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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

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Abstract

The effect of oxygen transfer rate on the synthesis of mycosubtilin, a non ribosomal lipopeptide antifungal biosurfactant, was investigated in the Respiration Activity MOnitoring System (RAMOS) for two *Bacillus subtilis* strains. These cultures were performed under definite oxygen-limited conditions without the adding of any anti-foam in the culture medium. By using four different filling volumes in the shaken bioreactors, different levels (20, 14, 9 and 7 mmol $O_2.l^{-1}.h^{-1}$) of oxygen-limited growth could be obtained. A 25-fold increase of the specific productivity of mycosubtilin was observed for *Bacillus subtilis* ATCC6633 in the case of the most severe oxygen limitation. But nearly no effect could be found with strain BBG100 carrying the constitutive P_{repU} promoter instead of the natural P_{myc} promoter. Transcript analysis of the *fenF* gene belonging to the *myc* operon indicated that the P_{myc} promoter regulation could be slightly oxygen sensitive. Additionally, different patterns of the synthetised mycosubtilin homologues were obtained for different level of oxygen-limited growths. At the present state of investigation, oxygen regulation was thus shown to act at different levels suggesting the existence of a complex regulatory system of NRPS lipopeptide synthesis in the natural *B. subtilis* ATCC6633 strain.

Introduction

Bacillus subtilis is known for its ability to produce several bioactive nonribosomal peptides (Grünewald and Marahiel, 2006) and among others, lipopeptides which are classified into three main families according to their chemical structure: surfactins, fengycins and iturins (Bonmatin et al., 2003, Schneider et al., 1999). The family of iturins composed of iturin A, A_L C, mycosubtillin, bacillomycin F, L, D, and LC. Most of them combine biosurfactant and antifungal activities. In particular, mycosubtilin which is a pore-forming lipopeptide (Maget-Danat and Peypoux, 1994) is considered as one of the most antifungal iturinic compounds. The first strain *Bacillus subtilis* ATCC6633 used in this work is a natural mycosubtilin and surfactin producer (Duitman et al., 1999). The second strain, BBG100, is a mycosubtilin overproducing derivative (Leclère et al., 2005).

The production process of lipopeptide is known to be influenced by environmental conditions such as pH, temperature, agitation and oxygen availability (Desai and Banat, 1997). In particular, oxygen availability changes metabolic pathways and fluxes in *Bacilli* (Çalik et al., 2000) affecting the synthesis of lipopeptide (Ohno et al., 1993, Jacques et al., 1999). Most of the studies investigating this effect were carried out in shake flasks with conventional agitation, filling volumes or plugs changing methods but no data on oxygen transfer rate (OTR) are available. In stirred bioreactors, only few references report the effect of the culture parameters on the production of non ribosomal lipopeptides, namely surfactin (Sen and Swaminathan, 1997) and surfactin/iturin A (Hbid et al., 1996). In the first article, the authors showed that a low agitation and high aeration rate favoured the synthesis of surfactin by strain *B. subtilis* DSM 3256. In the second article, the authors showed that, when the stirring rate decreases, *B. subtilis* S499 produces half as much surfactin and twice as much iturin. These latter experiments were performed in the presence of oxygen vectors that may have influenced the cell metabolism. No clear evidence of the effect of the OTR could be drawn from these

studies because of the absence of gas analysis and of the influence of the oxygen transfer conditions on the foaming. The lack of studies on this topic principally arise from the difficulty to manage the high foaming capacity of the culture medium during the production process in bioreactors, especially as the adding of synthetic or natural antifoam is not recommended because of the risk of interferences with the physiological state of the strain (Lee and Kim, 2004).

For the first time, our work investigates the effect of oxygen limitation on the lipopeptide biosurfactant synthesis under definite OTR conditions. The OTR was measured online using the Respiration Activity MOnitoring System (RAMOS) described previously (Anderlei et al., 2004). In our study, there was no need to add antifoam in the culture medium since the surface aeration strategy in shaken reactors decreased the broth foaming capacity. The effect of oxygen on the natural P_{myc} promoter of the *myc* operon encoding for the mycosubtilin synthetase was discussed. Indirect influence of OTR was pointed out with showing the different mycosubtilin homologues patterns obtained for the different oxygen-limited growths.

