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Optimization of Biosurfactant Lipopeptide Production from *Bacillus subtilis* S499 by Plackett-Burman Design

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Abstract

Bacillus subtilis S499 is well-known for its ability to produce two families of surfactant lipopeptides: Iturin A and Surfactin S1. Fermentation optimization for this strain was performed to amplify the surfactant production. Ten active variables were analyzed by two successive Plackett-Burman designs, consisting respectively of 12 and 16 experiments to give an optimized medium. The amount of biosurfactant lipopeptides in the supernatant of a culture carried out in this optimized medium was about five times higher than that obtained in nonoptimized rich medium. The analysis of the surfactant molecules produced in such optimized conditions has revealed the presence of a third family of lipopeptides: the fengycins.

The time-dependent production of these three families of molecules in bioreactors showed that surfactin S1 is produced during the exponential phase and iturin A and fengycins during the stationary phase.

Index Entries: *Bacillus subtilis*; biosurfactant; lipopeptide; statistical design; fengycin.

Introduction

Virtually all surfactants are chemically synthesized. Nevertheless, in recent years, much attention has been directed toward biosurfactants owing to their different advantages such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity

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and specific activity at extreme temperatures, pH and salinity, and the ability to be synthetized from renewable feedstocks *(1)*. However, applications of these biomolecules are limited by their cost price. The cost price can be reduced by optimizing biosurfactant production and by reducing the time of downstream processing. In this work, we propose a strategy for the amplification of biosurfactant production in the supernatant of *Bacillus subtilis* S499.

B. subtilis S499 coproduces two families of surfactant lipopeptides: surfactin S1 and iturin A *(2)*. These compounds can be distinguished by their chemical structure and properties. They consist of heptapeptides containing a β-hydroxy fatty acid for surfactins *(3)* or a β-amino fatty acid for iturins *(4)*. The length of the fatty-acid chain can vary from C-13 to C-16 for surfactins and from C-14 to C-17 for iturins, giving different homologous compounds and isomers (*n*, *iso*, and *anteiso*) for each lipopeptide *(5,6)*. Surfactins are especially potent surface-active compounds *(7–9)* exhibiting some biological properties such as cytolytic activity *(10)* and antiviral properties *(11)*. Iturin A is a strong antifungal agent *(12)*. Both show hemolytic activities. Surfactin S1 and Iturin A interact in synergism on biological *(13)* and surface-active properties *(14)*.

In this article, fermentation optimization of *B. subtilis* S499 was performed to amplify the biosurfactant production. Ten active variables were analyzed and optimized by two successive Plackett-Burman designs *(15)*, consisting respectively of 12 and 16 experiments. The time-dependent production of lipopeptides in optimized conditions was analyzed.

Materials and Methods

Strain

The strain *B. subtilis* S499 was supplied by L. Delcambe (CNPEM, Liège, Belgium). This strain was collected in Ituri, Congo (formerly Zaire) *(16)*.

Lipopeptide Production and Extraction

The strain was cultivated in 863 medium (glucose 20 g/L, peptone 10 g/L, yeast extract 10 g/L) at 30°C and pH 7 or under the culture conditions defined by the statistical designs (*see* Experimental Design). All the experiments were performed in 1-L shake flasks containing 500 mL of culture medium. Incubation was carried out on a rotary shaker using 10% of the seed culture performed in the same culture conditions. Cells were harvested after 72 h of culture by centrifugation. Lipopeptides were extracted by solid-phase extraction on bond elut C18 (1 g; Varian, CA) as previously described *(17)*.

Hemolytic Activity

Hemolytic activity was determined as described by Quentin et al. *(18)*. Lipopeptide extracts were dissolved in 400 µL of methanol. Fractions of

5, 10, 20, 30, 40, 50, 80, 100, and 200 µL were dried under vacuum. These dried samples were dissolved in 20 µL of methanol, mixed with 980 µL of a suspension of sheep erythrocytes (Diagnostic Pasteur, Belgium) diluted in Nacl 0.9% (final cellular concentration about 5.10^7 cells/mL) and incubated at 37°C for 30 min. Samples were then centrifuged at 17,000*g* for 20 min. The experimental data were corrected for the release of hemoglobin observed in the absence of lipopeptides. The 100% hemolysis value was obtained by diluting 0.1 mL of the erythrocyte suspension with 0.9 mL of distilled water. One hemolytic unit (HU) is the lipopeptide quantity giving 50% of hemolysis.

