scale production was the selection of an industrially suitable medium (*e.g.* low cost and sufficient production of rhamnolipids), which was achieved by use of the database ActyMedDat (Figure 1). Downstream from the selection of a suitable industrial medium, the use of the medium was tested in the scaling up to pilot scale bioreactors. Finally, the produced compounds were tested for stability and were delivered to the other partners for micro- and mesocosm applications. The flowchart of the process of production is described in Figure 2.

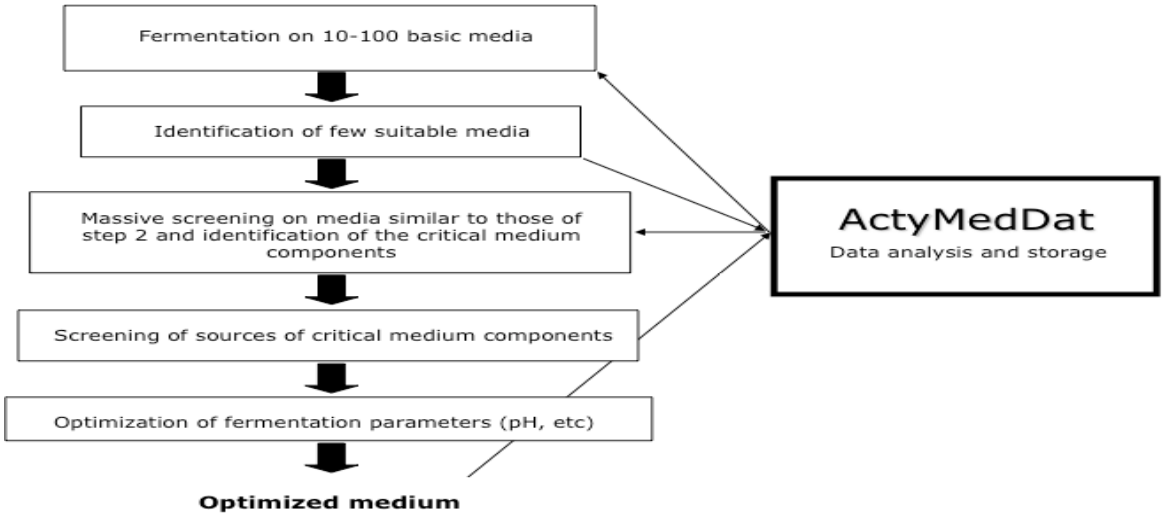


Figure 1 Schematic description of the approach of the ActyMedDat protocol

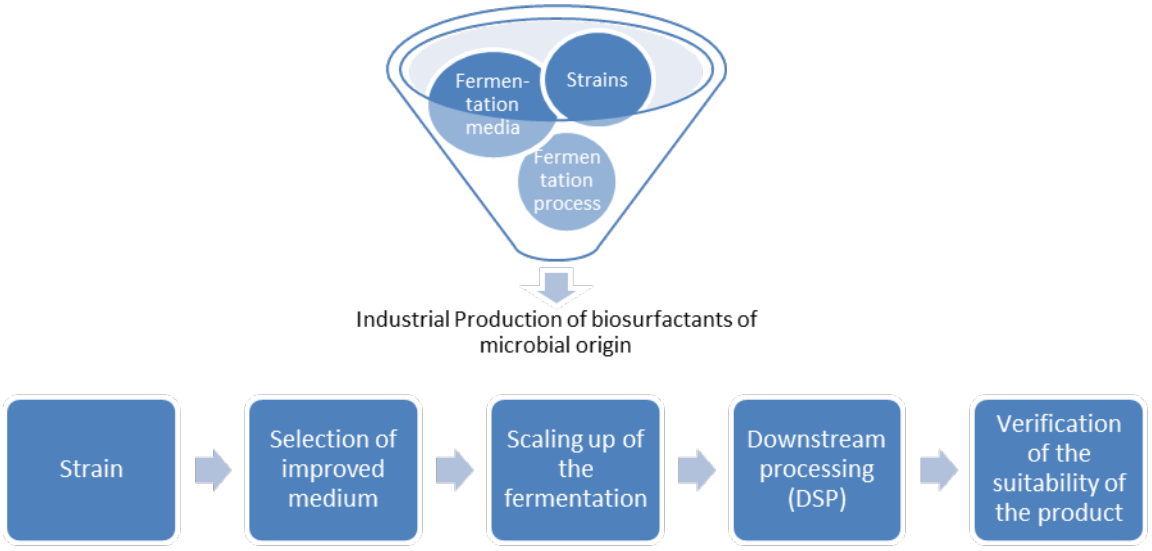


Figure 2 Flowchart of the upscaling approach used by Actygea

By the date of production of this report only two strains had been fully processed by this approach (see section 6). *Pseudomonas aeruginosa* DS10, and *Candida bombicola* were received from the

University of Ulster, and are able to produce rhamnolipids and sophorolipids respectively (biosurfactants). Reference productivity in flask was *ca.* 1.5 g/L for strain DS10 (Data from the University of Ulster) and *ca.* 5 g/L of rhamnolipids in bioreactor (Ibrahim Banat, personal communication). *Candida bombicola* was able to produce from 3 to 20 g/L (depending on the carbon source used in fermentation) of sophorolipids in flask fermentation. Productivity reported for small scale bioreactors was around 100 g/L (Konstantina Tsaousi, personal communication).

4 Biosurfactant producing strains and physicochemical characterisation of their products [UU]

From a collection of specialised microorganisms available in Ulster and able to produce biosurfactants 10 strains were examined. In Table 2 are presented the results of the produced biosurfactants of each strain and their physicochemical properties.