Materials and Methods

Strains and growth conditions

B. subtilis ATCC6633, a mycosubtilin and surfactin producer, and the derivative BBG100 were tested in this study. BBG100 was previously obtained in our laboratory by replacing the native promoter P_{myc} of the mycosubtilin synthetase operon by a constitutive promoter P_{repU} originating from *Staphylococcus aureus* plasmid pUB110. The preculture medium (Clark, 1981) was inoculated with a loopful of cells conserved at –80°C. Cells were grown in Erlenmeyer flasks at 30°C with a shaking speed of 140 rpm. Cells were then alternatively

transferred to RAMOS flasks or Erlenmeyer flasks containing the modified Landy medium pH 7.0 composed as follows : Glucose, 20 $g.l^{-1}$; $(NH_4)_2SO_4$, 2.3 $g.l^{-1}$, Glutamic acid, 2 $g.l^{-1}$; K_2HPO_4, 1 $g.l^{-1}$; $MgSO_4$ 0.5 $g.l^{-1}$; KCl, 0.5 $g.l^{-1}$; $CuSO_4$, 1.6 $mg.l^{-1}$; $Fe_2(SO_4)_3$, 1.2 $mg.l^{-1}$; $MnSO_4$, 0.4 $mg.l^{-1}$. Culture medium was buffered with MOPS (3-(N-morpholino)propanesulfonic acid) 100 mM and no yeast extract was added. Before inoculation, the preculture was centrifuged and washed in 9 $g.l^{-1}$ NaCl solution. The starting optical density of the main culture was 0.25 corresponding to a biomass concentration of 0.8 $g(D.W).l^{-1}$. The optical density was measured at 600 nm using a Kontron Uvicon 922. The cultures led in Erlenmeyer flasks were realised in triplicate.

RAMOS system

The OTR was measured online using the RAMOS (Anderlei et al., 2004). By using four different filling volumes (FV) of 10 ml, 25 ml, 42 ml and 60 ml corresponding to filling volume ratios (FVR) of 0.04, 0.1, 0.17 and 0.24, four levels of oxygen transfer capacity could be obtained in 250 ml RAMOS flasks for strain ATCC6633. Three non-monitored shake flasks were cultivated simultaneously under same conditions and used for sampling. Each sample flask was only used once in order to avoid the modification of the flask filling volume. The shaker was operated at 175 rpm, 50 mm shaking diameter and the temperature set at 30°C. Similar experiments were conducted with strain BBG100 by using 10, 42 and 60 ml filling volumes.

Analysis

Culture samples were centrifuged at 10,000g for 10 min. A volume of 1 ml of the supernatants was purified through C18 Maxi-Clean cartridges (Alltech) following the protocol described previously (Guez et al., 2007)[a] for lipopeptide HPLC analysis. Purified mycosubtilins and surfactins used as standards were supplied by Sigma. The retention time and second derivative of the absorption spectrum between 200 and 400 nm (Diode Array

PDA 996, Waters) were used to identify the eluted molecules (Millenium Software, Waters). Determination of the retention time of the major C-16 and C-17 mycosubtilin homologues was done with injecting purified fractions in HPLC (Guez, 2007[b]). The identification of these homologues was done with performing MALDI-MS analysis (Brucker Ultraflex tof, Brucker Daltonics) as described previously (Leclère et al., 2005).

Glucose concentrations were determined using a Dionex HPLC system with an organic-acid-resin column (300 x 8 mm, CS-Chromatography) at 60°C. Samples were prefiltered on 0.45 µm Millex-HV (Millipore). Sulphuric acid 1 mM mobile phase was pumped at a flow rate of 0.8 $ml.min^{-1}$.

Enzymatic assay was used to analyse the L-glutamic acid (Enzytec Fluid, Scil Diagnostic, Germany).

RNA isolation and reverse transcription

Culture samples were taken after two doubling times (n=2) which corresponded to the early exponential growth and four doubling times (n=4) which corresponded to the beginning of the oxygen-limited growth. Data corresponding to n=2 and n=4 originates from two independent duplicate sets in order to avoid the modification of the flasks filling volume. A volume corresponding to 1.10^9 cells was sampled and added to the Ambion Ribopure RNALater solution (v/v), mixed thoroughly during 10 s and centrifuged 10 min at -9°C and 11 000 g. The supernatant was discarded and the pellet stored at -80°C.

