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Evaluation of Cell Recycle on *Thermomyces lanuginosus* Xylanase A Production by *Pichia pastoris* GS 115

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Abstract

This work aims to evaluate cell recycle of a recombinant strain of *Pichia pastoris* GS115 on the Xylanase A (XynA) production of *Thermomyces lanuginosus* IOC-4145 in submerged fermentation. Fed-batch processes were carried out with methanol feeding at each 12 h and recycling cell at 24, 48, and 72 h. Additionally, the influence of the initial cell concentration was investigated. XynA production was not decreased with the recycling time, during four cell recycles, using an initial cell concentration of 2.5 g/L. The maximum activity was 14,050 U/L obtained in 24 h of expression. However, when the initial cell concentration of 0.25 g/L was investigated, the enzymatic activity was reduced by 30 and 75% after the third and fourth cycles, respectively. Finally, it could be concluded that the initial cell concentration influenced the process performance and the interval of cell recycle affected enzymatic production.

Index Entries: Xylanase A; *Pichia pastoris*; cell reutilization; heterologous expression.

Introduction

Xylanases are becoming one of the major groups of industrial enzymes, finding significant application in the paper and pulp industry, food and animal feed industries, and textile industry (*1*). In the pulp and paper industry, the commitment to remove chlorine from the production of the pulps and subsequently to completely eliminate chlorine compounds, producing totally chlorine-free (TCF) pulps, require the study and optimization of new bleaching sequences. The use of xylanases as bleaching

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boosters is a recent application of biotechnology to the paper industry *(2)*. The positive results using these enzymes, which significantly increase fiber bleachability, seem to be a consequence of the enzymatic removal of xylan from the fiber surface, which facilitates chemical bleaching of lignin-derived substances. In the food industry, xylanases are used to accelerate the baking of cookies, cakes, crackers, and other foods by helping to break down polysaccharides in the dough. In the animal feed industry, xylanases improve the digestibility of wheat by poultry and swine, by decreasing the feed viscosity *(2,3)*.

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in plant cell walls. Endo-β-1, 4-D-xylan xylanohydrolase, generally called xylanases, are the key enzymes, because they depolymerize the backbone by cleaving the β-(1,4) glycosidic bonds between the D-xylose residues in the main chain to produce short xylooligosaccharides *(4)*.

Xylanases are produced by several microorganisms mainly by fungi. Damaso *(5)* demonstrated that *Thermomyces lanuginosus* IOC 4145 secreted cellulase-free xylanase in submerged and solid-state fermentations using corncob as substrate, obtaining the best results with the latter mode of operation *(6,7)*. However, the scale-up of solid-state fermentation is not completely efficient, as a result of the process problems, such as mass and heat transfer limitations *(7)*. Additionally, the enhanced enzyme production and the industrial use of thermostable xylanases and cellulase-free preparations could be facilitated using a heterologous expression system that produces large amounts of secreted protein with an organism that can be grown in an industrial scale fermentor *(7)*.

The methylotrophic yeast *Pichia pastoris* has been developed to be an outstanding host for the production of foreign protein because its alcohol oxidase promoter was isolated and cloned *(7)*. In comparison with other eukaryotic expression systems, it offers many advantages. *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. Its expression system uses the methanol-induced alcohol oxidase (AOX1) promoter, which controls the gene that codes the expression of alcohol oxidase, the enzyme which catalyses the first step in the metabolism of methanol. The most important features of the system are that proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium *(8)*.

Our group has first cloned and expressed the gene of *T. lanuginosus* IOC-4145 in the expression system of *P. pastoris* GS 115, with the aim to produce cellulase-free XynA by submerged fermentation *(5)*. This strategy was adopted to facilitate the bioprocess monitoring when compared with solid state fermentation, as well as its downstream separation, because the recombinant yeast produces fundamentally the enzyme of interest *(8)*.

This work aims to evaluate cell recycle of a strain of *P. pastoris* GS115 bearing the *xilanase A* gene of *T. lanuginosus* IOC-4145, by submerged fermentation. Additionally, the influence of the initial cell concentration and

the feeding interval were investigated on the enzymatic production, to evaluate the strain stability to repeated batch operations.

Materials and Methods

Chemicals

All chemicals utilized were of analytical grade. Birchwood xylan and biotin were obtained from Sigma-Aldrich. Methanol and yeast nitrogen base (YNB) were purchased from Merck and Difco, respectively.

