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Influence of the Production of Two Lipopeptides, Iturin A and Surfactin S1, on Oxygen Transfer During *Bacillus subtilis* Fermentation

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ABSTRACT

Bacillus subtilis produces three types of lipopeptides: Iturins, Fengycins, and Surfactins. These amphiphilic molecules influence the volumetric oxygen transfer (K_La) like chemical surfactant. K_La values were from two- to fivefold lower when both Iturin A and Surfactin S1 were coproduced, and from 0.57- to 0.8-fold lower when only Iturin A was produced. The addition of an oxygen vector (*n*-Dodecane) during fermentation increased K_La values (7.5-fold) and biomass production (twofold), and decreased Iturin A and Surfactin S1 production. However, at high stirring conditions and in the presence of *n*-Dodecane, Iturin A production was improved.

Index Entries: *B. subtilis*; Iturin; Surfactin; oxygen transfer; oxygen vector.

INTRODUCTION

Bacillus subtilis produces three types of cyclic lipopeptides: Iturins *(1)*, Fengycins *(2)* (or Plipastatins *[3]*), and Surfactins *(4)*. Peptidic moiety of these compounds are composed of seven L- and D- α-amino acids for Iturins and Surfactins and 10 L- and D- residues for Fengycins. Lipidic moiety is a fatty acid with an amino group in Iturins and a hydroxy group in Surfactins or in Fengycins. The length of the fatty acid chain can vary from C-13 to C-17, giving different homologous compounds and isomers (*n*, iso, and anti-iso) for each lipopeptide. Iturin group is made up of Iturin A *(1)*, Mycosubtilin *(5)*, Bacillomycin L *(6)*, D *(7)*, and F *(8)*. All these molecules show hemolytic and antifungal activities. Three types of

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Fengycins have been described, Fengycin A and B *(2)* and Lipobacillin *(9)*. These molecules are only active against filamentous fungi, and they have a less important hemolytic activity than Iturins. The Surfactin group is made up of Surfactin S1, Surfactin [Ile7] *(10)*, Surfactin [Val7] *(11)*, Surfactin [Ala4] *(12)*, and Halobacillin *(13)*. Surfactin S1 shows antibiotic and antiviral activities and inhibition of fibrin clot formation *(4)*.

The amphiphilic structure of these lipopeptides gives them a surfactant behavior. Surfactin S1 is one of the most efficient biosurfactants known. At their critical micellar concentration, Surfactin S1 and Iturin A lower surface tension of water, from 72 to 31 mN/m and 54.5 mN/m, respectively *(14)*. They rapidly spread over the air–water interface at 90 mol/Å^2 for iturin A *(15)* and 300 mol/Å^2 for surfactin S1 *(16)*. No interfacial studies have been available until now for Fengycins.

Iturins and Fengycins are produced during the stationary phase of *B. subtilis* growth, contrary to Surfactins, which are produced during the exponential growth phase. Some *B. subtilis* strains produce only one type of lipopeptide, and others coproduce two or the three types of these molecules (*17*; Hbid et al., unpublished results). Different authors have investigated the production conditions of Iturins and Surfactins. Little information is, however, available about the influence, on the one hand, of oxygen on production of these lipopeptides and, on the other hand, of these lipopeptides on the volumetric oxygen transfer coefficient (K_La). Two contradictory results can be pointed out concerning the influence of oxygen on lipopeptide production: Shoda and coworkers have demonstrated *(18,19)* that, in flask or in fermentor, deficiency in dissolved oxygen had no adverse effect on Iturin A synthesis by the *B. subtilis* NB22 strain. However, our group has shown that a higher shaking rate has a positive influence on lipopeptide production by the strain *B. subtilis* S499 in flasks *(9)*. In addition, it is well known that surfactants affect oxygen transfer in fermentation and reduce, at high concentrations, the volumetric oxygen transfer coefficient (K_La) *(20)*.

In this article, we study the influence of lipopeptides from *B. subtilis* on the oxygen transfer in fermentation broth and during fermentation. We also study the influence of an oxygen vector (*n*-Dodecane) on the oxygen transfer during the fermentation and on biomass and lipopeptide production. These emulsifier oxygen vectors were used with success for *Aerobacter aerogenes* fermentation *(21)* and penicillin production *(22)*.

MATERIALS AND METHODS

Strains

The strain *B. subtilis* S499 was a gift from L. Delcambe (CNPEM, Liège, Belgium). Strain ATCC 13952 was purchased from American Type Culture Collection (ATCC).

