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Effect of moisture content in polyurethane foams as support for solid-substrate fermentation of *Lecanicillium lecanii* on the production profiles of chitinases

María del Carmen Marin-Cervantes, Yoyi Matsumoto, Laura Ramírez-Coutiño, Zaizy Rocha-Pino, Gustavo Viniegra, Keiko Shirai *

Universidad Autonoma Metropolitana, Biotechnology Department, Laboratory of Biopolymers, Avenue San Rafael Atlixco No. 186. Col. Vicentina, C.P. 09340, Mexico City, Mexico

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Abstract

Finely minced (MPUF) and roughly cut (CPUF) polyurethane foam was used as inert support for the growth and chitinases production of *Lecanicillium lecanii* by solid-substrate fermentation. *L. lecanii* growths on CPUF produced loose and disperse mycelia throughout the polymer honeycomb, but MPUF resulted in dense aggregates at the ends of the polymer branches. Despite similar growth rates, μ, and maximum biomass concentration, X_{max}, there were significant differences in the enzyme production. Highest enzyme titers (e_{max}) without glucose supplementation showed best results for >85% moisture content. e_{max}, of exo-chitinase was 45-fold higher in CPUF than MPUF. Endochitinases, e_{max}, were similar for CPUF and MPUF. Catabolic repression of enzyme production depended on moisture level, being stronger for lower moisture contents for exo-chitinases and milder or insignificant for endo-chitinases. Biomass yield coefficients of enzymes, $Y_{e/x}$, were higher with MPUF than with CPUF for endo-chitinases, but the reverse was found for exo-chitinases.

Keywords: *Lecanicillium lecanii*; Polyurethane foam; Chitinases; Entomopathogenic fungi

1. Introduction

Verticillium are gathered mycoparasitic and entomopathogenic species that produce extracelullar enzymes, such as chitinases. Several strains of *Lecanicillium lecanii* and *Verticillium fungicola* were evaluated as chitinase producers, and it is reported the activity to induce morphological changes in phytopathogen fungi [1].

The strains of *Verticillium fungicola* USDA 4519 and those of *Lecanicillium lecanii* USDA 974, USDA 2460, and ATCC 26854 showed the highest activities. Natural polymeric substrates, such as chitin, have been used to support growth of *Lecanicillium lecanii* as source of nutrients as well as inducer [2]. Chitin is not degraded inside the cell due to its insolubility, size, molecular complexity and heterogeneous composition but fungi secrete chitinases with different specificity, endochitinases and exochitinases, which are able to transform or hydrolyse chitin [3].

Verticillium lecanii (*Lecanicillium lecanii*) has been cultivated in solid substrate fermentation (SSF) using chitin as carbon source. SSF produced a highly concentrated enzymatic extract with very active chitinases [2]. The readily available sugar cane bagasse was used as support for fungal growth in that study; however, such SSF showed problems of substrate sterilization, temperature and pH control, as well contamination of the system, which was mainly detected at long process time. Besides, the enzymatic extracts carried impureness from the organic support, which involved a harder purification process.

The substrates in SSF, which are usually by products of agro-industry, have some disadvantages, such as excessive thickness of the substrate layer, low porosity, or inadequate internal structures that disturbed the aeration, heat removal and inefficient nutrient uptake. In most of these systems it is not possible to separate residual solid substrate from biomass,

* Corresponding author. Tel.: +52 5 5804 49 21; fax: +52 5 5804 47 12.
E-mail address: smk@xanum.uam.mx (K. Shirai).

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Table 1
Media composition of solid substrate fermentation of *L. lecanii* ATCC 26854 using polyurethane foam

Media	Initial moisture content (%)	PUF (%)	Nutrients (%)	PUF/nutrients	H_2O/solids	Initial a_w	
						Minced	Cut
PG[a]	75	21.14	3.8	5.5	3	0.991	0.985
PG[a]	85	10.64	4.35	2.4	5.7	0.988	0.989
PG[a]	90	4.85	4.14	1.2	10.1	0.995	0.995
NG[b]	75	21.71	3.27	6.6	3	0.993	0.993
NG[b]	85	11.28	3.71	3.0	5.7	0.993	0.993
NG[b]	90	5.43	3.57	1.5	10.1	0.999	0.993

[a] PG Czapeck media with added colloidal chitin and glucose.
[b] NG Czapeck media with added colloidal chitin.

therefore, direct estimation of growth is not attainable, and instead, that is usually measured by indirect methods based on protein, ATP, glucosamine or CO_2 concentrations.

