Effect of Dilution Rate and Methanol-Glycerol Mixed Feeding on Heterologous *Rhizopus oryzae* Lipase Production with *Pichia pastoris* Mut$^+$ Phenotype in Continuous Culture

Christian Canales, Claudia Altamirano, and Julio Berrios

School of Biochemical Engineering, Pontificia Universidad Católica de Valparaíso, Av. Brasil 2085, Valparaíso, Chile

The induction using substrate mixtures is an operational strategy for improving the productivity of heterologous protein production with Pichia pastoris. *Glycerol as a cosubstrate allows for growth at a higher specific growth rate, but also has been reported to be repressor of the expression from the AOX1 promoter. Thus, further insights about the effects of glycerol are required for designing the induction stage with mixed substrates. The production of* Rhizopus oryzae *lipase (ROL) was used as a model system to investigate the application of methanol-glycerol feeding mixtures in fast metabolizing methanol phenotype. Cultures were performed in a simple chemostat system and the response surface methodology was used for the evaluation of both dilution rate and methanol-glycerol feeding composition as experimental factors. Our results indicate that productivity and yield of ROL are strongly affected by dilution rate, with no interaction effect between the involved factors. Productivity showed the highest value around 0.04–0.06 h^{-1}, while ROL yield decreased along the whole dilution rate range evaluated (0.03–0.1 h^{-1}). Compared to production level achieved with methanol-only feeding, the highest specific productivity was similar in mixed feeding (0.9 UA g-biomass^{-1} h^{-1}), but volumetric productivity was 70% higher. Kinetic analysis showed that these results are explained by the effects of dilution rate on specific methanol uptake rate, instead of a repressor effect caused by glycerol feeding. It is concluded that despite the effect of dilution rate on ROL yield, mixed feeding strategy is a proper process option to be applied to* P. pastoris *Mut$^+$ phenotype for heterologous protein production.* © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 31:707–714, 2015

Keywords: Pichia pastoris, Mut$^+$, heterologous protein production, methanol glycerol mixed feeding, chemostat culture, response surface methodology

Introduction

The methylotrophic yeast *Pichia pastoris* is an attractive system for heterologous protein production, where most proteins are produced under the transcriptional control of the promoter of endogenous enzyme alcohol oxidase 1 (AOX1). One of its advantages is the tight control performed by the promoter, which is induced by methanol and it is repressed by glucose and glycerol. This make this system one of the most regulated and powerful promoters used in biotechnological applications.[1]

Along with the phenotypic and genotypic features of *P. pastoris*,[2] an efficient expression also depends on factors related to bioprocess development.[3–5] During the induction stage, methanol has a dual role as a carbon-energy source and as the inducer molecule. Thus, the process requires maintaining the methanol concentration above a level able to allow full induction from the promoter, but below the cell growth inhibition level.[6,7] These facts affect the kinetics and productivity of the system and they are the basis developing fed-batch culture strategies at high cell density, such as those with constant specific growth rate (μ),[8] or constant methanol concentration using an automatic control system.[9,10]

The induction stage can also be operated using feeding mixtures of methanol-glycerol substrates. Despite of the repressor effect of glycerol, the feasibility of this process option lies in the ability of methylotrophic yeasts to simultaneously consume substrate mixtures in carbon limited culture.[11,12] Based on this, several operational strategies have been developed in order to increase the productivity of the induction stage when applied to *P. pastoris* Mut^s, a slow methanol metabolization phenotype.[13–15]

On the other hand, methanol-glycerol mixed feeding has only been occasionally reported using the fast methanol metabolization phenotype (Mut$^+$). Lin et al.[16] reported an efficient angiostatin production process at high cell density using constant feeding of glycerol and automatic control of methanol concentration. Glycerol in mixed feeding increased

intracellular yield of heterologous protein production, as observed by Zhang et al.[17] on fed-batch cultures. Jungo et al.[18] determined in transient culture that the methanol-glycerol proportion did not affect the productivity related to that obtained with methanol as only carbon source. However, Hellwig et al.[19] in operations with controlled methanol concentration and constant glycerol feeding reported a decrease in production of the antibody fragment scFv4813, as compared to that produced with methanol only, indicating that glycerol represses the expression of the AOX1 promoter. The decreased productivity caused by glycerol feeding has been also observed on Mut^s phenotype,[20] but the evidence reported shows that methanol-glycerol mixed feeding operation applied to Mut$^+$ phenotype would have a higher productive potential. Nevertheless, this strategy has scarcely been applied to Mut$^+$, and has not been fully explored on a wider set of options of the main variables of the process.

Statistical experimental design application allows evaluating the variable of interest throughout the whole resulting factors combinations to obtain quantitative information about the effect of individual factors, as well as their interactions.[21] In addition to statistical experimental design, the effective control of the variables involved is a major task in order to precise determination of the effects of interest. In this regard, chemostat operation of a continuous culture is a powerful experimental tool for controlling μ by determining the dilution rate (D).[22] Therefore, in steady-state it provides high precision data, allowing reliable characterization and analysis of the system behavior at a given set of process conditions.

In heterologous protein production on *P. pastoris* with AOX1 promoter, continuous culture has been proposed as an operational strategy since the original work developed at The Salk Institute Biotechnology/Industrial Associates (S.I.B.I.A.).[13,23] As a research tool, it has been used for the kinetic analysis of growth and product formation on methanol as the only carbon source,[24] as well as to analyze the metabolic flux profiling of Mut^s phenotype growing on mixtures of methanol and glycerol.[25]

In this work, a study of kinetic cell growth and heterologous protein production with methanol-glycerol mixed feeding in continuous culture operated in the simple chemostat mode was performed. The objective was to determine the effects of mixture composition and dilution rate on the production of heterologous protein in the Mut$^+$ phenotype by applying the response surface methodology (RSM) and using a *Rhizopus oryzae* lipase (ROL)-producing strain of *P. pastoris* producing as a model system.

