BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Production of surfactin and fengycin by *Bacillus subtilis* in a bubbleless membrane bioreactor

**François Coutte · Didier Lecouturier ·
Saliha Ait Yahia · Valérie Leclère · Max Béchet ·
Philippe Jacques · Pascal Dhulster**

Abstract Surfactin and fengycin are lipopeptide biosurfactants produced by *Bacillus subtilis*. This work describes for the first time the use of bubbleless bioreactors for the production of these lipopeptides by *B. subtilis* ATCC 21332 with aeration by a hollow fiber membrane air–liquid contactor to prevent foam formation. Three different configurations were tested: external aeration module made from either polyethersulfone (reactor BB1) or polypropylene (reactor BB2) and a submerged module in polypropylene (reactor BB3). Bacterial growth, glucose consumption, lipopeptide production, and oxygen uptake rate were monitored during the culture in the bioreactors. For all the tested membranes, the bioreactors were of satisfactory bacterial growth and lipopeptide production. In the three configurations, surfactin production related to the culture volume was in the same range: 242, 230, and 188 mg l^{-1} for BB1, BB2, and BB3, respectively. Interestingly, high differences were observed for fengycin production: 47 mg l^{-1} for BB1, 207 mg l^{-1} for BB2, and 393 mg l^{-1} for BB3. A significant proportion of surfactin was adsorbed on the membranes and reduced the volumetric oxygen mass transfer coefficient. The degree of adsorption depended on both the material and the structure of the membrane and was higher with the submerged polypropylene membrane.

F. Coutte · D. Lecouturier (✉) · S. Ait Yahia · V. Leclère ·
M. Béchet · P. Jacques · P. Dhulster
ProBioGEM, Laboratoire de Procédés Biologiques,
Génie Enzymatique et Microbien, Polytech'Lille,
Univ Lille Nord de France, USTL,
Avenue du Professeur Langevin,
F-59655 Villeneuve d'Ascq Cedex, France
e-mail: didier.lecouturier@univ-lille1.fr

Introduction

Biosurfactants are potential future candidates to replace chemically synthesized surfactants in the food, cosmetics, health care industries (Desai and Banat 1997), and agricultural chemicals (Ongena and Jacques 2008). *Bacillus subtilis* produces three families of biosurfactant lipopeptides, i.e., surfactin, iturin, and fengycin or plipastatin, depending on the strains (Bonmatin et al. 2003; Ongena and Jacques 2008). Surfactin is one of the most powerful biosurfactants known (Deleu et al. 1999, 2003; Peypoux et al. 1999). It has several advantages over chemical synthetic surfactants, such as low critical micellar concentration (CMC) and biodegradability. It is especially suited to environmental applications such as bioremediation and dispersion of oil spills (Peypoux et al. 1999; Deleu et al. 2003; Kosaric 2005; Mulligan 2005). Surfactin also displays antiviral and antimycoplasmic activities (Vollenbroich et al. 1997). Fengycin and plipastatin are strong antifungal compounds (Ongena et al. 2005). Moreover, surfactin and fengycin are able to induce systemic resistance in plants (Ongena et al. 2005; Ongena et al. 2007), which makes them interesting for plant protection.

The production of lipopeptides by *B. subtilis* in bioreactors is commonly described (Cooper et al. 1981; Davis et al. 2001; Guez et al. 2007). *B. subtilis* is considered to be a strict aerobe, but several groups have demonstrated that this bacterial species is able to grow even with a severely limited oxygen supply. Typical indicators of this fermentative

process were lactate and 2,3-butanediol (Nakano et al. 1997). It was found that the supply of sufficient dissolved oxygen (DO) played a crucial role in the efficiency of lipopeptides production. This is particularly the case for surfactin production (Hbid et al. 1996; Davis et al. 1999; Jacques et al. 1999). Guez et al. (2008) recently showed that the synthesis of the lipopeptides surfactin and mycosubtilin in *B. subtilis* ATCC 6633 depends closely on the oxygen supply.

Both the rapid stirring and the aeration required to supply sufficient quantities of oxygen for aerobic growth generate high amounts of foam. Foam can be suppressed by the addition of chemical antifoams, but the latter may affect the strain physiology and hinder the recovery of pure products (Lee and Kim 2008). In several bioprocesses, foam formation was exploited for lipopeptide extraction (Guez et al. 2007), leading to a high yield of extracted lipopeptide in small bioreactors. However, this technique encounters several problems when scaled-up: high volume of foam collected and the use of intense cooling processes. In this work, an alternative way of producing lipopeptide without foam formation was developed.

A conventional fermenter was modified by integrating a hollow fiber membrane air–liquid contactor as the aeration system, thus avoiding air sparging and keeping the lipopeptides in the broth. A membrane-based contacting device is often composed of a hydrophobic microporous membrane with the gas flowing on one side and the liquid flowing on the opposite side of the fiber without either phase being dispersed into the other one. The pores remain gas-filled; oxygen is transported through the pores by gaseous diffusion (Gabelman and Hwang 1999; Pankhania et al. 1999). Membrane contactor technologies have been developed essentially with hollow fibers (Ahmed and Semmens 1996; Gabelman and Hwang 1999) and have been used for different applications, such as blood oxygenation (Khoshbin et al. 2005), wastewater treatments (Brindle and Stephenson 1996; Pankhania et al. 1999; Wei et al. 2006), or for bubble-free oxygenation of animal cell cultures (Schneider et al. 1995), but have never been used for biosurfactant production.

In the present work, the production of surfactin and fengycin by *B. subtilis* was carried out for the first time in a bubbleless bioreactor using a hollow fiber membrane as air–liquid contactor. Three different configurations were tested: external aeration module in polyethersulfone (reactor BB1) or in polypropylene (reactor BB2) and submerged module in polypropylene (reactor BB3). Growth, glucose consumption, lipopeptide production, and volumetric mass transfer coefficient of oxygen (K_La) were analyzed. Surfactin adsorption on the external hollow fiber membrane and its influence on the K_La were also established.