Organism and Growth Conditions

P. pastoris GS115 containing xylanase gene was obtained as reported by Damaso *(6)*. *P. pastoris* was maintained in minimal dextrose (MD) medium (in w/v: 1% glucose, 4×10^{-5}% biotin, 1.34% YNB, and 1.5% agar) for 4 d at 30°C and stocked at 4°C. For cell growth, cells were cultured in 1000-mL conical flasks containing 200 mL of buffered minimal glycerol (BMG) medium (100 m*M* potassium phosphate buffer pH 6.0 in w/v: 1.34% YNB and 4×10^{-5}% biotin in v/v: 1% glycerol), at 30°C and 250 rpm, until a cell concentration of about 9 g/L was reached. Cells were then harvested by centrifugation at 7800*g* for 18 min and ressuspended in expression medium.

Expression Medium

After centrifugation, cells were ressuspended in 20 mL of buffered minimal methanol (BMM) medium (100 m*M* potassium phosphate buffer pH 6.0 in w/v: 1.34% YNB and 4×10^{-5} % biotin, in v/v: 0.5% methanol) with an initial cell concentration of 0.25 or 2.5 g/L and incubated in 125-mL conical flasks at 30°C and 250 rpm (throw = 5 cm). At each 12 h, the pH was adjusted to 6.0 and methanol was added to a final concentration of 0.5%. The conditions of all experiments are shown in Table 1. The experiments were carried out in duplicate.

Enzyme Assays

Xylanase activity was assayed with Birchwood xylan as substrate. The solution of xylan (1% w/v) pH 6.0 and the enzyme at appropriated dilution were incubated at 75°C for 3 min, and the reducing sugars were determined by the Somogyi-Nelson procedure *(9)* by measuring absorbance at 540 nm. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars (expressed as xylose) per minute under the assay conditions.

Protein Assays

Protein concentration was measured by the method of Lowry with bovine serum albumin as standard *(10,11)*.

Table 1
Experimental Conditions Carried Out in 125-mL Conic Flasks for Recombinant XynA Production by *P. pastoris* GS115

Parameters	LCC 24 h	HCC 24 h	HCC 48 h	HCC 72 h
Interval of cell recycle (h)	24	24	48	72
Initial cell concentration (g/L)	0.25	2.50	2.50	2.50
Number of cycles evaluated	5	4	4	3

LCC, lower cell concentration; HCC, higher cell concentration.

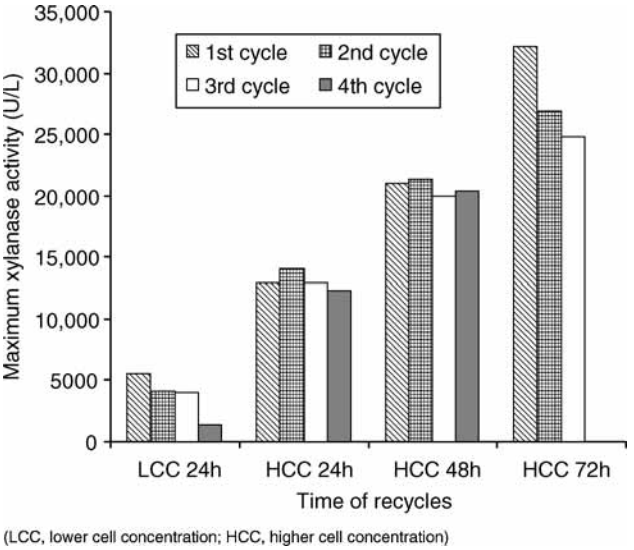

Fig. 1. Maximum XynA activity at the end of each cycle.

Results and Discussion

Aiming at investigating the effect of the inoculum size on the production of XynA by *P. pastoris* GS115, two initial cell concentrations were evaluated as 0.25 and 2.5 g/L. In the experiment carried out with X_o of 0.25 g/L and an interval cell recycle of 24 h, the cell growth was maintained for 96 h (four cycles). After that, *P. pastoris* stopped growing, and the maximum xylanase activity reached was 5550 U/L, in the first cycle. However, XynA production was not constant. There was a reduction of approx 30 and 75% in the maximum xylanase activity in the third and fourth cycles, respectively, in relation to the first one (Fig. 1), which corresponded to a decrease of approx 27 and 34% in the yeast productive capacity (Fig. 2).