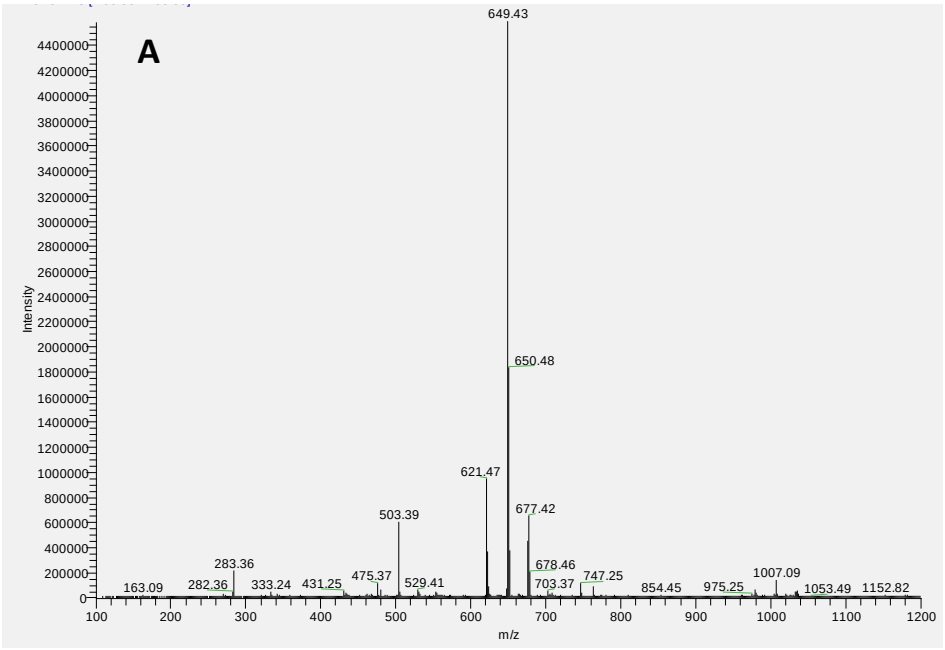
Table 2 Biosurfactant producing strains; EI: emulsification index

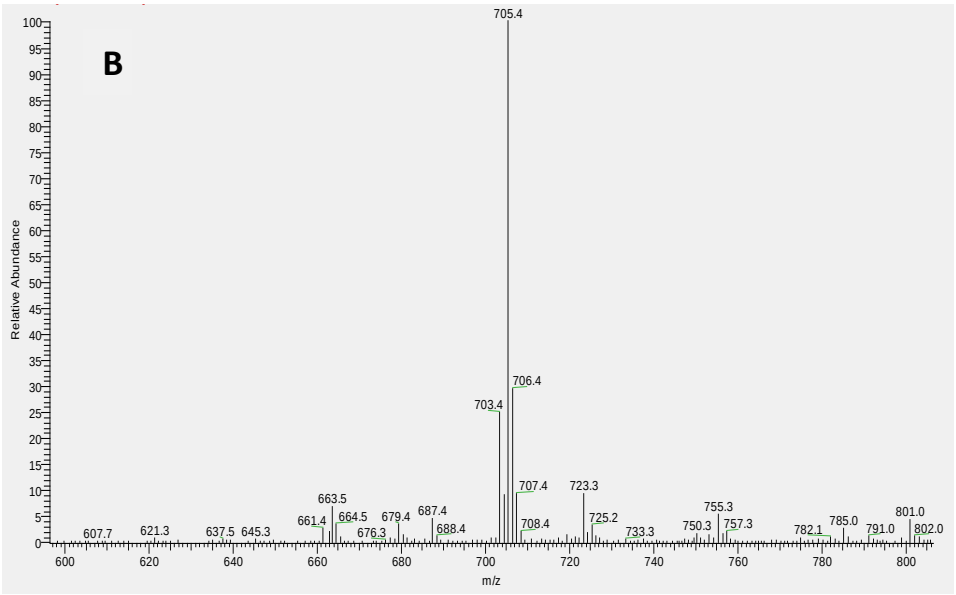
Strain	Biosurfactant (main*)	Yield (g/L)	Surface tension (mN/m)	EI $_{24\ kerosene}$(%)
Pseudomonas aeruginosa PAO1	Rhamnolipids (Rha-Rha-C_{10}-C_{10})	2-3	24-29	53-64
Pseudomonas aeruginosa ST5	Rhamnolipids (Rha-Rha-C_{10}-C_{10})	1.5-2.5	25-29	52-65
Pseudomonas aeruginosa DS10	Rhamnolipids (Rha-Rha-C_{10}-C_{10})	1.5-3	25-31	52-61
Burkholderia thailandensis E264	Rhamnolipids (Rha-Rha-C_{14}-C_{14})	2-4	26-33	67-70
Burkholderia glumae	Rhamnolipids (Rha-Rha-C_{14}-C_{14})	1.3-2.2	28-30	65-72
Burkholderia plantarii	Rhamnolipids (Rha-Rha-C_{14}-C_{14})	0.8-1.1	26-27	60-67
Burkholderia kururiensis	Rhamnolipids (Rha-Rha-C_{14}-C_{14})	1.9-3.1	30-33	60-63
Candida bombicola	Sophorolipids	20-100	36-39	48-53
Pseudozyma aphidis	Mannosylerythritol lipids	**	**	**
Bacillus sp.	Surfactin	0.2-0.8	24-31	58-65

*Most abundant congener produced by each strain.
** The production of mannosylerythritol lipids (MEL's) by *Pseudozyma aphidis* were detected by ESI-MS analysis after Solid Phase Extraction (SPE), though the measurement of the produced amount and the evaluation of their physicochemical properties is still difficult since MEL's are contained in the oil phase and the solvent extraction is not efficient.

All fermentations were carried out, initially in 1L flasks and then in 5-L bioreactor, in MSM with the following composition in g/L: $NaNO_3$, 2.0; Na_2HPO_4, 0.9; KH_2PO_4, 0.7; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001; trace element solution, 1.0 mL. The trace element solution was prepared as follows (g/L): $ZnSO_4 \cdot 7H_2O$, 0.70; $CuSO_4 \cdot 5H_2O$, 0.50; $MnSO_4 \cdot H_2O$, 0.50; H_3BO_3, 0.26; $MoNa_2O_4 \cdot 2H_2O$, 0.060. Glycerol was used as carbon source in *Pseudomonas, Burkholderia* and *Bacillus* fermentations, soybean oil in *Pseudozyma* fermentations and glucose for sophorolipids production. Solvent extractions were carried out at the end of the fermentations. For the detection of the produced

biosurfactants ESI-MS analysis are performed. *Pseudomonas aeruginosa* species are producing on average 2.5 g/L of a mixture of mono- and di- rhamnolipids (Figure 3A), which are reducing the surface tension to 24-29 mN/m and have an emulsification index of 52-65 %. *Burkholderia* is producing also a mixture or rhamnolipids (Figure 3B) that consist mainly by di-rhamnolipids with slightly higher emulsification index (60-72 %). The crude extract yield of sophorolipids produced by *Candida bombicola* (Figure 3C) is in the range of 20-100 g/L. The production of mannosylerythritol lipids (MEL's) by *Pseudozyma aphidis* were detected by ESI-MS analysis (Figure 3D) after Solid Phase Extraction (SPE), though the measurement of the produced amount and the evaluation of their physicochemical properties is still difficult since MEL's are contained in the oil phase and the solvent extraction is not efficient. *Bacillus* is producing surfactin, which is a high molecular weight cyclic lipopeptide (Figure 3E). The low yield that is produced by this strain could be improved by the use of other carbon sources.