Authors recently mentioned that a natural isolate like ATCC6633 was difficult to transform (Duitman et al., 2007). In the frame of this work, it should be added that RNA isolation of ATCC6633 is also difficult to perform with respect to the high resistance of its cell wall. For isolation of RNA, the cell pellets were resuspended in 50 µl of a 10 $mg.ml^{-1}$ lysosyme solution. After 10 min of incubation at 37°C, 350 µl of RNAWiz phenolic solution were added. Mechanical cell lysis in the presence of 250 µl of zirconium beads was led for 10 min

at maximal speed (Mixer Mill 200). The lysate was then centriguged 5 min at 12 000 g and 4°C. After having discarded the beads, 0.2 volume of chloroform was added. The mixture was incubated 10 min at room temperature and centrifuged 5 min at 12 000 g and 4°C. The aqueous phase was retrieved and 0.5 volume of ethanol added. Purification of RNA was then completed following the Ribopure Ambion protocole. The quantity of total RNAs was measured with a Nanodrop ND-1000 spectrophotometer. The quality was estimated with calculating the RNA 23S/16S ratio with a capillary electrophoresis (RNA 6000 NanoAssay, Agilent 2100 bioanalyzer).

The reverse-transcription was done with mixing 3 µg of total RNA and 0.5 µl hexamers (Invitrogen) to a final volume of 8.2 µl in H_2O. The mixture was heated at 65°C for 10 min and cooled at 4°C before the adding of 3 µl of 5X First Strand Buffer (Invitrogen), 1.5 µl of 0.1M DTT (Invitrogen), 0.3 µl of 25 mM dATP, dGTP, dCTP, dTTP (Amersham), 1 µl of Rnase Inhibitor and 1 µl of SuperScript II (200 U/µl, Invitrogen). Incubation of this mixture at 42°C during 1 h was followed by the adding of 0.5 µl SuperScript II and a subsequent incubation at 42°C during 45 min. To estimate the level of expression of the *myc* gene coding for the mycosubtilin synthetase, a 398 bp PCR product belonging to the *fenF* locus of the *myc* operon was amplified using the following pair of oligonucleotides : 5'-CAAAATGCAGATCCTGAGCA-3' and 5'-GGCATAGTCATGTGCGTTTG-3'. PCR products were analysed by electrophoresis on 1.5% agarose gel and the Median-based Trimmed Mean density (MTM) values of the bands were obtained thanks to Arrayvision scanner (Qiagen). MTM values of each band corresponding to *fenF* products were normalised with the MTM values of a 330 bp amplified product belonging to *rplL*, a housekeeping gene of *B. subtilis* coding for the ribosomal protein L12. Results were rescaled with respect to the experiment led at FVR=0.05. The following pair of oligonucleotides 5'-GCTTCCGTTAAAGAAGCAACTG-3' and 5'-AGAAGCGCCAACTTCTTCAA-3' were

used for *rplL*. The PCR mixture was done with the following reagents: cDNA, 0.5 µL; 10X buffer, 2,5 µl; dNTP (2.5mM), 1µl; $MgCl_2$ (25mM), 1.5 µl; forward primer, 0.5 µL; reverse primer, 0.5 µL, completed to 25µl with H_2O. 0.2 µl of Taq Gold (5U/µl) are then added to the mixture. The amplification conditions were 5 min at 94°C followed by 30 cycles: 30 s at 94°C, 30 s at 56°C, 60 s at 72°C and one cycle at 72°C for 5 min before conservation of the PCR product at 4°C.

Results

OTR-monitored cultures of ATCC6633 were first led in RAMOS flasks. Figure 1 shows the oxygen transfer rate (OTR) evolution. After a period of latency of 10 h due to the absence of yeast extract in the culture medium, the oxygen transfer rate increased until the maximal oxygen transfer rate was reached. RAMOS flasks with filling volumes of 60, 42, 25 and 10 ml allowed oxygen-limited growths at 7, 9, 14 and 20 mmol $O_2.L^{-1}.h^{-1}$ respectively. A decrease in the OTR then occurred successively for the experiments at a FV of 10, 25 and 42 ml because of the complete consumption of the carbon source. In case of the most severe oxygen-limitation obtained at a FV of 60 ml, the experiment was ended before total depletion of the carbon source. HPLC analysis showed that 0.45 $g.l^{-1}$ of glucose and 0.056 $g.l^{-1}$ of glutamic acid remained in the culture medium at 48 h (table 1). *B. subtilis* ATCC6633 and BBG100 showed similar pH evolution for the different oxygen-limited growths. The pH dropped from 7.0+/-0.04 to respectively 6.06+/-0.03 and 5.98+/-0.11 after 48 hours of culture (Table 1).

