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New integrated bioprocess for the continuous production, extraction and purification of lipopeptides produced by *Bacillus subtilis* in membrane bioreactor☆

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ABSTRACT

Recently, a bubbleless membrane bioreactor (BMBR) has been successfully developed for biosurfactant production by *Bacillus subtilis* [1]. In this study, for the first time, continuous culture were carried out for the production of surfactin in a BMBR, both with or without a coupled microfiltration membrane. Results from continuous culture showed that a significant part of biomass was immobilized onto the air/liquid membrane contactor. Immobilized biomass activity onto the air/liquid membrane contactor was monitored using a respirometric analysis. Kinetics of growth, surfactin and primary metabolites production were investigated. Planktonic biomass, immobilized biomass and surfactin production and productivity obtained in batch culture (3 L) of 1.5 days of culture were 4.5 g DW, 1.3 g DW, 1.8 g and 17.4 mg L^{-1} h^{-1}, respectively. In continuous culture without total cell recycling (TCR), the planktonic biomass was leached, but immobilized biomass reached a steady state at an estimated 6.6 g DW. 11.5 g of surfactin was produced after 3 days of culture, this gave an average surfactin productivity of 54.7 mg L^{-1} h^{-1} for the continuous culture, which presented a surfactin productivity of 30 mg L^{-1} h^{-1} at the steady state. TCR was then investigated for the continuous production, extraction and purification of surfactin using a coupled ultrafiltration step. In continuous culture with TCR at a dilution rate of 0.1 h^{-1}, planktonic biomass, immobilized biomass, surfactin production and productivity reached 7.5 g DW, 5.5 g DW, 7.1 g and 41.6 mg L^{-1} h^{-1} respectively, after 2 days of culture. After this time, biomass and surfactin productions stopped. Increasing dilution rate to 0.2 h^{-1} led to the resumption of biomass and surfactin production and these values reached 11.1 g DW, 10.5 g DW, 7.9 g and 110.1 mg L^{-1} h^{-1}, respectively, after 3 days of culture. This study has therefore shown that with this new integrated bioprocess, it was possible to continuously extract and purify several grams of biosurfactant, with purity up to 95%.

1. Introduction

The market for chemical surfactants or biosurfactants is constantly increasing worldwide. Almost all surfactants currently in commercial use are chemically derived from petroleum; a lot of interest has now focused on microbial surfactants, especially those produced by *Bacillus subtilis*, including surfactin, since they are gaining recognition as an alternative to chemical surfactants [2] and agricultural chemicals [3]. Surfactin is one of the most powerful biosurfactants known. It lowers the surface tension of water from 72 to 27 mN/m at a concentration even as low as 10 μM [4]. It displays several advantages over chemical synthetic surfactants, such as low critical micellar concentration (CMC), biodegradability and is thus especially well studied for environmental applications [5]. It also displays antiviral and antimycoplasma activities [4]. Moreover, surfactin is able to induce systemic resistance in plants [3,4].

The high production costs of surfactin have been an obstacle to commercial applications and numerous efforts have been made to lower them. The first bottleneck was the efficiency and the selectivity of the biocatalyst to get a high level of a selected biosurfactant such as surfactin. This was solved by obtaining mutant strains such as *B. subtilis* BBG131, an overproducing surfactin strain [6]. The second obstacle for the commercialization of these surface-active compounds is the intense foaming produced during aerobic sparged culture [7], so the recovery and purification from complex fermentation broth remained difficult [8]. This barrier explains also why the continuous production of this compound is not well studied. Recently, we have demonstrated that the lipopeptides from *B. subtilis* can be produced without foaming, using a bubbleless membrane bioreactor (BMBR) [1]. BMBRs have been studied and used

