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Fungal biofilm reactor improves the productivity of hydrophobin HFBII

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ABSTRACT

Production and purification of hydrophobin HFBII has recently been the subject of intensive research, but the yield of production needs to be further improved for a generic use of this molecule at industrial scale. In a first step, the influence of different carbon sources on the growth of *Trichoderma reesei* and the production of HFBII was investigated. The optimum productivity was obtained by using 40 g/L lactose. Carbon starvation and excretion of extracellular enzyme were determined as two main conditions for the production of HFBII. In the second phase, and according to the physiological mechanisms observed during the screening phase, a bioreactor set up has been designed and two modes of cultures have been investigated, *i.e.* the classical submerged fermentation and a fungal biofilm reactor. In this last set-up, the broth is continuously recirculated on a metal packing exhibiting a high specific surface. In this case, the fungal biomass was mainly attached to the metal packing, leading to a simplification of downstream processing scheme. More importantly, the HFBII concentration increased up to 48.6 ± 6.2 mg/L which was 1.8 times higher in this reactor configuration and faster than the submerged culture. X-ray tomography analysis shows that the biofilm overgrowth occurs when successive cultures are performed on the same packing. However, this phenomenon has no significant influence on the yield of HFBII, suggesting that this process could be operated in continuous mode. Protein hydrolysis during stationary phase was observed by MALDI-TOF analysis according to the removal of the last amino acid from the structure of HFBII after 48 h from the beginning of fermentation in biofilm reactor. Hopefully this modification does not lead to alternation of the main physicochemical properties of HFBII.

1. Introduction

Trichoderma reesei is a saprophytic fungus naturally occurring in soils and can be easily cultivated in laboratory conditions over a broad range of temperatures (20–30 °C) and at relatively low pH [1]. *T. reesei* has been widely studied for its secretion efficiency and more precisely for the production of extracellular enzymes such as cellulases and hemi-cellulases. The ability to use a variety of carbon sources [2], and its growth and secretion capabilities [3] remark this fungus as a powerful microbial cell factory.

Beside cellulolytic enzymes, *T. reesei* is able to produce hydrophobins, a family of proteins with high surface activity [4]. Hydrophobins exhibit a negative effect when present in carbonated beverages contaminated by molds, leading to gushing [5,6]. Nevertheless, due to its outstanding surface-active properties, several positive potential applications such as antifouling agent, drugs formulation, and stabilization of emulsions have been proposed for this family [7]. Class II hydrophobins HFBI and HFBII produced by *T. reesei* have been structurally studied by many authors [8,9]. On one hand, HFBI is bound to the mycelium and can be easily produced in submerged culture to a titer ranging from 1 to 2 g/L. On the other hand, HFBII is mainly secreted to the extracellular medium in much lower amount, *i.e.* around 30 mg/L with wild type *T. reesei* and around 200 mg/L with genetically engineered *T. reesei* (Table 1).

Although genetically engineered strains lead to a significant improvement of the titer, severe foaming issues are observed

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Table 1
The effect of lactose concentration on HFBII production in shake flask.

Lactose concentration (g/L)	10	20	30	40
HFBII (mg/L)	2.7 ± 0.5	14.3 ± 0.9	17.7 ± 0.9	20.0 ± 0.8

considering the massive release of HFBII into the extracellular medium. Improvement in production of HFBII can be done by optimizing the medium composition. Lactose has been found to be a strong promoter of cellulolytic activity and HFBII production through expression of the *hfb2* gene and repression of *hfb1* [10–12]. On the opposite, glucose has been found to promote the production of HFBI [13].

Beside the optimization of the cultivation medium, alternative bioreactor design can also be proposed. In fact, cultivation in submerged bioreactor presents several drawbacks, such as mass transfer limitation when viscosity is increased by mycelium growth, shear stress, and foaming when biosurfactants are produced. Solid-state fermentation (SSF) is an affordable alternative to submerged fermentation, but is generally difficult to optimize and scale-up [14]. However, SSF is recognized as a technique closer to the physiology of fungi and promote sporulation and excretion of proteins and secondary metabolites [15]. These solid-state physiology mechanisms can be attributed to the limitation in water activity and the formation of aerial structures after attachment of fungi to the solid substrate.

In this work, a fungal biofilm reactor has been evaluated as a new cultivation platform for the optimization of HFBII production by *T. reesei*. This reactor involves the continuous recirculation of the cultivation medium onto a metal structured packing exhibiting a high specific surface. This bioreactor set-up has been previously proposed as a scalable biofilm reactor design [16,17] and has been successfully used to improve lipopeptides production by *Bacillus subtilis* [18]. The design of the bioreactor has been optimized in order to promote the natural binding of the fungal biomass on the packing. Three main technological advantages are expected from this bioreactor configuration, *i.e.* the alleviation of foam formation since oxygen transfer is not ensured by direct bubbling of compressed air into the liquid phase, the alleviation of mass transfer limitation due to the increase of viscosity of the broth, and the excretion intensification of proteins and secondary metabolites to the extracellular medium considering the particular physiology exhibited by the aerial hyphae formed onto the structured packing [19,20]. These technical advantages have been evaluated by comparison with classical submerged fermentation during the production of hydrophobin HFBII. The possibility to extent the use of biofilm reactor in continuous mode has also been assessed.