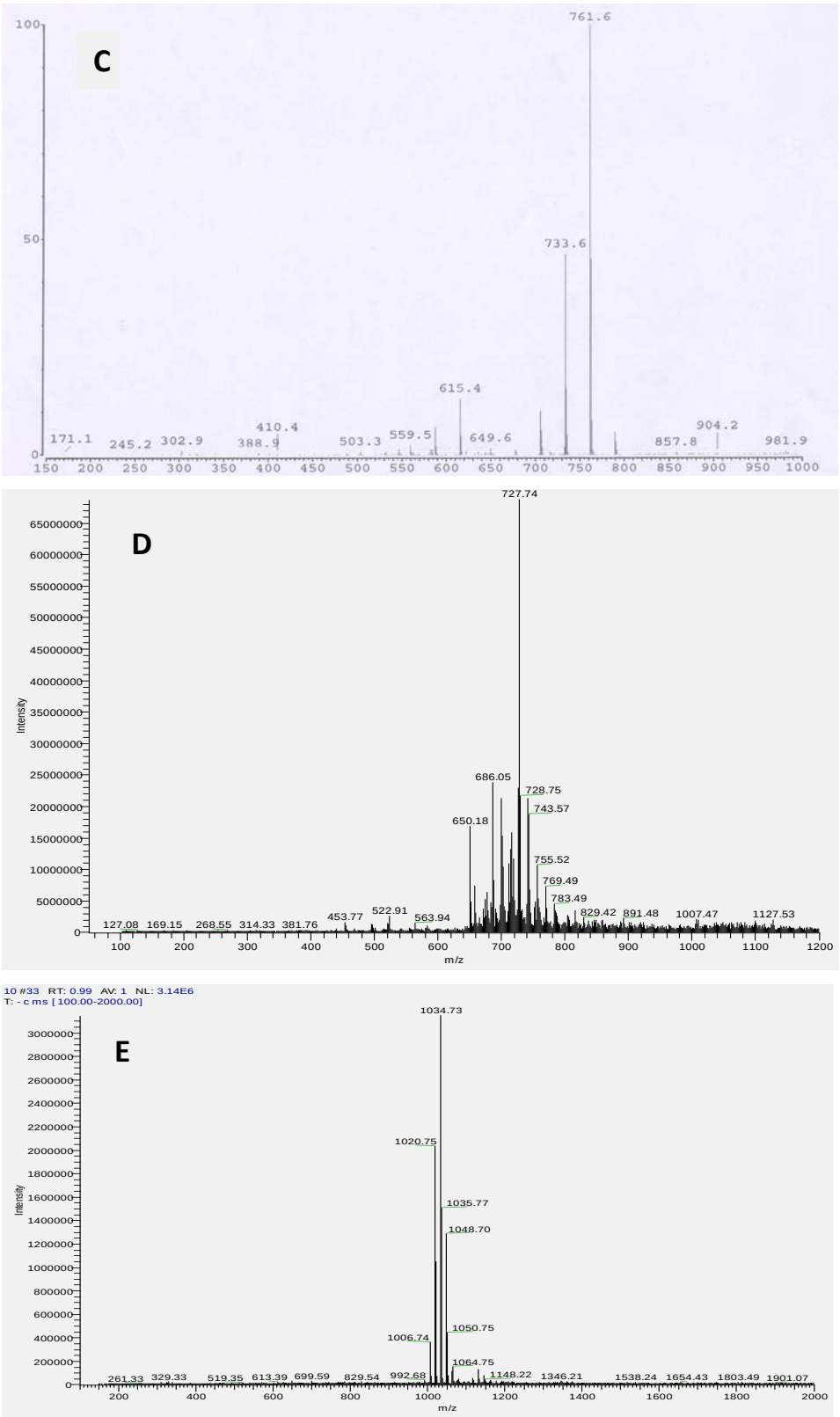


Figure 3 **ESI-MS analysis for the detection of the produced biosurfactants**
(A) rhamnolipids by *P.aeruginosa*, (B) rhamnolipids by *Burkholderia,* (C) sophorolipids by *C. bombicola,* (D) mannosylerythritol lipids by *P. aphidis* and (E) surfactin by *Bacillus*

Pseudomonas aeruginosa ST5, *Pseudomonas aeruginosa* DS10 and *Candida bombicola* strains had been delivered to ACTYEA to develop an optimised and up-scaled production of biosurfactants.

5 Biosurfactant production with *Bacillus subtilis* R39 strain [UNIBO]

5.1 Effect of the N source and C/N ratio

At decreasing C/N ratio, both the growth and biosurfactant production progressively decreased (Figure 4, left: "SN"). In particular, SN13 (sodium nitrate at C/N ratio 13) was the only condition under which biosurfactants were produced leading to 20% EI_{24} and to a drop collapse scored 1 (data not shown); in all other conditions no EI_{24}, nor any drop collapse effect occurred. When urea replaced sodium nitrate as the sole nitrogen source (Figure 4, right: "Ur"), no significant effect of C/N ratio was evident on growth, although growth was lower than with sodium nitrate. Differently from what observed with sodium nitrate, the biosurfactant production in the presence of urea increased at lower C/N value. However, while also in this case drop collapse never exceeded a score of 1, the EI_{24} with urea was 16%, that is substantially lower than 20% occurring in SN13. According to these results, the employment of sodium nitrate 2.0 g/L together with glucose 10g/L, i.e. with a C/N ratio of 13, resulted in the highest production biosurfactants; therefore these conditions were used to optimize the fermentation process.