Experimental Design

Two successive designs of respectively 12 and 16 experiments are shown in Tables 1 and 2. The experiments were carried out as described by Plackett and Burman *(15)*. Each column represents a different experimental trial and each row represents different variables. Each variable was tested at two levels, a higher (+) and a lower (–) level as in Tables 1 and 2. Four centerpoint replications were performed for each design at the mid-level of each variable to estimate the experimental error. The trace element solution in the first design contained/L: 1 mg $CUSO_4$; 5 mg $FeCl_3 \cdot 6H_2O$; 4 mg $Na_2MoO_4 \cdot 2H_2O$; 2 mg KI; 0.14 g $ZnSO_4 \cdot 7H_2O$; 10 mg H_3BO_4; 0.4 g $MnSO_4 \cdot H2O$; and 10 g citric acid. In the second design, no manganese was added to the solution and a second salt solution was used. It contained/L: 0.4 g $MnSO_4 \cdot H_2O$ and 50 g $MgSO_4$. Zwitterionic buffers were used for maintaining the pH values (pH 6 and 6.5, MES; pH 6.5, 7, and 7.5, BES; pH 8, EPPS).

Data Analysis

The main effect (m_i) of the variable upon the measured response (Y_j) was calculated by:

$$m_i = [\Sigma^n{}_1 \text{ (signe)}i,jY_j]/(n/2)$$

where n is the number of experiments.

The experimental error was evaluated by the main effect estimate:

$$\text{CL} = \pm tSE/(n/4)^{0.5}$$

where t is the student variable and SE, the standard error. The confidence level chosen was 98%.

Lipopeptide Production in Fermentor

The strain was cultivated in optimized conditions in a 20-L fermentor equipped with a mechanical foam-breaking system (Fundafoam from Chemap, Volketswil, Switzerland). All fermentation parameters (pH, temperature, and stirring) were controlled with a Biolafitte regulation unit. Preculture (500 mL) was carried out in a 1-L shake flask, in the same culture

Table 1
First Plackett-Burman Design for the Study
of *B. subtilis* Biosurfactant Production by 12 Experiments and 4 Centerpoint Replications

Variables	Lower level (–)	Mid level (0)	Higher level (+)	Experimental trials															
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Temperature	30°C	35°C	40°C	+	+	–	+	+	+	–	–	–	+	–	–	0	0	0	0
pH	6	7	8	+	–	+	+	+	–	–	–	+	–	+	–	0	0	0	0
Shaking	100 rpm	150 rpm	200 rpm	–	+	+	+	–	–	–	+	–	+	+	–	0	0	0	0
KH_2PO_4[a]	0.1	1	1.9	+	+	+	–	–	–	+	–	+	+	–	–	0	0	0	0
Trace elements[b]	1	5	9	+	+	–	–	–	+	–	+	+	–	+	–	0	0	0	0
Sucrose[a]	2	10	18	+	–	–	–	+	–	+	+	–	+	+	–	0	0	0	0
$(NH_4)_2SO_4$[a]	0.5	1.5	2.5	–	–	–	+	–	+	+	–	+	+	+	–	0	0	0	0
Peptone[a]	2	10	18	–	–	+	–	+	+	–	+	+	+	–	–	0	0	0	0
Yeast extract[a]	2	10	18	–	+	–	+	+	–	+	+	+	–	–	–	0	0	0	0

[a]Concentrations in g/L. [b]Concentrations in ml/L.

