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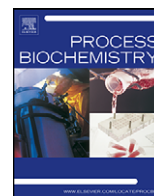
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# Lipopeptide overproduction by cell immobilization on iron-enriched light polymer particles

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## ABSTRACT

The study concerns surfactin and/or fengycin batch production by immobilized cells of *Bacillus subtilis* ATCC 21332. Light carriers designed for a three phase inverse fluidized bed biofilm reactor (TPFIBR) were used. With respect to the biofilm reactor development, a new support based on iron grafting onto polypropylene foams has been proposed. A suspension solid-state grafting process was applied to graft ferric acetylacetonate onto polypropylene (PP) foams with a density of 0.3–0.7 g/cm<sup>3</sup>. The iron contents grafted onto PP increased with the reaction time and then it tended to level off. The iron contents at 7.5 and 10 h are 0.74 and 0.75 wt%, respectively. It was specified that the equilibrium was reached at 7.5 h. Influence of particles on lipopeptide production was analyzed in two kinds of experiments: preliminary colonization step of particles, followed by production step in modified culture medium (named in this work colonization step) or direct addition of pellets in culture medium (named production step). All PP+ iron pellets promoted biomass enhancement. The production yield was modified for all types of PP supports, containing respectively 0, 0.35 and 0.75% of iron. The immobilized cultures produced 2.09–4.3 times more biosurfactants than planktonic cells. In production experiments addition of carriers seemed to modify the ratio between surfactin and fengycin with an enhancement of the fengycin production. The highest concentration of fengycin was obtained with addition of pellets containing 0.35% of iron.

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## 1. Introduction

In literature, numerous studies concerning free cells productivities of biosurfactants have been reported. Several parameters such as carbon sources, temperature, amount of inorganic ions, and addition of solid carriers may trigger both strain growth and lipopeptide synthesis. In the present work, we focused on evaluating various bioreactor parameters and systems with respect to efficient lipopeptide production by *Bacillus subtilis* ATCC 21332 immobilized bacteria on light carriers designed for use in three phase inverse fluidized bed biofilm reactor (TPFIBR). The *B. subtilis* ATCC 21332 is known to produce a single surfactin lipopeptide [1,2], but this strain was recently proved to be a co-producer of surfactin and fengycin [3]. However, the lipopeptide surfactin is one of the most powerful biosurfactants, while fengycins show more strong antifungal activity in combination with surfactins [4]. The use of immobilized bacteria, leading to high cell concentrations within the reactor, seemed a promising method for improving reactor performance and selectivity [5,6–8]. With respect to the cell's immobilizing performance various

parameters such as solid supports, culture medium, oxygen transfer and ions concentrations of metals are object of analysis. According to Wei and Chu [9,10], surfactin concentration was enhanced when iron concentration in the medium raised 4 μM and when Mn<sup>2+</sup> was added. Wei et al. [11] determined with experimental design plan that Mg<sup>2+</sup>, K<sup>+</sup>, Mn<sup>2+</sup> and Fe<sup>2+</sup> were the more significant factors affecting production of surfactin. Yeh et al. [12], Davies et al. [1] and Lee and Kim [13], studied the influence of oxygen concentration on the synthesis of the biosurfactant. Yeh et al. [14] found that the oxygen volumetric mass transfer coefficient ( $K_La$ ) was highly correlated with the performance of surfactin production. For planktonic cultures, limitation of O<sub>2</sub> enhances the production while surfactin is no more produced under static conditions. The highest surfactin productivity was achieved when the fermentation was carried out at a  $K_La$  value of 0.0132 s<sup>-1</sup>. The focused inverse fluidized bed reactor shows high mass transfer at relatively low gas flow rate ( $K_La > 0.016$  s<sup>-1</sup> [15,16]). This type of reactor has found a privileged place in biotechnology and various industrial-scale applications do exist: ethanol production, aerobic and anaerobic wastewater treatment [17–19]. The main difficulty during the production process in aerated and stirred bioreactors lies in the high foaming properties of the surface-active compounds. Yeh et al. [12] showed that activated carbon or expanded clay added to the liquid medium led

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to increase of surfactin production up to 2150 and 3300 mg L<sup>-1</sup>, respectively (22- and 33-fold increase when compared to the control experiment). Diimitrov et al. [7] were tested different solids surfaces, including polypropylene (PP), with respect to propose a method to improve the biofilm growth on different polymer materials by modifying their surface properties. The ability of two aerobic bacteria strains: *Pseudomonas aeruginosa* O1 and *B. subtilis* CIP 5265 to grow on various non-coated and coated polymer materials were investigated also. It was found that supports with modified surface show higher biofilm development rate and better surface colonization. The influence of the surface free energy on the detachment force and correspondingly on the biofilm formation was demonstrated.