Materials and methods

Microorganism and preculture conditions

For each culture and inoculum, *B. subtilis* ATCC 21332 was taken from a −80°C frozen stock (Gancel et al. 2009) and transferred onto solid Luria–Bertani medium. A single colony was withdrawn and inoculated into a 500-ml flask containing 50 ml of Landy's medium (Guez et al. 2008), then incubated at 30°C with shaking at 130 rpm for 8 h. This first preculture was then used to inoculate a second series of precultures which were carried out in 1-l flask containing 100 ml of Landy's medium at 30°C and shaken at 130 rpm. Cells were harvested at the end of the exponential growth phase when the optical density at 600 nm (OD_{600nm}) ranged between 4 and 6, centrifuged 10 min at 5,000×g and washed once with saline solution containing 0.9% of NaCl. The pellet was then resuspended in 50 ml of Landy's medium, and *B. subtilis* cells were used to inoculate 2.5 or 3 l of Landy's medium at a starting OD_{600nm} of about 0.2.

Bioreactor setup and batch fermentation conditions

Four bioreactor designs were developed for lipopeptide production using Bioflo 3000 bench-top fermenters from New Brunswick Scientific (New Brunswick Scientific, Edison, MA, USA). The software used for controlling the process and acquiring data was AFS Biocommand from New Brunswick Scientific. One bioreactor was used with gas sparging aeration, designated as Foaming Bioreactor (FB; Fig. 1), and the other ones with either an external hollow fiber membrane aeration module, designated as Bubbleless Bioreactor (BB1; BB2; Fig. 1) or submerged hollow fiber membrane aeration (BB3; Fig. 1). Two different external membrane modules were used for aeration. A polyethersulfone one, supplied by GE Health-care, was used for the BB1 (pore size 0.65 μm; area 2.5 m²; Amersham Biosciences Corp., Westborough, USA), and a polypropylene one, supplied by MEDOS, was used for the BB2 (pore size 0.2 μm; area 1.9 m²; Hillite 7000, MEDOS Medizintechnik AG, Stolbeg, Germany). A polypropylene submerged hollow fiber module, supplied by Spectrum Labs, was used for the BB3 (pore size 0.05 μm; area 0.375 m²; JM Separations, HG Eindhoven, The Netherlands). The characteristics of membranes and bioreactors are listed in Table 1. In the case of BB1, the culture flowed inside of the hollow fibers at 0.021 m s⁻¹. Air pressure on the shell side was 0.5 barg. In the case of BB2, the culture flowed outside of the hollow fibers at 0.011 m s⁻¹, and air flowed inside of the fiber and was freely vented to the atmosphere. For these two external membrane modules, the pressure drop between inlet and

Fig. 1 Schematic representation of bioreactors used in this study for the production of lipopeptides by *B. subtilis*. *FB* foaming bioreactor, *BB1* and *BB2* bubbleless bioreactor 1 and 2 using an external hollow fiber membrane module, *BB3* bubbleless bioreactor 3 using a submerged hollow fiber membrane. *A* air inlet, *B* air outlet
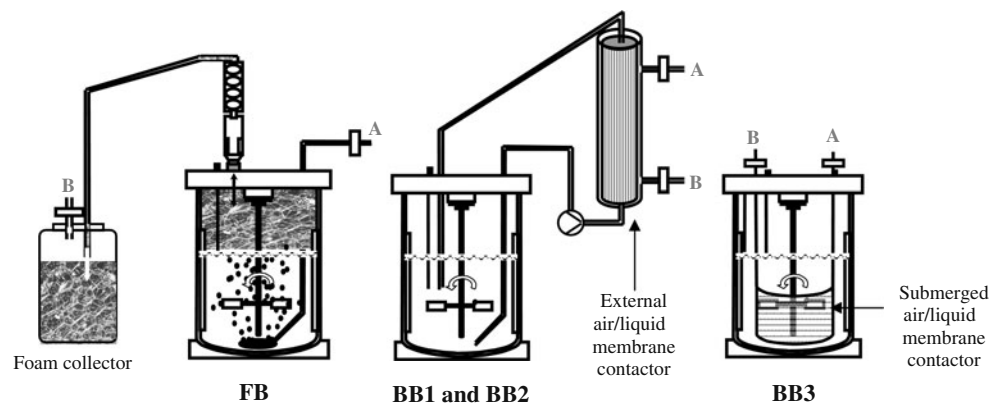
outlet of the culture broth in the membrane was negligible (<0.1 barg). In the case of the BB3, over pressure in the bioreactor was 0.1 barg. Membranes and all connections were sterilized at 121°C for 20 min. All bioreactor cultures were carried out at 30°C, and the aeration rate was fixed at 1 vvm. The pH was maintained at 7 with an automatic addition of either 3 M NaOH or 0.66 M H_3PO_4 solution to the bioreactor. The experiments were repeated twice.

Membrane washings

After the fermentation, the various membranes were washed twice with 3 l of distilled water at pH 7 and 30°C for 1 h and twice with 3 l of 0.1 M NaOH solution at pH 10 and 50°C for 1 h. The value of K_La was measured after the first washing at 30°C, then again after the first washing at 50°C. After each washing, a sample was analyzed by high performance liquid chromatography (HPLC) to quantify the surfactin or fengycin desorbed from the membrane. Finally, the membranes were regenerated with a 0.5 M NaOH treatment at 50°C during 1 h, then with a 1.3 mM NaOCl at 50°C during 1 h, and rinsed with distilled water at 20°C until the pH reached about 7, and then stored at 4°C. The

efficiency of the membrane cleaning was checked by measuring the K_La value.

Analytical methods

The optical density was measured at 600 nm with a SECOMAN Prim spectrophotometer (SECOMAN, Domont, France). Total biomass was determined by dry weight measurement (D.W.).

