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The effect of glycerol mixed substrate on the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system

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ABSTRACT

A recombinant *Rhizopus oryzae* lipase producing Mut^s *Pichia pastoris* strain was used as a model organism to study the effect of mixed substrates (glycerol and methanol) on the specific product productivity. Different fed-batch cultivations were performed under three constant specific growth rates (0.02, 0.05 and 0.1 h^{-1}), maintaining a constant methanol concentration of 2 $g\,l^{-1}$.

At the lowest μ tested (0.02 h^{-1}), the specific productivity was 1.23 and 1.61 fold higher and the specific methanol consumption rate (q_{sMeOH}) was 3 and 3.5 fold higher than values obtained when μ was 0.05 and 0.1 h^{-1}, respectively. This implies a relation between the q_{sMeOH} and the specific productivity, yielding higher specific productivities whenever the consumption of methanol is higher. Although glycerol was maintained under limiting conditions in all μ tested, when the relation between the μ_{Gly} and μ_{MeOH} was larger than 4, an important decrease on the maximal activity value was observed.

Finally, a comparison under the same conditions using glycerol or sorbitol as co-substrates was also performed, obtaining better specific productivity when sorbitol was used. In addition, protease activity was detected when glycerol was used as co-substrate.

1. Introduction

Pichia pastoris is a methylotrophic yeast which is a well-known host for the commercially interesting production of heterologous proteins. There are three phenotypes in the recombinant *P. pastoris* strains according to their ability to metabolise methanol: Mut$^+$, which corresponds to the *P. pastoris* wild type, where both alcohol oxidase genes (*AOX1* and *AOX2*) are active; Mut^s where only *AOX2*, which is responsible for 15 per cent of the AOX activity, is active; Mut$^-$ where *AOX1* and *AOX2* are disrupted. The Mut^s strains display slower growth on methanol than the Mut$^+$ strains due to deletion of the *AOX1* gene.

The use of methanol as sole carbon source presents some operational problems in *P. pastoris* Mut$^+$ phenotype. Storage of a large amount of methanol in explosion-proof facilities is expensive [1]. The high heat of combustion of methanol (−727 kJ C-mol^{-1}), requires a rapid and efficient cooling system, an increase of culture temperature can affect the productivity and quality of the recombinant protein [2]. The high specific oxygen consumption in high cell density *P. pastoris* cultures provokes oxygen transfer problems.

These problems can be reduced using a *P. pastoris* Mut^s phenotype because the deletion of the *AOX1* gene reduces the methanol consumption rate and the associated operational problems observed with Mut$^+$ phenotype, and sometimes reach higher expression levels of foreign proteins than Mut$^+$ strains [3]. On the other hand Mut^s is not as sensitive as Mut$^+$ to high transient methanol concentrations which make the bioprocess easier to control and enables scale up [4]. However, Mut^s phenotype fermentation results in long induction times with low growth rates.

For both phenotypes the use of additional carbon sources can minimize these problems. Glycerol is one of the most frequently used co-substrates. The enthalpy of combustion of glycerol is −549.5 kJ C-mol^{-1} reducing the heat of the bioprocess compared with methanol alone [5]. Moreover, oxygen consumption is also reduced because the oxidation of glycerol demands less oxygen, minimizing the operational problems of Mut$^+$ phenotype using methanol as carbon source [6]. Although these advantages are minimized using Mut^s phenotype, mixed carbon sources are specially recommended in this phenotype to improve the specific growth rate (μ) of the Mut^s strains and the expression levels of foreign genes increasing the productivity of the bioprocess.

However, glycerol is reported to repress the expression of alcohol oxidase and subsequently the expression of the target protein [7]. Thus, the rational design of operational strategies for the addition of both substrates in fed-batch fermentation, while avoiding glycerol repression, is the key point of the bioprocess to maximize its productivity. The first feeding strategies were developed in Mut$^+$ phenotype [8–10]. Jungo and co-workers proposed a fed-batch fermentation at a constant μ of 0.06 h^{-1}, by a pre-programmed exponential feeding rate with a methanol–glycerol ratio of 65 per

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Nomenclature

q_{sMeOH}	specific methanol consumption rate [g methanol g biomass^{-1} h^{-1}]
q_{sGly}	specific glycerol consumption rate [g glycerol g biomass^{-1} h^{-1}]
q_p	specific lipase production rate [AU g biomass^{-1} h^{-1}]
F	feed-flow rate [l h^{-1}]
V	total volume of the culture [l]
S	glycerol concentration inside the bioreactor [g l^{-1}]
So	inlet glycerol concentration [g l^{-1}]
X	biomass concentration [g l^{-1}]
$Y_{P/X}$	production yield [AU g biomass^{-1}]
$Y_{x/s}$	biomass/glycerol yield [g biomass g glycerol^{-1}]
μ	specific growth rate [h^{-1}]
t	time [h]
t_0	initial induction phase time [h]
Δt	time interval [h]
ROL	*Rhizopus oryzae* lipase

cent in the feed solution as the best conditions to maximize avidin productivity [2]. A similar operational strategy but maintaining a residual methanol concentration between 1 and 2 g l^{-1} was implemented in Mut^s phenotype by d'Anjou and Daugulis [11]. They tested two different μ (0.03 and 0.07 h^{-1}) obtaining the highest production of sea raven AFP protein at the lowest μ. Compared with Mut$^+$ phenotype the optimal μ is lower.