Oxygen availability played an important role in the lipopeptide synthesis by ATCC6633. The mycosubtilin production increased with the strength of the oxygen limitation from 2.4 to 45.4 $mg.l^{-1}$ (figure 2A). This result was amplified for the mycosubtilin specific productivity as the increase ranged from 0.001 to 0.025 $mg.g(DW)^{-1}\ h^{-1}$ (table 1). In the case of the BBG100 strain carrying the constitutive P_{repU} promoter instead of the native P_{myc}, the mycosubtilin production remained quite constant in the range of 70 to 80 $mg.l^{-1}$ (figure 2B) and the specific

productivity in the range of 0.033 to 0.035 mg.g(DW)$^{-1}$.h^{-1} (table 1). Considering the experiments where the carbon sources were totally depleted before the end of the culture, no diminution of the mycosubtilin production was observed during the culture (fig 2A) whereas this phenomenon occurred in the case of surfactin (figure 2C) rending the surfactin production kinetics difficult to analyse. Nevertheless, the surfactin production by the mycosubtilin overproducing strain BBG100 (figure 2D) was reduced in comparison to ATCC6633.

Oxygen dependent regulation of the native P_{myc} promoter of ATCC6633 was inspected through a transcript analysis performed on the *fenF* gene that belongs to the *myc* operon coding for the mycosubtilin synthetase. Figure 3 shows comparative RT-PCR results obtained for different oxygen-limited growths led in 500 ml non-monitored Erlenmeyer flasks filled with 20, 50, 100 and 200 ml corresponding to FVR of 0.05, 0.1, 0.2, and 0.4. At n=2, MTM ratios of *fenF* were quite similar for the different FVR indicating the absence of transcriptional regulation of the P_{myc} promoter in the early exponential growth, *i.e* before oxygen-limited growth. At n=4 and a FVR ranging from 0.05 to 0.2, the expression of *fenF* slightly increased whereas the mycosubtilin specific productivity was also shown to increase from 0.002 to 0.029 mg.g(DW)$^{-1}$.h^{-1} (figure 4). Compared to FVR=0.1 or FVR=0.2, the *fenF* expression then decreased at FVR=0.4. In this experiment, the mycosubtilin specific productivity interestingly dropped to 0.014 mg.g(DW)$^{-1}$.h^{-1}. This result showed that the positive effect of oxygen-limited growth on the mycosubtilin synthesis can be inverted in the case of too severe oxygen limitations.

Different mycosubtilin homologues patterns were obtained for the different oxygen-limited growths in Erlenmeyer flasks at a FVR of 0.05, 0.1, 0.2 and 0.4 (figure 4). Patterns corresponding to FVR of 0.2 and 0.4 showed a quite constant distribution of the mycosubtilin C-16 and C-17 homologues. Patterns corresponding to FVR of 0.05 or 0.1 showed an increase of the C-16 mycosubtilin and a slight decrease of the C-17 mycosubtilin. The high standard

deviations determined for the FVR=0.05 sample result from the low level of mycosubtilin produced in these conditions.

Discussion

Among the environmental factors affecting the production of lipopeptide biosurfactants, authors pointed out the importance of the pH for microorganism in general (Desai et al., 1997) and *B. subtilis* ATCC6633 in particular (Guez, 2007)[b]. Effects of the pH were therefore largely reduced in this study with buffering the medium with MOPS 100 mM.

Combined results obtained in RAMOS and Erlenmeyer flasks showed that the gain in mycosubtilin productivity could be lost in the case of too severe oxygen limitations. Indeed, an increase of the rate of mycosubtilin synthesis was observed when the strength of definite oxygen limitation increased from 0.04 to 0.24. Further experiments led in non-monitored flasks between FVR of 0.05 and 0.2 confirmed this result. These latter experiments allowed the testing of a higher FVR of 0.4 which led to a dramatic drop in the lipopeptide synthesis.