Lipopeptide production was quantified by HPLC. Samples of fermentation broth and of washing solutions were centrifuged at 13,000×g for 15 min at 4°C. The lipopeptides were quantified in the supernatant. The samples were filtered through 0.2-μm cellulose filters and diluted with 100% MeOH before analysis by HPLC (Waters Corporation, Milford, MA, USA) using a C_{18} column (5 μm, 250× 4.6 mm, VYDAC 218 TP, Hesperia, CA, USA). Analyses of lipopeptides were performed as described in Gancel et al. (2009).

Glucose, lactate, and 2,3-butanediol concentrations were analyzed in the filtered samples by HPLC Spectra SYSTEM P1000 XR supplied by Thermoelectron Corporation (Thermo Fisher Scientific Inc., Waltham, MA, USA) using a Fast Fruit Juice column (150×7.8 mm, WATERS) according to Guez et

Table 1 Fermentation and bioreactors characteristics

Characteristics	Foaming bioreactor (FB)	Bubbleless bioreactor 1 (BB1)	Bubbleless bioreactor 2 (BB2)	Bubbleless bioreactor 3 (BB3)
Supplier	–	GE-Healthcare	Medos	Spectrum Labs
Membrane area (m²)	–	2.5	1.9	0.375
Pore size (μm)	–	0.65	0.2	0.05
Fiber composition	–	Polyethersulfone	Polypropylene	Polypropylene
Membrane structure	–	External module	External module	Submerged fibers
Agitation speed (rpm)	160	160	160	500
Medium volume (l)	3	3	3	2.5
Broth flow velocity (m s⁻¹)	–	0.021	0.011	–
Air flow side	–	Outside the fibers	Inside the fibers	Inside the fibers

al. (2008). The lipopeptide production yield ($Y_{surfactin/glucose}$ or $Y_{fengycin/glucose}$) was defined as the amount of lipopeptide produced per amount of glucose consumed.

Measurement of the specific oxygen uptake rate and the oxygen volumetric mass transfer coefficient (K_La)

For each membrane contactor, the volumetric oxygen mass transfer coefficient was measured under the conditions similar to the fermentation processes, except that the Landy's medium was replaced by distilled water at pH 7.

DO was measured with a submerged probe supplied from Mettler Toledo (Viroflay, France), and both the O_2 consumption and CO_2 production were monitored by a 4100 Gas Analyser (Servomex, Crowborough, England) and were related to the biomass. Here, the apparent K_La in the bioreactor was measured by the static gassing out method as described earlier (Trible et al. 1995; Yeh et al. 2006). Either the vessel or the membrane was flushed with N_2 in order to decrease DO to 0%. Then the N_2 flow was stopped, and the medium was aerated at previously defined flow rate, and the variation of DO concentration was monitored with respect to time. K_La values were obtained by plotting $\ln((C^*-C_1)/(C^*-C_2))$ versus time.

Surfactin adsorption assays on hollow fibers

Surfactin solution was prepared from the broth of a batch culture of *B. subtilis* BBG130 (Coutte et al. 2010) in 3-l flasks. The strain was cultured as described above for preculture in Landy's medium. The culture broth was centrifuged 15 min at 13,000×g, and the supernatant was ultrafiltered through a regenerated cellulose 10-kDa membrane (Millipore SA, Molsheim, France). Surfactin was washed four times by diafiltration. During the four washings, the surfactin solution was diafiltered to obtain 10% of the initial retentate volume and fed to the initial volume with distilled water. Then methanol was added to the retentate till the concentration of methanol reached about 70% (*v/v*), and the solution was filtered through the 10-kDa membrane. The final filtrate was concentrated by evaporation using a Heidolph VV2000 Rotavapor (Heidolph Instruments Gmbh & Co, Schwabach, Germany) and quantified by HPLC. Surfactin adsorption assays were carried out in the membrane bioreactors at pH 7 and 30°C during 90 min with a solution of surfactin of 200 ± 10 mg l^{-1} in distilled water. The adsorption of surfactin at the solid–liquid interface of the hollow fiber membrane was evaluated by measuring residual surfactin in the solution by HPLC (Chen and Juang 2008). The experiments were repeated three times. At the end of the experiment, the membrane was washed as described previously. The K_La was measured before the adsorption

experiment and after the first washing with water at 30°C as previously described.

Results

Growth analysis

In the foaming bioreactor and in the bubbleless bioreactors, a typical time-course profile was observed for a batch culture of *B. subtilis*, i.e., after a short exponential phase of less than 6 h, during which dissolved oxygen decreased and dropped to zero (Fig. 2), a constant growth rate was observed (data not shown). Finally, the quantity of biomass leveled off between the 24th and the 36th hour. After 36 h of culture in the FB, the amount of biomass in the broth reached a maximum of 11 g D.W. (Table 2). Foaming began from the sixth hour outward, causing the bioreactor to overflow. The biomass extracted from the bioreactor with foam in 1.13 l of broth was 1.4 g D.W., resulting in a global biomass production of 12.4 g D.W. for the FB. In the bubbleless bioreactors, the maximum amount of suspended biomass reached between 24 and 36 h of culture with values of 7.2, 3.8, and 8.1 g D.W. for the BB1, BB2, and BB3, respectively. However, the washings of the membranes showed that a part of biomass was adsorbed onto the membranes. Membrane washings released 2.1 g D.W. of biomass for the BB1, 1.2 g D.W. for the BB2, and 0.3 g D.W. for the BB3, resulting in a total biomass produced of 9.3, 5, and 8.4 g D.W. for the BB1, BB2, and BB3, respectively.
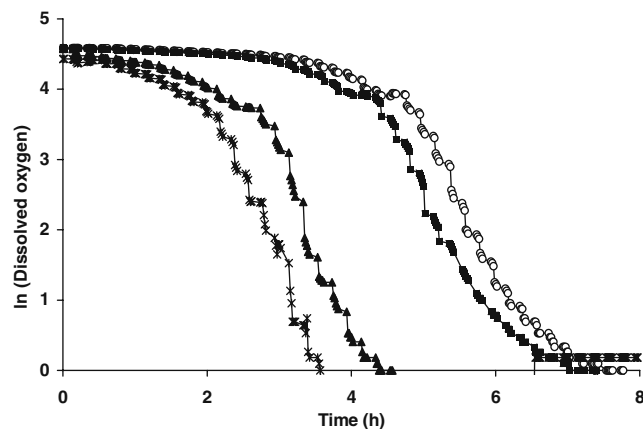

