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Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21

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HIGHLIGHTS

- Demonstration that k_La is the key parameter controlling lipopeptide production.
- Determination of k_La ranges favorable for surfactin mono-production.
- Determination of k_La ranges favorable for mixed production of surfactin and fengycin.
- Demonstration that power dissipation influences indirectly lipopeptide production via k_La.
- Establishment of correlations between k_La and lipopeptide production and selectivity.

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ABSTRACT

The influence of power dissipation and volumetric oxygen transfer coefficient k_La on *Bacillus subtilis* productivity of lipopeptides surfactin and fengycin was studied in shake flasks in view of scaling-up of this fermentation process. The experiments performed with different flask sizes, relative filling volumes, and shaking frequencies confirmed clearly that lipopeptide production changed in function of power dissipation, via interfacial gas–liquid contact surface and oxygen supply. It was demonstrated that k_La is the key parameter controlling the productivity and the selectivity of the bioreaction. Varying the oxygen transfer conditions, the synthesis could be oriented to mixed production or to surfactin mono-production. The fraction of surfactin towards total lipopeptides produced and the maximal surfactin production both increased with k_La increase (surfactin concentration about 2 g L^{-1} at k_La = 0.04–0.08 s^{-1}), while the maximal fengycin production (fengycin concentration about 0.3 g L^{-1}) was obtained at moderate oxygen supply (k_La = 0.01 s^{-1}).

1. Introduction

Bacillus subtilis produces three different families of lipopeptides: surfactin, iturin and fengycin. Surfactin is considered as one of the most potent biosurfactant and shows antiviral properties. Iturin and fengycin are strong antifungal compounds (Jacques, 2011). In addition, surfactin and fengycin are able to induce systemic resistance in plants and could be used in a next future as biocontrol agent of plant diseases (Ongena and Jacques, 2008). The actual level of knowledge of the biosynthesis of these lipopeptides and its regulation mechanism allows developing different techniques to overproduce the main active compounds and to reach yields that are compatible with industrial development of such compounds (Jacques, 2011). A lot of studies have pointed out different environmental factors for their effect on lipopeptide production using planktonic and immobilized cells. Several studies demonstrated that the production of lipopeptides is strongly influenced by oxygen transfer conditions. In these studies the authors analyzed this effect by different ways. Phae and Shoda (1991) and Ohno et al. (1993) have demonstrated that in flasks and in fermenters, deficiency in dissolved oxygen has no adverse effect on iturin production by *B. subtilis* NB22 strain. Hbid et al. (1996) have shown the negative influence of the addition of an oxygen vector, the *n*-dodecane, on the production of surfactin and iturin A in bioreactor. Results of Sen and Swaminathan (1997) indicated that a low agitation and a high aeration rate favored, in bioreactor, the biosynthesis of surfactin by *B. subtilis* 3256 while Jacques et al. (1999) have demonstrated, in flasks, that a high shaking rate is beneficial for a good production of the total amount of lipopeptides by *B. subtilis* S499. Yeh et al. (2006) have proposed for surfactin production an innovative bioreactor equipped with a foam collector and a cell recycling system. In the used modified jar fermentor, they have observed a clear positive effect of the volumetric oxygen transfer

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coefficient, k_La, on the production of surfactin by *B. subtilis* ATCC 21332. By using Respiratory Activity Monitoring System, Guez et al. (2008) have demonstrated that low oxygen transfer rates are more favorable for mycosubtilin production and productivity in flasks. Recently, Shih et al. (2009) have shown that increasing power consumption by increasing agitation rate or modifying baffle design had contradictory effect on iturin production. It should be mentioned that very few of the published works tried to correlate the lipopeptide biosynthesis with chemical engineering parameters such as k_La or power consumption used in scaling-up of aerobic fermentation. In addition, no data exist concerning the influence of oxygen transfer conditions on fengycin production. In our laboratory, we have recently isolated a spontaneous mutant strain of *B. subtilis* ATCC 21332, called BBG21, which co-produces surfactin and fengycin. We thus decided to analyze in shake flasks the production of these lipopeptides in different oxygen transfer conditions. Indeed, studying the bubble-less process of oxygenation from continuous gas phase to a formed liquid surface in flasks is a good way to understand the relation between energy supply, gas–liquid mass transfer and fermentation production and to facilitate the scale-up of lipopeptide production in bioreactor. Such data could be also of interest to design bioreactor onto its selectivity, as well as for the development of adequate mathematic model. This strategy was used by several authors to characterize for various bioreactions typical engineering parameters such as: mass transfer (Maier et al., 2004; Xu and Zhong, 2011), power input (Büchs et al., 2000), hydro-mechanical stress and fluid movement (Peter et al., 2006), and the influence of the power dissipation on cell response (Mehmood et al., 2010). Working in various bioreactors (flasks, parallel fermenter and 5 L fermenter) Rahulan et al. (2011) have demonstrated that the information obtained in flasks could be useful to design a strategy to improve the studied bioreaction in a large-scale fermentors.

