IMPLEMENTATION OF A METAL STRUCTURED PACKING IN A FUNGAL BIOFILM REACTOR FOR THE PRODUCTION OF A RECOMBINANT PROTEIN BY ASPERGILLUS ORYZAE

Q. ZUNE*, DELEPIERRE A*; D. TOYE, P. J. PUNT*** F. DELVIGNE******
*Unit of BioIndustry, Gembloux AgroBioTech (ULg). Passage des Déportés, 2; 5030 Gembloux.
**Laboratory of Chemical Engineering, ULg. Allée de la Chimie, 3/6c. B-4000 Liege.
***Wageningen Centre for Food Sciences, P.O. Box 557, 6700 AN Wageningen (The Netherlands).
****Unit of BioIndustry, Gembloux AgroBioTech (ULg). F.R.S-F.N.R.S Belgium. Passage des Déportés, 2; 5030 Gembloux

INTRODUCTION

Fungi are eukaryotic microorganisms exhibiting a high secretive power of various secondary metabolites and the ability to perform post-translational modifications during protein synthesis. In the field of fermentation industry, they are ideal hosts for secondary metabolites and recombinant proteins production. At an industrial scale, these latter are mainly produced in submerged culture or in solid-state culture. A stirred tank bioreactor used in the submerged culture mode allows biomass growth as free filamentous mycelium suspended in the liquid broth. Despite its simple implementation and its easiness of process control, mycelium growth increases the viscosity of the liquid medium and mechanical agitation induces high shear stress (1). In the solid-state fermentation mode, biomass grows on the surface of particles covering several holed trays arranged in a multi stage vessel. Particles are organic solid residues from industrial and agricultural processes such as wheat bran. They have functions of support and substrate for biomass growth (2). Absence of free water, great oxygen availability and solid-state medium physiology enhances metabolites secretion compared with the submerged culture mode. Nevertheless, solid-state cultures involve a complex heat removal and fastidious downstream process operations. Recently, fungal biofilm reactor combining advantages from submerged and solid-state fermentation has demonstrated its efficiency for secondary metabolites production. In this culture mode, fungal biofilm grows on inert support immerged in a liquid broth containing all nutriments required for the fermentation. In previous studies, secondary metabolites such as hydrophobin production by Trichoderma reseei or alpha-pyrone production by Trichoderma harzanium were performed in fungal biofilm reactor and exhibited higher yields compared with submerged culture in the same conditions (unpublished data). The authors observed also a lower viscosity of the medium during the fermentation and an easier recovery of metabolites. In this work, we try to study the fungal biofilm reactor for the production of a recombinant protein from a Aspergillus oryzae modified strain. This latter contains a reporter gene system under the control of a promoter specifically induced in solid-state medium physiology (3). The recombinant protein is a fusion protein including the amino acids sequence of the green fluorescent protein (GFP), allowing a simple detection and quantification. The results gained in the fungal biofilm reactor were compared with those of a submerged culture carried out in the same conditions.

MATERIAL AND METHODS

Microbial strain and medium

The Aspergillus oryzae ATCC 16868 strain carries a reporter gene system under the control of a promoter specifically induced in solid-state condition,

pglaB (4). The reporter gene, considered as the recombinant protein, is a fusion protein containing a truncated sequence of the glucoamylase A (glaA) linked to the sequence of the green fluorescent protein (gfp). A positive control performed with the wild strain of A. oryzae permits to reveal the reliability of the construction in a western blot analysis. The blotting with a primary antibody anti-GFP shows a first band at 70 kDa corresponding to the fusion protein and a second band at 23 kDa corresponding to a truncated GFP.

Petri dish with PDA medium is inoculated with fungal spores and incubated for 1 week at 30°C and stored at 4°C for up 2 weeks before harvest by addition of 9 mL of peptone water and scraping with a sterile syringe needle. The spores from one petri dish are counted using a Burker cell in order to inoculate the liquid medium (soluble starch 5 g L-1, casein peptone 5 g L-1,yeast extract 5 g L-1, chloramphenicol 100 mg L-1, one drop of an antifoaming agent KS911) to the target density of 2-5 E+8 spores / L.