Alternatively, SSF are also carried out on inert support materials, such as ion exchanger resins (i.e. Amberlite) and polyurethane foams (PUF) where the nutrients from a liquid media are absorbed [4]. The use of inert supports allows direct biomass determination, cleaner enzymatic extractions and more homogeneous aeration [5]. PUF have been used as a suitable support for SSF since it presents high porosity, low density and relatively high water absorption. PUF have an adequate pore size which provides a satisfactory environment for fungal growth. Moreover, the biomass and support are easily separated into the enzymatic extract with few impurities, which facilitates further purification [5–7].

The research in this area has been focused in the past years on how to improve chitinases productivity by the fungi, however, there is no information, at least to the best of our knowledge, on the relationships between size and shape of foams as supports for chitinases production considering factors such as moisture content and catabolite repression by glucose addition in solid state fermentation.

2. Materials and methods

2.1. Organism and cultivation conditions

Lecanicillium lecanii (former *Verticillium lecanii*) ATCC 26854 (American Type Culture Collection, USA) was stored on potato dextrose agar slants at 4 °C until needed. The spore suspension was obtained by mechanical agitation with a solution of 0.1% (v/v) of tween 80 at a concentration of 10^7 spores/ml [2].

2.2. Chitin

Chitin was obtained from shrimp head waste silage that contained residual proteins and ashes, 14 and 8% (dry weight basis), respectively [8]. For addition into the media, colloidal chitin was prepared according to earlier reported methodology [9].

2.3. Media

The composition of modified Czapeck medium was: $NaNO_3$ 3.73 g/l, K_2HPO_4 3.0 g/l, $MgSO_4$ 0.5 g/l, $FeSO_4$ 0.096 g/l, KCl 0.5 g/l. Colloidal chitin (36 g/l) was used as sole carbon source and this medium is referred as NG (non-glucose). A second medium PG (plus glucose) was used. PG contained glucose (15 g/l) and colloidal chitin (23.3 g/l). The pH of the media was maintained to 6 by addition of either HCl or NaOH (Table 1). The C/N, C/P, C/S molar ratios of the media were 5.5, 32 and 110, respectively [10].

2.4. Polyurethane foam

PUF were used in two sizes: (i) minced (M) and (ii) cut into cubes (C). The minced PUF (M) were prepared by using a Warring blender and meshed until a 0.3–0.5 cm particle size range, then washed and dried overnight at 70 °C. PUF were also cut into cubes (C) with approximately 0.5 cm × 0.5 cm × 0.5 cm cubic sizes, then washed and dried overnight at 70 °C.

2.4.1. PUF pore-size characterization

Digital image analysis was carried out to measure the interstitial spacing in both minced and cut in cubes foams. A microscope (Olympus BX50, Japan) was used to view the pore structure of the PUF in cross section of the foams. High resolution photographs were taken using a cooled charge-coupled device camera, and image analyses were performed using Image-Pro Plus software (Media Cybernetics, Maryland, USA). Digital photographs were analyzed on areas with dimensions of 8.95 mm × 7.21 mm by 4× magnification. The photographs were taken randomly, from 120 to 140 measurements were carried out for each type of PUF (minced and cut).

2.4.2. Porosity measurement

PUF in powder was prepared with a mincer (Thomas Scientific, model 3379-E) and meshed until a particle size of 1 mm. Foams minced or cut, as well as powdered as a control sample, were placed in a measuring cylinder without leaving any free spaces between the foams, and then weighed. Subsequently, the void fraction (ε) in the foam was measured by weighing PUF minced or cut referred to powder foam. This procedure was repeated three times for MPUF and CPUF samples.

The porosity (ϕ) was calculated by means of the following Eq. (1):

$$\phi = 1 - \varepsilon \quad (1)$$

where $\varepsilon = (W/W_0)$. W_0 is the weight of PUF in powder, W is the weight of PUF, minced or cut, and ϕ is the porosity. Then when $W \leq W_0$, the porosity (ϕ) ≥ 0.

2.4.3. Density and water storage capacity

The foams were dried to constant weight at 70 °C for 24 h and then cooled in a dessicator and their masses were recorded. Each foam volume was calculated considering height and diameter. PUF densities were calculated by dividing mass by volume. Water storage capacity was calculated by dividing the difference of wet and dry weights by the dry weight and multiplying by 100. Each determination was carried out by triplicate.

2.5. Solid substrate fermentation (SSF)

The culture media, PG and NG, as described above, and inoculum were absorbed into polyurethane foams with two sizes: minced (MPUF) or cut (CPUF). Three ratios of water:nutrients:support were evaluated, corresponding to 75, 85 and 90% of moisture content. SSF was carried out in 250 ml flasks closed with cotton plugs. Fermentation was carried out at 25 °C, during 7 days