According to these previous results, we choose in this work to study the effect of light PP solid carriers coated with Fe<sup>2+</sup>. A batch stirred tanks reactors were used as a model and also a control. The particles have densities lower than the liquid phase, generally for TPFIBR, between 0.3 and 0.7 g/cm<sup>3</sup>. Moreover, the particles should support a temperature of 130 °C which permit sterilization. Polypropylene, one of the general plastics, is capable of being used at high temperature repeatedly but the mass density of original PP pellets is 0.9 g/cm<sup>3</sup>, higher than the required densities of the biofilm carriers. Xu et al. [20] showed that the mass densities of polypropylene could be regulated easily to 0.3–0.7 g/cm<sup>3</sup> by using a supercritical CO<sub>2</sub> assisted foaming process while retaining the thermal stability of polypropylene.

## 2. Materials and method

### 2.1. Bacterial strain and culture conditions

*B. subtilis* ATCC 21332 from the collection of ProBioGem Laboratory and the same, recently obtained from the American Type Culture Collection, was used. The strain ATCC 21332, was recently proved to be a co-producer of surfactin and fengycin [3]. The strain was cultivated in modified Landy base medium at pH 7 [21] buffered with MOPS 100 mM without or with glutamic acid (respectively named Lm1 and Lm2 media). Cells were cultivated overnight in Lm2 medium at 30 °C then washed and concentrated 10 times by centrifugation. The main cultures were inoculated into 1000 mL flasks containing 200 mL of Lm1 (colonization experiment) or Lm2 (production experiment) medium, in order to obtain 0.5 as initial optical density (at 600 nm). In colonization experiments, Lm1 medium was first used to avoid the synthesis of lipopeptides. Particles were allowed to be coated by the strain at 40 rpm for 48 h. The supernatant was then discarded and both free cells and coated beads were put in 200 mL of Lm2 medium for 48 h at 140 rpm, 30 °C to produce biosurfactants. In production experiments, pellets were directly added in Lm2 medium and lipopeptides were produced as explained above during 48 h. All experiments were achieved in batch conditions. With respect to reproduce hydrodynamic and mass transfer conditions of TPIFB [5,15], the concentration of particles was equal to 1 g per 15 mL of culture medium and the aerobic fermentation, namely the production of surfactin, was realised under O<sub>2</sub>-sufficient conditions using 1000 mL flasks containing 200 mL of medium. All samples were triplicate.

### 2.2. Quantitative analysis of biomass

At the end of the production, pellets were sonicated with 4 mL of NaOH (0.1 M) during 5 min at 20 kHz, and proteins were quantified by Lowry method. After centrifugation, planktonic biomass was quantified by the same method.

### 2.3. Quantitative analysis of lipopeptides

Preliminary tests showed that *B. subtilis* ATCC 21332 produced two kinds of lipopeptides, surfactin and fengycin. At the end of the culture, 1 mL of supernatant was purified on C<sub>18</sub> column (Extract - clean SPE 500 mg, Altech) and eluted in methanol.

The surfactin concentration was determined by reverse phase C<sub>18</sub> HPLC (600 s, Waters, USA) equipped with a Merck C<sub>18</sub> column (5 µm, Merck, Germany) as previously described [8,21,22]. The standard of surfactin was purchased from Sigma (USA). The Fengycins were eluted during 40 min under a gradient ACN/H<sub>2</sub>O/TFA from 45/55/0.1 to 55/45/0.1 at 0.6 mL/min. The standard of fengycins was kindly provided by Dr M. Deleu from the Agricultural University of Gembloux (Belgium) and gave peaks between 10 and 25 min. Peaks were selected through calibration and spectra were analyzed using values of second derivative which give two major peaks at 213 and 236 nm associated with a minor peak at 290 nm [21,23].

### 2.4. Biofilm carriers

Foamed polypropylene pellets were used as biofilm carriers in this work.