Table 2
Second Plackett-Burman Design for the Study
of *B. subtilis* Biosurfactant Production by 16 Experiments and 4 Centerpoint Replications

Variables	Lower level (–)	Mid level (0)	Higher level (+)	Experimental trials																	
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Temperature	27°C	30°C	33°C	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	0	0	0	0
pH	5.5	6.5	7.5	–	–	+	+	–	–	+	+	–	–	+	+	–	–	+	+	0	0	0	0
Shaking	150 rpm	175 rpm	200 rpm	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	0	0	0	0
KH_2PO_4[a]	0.1	1	1.9	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	0	0	0	0
Trace elements[b]	1	5	9	–	+	+	–	+	–	–	+	–	+	+	–	+	–	–	+	0	0	0	0
Sucrose[a]	10	20	30	–	–	+	+	+	+	–	–	+	+	–	–	–	–	+	+	0	0	0	0
$MnSO_4$ $MgSO_4$[b]	1	5	9	–	+	+	–	–	+	+	–	+	–	–	+	+	–	–	+	0	0	0	0
Peptone[a]	10	20	30	–	+	–	+	+	–	+	–	+	–	+	–	–	+	–	+	0	0	0	0
Yeast extract[a]	1	4	7	+	–	–	+	–	+	+	–	–	+	+	–	+	–	–	+	0	0	0	0

[a]Concentrations in g/L. [b]Concentrations in ml/L.

conditions, for 16 h. Chemicals and solvents were of analytical grade. Water was of Milli-Ro quality (Millipore, MA). pH and O_2 probes were purchased from Ingold. Biomass was estimated with optical density (OD) measurement at 600 nm.

Lipopeptide Purification and Quantification

Lipopeptide crude extracts were solubilized in 250 µL of methanol and loaded (50 µL) on a PepRPC HR5/5 column (FPLC, Pharmacia, Uppsala, Sweden) as previously described *(17)*. Main homologous compounds of each lipopeptide family were purified by using the technique proposed by Razafindralambo et al. *(8)*, and used as a standard for following the lipopeptide production. Amino-acid analyses of purified fengycins were carried out by high-performance liquid chromatography (HPLC) (PICO-TAG). Molecular weight of purified fengycin was established by electrospray mass spectrometry (MS) measurements using a VG Platform, Fisons Instruments (Manchester, UK).

Results

Optimization of Biosurfactant Production

For rapid identification of the main variables affecting the biosurfactant production of *B. subtilis* S499, two successive Plackett-Burman designs of 12 and 16, respectively, experiments were carried out with 4 centerpoint replications in each case. The variables and their levels were selected from literature data and are shown in Tables 1 and 2. The surfactant production was evaluated by measuring the hemolytic activity of the supernatant. This activity is similar for lipopeptides with the same fatty acid (concentration giving 50% of hemolysis: surfactin *iso*-C15, 26.41 µ*M*; iturin A *iso*-C15, 19.66 µ*M*). In addition, Iturin A and Surfactin S1 interact in synergism on their hemolytic activities like on their surface-active properties *(14,19)*. The estimations of the main effects (m_i) and the experimental error were given in Figs. 1 and 2. The first design revealed a significant negative effect of temperature and pH, and a significant positive effect of shaking, peptone, and yeast extract concentrations. In the second design, the variables and their levels were modified to specify the results obtained in the first design. The levels of temperature, pH, shaking, phosphate, sucrose, peptone, and yeast-extract concentrations were changed. The effect of two ions (Mg and Mn) were separately analyzed from the other trace elements. This second design revealed a significant positive effect of pH, shaking, phosphate, and peptone concentrations.

This Plackett-Burman optimization allowed us to define new cultural conditions adapted for high-surfactant production: temperature, 30°C; pH 7.0; shaking, 200 rpm; KH_2PO_4, 1,9 g/L; [trace elements], 1 mL/L; Sucrose, 20 g/L; [Peptone], 30 g/L; Mn, Mg, 9 mL/L; Yeast extract: 7 g/L.

