MICROBIAL CELL FACTORIES

RESEARCH

Open Access

Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*

Roland Prielhofer[1], Michael Maurer[2,3], Joachim Klein[4], Jana Wenger[4], Christoph Kiziak[4], Brigitte Gasser[1,2*] and Diethard Mattanovich[1,2]

Abstract

Background: Inducible high-level expression is favoured for recombinant protein production in *Pichia pastoris*. Therefore, novel regulated promoters are desired, ideally repressing heterologous gene expression during initial growth and enabling it in the production phase. In a typical large scale fed-batch culture repression is desired during the batch phase where cells grow on a surplus of e.g. glycerol, while heterologous gene expression should be active in the feed phase under carbon (e.g. glucose) limitation.

Results: DNA microarray analysis of *P. pastoris* wild type cells growing in glycerol-based batch and glucose-based fed batch was used for the identification of genes with both, strong repression on glycerol and high-level expression in the feed phase. Six novel glucose-limit inducible promoters were successfully applied to express the intracellular reporter eGFP. The highest expression levels together with strong repression in pre-culture were achieved with the novel promoters P_{G1} and P_{G6}.

Human serum albumin (HSA) was used to characterize the promoters with an industrially relevant secreted protein. A P_{G1} clone with two gene copies reached about 230% of the biomass specific HSA titer in glucose-based fed batch fermentation compared to a P_{GAP} clone with identical gene copy number, while P_{G6} only achieved 39%. Two clones each carrying eleven gene copies, expressing HSA under control of P_{G1} and P_{G6} respectively were generated by post-transformational vector amplification. They produced about 1.0 and 0.7 g L^{-1} HSA respectively in equal fed batch processes. The suitability in production processes was also verified with HyHEL antibody Fab fragment for P_{G1} and with porcine carboxypeptidase B for P_{G6}. Moreover, the molecular function of the gene under the control of P_{G1} was determined to encode a high-affinity glucose transporter and named *GTH1*.

Conclusions: A set of novel regulated promoters, enabling induction without methanol, was successfully identified by using DNA microarrays and shown to be suitable for high level expression of recombinant proteins in glucose-based protein production processes.

Keywords: *Pichia pastoris*, Heterologous protein production, Glucose-limited fed batch cultivation, Inducible promoter, High-affinity glucose transporter

* Correspondence: brigitte.gasser@boku.ac.at
[1]University of Natural Resources and Life Sciences, Department of Biotechnology, Muthgasse 18, Vienna 1190, Austria
[2]Austrian Centre of Industrial Biotechnology (ACIB GmbH), Muthgasse 11, Vienna 1190, Austria
Full list of author information is available at the end of the article

Background

The methylotrophic yeast *Pichia pastoris* is widely used as a production platform for heterologous proteins. Latest developments in strain engineering for improved protein folding and secretion and glyco-engineering have recently been reviewed by Damasceno et al. [1].

Another important target for strain development is the promoter driving expression of the heterologous gene. A summary of the most important promoters of non-methylotrophic and methylotrophic yeasts is provided by Mattanovich et al. [2]. While production of recombinant proteins in *P. pastoris* has been successfully achieved under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}), regulated promoters have several advantages: they enable initial biomass gain without product formation and allow tuning of the production process. Additionally, a potential impact of product accumulation on growth or viability of the cells can be prevented by decoupling growth from the production phase.

However, today's available regulated promoters of *P. pastoris* have drawbacks. Many of them derive from methanol utilization pathway genes, which are generally repressed by glucose and/or ethanol and strongly induced by methanol. P_{AOX1} induces high-level expression of its encoded alcohol oxidase 1, which catalyzes the oxidation of methanol to formaldehyde [3]. Its weaker homolog P_{AOX2} has been used for protein production as well [4]. Another strong promoter of this pathway is P_{FLD1}, its gene formaldehyde dehydrogenase is either induced by methylamine or methanol [5]. The promoter of dihydroxyacetone synthase, P_{DAS}, was reported to be similarly regulated and even stronger than P_{AOX1} [3], however it is not commonly used for protein production. Methanol and methylamine are both highly flammable and hazardous to health, so safety precautions are required for their industrial use. In addition to that, methanol consumption is technically disadvantageous because it causes high heat evolution and an increased oxygen demand during the fed batch phase [6].

The P_{ICL1} promoter controls the expression of isocitrate lyase and is regulated by the carbon source used for cell growth. No detectable promoter activity is present when cells are growing on glucose, while it gets turned on when cells are stationary or growing on ethanol [7]. Hence, this promoter might be an alternative for some applications, but its regulatory properties are poor. *PHO89* is a regulated sodium phosphate symporter and its promoter was investigated and shown to produce reasonable amounts of protein [8]. Cells must be phosphate-limited for the full activation of P_{PHO89}, and an increase in product titer was even shown in phosphate-limited stationary phase. Additionally, an impact on growth was reported and reduced cellular fitness can be assumed in these conditions.

On the other hand, constitutive promoters might be advantageous for the over-expression of genes or to co-express helper factors and marker genes. The widely-used P_{GAP} controls the expression of glyceraldehyde-3-phosphate dehydrogenase at a high basal level [9]. Its productivity can be influenced by controlling the growth rate at the optimal activity of P_{GAP} [10], and by a decrease of available O_2 levels [11]. The promoter of the translational elongation factor EF-1 alpha gene, P_{TEF1}, was analyzed and showed a tighter growth-associated regulation than P_{GAP} [12].

A promoter library of P_{AOX1} was generated, leading to a few variants that were slightly stronger than wild type P_{AOX1}, and a number of variants with altered regulatory properties, some of them being active without methanol [13]. Another library approach was done for P_{GAP} by mutation and clones expressing yeast-enhanced green fluorescent protein (yEGFP) under the control of obtained variants produced 8 to 218% of fluorescence intensity compared to the wild type promoter [14]. Potential promoter libraries can also be deduced from microarray data and rational considerations. Focussing on highly transcribed genes in general, 15 promoters were selected for characterization and the promoter of the thiamine biosynthesis gene P_{THI11}, which is regulated by the availability of thiamine in the growth medium, was discovered [15].

As described above, the number of strong promoters with advantageous properties for protein production is limited in *P. pastoris*. This work was designated to identify novel promoters with both, high expression and an optimal regulation in production process conditions. Equally important, the addition of inducers was to be avoided, because their use is often associated with extra costs and safety precautions in large scale fermentation processes.

