2007

Evaluation of Metals in a Defined Medium for *Pichia pastoris* Expressing Recombinant β-Galactosidase

Bradley A. Plantz
University of Nebraska - Lincoln, bplantz2@unl.edu

Kenneth W. Nickerson
University of Nebraska - Lincoln, knickerson1@unl.edu

Stephen D. Kachman
University of Nebraska - Lincoln, steve.kachman@unl.edu

Vicki L. Schlegel
University of Nebraska - Lincoln, vschlegel3@unl.edu

Published in *Biotechnology Progress* 23:3 (2007), pp. 687–692.
doi: 10.1021/bp060332t

Submitted November 2, 2006; accepted February 21, 2007; published online March 31, 2007.

Evaluation of Metals in a Defined Medium for *Pichia pastoris* Expressing Recombinant *β*-Galactosidase

Bradley A. Plantz, Kenneth Nickerson, Stephen D. Kachman, and Vicki L. Schlegel

School of Biological Sciences, Beadle Center, University of Nebraska, Lincoln, Nebraska 68583-0666, and Department of Statistics, 340 Hardin Center, University of Nebraska, Lincoln, Nebraska 68583-0963

Corresponding author — V. L. Schlegel, vschlegel3@unl.edu

Abstract

Culture growth and recombinant protein yield of the *Pichia pastoris* GS115 methanol utilization positive system were studied in response to the types and levels of metals present in the growth medium and the supplemental salts typically used for these fermentations. Magnesium and zinc were both required to support cell growth but at significantly reduced levels compared to the control. However, supplementation with calcium, cobalt, iron, manganese, iodine, boron, and molybdenum were not required to sustain cell mass. When the medium was reformulated with only zinc and magnesium, the cells grew to 12–15 generations, which are expected for high cell density fed-batch fermentations. Product yields of the recombinant protein *β*-galactosidase were significantly influenced by the trace metal concentrations. By using response surface and full factorial designs, maximum protein yield occurred when the concentration of zinc salt was limited to the level necessary only to support cell mass while protein yield positively correlated to increasing levels of the remaining trace metal salts. These studies are the first to show that excess trace metals must be optimized when developing *P. pastoris* based fed-batch fermentations.

Introduction

Maximal product yields and improved process stability have been achieved for the commercial *Pichia pastoris* production of recombinant proteins by applying unstructured models to the fermentation protocol

(*1–3*). Because recombinant protein production places an energy and carbon burden on the host organism by forcing energy and amino acid precursors away from cell mass synthesis, thus depleting these pools (*4–7*), unstructured models relating recombinant protein production to growth rate undoubtedly influence the flux of carbon (from methanol when using the mut$^+$ host system) through these two pathways. The unstructured model proposed by Zhang et al. (*2*) assumes a quasi-steady state between recombinant protein yield and cell growth. That is, the yield of recombinant protein will be constant provided that the specific growth rate remains constant and the growth environment is not overly affected by changes in the cell mass. Yet our studies showed that the *P. pastoris* production of two recombinant proteins, ovine interferon-τ (secreted) and β-galactosidase (cytosolic), were limited by time post-induction of the alcohol oxidase 1 (AOX1) promoter (*8*). We also noted that the culture growth medium, basal salts medium (BSM) versus FM22, and the *Pichia* trace metal (PTM) salt supplements, PTM1 versus PTM4, affected product yields (for formulations see ref 9). These observations were attributed to a limitation caused by metabolite pool depletion rather than by inhibiting metabolic intermediates accumulating in the supernatant.

It is well-known that metals such as magnesium, calcium, and some of the transition metals play an important, though not fully understood, role in cell metabolism primarily as structural components of metalloenzymes. Carbon flux demands of recombinant protein expression may be influenced by the level and composition of the metal supplements as shown by previous studies (*8*). Production was enhanced by the addition of citrate (*3*, *8*), which chelates divalent cations and affects their biological availability in the presence of phosphate. Brady et al. (*13*) also observed an increase in the yield of the malaria vaccine antigen P30P2MSP119 when the PTM salt level was reduced en masse for the purpose of minimizing metallophosphate precipitation. However, the basal medium and supplemental salts originally developed for the conversion of methanol to single cell protein (*10*) has remained basically unchanged (*9*, *11*, *12*). Other than a particular species of anion, e.g., potassium phosphate versus phosphoric acid, the main formulation differences have only occurred at the level of metal ions present.