Materials and Methods

Microorganism

The strain *P. pastoris* Mut$^+$ X-33/pPICZαA-ROL, transformed with the cloning vector pPICZαA, which contains single copy of the gene encoding ROL, was used in this study. The strain was provided by Dr. Pau Ferrer of the Universitat Autònoma de Barcelona, Spain, and the strain construction is described elsewhere.[26]

Cell bank preparation

A cell bank was generated by culturing on yeast extract-peptone-dextrose (YPD) media containing per liter of distilled water: 10 g yeast extract, 20 g peptone, and 10 g glucose. Growth occurred at 30°C for 24 h. Thereafter, cells were subcultivated on agar YPD-Zeocin 1 mL L^{-1} during 48 h. From a grown colony, an inoculum was transferred to 50 mL of broth YPD-Zeocin 1 mL L^{-1}, and it was incubated on orbital shaker for 24 h, at 30°C and 200 rpm. The cells were centrifuged twice at 5,000 rpm and resuspended in sterile distilled water. The washed cells were resuspended in YP (yeast extract-peptone) media and were dispensed in cryovials containing glycerol, forming a cellular bank cryopreserved on glycerol 120 g L^{-1} and stored at −80°C.

Inoculum preparation

For each continuous culture, a cryovial was thawed at room temperature and inoculated into 250 mL flasks with 50 mL of buffered minimal glycerol media and 100 mM of phosphate buffer containing per liter: 10 g yeast extract, 20 g peptone, 0.4 mg biotin, and 10 g glycerol. Incubation then followed for 18 h at 30°C and 200 rpm. The cells were centrifuged at 5,000 rpm for 10 min, suspended in 100 mM phosphate buffer, washed twice with distilled water, and used as an inoculum for the bioreactor.

Continuous cultures and operational conditions

Cultures were performed in a 1.5 L Sartorius Biostat A plus (Melsungen, Germany), using 1 L of culture medium, and equipped with polarographic dissolved oxygen probe Oxyferm FDA (Hamilton, Switzerland), pH probe EasyFerm Plus (Hamilton, Switzerland), and two Rushton turbines. The operation temperature was 30°C and pH 5.5 was automatically controlled by the addition of 3 N NaOH. Air flow rate was 1 L min^{-1} and agitation rate set at 700 rpm. The culture medium composition per liter was: 1.7 g YNB (yeast nitrogen base) Difco BD without amino acids and without nitrogen source, 5 g ammonium sulfate, and 5 g of glycerol as carbon source during the initial batch phase. The reactor with the medium was autoclaved at 121°C for 20 min, except the YNB which was sterilized by microfiltration 0.22 μm. Compared to BSM traditional production medium, YNB medium does not exhibit problems for high ionic strength[27] or the insolubility compounds.[5] The risk of nitrogen limitation is also reduced because the medium contains ammonium sulfate instead of ammonia.[28]

The culture began with a batch stage during 8 h with glycerol as the sole carbon source. During the continuous operation, the volume was controlled by overflow output. Inlet flow was handled with a variable speed peristaltic pump (Masterflex, Cole Parmer). The steady-state was achieved after continuous operation for at least 4 residence time (τ), and all results are obtained during the steady-state operation between 4 and 5τ. The feeding stream was YNB medium with the same composition per liter of the batch stage, but incorporating the methanol-glycerol mixture, with the total concentration of carbon source at 12 g L^{-1} which was constant in all the experiments.

Analytical methods

Biomass concentration (X) was measured by dry weight. For this, 10 mL of culture was centrifuged at 5,000 rpm for 10 min, and resuspended in distilled water. The washing procedure was repeated two times and the cell suspension was

Table 1. Central Composite Experimental Design, with Two Variables (Coded and Actual Values), Applied for Testing the Process Production of ROL Using Mixed Feeding of Methanol and Glycerol on Chemostat, with *P. pastoris* Mut$^+$

	Coded Variables		Actual Variables		Responses		
Run	x1	x2	Methanol (%)	D (h^{-1})	q_P (UA g-biomass^{-1} h^{-1})	Q_P (UA L^{-1} h^{-1})	$Y_{P/X}$ (UA g-biomass^{-1})
1	−1	−1	52	0.040	0.77	3.00	19.4
2	1	1	83	0.090	0.61	1.08	6.8
3	0	0	68	0.065	0.91	2.21	14.0
4	α	0	90	0.065	0.84	1.95	13.0
5	−1	1	52	0.090	0.54	1.89	6.0
6	−α	0	45	0.065	0.69	2.47	10.7
7	0	0	68	0.065	1.03	2.53	15.9
8	0	0	68	0.065	0.90	2.47	13.8
9	1	−1	83	0.040	0.84	3.00	21.1
10	0	−α	68	0.030	0.72	2.85	24.1
11	0	α	68	0.100	0.48	1.50	4.76

placed in capsules, dried at 100°C until constant weight. Over the transient state, biomass was monitored by measuring the optical density at 600 nm.

Methanol was measured by GC-FID chromatograph (Clarus 600, PerkinElmer), using a capillary column Supelco Equity-1, N$_2$ mobile phase 5 mL min^{-1}, at 200, 80, and 200°C for the injector, oven and detector, respectively.

Glycerol was measured by HPLC-IR (PerkinElmer Series 200), with a column Aminex HPX-87H using 4 mM H$_2$SO$_4$ as mobile phase at 0.6 mL min^{-1}.

Extracellular lipase activity was determined from hydrolysis of *p*-nitrophenylbutyrate 4 mM at 30°C and pH 7.0, measuring on-line the formation of product at 348 nm.[29] One unit of activity (UA) is defined as the amount of enzyme which liberates 1 µmol of *p*-nitrophenol in 1 min.

Response surface experimental design

The variables studied were D between 0.03 and 0.1 h^{-1}, and methanol percentage composition in the feed from 45 to 90%. A two-variable central composite design was applied with $\alpha = 1.4$, considering one experimental block and three replicates of the central point. The coded and actual variables of the design are shown in Table 1.