#### 2.4.1. PP foaming process using supercritical carbon dioxide

The polypropylene (PP) pellets with an average diameter of 3–4 mm and a mass density of 0.9 g/cm<sup>3</sup> were supplied by Shanghai Petrochemical Company. The mass-average molar mass of the PP was 188,700 g/mol. Its polydispersity index and crystallinity were 5.1 and 47%, respectively.

In order to reduce the mass density of original PP pellets (from 0.9 to 0.3–0.7 g/cm<sup>3</sup>), an environmental-friendly technique previously described was used to prepare PP microcellular foams using supercritical carbon dioxide (scCO<sub>2</sub>) as a foaming agent [20]. The CO<sub>2</sub> (purity 99.9%) was obtained from Air Product Co., Shanghai, China. Briefly, a high-pressure stainless steel vessel with internal volume of 100 cm<sup>3</sup> was used. About 20 g PP pellets were placed in the high-pressure vessel and then washed with low-pressure CO<sub>2</sub>. Thereafter, the high-pressure vessel was immersed in a oil bath with a controlling accuracy of ±0.2 °C and rapidly heated to 157 °C. A given amount of CO<sub>2</sub> was charged to a pressure of 15 MPa, which was measured with accuracy of ±0.01 MPa by a pressure transducer of Shenzhen MSI/JL Electronics Co., China. The CO<sub>2</sub> loading was achieved by a DZB-1A syringe pump of Beijing Satellite Instrument Co., China, with an accuracy of 0.01 cm<sup>3</sup>. After the sorption of CO<sub>2</sub> into the PP pellets reached at equilibrium, the CO<sub>2</sub> in the high-pressure vessel was released from the foaming pressure to the ambient one. The average depressurization rate was controlled at 15 MPa/s. The foamed PP pellets were then taken out for subsequent analysis and application.

#### 2.4.2. Physical–chemical characterization of carriers

The cell morphologies of the foamed PP samples were characterized by a JSM-6360LV scanning electron microscopy (SEM, Fig. 1). The samples were immersed in liquid nitrogen for 10 min and then fractured. The SEM scanned fractured surfaces. The average cell size and cell density was calculated through the analysis of the SEM photographs by the software Winroof.

The mass densities of foamed PP samples  $\rho_f$  were measured according to ASTM D792-00 involving weighing polymer foam in water using a sinker. And  $\rho_f$  was calculated as follows:

$$\rho_f = \frac{a}{a + w - b} \rho_{\text{water}} \quad (1)$$

where  $a$  is the apparent mass of specimen in air without sinker,  $b$  the apparent mass of specimen and sinker completely immersed in water and  $w$  is the apparent mass of the totally immersed sinker.

#### 2.4.3. Grafting process of ferric acetylacetonate onto PP

Three types of particles were used containing 0, 0.35 and 0.74% of iron (w/w) benzoyl peroxide (BPO) (chemical-grade) was purchased from Shanghai Chemical Co. and purified twice in chloroform before use. Ferric acetylacetonate {[CH<sub>3</sub>COCH=C(O)-CH<sub>3</sub>]<sub>3</sub>Fe} with a purity of 97% was purchased from Sigma-Aldrich Co.

A suspension solid-state grafting process was applied by using water as a dispersion medium, BPO as an initiator and a small amount of toluene as a swelling agent. In the typical procedure, reaction was carried out in a 250-mL three-necked

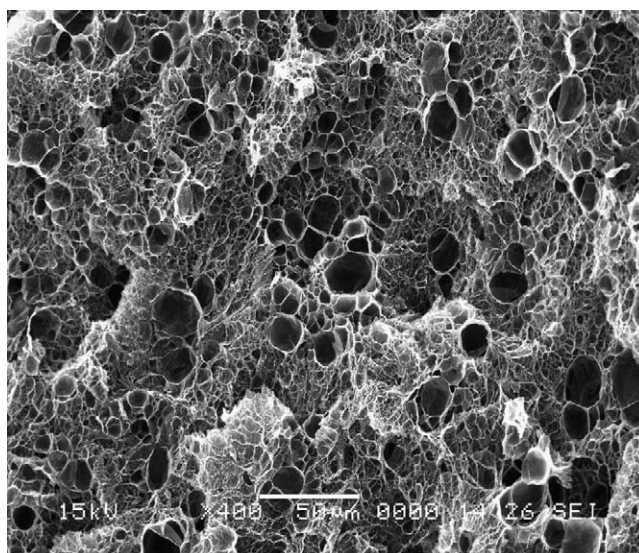


Fig. 1. SEM micrographs of foamed PP.