Fig. 2 Analysis of the dissolved oxygen decreasing during the first 8 h of batch culture of *B. subtilis* ATCC 21332. Data were from AFS Biocommand Interface acquired from the Mettler Toledo DO sensor. A logarithmic representation is shown for dissolved oxygen. *Circle* FB; *filled triangle* BB1 (external PES membrane, 2.5 m², 0.65 μm); *filled square* BB2 (external PP membrane, 1.9 m², 0.2 μm); *filled star* BB3 (submerged PP membrane, 0.375 m², 0.05 μm)

Table 2 Growth rate and lipopeptide production of the different fermentation processes

Analysis	Foaming bioreactor (FB)	Bubbleless bioreactor 1 (BB1)	Bubbleless bioreactor 2 (BB2)	Bubbleless bioreactor 3 (BB3)
Growth analysis				
Maximum suspended biomass (g D.W.)	11	7.2	3.8	8.1
Extracted biomass from the foam (g D.W.)	1.4	–	–	–
Desorbed biomass during washing (g D.W.)[a]	–	2.1	1.2	0.3
Total biomass produced (g D.W.)	12.4	9.3	5.0	8.4
Total amount of glucose consumed (g)	58.6	56.7	57	40
Surfactin production analysis				
Amount of surfactin produced in the broth (mg)	60	123	560	319
Amount of surfactin produced in the foam (mg)	714	–	–	–
Amount of surfactin desorbed from the hollow fiber after washing[a] (mg)	–	604	130	150
Total amount of surfactin produced (mg)	774	727	690	469
Surfactin production yield $Y_{P/S}$ (mg g^{-1})	13.2	12.8	12.1	11.7
Fengycin production analysis				
Amount of fengycin produced in the broth (mg)	–	30	566	982
Amount of fengycin produced in the foam (mg)	43	–	–	–
Amount of fengycin desorbed from the hollow fiber after washing[a] (mg)	–	112	66	0
Total amount of fengycin produced (mg)	43	142	622	982
Fengycin production yield $Y_{P/S}$ (mg g^{-1})	0.73	2.51	10.5	24.4

All the values are mean data of duplicates (standard deviations were essentially within 2–15%). Fermentation volumes were 3 l for FB, BB1 (external PES membrane, 2.5 m^2, 0.65 μm), BB2 (external PP membrane, 1.9 m^2, 0.2 μm), and 2.5 l for the BB3 (submerged PP membrane, 0.375 m^2, 0.05 μm) as described in Table 1

[a] After two washings with water at 30°C and two washings with 0.1 M NaOH pH 10.0 at 50°C, as described in Materials and Methods section

Lipopeptide production analysis

In the FB, surfactin and fengycin were continuously extracted by foaming. Their concentrations in the broth remained near the CMC (20 mg l^{-1}) during the whole culture (data not shown). The foam analysis revealed that surfactin was produced after the end of the exponential growth phase and up to the stationary phase. The fengycin production began after the 12th hour till the end of the culture (Fig. 3). After 72 h of culture, the amount of surfactin produced, calculated from foam and broth analysis, was 774 mg and that of fengycin was 43 mg (Table 2). The amounts of surfactin and fengycin produced per amount of glucose consumed (respectively, $Y_{surfactin/glucose}$ and $Y_{fengycin/glucose}$) were, respectively, 13.2 and 0.73 mg.g^{-1}.

In all the bubbleless bioreactors, no foaming occurred. Final amounts of surfactin measured in the broth of the BB1, BB2, and BB3 were 123, 560, and 319 mg, respectively (Table 2). However, the washings of the membranes revealed that a large part of surfactin was adsorbed onto the membranes. The washing showed that in the case of the BB1 and BB3, most of the surfactin was adsorbed into the membranes. It is obvious in these cases that the surfactin kinetics in the broth can safely be left out

from this work. The washing of the membranes used in the BB1, BB2, and BB3 released 604, 130, and 150 mg of surfactin, respectively. Finally, the total amount of surfactin produced was 727 mg for the BB1, 690 for the BB2, and
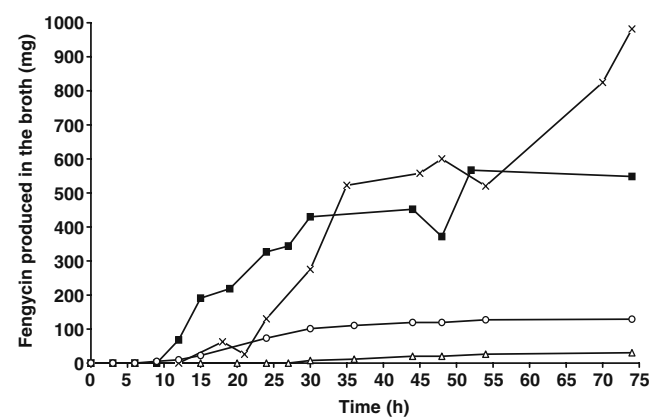

Fig. 3 Analysis of the amount of fengycin produced in the broth or in the foam during batch culture of *B. subtilis* ATCC 21332. Volumes of fermentation broth in FB, BB1 were 3 l and 2.5 l in BB2. *Circle* FB; *filled triangle* BB1 (external PES membrane, 2.5 m^2, 0.65 μm); *filled square* BB2 (external PP membrane, 1.9 m^2, 0.2 μm); *times symbol* BB3 (submerged PP membrane, 0.375 m^2, 0.05 μm). Values were an average from a duplicate set; standard deviations were essentially within 2–15%

469 mg for the BB2, resulting in a $Y_{surfactin/glucose}$ for surfactin quite similar for the FB and the BBs, with an average of 12.2 mg g^{-1}.