Lipopeptide Production and Extraction

Strains were cultivated on Jacques et al. medium *(9)*, in a 20-L fermentor, equipped with a mechanical foam breaking system (from AEG). The medium contained per liter: 30 g peptone, 20 g saccharose, 7 g yeast extract, 1.9 g KH_2PO_4, 0.001 mg $CuSO_4$, 0.005 mg $FeCl_3 \cdot 6H_2O$, 0.004 mg Na_2MoO_4, 0.002 mg KI, 3.6 mg $MnSO_4 \cdot H_2O$, 0.45 g $MgSO_4$, 0.14 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg H_3BO_3, 10 mg citric acid. Culture volume was 12 L. All fermentation parameters (pH, temperature, stir-

ring, and pO_2) were regulated with a Biolafitte regulation unit. Precultures were done in 500-mL into 1-L flasks, in the same medium, at 30°C and 200 rpm, for 16 h. Culture was carried out at 30°C, pH 7.0, adjusted with NaOH (3*N*) and H_3PO_4 (3*N*), 200 rpm, and 0.3 vvm (air volume/culture volume/minute). At 72 h of culture, cells were harvested and supernatant acidified to pH 2.0 in order to precipitate lipopeptides. Pellet was collected by centrifugation and extracted overnight with methanol. Extract was evaporated to dryness, resuspended in water, and lyophilized.

Universal peptone M66 from Merck was used. Chemicals and solvents were of analytical grade. Water was of Milli-Ro quality. pH and pO_2 probes were purchased from Ingold.

Lipopeptide Measurement

Lipopeptide extract was dissolved in methanol and subjected to high-performance liquid chromatography (HPLC) analysis, on a C_{18} column (5 µm, 250/4 mm, Lichrocart, Merck). Each group of lipopeptides was separately quantified. Surfactin was analyzed with the solvent: acetonitrile/water/ trifluoroacetic acid (TFA), 73/27/0.1 (v/v/v), and Iturin A with the solvent acetonitrile/water/ TFA, 40/60/0.1 (v/v/v). Solvents were of HPLC grade. TFA was from Sigma (St. Louis, MO).

K_La Measurement

K_La measurement was carried out in the Biolafitte fermentor of 20 L with a working volume of 12 L (internal diameter D = 21.5 cm, liquid height H_l = 37 cm). The fermentor was equipped with three Rushton turbines with a diameter of 10 cm. K_La was calculated either with the static gassing-out method, in the case of increasing lipopeptide concentrations in the culture medium, or in distilled water; or in the case of increasing peptone concentrations; or with the dynamic gassing-out method, during fermentation with or without *n*-Dodecane. All K_La measurements were carried out at 30°C and pH 7.0.

Fermentation of *B. subtilis* with *n*-Dodecane

Fermentation was carried out as described, except that 1 L of *n*-Dodecane was added to 12 L of culture broth before sterilization.

Biomass Measurement

Biomass was estimated with optical density (OD) measurement at 600 nm. When *n*-Dodecane was present, it was eliminated by centrifugation at 27,000*g* for 10 min, and cells were washed once with fresh medium and then resuspended in fresh sterile medium before OD measurement.

n-Dodecane Measurement

Concentration of *n*-Dodecane was followed by gas chromatography (GC) after extraction from culture medium with cyclohexane. Analyses were performed on a CP-Sil 8CB capillary column 25 m to 0.32 mm (Chrompack), in a Hewlett-Packard chromatograph, equipped with an FID detector. *n*-Decane was used as an internal standard for extraction and GC analysis. *n*-Dodecane and *n*-Decane were from Acros chimica and Sigma, respectively.