The response variables considered were specific productivity of ROL $q_p = $ UA $\times D/X$, volumetric productivity of ROL $Q_p = $ UA $\times D$, and yield production of ROL $Y_{P/X} = $ UA/X.

The results for each productive response (*y*) were modeled by quadratic polynomial equations of the form

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2 + \beta_4 x_1^2 + \beta_5 x_2^2 \quad (1)$$

with β representing the model coefficients and x the factors.

The analysis of significance of the model was performed by analysis of variance test (ANOVA) using the *P*-value and Fisher coefficient (*F*-value) for 95% confidence interval. The adjustment was estimated based on the coefficient adjusted-R^2. Design Expert 8.0.7.1 trial version software was used to perform the statistical calculations.

Specific methanol uptake rate (q_{Meth}), and specific glycerol uptake rate (q_{Gly}) were calculated based on a bioreactor methanol mass balance according to

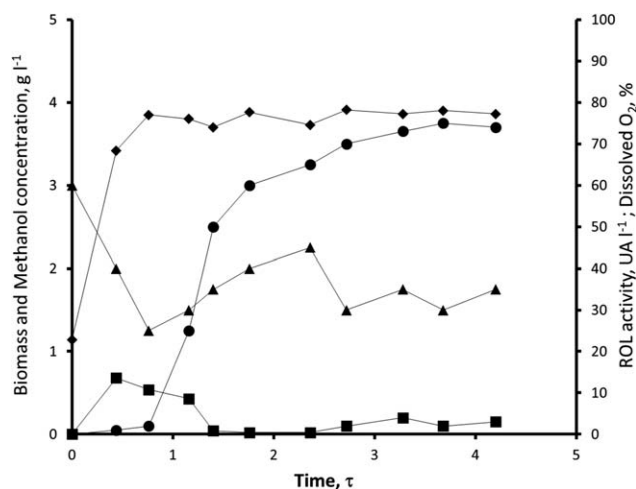$$q_{Meth} = D \cdot \frac{(Met_{in} - Met_{out})}{X} \quad (2)$$

Figure 1. Fermentation process of run *n*° 1 of the experimental central composite design (Table 1).

ROL activity (•), biomass (◆), dissolved oxygen (▲), and methanol (■). The culture was performed at dilution rate of 0.04 h^{-1} with 52% methanol fed (concentration of methanol in the feeding stream 6.2 g L^{-1}).

$$q_{Gly} = D \cdot \frac{(Gly_{in} - Gly_{out})}{X} \quad (3)$$

Results and Discussion

Biomass production and specific methanol uptake rate

In this work, the production of heterologous protein and the cell growth kinetics were assessed at low cell density. This experimental fermentation approach avoids conditions such as usage of antifoam that might affect lipase activity measurements or production formaldehyde that causes inhibition of cell growth.[30]

In all cultures, the dissolved oxygen tension levels were higher than 20%, with moderate foaming appearing only late in the culture time. Oxygen uptake rate (OUR) ranged 0.1–0.13 g-O$_2$ L^{-1} h^{-1} that allowed operation with atmospheric air and the agitation rate set at 700 rpm. Glycerol concentration was below the detection limit (0.050 g L^{-1}) over the whole experimental range, and lower than the K_s value (0.178 g L^{-1} [28]) suggesting cell growth is limited by glycerol. The time evolution of a continuous fermentation is shown in Figure 1 as an example of parameter variation during the production process.

The steady-state methanol concentration (data not shown) was lower than 0.020 g L^{-1} at 0.03 h^{-1}, and rose up to 5–7 g L^{-1} at higher D (>0.09 h^{-1}). At intermediate D values (around 0.06 h^{-1}), the methanol concentration reached 2–3 g L^{-1} into the concentration range that is not growth inhibitory,[7] in agreement with the methanol level reported as optimal for full expression of AOX1 promoter.[31] The biomass concentration ranged between 1.8 and 3.9 g L^{-1}, and was strongly affected by the operational variables (Figure 2a). Because glycerol was totally consumed, its contribution to biomass formation was proportional to glycerol concentration fed according to the expression $Gly_0 \times Y_{X/G}$, considering a constant yield within the D range tested.[32] Methanol consumption also contributed to biomass cell growth along D, but its contribution was lower because of lower biomass yield of methanol as a carbon source (0.27 g-biomass g-methanol^{-1} vs. 0.54 g-biomass g-glycerol^{-1}).

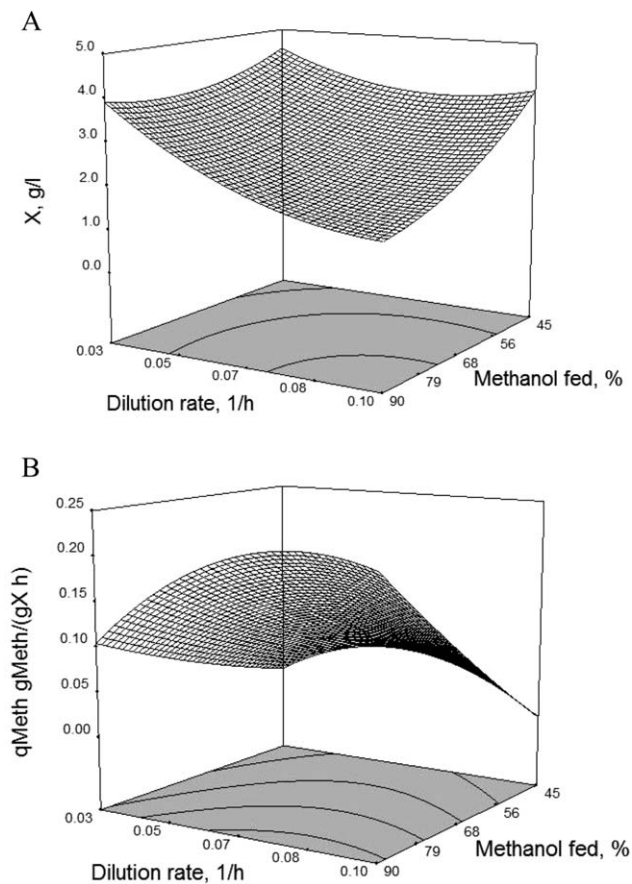Methanol specific uptake rate (q_{Meth}) showed a curved response with a maximum consumption of 0.2 g-methanol g-biomass^{-1} h^{-1} (Figure 2b). Glycerol feed decreased q_{Meth} along with decreasing % methanol fed. This change in q_{Meth} suggests that methanol consumption ability is dependent on glycerol when carbon sources are consumed simultaneously. Linked to this effect is the fact that methanol is consumed even at $\mu = 0.1$ h^{-1}, which is higher than μ_{max} for that carbon source (0.06 h^{-1}) for this strain grown on methanol as single carbon source.[33] Changes in methanol uptake rate were previously reported by Egli et al.[12] where substrate consumption effects that arise from feeding glucose-methanol mixtures to methylotrophic yeasts was of interest. Simultaneous activation of different metabolic carbon pathways possibly modifies the methanol consumption as was observed in this study, and as it has been determined by metabolic flux analysis when glycerol-methanol mixtures are fed.[25]