Considering the oxygen positive effect, a 25-fold increase of the mycosubtilin productivity was observed at a FVR of 0.24 compared to a FVR of 0.04. Authors previously observed an increase of the iturin A production rate as the stirring decreased in the bioreactor, *i.e.* as the oxygen transfer was decreased (Hbid et al., 1996). It is known that iturinA and mycosubtilin have almost the same structure while the amino acids at positions 6 and 7, D-Ser→L-Asn, are inverted in iturin A. Consequently, the positive oxygen regulation observed for the mycosubtilin synthesis in ATCC6633 could be found in the iturin A producing strain, *B. subtilis* S499.

In oxygen-monitored flasks, no effect of oxygen could be found for the BBG100 strain carrying the constitutive P_{repU} promoter instead of the native P_{myc}. It was suggested that oxygen metabolism could interfere in the regulation of the native P_{myc} promoter. At the

beginning of the oxygen-limited growths of ATCC6633, a constant expression of *fenF* was found at FVR of 0.05, 0.1 and 0.2. Interestingly, an increase of the expression of *fenF* was observed at a FVR of 0.1–0.2 compared to a FVR of 0.05 with respect to the increasing mycosubtilin specific productivity. Furthermore, a slight decrease of *fenF* expression was observed at FVR of 0.4 with respect to the low value of the mycosubtilin synthesis. It was recently demonstrated by pulse-chase experiments that the incorporation of C-10 to C-16 fatty acid moieties in mycosubtilin by the acyl ligase domain of the mycosubtilin synthetase was not specific (Hansen et al., 2007). These results suggested that the intracellular pool of fatty acid homologues could modulate the synthesis of mycosubtilin homologues. In our experiments, the different mycosubtilin homologues patterns obtained for the different oxygen-limited growths could express an indirect influence of OTR on the fatty acid synthesis and thus on the lipopeptide synthesis.

It was also shown in RAMOS experiments that surfactin production by the mycosubtilin overproducing strain BBG100 was lowered in comparison to ATCC6633. A study showed that the expression of both lipopeptides was clearly not coordinated as the *srfA* operon was mainly regulated by the response regulator ComA and the *myc* operon was primarily regulated by the transition state regulator AbrB (Duitman et al., 2007). However mycosubtilin and surfactin synthetases require an identical cofactor, the 4'-phosphopantetheinate, which transfer is catalysed by the product of the gene *sfp* which encode a 4'-phosphopantetheinyl transferase. This activation step could be limiting for surfactin synthetase when mycosubtilin synthetase is overproduced.

To end with, the diminution of surfactin concentration observed during the RAMOS culture at a FV of 10, 25 and 42 ml is not yet explained. It could result from a re-use of the surfactin as a substrate by the strain since it appeared only in culture where carbon source depletion was previously observed.

Conclusion

This study showed that definite oxygen metabolism effectively modulates the synthesis of a NRPS lipopeptide in *B. subtilis* ATCC6633. For the first time, the difficulty of monitoring the bacteria respiration during the production process of a biosurfactant was overcome. It highlights in particular the high potential of RAMOS system for physiological studies on biosurfactant synthesis. Providing knowledge of the effect of OTR on mycosubtilin synthesis by *B. subtilis* ATCC6633 allowed equalising the specific productivity obtained by a genetically modified derivative of ATCC6633 by simply manipulating an external parameter as OTR.

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Figure 1: Evolution of oxygen transfer rate (OTR), expressed in mol $O_2.l^{-1}.h^{-1}$, during growth of *B. subtilis* ATCC6633 in RAMOS flasks with a filling volume (FV) of 10 ml (-♦-), 25 ml (-■-), 42 ml (-▲-) and 60 ml (-●-). Displayed data corresponds to four experiments led simultaneously. The experiment with a FV of 42 ml is representative of a triplicate set.

Figure 2: Evolution of the mycosubtilin (A and B) and surfactin (C and D) production expressed in $mg.l^{-1}$ during growth-limited cultures of *B. subtilis* ATCC6633 and BBG100 in RAMOS flasks with different filling volumes of 10 ml (-♦-), 25 ml (-■-), 42 ml (-▲-), 60 ml (-●-). For each FV condition, displayed kinetics data corresponds to one RAMOS and three non-monitored flasks experiments led simultaneously.