Conforming to the above aspects, the aim of this study was to investigate the production capacity of surface aerated planktonic cells of *B. subtilis* BBG21 and the relations between lipopeptide production, power dissipation, and k_La, and to analyse the selectivity of the fermentation process.

2. Methods

2.1. Culture and production conditions

In this study, *B. subtilis* BBG21, a spontaneous mutant strain of ATCC 21332, was used. Cultures were performed in Erlenmeyer flasks of different sizes (50, 100, 250, 500 and 1000 mL) at various shaking frequency conditions (150, 200 and 250 min^{-1}). Different relative filling volumes (*Rv*, volume of liquid medium/volume of flask) from 0.05 to 0.4 mL mL^{-1} were tested in each kind of flask. The cultures were performed for 24, 48, 72 and 96 h at 30 °C in Landy medium buffered at pH 7 with 100 mM MOPS. All data presented are the means of triplicate determination experiments ($n = 3$).

2.2. Analytical measurement methods

2.2.1. Quantitative analysis of lipopeptides

The concentrations of surfactin and fengycin were determined by reverse phase HPLC (600 s, Waters, USA) equipped with a C18 column (5 mm, Merck, Germany) as previously described (Gancel et al., 2009; Chtioui et al., 2010; Coutte et al., 2010). The standard of surfactin was purchased from Sigma (USA) with purity of 98%. The standard of fengycin (95% purity) was kindly provided by Dr. M. Deleu from the Agricultural University of Gembloux (Belgium). The average standard deviation for all experiments was 7.1% for surfactin concentrations and 16.7% for fengycin ones. The higher dispersion of fengycin concentrations could be explained by the fact that at many of studied conditions fengycin concentrations were relatively low. The average standard deviation for fengycin concentrations higher than 100 mg L^{-1} was 7.4%.

2.2.2. Oxygen transfer and mean power dissipation quantifications

To determine volumetric gas–liquid mass transfer coefficient k_La in shake flasks, an empirical correlation was used:

$$K_La = 6.67 \times 10^{-6} N^{1.16} V_L^{-0.83} d_0^{0.38} d^{1.92} \quad (1)$$

where N is the shaking frequency (min^{-1}), V_L the working volume (mL), d_0 the shaking diameter (cm), and d the maximum inner shake flask diameter (cm). This correlation was proposed by Maier (2002) for standard glass flasks with hydrophilic walls, nominal flask volume of 50–1000 mL, shaking diameters of 1.25–10 cm, relative filling volumes of 0.04–0.2 mL mL^{-1}, and shaking frequencies of 50–500 min^{-1}, and was used also by Mehmood et al. (2010) and Seletzky et al. (2007).

The power input P was calculated according to the equation proposed by Büchs et al. (2000) using the modified Newton number Ne_{mod}:

$$P = Ne_{mod} N^3 d^4 V_L^{0.33} \rho \quad (2)$$

$$Ne_{mod} = 70\mathrm{Re}^{-1} + 25\mathrm{Re}^{-0.6} + 1.5\mathrm{Re}^{-0.2} \quad (3)$$

with the Reynolds number:

$$\mathrm{Re} = \frac{N d^2 \rho}{\mu} \quad (4)$$

where ρ (kg m^{-3}) is liquid density, and μ (Pa s) the dynamic viscosity (water values at 30 °C).

The mechanical mean power dissipation P/V_L and volumetric mass transfer coefficient k_La were estimated for each kind of flask, for different relative filling volumes (*Rv* of 0.05, 0.1, 0.2, 0.3 or 0.4 mL mL^{-1}) and agitation conditions (150, 200 or 250 min^{-1}).

3. Results and discussions

3.1. Influence of flasks size, relative filling volume and shaking frequency on lipopeptides production

B. subtilis BBG21 was first cultivated at two different agitation conditions (150 and 250 min^{-1}) in 1000 mL Erlenmeyer flasks filled with 50 mL of Landy medium. Kinetics of lipopeptide production was analyzed during 96 h. These first experiments revealed that surfactin and fengycin showed complete different behaviour in function of agitation. The increase of agitation speed leaded to an increase of surfactin and decrease of fengycin productions. In addition, maximal concentrations of lipopeptides were observed at different time depending of the lipopeptide and culture conditions. Hence, to compare surfactin and fengycin production at different studied conditions the obtained maximal concentrations of these lipopeptides were used.

The influence of the flask size and the agitation conditions on surfactin and fengycin productions at constant relative filling volume of 0.05 mL mL^{-1} is presented in Fig. 1. The obtained results show clearly that, at the studied conditions, *B. subtilis* produced much more surfactin than fengycin. The production of both lipopeptides and the selectivity of lipopeptide synthesis were influenced by the shaking frequency and the size of flasks probably due to different hydrodynamic conditions. The production of surfactin increased with the decrease of the flask size and the increase of agitation, while the fengycin production was favoured at the lower shaking frequencies and higher flask sizes (Fig. 1).