The purpose of this study was to determine the growth metal requirements for *P. pastoris* with applications to high cell density fermentation processes designed to express recombinant proteins. Minimum metal requirements to sustain cell yield were first established for cultures grown on medium containing either glucose or methanol. The effects of the metals on product yield of the recombinant β-galactosidase protein were then determined through the application of response

surface and full factorial statistical designs. Several indirect benefits can be realized by such studies, including (a) lowered costs incurred by maintaining unnecessary metals; (b) reduced equipment repair and maintenance due to metal precipitates, such as the highly abrasive metal phosphates that can cause wear on a fermentor's moving parts; (c) prevention of potential downstream processing problems caused from interfering excess metals, as noted by Brady et al. (*13*); (d) reduction or elimination of metal effluent disposals, which can contain metals that negatively impact the environment, such as copper, zinc and molybdenum; and (e) deterrence of altered cell physiology due to excess metals that provide selective pressure for yeast morphology differentiation, which in turn can divert metabolic resources from growth and recombinant expression (*14*). These reasons collectively support eliminating or reducing non-essential metal ions in *P. pastoris* production of recombinant proteins.

Materials and Methods

Strains. *Pichia pastoris* GS115 his^- was purchased from Invitrogen (Carlsbad, CA). A methanol utilization positive strain expressing β-galactosidase was derived by transforming the plasmid pSAOH5 containing the lacZ expression cassette (*15*), which was a generous gift from James Cregg (Keck Graduate Institute, Claremont, CA). After screening for the expression of β-galactosidase and for stability of the lacZ cassette, a single colony was selected and designated as BP5.

Growth Medium and Culture Conditions. Bacteriological grade nutrients were used for routine cultivation. Inoculum cultures were grown on medium containing (per liter) 5 g yeast extract, 10 g peptone, and 20 g glycerol and adjusted to pH 5.5. Solutions, buffers, and media used for the testing of metal requirements were prepared with double deionized water (>10 MΩ) and American Chemical Society or higher grade chemicals. Preparation of the basal salts medium and supplemental salts were based, respectively, on the FM22 and PTM4 formulations described by Stratton et al. (*9*). The FM22 was modified to contain (per liter) 4.3 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 1.4 g K_2SO_4, 1.2 g $MgSO_4 \cdot 7H_2O$, 10 mM 2-[*N*-morpholino]- ethanesulfonic acid, 10 mM sodium citrate, and either glucose or methanol as the carbon source. As this was a significant departure from FM22, this medium was designated *Pichia* nitrogen base 1 (PNB1). The PTM4 stock solution contained (per liter) 1.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H2O$, 0.20 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.50 g $CaSO_4 \cdot 2H_2O$, 0.50 g $CoCl_2$, 7.0 g $ZnSO_4 \cdot 7H_2O$,

22 g $FeSO_4 \cdot 7H2O$, and 0.20 g biotin. As supplementation levels of PTM salts are arbitrary, a 1X concentration of the PTM4 was set at 2 mL L^{-1}. For the culture growth studies, PNB1 and PTM4 solutions were used as described except the metal under evaluation was diluted as indicated in Results. To minimize the risk of oxygen limitation, wide-mouth 250-mL Erlenmeyer flasks with 50 mL of medium were used.

Diluents and Reagents. A solution of 0.85% NaCl, 10 mM sodium citrate, and 10 mM 2-[*N*-morpholino]ethanesulfonic acid adjusted to pH 5.5 served as the diluent and buffer for washing the cells. Z-buffer was prepared for the Miller assay by combining (per liter) 8.5 g Na_2HPO_4, 5.5 g NaH_2PO_4, 0.75 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, and 2.7 mL β-mercaptoethanol. *o*-Nitrophenyl-β-D-galactopyranoside (ONGP) was added to the Z-buffer at a final concentration of 4 mg L^{-1}.

Analytical Methods. Culture densities were determined by measuring the optical density at 600 nm while β-galactosidase yields were monitored according to Miller (*16*). Briefly, triplicate samples of fresh or fresh frozen cell mass were diluted to 0.05 optical density units (ODU) L-1 in Z-buffer. Chloroform (20 μL) and 0.1% sodium dodecyl sulfate (10 ±L) were added to 1 mL of cell suspension and mixed briefly. The samples were equilibrated at 28 ± 1 °C for 5 min, and 0.2 mL of ONGP solution was added to each. The reaction was stopped with 500 μL of 1 M Na_2CO_3, and the incubation time was recorded. The absorbance at 420 nm was measured with a SpectraMax Plus (Molecular Devices, Sunnyvale, CA) spectrophotometer. The background activity of β-galactosidase was determined from *P. pastoris* cultures grown on glucose. The yield of β-galactosidase is reported as Miller Units normalized to culture density (MU ODU^{-1}).