Figure 3: Effect of different oxygen-limited growths obtained in flasks with different filling volume ratios (FVR) of 0.05, 0.1, 0.2 and 0.4 on the expression of the *fenF* gene of *B. subtilis* ATCC6633. The culture medium was the modified Landy medium buffered with MOPS 100 mM at pH 7.0. Comparative RT-PCR gels for *fenF* and *rplL* and the corresponding *rplL* normalised MTM ratios for *fenF* are presented for doubling times of 2 and 4 (□). Experiments were realised in duplicates (□ and ■).

Figure 4: Evolution of the mycosubtilin productivity, expressed in $mg.g(DW)^{-1}.h^{-1}$, and mycosubtilin homologues C-16 (▯) and C-17 (▮) patterns for cultures of *B. subtilis* ATCC6633 led at FVR of 0.05, 0.1, 0.2 and 0.4. Data originated from a triplicate set.

Table 1: Measured values of the pH, optical density at 600 nm, glucose, glutamic acid and mycosubtilin concentrations after 48 h of culture at 30°C and shaking at 175 rpm in the

RAMOS. Displayed data corresponds to 4 experiments led simultaneously at different filling volumes of 10, 25, 42 and 60 ml.

Appendix 1: MALDI-MS spectrum of the partially purified C-16 and C-17 mycosubtilin homologues. Identification of the $[M+K^+]$ ions of C-16 and C-17 mycosubtilin homologues was done according to their calculated m/z values, 1109.539 and 1123.554 uma.

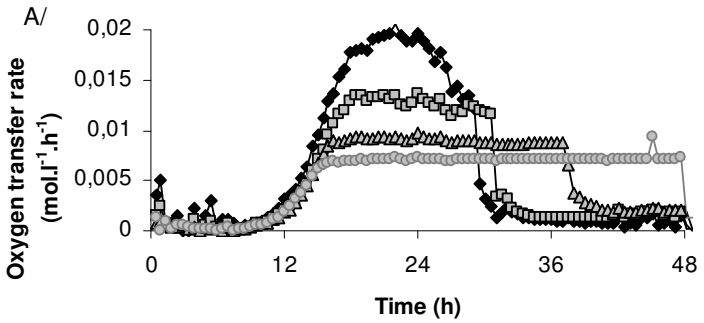

A/
0,02
0,015
0,01
0,005
0
Oxygen transfer rate (mol.l-1.h-1)
0
12
24
36
48
Time (h)

Figure 1

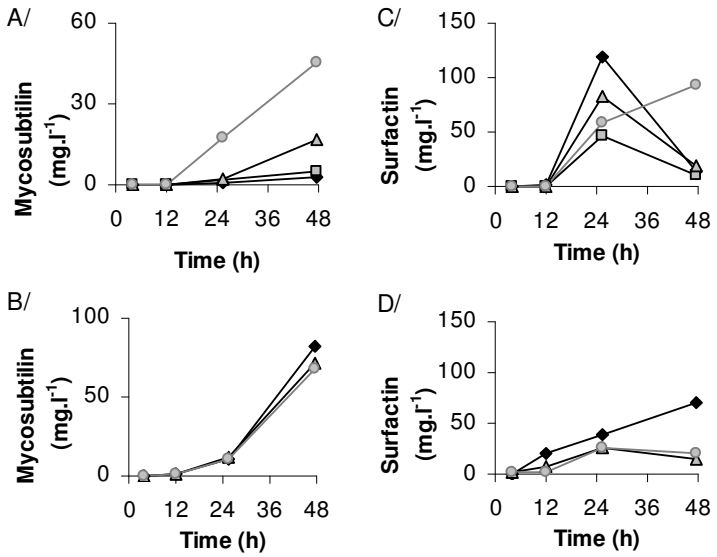

A/
Mycosubtilin (mg.l-1)
60
30
0
0 12 24 36 48
Time (h)
C/
Surfactin (mg.l-1)
150
100
50
0
0 12 24 36 48
Time (h)
B/
Mycosubtilin (mg.l-1)
100
50
0
0 12 24 36 48
Time (h)
D/
Surfactin (mg.l-1)
150
100
50
0
0 12 24 36 48
Time (h)