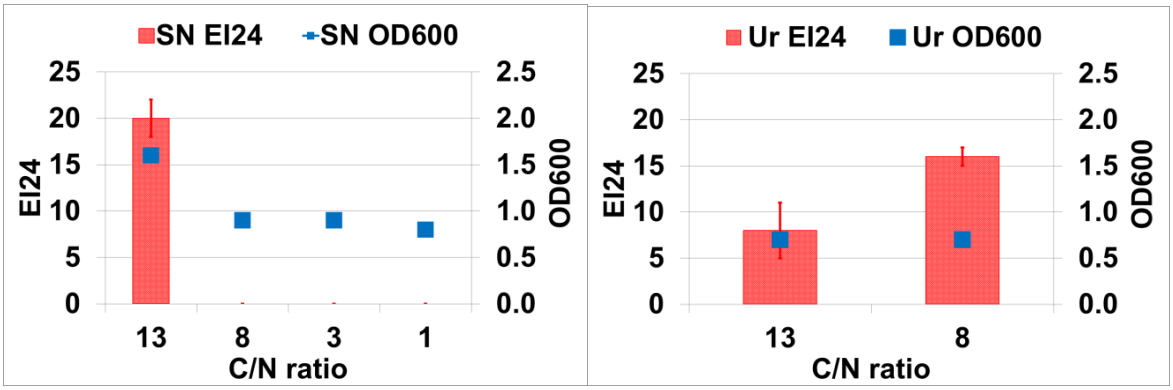


Figure 4 Biosurfactant production (EI24) and growth (OD600) of strain R39 after 24h cultivation on different N source (sodium nitrate "SN", urea "Ur") and C/N ratios

5.2 Optimisation of cultivation and biosurfactant production in fermenter

The conditions used and the results obtained in terms of time required to reach the end of the exponential growth phase (according to OD_{600}, glucose consumption and dissolved oxygen concentration) and the recorded OD_{600}, pH, drop collapsing activity (DC), Emulsification Index (EI_{24}) and foam formation are summarized in Table 3 (IFT values are not reported, since no significant decreases in IFT were observed, compared to the negative control, under any condition). In particular, the following parameters were primarily investigated, as they are known to influence the physiology of microorganisms and the rheological properties of the medium (Hsu and Wu, 2002):

- the growth of the inoculum (one-step or two-step growth),
- the dissolved oxygen (DO) concentration in the medium (not controlled, 95% and 60% of saturation),
- the strategies for oxygen supplementation (air, pure oxygen, air enrichment with oxygen), and
- the control of pH (none, via supplementation of acid and alkaline solutions, or by increasing the buffering capacity of the medium).

The standardization of the inoculum is needed for both for the reliability and repeatability of the results obtained; in addition, the inoculum should guarantee a prompt set on of growth phase in the bioreactor to limit operational costs and increase productivity. In aerobic fermentation oxygen at low concentrations can become the limiting factor, thus influencing the growth rate of the microorganism and its physiological state (Chopra *et al.,* 2015; Yezza *et al.,* 2004). This is crucial in the production of primary metabolites such as biosurfactants produced by strain R39.

The control of pH might be decisive in maintaining the best conditions for biosurfactant productions (Hsu and Wu, 2002). Biosurfactants lead to foaming, which in turn hampers the maintenance of defined conditions and complicate downstream applications (Amani *et al.,* 2010). The salinity or ionic strength of the medium can control foam production (Rizzo *et al.,* 2014), and so does the mechanical effect of aeration and stirring (Hoeks *et al.,* 2003). The additional supplementation of oxygen might influence both the rheology of the system and the physiology of the biomass and has noteworthy impact on the costs of the process. All the values set in the reported experiments were in the range of those used in analogous scale up studies (Chopra *et al.,* 2015; Shuckla *et al.,* 2001; Hoeks *et al.,* 2003; Yezza *et al.,* 2004).

Foam formation and pH, stirring and air flow profiles during representative fermentations are represented in Figure 5.

Table 3 Conditions of operation and results (strain R39 growth and biosurfactant production) of each fermentation

Test	F1	F2	F3	F4	F5	F6	F7	F8	F9
Inoculum preparation	1-step growth		2-steps growth						
pH control	7.0[(a)]	7.0[(a)]	7.0[(a)]	None [(b)]	Buffered medium [(c)]	None [(b)]	Buffered medium [(c)]	Buffered medium [(c)]	7.0[(a)]
O_2 Conc. (% sat.)	n.c.[(d)]	95%[(e)]	n.c.[(d)]	60%[(e)]	60%[(e)]	60%[(e)]	60%[(e)]	60%[(e)]	60%[(e)]
Gas	Air	Air	Air	Air	Air	O_2	O_2	Air+O_2	O_2
End exp. growth (h)	48	42	24	21	22	23	24	22	22
Final OD_{600}	2.6	2.1	2.4	2	2.5	2.4	2.5	2.5	2.1
Final pH	7.1	7	7	8.8	7.2	8.3	7	7.4	7.4
Final DC[(f)]	2	1	0	0	1	1	0	2	1
Final EI (%)	10±4	44±1	42±1	35±5	55±2	53±2	55±2	52±1	59±5
Foam[(g)]	+++	+++	++	+++	-	++	+	-	+++

(a): addition of acid and alkaline solutions to non-buffered MSMg during fermentation
(b): No addition of acid and alkaline solutions to non-buffered MSMg during fermentation
(c): use of MSMgP medium with high buffering capacity with no addition of acid and alkaline solutions during fermentation
(d): n.c. – not controlled; constant stirring rate (300 rpm) and air flow (0.3 vvm)
(e): cascade control of stirring rate (300 to 700 rpm) and gas flows: air (0.3 to 1.0 vvm), pure oxygen (0.02 to 0.06 vvm) or pure oxygen and air (0 to 0.05 vvm and 0.3 to 1.0 vvm, respectively). Stirring had the priority over gas flow. When the gas purged was composed of pure oxygen and air the first had the priority over the latter.
(f): scores from 0 (same as negative control, i.e. distilled water) to 3 (same as positive control, i.e. 0.5% SDS)
(g): see Figure 5 for representative foam levels