A typical production process under the control of P_{GAP} uses glycerol in the batch phase, and a constant glucose fed batch for 100 hours to reach more than 100 g L^{-1} cell dry weight [16]. In order to identify potential inducible promoters in the course of this process, we used DNA microarray analysis to compare gene expression patterns of glycerol-excess (=batch growth phase) and glucose-limited (=fed batch production phase) conditions. The expression capacity of selected promoter targets was characterized with model proteins and verified in fed batch processes.

Results and discussion

Identification of novel promoters with desired induction properties

A typical *P. pastoris* protein production process avoiding methanol induction starts with a glycerol batch (surplus of carbon source) which is followed by a glucose fed

batch (limit of carbon source) [10]. DNA microarrays were used to analyze gene expression patterns and to identify potential promoters for this cultivation strategy. In order to eliminate growth rate related effects, glucose-limited conditions were analyzed in chemostat cultivation where the growth rate, similar to that in the batch phase, was fixed by controlling the dilution rate at 0.1 h^{-1}.

The microarray data was mined for genes with both, high difference in expression level between repressed and induced state (fold change) as well as high signal intensity in the induced state to identify potent promoters for inducible high-level protein production in *P. pastoris*. Six potential promoters (abbreviated as P_{G1}, P_{G3}, P_{G4}, P_{G6}, P_{G7} and P_{G8}, see Figure 1 and Table 1) were considered for further characterization.

Verification of promoter strength and regulation

At first, the strength and regulation of the novel promoters were assayed with the intracellular reporter protein eGFP in small scale screening cultures. Both, repressive conditions in pre-culture (glycerol excess) and induced ones during main culture (glucose limit) were analyzed during the screening. In order to simulate fed batch like conditions in screenings, we had to adapt the screening strategy. Instead of usual feedings with certain amounts of glucose which lead to repeated batch phases, we used slow glucose releasing polymer particles (12mm feed beads, Kuhner, CH), liberating glucose at a non-linear rate of $1.63 \cdot t^{0.74}$ mg per disc (t = time [h]), which equals to 28.6 mg per disc after 48 hours.

As shown in Figure 2, P_{G1} and P_{G6} had superior properties in terms of both, regulation and induction strength. In order to visualize gene dosage effects, genomic DNA of several clones was isolated and analyzed by real-time PCR to determine the gene copy number (GCN) of eGFP. Compared to a P_{GAP} clone with one gene copy, the specific fluorescence of P_{G1} and P_{G6}

Figure 1 Microarray data (red channel) of identified target genes in comparison to GAP. Bars represent relative expression levels in glycerol excess (batch phase, blue bars on the left side) and in glucose limit (chemostat cultivation, red bars on the right side). Numbers in the right column represent the fold change of signal intensity between glucose limit and glycerol excess conditions.

Table 1 Identified promoter candidates

Promoter	Gene	*P. pastoris* gene identifier (strain GS115)
P_{GAP}	GAP	PAS_chr2-1_0437
P_{G1}	G1	PAS_chr1-3_0011
P_{G3}	G3	PAS_chr4_0550
P_{G4}	G4	PAS_chr4_0043
P_{G6}	G6	PAS_chr2-1_0853
P_{G7}	G7	PAS_chr1-4_0570
P_{G8}	G8	PAS_chr1-3_0165

controlled expression of eGFP (normalized to GCN) were induced from almost zero in batch phase to about 150% and 100% after 48 h screening culture, respectively. The other promoters P_{G3}, P_{G4}, P_{G7}, and P_{G8} still showed a good regulation and induction strength suitable for inducible protein expression, with expression strengths spanning a spectrum of about 20% to 120% relative to P_{GAP} (Figure 2A). The next step was to investigate the induction behaviour of the novel promoters in more detail.
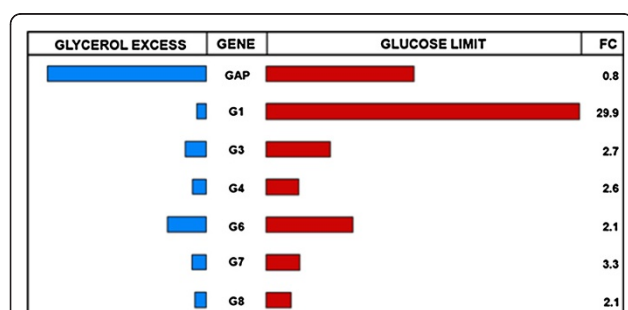
Analysis of the glucose dependent regulation

The induction behaviour of the novel promoters was characterized in screenings with eGFP producing clones in YP media containing different amounts of glucose (ranging from 20 to 0.002 g L^{-1}). The cells were cultivated for 5–6 hours and eGFP expression was analyzed by flow cytometry.
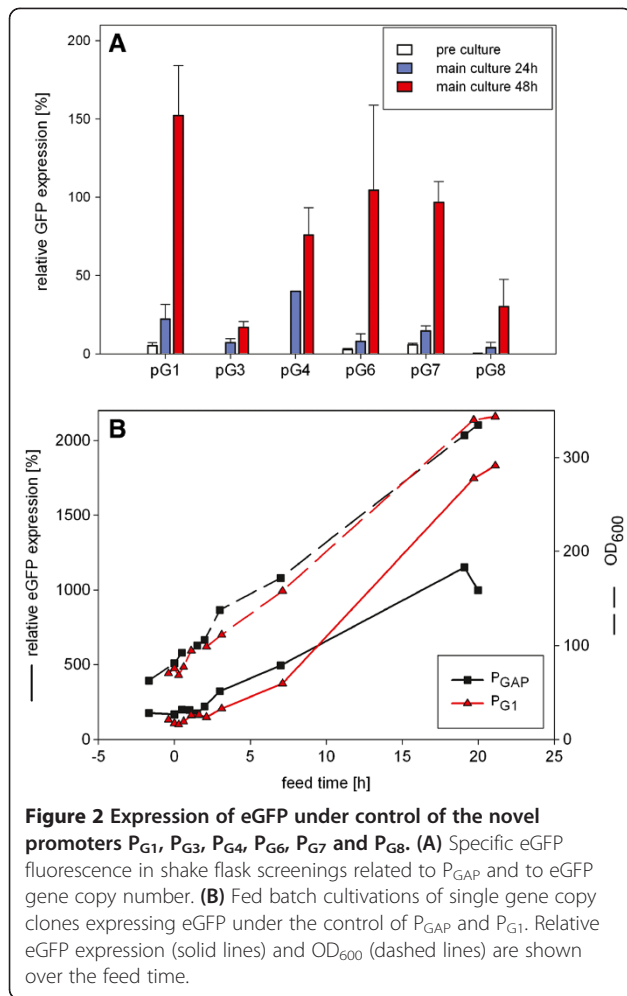
Promoters P_{G1} and P_{G7} showed a flat induction course leading to full activity only with less than 0.05 g L^{-1} glucose. That is clearly different to P_{G3}, P_{G4} and P_{G6}'s steeper regulation pattern which reach their top activity already at around 4 g L^{-1} glucose (Figure 3). In other words, P_{G1} is not only the strongest but also most tightly repressed by glucose among the promoters tested here.