Statistical Analysis. The effects of metal dilution and comparison of the treatment groups relative to positive controls were evaluated with analysis of variance (ANOVA) at the 5% significance level ($p < 0.05$). Orthogonal contrast, and in some instances an evaluation of the trend with respect to dilution factor, was then used to determine the dilution that resulted in a significant reduction in culture density. Statgraphics version 4.0 (Rockville, MD) was used to design the response surface and factorial experiments and to analyze all results. Response surface studies were completed with a central composite 2^3 + star that was rotatable with respect to the center point. Two additional center point replicates were added to increase the error degrees of freedom. The factorial experiments included two replications of each treatment combination.

Results

Metals: Influence on Cell Yield. Cell yield for each of the glucose- and methanol-grown cultures was measured as magnesium was diluted stepwise to exclusion in the PNB1 medium (Table 1). One-way ANOVA was then used to determine whether the dilution series and control flasks were significantly different (glucose, 30.5 ± 0.7 ODU; methanol 14.0 ± 1.4 ODU), whereas orthogonal contrast was used to determine the concentration of magnesium that first limited growth. The growth limiting concentration for the glucose- and the methanol-grown cultures was, respectively, 3,000 and 12,000 μg mL-1 (indicated by the asterisks in Table 1). Each subsequent dilution step resulted in further reduction in cell mass yield, thereby substantiating the observed growth-limiting concentrations ($p < 0.05$). This experimental design was completed to evaluate the PTM4 supplemental metals, manganese, calcium, iron, copper, and zinc. Cell mass on glucose and methanol were significantly effected by zinc and manganese and by zinc, iron, and copper, respectively, ($p < 0.05$), at a given dilution (Table 1). Similar to the magnesium studies, increasing zinc dilutions continued to reduce cell mass yield for both the glucose and methanol series, confirming the validity of the results. However, continued dilutions of manganese did not significantly reduce cell yield of the glucose-grown cultures. For the methanol series, a significant reduction in yield occurred only when both iron and copper were completely excluded. Because manganese, iron, and copper influenced cell mass yields for either the glucose or methanol-grown cultures but not for both, these results were also considered inconclusive and additional testing was required.

The micro-trace elements boron, cobalt, iodine, and molybdenum were tested by singular exclusion or by excluding the entire group. The growth results were then compared against the respective controls by using the *t*-test (Table 1). Exclusion of boron resulted in lower cell densities, but the validity of the test was questionable because of the significant difference between the SDs ($p < 0.05$) of the control and the test sample at the 95% confidence interval. Furthermore, if boron was growth-limiting, a significant reduction in growth should have resulted when the entire micro-trace elements were excluded as a group, which did not occur. Cell mass was not affected when the remaining trace elements cobalt, iodine, and molybdenum were excluded individually or as a group.

Extending the generations by multiple passages required for bench top fed-batch further depletes the metal reserves stored within the cell. An extended generation experiment was therefore completed to determine whether supplements of iron, manganese, copper, or the

remaining trace metals were necessary to support cell yield expected for a bench-top fermentation. The PNB1 test growth medium was reformulated to include only magnesium, zinc, and biotin in addition to the basal nutrients, while the control growth medium consisted of PNB1 and a PTM4 supplement. Two passages through a 50-mL shake flask at a starting OD_{600} of 0.1 (each passage) resulted in generations of 15 and 12 (Table 2) relative to the glycerol- and methanol grown cultures. Densities of the cultures grown on the simplified PBN1 medium containing either glucose or methanol (Table 2) were not significantly different from the controls ($p > 0.05$).

Metals: Influence on Recombinant *β*-Galactosidase Yield. Product levels of recombinant *β*-galactosidase were then analyzed in response to different treatment levels of zinc, magnesium, and the PTM salts via a response surface design. Because *β*-galactosidase on a per cell basis does not accumulate monotonically but instead reaches a maximum prior to stationary phase (*8*), the time point that maximum product yield occurs had to be established. The 18 shake flasks prepared for the response surface design were also used to complete the time course study. Product yield was measured immediately after the culture was transferred to PNB1 containing methanol ($T = 0$) and sampled periodically through to 170 h. Cell yield did not increase during the first 8 h as the culture was adapting to methanol (Figure 1). After the lag phase, the 18 cultures grew exponentially at a specific growth rate of 0.0820 h^{-1} and reached a maximum mean of 3.4 ± 0.8 ODU by 57 h. Cell densities continued to increase through to 170 h but at the reduced specific growth rate of 0.0068 h^{-1}, reaching a mean cell density of 8.6 ± 4.5 ODU. As expected, the product yield maxima did not correlate with the culture density maxima (*8*). A short plateau in *β*-galactosidase yield was reached at 33 h (525 ± 243 MU ODU^{-1}) to 47 h (512 ± 170 MU ODU^{-1}), while the culture was still growing at the faster specific growth rate. Although the two maximal product yields were not significantly different, the 33 h samples were selected for response surface analysis because the higher variability within these replicates suggested greater treatment effects.