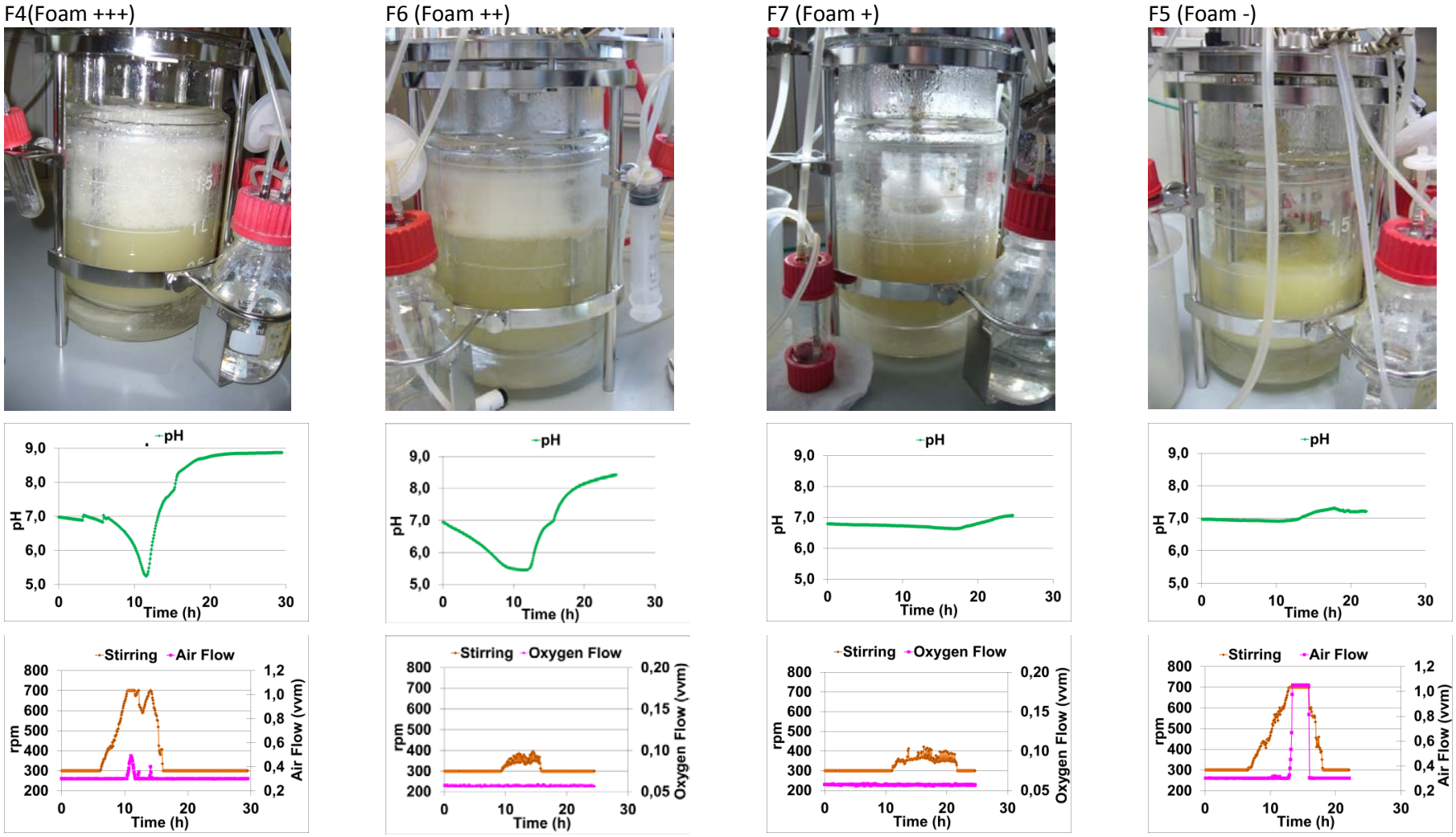


Figure 5 **Foam accumulation, pH, stirring and gas flow profiles of 4 representative fermentations**

Fermentations performed using the inoculum grown with the one-step procedure, pH controlled at 7.0 and air purging (F1, F2) resulted in long lag phases (15-20h) with exponential growth phase ending after 42-48h, when production of surface active molecules was evident from drop collapse activity, EI_{24} and the accumulation of a very thick layer of foam above the medium. Among these, slightly shorter lag phase and earlier end of exponential growth phase were observed in F2, were dissolved oxygen concentration was maintained at 95% saturation with air, indicating a faster growth. Maintenance of high dissolved oxygen concentration (95%, F2) also resulted in a slightly lower drop collapse activity and a remarkably higher EI_{24}, suggesting that a mixture of different surface active molecules may be produced under different growth conditions.

The use of a two-step growth of the inoculum was performed in all the following fermentations. The use of such an inoculum in fermentation F3 under the same conditions of F1 (pH 7.0, air purging with no control of dissolved oxygen concentration) brought forward the onset of the exponential growth-phase by reducing the lag-phase down to 8-10h and the fermentation time to 24h. Despite no drop collapse effect was observed, a relevant increment of EI_{24}, taking place during exponential growth-phase, occurred along with large foam accumulation. Since dissolved oxygen concentration during exponential growth phase was 0%, an additional fermentation was performed under the same conditions by setting the dissolved oxygen concentration control to 60% of saturation with air. However, the large formation of foam on the surface medium interfered with pH control, causing an uncontrolled large addition of acid and alkaline solutions and the abortion of the process (data not shown). To avoid process abortion and to assess the effect of pH regulation, pH was not controlled in fermentation F4, while keeping dissolved oxygen concentration control to 60% of saturation with air. Growth occurred rapidly, while pH progressively decreased down to 5.2 in the first 10h of cultivation and subsequently increased to 8.8 during exponential growth phase. A huge foam layer accumulated in the headspace of the vessel during the exponential growth-phase, accompanied by EI_{24} increase to 35% at the end of the process.

A different strategy for pH control was then adopted in the following fermentation (F5), where medium MSMgP, having a strong buffering capability at pH 7.0, was employed in combination to dissolved oxygen concentration control at 60% of saturation with air. This resulted in a higher EI_{24} (55%) and, most importantly, in a negligible foam accumulation. A fermentation was also performed by purging pure oxygen instead of air to control dissolved oxygen concentration at 60% saturation in a not buffered medium (F6), in order to evaluate if reduced gas flows allow to reduce foam formation. The pH trend was the same as in F4, first decreasing to approximately 5 and then rising above 8. A high EI_{24} was reached and a limited reduction in foam accumulation was achieved. Thus, additional fermentations were performed with MSMgP by purging either oxygen or oxygen-enriched air (F7, F8). Results were comparable with those obtained in F5 both in terms of EI_{24} (52-55%) and foam accumulation (none or negligible). Finally, to confirm that foam reduction is mainly due to the occurrence of high phosphates concentration in the medium (use of MSMgP) rather than pH control itself or low gas purging flows, an additional fermentation was carried out controlling the pH with the ordinary pH control system and purging pure oxygen in the low phosphate medium F9. While a high EI_{24} was obtained (59%), a remarkable layer of foam accumulated.