The effect of different methanol–glycerol ratios at constant feeding rate in fed-batch cultures has been studied in the production of mouse α-amylase [12]. Another strategy applied to the production of porcine follicle-stimulating hormone was to implement an exponential feeding rate with a constant methanol–glycerol ratio to the feed [13].

Files and co-workers tested the effect of different glycerol feed rates with constant methanol feeding rates of 1.8 g h^{-1} l^{-1} for the production of human cystatin-C [14]. Off-line methanol concentration control for the production of hepatitis B surface antigen [15] and on-line with a manual variable glycerol feeding-rate [16] has also been implemented for the production of angiostatin.

Recombinant *Rhizopus oryzae* lipase (ROL) production under AOX promoter has been widely studied from a bioprocess engineering optimization point of view with both phenotypes using methanol as the sole carbon source in conventional fed-bath fermentations [17,18] and alternative operational strategies [19]. Previous works have demonstrated that sorbitol is an excellent co-substrate to increase the production of ROL, minimizing operational problems [20,21]. However, its low maximum specific growth rate (μ_{max}) (0.02 h^{-1}) in *P. pastoris* fermentation is the cause of low productivities. The objective of this work is to test the glycerol as co-substrate in the production of ROL under a partial on-line monitoring and control of the bioprocess to improve the reproducibility of the process on Mut^s phenotype. It will allow to study the influence of specific growth rate modulated by a pre-programmed exponential glycerol feeding rate at a constant set-point of methanol concentration and to understand the influence of glycerol on recombinant ROL production in mixed substrates using a Mut^s phenotype.

2. Materials and methods

2.1. Strains

In this work we used a Mut^s strain having single copy of the ROL gene derived from *P. pastoris* KM71 (*arg4 his4 aox1Δ::SARG4 AOX2*; [3]) with its histidine auxotrophy reverted as described elsewhere [17]. Also a wild type *P. pastoris* KM71 (Mut^s) with its histidine auxotrophy reverted was used as control.

2.2. Inoculum preparation

Pre-inocula for bioreactor culture was grown for 30 h in 1 l baffled shake flasks at 30 °C, 200 rpm, in YPD medium containing per litre of distilled water: 10 g yeast extract, 20 g peptone, 20 g glucose and 1 ml of zeocin (100 mg ml^{-1}). Shake flask contained 200 ml of YPD medium. The culture was centrifuged at 4000 × *g*, the harvested cells were resuspended in sterile water (30 ml) and used to inoculate a 5-l bioreactor.

2.3. Fed-batch cultivation set up and operational conditions

The basal salt synthetic medium for fed-batch cultivations is similar to previously reported [21].

Cells were cultured in a 5 l Braun Biostat B bioreactor (Braun Biotech, Melsungen, Germany) and the cultivation conditions were: initial volume 3.2 l, stirring rate 800 rpm, temperature 30 °C, pH controlled at 5.5 by adding NH_4OH 30 per cent (v/v) during the batch phase, and KOH (5 M) during the induction phase, dissolved oxygen above 30 per cent air saturation, with a constant air flow rate between 0.5 and 8 l min^{-1}. The cultivation started with a 40 g l^{-1} of glycerol batch phase. Secondly, the induction phase was started with a pre-programmed exponential feeding rate of glycerol with the objective of maintaining the specific growth rate at a constant value throughout the fed-batch induction phase. Concurrently, the methanol set-point was maintained using a predictive control algorithm coupled with a PI feedback controller previously described [18].

In reference to glycerol addition, a quasi-steady state is assumed for the residual substrate concentration. Thus, from fed-batch substrate balance, the feeding rate $F(t)$ for a fixed μ can be expressed by Eq. (1).

$$F(t) = \frac{\mu \cdot (X(t_0) \cdot V(t_0))}{Y_{X/S} \cdot S_0} \cdot \exp(\mu(t - t_0)) \quad (1)$$

This feeding rate equation can be applied if V, X, and $Y_{x/s}$ are known at t_0 and the yield can be assumed as constant throughout the cultivation. At the beginning of the induction phase (t_0), the biomass concentration was around 20 g l^{-1}, the culture volume was 3.3 l, and the glycerol feed concentration was fixed at 550 g l^{-1}. The feeding medium contained per litre: 550 g of glycerol, 204 g NH_4Cl, 2 ml of biotin solution and 5 ml of trace salts solution. The biomass/glycerol yield was considered to be constant at 0.5 g g^{-1} under these conditions. For programming reasons, the feeding rate (Eq. (1)) was expressed as a function of the previously added feed rate (Eq. (2)).

$$F(t + \Delta t) = F(t) \cdot \exp(\mu \cdot \Delta t) \quad (2)$$

The equation only requires the initial value of $F(t_0)$ when the induction phase starts and the time between two additions (Δt: 1 min).

According to the methanol addition, this was programmed using a control algorithm previously developed to maintain a constant concentration in the bioreactor by adding a solution of pure methanol with 5 ml l^{-1} of trace salts solution and 2 ml l^{-1} of biotin solution [18]. The methanol set-point was fixed at 2 g l^{-1} during the induction phase. Methanol concentrations were monitored using an on-line methanol sensor (Raven Biotech, Vancouver, BC) immersed in the culture medium. Both carbon sources were added in the induction phase by two automatic microburettes MicroBU-2031 from Crison Instruments (Alella, Barcelona, Spain).