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Nomenclature

BMBR	bubbleless membrane bioreactor
PES	polyethersulfone
RC	regenerated cellulose
DW	dry weight
TCR	total cells recycling
MWCO	molecular weight cut-off
D	dilution rate
X_{pb}	planktonic biomass (g DW L^{-1})
X_{ad}	adhered biomass (g DW L^{-1})
X_{db}	desorbed biomass (g DW L^{-1})
OUR_{tot}	total oxygen uptake rate (mmol L^{-1} h^{-1})
OUR_{spe}	specific oxygen uptake rate (mmol g DW h^{-1})
$OUR_{spe\ pb}$	specific oxygen uptake rate of planktonic biomass (mmol g DW h^{-1})
$OUR_{spe\ ad}$	specific oxygen uptake rate of adhered biomass (mmol g DW h^{-1})
CPR_{tot}	total carbon dioxide production rate (mmol L^{-1} h^{-1})
V	volume of the culture in bioreactor (L)
a	membrane area (m^2)
K_La	volumetric oxygen transfer coefficient (h^{-1})

in wastewater treatment; most of them are characterized by the development of a biofilm on the membrane surface, and are also named Membrane Aerated Biofilm Reactor [9–11].

The aim of our study was to elaborate a new and original integrated bioprocess for continuous production and extraction of the lipopeptides. Coupled to the BMBR [1], a combined system of a microfiltration membrane and an ultrafiltration membrane was used to separate and concentrate continuously the biomass from the culture medium and to extract the lipopeptides produced. The use of coupled microfiltration membrane for total cell recycling (TCR) was well known, at a large scale, to improve the efficiency of several microbial bioprocesses, particularly with yeast culture for production of alcohol [12–14]. This improvement of efficiency was correlated with the concentration of the cells in the bioreactor which reached high concentration of at least 100 g DW L^{-1} [12,13]. Moreover in a continuous culture the use of high dilution rate improves the productivity compared to the batch [13,14]. Coupled to this microfiltration step, an ultrafiltration membrane was selected as an efficient method for the concentration and purification of the lipopeptides. Several other methods have been studied for the recovery of surfactin such as using extreme foaming [8,15], but ultrafiltration (UF) is also well investigated. Indeed, surfactant molecules above their CMC, associate to form supramolecular structures such as micelles or vesicles. Surfactin micelles can easily be retained by UF membrane with sufficiently low weight cut-offs (MWCOs) [16–21].

All the different methods gave satisfactory results in the recovery and purification of surfactin, but owing to the intense foaming most of them were carried out after and not during the fermentation process. The continuous integrated process developed in this work will demonstrate, for the first time, the feasibility of integrating these two purification steps. The continuous integrated bioprocess performance was analyzed and compared to batch and continuous cultivation in BMBR. The influence of dilution rate on biomass growth and lipopeptide productivity was studied. The formation of a biofilm on the surface of the air/liquid membrane contactor was monitored using respirometric analysis. Substrate consumption and primary metabolite formation were analyzed to highlight the limiting factors of the system.

2. Materials and methods

2.1. Microorganism and preculture conditions

For each inoculum and culture in a classical aerated bioreactor or in a BMBR, *B. subtilis* strains 168 (wild-type) and its derivative BBG131 were taken from a −80 °C frozen stock and the precultures were done as described in our previous study [1]. Each culture was done in Landy medium complemented with 16 mg L^{-1} tryptophan at 37 °C and pH 7.0. The strain BBG131 used in this study is a surfactin overproducer obtained in our previous study [6] from a *B. subtilis* 168 Sfp^+ derivative by (i) replacement of the native promoter of the surfactin operon by a constitutive one originating from the replication gene *repU* of *Staphylococcus aureus* plasmid pUB110 and (ii) the disruption of the *ppsA* gene, from the operon coding for plipastatin (fengycin) synthetase.