Surface responses of ROL production

The fermentation approach applied in this work allows a clear evaluation of ROL production, since it avoids factors associated with cell death that cause degradation of heterologous protein produced,[33] that can be observed in high cell density process.[34] This experimental approach also minimizes the effect of process conditions such as high agitation rate and oxygen enrichment,[35] that can lead to uncontrolled variables into the experimental design. Quadratic model was selected to analyze the ROL response surfaces. ANOVA of the surfaces are shown in Table 2. The F-statistics is the quotient between the response variability caused by the treatments and that caused by random error. Accordingly, combination of high F-value and low probability of P-value (<0.05) indicate a high significance of the resulting quadratic models. The determination coefficients (R^2) indicate that the quadratic models fit satisfactorily well to the experimental results (Table 3).

The specific ROL productivity surface is shown in Figure 3a. There is a region of high productivity up to $D = 0.06$ h^{-1}, and a severe decrease in productivity at higher D values (0.06–0.10 h^{-1}). The productive behavior was influenced by D with no interaction effects between the variables. In the model of specific productivity of ROL (Table 3), the interaction coefficient (β_3) is negligible and not significant (P-value 0.92). The contour analysis of specific ROL productivity shows the presence of a region containing a maximum in the D range from 0.05 to 0.06 h^{-1}. In this experimental area, q_{Meth} reaches around 0.12 g-methanol g-biomass^{-1} h^{-1} (Figure 2b). This maximum region was obtained in the D level in which the methanol concentration was 2–3 g L^{-1}. The D range of maximum q_p (0.05–0.06 h^{-1}) was higher than that reported as μ optimal to heterologous protein production with *P. pastoris* using methanol as the only carbon source (0.01–0.03 h^{-1}).[35,36] The present result is in agreement with previously reported optimal μ to intracellular yield of Heavy-chain fragment C of botulinum neurotoxin serotype C produced using fed-batch mixed

Figure 2. **Effect of dilution rate and methanol % fed at steady state on biomass concentration (A) and specific methanol uptake rate (B).**

The continuous cultures with methanol-glycerol feeding were performed according to the central composite design (Table 1).

Table 2. ANOVA Results of the Model Surfaces Responses for Heterologous Production of ROL (Eq. 1), Considering Dilution Rate and % Methanol Feeding as Factors, on Chemostat with *P. pastoris* Mut$^+$

Response Model	Sum of Squares	Degrees of Freedom	Mean Square	F-Value	P-Value Prob $> F$
q_p	0.28	5	0.056	20.94	0.0023
Q_P	4.24	5	0.85	42.40	0.0004
$Y_{P/X}$	393.73	5	78.75	131.72	<0.0001

Table 3. Coefficients of Response Models (Eq. 1) for ROL Production on Chemostat with *P. pastoris* Mut$^+$

Coefficient	Response		
	q_p	Q_p	$Y_{P/X}$
β_0	-1.679	0.373	3.200
β_1	0.049	0.066	0.781
β_2	31.447	30.989	-232.466
β_3	0.003	-0.509	-0.534
β_4	-0.0003	-0.0003	-0.005
β_5	-275.113	-164.0	-50.245
R^2	0.952	0.942	0.986

feeding strategy, with automatic control of methanol concentration.[17]

The response surface of Q_p is presented in Figure 3b. ANOVA analysis revealed that linear (β_2) and quadratic (β_5) coefficient of D are significant (*P*-value < 0.013). To this response, the effect of D depends on the level of methanol due to the existence of a moderate interaction effect (*P*-value 0.20). At higher levels of methanol feeding, Q_p decreased sharper than at lower levels of methanol feeding, similar to that observed for the production of biomass (Figure 2a). Stoichiometric calculations showed that at 0.09 h^1, glycerol consumption explains 90% of the formed biomass at 52% methanol fed, while the biomass production attributable to glycerol consumption decreases to 65% at 83.5% of methanol in the feeding. Thus, the variation of Q_p is consistent with the production of biomass in the experimental conditions.

Figure 3c shows the strong effect of D on the $Y_{P/X}$ response. ANOVA of the model shows that there are no significance to both, interaction (β_3, *P*-value 0.54) and quadratic D terms (β_5, *P*-value 0.85). The highest $Y_{P/X}$ is achieved at lower D according to higher biomass production. YP/X represents the specific ability of the strain for ROL production. This expression potential depends on AOX1 promoter activity, and at the experimental conditions, it could be affected by D and not by methanol % feeding. The highest activity measured at steady state was 95 UA L^{-1} at $D = 0.03$ h^{-1}. With a further increase of D the ROL activity decreased gradually to 40 UA L^{-1} at $D = 0.06$ h^{-1}, and to 10 UA L^{-1} at $D = 0.1$ h^{-1}.