2. Materials and methods

2.1. Fungal strain and cultivation medium

T. reesei MUCL 44908 (purchased from BCCM/MUCL (Agro)Industrial Fungi and Yeast Collection company) was used in this study. The culture medium consisted of: peptone 4.0 g/L, yeast extract 1.0 g/L, KH_2PO_4 4.0 g/L, $(NH_4)_2SO_4$ 2.8 g/L, $MgSO_4{\cdot}7H_2O$ 0.6 g/L, $CaCl_2$ 0.6 g/L, $CoCl_2{\cdot}6H_2O$ 4.0 mg/L, $MnSO_4{\cdot}H_2O$ 3.2 mg/L, $ZnSO_4{\cdot}7H_2O$ 6.9 mg/L and $FeSO_4{\cdot}7H_2O$ 10.0 mg/L. The medium was supplemented with either lactose, galactose or glucose with the initial concentration of 40 g/L.

2.2. Bioscreen analysis of the carbon source effect

The growth curves with different carbon sources were obtained using spectrophotometric/turbidimetric measurements (Bioscreen C) [21]. The cultivation medium was prepared in a microplate specifically designed by the bioscreen supplier in order to avoid evaporation. Additional measure taken in order to limit evaporation was to fill the wells located at the periphery of the microplate with sterile medium. For inoculation, the spores were collected from surface culture of *T. reesei* on the petri dishes with the medium of Malt Extract Agar (MEA) and placed into the test tubes containing fresh medium culture (1×10^5 *T. reesei* spores/mL). The latter was used as the overnight culture and was added to the fresh medium after 12 h. The microplate reader was programmed at 25 °C with an acquisition frequency tuned in order to read automatically the optical density (OD) during 96 h at a wavelength of 620 nm. During cultures, microplate was maintained under orbital shaking. A model based on the area under the kinetic curve (AUKC) [22] was applied for the characterization of the growth rate during the culture of *T. reesei* in microplate. Growth was followed online by measuring absorbance (Bioscreen C). Based on the evolution of absorbance, AUKC was calculated by using the *trapz* function of MatLab on the basis of 3 h periods. After that, the relative AUKC (rAUKC) was estimated by dividing each AUKC with time and the change in rAUKC, namely ΔrAUKC, was calculated. Increasing ΔrAUKC is correlated to a higher growth rate.

2.3. Bioreactor set up

A first set of cultures was carried out in a lab-scale 2 L bioreactor (working volume: 1 L, Biostat B-Twin, Sartorius). Temperature was maintained at 30 °C during the whole culture (remote control by the MFCS/win 3.0 software). Submerged culture was carried out by using a mechanical stirring system (Rushton disk turbine with 6 blades running at 800 min^{-1}). For biofilm reactor, the stirring system was removed and the headspace of the reactor above the liquid surface was filled with a metal structured packing (Sulzer, Chemtech) (Fig. 1A). A peristaltic pump ensured the continuous recirculation of the medium at a flow rate of 26 L/h (connection made with silicone tubing with an internal diameter of 5 mm). The recirculated medium is distributed at the top of the packing by 5 injector tubes distributed evenly (inner diameter of 5 mm). Air was supplied under the packing at a flow rate of 1 L/min. The air injection system was located above the liquid surface in order to avoid foam formation during HFBII production. Since the bottom part of the reactor is filled with liquid medium, all the standard probes can be used as well as the corresponding regulation loops (temperature, pH and dissolved oxygen).

In the second experiment, the biofilm reactor was scaled up to 10 L working volume (Biolafitte bioreactor with a total volume of 20 L). As for the lab-scale biofilm reactor, the headspace was filled with a stainless steel structured packing made of several corrugated sheets (Sulzer, Chemtech) (Fig. 1B). A peristaltic pump ensured the continuous recirculation of the medium at a flow rate of 26 L/h. Air was supplied under the packing at a flow rate of 10 L/min. After 48 h of cultures, 8 liters were removed and replaced by a fresh medium. This mode of operation was considered as an intermittent feeding and repeated two times.

2.4. Analytical methods

2.4.1. Growth, substrate and metabolites dynamics

Lactose consumption during the fermentation was tracked by the enzymatic Lactose assay kits (Abcam ab83384) following the manufacturer instructions and also by a LC-20AT modular HPLC system (Shimadzu, Kyoto, Japan) [23]. Analyses were performed in triplicate.

For determination of dry matter, forty milliliter of the culture medium was filtered through a Whatman filter No. 4, and was