When X_o of 2.5 g/L was evaluated on the production of XynA, the cell growth was maintained throughout all cycles with a 24 h interval cell recycles (Fig. 3A). The maximum xylanase activity (14,050 U/L) was achieved in the second cycle, decreasing only 10% in the following cycle (Fig. 4A). Comparing these results with those obtained with X_o of 0.25 g/L it was

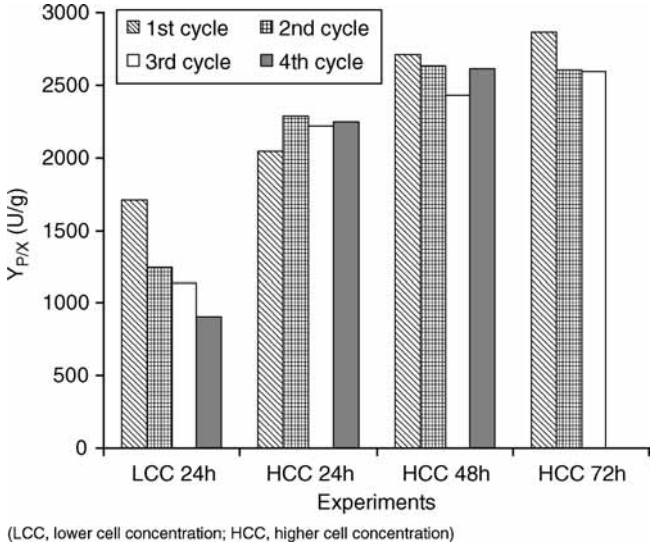

Fig. 2. Productive capacity of *P. pastoris* GS 115 throughout the cycles.

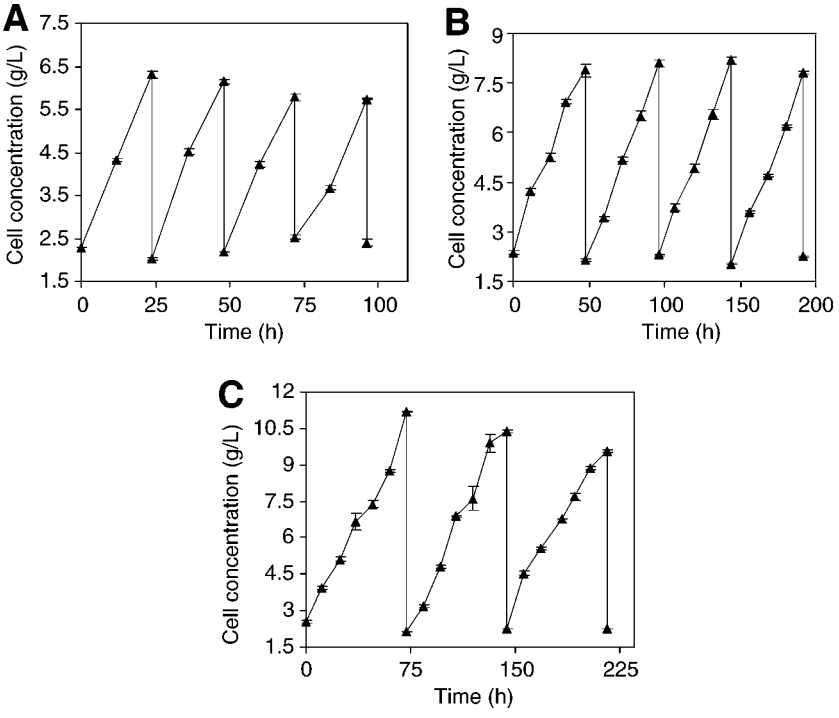

Fig. 3. Growth of *P. pastoris* GS115 in BMM medium throughout the cycles. Cell recycles with intervals of: **(A)** 24 h, **(B)** 48 h, and **(C)** 72 h.

possible to conclude that the initial cell concentration influenced the bioprocess performance. Therefore, X_o = 2.5 g/L was chosen for the next experiments. In addition, all experiments performed with X_o = 2.5 g/L did