The treatment level coded and corresponding natural values for the response surface analysis are listed in Table 3. The resulting model of the 33 h data was reduced by excluding statistically non-significant factors unless the quadratic term included the main effect (Table 4). Due to higher variability inherent to shake flask experiments, p_{crit} was set at $\alpha = 0.20$ to determine whether the treatments significantly impacted product yield. Product yield increased with decreasing zinc levels ($p = 0.0073$) (Figure 2a and c) but were unaffected by magnesium

(p = 0.7126) (Figure 2a and b). A quadratic relationship resulted between the PTM salt level and magnesium (p = 0.1242), as indicated by the saddle shaped curve in Figure 2b, with the maximal yield produced near the midpoint of the PTM treatment levels. A similar quadratic relationship occurred between the PTM salts and zinc (Figure 2c) but the relationship was not significant (p = 0.8260).

Two replications of a full factorial 2^3 design were completed to definitively determine an optimal PTM level and its interaction with magnesium. Zinc was supplemented at a concentration of 0.056 mg mL^{-1}, a level that was expected to limit growth, while magnesium and the PTM salts were increased stepwise from 8.1 (-1) to 234 mg L^{-1} (1) and from 0.02 (-1) to 2 μL L^{-1} (1), respectively, each at three levels. An additional time course study was completed to determine the time point at which product yield maximized. The cultures grew at a mean specific growth rate of 0.031 h^{-1}, resulting in a mean culture density of 19.3 ± 0.8 ODU that maximized at 72 h. No additional samples were collected after this time point. The low SD for the final time point cell yield data indicated that no flask was limited by the treatment levels. Product yield again reached a plateau but at slightly earlier times compared to the previous studies, i.e., from 24 h (1492 ± 174 MU ODU^{-1}) to 34 h (1507 ± 130 MU ODU^{-1}). The 24 h samples were selected for analysis because the SD within this group was higher than for the 34 h samples. The ANOVA model with non-significant treatments removed is listed in Table 5, and the results are shown graphically in Figure 3. There was no magnesium interaction or significant effect on product yield by magnesium, which confirms the response surface analysis results for this salt. The PTM salt concentration positively correlated with β-galactosidase yield (p = 0.032). The yield (J) equation for β-galactosidase can thus be simplified to

$$J = 1492 + 107(\text{PTM salts})$$

where PTM salts is the volume in microliters of the salts solution added per liter of FM22 basal medium.

Discussion

Magnesium and certain transition metals, e.g., iron, zinc, copper, manganese, etc., are essential metals that serve as structural components for proteins, as cofactors, and as enzyme active sites in cellular systems. Yet excess metals can inhibit culture growth and metabolic processes. While published and accepted growth medium formulations are

commonly used in *P. pastoris* high cell density fed-batch fermentation (*9*), the choice of basal medium (FM22 vs BSM) and PTM salts (PTM1 vs PTM4) and the level of PTM salts supplementation are arbitrary (*2*, *3*, *17—19*). The purpose of this research was thus to determine the types and levels of metals required to support cell mass for high cell density fed-batch fermentations and the concentration of metals that significantly influenced recombinant β-galactosidase yields.

The two most commonly used *P. pastoris* expression systems are the methanol utilization positive and pGAPZ strains that utilize, respectively, the AOX1 promoter (*20*) and the glyceraldehyde- 3-phosphate dehydrogenase promoter (*21*). For this reason medium metal requirements were evaluated with either glucose or methanol serving as the carbon source. Increased dilutions of only magnesium and zinc (Table 1) significantly limited cell growth on either carbon source. This observation was confirmed by the extended growth experiments (Table 2) in which FM22 with the complete complement of metals was compared to a modified formulation that excluded iron, manganese, copper, calcium, cobalt, boron, and molybdenum. The modified growth medium containing only magnesium and zinc was capable of sustaining the number of generations required for a bench-top *P. pastoris* fermentation (i.e., 8.2 generations) with an initial 2 L volume, a final 4 L volume, 120 mL of inoculum (OD_{600} = 40), and an expected final culture density of OD600 450. This study did not test for the absolute minimum metal levels and did not indicate that the remaining trace metals are not required. Rather, the data suggest that a sufficient quantity of the nutrients were provided from other sources. For instance, the sodium citrate certificate of analysis (J. T. Baker, lot V23627) states that the product contains 0.002% calcium and <3 ppm Fe.