Fengycin production was observed in the broth of the bubbleless bioreactors, especially in the BB2 and the BB3. It began during the 12th hour of culture and increased strongly during the linear growth phase as shown in Fig. 2. In the BB1, this production remained quite low, but it reached a value of 566 mg in the BB2 and 982 mg in the BB3. Only a small quantity of fengycin was desorbed when washing the membranes. The amounts of fengycin desorbed from the membrane of the BB1 and the BB2 was 112 and 66 mg, resulting in $Y_{fengycin/glucose}$ of 2.51 and 10.5 mg g^{-1}. No fengycin was desorbed from the membrane of the BB3, but the global amount of fengycin produced and $Y_{fengycin/glucose}$ were significantly higher than those obtained from the other bioreactors, 982 mg and 24.4 mg g^{-1}, respectively.

Specific oxygen uptake rate and oxygen volumetric mass transfer coefficient (K_La) analysis

The apparent specific OUR of the processes was at its maximum during the exponential phase, at 12 mmol h^{-1} g D.W.$^{-1}$ in the FB, 11 mmol h^{-1} g D.W.$^{-1}$ in the BB1, 17 mmol h^{-1} g D.W.$^{-1}$ in the BB2, and 4 mmol h^{-1} g D.W.$^{-1}$ in the BB3. After the end of this phase, the specific OUR dropped to 2 mmol h^{-1} g D.W.$^{-1}$ in the FB, 3 mmol h^{-1} g D.W.$^{-1}$ in the BB1 and the BB2, and 1 mmol h^{-1} g D.W.$^{-1}$ in the BB3. Oxygen volumetric mass transfer coefficient (K_La) is an important indicator of the biofouling of the membrane during the fermentation process. The measurement of the K_La after the fermentation process and before washing the membranes revealed that the K_La decreased and reached near 4.07 h^{-1} in the BB1, 0.9 h^{-1} in the BB2, and 1.75 h^{-1} in the BB2 (Table 3). Multiple washing with pH 7 water at 30°C and 0.1 M NaOH solution at 50°C partially restored the oxygen transfer for the BB1 membrane and restored the initial

K_La of the membrane of the BB2 (Table 3). In the BB3, the initial K_La value could not be restored either by water or 0.1 M NaOH washings. However, the membrane could be cleaned by a 0.5 M NaOH and NaOCl at 50°C treatment.

Organic acid and 2,3-butanediol production

The effect of the oxygen limitation in our experiments was evaluated by focusing on production of the organic acid and 2,3-butanediol (Fig. 4). Lactate and 2,3-butanediol were produced during the growth in the FB at maximal concentrations of 0.65 g l^{-1} (Fig. 3). We found similar concentrations of lactate in BB1, but 2,3-butanediol concentration remained very low. On the contrary, lactate and 2,3-butanediol were both higher in BB2 (0.9 and 0.8 g l^{-1}, respectively) and considerably higher in the BB3 (3.5 g l^{-1} after 21 h of culture and 2.6 g l^{-1} after 47 h of culture, respectively).

Surfactin adsorption on hollow fiber: effect on K_La

Surfactin is known for its ability to adsorb onto both hydrophilic and hydrophobic surfaces (Shakerifard et al. 2009). Its adsorption on both hollow fiber membranes used as air–liquid contactors was thus studied with overcritical micellar concentration solutions in water at pH 7. Results are shown in Fig. 5. For the 2.5 m^2 PES membrane of the BB1, surfactin adsorbed in few minutes, and then the residual concentration stabilized at 35 mg l^{-1}. For the 1.9 m^2 PP membrane of the BB2, the result was 120 mg l^{-1} and 140 mg l^{-1} for the 0.375 m^2 PP membrane of the BB3. The amount of surfactin adsorbed on the microfiltration polyethersulfone membrane reached 200 mg m^{-2} (Table 4). In contrast, the adsorption capacity of the ultrafiltration polypropylene membrane was more than twice as high (530 mg m^{-2}). These results are similar to those obtained in cultures. The evolution of the oxygen volumetric mass transfer coefficient after each adsorption experiment is presented in Table 4. A decrease in K_La by a factor of about

**Table 3** Membrane colonization, surfactin adsorption, and their effect on K_La

	Foaming bioreactor (FB)	Bubbleless bioreactor 1 (BB1)	Bubbleless bioreactor 2 (BB2)	Bubbleless bioreactor 3 (BB3)
Cells desorbed (g m^{-2})	–	0.84 (SD 0.25)	0.63 (SD 0.14)	0.8 (SD 0.19)
Surfactin desorbed[a] (mg m^{-2})	–	241.8 (SD 10.2)	68.4 (SD 5.41)	400.2 (SD 13.9)
Initial K_La (h^{-1})	27.29 (SD 0.17)	39.36 (SD 0.09)	8.99 (SD 0.04)	28.24 (SD 0.15)
K_La after fermentation (h^{-1})	–	4.07 (SD 0.01)	0.91 (SD 0.01)	1.75 (SD 0.06)
K_La after washing[a] (h^{-1})	–	15.47 (SD 0.09)	8.90 (SD 0.13)	4.46 (SD 0.51)

Values are mean data from two experiments. BB1, external PES membrane, 2.5 m^2, 0.65 μm; BB2, external PP membrane, 1.9 m^2, 0.2 μm; BB3, submerged PP membrane, 0.375 m^2, 0.05 μm

SD standard deviation

[a] After two washings with water at 30°C and two washings with 0.1 M NaOH pH 10 at 50°C, as described in Materials and Methods