Operating procedures for semi-solid and submerged culture

The submerged culture is carried out in a classical stirred tank bioreactor (Fig1A). Aeration of 0,25 vvm, agitation of 800 rpm, pH 6 and temperature of 30°C are maintained for the 72 hours of fermentation. The fungal biofilm reactor is a 2L stirred bioreactor without its agitation axis. This latter is replaced by a stainless steel structured packing having the function of an inert support with high specific area (700 m^2/m^3). Two types of fungal biofilm reactor are considered in this work. In the first one, the support is totally immerged in the liquid medium during all the culture (Fig1B). In the second one (Fig1C), the support is totally immerged in the liquid medium during the 24 first hours of culture (phase I). Then, a fraction of the liquid phase is transferred into an intermediary vessel (Fig1D) in order to perform a continuous recirculation of the liquid on the support thanks to a peristaltic pump (phase II). The recirculated liquid asperses the metal packing from two holes inserted in the lid of the bioreactor with a flow rate of 18 L h-1. Aeration flow rate of 1 vvm, temperature of 30°C and pH 6 are maintained for each fermentation run of 72 hours. Fermentation broths are inoculated with the same spores density from the same petri dish.

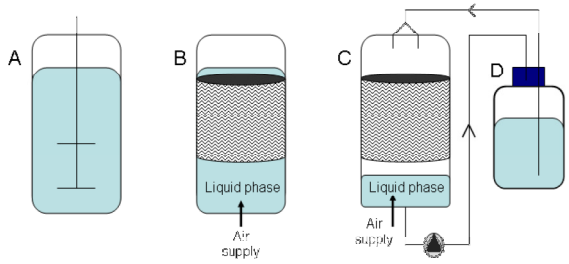

Figure 1 : scheme of the cultivation systems. A) Stirred tank bioreactor for the submerged culture. B) Fungal biofilm reactor with a total immersion of the support. C) Fungal biofilm reactor with recirculation of the liquid phase on the support. D) Intermediary vessel.

Mass balance

At the end of each fermentation run, we measured the residual volume, the weight of the fungal biofilm attached to the packing and its dry matter. The fungal biofilm is scrapped from the metal packing and submitted to a dry matter quantification.

Biochemical analysis

Supernatant of the liquid phase is sampled during the culture in order to follow kinetic parameters such as carbon source, alpha-amylase activity and total protein content. Each sample collected from the liquid medium is filtered on a 0,20 µm filter mesh and stored at -20°C before further analysis. A spectrometric method is used to determine residual glucose and starch in the culture supernatant. Alpha-amylase enzymatic activity is quantified by a standard spectrometric method (DNS) based on the assay of glucose and maltose amount produced by enzymatic hydrolysis of soluble starch. Total protein content in the culture supernatant is performed by Bradford assay.

Recombinant protein detection and quantification

Quantification of the recombinant protein in the extracellular medium is estimated by spectrofluorimetry (λem 485 nm and λex 535 nm). A western blot analysis with a primary antibody anti-GFP confirms spectrofluorimetry results since eventual occurrence of denatured GFP in the culture supernatant does not emit fluorescence.

RESULTS

In the following paragraphs, we name the fungal biofilm reactor with total immersion of the packing by "immerged BfR" and the fungal biofilm reactor with liquid recirculation on the packing by "aspersed BfR".

A metal structured packing to support mycelium growth as a fungal biofilm

The total immersion of the packing in the liquid medium allows spores adhesion on the whole surface. After their germination, elongation and branching of mycelial hyphae leads to a homogeneous colonization of the metal packing (Fig2A and 2B). The low shear effect of liquid phase prevents detachment of the fungal biofilm and keeps liquid phase perfectly limpid during all the culture. In the aspersed BfR, 80 % of the liquid phase is transferred in the intermediary vessel after 24 hours of culture. The transferred liquid phase is recirculated on the form of a liquid film flowing on the surface of the packing. The liquid distribution does not asperse the whole cross sectional area of the structured metal packing. It induces preferential flow paths inside the support leading to conidiation on external corrugated sheets not wet by the liquid film (Fig2A). The fungal biofilm distribution can be visualized inside the structured packing by the non-invasive imaging technique X-ray tomography (Fig2C). The reconstructed image from a cross sectional area located in the middle of packing height shows an equally distributed biofilm on the whole surface as well as the absence of clogging between the corrugated sheets. The dry matter of the fungal biofilm at the end of the culture is slightly higher in the aspersed BfR (2,96 %) than in the immerged BfR(2,60%).

BfR effect on growth kinetic

Presence of soluble starch in the liquid medium involves a first step of hydrolysis in glucose monomers in order to allow carbon source assimilation, followed by a second step of glucose consumption inducing exponential growth phase of the mycelium (Fig3A).