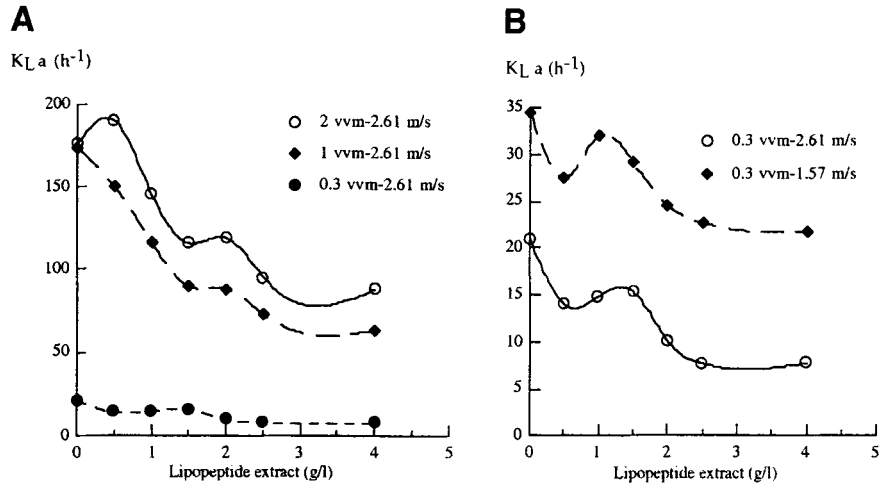

Fig. 1. **(A)** Influence of the concentration (g/L) of a lipopeptide extract from strain S499 on K_La in culture medium. Experiences were carried out at the same peripheral speed (2.61 m/s) and at different aeration rates, 0.3, 1, and 2 vvm. One gram of lipopeptide extract contains 23.2 mg of Surfactin S1 and 0.43 mg of Iturin A. Measurement at t = o in each condition was repeated three times. Standard deviation of K_La was ± 2.5%. **(B)** Influence of lipopeptide concentration (g/L) of a lipopeptide extract from strain S499 on K_La in culture medium at the same aeration rate 0.3 vvm and at two peripheral speeds, 2.61 and 1.57 m/s.

RESULTS

Influence of Crude Lipopeptide Extracts on the Volumetric Oxygen Transfer Coefficient (K_La) in the Culture Medium

Crude lipopeptide extract from *B. subtilis* S499, containing principally Iturin A and Surfactin S1, was added to sterile culture medium at different concentrations. K_La was measured in different conditions of aeration rate and peripheral speed. Results are shown in Fig. 1A,B.

Increasing lipopeptide concentrations decrease K_La, whatever the aeration rate and the peripheral speed. This decrease is more important for low lipopeptide concentrations. K_La was between 2- and 2.9-fold lower according to the aeration rate and the peripheral speed when 48 g of lipopeptide extract were added to 12 L of medium.

In Fig. 1B, it was surprisingly noticed that values of K_La were 2- to 2.5-fold lower for a high peripheral speed (2.61 m/s) than for a low one (1.57 m/s), with or without lipopeptides in the medium. This result was only pointed out at low aeration rates (0.3 and 1 vvm).

Similar K_La measurements were made in the same conditions, but only with solutions of peptone in distilled water. Results demonstrate (data not shown) that, in water, peptone (30 g/L) decreases K_La values 2- to 3.6-fold like a surfactant. The addition of an antifoaming agent (0.1%) restores the K_La value obtained in pure distilled water. At low aeration rates, K_La values were also higher for a low peripheral speed than for a high speed.

Table 1
Influence of Iturin A (48.56 mg/L)
on the Volumetric Oxygen Transfer Coefficient K_La in the Culture Medium

	0.3 vvm		1 vvm		2 vvm	
K_La (h^{-1})	1.57 m/s	2.61 m/s	1.57 m/s	2.61 m/s	1.51 m/s	2.61 m/s
Before adding Iturin A	36.4	25.3	79.6	164.2	104.7	181.3
After adding Iturin A	30.5	23.7	65.9	92.9	70.8	104.6

Influence of Iturin A Extract on K_La

The same experimentation was performed with a lipopeptide extract from the supernatant of *B. subtilis* ATCC 13952 strain fermentation. This extract only contained Iturin A. Iturin A also diminished K_La as demonstrated, but the decreasing factor was lower (from 0.8 at 0.3 vvm and 2.61 m/s to 0.57 at 2 vvm and 2.61 m/s) than lipopeptide extract containing Iturin A and Surfactin S1. Results are shown in Table 1.

Influence of Lipopeptide Extract on K_La in Distilled Water

In order to avoid the peptone effect, influence of lipopeptide extract on oxygen transfer was evaluated in the same conditions, but in sterile distilled water. Results are shown in Fig. 2A,B. Dramatic reduction of the volumetric oxygen-transfer coefficient (K_La) was noticed for high peripheral speed, 2.61 m/s (5.2-fold lower at 1 vvm and 3.35-fold lower at 2 vvm). The reduction factor was less important for lower peripheral speeds, 1.4–1.8 (for 1 and 2 vvm, respectively) at 1.57 m/s, and 1.7–2 (for 1 and 2 vvm, respectively) at 1.04 m/s.