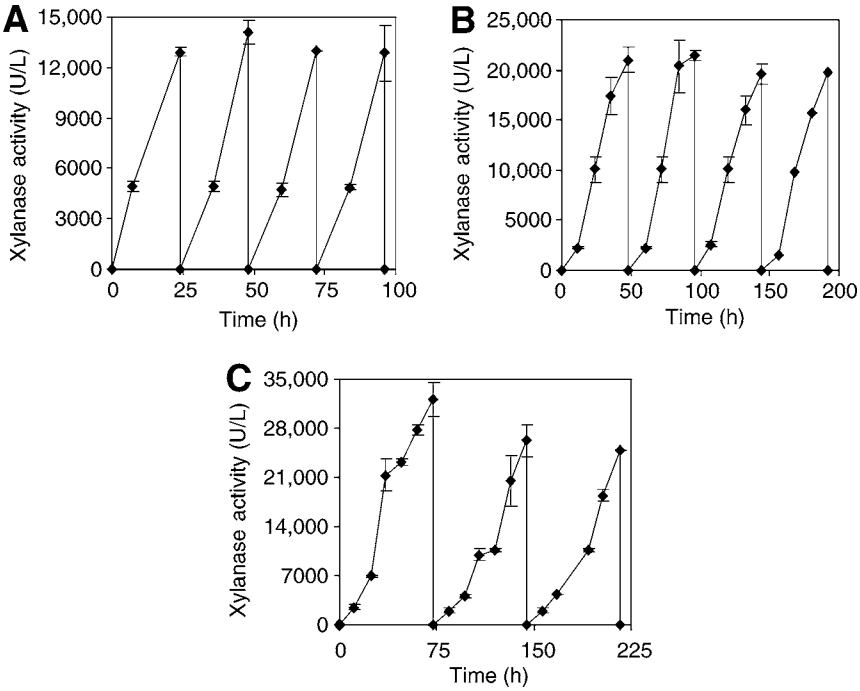

Fig. 4. XynA production by recombinant *P. pastoris* GS115 with cell recycling intervals of **(A)** 24 h, **(B)** 48 h, and **(C)** 72 h.

not have significant decrease in the productive capacity, as was observed when $X_o = 0.25$ g/L was evaluated (Fig. 2).

After selecting the most appropriate initial cell concentration, the influence of the interval of cell recycle on the XynA production was assayed. Additionally, the maximum number of recycles that maintain *P. pastoris* stability was determined. In the experiments with cell recycle at each 48 h, the maximum xylanase activity was obtained at the end of the second cycle was 21,388 U/L (Fig. 4B). Thus, it was concluded that four recycles are the limit when 48-h interval was adopted.

In the experiments carried out with a 72 h interval cell recycle, the xylanase activity did not remain constant, with a maximum value of 32,140 U at the end of the first cycle, and a reduction of 19 and 23% in the following cycles (Fig. 4C). The cell growth showed decreases of 8 and 15% in the second and third cycles, respectively (Fig. 3C). However, the productive capacity ($Y_{P/X}$) had a reduction of only 10%.

The loss of the capacity of growth and consequently of production, observed after three or four cell recycles, may be associated with the phenomenon of senescence, which is a consequence of termination of replication and is therefore intimately linked to cell division and hence the cell cycle, as pointed out by Powell *(12)*.

The response parameters of each experiment are displayed in Table 2. It can be observed that volumetric productivity (Q_P) and yield of product

Table 2
Comparison of the Process Parameters Between the Experiments With Different Initial Cell Concentration and Cell Recycle Times on the XynA Production of *T. lanuginosus* IOC-4145 by *P. pastoris* GS115

Parameters	LCC 24 h	HCC 24 h	HCC 48 h	HCC 72 h
Maximum enzymatic activity (U/L)	5550 ± 10	14,050 ± 700	21,020 ± 1290	32,140 ± 2370
Q_P (U/Lh)[a]	151.6 ± 88	549.6 ± 24	425.2 ± 19.2	385.1 ± 54
$Y_{X/S}$ (g/g)[a]	0.429 ± 0.04	0.473 ± 0.05	0.371 ± 0.02	0.339 ± 0.03
$Y_{P/X}$ (U/g)[a]	1129 ± 594	3551 ± 329	3563 ± 339	3437 ± 262
$Y_{P/S}$ (U/g)[a]	459.8 ± 267	1667 ± 72.6	1290 ± 58.5	1167 ± 165.2
Total time (h)	96	96	196	216

[a]Average values of the parameters for all cycles and their standard deviation.
LCC, lower cell concentration; HCC, higher cell concentration.

(xylanase activity) in relation to substrate consumed ($Y_{P/S}$) showed considerable differences between experiments with initial cell concentrations of 0.25 and 2.50 g/L, independent of the recycling.

The average values of the volumetric productivity (Q_P) at the end of all cycles for each experiment were 549.6 , 425.2 , and 385.1 ULh for cycles of 24, 48, and 72 h, respectively (Table 2). Comparing the results of volumetric productivity, yield of cell growth in relation to methanol consumed, yield of product (xylanase activity) in relation to cell growth and yield of product in relation to methanol consumed (Table 2), it is possible to conclude that a 24 h interval cell recycle, using X_o of 2.5 g/L, seems to be the best choice for XynA production. These results suggest that the continuous fed batch operation should be the next step to be adopted for XynA production.

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