2.2. Batch fermentation in aerated bioreactor

The batch cultivation of *B. subtilis* 168 was investigated in a bubble free aerated bioreactor to determine its growth and respirometric parameters, such as oxygen uptake rate (OUR). These were compared to those obtained by the strain *B. subtilis* BBG131 in a BMBR. *B. subtilis* 168 was cultivated at 37 °C, in 3 L of Landy medium in a stirred tank reactor Bioflo 3000 bench-top fermenter from New Brunswick Scientific (New Brunswick Scientific, Edison, MA, USA). Bioreactor cultures were performed with an aeration rate fixed at 1 vvm. The agitation rate was fixed at 300 rpm to reach a volumetric oxygen mass transfer coefficient (K_La) close to that obtained with the BMBR (40 h^{-1}). The pH was maintained at 7.0 by automatic addition of 3.0 M NaOH or 0.66 M H_3PO_4 solution to the bioreactor through a peristaltic pump. During fermentation, pH and dissolved oxygen in the broth were monitored and both the O_2 consumption and CO_2 production were recorded by a 4100 gas analyser (Servomex, Crowborough, England). The broth was periodically sampled for monitoring bacterial growth. Experiments were performed in triplicate and mean values are presented.

2.3. Batch fermentation in bubbleless membrane bioreactor (BMBR)

The batch cultivation of *B. subtilis* BBG131 was investigated in a BMBR to determine its kinetic parameters compared to those obtained in continuous culture. In order to propose an integrated bioprocess to produce surfactin, the culture conditions and BMBR setup were chosen taking into account the results previously obtained in batch fermentation [1]. The BMBR configuration used is the BB1 [1]. In this configuration, *B. subtilis* BBG131 was cultivated under the same conditions as described above for the batch fermentation of *B. subtilis* 168, the usual aeration being just replaced by a bubbleless aeration through a coupled external hollow fiber membrane aeration M1 (area 2.5 m^2) supplied by GE-Healthcare (polyethersulfone (PES); 2.5 m^2; 0.65 μm; Amersham Biosciences Corp., Westborough, USA). Membranes, tanks and all connections were sterilized at 121 °C for 20 min. The broth was periodically sampled for quantification of bacterial growth, surfactin production, substrates and primary metabolites concentrations. Experiments were performed in duplicate and mean values are presented.

2.4. Integrated bioprocess set-up for continuous production, extraction and purification of lipopeptides

Continuous cultures were done with or without TCR. Continuous cultivations were carried out under the same culture conditions as the batch cultivation in bioreactor, starting by continuously feeding fresh Landy's medium into the bioreactor at a constant flow rate. The continuous process was divided in two or three main stages. In phase I, cells were grown in preliminary batch culture until the dissolved oxygen decreased to 0 mg L^{-1} (during about 8 h for the total cell-recycling process and during about 15 h for the process without TCR). In phase II, the cells were grown in a continuous culture (feed flow rate = 0.3 L h^{-1}) where the volume V was constant because of the overflow of medium at a flow rate up to 0.3 L h^{-1}.

For TCR process, as described in Fig. 1, the bioreactor was continuously fed with fresh medium. The effluent culture flowed inside the hollow fibers of a cross-flow microfiltration membrane M2 (Healthcare, PES; 0.41 m^2; 0.2 μm; Amersham Biosciences Corp., Westborough, USA) at 0.5 L min^{-1}. This membrane concentrated the biomass by feeding back all the cells to the bioreactor. During the batch phase, the retentate outlet of the microfiltration membrane was opened while the permeate one was closed in order to limit the clogging of the membrane. The permeate containing residual substrates and metabolites, such as organic acids and surfactin, was then collected within the tank 2. To maintain the volume V of the bioreactor constant, broth was extracted at a same flow rate as the feeding, the pump being regulated by a signal from a balance controller (Ohaus Corporation, Pine Brook, USA), which measured the mass of the bioreactor and controlled the peristaltic pumps of the process. Finally, the residual substrates and metabolites were separated from the lipopeptides by an ultrafiltration membrane M3 (0.1 m^2; regenerated cellulose (RC), 10 kDa; Sartorius AG, Göttingen, Germany). To maintain a constant volume in the tank 2, the overflow rate of the ultrafiltrate in the tank 3 was regulated in the same way as the feed rate. After 44 h of continuous culture, the feed flow rate and microfiltration overflow rate were increased up to 0.6 L h^{-1}. In case of process