The lack of curvature on variable D in the $Y_{P/X}$ response surface differs from the observed for q_p and Q_p. While the response surface of q_p shows a maximum region inside the experimental area, the highest response of Q_p it is shifted toward lower values of D, showing a wide area of high volumetric productivity. The displacement in the highest q_p and Q_p responses is not an exclusive effect of mixed feeding in *P. pastoris*. In the strategy with methanol-only feeding, it has also been seen that values of the variables that maximize Q_p do not coincide with those for maximum q_p response, as noted by Zhang et al.[37] who studied the effect of biomass concentration and D on the continuous production of recombinant interferon. This provides the option to design production processes depending on the desired higher response, either for q_p or Q_p.

Figure 4a shows how q_{Meth} was related to a downward trend of Q_p response, while the highest q_p was obtained at intermediate q_{Meth} levels around 0.12 g-methanol g-biomass^{-1} h^{-1}. In contrast, q_{Gly} had a weak effect on Q_p response, while no effect was detected on q_p response (Figure 4b). Thereby, specific glycerol feeding rate on the range 0.030–0.150 g-glycerol g-biomass^{-1} h^{-1} has a moderate
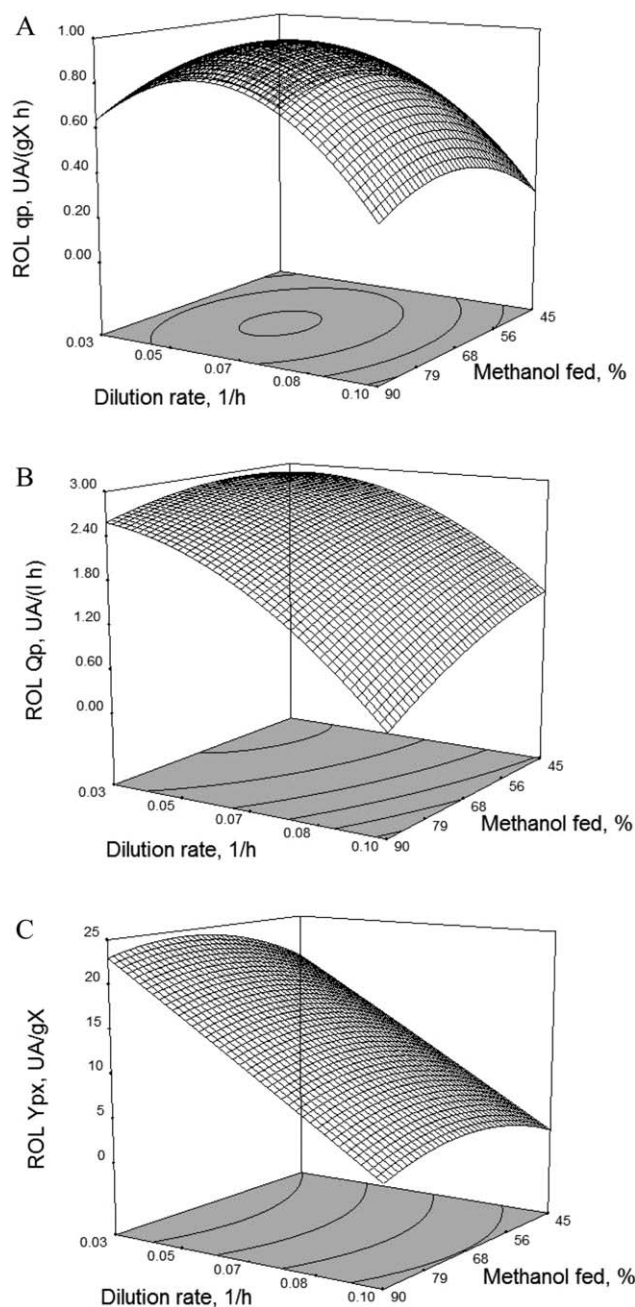

Figure 3. Response surface of the effect of dilution rate and methanol fed at the steady state on specific productivity (A), volumetric productivity (B), and product yield (C) of ROL activity.

The continuous cultures with mixed methanol glycerol feeding were performed according to the central composite design (Table 1).

effect on productivity of ROL. Since q_{Gly} depends on D, the effect of q_{Gly} can be explained as a consequence of changes in D, in agreement with the effect of D on Q_p response (Figure 3b). These findings indicate that productive responses observed for q_p and Q_p, would be mainly the effect of q_{Meth}. The preceding also shows that considering the feeding used in this study, glycerol feeding rate per bioreactor volume ranged between 0.07 and 0.51 g-glycerol L^{-1} h^{-1} has little effect on ROL Q_p and null effect on ROL q_p. The lack of effect of glycerol feeding on q_p was also observed by Jungo et al.[18] who studied the effect of the composition feeding at

$D = 0.06$ h^{-1} on avidin heterologous production. In this work, the same behavior was observed on a wider range of D. This make possible to operate the induction stage with substrate mixed fed without the risk of glycerol repression.