Conversion of the shake flask data (Table 1) relative to a process fermentor (500 ODU) results in 645 mg L^{-1} magnesium heptahydrate and 127 μg L-1 zinc dehydrate requirements, which represents, respectively, a 18- and 23-fold reduction compared to FM22 and BSM formulations provided by Stratton et al. (*9*). The conversion of zinc chloride is complicated by the choice of PTM1 versus PTM4 and also by the level of supplementation. On the basis of the original 1X concentration of PTM4 used in our dilution studies, the concentration of zinc required for a process fermentation is 14 mg L-1. Compared to the actual growth requirement concentration, zinc is present in 110-fold excess. When the calculation is applied to the level of PTM1 added to the batch medium described by Zhang et al. (*2*), a 685-fold excess of zinc was used. A minimum requirement for the remaining trace metals was not determined, and therefore an estimate of fold excess for the reported processes cannot be calculated. Still, lower iron and calcium levels are

warranted because of the metal precipitates that form at the *P. pastoris* cultivation pH of 5.0—6.2.

Magnesium did not substantially impact the production of β-galactosidase on a per cell basis (Figures 2a and 3). As such, magnesium can be reduced to levels required to support cell mass only. Zinc can also be lowered to a concentration that supports cell mass only due to the negative correlation between β-galactosidase yield and zinc (Figure 2b). Supplementation with PTM salts was not needed to sustain cell growth, but PTM salts positively influenced β-galactosidase yield as shown in Figures 2b,c and 3. However, the supplementation level conversion from shake flask to high cell density fermentor is 2 to 52 μL L^{-1}, which is a 38-fold reduction from our baseline formulation.

Conclusions

This work was the first to evaluate the influence of metals on both cell growth and product yield for *P. pastoris* expression systems. When optimizing a fermentation process, the objective is not solely to maximize yield but also to decrease production costs, including indirect issues such as compliance to regulatory agency requirements, impacts to downstream processing steps, effects of the medium constituents to equipment maintenance, etc. Clearly, reduction or elimination of trace metals potentially impacts all of these intangibles. It can be concluded from this work that the level of metals as published by Stratton et al. (*9*) are in excess for the *P. pastoris* production of β-galactosidase, some of which over 2 orders of magnitude. Although it is expected that medium requirements will be dependent on the type of protein expressed, these studies show that excess trace metals must be considered when optimizing *P. pastoris* fedbatch fermentations.

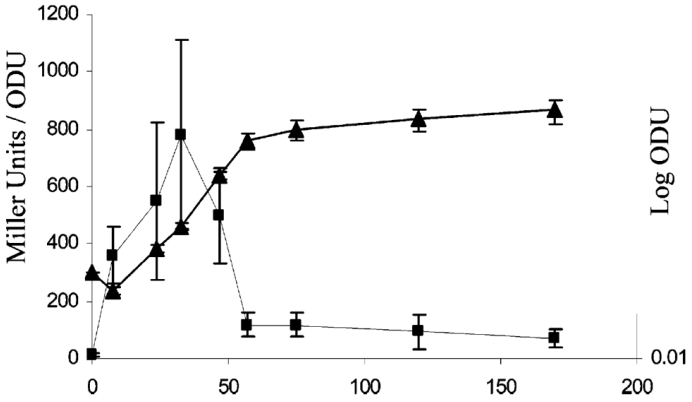
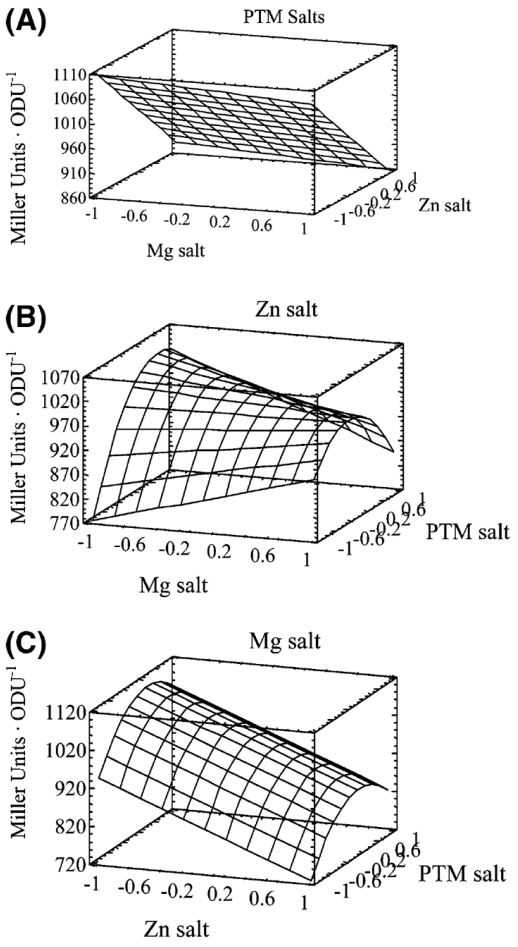