Based on these regulatory features we intended to characterize the functions of the genes under control of the P_G promoters. At the time of their identification, no or only putative functions were assigned to the underlying genes aside from G1. Therefore, we used NCBI Conserved Domain search to analyze the protein sequences in order to identify putative gene functions.

The gene under the control of P_{G1} was previously functionally clustered with *K. lactis* high-affinity glucose transporter *HGT1* [17]. It contains two major facilitator superfamily domains, same as the G7 gene, which is therefore assumed to be a glucose transporter too. For *Saccharomyces cerevisiae*, it was reported that hexose transporters underlie complex regulation patterns and are expressed in dependence of glucose concentration [18]. The regulation pattern exhibited by the promoters

Figure 2 Expression of eGFP under control of the novel promoters P_{G1}, P_{G3}, P_{G4}, P_{G6}, P_{G7} and P_{G8}. (A) Specific eGFP fluorescence in shake flask screenings related to P_{GAP} and to eGFP gene copy number. **(B)** Fed batch cultivations of single gene copy clones expressing eGFP under the control of P_{GAP} and P_{G1}. Relative eGFP expression (solid lines) and OD_{600} (dashed lines) are shown over the feed time.

P_{G3}, P_{G4}, P_{G6} and P_{G8} might be associated with a role in central metabolism. An AKR (aldo keto reductase) domain was found in the gene expressed under P_{G3}. The genes controlled by P_{G4} and P_{G6} are both putative aldehyde dehydrogenases, predicted to be localized in the cytosol and in the mitochondria, respectively. The Gti1/Pac2 family domain found in G8 plays a role in gluconate uptake upon glucose starvation and in sexual development in *Schizosaccharomyces pombe*.

We could show the need of an explicit glucose limit for full activity of the novel promoters, which is most pronounced for P_{G1}. This demonstrates that the microarray data-based promoter selection is excellently suited to select for promoters with features relevant for bioprocesses, and secondly indicates the novel promoter´s advantages in fed batch fermentation.
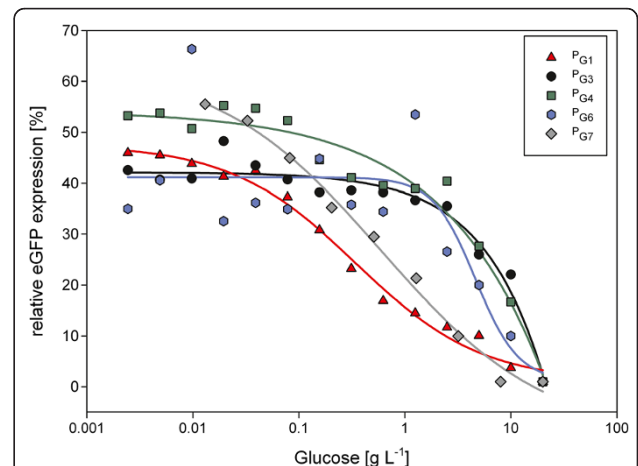
To prove this statement, the application of the strongest and most promising promoter P_{G1} was tested in a fed batch fermentation where truly glucose-limited conditions are present [10]. A single gene copy clone expressing eGFP was chosen for comparison to an equivalent single gene copy clone of eGFP under the control of P_{GAP}.
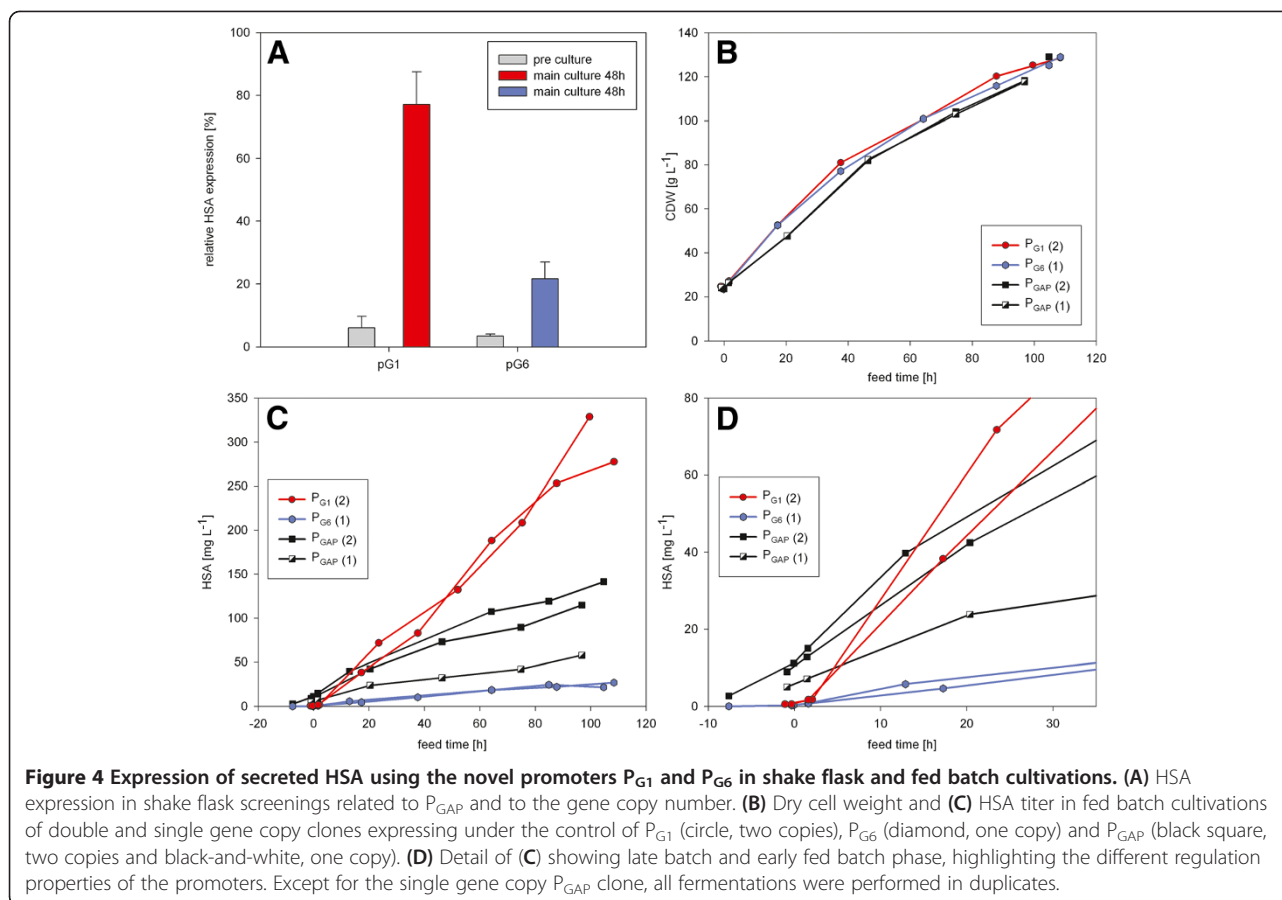
Thereby we could show that the P_{G1} promoter remains repressed during the batch phase and that its induction during fed batch clearly exceeds the strength of P_{GAP}. Relative eGFP expression (fluorescence related to the culture volume) and OD_{600} over the feed time are shown in Figure 2B.