Figure 2

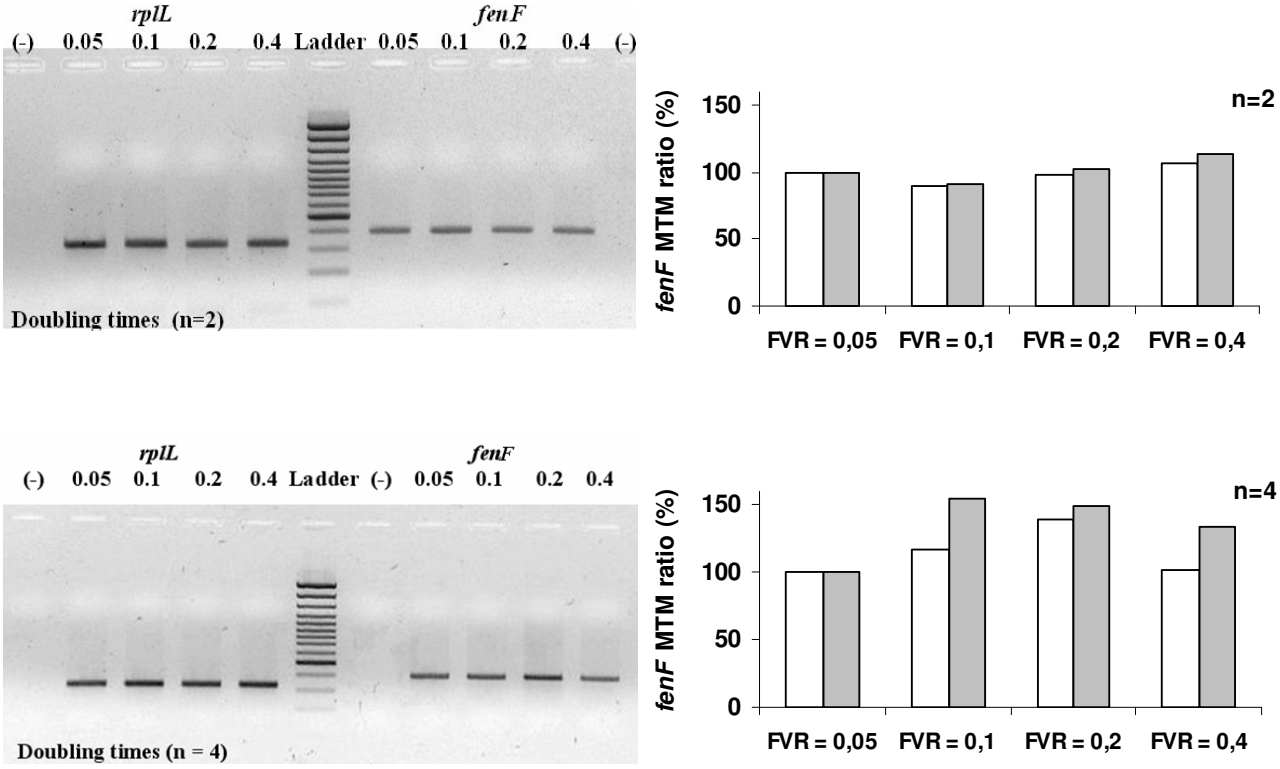

rplL
fenF
(-) 0.05 0.1 0.2 0.4 Ladder 0.05 0.1 0.2 0.4 (-)
Doubling times (n=2)
fenF MTM ratio (%)
150
100
50
0
n=2
FVR = 0,05
FVR = 0,1
FVR = 0,2
FVR = 0,4
rplL
fenF
(-) 0.05 0.1 0.2 0.4 Ladder (-) 0.05 0.1 0.2 0.4
Doubling times (n = 4)
fenF MTM ratio (%)
150
100
50
0
n=4
FVR = 0,05
FVR = 0,1
FVR = 0,2
FVR = 0,4