Figure 1. Culture cell mass and β-galactosidase yields versusproduction time. After the derepression of the AOX1 promoter by transitioning the culture from growth glycerol to methanol (time 0), samples were collected at the indicated time points and analyzed for culture density (□) or yield of â-galactosidase (□) as determined by the Miller assay. Error bars indicate 1 SD of the mean.

Figure 2. Yield of β-galactosidase related to levels of cation groups. Response surfaces when (A) magnesium and zinc salts, (B) PTM and magnesium salts, and (C) PTM and zinc salts treatment levels are varied.



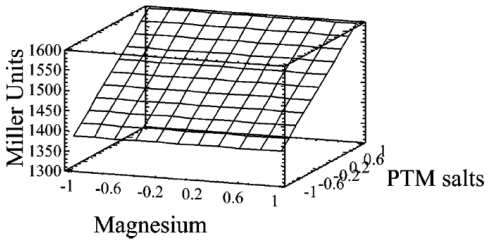


Figure 3. Yield of β-galactosidase from 2^3 full factorial experimental design when non-significant treatments and interactions are removed from the analysis.

Table 1. Cell Yield of Culture Grown on Medium Containing Limited Trace Metals

		glucose		methanol	
metal	μg L^{-1} metal	ODU[a]	p value[b]	ODU[a]	p value[b]
Mg^{+}	0	3.4 ± 0.1	0.0001	0.2 ± 0.0	0.0003
	3,000	28.1 ± 0.1[c]		4.9 ± 0.1	
	12,000	32.0 ± 0.0		9.3 ± 0.4[c]	
	80,000	31.5 ± 0.7		12.3 ± 0.2	
Zn^{2+}	0	3.0 ± 0.2	0.0001	2.2 ± 0.0	0.0002
	1.4	4.1 ± 0.0		5.5 ± 0.1[c]	
	14	22.5 ± 0.7[c]		12.5 ± 2.1	
Mn^{2+}	0	32.0 ± 0.	0 0.0024	10.5 ± 0.7	0.1305
	0.06	32.5 ± 0.7		11.5 ± 0.7	
	0.6	31.5 ± 0.7[c]		12.0 ± 1.4	
Ca^{2+}	0	34.5 ± 0.7	0.3290	10.9 ± 4.4	0.7100
	0.01	35.0 ± 1.4		13.0 ± 2.8	
	0.1	34.5 ± 0.7		11.0 ± 2.8	
$Fe^{2+(3+)}$	0	36.5 ± 0.7	0.1510	10.5 ± 0.7[c]	0.0036
	0.044	35.5 ± 0.7		12.0 ± 1.4	
	4.4	37.0 ± 0.0		13.5 ± 0.7	
Cu^{2+}	0	34.5 ± 0.7	0.3200	6.0 ± 0.0[c]	0.0024
	0.004	35.0 ± 1.4		11.5 ± 0.7	
	0.4	34.5 ± 0.7		11.1 ± 0.1	
Co^{2+}	0	35.0 ± 0.7	0.2730	14.0 ± 0.0	0.9960
I	0	35.8 ± 0.4	0.5980	13.5 ± 0.7	0.7220
B	0	34.0 ± 0.0	0.0001	13.0 ± 1.4	0.6200
Mo	0	35.0 ± 0.7	0.2730	13.5 ± 0.7	0.7220
Co^{2+}, I, B, Mo	excluded as a group	36.0 ± 0.7	0.4230	13.0 ± 0.3	

a. Results presented as mean ± SD; n = 3.
b. ANOVA comparing within-group differences between treatment and control cultures with a p_{crit} = 0.05. Cell yield of control cultures: magnesium with glucose as the carbon source, 30.5 ± 0.7 ODU; all other metals and glucose, 36 ± 0.1 ODU; Methanol as carbon source, 14 ± 1.4 ODU.
c. First dilution that was significantly reduced from the control flasks.