Expression of secreted human serum albumin

P_{G1} and P_{G6} were further selected to assay the production of a secreted protein under their control. Human serum albumin (HSA) is efficiently produced in *P. pastoris*. Therefore it can be regarded as an industrially relevant secreted reporter protein. Its expression under the control of P_{G1}, P_{G6} and P_{GAP} was screened in shake flasks with glucose-limited conditions (through the use of feed beads) in the main culture. During the glycerol (batch) pre-culture, both P_{G1} and P_{G6} promoters remained well repressed. As seen before, during main culture expression under P_{G1} was stronger than under P_{G6} and, in relation to gene copy number, reached around 77% of the biomass specific HSA yield of cells expressing under P_{GAP}, while P_{G6} produced about 22% of P_{GAP} (Figure 4A). However, the novel promoters might not show their full potential in shake flask screenings, since these conditions are not strictly glucose limited during the entire production phase.

To exploit the full potential of the novel promoters, glucose limited fed batch cultivations were performed. Based

Figure 3 Induction behaviour of the novel promoters. Specific eGFP fluorescence of clones expressing eGFP under the control of P_{G1}, P_{G3}, P_{G4}, P_{G6} and P_{G7} in media containing different amounts of glucose. Data is related to P_{GAP}, normalized to 1.0 at the highest glucose concentration of 20 g L^{-1} and plotted against the logarithmic glucose concentration (trend line calculation: four parameter logistic curve). The glucose concentration given on the x-axis refers to the glucose set point at the beginning of the cultivation (serial dilutions ranging from 20 to 0.002 g L^{-1}). This screening setup and data processing points out relative promoter activities, thereby showing the kinetics of induction, but does not allow comparison of promoter strength.

Figure 4 Expression of secreted HSA using the novel promoters P_{G1} and P_{G6} in shake flask and fed batch cultivations. (A) HSA expression in shake flask screenings related to P_{GAP} and to the gene copy number. **(B)** Dry cell weight and **(C)** HSA titer in fed batch cultivations of double and single gene copy clones expressing under the control of P_{G1} (circle, two copies), P_{G6} (diamond, one copy) and P_{GAP} (black square, two copies and black-and-white, one copy). **(D)** Detail of **(C)** showing late batch and early fed batch phase, highlighting the different regulation properties of the promoters. Except for the single gene copy P_{GAP} clone, all fermentations were performed in duplicates.

on the screening results, one clone each expressing sufficient amounts of HSA under the control of P_{G1} and P_{G6} was selected. Those clones, harbouring two and one gene copies respectively, were compared with their respective gene copy equivalent P_{GAP} clones (Figure 4B, 4C and 4D). Dry cell weight (DCW) and HSA titers are summarized in Table 2. HSA titers of P_{GAP} clones correlate with their respective gene copy number. Again, the P_{G1} clone showed superior properties - it clearly outperformed the P_{GAP} clone with the same gene copy number and produced about 230% of the biomass specific product yield compared to P_{GAP}. P_{G6} produced about 39% of the biomass specific HSA yield of its gene copy equivalent P_{GAP} clone. Besides,

PGAP-driven expression was already active in the batch phase, and more than 5% of the final HSA amount was already present at the batch end for both clones expressing under its control. While this is not an issue in case of HSA, it is a clear disadvantage compared to inducible promoters such as P_{G1} in case of toxic or difficult to express products. Figure 4C shows HSA titer over the feed time, and the unique repression/induction efficiency of P_{G1} is clearly pointed out in the first hours (Figure 4D). Both, P_{G1} and P_{G6} showed good repression in the batch phase and induction by the glucose limited feed.

To verify that the novel promoters also exhibit their superior regulatory properties and expression capacity

Table 2 Summary of fed batch cultivations of *P. pastoris* expressing HSA under the control of P_{G1}, P_{G6} and P_{GAP}

Promoter	GCN	Batch end			Fed batch end			
		DCW	HSA	HSA/DCW	DCW	HSA	HSA/DCW	% HSA/DCW
		[g L^{-1}]	[mg L^{-1}]	[mg g^{-1}]	[g L^{-1}]	[mg L^{-1}]	[mg g^{-1}]	of P_{GAP}
P_{G1}*	2	24.3	0.5	0.0	126.9	303.1	2.4	231.2
P_{G6}*	1	23.9	0.3	0.0	127.1	24.3	0.2	38.9
P_{GAP}*	2	23.8	10.1	0.4	123.7	128.2	1.0	
P_{GAP}	1	24.2	5.0	0.2	117.7	57.8	0.5	

* performed in duplicates.