Figure 3

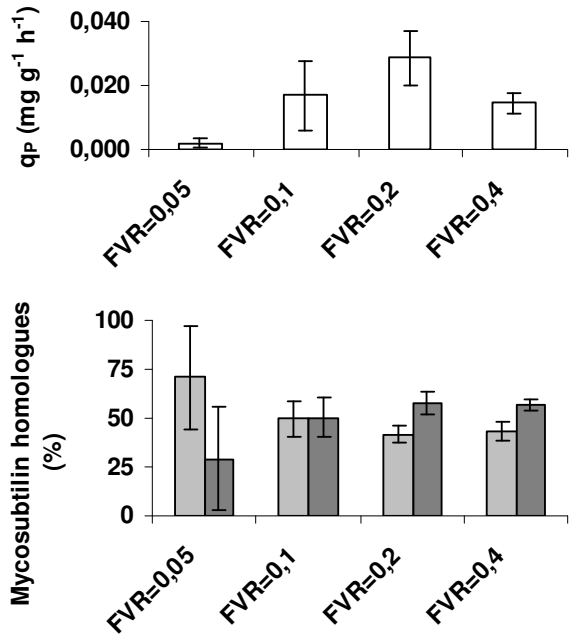
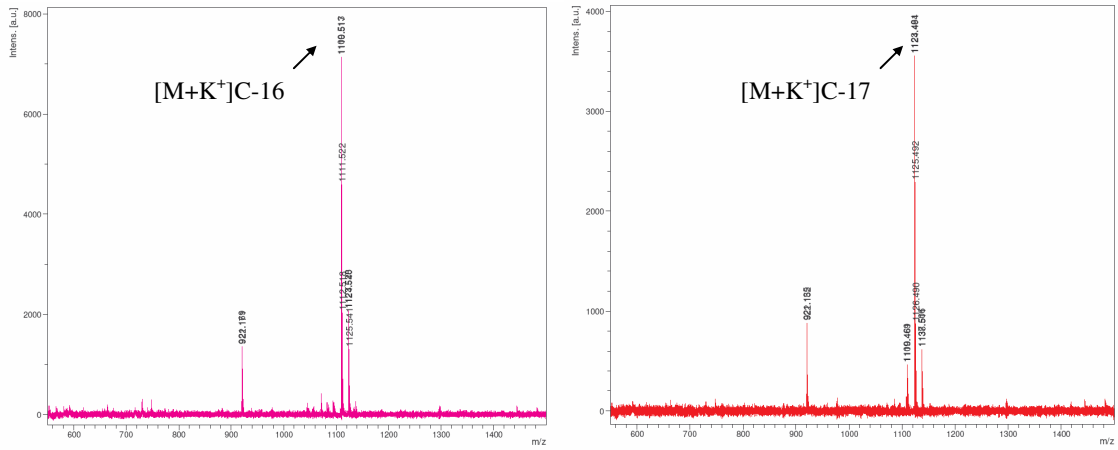

q_P (mg g^{-1} h^{-1})
0,040
0,020
0,000
FVR=0,05
FVR=0,1
FVR=0,2
FVR=0,4
Mycosubtilin homologues (%)
100
75
50
25
0
FVR=0,05
FVR=0,1
FVR=0,2
FVR=0,4

Figure 4


[M+K+]C-16
Intens. [a.u.]
8000
6000
4000
2000
0
1110.513
1111.522
922.189
600
700
800
900
1000
1100
1200
1300
1400
m/z
[M+K+]C-17
Intens. [a.u.]
4000
3000
2000
1000
0
1124.484
1125.492
922.158
1109.469
1138.506
600
700
800
900
1000
1100
1200
1300
1400
m/z

Appendix 1

Table 1

Strain	Filling Volume (ml)	pH	OD	Glucose ($g.l^{-1}$)	Glutamic acid ($g.l^{-1}$)	mycosubtilin ($mg.l^{-1}$)	q_P ($mg.g\ (DW)^{-1}\ h^{-1}$)
6633	10	6.02	15.2	0	0.017	2.8	0.001
	25	6.06	14.9	0	0.021	4.7	0.002
	42	6.08	18.1	0	0.034	16.9	0.006
	60	6.07	11.2	0.45	0.056	45.4	0.025
BBG100	10	5.96	15.7	0.07	0.016	82.2	0.033
	42	5.88	12.2	2.8	0.039	72	0.037
	60	6.10	12.3	2.4	0.028	67.9	0.035