Comparative production of ROL using methanol and mixed feeding

Productivity and yield of ROL with mixed feeding at optimal conditions was compared with the conventional operation
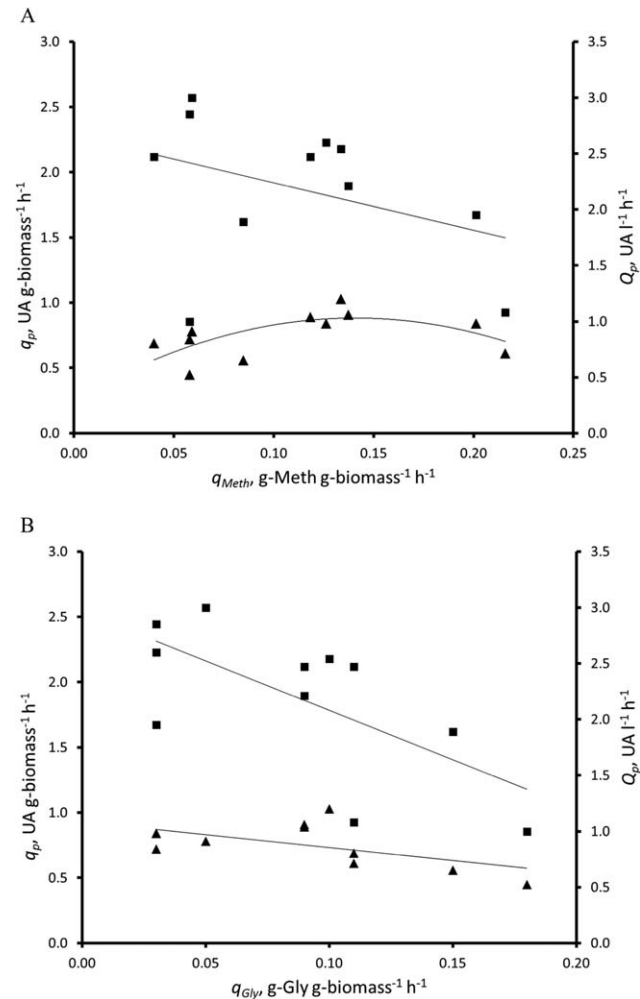

Figure 4. **Variation of specific (▲) and volumetric (■) productivity of ROL produced by *P. pastoris* Mut$^+$ vs. methanol specific consumption rate (A) and vs. glycerol specific consumption rate (B).**

Data were taken at steady state during continuous culture according to central composite experimental design (Table 1). Specific substrate consumption rates were calculated according to Eqs. (2) and (3).

using methanol-only feeding. Continuous fermentations with methanol-only feeding were carried out at $D = 0.02$ h^{-1}, reported as µ optimal to heterologous protein production with *P. pastoris* Mut$^+$ growing on methanol as the only carbon source.[35,36] In order to test the effect of glycerol on the feeding at 0.05 h^{-1}, additional continuous experiment with methanol-only feeding were carried out at 0.05 h^{-1}. Results of comparative experiments are presented in Table 4. Similar q_P were reached between induction operations with methanol only at 0.02 h^{-1} and mixed feeding (0.91 vs. 0.98 UA g-biomass^{-1} h^{-1}). However, mixed feeding Q_P was 60% higher than methanol-only feeding (2.75 vs. 1.60 UA L^{-1} h^{-1}). The increased biomass production with mixed feed follows from the easily assimilable glycerol carbon source. It can be noted that productive responses in both conditions, mixed and methanol-only feeding, are a result of the same level of q_{Meth} (0.103–0.106 g-methanol g-biomass^{-1} h^{-1}).

The effect of glycerol feeding is revealed by comparing operations at 0.05 h^{-1}. It is seen that productivity and yield is higher than that obtained with methanol-only feeding. However, q_{Meth} is only one third with mixed feeding. Thus, glycerol feeding modifies the productive response of D, moving the maximum q_P (0.02 h^{-1}) to higher D level (0.05 h^{-1}), as a consequence of substrate cometabolism.

The two distinct feeding strategies (methanol-only and mixed), operated at different conditions, led to similar specific ROL productivity under similar q_{Meth}. This suggests that the observed effect of q_{Meth} on q_P for mixed feeding (Figure 4a) would be also valid on the induction process with methanol-only feeding. Thus, it would exist a pattern effect of methanol consumption kinetics on ROL q_P regardless of the presence of cosubstrate.

In heterologous protein production with *P. pastoris* Mut$^+$, kinetic variables such as those related with substrate consumption are of great interest. Even, q_{Meth} has been reported as basic criteria to design induction operation of high productive protein production process.[38] It has recently been reported that the implementation of methanol feeding based on constant q_{Meth} in fed-batch operation achieves a more stable production profile of galactose oxidase, with similar volumetric productivity to that obtained at pre-programmed feeding.[39]

In the present kinetic study, the composition of the mixture feeding considered as a process variable, together with continuous cultivation, allowed for assessing the effect of feeding rate without changing cell growth rate. Thus, it was possible to identify and analyze the effects of variables independently from each other, which would not be possible in fed-batch operation. The decreasing of the $Y_{P/X}$ response with mixed feeding operation has also been observed by Hellwig et al.[19] at a similar range of specific glycerol consumption rates. This effect might be caused by an increase of D rather than the repression by glycerol feeding.

Table 4. Comparison of Heterologous Production Processes of ROL Using Mixed Feeding and Methanol only on Chemostat, with *P. pastoris* Mut$^+$

			Productive Responses		
Feeding Operation	D (h^{-1})	q_{Meth} (g g-biomass^{-1} h^{-1})	q_P (UA g-biomass^{-1} h^{-1})	Q_P (UA L^{-1} h^{-1})	$Y_{P/X}$ (UA g-biomass^{-1})
Methanol-glycerol (71–29%)*	0.05	0.120	0.97	2.70	18.90
Methanol-glycerol (71–29%)	0.05	0.106	0.98	2.75	19.64
Methanol	0.05	0.318	0.45	0.50	9.09
Methanol	0.02	0.103	0.91	1.60	45.71

*Model predicted.

From the standpoint of process development, the design of fermentation strategies with *P. pastoris* to exploit the maximum protein production potential is the main challenge in the bioprocess research. In a previous process variables study performed with methanol-only fed-batch, the highest production of trypsinogen was achieved with Mut^+ phenotype (15.2 vs. 8.8 mg g-biomass^{-1}) regardless of the variable conditions in which the process with Mut^+ was carried out.[40] This shows that the maximal potential productivity of the Mut^+ phenotype is difficult to determine in fed-batch operations due to dynamic nature of the process and the fast methanol metabolism by the strain. Specific cell growth rate correlates well with phytase production in Mut^S phenotype (optimal μ 0.013–0.017 h^{-1}),[41] showing that the productivity improvement by means of increasing of μ is a fully valid strategy even in the low range of specific growth rate.