Fig. 4 Lactate and 2, 3-butanediol production during *B. subtilis* batch cultures in Landy's medium at 30°C, pH maintained at 7. Values were an average from a duplicate set; standard deviations were essentially within 2–15%. *Solid lines* lactate; *dotted lines* 2.3-butanediol. *Circle* FB (3 l); *filled triangle* BB1 (3 l, external PES membrane, 2.5 m², 0.65 μm); *filled square* BB2 (3 l, external PP membrane, 1.9 m², 0.2 μm); *times symbol* BB3 (2.5 l, submerged PP membrane, 0.375 m², 0.05 μm)
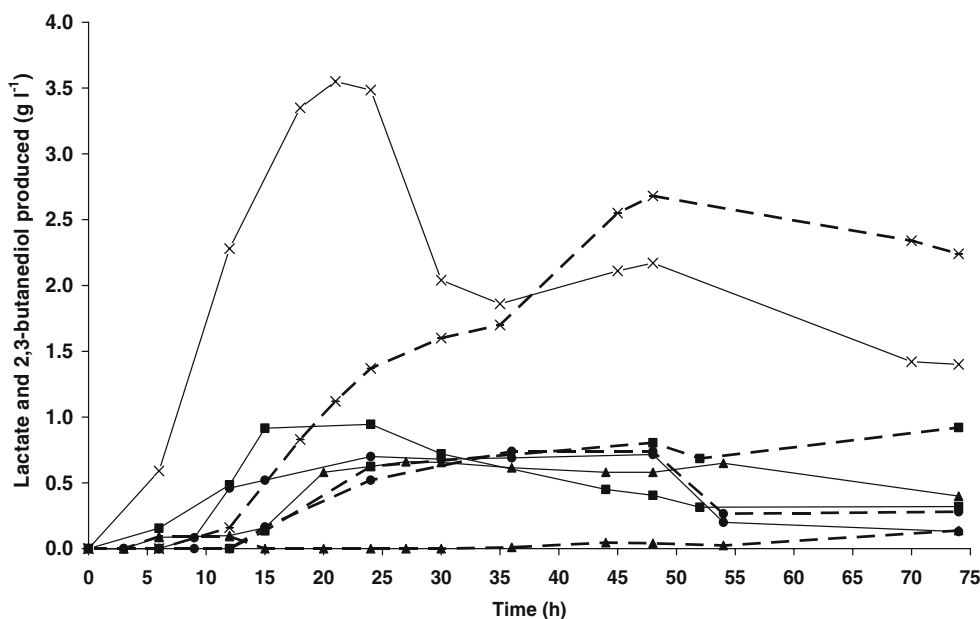
1.5 was observed for the microfiltration membrane of the BB1. No decrease was observed with the microfiltration membrane of the BB2. With the ultrafiltration membrane of the BB3, the $K_L a$ decreased fivefold after 90 min of contact with the surfactin solution. These results highlight the importance of membrane structure, composition, and pore size on the effect of surfactin adsorption on oxygen transfer.

Discussion

In this study, lipopeptide production in bubbleless membrane-aerated bioreactors was compared to lipopep- tide production in a conventional air-sparging aerated bioreactor.

The bubbleless bioreactors readily support bacterial growth and lipopeptide production without foam formation. During the linear growth phase, which began with O_2 depletion in the broth, the biomass grew with a comparable rate in the FB, BB1, and BB2. In the BB1 and BB2, the large membrane surface was colonized by cells, constituting, respectively, 23% and 24% of the biomass produced at the end of the culture. This reduced the apparent growth rate in the broth. In the BB3, the adhered biomass was reduced. The cell colonization could have been limited by a lower membrane surface/bioreactor volume ratio and higher shear forces on the

Fig. 5 Adsorption of surfactin on the different hollow fiber membranes at 30°C. The amount of surfactin adsorbed onto the surface (milligram per square meter) of hollow fiber at any time was presented. Surfactin at 200 mg l⁻¹ was put into contact with the hollow fiber during an oxygenation experiment as used in the previously described fermenta- tion of *B. subtilis*. Values were an average from three experiments. *Filled triangle* microporous PES membrane (2.5 m², 0.65 μm); *filled square* microporous PP membrane (1.9 m², 0.2 μm); *times symbol* ultrafiltration PP membrane (0.375 m², 0.05 μm)
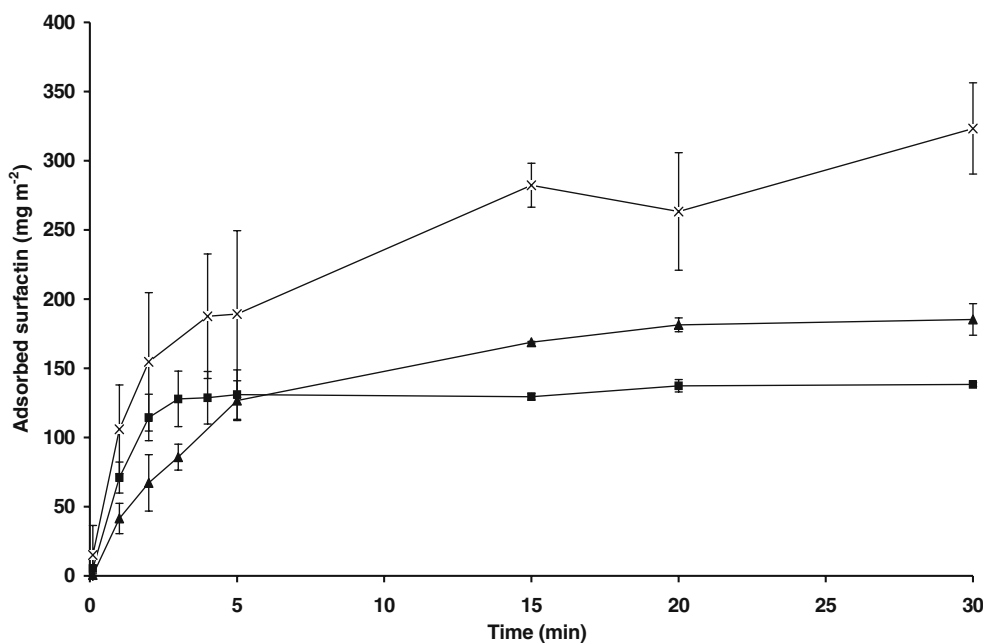
Table 4 Evolution of oxygen mass transfer after surfactin adsorption on membrane