glass flask, where the total volume of reactive was about 150 mL. The reactor was equipped with a reflux condenser and a helix stirrer. This flask was immersed in a homemade electronically controlled temperature oil bath. The temperature of the latter was measured with a calibrated mercury thermometer with accuracy of  $\pm 0.02$  °C and was controlled with an accuracy of  $\pm 0.2$  °C. The foamed PP pellets, water (120 mL) and toluene (1 mL) were charged to the flask.

The reactor was heated to 90 °C and kept at the temperature for swelling PP for 1 h. Then, the reaction was continued during 7 h. The ferric acetylacetonate (2 g) and BPO (0.4 g) were divided into three equal parts and added into the reactor after 0, 1.5 and 3 h, respectively. After filtration, PP particles were purified by Soxhlet extraction in acetone for 24 h and dried in a vacuum oven at 80 °C for 10 h in order to eliminate unreacted ferric acetylacetonate and other small molecules.

#### 2.4.4. Grafted PP samples

Iron content in the grafted PP was determined by Inductively Coupled Plasma (ICP, TJA IRIS 1000) analysis. The percentage of grafted ferric acetylacetonate in the PP was then deduced.

#### 2.4.5. Quantitative analysis of iron onto particles

PP powder was used to verify how much ferric acetylacetonate or iron could be grafted onto pellets. The PP powder with particles smaller than 0.15 mm in diameter was prepared by grinding the above pellets with liquid nitrogen as a coolant.

The grafting process was carried out with powder as described above. After purification, the iron content grafted onto PP was determined by using Inductively Coupled Plasma (ICP).

Foamed PP pellets surface was also submitted to iron content analysis. For each sample, the iron content was tested for three times and the average value was adopted.

### 3. Results and discussion

Fig. 1 shows the cell morphology of foamed PP. According to the SEM micrograph, the average cell diameter was calculated to be 7.2  $\mu\text{m}$  and cell density  $2.5 \times 10^8$  cells/cm<sup>3</sup>. The density tests showed that the mass densities of foamed PP were about 0.5 g/cm<sup>3</sup>, which obviously fits the requirement for pellets used in an inverse fluidized bed (mass densities between 0.3 and 0.7 g/cm<sup>3</sup>).

In order to introduce iron on the surface of pellets, ferric acetylacetonate  $\{[\text{CH}_3\text{COCH}=\text{C}(\text{O}-\text{CH}_3)_3\text{Fe}]\}$  was chosen as a component to be grafted by using a suspension solid-state grafting process. Fig. 2 shows the evolution of the iron content as well as ferric acetylacetonate content grafted onto the PP powder as a function of the time. During the first hours, the iron and ferric acetylacetonate contents grafted onto PP both increased with the reaction time and then it tended to level off. The iron content at 7.5 and 10 h are 0.74 and 0.75 wt%, respectively. It was specified that the equilibrium was reached at 7.5 h. The percentage of grafted

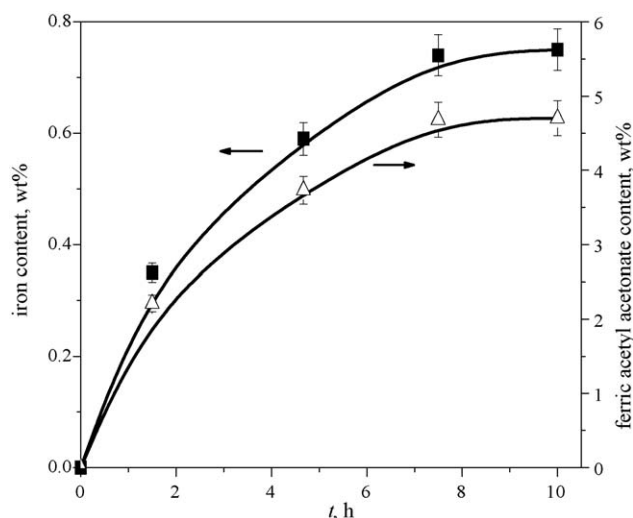


Fig. 2. Evolution of iron content as well as ferric acetylacetonate content grafted onto PP powder as a function of time.