The results obtained suggest that the **use of strongly buffered medium seems to be a most promising approach to limit foam production and assure a significant biosurfactant production,** whatever the gas purged (air, oxygen or oxygen-enriched air). It is possible that the higher ionic strength supplied by MSMgP was responsible for the containment of foam. Future work will aim to characterizing the biosurfactants produced and to further optimize the fermentation process to increase production (e.g., fed-batch addition of substrate).

6 Large scale production [ACTYGEA]

6.1 Suitability for large scale production: selection of an industrial medium (ActyMedDat) for the production of rhamnolipids

In the first year of activity, *Pseudomonas aeruginosa* strains ST5 and DS10 were chosen based on their performance on different fermentation media (data not shown). From this screening, strain DS10 was the one, which most fitted the industrial production. Strain DS10 was fermented on sixteen different fermentation media selected from the database ActyMedDat (according to the principles of the database ActyMedDat). From this first screening, productivities ranging from 1.5 g/L (control) to 16 g/L of rhamnolipids were detected (see Figure 6 for partial results of screening). The results reported in Figure 1Figure 6 show that a high number of industrial media available in the database ActyMedDat are suitable for the production of rhamnolipids. In particular, the strain was able to reach a maximum productivity of rhamnolipids of 16-17 g/L, which is comparable to the level of the control sample (K, 19 g/L). However, among the selected media, some show the production of potential antimicrobial agents (not shown), indicated by a blue-green colour in the culture, which should be avoided in a preparation for *in*-field application.

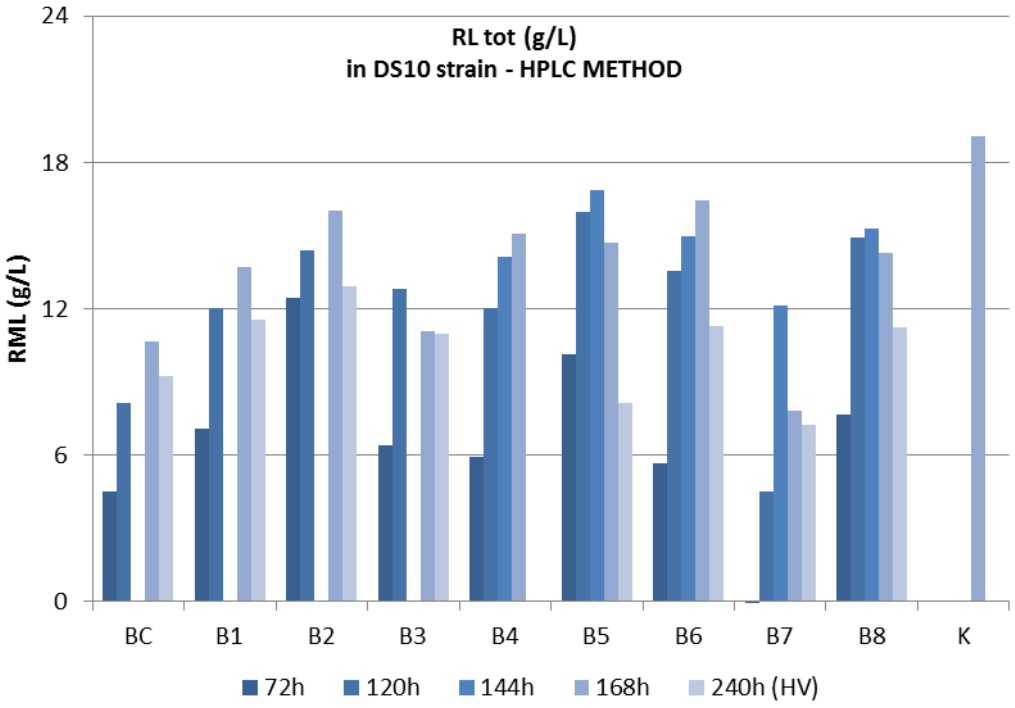


Figure 6 **Production of rhamnolipids in strain *Pseudomonas aeruginosa* DS10 after different cultivation times in different media from the database ActyMedDat**
All media were added with 40 g/L soybean oil. Rhamnolipid concentration in fermentation broths was estimated by HPLC and quantified by comparison to a commercial standard (Sigma-Aldrich). As control, a commercial preparation of rhamnolipids for industrial purposes was used (K).

Therefore, media indicating potential production of antimicrobial agents were discarded and the remaining media were analyzed according to the ActyMedDat protocol. The analysis of the selected media evidenced a particular importance of the peptone component on productivity. The second step of the ActyMedDat approach identified medium Acty333 as the best performer in rhamnolipid production with peaks reaching 20-25 g/L in flask fermentation (Figure 7).