Influence of *n*-Dodecane on K_La

In order to evaluate the effect of an oxygen vector on K_La values, oxygen transfer was evaluated in the presence of a high concentration (4 g/L) of the lipopeptide extract from *B. subtilis* S499 and with or without added *n*-Dodecane (8.3%). Results are shown in Table 2. *n*-Dodecane increases the per-liter K_La with a factor from 1.2 to 2.6.

Influence of *n*-Dodecaneon Lipopeptide and Biomass Productions

To elucidate the influence of *n*-Dodecane on lipopeptide and biomass productions, fermentations were carried out with the strain *B. subtilis* S499, with or without *n*-Dodecane (8.3%). Biomass, lipopeptide concentration, K_La, and *n*-Dodecane concentrations were followed. Results are summarized in Fig. 3 and in Table 3. Dodecane addition to culture medium increased biomass production more than twofold, whereas K_La was 7.5-fold higher. K_La values continuously decreased during the culture without oxygen vector and were 3.6-fold lower after 48 h of fermentation. An opposite phenomenon was observed in the presence of *n*-Dodecane. In this last case, K_La was relatively stable during the first hours of the culture but continuously increased afterward. This effect is probably owing to *n*-Dodecane globule stabilization caused by biosurfactant production. Lower Iturin A and Surfactin S1 concentrations were observed in the presence of the oxygen vector.

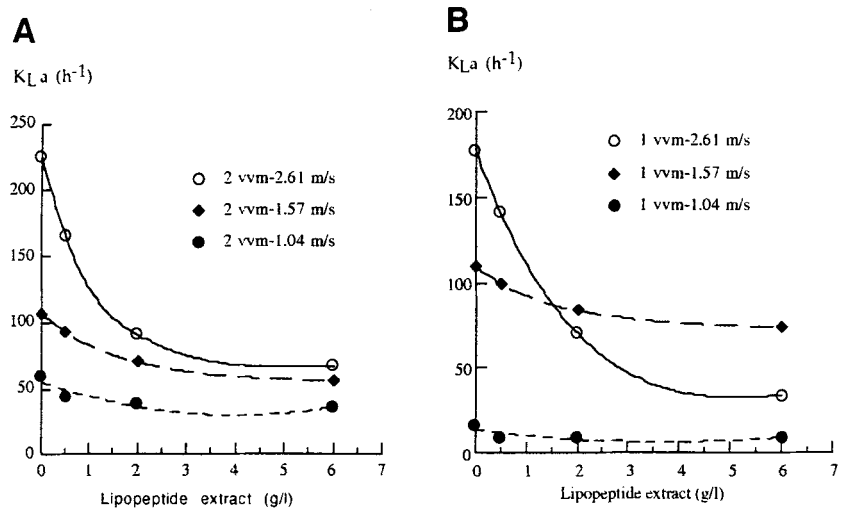

Fig. 2. **(A)** Influence of lipopeptide extract (strain S499) on K_La in distilled water at 2 vvm and at different peripheral speeds. **(B)** Influence of lipopeptide extract (strain S499) on K_La in distilled water at 1 vvm and at different peripheral speeds.

Table 2
Effect of *n*-Dodecane Addition (8.3%) on the per Liter K_La in a Culture Medium with High Lipopeptide Extract Concentration (4 g/L)

	0.3 vvm		1 vvm		2 vvm	
K_La (h^{-1})	1.57 m/s	2.61 m/s	1.57 m/s	2.61 m/s	1.51 m/s	2.61 m/s
Without *n*-Dodecane	1	0.6	6.1	5.3	4.9	7.3
With *n*-Dodecane	1.2	1.6	7.9	9.1	9.7	14

Another fermentation was then performed with *n*-Dodecane, but at a higher peripheral speed (1.57 m/s). Fermentation control (without *n*-Dodecane) could not be carried out because of the excess of foam formed. As shown in Table 3, biomass and K_La were more than twofold higher than at lower peripheral speed. Surfactin S1 production was not improved by a higher stirring, but Iturin A production was multiplied by 4.