Table 1

Biomass obtained when lipopeptides were quantified, in mg of proteins per litre of medium (96 h in colonization experiments, 48 h in production experiments).

	Colonization	Production
Planktonic	397 $\pm$ 12	672 $\pm$ 35
+ PP Fe 0%	414 $\pm$ 10	572 $\pm$ 41
+ PP Fe 0.35%	936 $\pm$ 40	819 $\pm$ 37
+ PP Fe 0.74%	1041 $\pm$ 100	870 $\pm$ 55

ferric acetylacetonate was subsequently calculated to be 4.7%. However, ferric acetylacetonate was difficult to penetrate into foamed pellets and the component can only graft on the surface of the PP pellets. The final product is heterogeneous: the colour of the outer surface appeared red whereas inner polymer remained white. The surface of grafted PP foamed pellets was cut and submitted to ICP analysis and the average iron content was determined to be also 0.74 wt%. Considering that only the outer surface of the PP pellets was used as the biofilm carriers, the grafted PP foamed pellets prepared in this way were suitable for the biofilm carriers. In Fig. 2, PP powder was used to investigate the kinetics of the grafting reaction. However, in practice, foamed PP pellets were grafted onto ferric acetylacetonate and used as the carriers. The grafted content of ferric acetylacetonate on the outside surface of the foamed PP pellets was determined as following: the outside surface of the foamed PP pellets was cut and was subject to ICP for analyzing the grafted content of the ferric acetylacetonate. The grafted content of ferric acetylacetonate on the outside surface of the grafted foamed PP pellets was equal to that of the grafted PP powder at the same reaction conditions.

Three types of particles containing 0–0.74 iron mass % were used in this study. The results obtained for total biomass (free and immobilized cells) are summarized in Table 1. The growth in Lm1 medium, at low agitation rate (40 rpm), restricted the final biomass in planktonic cultures and when no iron was present in pellets. The strain was not able to coat pure hydrophobic PP particles (biomass reached the planktonic cultures level), whereas iron PP pellets promoted the biofilm formation. In fact, all PP+ iron pellets promoted biomass enhancement. The colonization seemed to be useless as final biomass obtained with iron containing particles were the same in production and colonization experiments.

The maximal production, for each experimental condition, was reached after 48 h of fermentation (data not shown). Lipopeptides syntheses obtained at this time, in Lm2 medium, are shown in Table 2. In planktonic culture, the colonization step decreased the lipopeptides synthesis. In the production experiments, the three types of PP particles both enhanced surfactin and fengycin. The production yield was modified for all types of PP supports,

Table 2

Lipopeptides synthesis in mg/L after 48 h in Lm2 medium. In colonization experiments pellets are allowed to be coated by the strain in Lm1 medium then Lm2 medium was added. In production experiments, pellets are directly added in Lm2 medium.

	Surfactin	Fengycin
Colonization		
Planktonic culture	50	166
PP+ iron 0%	nd	nd
PP+ iron 0.35%	215	609
PP+ iron 0.74%	183	336
Production		
Planktonic culture	143	326
PP+ iron 0%	300	310
PP+ iron 0.35%	390	680
PP+ iron 0.74%	310	510

containing respectively 0, 0.35 and 0.75% of iron. The immobilized cultures produced 1.75–3.8 times more biosurfactants than planktonic cells. In production experiments addition of carriers modified the ratio between surfactin and fengycin. The highest concentration of fengycin was obtained with addition of pellets containing 0.35% of iron.

#### 4. Conclusion

Three types of particles with different iron concentration were tested. The results showed that addition of solids carriers in media increased the lipopeptide production from 3.67 to 4.3 times in colonization experiments and 2.09 to 2.7 times in production experiments. The ratio between surfactin and fengycin remained constant in planktonic cultures and colonization experiments (25–75%). In production experiments, the production yield and ratio between fengycin and surfactin were modified. When cells were immobilized on PP particles+ iron 0.35%, the surfactin and fengycin production were increased respectively 2.7 and 2.09 times in comparison to the free cells culture. In conclusion, the results obtained in this study suggest that by using immobilized cells and various ratio of iron we can control the selectivity of the bioreaction concerning the fengycin or surfactin production.

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