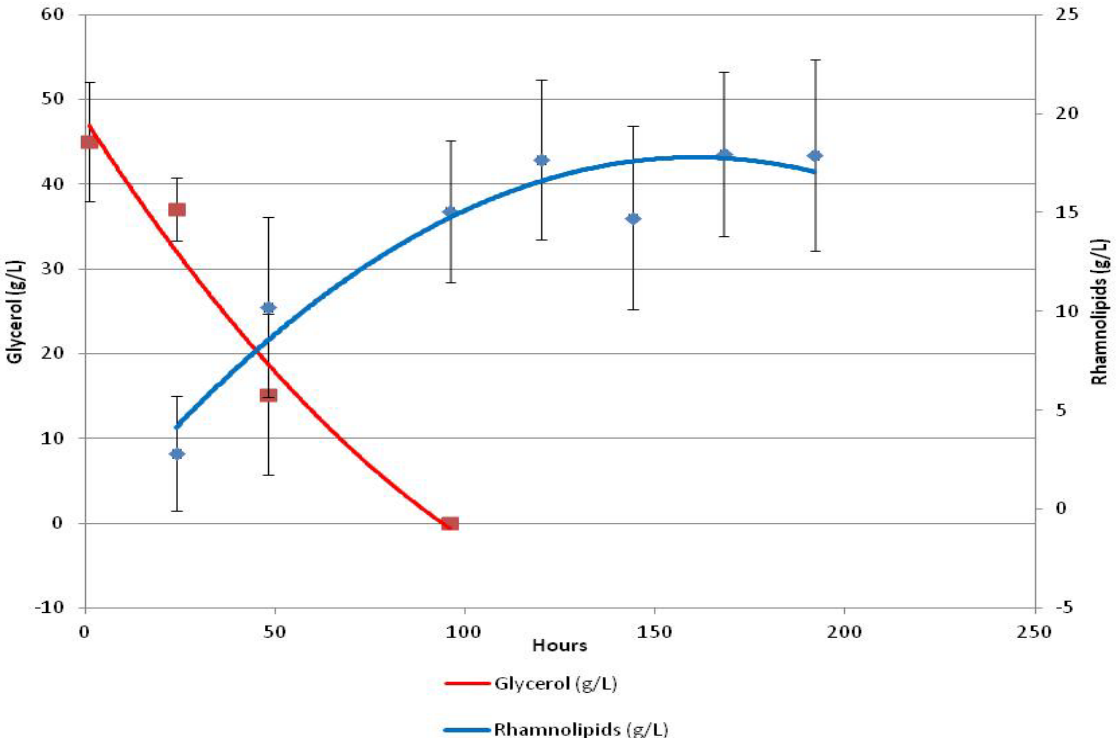


Figure 7 Typical rhamnolipid production trend for strain DS10 in medium Acty333

6.2 Suitability for large scale production: selection of an industrial medium (ActyMedDat) for the productionof sophorolipids

It is reported in the literature, that sophorolipids are preferentially produced (or production is directly induced) in the presence of oil in the cultivation medium (De Oliveira M.R., *et al;* 2014). The cultivation of *Candida bombicola* was therefore preferentially performed on media containing oil. The first screening identified medium Acty218 as able to induce production of sophorolipids at a concentration of *ca.* 50 g/L in flask fermentation (Figure 8). The second screening of media showed that the production of sophorolipids by *Candida bombicola* was optimal if the main carbon source was glucose, while glycerol hampered the production of sophorolipids (Figure 8).

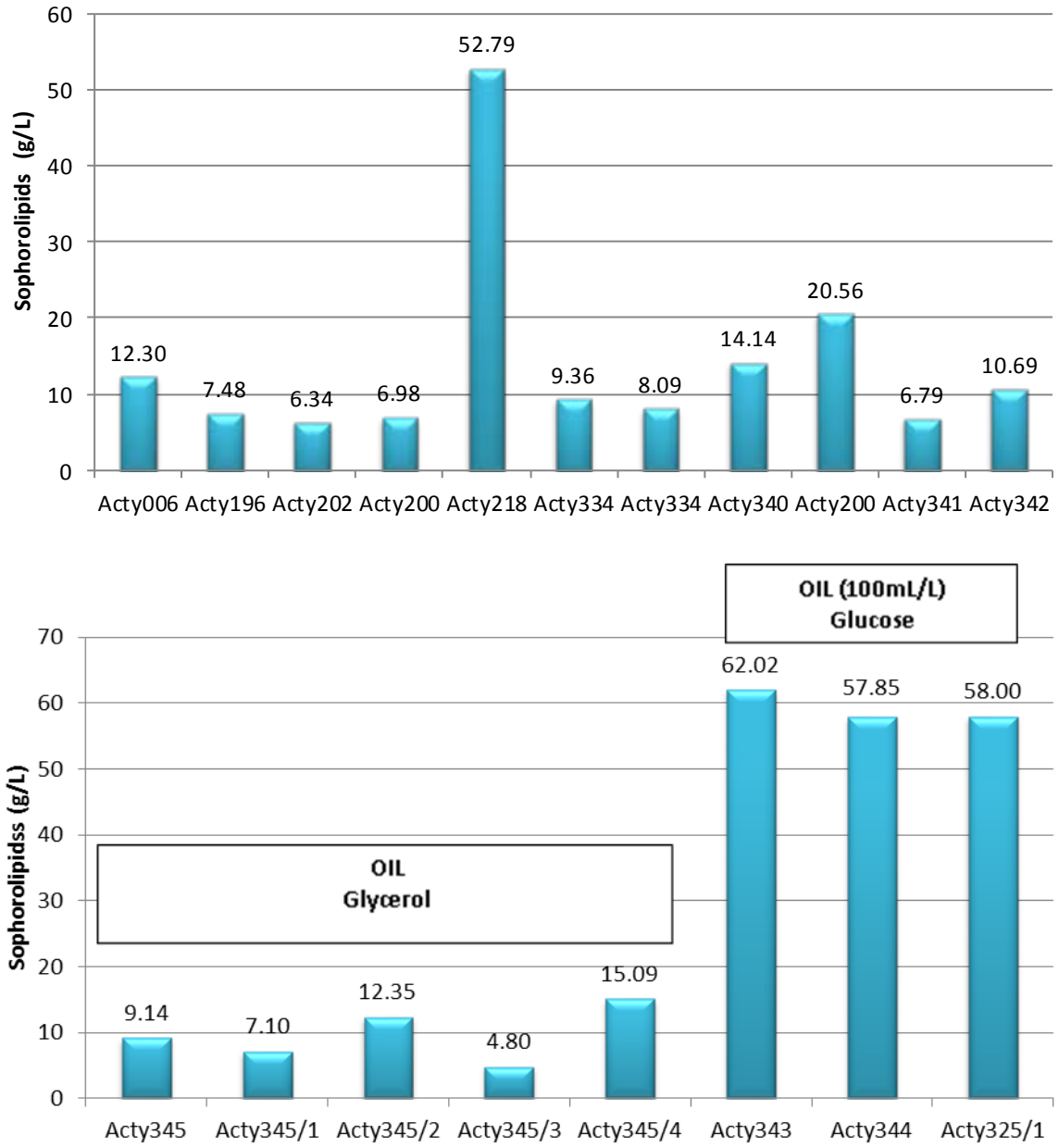


Figure 8 **Maximum sophorolipid production in strain *Candida bombicola* in different media of the database ActyMedDat**

Sophorolipids were efficiently produced if glucose was added to the medium as a ready to use carbon source, while glycerol hampered production. Sophorolipid concentration in fermentation broths was determined using HPLC.

A typical in-flask fermentation trend is reported in Figure 9. Medium Acty343 with the addition of 100 g/L soybean oil was therefore chosen for scaling-up into bioreactors.