Table 3 Summary of fed batch cultivations of GCN amplified HSA expressing clones under the control of P$_{G1}$ and P$_{G6}$

Promoter	GCN	Batch end			Fed batch end		
		DCW	HSA	HSA/DCW	DCW	HSA	HSA/DCW
		[g L^{-1}]	[mg L^{-1}]	[mg g^{-1}]	[g L^{-1}]	[mg L^{-1}]	[mg g^{-1}]
P$_{G1}$	11	18.9	0.2	0.0	114.0	1060.8	9.3
P$_{G6*}$	11	22.5	0.3	0.0	110.8	728.7	6.6

* performed in duplicates.

in industrially relevant conditions, we elevated HSA gene copy number by post-transformational vector amplification as described previously [19]. Thereby, we were able to produce more than 1 g L^{-1} HSA under the control of P$_{G1}$ with a clone harbouring 11 gene copies, which corresponds to the 3.4-fold titer of its two copy clone (Table 3). Again, P$_{G1}$ outperformed a comparable clone with the same gene copy number under the control of the P$_{GAP}$ promoter, which produced 607 mg L^{-1} HSA in a similar fermentation [19]. High level HSA production was also achieved with an amplified clone expressing HSA (11 gene copies as well) under the control of the weaker P$_{G6}$ promoter, which produced more than 720 mg L^{-1} HSA (Table 3). This titer is approximately 30-fold higher than the titer reached with the P$_{G6}$ single copy clone (24 mg L^{-1}), thus indicating that multiple copies of expression cassettes under control of a weaker promoter can also lead to high productivities. One possible explanation of this effect could be that the ratio of a repressing protein to promoter copy number and thus repressor binding sites is decreased in the amplified clones, therefore leading to higher transcription.

Additionally, the porcine enzyme carboxypeptidase B (CpB) that is used for human insulin production, and an antibody Fab fragment were produced under the control of P$_{G1}$ and P$_{G6}$ respectively, and exceeded the production levels compared to P$_{GAP}$. Thereby we verified again the suitability of these promoters in standard glucose based production processes.

P$_{G1}$ activity depends on specific growth rate

We elucidated the expression activity of P$_{G1}$ at different growth rates using an HSA clone with two gene copies under its control. It was cultivated in chemostat with different dilution rates and the highest specific product formation was found at a growth rate of about 0.07 h^{-1} (Figure 5). This clearly differs to the profile obtained with P$_{GAP}$ in [10], where the highest specific product formation was obtained only at higher growth rates. Growth rate dependency may be utilized to optimize space-time yield or other parameters in the production processes [10].

Knock out of G1

Furthermore, we decided to clarify the function of the gene PAS_chr1-3_0011, which underlies the control of the promoter P$_{G1}$. It contains 12 transmembrane domains (predicted by TMHMM Server v. 2.0), two Major Facilitator Superfamily (MFS) and other transporter domains. Based on the sequence homology to the *K. lactis* high-affinity glucose transporter *HGT1*, the gene controlled by P$_{G1}$ was expected to have a function in glucose transport [17], [20]. Strong activity of its promoter at very low glucose concentrations further strengthened this assumption. For further verification, the gene was disrupted using the split marker cassette technique (primers given in Additional file 1: Table S1) as described by Heiss et al. [21]. Similar as described by Jørgensen and his colleagues [22], we compared the glucose uptake of the wild type and a G1 knock out clone in glucose-limited chemostat cultivations at different growth rates. The glucose saturation constants were calculated from the residual glucose concentrations (Table 4) and a K$_S$ of 9.7, 23.1 and 69.3 μM was obtained for three different dilution rates (μ=0.14, 0.1 and 0.05 h^{-1}) for the wild type. Changing K$_S$ values are observed for the whole cell in different conditions, which is due to the differential regulation of its several transporters. A reduced
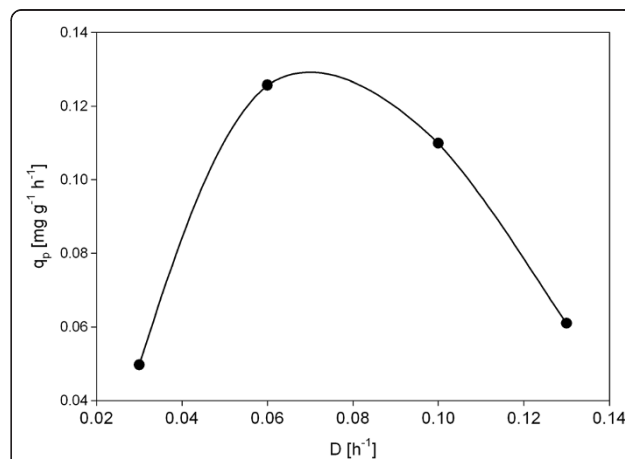
Figure 5 Correlation of specific productivity to specific growth rate using P$_{G1}$. Specific product formation rate (q$_p$) observed in chemostat cultivation at different dilution rates of a clone expressing HSA under the control of P$_{G1}$ as well as the respective trend curve (spline curve).

Table 4 Glucose substrate saturation constants of a wildtype and a G1 knock out clone

	G1 k. o., μ_{max} = 0.18 h^{-1}			Wildtype, μ_{max} = 0.18 h^{-1}		
D [h^{-1}]	S [μM]	X [g L^{-1}]	K_S [μM]	S [μM]	X [g L^{-1}]	K_S [μM]
0.14	316.4	29.5	90.4	33.9	30.5	9.7
0.10	123.8	31.3	99.0	28.9	32.1	23.1
0.05	79.9	31.3	207.8	26.6	30.8	69.3

Residual glucose (S), dry cell weight (X) and substrate saturation constant (K_S) of a wild type and a G1 knock out (k. o.) clone at different dilution rates in chemostat cultivations.

capacity of glucose uptake at low specific growth rates has been reported before [22]. The G1 knock out clone appeared to have much higher saturation constants of 90.4, 99.0 and 207.8 μM at the same dilution rates, which was also described for the high-affinity glucose transporter disruption in *Aspergillus niger* [22]. The knock out clone does not display the low K_S values of the wild type, so that the gene PAS_chr1-3_0011 was clearly identified as a high-affinity glucose transporter. As the short name *HGT1* is used as an alias for a peptide transporter in *S. cerevisiae* we suggest the short name *GTH1* (glucose transporter with high affinity) for this *P. pastoris* gene.