Oxygen mass transfer (K_La) study	Bubbleless bioreactor 1 (BB1)	Bubbleless bioreactor 2 (BB2)	Bubbleless bioreactor 3 (BB3)
Desorbed surfactin (mg m^{-2})[a]	200.5 (SD 0.9)	133.9 (SD 10.8)	530.3 (SD 28.0)
Initial K_La (h^{-1})	39.36 (SD 0.09)	8.99 (SD 0.04)	28.24 (SD 0.15)
K_La after surfactin adsorption (h^{-1})	25.53 (SD 0.07)	7.61 (SD 0.02)	2.57 (SD 0.02)
Relative K_La decrease (%)	35	15	91

Values are mean data from three experiments

SD standard deviation

[a] After two washings with water at 30°C and two washings with 0.1 M NaOH pH 10 at 50°C, as described in Materials and Methods

membrane surface. However, the total amounts of biomass produced in the BB3 and BB1 at the end of the culture, respectively, 9.3 and 8.4 g D.W., were quite similar to that in the FB. Biomass yields per amount of glucose were comparable to those found in other studies of *B. subtilis* ATCC 21332 in batch culture at 30°C, using Cooper or MSI medium (Davis et al. 1999; 2001; Yeh et al. 2006).

No foam was produced in any of the bubbleless bioreactors designed and used in this study in spite of significant production of lipopeptides. The total amounts of lipopeptides produced in the bubbleless bioreactors were higher than those produced in the FB. Surfactin production in the BB1 and the BB2 was similar to that in the FB, as indicated by the $Y_{surfactin/glucose}$, which was on average 12.4 mg g^{-1}. These results were higher than or close to those published in previous works (Davis et al. 1999, 2001; Yeh et al. 2006; Isa et al. 2007). In the BB3, surfactin production was lower than in the other bioreactors, but fengycin production was surprisingly high (982 mg after 72 h of culture). No comparable value of fengycin production in bioreactor has been published in the literature. The production of fengycin could be enhanced by the low oxygen transfer in the BB3, which was half that in the FB. The oxygen transfer limitation in this bioreactor was demonstrated by the low specific OUR and also by the high production of end products typical for conditions with limited oxygen, i.e., lactate and 2,3-butanediol, as previously demonstrated (Nakano et al. 1997). In fact, a very small part of the biomass adhered to the aeration membrane in the BB3 resulting in a strong oxygen limitation for the majority of the cells in the broth. On the other hand, a great part of the biomass in the BB1 and the BB2 was fixed on the membrane and benefited from a much higher oxygen supply, which could promote surfactin production. To verify the effect of oxygen limitation on fengycin synthesis, we investigated this production under definite oxygen transfer rate conditions as described by Guez et al. (2008). Experiments done in nonmonitored flasks between filling volume ratio of 0.1, 0.3, and 0.6 confirmed this result (data not shown).

The ability of surfactin to adsorb onto different surfaces was recently demonstrated by Shakerifard et al. (2009). The large fraction of surfactin adsorbed on aeration membranes, with differences in the fixed amounts depending on the kind of membrane, confirmed these results. The adsorption of surfactin at the solid/liquid interface of the hollow fiber membrane is quite a rapid phenomenon. Adsorption was very high on the ultrafiltration PP membrane. As a consequence of surfactin adsorption, surfactin concentration remained very low in the broth of the bubbleless bioreactors. However, we found in adsorption assays that it was possible to saturate the membrane surface so that surfactin accumulated in the medium.

Cell-free adsorption assays demonstrated that surfactin adsorption is the main cause of the K_La decrease for the BB3. K_La was reduced to 92% of the initial value. Several authors have also reported that surfactin could be organized in large rod micelles with different size ranging from 10 to 115 nm (Maget-Dana and Ptak 1992; Ishigami et al. 1995; Deleu et al. 1999). It is also possible that the biggest micellar structures of surfactin block the 50-nm pores of the ultrafiltration membrane of the BB3. This phenomenon should be less significant with microfiltration membrane where pores are larger. For this membrane, the K_La decrease results rather from the strong cell adhesion observed. Fengycin was only weakly adsorbed on the different membranes and especially on the BB3 membrane. The lack of fengycin adsorption could be explained by a competition between surfactin and fengycin for the adsorption on the support because (a) surfactin is usually produced earlier than fengycin and (b) surfactin has a higher adsorption rate than fengycin, as previously shown at the dodecane/water interface (Deleu et al. 1999).

In this study, an innovative process of biosurfactant production has been presented. Bubbleless membrane-aerated bioreactors have been designed to prevent foam formation. First, satisfactory microbial growth and lipopeptide synthesis were obtained in the three tested bubbleless bioreactors without foam formation. Secondly, PP submerged membrane bioreactor triggered a very high level of fengycin production with *B. subtilis* ATCC 21332.

In spite of a high surfactin adsorption, high lipopeptide concentrations could be obtained in the culture broth which allows the use of a large variety of downstream extraction

processes. Further investigation should be carried out to characterize more precisely the effects of environment and membrane nature on the adsorption of lipopeptides to the membranes.