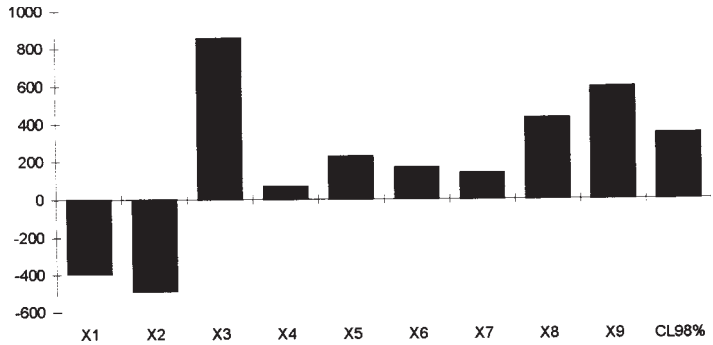

Fig. 1. Estimation of the main effects of each variable on the biosurfactant production for the first design. X1, temperature; X2, pH; X3, shaking; X4, KH_2PO_4; X5, trace elements; X6, sucrose; X7, $(NH_4)_2SO_4$; X8, peptone; X9, yeast extract; C.L., confidence level.

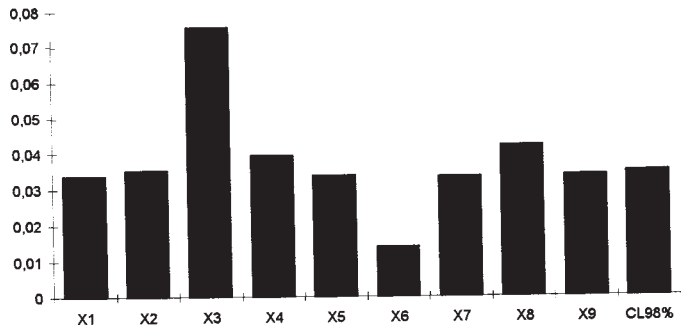

Fig. 2. Estimation of the main effects of each variable on the biosurfactant production for the second design. X1, temperature; X2, pH; X3, shaking; X4, KH_2PO_4; X5, trace elements; X6, sucrose; X7, Mn-Mg; X8, peptone; X9, yeast extract; C.L., confidence level.

Analysis of the Lipopeptides Produced

Lipopeptides are the main surfactant molecules produced by *B. subtilis*. The type and the amount of lipopeptides produced in the optimized conditions were determined by HPLC analyses followed by amino-acid analysis and molecular weight determination by MS. Three groups of molecules were found. Two of them corresponded to the families of Iturin A and Surfactin S1 that are normally produced by this strain. The amino-acid analyses and the molecular weight of the third group of molecules (Table 3) revealed the presence of another family of lipopeptide that had never been found before in the supernatant of this strain: fengycins *(20)*. Complete structure determination of the two main fengycins (Fengycin A-C16 and B-C17) produced was carried out by MS and 2D-NMR (data not shown). Surfactant lipopeptide production obtained in a fermentor under these optimized conditions was compared with that obtained in a nonoptimized medium. Results given in Table 4 confirm the efficiency of the optimization process.

Table 3
Amino-Acid Composition, Molecular Weight,
and Identification of the Main Different Molecules of Fengycins Purified by HPLC

Peak	Amino-acid composition	Molecular weight	Identification
1	3 Glx, 2 Tyr, 1Orn, 1 Ile, 1 **Ala**, 1 Pro, 1 allo-Thr	1435	Fengycin A-C14
2	3 Glx, 2 Tyr, 1Orn, 1 Ile, 1 **Ala**, 1 Pro, 1 allo-Thr	1449	Fengycin A-C15
3	3 Glx, 2 Tyr, 1Orn, 1 Ile, 1 **Val**, 1 Pro, 1 allo-Thr	1477	Fengycin B-C15
4	3 Glx, 2 Tyr, 1Orn, 1 Ile, 1 **Ala**, 1 Pro, 1 allo-Thr	1463	Fengycin A-C16
5	3 Glx, 2 Tyr, 1Orn, 1 Ile, 1 **Ala**, 1 Pro, 1 allo-Thr	1477	Fengycin A-C17
6	3 Glx, 2 Tyr, 1Orn, 1 Ile, 1 **Val**, 1 Pro, 1 allo-Thr	1505	Fengycin B-C17