Table 2. Cell Yields of Cultures Grown on the Reformulated PBN1 Medium[a]

	glucose		methanol	
	metals excluded[b]	positive control[c]	metals excluded[b]	positive control[c]
passage 1[d] (ODU)	13.3 ± 1.5	14.3 ± 0.6	6.2 ± 0.0	6.3 ± 0.3
passage 2[d] (ODU)	20.0 ± 1.0	20.3 ± 1.2	5.9 ± 0.2	5.7 ± 0.4
total generations	14.7 ± 0.2	14.8 ± 0.1	12.0 ± 0.1	12.0 ± 0.2

a. Results presented as mean ± range; n ± 3.
b. Formulation described within the text, metals excluded: iron, manganese, copper, calcium, cobalt, boron, molybdenum.
c. PNB1 and PTM4 salts.
d. Passage 1 was inoculated with cells grown on YPD, washed twice to remove residual nutrients and then adjusted to a starting OD_{600} of 0.1. Passage 2 was inoculated from a volume of cells taken directly from passage 1 so that again, the starting OD_{600} was 0.1.

Table 3. Coded and Natural Values for Evaluation of Metal Treatment by Response Surface Analysis

	natural values		
coded value	$ZnSO_4 \cdot H_2O$ (μg L^{-1})	$MgSO_4 \cdot 7H_2O$ (mg L^{-1})	PTM salts (μL L^{-1})
–1.68	1.4	1.2	0.02
–1.0	30	25	0.42
0.0	71	59	1.0
1.0	110	94	1.6
1.68	140	120	2.0

Table 4. ANOVA Table for β-Galactosidase Yield at 33 h; Response Surface Design[a]

source	sum of squares	degrees of freedom	mean square	F ratio	p value
A: Mg	2.221×10^3	1	2.221×10^3	0.14	0.7126
B: Zn	1.620×10^5	1	1.620×10^5	10.38	0.0073
C: PTM salts	3.496×10^4	1	3.496×10^4	2.24	0.1603
AC	4.263×10^4	1	4.263×10^4	2.73	0.1242
CC	1.316×10^4	1	1.316×10^4	8.44	0.0132
total error	1.872×10^5	12	1.560×10^4		
total correlation	5.606×10^5	17			

a. Excluded variables: Mg x Mg (p = 0.6302); Mg x Zn (p= 0.6687); Zn x Zn (p = 0.3026); Zn x PTM (p = 0.8260).

Table 5. ANOVA Table for β-Galactosidase Yield at 24 h; 2^3 Design[a]

source	sum of squares	degrees of freedom	mean square	*F* ratio	*p* value
PTM salts	1.381×10^5	1	1.381×10^5	5.65	0.0329
PTM salts x magnesium	3.125×10^2	1	3.125×10^2	0.01	0.9120
PTM salts x PTM salts	2.8477×10^4	1	2.8477×10^4	1.15	0.3070
total correlation	5.122×10^5	17			

a. Excluded variables: Mg salts (p = 0.2956), Mg x Mg (p = 0.3172).

References and Notes

(1) Kobayashi, K.; Kuwae, S.; Ohya, T.; Ohda, T.; Ohyama, M.; Tomomitsu, K. High level secretion of recombinant human serum albumin by fed—batch fermentation of the methylotrophic yeast, *Pichia pastoris*, based on optimal methanol feeding strategy. *J. Biosci. Bioeng.* 2000, *90*, 280—288.

(2) Zhang, W.; Bevins, M.; Plantz, B.; Smith, L.; Meagher, M. Modeling *Pichia pastoris g*rowth 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.

(3) Sinha, J.; Plantz, B.; Zhang, W.; Gouthro, M.; Schlegel, V.; Liu, C—P; Meagher, M. Improved production of recombinant ovine interferon—τ by mut$^+$ strain of *Pichia pastoris* using an optimized methanol feed profile. *Biotechnol. Prog.* 2003, *19*, 794—802.

(4) Diaz—Ricci, J. C.; Tsu, M.; Bailey, J. E. Influence of expression of the pet operaon on intracellular metabolic fluxes of *Escherichia coli*. *Biotechnol. Bioeng.* 1992, *39*, 59—65.