The goal of a strategy with mixed feeding using Mut^+ strains is to increase the heterologous protein productivity by increasing μ but avoiding the adverse effects of the fast metabolization of methanol. In this work, the chemostat allowed covering a range of *D* for the characterization of the potential productivity. Our results show that mixed feeding has the same potential of q_P that the one achieved with the methanol-only feeding. Compared to the latter, the highest q_P is achieved at higher *D* and at the same level of q_{Meth} (around 0.1 g-methanol g-biomass^{-1} h^{-1}), but with a noticeable increased volumetric productivity. Thus, mixed feeding could be applied to production process in which, by operational criteria, either the cultivation time or Q_P are the key consideration of the process.

Other easily assimilable substrates such as glucose have been used as growth substrate on batch cellular growth stage to produce phytase.[42] A study shows *P. pastoris* Mut^+ on continuous operation with high trypsinogen productivity, using glucose as cosubstrate with a 60% of methanol,[43] even higher the percentage used in the present study. Evidence shows that the own dynamics of the carbon limited cell growth, removes the substrate repression effect, therefore, mixed feeding could be applied using substrates recognized as repressors, other than glycerol. Furthermore, since biodiesel industry development has pushed down the glycerol market price,[44] its utilization is further attractive from economy process perspective. Hence, it is expected that glycerol remains an interest issue presently and in the future, to heterologous protein production with *P. pastoris*.

Conclusions

In this work, the heterologous protein production with mixed feeding strategy in *P. pastoris* Mut^+ phenotype was analyzed by means of the application of a fundamental fermentation approach. It was demonstrated that the heterologous protein production is strongly dependent of *D* variable, while within 40–90% range of methanol in glycerol mixes, the feeding composition showed a weak effect. The experimental design revealed that variables did not exhibit interaction effects on the range of conditions tested, although the production followed a different response pattern.

Compared to methanol-only feeding, the volumetric productivity with mixed feeding was higher. Mixed feeding showed a maximum specific productivity in a similar way to that achieved with methanol-only feeding. However, mixed feeding moved the maximum specific productivity

to higher values of *D*. This effect allows operating the induction within a *D* range of 0.04–0.06 h^{-1} keeping a high productivity.

Although the strong effect of *D* on $Y_{P/X}$ that caused a decrease in ROL volumetric and specific productivities when compared to the obtained with methanol-only feeding, mixed feeding strategy can potentially be used in high cell density operations for heterologous protein production using *P. pastoris* Mut^+ phenotype.

Literature Cited

1. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev*. 2000;24:45–66.
2. Macauley-patrick S, Fazenda ML, Mcneil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*. 2005;22:249–270.
3. Jahic M, Veide A, Charoenrat T, Teeri T, Enfors S. Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. *Biotechnol Prog*. 2006;22:1465–1473.
4. Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. *Biochem Eng J*. 2012;64:91–105.
5. Cos O, Ramón R, Montesinos JL, Valero F. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. *Microb Cell Fact*. 2006;5:17.
6. Katakura Y, Zhang W, Zhuang G, Omasa T, Kishimoto M, Goto Y, Suga K. Effect of methanol concentration on the production of human B2-glycoprotein I domain V by a recombinant *Pichia pastoris*: a simple system for the control of methanol concentration using a semiconductor gas sensor. *J Ferment Bioeng*. 1998;86:482–487.
7. Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. *Biotechnol Bioeng*. 2000;70:1–8.
8. Schenk J, Balazs K, Jungo C, Urfer J, Wegmann C, Zocchi A, Marison I, von Stockar U. Influence of specific growth rate on specific productivity and glycosylation of a recombinant avidin produced by a *Pichia pastoris* Mut$^+$ strain. *Biotechnol Bioeng*. 2008;99:368–377.
9. Cos O, Ramon R, Montesinos JL, Valero F. A simple model-based control for *Pichia pastoris* allows a more efficient heterologous protein production bioprocess. *Biotechnol Bioeng*. 2006; 95:145–154.
10. Gurramkonda C, Polez S, Skoko N, Adnan A, Gäbel T, Chugh D, Swaminathan S, Khanna N, Tisminetzky S, Rinas U. Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. *Microb Cell Fact*. 2010;9:31.
11. Egli T, Kappeli O, Fiechter A. Regulatory flexibility of methylotrophic yeasts in chemostat cultures: simultaneous assimilation of glucose and methanol at a fixed dilution rate. *Arch Microbiol*. 1982;131:1–7.
12. Egli T, Bosshard C, Hamer G. Simultaneous utilization of methanol-glucose mixtures by *Hansenula polymorpha* in chemostat: influence of dilution rate and mixture composition on nutrient utilization pattern. *Biotechnol Bioeng*. 1986;23:1735–1741.
13. Brierley R, Bussineau C, Kosson R, Melton A, Siegel R. Fermentation development of recombinant *Pichia pastoris*

expressing the heterologous gene: bovine lysozyme. *Ann NY Acad Sci*. 1990;589:350–362.

14. Loewen MC, Liu X, Davies PL, Daugulis A. Biosynthetic production of type II fish antifreeze protein: fermentation by *Pichia pastoris*. *Appl Microbiol Biotechnol*. 1997;48:480–486.

15. Files D, Ogawa M, Scaman CH, Baldwin SA. A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-C. *Enzyme Microb Technol*. 2001;29:335–340.

16. Lin J, Panigraphy D, Trinh LB, Folkman J, Shiloach J. Production process for recombinant human angiostatin in *Pichia pastoris*. *J Ind Microbiol Biotechnol*. 2000;24:31–35.