DISCUSSION

In preliminary experiments, we studied in flasks, the optimization of culture conditions for biomass and lipopeptide productions from *B. subtilis* S499 strain, using Plackett-Burman design of experiments. Results show that good yields of both biomass and lipopeptides were obtained when cultures were vigorously shaken *(9,23)*. Extrapolation of optimal medium to control condition of aeration in the fermentor was confronted by two problems: the dramatic reduction of oxygen transfer in the presence of biosurfactants (Iturin A and Surfactin S1) and foam formation. In this work, we have studied the influence of lipopeptides on the oxygen transfer, in culture medium and in water. These experiments were performed with

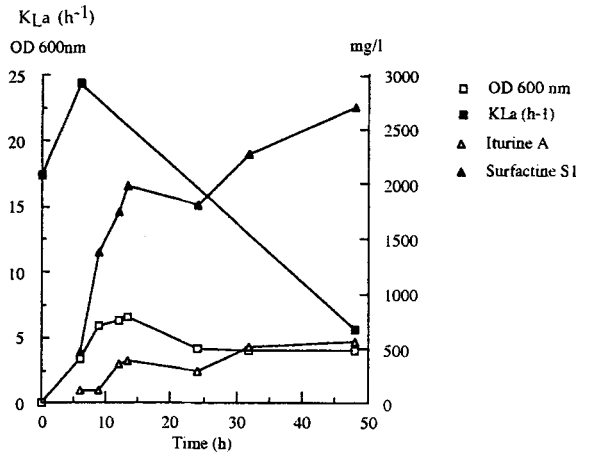

Fig. 3. Growth ($OD_{600\,nm}$) and evolution of K_La, Iturin A, and Surfactin S1 concentrations as a function of time during fermentation of *B. subtilis* S499 at 0.5 vvm and 1.57 m/s in the absence of *n*-Dodecane.

Table 3
Optical Density (600 nm), per Liter K_La/h, concentrations (mg/L) of Surfactin S1 and Iturin A, After 48 h of Culture of *B. subtilis* S499, With or Without *n*-Dodecane[a]

	Culture conditions		
Parameters	0.5 vvm, 1.04 m/s without *n*-Dodecane	0.5 vvm, 1.04 m/s with *n*-Dodecane	0.5 vvm, 1.57 m/s with *n*-Dodecane
$OD_{600\,nm}$	4	9.5	22.6
K_La/h	0.8	5.8	11.8
Surfactin S1, mg/L	2710	1221	1180
Iturin A, mg/L	561	298	1388

[a] Cultures with *n*-Dodecane were performed at two peripheral speeds (1.04 and 1.57 m/s).

two types of lipopeptide extracts, the first containing a mixture of Iturin A and Surfactin S1 (with a larger proportion of the latter) and the second containing only Iturin A. In both cases, lipopeptides decrease K_La as pointed out for other surfactants *(20)*, but lipopeptide extract containing a majority of Surfactin S1 decreases K_La strongly in all aeration cases in comparison to lipopeptide extract containing only Iturin A. This effect could be owing to the high surface activity of Surfactin S1, which forms a stable film covering a large interfacial area and reduces oxygen (or gas) transfer into the medium.

We also pointed out, by using the two types of lipopeptide extract and peptone, an inverse relationship between K_La values and peripheral speed at low aeration rate (0.3 and 1 vvm) (Figs. 1B, 2B). One hypothesis can be advanced. Increasing stirring causes an increase of specific area (a in K_La), but also disturbs the liquid–liquid micelles formation and, thus, enhances the adsorption rate of lipopeptides at the new air–water surface. This effect leads to a high decrease in K_La. Addition of *n*-Dodecane to the medium eliminates this phenomenon because of its antifoam properties and adhesion of lipopeptides to the *n*-Dodecane–water interface.

Addition of *n*-Dodecane to the culture medium increases oxygen transfer. A higher increasing factor was noticed for a higher agitation rate because of higher emulsion dispersion. Addition of *n*-Dodecane to the fermentation medium increases biomass production, but decreases lipopeptide production. This effect is in conformity with results obtained in the case of addition of *n*-Hexadecane (4%) to the culture medium of strain *B. subtilis* ATCC 21332 *(24)*. In this case, no Surfactin was detected. On the contrary, this result is not in agreement with the optimized conditions (high shaking) that we have previously determined for lipopeptide production. This result is, however, confirmed at a high aeration rate. In this condition, very good yield of Iturin A was obtained. Contrary to Shoda's group, who shows that oxygen starvation does not influence Iturin A production in the strain *B. subtilis* NB22 *(18,19)*, we go on to postulate that a good oxygen transfer is neccessary to improve Iturin A production. Moreover, addition of *n*-Dodecane appears to be a good technology for Iturin production at a larger scale without foam formation.

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