Conclusions

Efficient regulated promoters cannot necessarily be found by classical batch screening approaches. Simulating production conditions in lab scale and searching the promoter space offers a new target oriented approach. We could show here that the cultivation of *P. pastoris* in conditions where repression or induction are desired, followed by the analysis of transcript levels with DNA microarrays offers a potent opportunity to find new, strong and regulated promoters.

Six novel promoters were identified and further characterized. All of them are activated by carbon source depletion. The new promoters provide a tool box for expression of recombinant genes and are thus well suitable for protein production processes. P_{G1} had the most favourable repression kinetics and exceeded the expression levels of the well-established constitutive GAP promoter in glucose limited fed batch cultures by more than twofold. The molecular function of the gene under its control was identified as high-affinity glucose transporter and named *GTH1*.

Materials and methods
Strains and cultivation
Escherichia coli DH10B (Invitrogen) was used for subcloning. It was routinely cultivated in petri dishes or shake flasks using LB media supplemented with 25 μg mL^{-1} Zeocin. A wild type *Pichia pastoris* strain CBS2612 which can grow on minimal media supplemented with biotin, was used for protein expression in this work.

The main culture for screenings was either done with YP or BM media and glucose feed beads (12 mm, Kuhner, CH) which provided the carbon source.

YP media contained 20 g L^{-1} peptone and 10 g L^{-1} yeast extract, which can be supplemented with 12.6 g glycerol or 20 g glucose to obtain YPG and YPD, respectively. For cultivation on plates, 5 g L^{-1} agar-agar was added to the liquid medium. BM media was based on YP, supplemented with 13.4 g L^{-1} yeast nitrogen base (Cat.No. 291940, Becton Dickinson, FR) with ammonium sulfate, 0.4 mg L^{-1} biotin and 100 mM potassium phosphate buffer pH 6.0.

Identification of novel inducible promoters

a) Bioreactor cultivations
   Fermentations for the identification of promoter candidates were done in 3.5 L working volume bioreactors (Minifors, Infors, CH) in three biological replicates. Cells were grown for about 24 h in batch on glycerol medium, followed by an exponential feed phase on glycerol fed batch medium calculated as described by Resina et al. [23] with a specific growth rate of μ= 0.1 h^{-1} and a substrate yield coefficient of $Y_{X/S}$ of 0.5 g g^{-1}. Sequentially, chemostat cultivation (D = μ = 0.1 h^{-1}) with high density glucose medium was performed.
   Glycerol batch medium contained per liter: 2 g citric acid monohydrate, 39.2 g glycerol, 20.8 g NH$_4$H$_2$PO$_4$, 0.5 g MgSO$_4$· 7H$_2$O, 1.6 g KCl, 0.022 g CaCl$_2$· 2H$_2$O, 0.8 mg biotin and 4.6 mL PTM1 trace salts stock solution. HCl was added to set the pH to 5.0.
   Glycerol fed-batch medium contained per liter: 632 g glycerol, 8 g MgSO$_4$· 7H$_2$O, 22 g KCl, and 0.058 g CaCl$_2$· 2H$_2$O.
   High-density chemostat medium contained per liter: 2 g citric acid monohydrate, 99.42 g glucose monohydrate, 22 g NH$_4$H$_2$PO$_4$, 1.3 g MgSO$_4$· 7H$_2$O, 3.4 g KCl, 0.02 g CaCl$_2$· 2H$_2$O, 0.4 mg biotin and 3.2 mL PTM1 trace salts stock solution. HCl was added to set the pH to 5.0.
   PTM$_1$ trace salts stock solution contained per liter: 6.0 g CuSO$_4$· 5H$_2$O, 0.08 g NaI, 3.36 g MnSO$_4$· H$_2$O, 0.2 g Na$_2$MoO$_4$· 2H$_2$O, 0.02 g H$_3$BO$_3$, 0.82 g CoCl$_2$, 20.0 g ZnCl$_2$, 65.0 g FeSO$_4$· 7H$_2$O, 0.2 g biotin and 5.0 mL H$_2$SO$_4$ (95%-98%).

b) Microarray hybridization
   RNA purification and sample preparation as well as microarray hybridization (in-house designed *P. pastoris* specific oligonucleotide arrays, AMAD-ID: 034821, 8x15K custom arrays, Agilent) and data analysis were done as described by Graf et al. [24].

Characterization of promoter strength and regulation

a) Cloning

Cloning and transformation was done using the in-house vector pPuzzle [15], which contains a Zeocin resistance cassette for selection in both *E. coli* and yeast, an expression cassette for the gene of interest (GOI) consisting of a multiple cloning site and the *S. cerevisiae* CYC1 transcription terminator, and a locus for integration into the *P. pastoris* genome (3′ AOX1 region or rDNA locus). Promoter sequences (up to 1000 bps upstream of the start codon of their respective genes) were PCR-amplified from *P. pastoris* genomic DNA (primer sequences see Additional file 1: Table S1). The promoters were ligated into pPuzzle in front of the start codons of the model proteins, using the ApaI and the SbfI restriction sites of the multiple cloning site of the vector. Vectors expressing the respective model protein under control of P_{GAP} were used as controls throughout the study. For the expression of heterodimeric HyHEL antibody Fab fragment (HyHEL Fab), the expression cassettes of light chain and Fab heavy chain (each under control of P_{G1}) were combined into one vector (using the strategy described in [27]).

HSA was secreted by its native secretion leader, while for CpB and HyHEL Fab the *S. cerevisiae* alpha mating factor signal sequence was used. To avoid positional effects on reporter gene expression levels, genome integration of the expression plasmids was targeted to either the 3′flanking region of the *AOX1* gene or the ribosomal DNA locus (rDNA, for multicopy integration) of *P. pastoris,* respectively.

Plasmids were linearized within the genome integration region prior to electroporation (2 kV, 4 ms, GenePulser, BioRad) into electrocompetent *P. pastoris*. Multicopy integration of HSA expressing clones was done as described by Marx et al. [19] and selected at higher Zeocin concentrations (up to 1000 μg mL^{-1}).