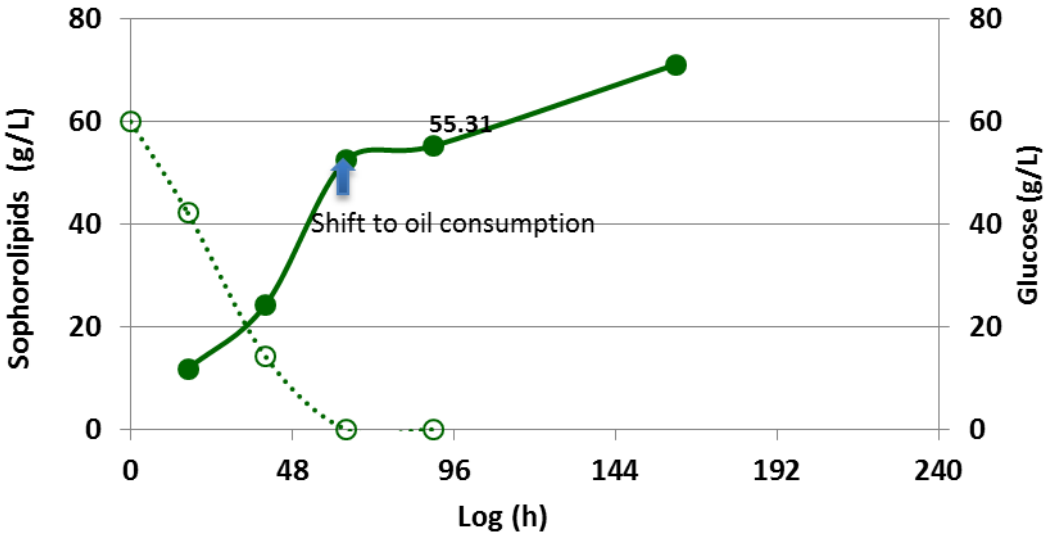


Figure 9 **Time course of Sophorolipid production (continuous line) and Glucose consumption (dotted line) in medium Acty343**

6.3 Suitability for large scale production: Scaling up of the production to the 30 L scale and identification of a suitable downstream processing method

The conditions for pilot scale fermentation (30 litres) were identified and were deemed acceptable. These conditions were extensively described in the deliverable D3.7.

For the preparation of rhamnolipids and sophorolipids for application into the environment, pathogenicity and production of antimicrobial agents were considered when dealing with *P aeruginosa*. Antimicrobial compounds (which are typically synthesized by *P. aeruginosa* (Sorensen RU1, and Klinger JD. 1971) and which appear as a bluish colour in the media (not shown) were reduced by selecting those media which did not show pigmentation during fermentation. The pathogenicity of the strains, together with the limitation imposed on the release in the environment of microorganisms, was further solved by sterilizing the fermentation broth at the end of fermentation. The same approach was applied to sophorolipids and the results were comparable (data not shown). For more details on the downstream processing refer to the description of deliverable D3.7.

6.3.1 Functional testing of the produced rhamnolipids and sophorolipids

The control of stability and efficacy of the products scaled to industrial pilot bioreactors and processed as above was performed by use of a standard Emulsification test according to Cooper and Goldenberg (1987). The test was performed using hexadecane and the Dansk Blend as the standard hydrocarbons and crude rhamnolipid or sophorolipid preparations (fermentation broths) as the source of biosurfactants. Samples were diluted 200 fold and the maintaining of the emulsification was observed at 1 day (24 hours). As summarized in Table 4, the rhamnolipid preparation, showed an emulsification of *ca*. 60% during 24 hours (dilution 200 fold, corresponding to *ca*. 0.08 g/L of rhamnolipids referred to the aqueous phase). The control sample (K) showed a lower emulsification (52%). The observation at 7, 14 and 30 days showed that the optimal dilutions for a long lasting emulsification, were in the range 100-200 fold (data not shown). Sophorolipids, showed an

emulsification of *ca*. 60% during 24 hours (dilution 200 fold, corresponding to *ca*. 0.4 g/L of sophorolipids referred to the aqueous phase).

Table 4 Emulsification index of the rhamnolipid and sophorolipid preparations

Sample	Hexadecane EI (%)	Crude Oil EI (%)	Notes
Rhamnolipids Actygea	63	57	Undiluted with crude oil
K	52	NA	Undiluted with crude oil
Sophorolipids Actygea	55	63	Undiluted with crude oil
SC2000	65	65	Undiluted with crude oil
SuperSolv	65	50	Undiluted with crude oil

Besides comparison with the commercial rhamnolipid preparation (K), samples produced in Actygea were compared also with the commercial surfactants SuperSolv and SC2000 (BioBased Europe). With hexadecane, the commercial surfactants displayed a stable emulsification index in all the considered dilutions (up to 600 fold), which lasted till the end of the observation period (30 days) (data not shown). The emulsification index was also calculated using Dansk Blend as the organic phase. The rhamnolipid preparations were effective only if undiluted. The undiluted samples were as effective as the SC2000 control, while performed better than the SuperSolv product (Table).

6.4 Outlook

The comparison of the quality and price of rhamnolipids and sophorolipids with marketed products of the same type revealed that rhamnolipids produced in Actygea are in the range of price proposed by the different suppliers. Concerns still remain on the purity of the rhamnolipids and on production with *Pseudomonas aeruginosa* (even if heat inactivated). Production with non-pathogen strain is advisable. Sophorolipids are produced at cheaper prices compared to Actygea. Investigation on how to reduce production prices still remains an issue. A more detailed analysis is provided in deliverable D3.7.

Several producers are investing in proprietary microorganisms both, “newly identified” or genetically improved - by means of the old mutagenesis and selection method, or through recombinant approaches. Concerning rhamnolipids, the potential for a real commercialisation is good since it is economically feasible and the fermentation process is relatively easy to perform. The selection of new derivatives of the DS10 strain could give further impulse to a good product. The use of new strains which have already been identified in the Kill•Spill consortium bears potential for the production of innovative rhamnolipids.

Concerning sophorolipids, the production is economically reasonable but still has a good margin of improvement. The same considerations about new strains as mentioned above apply also for sophorolipids.

The potential of the surfactants produced till now, stands in the fact that they are well known and characterized, and have a consistent story of success which warrants no limitation on their use for on-site applications.

In conclusion, the industrial production is considered feasible for these two classes of compounds and the compounds have been delivered to the partners of the project described in Table 5.

Table 5 Beneficiaries of the produced rhamnolipids and sophorolipids

Name of the partner	Amount of rhamnolipids / sophorolipids delivered (grams)
University of Copenhagen	30 (rhamnolipids)
Technical University of Crete (TUC)	15 (rhamnolipids)
University of Milan "Degli Studi"	7.5 (rhamnolipids)
University of Ulster	3.0 (rhamnolipids) 9.0 (sophorolipids)

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