References

Ahmed T, Semmens MJ (1996) Use of transverse flow hollow fibers for bubbleless membrane aeration. Water Res 30:440–446

Bonmatin JM, Laprévote O, Peypoux F (2003) Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity–structure relationships to design new bioactive agents. Comb Chem High T Scr 6:541–556

Brindle K, Stephenson T (1996) The application of membrane biological reactors for the treatment of wastewaters. Biotechnol Bioeng 49:601–610

Chen AL, Juang RS (2008) Extraction of surfactin from fermentation broth with *n*-hexane in microporous PVDF hollow fibers: significance of membrane adsorption. J Membrane Sci 325:599–604

Cooper DG, Macdonald CR, Duff SJ, Kosaric N (1981) Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol 42:408–412

Coutte F, Leclère V, Béchet M, Guez JS, Lecouturier D, Chollet-Imbert M, Dhulster P, Jacques P (2010) Effect of *pps* disruption and constitutive expression of *srfA* on surfactin productivity, spreading and antagonistic properties of *Bacillus subtilis* 168 derivatives. J Appl Microb. doi:10.1111/j.1365-2672.2010.04683.x

Davis DA, Lynch HC, Varley J (1999) The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. Enzyme Microb Tech 25:322–329

Davis DA, Lynch HC, Varley J (2001) The application of foaming for the recovery of surfactin from *B. subtilis* ATCC 21332 cultures. Enzyme Microb Tech 28:346–354

Deleu M, Razafindralambo H, Popineau Y, Jacques P, Thonart P, Paquot M (1999) Interfacial and emulsifying properties of lipopeptides from *Bacillus subtilis*. Colloid Surf A 152:3–10

Deleu M, Bouffioux O, Razafindralambo H, Paquot M, Hbid C, Thonart P, Jacques P, Brasseur R (2003) Interaction of surfactin with membranes: a computational approach. Langmuir 19:3377–3385

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol R 61:47–64

Gabelman A, Hwang ST (1999) Hollow fiber membrane contactors. J Membrane Sci 159:61–106

Gancel F, Montastruc L, Liu T, Zhao L, Nikov I (2009) Lipopeptide overproduction by cell immobilization on iron-enriched light polymer particles. Process Biochem 44:975–978

Guez JS, Chenikher S, Cassar JP, Jacques P (2007) Setting up and modelling of overflowing fed-batch cultures of *Bacillus subtilis* for the production and continuous removal of lipopeptides. J Biotechnol 131:67–75

Guez JS, Müller CH, Danzé PM, Büchs J, Jacques P (2008) Respiration activity monitoring system (RAMOS), an efficient tool to study the influence of the oxygen transfer rate on the synthesis of lipopeptide by *Bacillus subtilis* ATCC6633. J Biotechnol 134:121–126

Hbid C, Jacques P, Razafindralambo H, Mpoyo MK, Meurice E, Paquot M, Thonart P (1996) Influence of the production of two lipopeptides, iturin A and surfactin S1, on oxygen transfer during *Bacillus subtilis* fermentation. Appl Biochem Biotech 57–58:571–579

Isa MHM, Coraglia DE, Frazier RA, Jauregi P (2007) Recovery and purification of surfactin from fermentation broth by a two-step ultrafiltration process. J Membrane Sci 296:51–57

Ishigami Y, Osman M, Nakahara H, Sano Y, Ishiguro R, Matsumoto M (1995) Significance of beta-sheet formation for micellization and surface adsorption of surfactin. Colloid Surface B 4:341–348

Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, De Pauw E, Thonart P (1999) Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett–Burman design. Appl Biochem Biotech 77:223–233

Khoshbin E, Westrope C, Pooboni S, Machin D, Killer H, Peek GJ, Sosnowski AW, Firmin RK (2005) Performance of polymethyl pentene oxygenators for neonatal extracorporeal membrane oxygenation: a comparison with silicone membrane oxygenators. Perfusion 20:129–134

Kosaric N (2005) Biosurfactants and their application for soil bioremediation. Food Technol Biotech 39:295–304

Lee BS, Kim EK (2008) Lipopeptide production from *Bacillus* sp. GB16 using a novel oxygenation method. Enzyme Microb Tech 35:639–647

Maget-Dana R, Ptak M (1992) Interfacial properties of surfactin. J Colloid Interf Sci 153:285–291

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133:183–198

Nakano MM, Dailly YP, Zuber P, Clark DP (1997) Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J Bacteriol 179:6749–6755

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125

Ongena M, Jacques P, Touré Y, Destain J, Jabrane A, Thonart P (2005) Involvement of fengycin-type lipopeptides in the multifaceted biocontrol of *Bacillus subtilis*. Appl Microbiol Biot 69:29–38

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 9:1084–1090

Pankhania M, Brindle K, Stephenson T (1999) Membrane aeration bioreactors for wastewater treatment: completely mixed and plug-flow operation. Chem Eng J 73:131–136

Peypoux F, Bonmatin JM, Wallach J (1999) Recent trends in the biochemistry of surfactin. Appl Microbiol Biot 51:553–563

Schneider M, Reymond F, Marison U, von Stockar U (1995) Bubble-free oxygenation by means of hydrophobic porous membranes. Enzyme Microb Tech 17:839–837

Shakerifard P, Gancel F, Jacques P, Faille C (2009) Effect of different *Bacillus subtilis* lipopeptides on surface hydrophobicity adhesion of *Bacillus cereus* 98/4 spores to stainless steel and Teflon. Biofouling 25:533–541

Trible LA, Briens CL, Margaritis AA (1995) Determination of the volumetric mass transfer coefficient (k(L)a) using dynamic "gas out-gas in" method: analysis of errors caused by dissolved oxygen probe. Biotechnol Bioeng 46:388–392

Vollenbroich D, Ozel M, Vater J, Kamp RM, Pauli G (1997) Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. Biologicals 25:289–297

Wei C, Huang X, Wen X (2006) Pilot study on municipal wastewater treatment by a modified submerged membrane bioreactor. Water Sci Technol 53:103–110

Yeh MS, Wei YH, Chang JS (2006) Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus subtilis*. Process Biochem 41:1799–1805