17. Zhang W, Hywood KJ, Plantz BA, Schlegel V, Smith L, Meagher M. *Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement. *J Ind Microbiol Biotechnol*. 2003;30:210–215.

18. Jungo C, Marison I, von Stockar U. Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: a quantitative study based on concentration gradients in transient continuous cultures. *J Biotechnol*. 2007;128:824–837.

19. Hellwig S, Emde F, Raven NP, Henke M, van der Logt P, Fischer R. Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. *Biotechnol Bioeng*. 2001;74:344–352.

20. Boze H, Céline L, Patrick C, Fabien R, Christine V, Yves C, Guy M. High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*. *Process Biochem*. 2001;36:907–913.

21. Mandenius C-F, Brundin A. Bioprocess optimization using design-of-experiments methodology. *Biotechnol Prog*. 2008;24: 1191–1203.

22. Hoskisson PA, Hobbs G. Continuous culture-making a comeback? *Microbiology*. 2005;151:3153–3159.

23. Digan M, Lair S, Brierley R, Siegel R, Williams M, Ellis S, Kellaris P, Provow S, Craig W, Veliçelebi G, Harpold M, Thill G. Continuous production of a novel lysozyme via secretion from the yeast, *Pichia pastoris*. *Nat Biotechnol*. 1989;7:160–164.

24. Curvers S, Linneman J, Klauser T, Wandrey C, Takors R. Recombinant protein production with *Pichia pastoris* in continuous fermentation - kinetic analysis of growth and product formation. *Eng Life Sci*. 2002;2:229–235.

25. Solá A, Jouhten P, Maaheimo H, Sánchez-Ferrando F, Szyperski T, Ferrer P. Metabolic flux profiling of *Pichia pastoris* grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates. *Microbiology*. 2007;153:281–290.

26. Minning S, Serrano A, Ferrer P, Sola C, Schmidt RD, Valero F. Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. *J Biotechnol*. 2001;86:59–70.

27. Surribas A, Stahn R, Montesinos JL, Enfors SO, Valero F, Jahic M. Production of a *Rhizopus oryzae* lipase from *Pichia pastoris* using alternative operational strategies. *J Biotechnol*. 2007;130: 291–299.

28. Ghosalkar A, Sahai V, Srivastava A. Optimization of chemically defined medium for recombinant *Pichia pastoris* for biomass production. *Bioresour Technol*. 2008;99:7906–7910.

29. Cabrera Z, Gutarra M, Guisan JM, Palomo JM. Highly enantioselective biocatalysts by coating immobilized lipases with polyethyleneimine. *Catal Commun*. 2010;11:964–967.

30. Cregg J, Vedvick T, Raschke W. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology*. 1993; 11:905–910.

31. Arnau C, Ramon R, Casas C, Valero F. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. *Enzyme Microb Technol*. 2010;46:494–500.

32. D'Anjou MC, Daugulis AJ. A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. *Biotechnol Bioeng*. 2001;72:1–11.

33. Cos O, Resina D, Ferrer P, Montesinos JL, Valero F. Heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. *Biochem Eng J*. 2005; 26:86–94.

34. Hyka P, Züllig T, Ruth C, Looser V, Meier C, Klein J, Melzoch K, Meyer HP, Glieder A, Kovar K. Combined use of fluorescent dyes and flow cytometry to quantify the physiological state of *Pichia pastoris* during the production of heterologous proteins in high-cell-density fed-batch cultures. *Appl Environ Microbiol*. 2010;76:4486–4496.

35. Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C, Meagher M. Improved production of recombinant ovine Interferon- τ by Mut$^+$ strain of *Pichia pastoris* using an optimized methanol feed profile. *Biotechnol Prog*. 2003;115:794–802.

36. Potgieter TI, Kersey SD, Mallem MR, Nylen AC. Antibody expression kinetics in glycoengineered *Pichia Pastoris*. *Biotechnol Bioeng*. 2010;106:918–927.

37. Zhang W, Liu C-P, Inan M, Meagher M, Optimization of cell density and dilution rate in *Pichia pastoris* continuous fermentations for production of recombinant proteins. *J Ind Microbiol Biotechnol*. 2004;31:330–334.

38. Dietzsch C, Spadiut O, Herwig C. A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*. *Microb Cell Fact*. 2011;10:14.

39. Anasontzis GE, Salazar M, Spadiut O, Brumer H, Olsson L. Effects of temperature and glycerol and methanol-feeding profiles on the production of recombinant galactose oxidase in *Pichia pastoris*. *Biotechnol Prog*. 2014;30:728–735.

40. Viader-Salvadó JM, Fuentes-Garibay JA, Castillo-Galván M, Iracheta-Cárdenas MM, Galán-Wong LJ, Guerrero-Olazarán M. Shrimp (*Litopenaeus vannamei*) trypsinogen production in *Pichia pastoris* bioreactor cultures. *Biotechnol Prog*. 2013;29: 11–16.

41. Viader-Salvadó JM, Castillo-Galván M, Fuentes-Garibay JA, Iracheta-Cárdenas MM, Guerrero-Olazarán M. Optimization of five environmental factors to increase beta-propeller phytase production in *Pichia pastoris* and impact on the physiological response of the host. *Biotechnol Prog*. 2013;29: 1377–1385.

42. Hang H, Ye X, Guo M, Chu J, Zhuang Y, Zhang M, Zhang S. A simple fermentation strategy for high-level production of recombinant phytase by *Pichia pastoris* using glucose as the growth substrate. *Enzyme Microb Technol*. 2009;44:185–188.

43. Paulová L, Hyka P, Branská B, Melzoch K, Kovar K. Use of a mixture of glucose and methanol as substrates for the production of recombinant trypsinogen in continuous cultures with *Pichia pastoris* Mut$^+$. *J Biotechnol*. 2012;157:180–188.

44. Yang F, Hanna MA, Sun R. Value-added uses for crude glycerol-a byproduct of biodiesel production. *Biotechnol Biofuels*. 2012;5:13.