P. pastoris cells were first selected and cultivated in petri dishes on YPD agar and then inoculated in an YPG medium as pre-culture for screenings and fermentations. Antibiotic selection by Zeocin was applied on plates and in pre-culture at a concentration of 25 μg mL^{-1} or higher.

b) Expression screening

Expression of intracellular eGFP and the secreted proteins HSA, CpB and HyHEL Fab with the novel promoters in comparison to P_{GAP} was evaluated in shake flask screenings. All screenings were performed at 25°C and with shaking at 180 rpm. Single colonies were inoculated in YPG medium with selection pressure (Zeocin) for pre-culture. After approximately 24 hours, the pre-culture was used to inoculate the main culture with an optical density (OD$_{600}$) of 0.1 (for eGFP) or 1 (for HSA, CpB and HyHEL Fab) in 10 mL YP or BM medium, respectively. Glucose feed beads (12 mm, Kuhner, CH) were used to generate glucose-limiting growth conditions. Expression of eGFP was measured at the end of pre-culture and at 24 and 48 hours of the main culture. Culture supernatant of clones expressing secreted protein was harvested from the pre-culture and after 48 hours and cell density was determined by measuring wet cell weight or OD$_{600}$.

c) Comparative analysis of *P. pastoris* promoter activity

In order to analyze relative transcription strength of the P_G promoters at different glucose concentrations, a comparative promoter activity study using various glucose concentrations (ranging from 20 to 0.002 g L^{-1} glucose) was performed with eGFP expressing clones in 24-well plates (Cat. No. 7701–5110, Whatman, UK) covered with breath seal membranes (Cat. No. B-100, Excel Scientific, CA). Glucose concentrations of 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, 0.01, 0.005, 0.002 g L^{-1} were obtained by serial dilution in YP media, and represent the inital setpoints. The main culture was inoculated from YPG-Zeocin pre-culture with an OD$_{600}$ of 0.01 and samples were taken after 5–6 hours and analyzed by flow cytometry.

d) Fed batch cultivation

All fed batch fermentations were done in 1.0 L working volume bioreactors (SR0700ODLS, DASGIP, DE). The dissolved oxygen was controlled at DO = 20% with the stirrer speed (400 – 1200 rpm). Aeration rate was 18 L h^{-1} air, the temperature was controlled at 25°C and the pH was controlled at 5.85 for HSA [25] or pH 5.0 for the other proteins [10] with addition of ammonium hydroxide (25%). To start the fermentation, 300 mL batch medium was sterile filtered into the fermenter and a *P. pastoris* clone was inoculated from an overnight pre-culture with a starting optical density (OD$_{600}$) of 1. For the cultivation of clones expressing eGFP, the batch phase of approximately 25 h was followed by a fed batch phase with a feeding rate optimized according to [10]. HSA expressing strains were cultivated as described by Marx et al. [19], where the batch phase was followed by a constant feed of 2 g h^{-1} fed batch medium for 100 h, Carboxypeptidase B and HyHEL Fab expressing clones were cultivated similarly. Samples were taken during batch and fed batch phase, and analyzed for expression.

Glycerol batch and glucose fed batch media for eGFP, HyHEL Fab and Carboxypeptidase B expressing clones were exactly as described in [10], while for the production of HSA the media was described in [19].

e) Chemostat cultivation

A strain expressing HSA (2 GCN) under control of P_{G1} was tested for its growth rate dependent expression behaviour in chemostat at different dilution rates (D = 0.03, 0.06, 0.10, 0.13) [10].

For characterization of glucose uptake characteristics, the *P. pastoris* wild type strain and the strain deleted for PAS_chr1-3_0011 were cultivated in glucose limited chemostats at D = 0.05, 0.10, and 0.14 h^{-1}. Samples were taken rapidly as described below.

Analytical methods

a) Copy number determination with real-time PCR

Genomic DNA was isolated using the DNeasy Blood&Tissue Kit (Cat. No. 69504, Quiagen, DE). Gene copy numbers were determined with quantitative PCR using the SensiMix SYBR Kit (QT605-05, Bioline reagents, UK). The primers (supplementary Additional file 1: Table S1) and sample were mixed with the SensiMix and applied for real time analysis in a real-time PCR cycler (Rotor Gene, Qiagen, DE). All samples were analyzed in tri- or quadruplicates. Data analysis was performed with the two standard curve method of the Rotor Gene software. The actin gene *ACT1* was used as calibrator.

b) Determination of protein expression levels

A plate reader (Infinite 200, Tecan, CH) was used to determine eGFP fluorescence in fermentation samples. Therefore, samples were diluted to an OD$_{600}$ of 5 and fluorescence intensity was then related to the culture volume.

Expression of eGFP in screenings was analyzed by flow cytometry as described before [15]. Specific eGFP fluorescence referred to in this study is the fluorescence intensity related to the cell volume for each data point as described by Hohenblum et al. [26]. Then the geometric mean of the population´s specific fluorescence was normalized by subtracting background signal (of non-producing *P. pastoris* wild type cells) and related to expression under the control of P_{GAP}.

For quantification of HSA in shake flask and fermentation supernatants, the Human Albumin ELISA Quantitation Set (Cat. No. E80-129, Bethyl Laboratories, TX) was used. The HSA standard was applied with a starting concentration of 400 ng mL^{-1}. Dilution-, Blocking- and Washing buffer were based on TBS (50 mM Tris–HCl, 140 mM NaCl, pH 8.0) and completed with BSA (1% (w/v)) and/or Tween20 (0.05% (v/v)) accordingly.

HyHEL Fab was determined with ELISA as described previously [27].

CpB was quantified using an enzymatic assay based on the cleavage of hippuryl-L-arginine (Cat. No. H2508, Sigma, MO). Generation of hippuric acid was monitored at its absorbance maximum of 254 nm. Prior to the measurement, the samples were desalted with Zeba Spin columns (Thermo Fisher Scientific, IL) and activated with trypsin (Cat. No. T8345, Sigma, MO).

c) Determination of residual glucose

The D-Glucose Assay - GOPOD-Format (Megazymes, IE) was used to determine residual glucose of chemostat samples. Supernatant sampling was done by pumping culture broth out of the bioreactor by producing an overpressure, and its direct sterile filtration using a vacuum filter unit (Cat. No. 5141178, Whatman, UK). Glucose-limited cultivations usually go along with very low residual glucose concentrations in the supernatant, so the manufacturer's protocol was adapted for glucose concentrations from 10 to 100 mg L^{-1}. Briefly, the ratio of reaction buffer to sample was changed from 30:1 to 3:1.

Additional file

Additional file 1: Table S1. Primer Sequences. Sequences of oligonucleotides used for amplification of promoters, determination of gene copy numbers of the model protein expression cassettes, and generation of G1 disruption cassette (including verification of positive knock-outs).