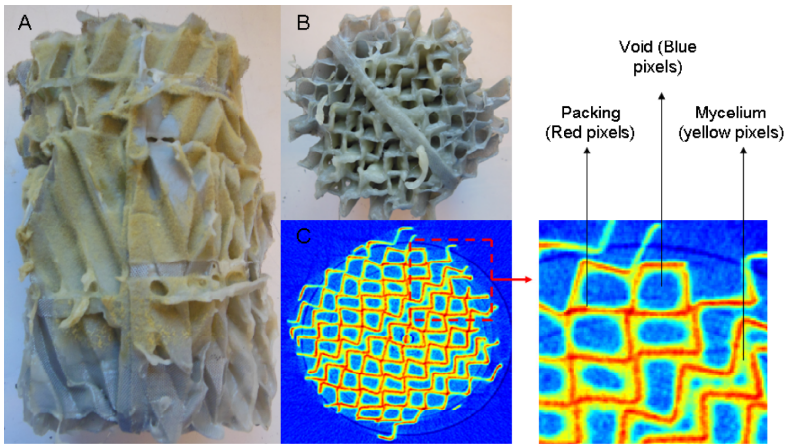

Figure 2 : Metal structured packing supporting fungal biofilm growth. A) Colonized packing at the end of a culture in aspersed BfR. B) Fig1A viewed from below. C) Reconstructed image from a X-ray tomography analysis of a cross sectional area of the packing.

We measure an increase of the alpha-amylase enzymatic activity after glucose depletion in each condition (Fig3B). BfR culture induces a delay of biomass exponential growth phase and exhibits a lower enzymatic activity compared with SC (Fig3B). The highest alpha-amylase activity reaches 17,6 ± 2,5 EU after 68h in submerged culture against 9,6 ± 0,2 and 14,1±0,5 EU after 88 and 78h in the immerged and aspersed BfR respectively (Tab1).

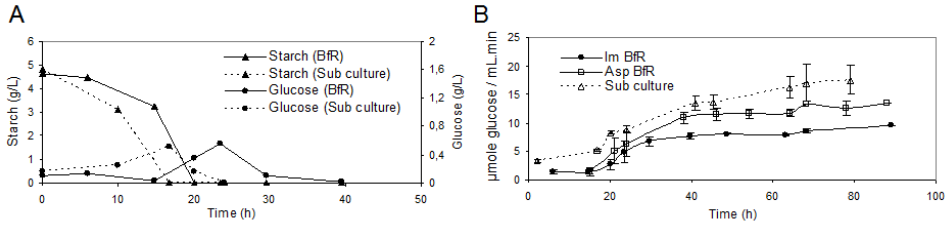

Figure 3 : A) Carbon source consumption in the submerged culture and the BfR. B) Enzymatic activity of alpha-amylase in the extracellular medium during the culture in each condition.

The highest total protein concentration reached during the culture in the extracellular medium is 81 ± 3,7 mg/L in the submerged culture (68h) against 29 ± 4,9 and 107 ± 8,1 mg/L for immerged (88h) and aspersed BfR (78h) respectively. The table 1 gives also protein productivity per gram of dry fungal biofilm for immerged and aspersed BfR, but not for the submerged culture in which we cannot give an accurate value of the dry biomass concentration because a great amount of biomass is attached to the bioreactor walls during the sampling.

Tableau 1 : Summary of the highest enzymatic activity, total protein concentration and protein per gram of dry fungal biofilm in the extracellular medium for each culture condition.

	Enzymatic activity*	Prot (mg / L)	Prot (mg / g DB*)
Submerged culture	17,6 ± 2,5	81 ± 3,7	/
Immerged BfR	9,6 ± 0,2	29 ± 4,9	4,3 ± 0,7
Aspersed BfR	14,1±0,5	107 ± 8,1	17,3 ± 1,3
	*Max, enzymatic unit EU [µmole glucose / ml enzyme.min]		* dry biomass

Recombinant protein GLA::GFP expression

The fluorescence intensity emitted by the GFP sequence of the recombinant protein in the culture supernatant is represented in the figure 4A for each culture condition. The productivity is higher in the submerged culture than in BfR and seems to be regular from the 24th hour until the 68th hour of culture. As already observed for alpha-amylase activity, productivity is higher in the aspersed BfR than in immerged BfR. The highest fluorescence intensity in the submerged culture is 2,08 and 1,47 times higher than immerged (88h) and aspersed BfR (78h) respectively.