Table 4
Biosurfactant Lipopeptide Production (mg/L)
by *B. subtilis* S499 Cultivated in Fermentor
in Nonoptimized (medium 868) and Optimized Medium

	In fermentor	
Lipopeptide	Medium 868	Optimized medium
Surfactin S1	154	826
Iturin A	134	657
Fengycins	31	95

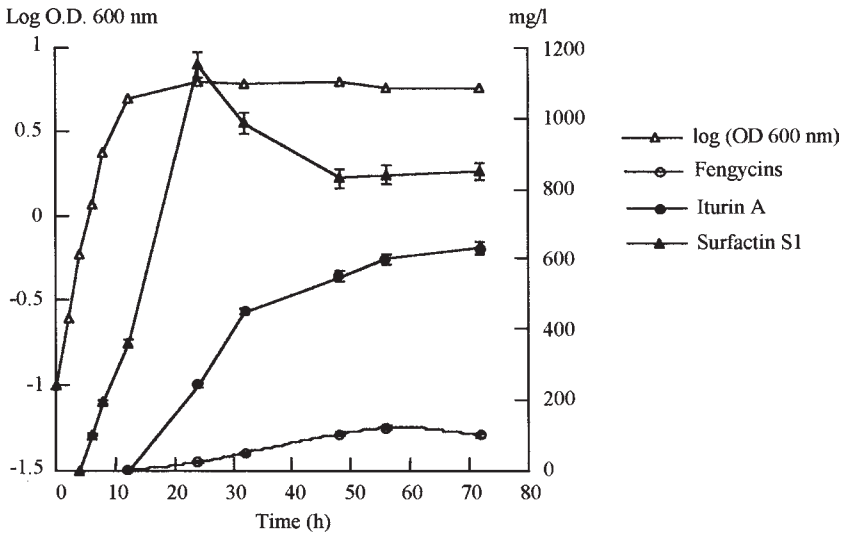

Fig. 3. Growth- and time-dependent lipopeptide production by *B. subtilis* S499 in optimized conditions.

Time-Dependent Lipopeptide Production

The time-dependent production of these three families of lipopeptides under optimized culture conditions was determined (Fig. 3). Surfactin S1 is produced during the exponential phase, Iturin A and Fengycins during the stationary phase.

Discussion

This work deals with the development of an optimization strategy for biosurfactant production by *B. subtilis* S499. The idea was to produce a maximal amount of biosurfactant by avoiding any downstream processing. *B. subtilis* S499 is particularly interesting because this strain produces a relatively high diversity of surfactant lipopeptide compounds that can show some synergism on their surface-active properties. The use of statis-

tical methods allows us quickly and efficiently to improve the amount of biosurfactant lipopeptides produced in the supernatant of *B. subtilis* S499 cultures. The highest productivity achieved under optimized conditions can reduce the cost price of the produced biosurfactant, which appears as a main limiting factor for their applications.

From a more fundamental point of view, this work also points out or confirms different culture parameters that need to be taken into account for the lipopeptide production by *B. subtilis* S499: temperature, pH, phosphate, peptone and yeast extract concentrations, and shaking. The results emphasize two points. First, the optimized conditions thus determined correspond to a relatively rich culture medium. Several authors have shown that surfactin production is quite high in a mineral medium. Different experiments have therefore been conducted in order to specify the amount of surfactin produced in mineral medium such as Landy medium *(21)*. The production of surfactin is, in all cases, below the level attained by us under optimized conditions (data not shown). These differences could reflect some disparities between the *B. subtilis* species concerning the regulation process of the lipopeptide production. Another interesting result is related to the shaking effect on the lipopeptide production. We have already shown in a preceding work *(22)* that oxygen is an important parameter to consider for the lipopeptide biosynthesis.

In addition, it has been shown that *B. subtilis* S499 coproduces three lipopeptide families: Surfactin S1, Iturin A, and Fengycin homologous compounds. This is the first example of a strain able to produce these three families. Fengycins have already been described as antifungal compounds showing a less important hemolytic activity *(20)*.

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