Author details
[1]University of Natural Resources and Life Sciences, Department of Biotechnology, Muthgasse 18, Vienna 1190, Austria. [2]Austrian Centre of Industrial Biotechnology (ACIB GmbH), Muthgasse 11, Vienna 1190, Austria. [3]University of Applied Sciences FH-Campus Vienna, School of Bioengineering, Muthgasse 18, Vienna 1190, Austria. [4]Lonza AG, Rottenstraße 6, Visp 3930, Switzerland.

References

1. Damasceno LM, Huang CJ, Batt CA: **Protein secretion in Pichia pastoris and advances in protein production.** *Appl Microbiol Biotechnol* 2012, **93**:31–39.
2. Mattanovich D, Branduardi P, Dato L, Gasser B, Sauer M, Porro D: **Recombinant protein production in yeasts.** *Methods Mol Biol* 2012, **824**:329–358.
3. Tschopp JF, Brust PF, Cregg JM, Stillman CA, Gingeras TR: **Expression of the lacZ gene from two methanol-regulated promoters in Pichia pastoris.** *Nucleic Acids Res* 1987, **15**:3859–3876.
4. Ohi H, Miura M, Hiramatsu R, Ohmura T: **The positive and negative cis-acting elements for methanol regulation in the Pichia pastoris AOX2 gene.** *Mol Gen Genet* 1994, **243**:489–499.
5. Shen S, Sulter G, Jeffries TW, Cregg JM: **A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast Pichia pastoris.** *Gene* 1998, **216**:93–102.
6. 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.
7. Menendez J, Valdes I, Cabrera N: **The ICL1 gene of Pichia pastoris, transcriptional regulation and use of its promoter.** *Yeast* 2003, **20**:1097–1108.
8. Ahn J, Hong J, Park M, Lee H, Lee E, Kim C, Lee J, Choi E, Jung J: **Phosphate-responsive promoter of a Pichia pastoris sodium phosphate symporter.** *Appl Environ Microbiol* 2009, **75**:3528–3534.
9. Waterham H, Digan M, Koutz P, Lair S, Cregg J: **Isolation of the Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter.** *Gene* 1997, **186**:37–44.
10. Maurer M, Kühleitner M, Gasser B, Mattanovich D: **Versatile modeling and optimization of fed batch processes for the production of secreted heterologous proteins with Pichia pastoris.** *Microb Cell Fact* 2006, **5**:37.
11. Baumann K, Maurer M, Dragosits M, Cos O, Ferrer P, Mattanovich D: **Hypoxic fed-batch cultivation of Pichia pastoris increases specific and volumetric productivity of recombinant proteins.** *Biotechnol Bioeng* 2008, **100**:177–183.
12. Ahn J, Hong J, Lee H, Park M, Lee E, Kim C, Choi E, Jung J: **Translation elongation factor 1-alpha gene from Pichia pastoris: molecular cloning, sequence, and use of its promoter.** *Appl Microbiol Biotechnol* 2007, **74**:601–608.
13. Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, Lin-Cereghino J, Kovar K, Cregg JM, Glieder A: **Promoter library designed for fine-tuned gene expression in Pichia pastoris.** *Nucleic Acids Res* 2008, **36**:e76.
14. Qin X, Qian J, Yao G, Zhuang Y, Zhang S, Chu J: **GAP promoter library for fine-tuning of gene expression in Pichia pastoris.** *Appl Environ Microbiol* 2011, **77**:3600–3608.
15. Stadlmayr G, Mecklenbrauker A, Rothmuller M, Maurer M, Sauer M, Mattanovich D, Gasser B: **Identification and characterisation of novel Pichia pastoris promoters for heterologous protein production.** *J Biotechnol* 2010, **150**:519–529.
16. Cos O, Ramón R, Montesinos J, 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.
17. Mattanovich D, Graf A, Stadlmann J, Dragosits M, Redl A, Maurer M, Kleinheinz M, Sauer M, Altmann F, Gasser B: **Genome, secretome and glucose transport highlight unique features of the protein production host Pichia pastoris.** *Microb Cell Fact* 2009, **8**:29.
18. du Preez JC, de Kock SH, Kilian SG, Litthauer D: **The relationship between transport kinetics and glucose uptake by Saccharomyces cerevisiae in aerobic chemostat cultures.** *Antonie Van Leeuwenhoek* 2000, **77**:379–388.
19. Marx H, Mecklenbräuker A, Gasser B, Sauer M, Mattanovich D: **Directed gene copy number amplification in Pichia pastoris by vector integration into the ribosomal DNA locus.** *FEMS Yeast Res* 2009, **9**:1260–1270.
20. Baruffini E, Goffrini P, Donnini C, Lodi T: **Galactose transport in Kluyveromyces lactis: major role of the glucose permease Hgt1.** *FEMS Yeast Res* 2006, **6**:1235–1242.
21. Heiss S, Maurer M, Hahn R, Mattanovich D, Gasser B: **Identification and deletion of the major secreted protein of Pichia pastoris.** *Appl Microbiol Biotechnol* 2012, doi:10.1007/s00253-00012-04260-00254.
22. Jørgensen TR, VanKuyk PA, Poulsen BR, Ruijter GJ, Visser J, Iversen JJ: **Glucose uptake and growth of glucose-limited chemostat cultures of Aspergillus niger and a disruptant lacking MstA, a high-affinity glucose transporter.** *Microbiology* 2007, **153**:1963–1973.
23. Resina D, Cos O, Ferrer P, Valero F: **Developing high cell density fed-batch cultivation strategies for heterologous protein production in Pichia pastoris using the nitrogen source-regulated FLD1 Promoter.** *Biotechnol Bioeng* 2005, **91**:760–767.
24. Graf A, Gasser B, Dragosits M, Sauer M, Leparc G, Tüchler T, Kreil D, Mattanovich D: **Novel insights into the unfolded protein response using Pichia pastoris specific DNA microarrays.** *BMC Genomics* 2008, **9**:390.
25. 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.
26. Hohenblum H, Borth N, Mattanovich D: **Assessing viability and cell-associated product of recombinant protein producing Pichia pastoris with flow cytometry.** *J Biotechnol* 2003, **102**:281–290.
27. Gasser B, Maurer M, Gach J, Kunert R, Mattanovich D: **Engineering of Pichia pastoris for improved production of antibody fragments.** *Biotechnol Bioeng* 2006, **94**:353–361.