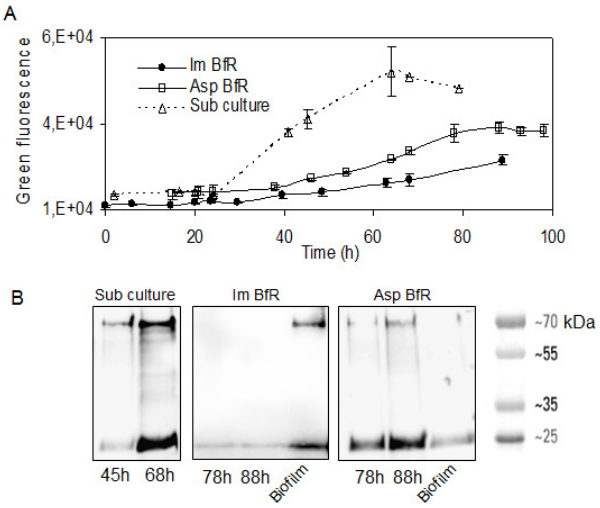

Figure 4 : Recombinant protein GLA::GFP quantification in extracellular medium based on fluorescence. A) Green fluorescence kinetic by spectrofluorimetry. B) Western blot analysis of GLA::GFP

As denatured GFP do not emit fluorescence, GLA::GFP were immunodetected in a western blot analysis (Fig4B). The two bands observed at 70 and 23 kDa most likely represent the GLA::GFP fusion protein and a proteolytically truncated GFP. Bands intensity are greater for the submerged culture than for both other conditions. Ratio between intensity of 70 kDa and 23 kDa band is higher in the submerged culture. We performed an extraction of the total protein content being entrapped in the thick mycelium attached to the packing. The western blot analysis reveals the presence of the recombinant protein in the fungal biofilm of the immerged and aspersed BfR.

DISCUSSION

In this study, the usefulness of the metal structured packing for BfR is its high specific area (3000 cm^2/L). The staggered arrangement of the corrugated gauze sheets allows to maximize exchanges between attached biomass, liquid and gas phases (5). In the BfR designed for this study, the absence of high shear stress caused by agitation in submerged culture prevents detachments of the attached biomass keeping thus the liquid phase perfectly limpid during all the culture. This parameter is attractive for the implementation of a continuous process. However, it is required to intensify oxygen and nutriments mass transfer as well as metabolites diffusion inside the fungal biofilm. We assume that the lower mass transfer in our BfR is responsible for the lower productivities relative to submerged culture. Indeed, a greater oxygen availability in the aspersed BfR increases the recombinant protein production relative to the immerged BfR, and presence of entrapped GLA::GFP in the fungal biofilm highlights a diffusion constraint. Despite successful attempts for secondary metabolites production with the same fungal BfR, recombinant protein production seems

to be more complicated because it would depend of physicochemical and physiological parameters. Effectively, the glaB promoter was supposed to be activated only under solid-state and fungal biofilm conditions. Until now, that has been demonstrated by several authors (4, 6, 7). However, all this studies comparing glaB expression between submerged culture and solid-state conditions were carried out at flask-scale. In the stirred tank bioreactor used in this study , the promoter of glaB could be more activated by other factors such as carbon starvation or shear stress than those specific of solid-state and fungal biofilm conditions. No published data in the literature mention study of glaB induction under submerged culture conditions carried out in laboratory or pilot-scale bioreactors. Further studies should considered use of other promoters for recombinant production in SSC systems.

CONCLUSION

In this study, we investigated the implementation of a metal structured packing for the production of a recombinant protein GLA::GFP from Aspergillus oryzae. Secondary metabolites production in fungal biofilm reactor exhibit higher productivities than submerged culture. Immersion of the packing in the liquid medium ensures an homogeneous colonization of the mycelium on the form of a fungal biofilm. However, we observe lower productivities of GLA::GFP in the extracellular medium compared with the submerged culture. Diffusion constraint and lower mass transfer could be responsible of lower productivities in BfR conditions at bioreactor-scale, as well as promoter selection used to induce the recombinant protein production. On the other side, persistence of a free liquid biomass of low viscosity is attractive for the implementation of a continuous process. Further studies will consider a 2-D proteomic comparison of the extracellular medium from BfR and submerged culture conditions in order to identify over-expressed proteins.

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