(5) Gonzalez, R.; Andrews, B. A.; Molitor, J.; Asenjo, J. A. Metabolic analysis of the synthesis of high levels of intracellular human SOD in *Saccharomyces cere* V*isiae* rh-SOD 2060 411 SGA122. *Biotechnol. Bioeng.* 2003, *82*, 152—169.

(6) Bentley, W. E.; Mirjalili, N.; Andersen, D. C.; Davis, R. H.; Kompala, D. S. Plasmid encoded protein: the principal factor in the metabolic burden associated with recombinant bacteria. *Biotechnol. Bioeng.* 1990, *35*, 668—681.

(7) Martinez, A.; York, S. W.; Yomano, L. P.; Pineda, V. L.; Davis, F. C.; Shelton, J. C.; Ingram, L. O. 1999. Biosynthetic burden and plasmid burden limit expression of chromosomally integrated heterologous genes (*pdc, adhB*) in *Escherichia coli*. *Biotechnol. Prog.* 1999, *15*, 891—897.

(8) Plantz, B. A.; Sinha, J.; Villarete, L.; Nickerson, K.; Schlegel, V. L. *Pichia pastoris* fermentation process optimization: Energy state and testing a growth associated model. *Appl. Microbiol. Biotechnol.* 2006, *72*, 297—305.

(9) Stratton, J.; Chiruvolu, V.; Meagher, M. High cell—density fermentation. In *Methods in Molecular Biology: Pichia Protocols*; Higgins, D. R., Cregg, J. M., Eds.; Humana Press: Totowa, NJ, 1998; pp 107—120.

(10) Hitzman, D. O.; Wegner, E. H. Methanol foam fermentation to single cell protein by microorganisms. U.S. Patent 3,982,998, 1976.

(11) Cregg, J. M.; Tschopp, J. F.; Stillman, C.; Siegel, R.; Akong. M.; Craig, W. S.; Buckholz, R. G.; Madden, K. R.; Kellaris, P. A.; Davis, G. R.; Smiley, B. L.; Cruze, J.; Torregrossa, R.; Velicelebi, G.; Thill, G. P. High—level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Bio/Technology* 1987, *5*, 479—485.
(12) Clare, J.; Rayment, F. B.; Ballantine, S. P.; Sreekrishna, K.; Romano, M. A. High—level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Bio/Technology* 1991, *9*, 455—460.
(13) Brady, C. P.; Shipm, R. L.; Miles, A. P.; Whitmore, M.; Stowers, A. High—level production and purification of P20P2MSP119, an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expression Purif.* 2001, *23*, 468—475.
(14) Harrison, J. J.; Rabiei, M.; Turner, R. J.; Badry, E. A.; Sproule, D. M.; Ceri, H. Metal resistance in *Candida* biofilms. *FEMS Microbiol. Ecol.* 2006, *55*, 479—491.
(15) Tschopp, J. F.; Brust, P. F.; Cregg, J. M.; Stillman, C. A.; Gingeras, T. R. Expression of the lacZ gene from two methanolregulated promoters in *Pichia pastoris*. *Nucleic Acid Res.* 1987, *15*, 3859—3876.
(16) Miller, J. In *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*; Cold Spring Harbor Laboratory Press: Woodbury, NY, 1992; pp 72—74.
(17) Werten, M.; Van Den Bosch, T.; Wind, R. D.; Mooibroek, H.; De Wolf, F. (1999) High—yield secretion of recombinant gelatins by *Pichia pastoris*. *Yeast* 1994, *15*, 1087—1096.
(18) Hong, F.; Meinander, N. Q.; Jönsson, L. J. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnol. Bioeng.* 2002, *79*, 438—449.
(19) Kobayashi, K.; Kuwae, S.; Ohya, T.; Ohda, T.; Ohyama, M.; Ohi, H.; Tomomitsu, K.; Ohmura, T. High—level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. *J. Biosci. Bioeng*. 2000, *89*, 55—61.
(20) Higgins, D. R.; Cregg, J. M. Introduction to *Pichia pastoris*. In *Methods in Molecular Biology: Pichia Protocols*; Higgins, D. R., Cregg, J. M., Eds.; Humana Press: Totowa, NJ, 1998; pp 1— 15.
(21) Waterham, H. R.; Digan, M. E.; Koutz, P. J.; Lair, S. V.; Cregg, J. M. Isolation of the *Pichia pastoris* glyceraldehyde—3—phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* 1997, *186*, 37—44.