Optimization of Specific Product Formation Rate by Statistical and Formal Kinetic Model Descriptions of an HSA Producing *Pichia pastoris* Mut^S Strain

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Pichia pastoris has proven to be an ideal host for eukaryotic protein expression. Numerous models have been developed to describe the heterologous product formation of this yeast, however the pH and temperature dependence has not been characterized yet. A linear full factorial design with a centre point was expanded with a second order Box-Behnken design towards increasing product yields to examine the influence of pH and temperature on the product formation of a human serum albumin (HSA) producing *P. pastoris* GS115 Mut^S strain in methanol-induced fermentations. Both the linear and quadratic form of pH had significant effects on the product yield, productivity and specific product formation rate, whereas the effect of temperature was less important. As the specific growth rate was independent of pH and temperature in the product forming experimental range, the volumetric productivity was determined mainly by the specific product formation rate. For this reason, the optimization of productivity requires the growth independent description of specific product formation rate. Beside a parabolic statistical model, an empirically based formula was fitted to the experimental results in order to characterize product formation. The empirical formula was composed of three separate terms i.e. the specific maximal product formation rate, a substrate inhibition kinetics module for $[H^+]$ and two Arrhenius equations describing the temperature dependence of product formation. The maximum specific product formation rate was measured at the optimum, predicted by the empirical formula (pH5.64 and 20.24 °C), which slightly exceeded the calculated value (0.370 mg HSA per g DW and h vs. 0.354 mg HSA per g DW and h).

Key words:
Pichia pastoris, product formation, model, pH dependence, temperature dependence

Introduction

The *Pichia pastoris* expression system is known as an ideal tool for recombinant protein production.[1,2] The methylotrophic yeast *P. pastoris* is capable of growing on a simple methanol salt medium in high cell densities[3,4] and can use methanol as sole carbon and energy source. Through its strong and tightly regulated alcohol-oxidase (AOX) promoters, *Pichia* is an extremely effective host for recombinant protein expression.[5,6,7] *P. pastoris* possesses a series of beneficial features such as low level secretion of endogenous proteins, efficient post-translational modifications,[8] acceptance of signal sequences of different origin for extracellular expression,[9,10] and lack of α(1,3)-mannosiltransferase activity which helps to avoid the hyperglycosylation of heterologous proteins.[11,12] Usually two variations of recombinant *P. pastoris* are used for recombinant protein production: the Mut$^+$ (Methanol utilisation type +) has intact AOX1 and AOX2 genes and can grow on methanol with similar rate as the wild type strain, whereas the Mut^S (Methanol utilisation type Slow) has a disrupted AOX1 and is capable to utilise methanol with the 1/6th – 1/10th efficiency of the wild type strain.[13] Although Mut$^+$ variant is more commonly used, in some cases Mut^S results in higher active heterologous protein concentration.[14,15,16] Moreover, the commonly used *P. pastoris* KM71 exists only in Mut^S form.[17] For these reasons, *P. pastoris* Mut^S fermentation deserves thorough investigations in order to increase protein expression efficiency.

Methanol serves as the inducer and as the sole carbon and energy source during the production phase of a *P. pastoris* fermentation. *Pichia* can assimilate methanol, but can not tolerate high methanol concentrations as a result of the accumulation of toxic oxidized products (ie. formaldehyde and hydrogen peroxide) of the methanol catabolism. Besides the direct negative effects of high methanol concentrations on cell metabolism, the efficient ex-

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pression of recombinant proteins also requires low substrate levels: the induction of AOX1 promoter is maximal among growth limiting substrate concentrations. Three empirical methods have been described in order to maintain the required limiting constant methanol level during fed-batch cultivations of *P. pastoris:* dissolved oxygen spike method,[18] application of organic vapour sensor for monitoring the methanol level, and use of pre-programmed feed rates.[19,20,21,22] To investigate the product formation rate of recombinant *P. pastoris*, we applied the latest method based on our earlier experiments.[23]

The fermentation parameters used widespread are from "*Pichia* Fermentation Process Guidelines" of Invitrogen Co. (San Diego, CA) and the influence of these are generally not evaluated on individual fermentations. *P. pastoris* is regularly cultivated at pH 5.0 and at 30 °C, however, this yeast is capable to grow on glycerol between pH 2.5 – 7.5 and at a wide temperature range,[24] although 30 – 32 °C is the upper limit of recombinant product formation. It is important to note that unless proteinase deficient strains are used, mostly alkalic and neutral proteases disrupt recombinant proteins in the fermentation broth, which suggests the application of low pH during product formation. Reduction of fermentation temperature to 23 °C was reported to enhance recombinant product formation.[25] This effect was explained by increased cell viability and synthesis rate dedicated to the higher AOX activity at this low temperature,[26] however, the influence of temperature on specific product formation has not been thoroughly studied yet. The purpose of this work was the systematic investigation of the influence of pH and temperature on the product formation of *P. pastoris* Mut^S in the range of pH 3.2 – 7.2 and 17 – 29 °C. It has been reported that unlike in *P. pastoris* Mut^+, the specific product formation rate is rather independent of growth rate in the Mut^S version in respect of methanol concentration.[27] By the statistical analysis of the correlation of pH and temperature with fermentation kinetic variables we wished to determine if the volumetric productivity is mainly determined by the specific growth rate or by the specific product formation rate. The procedure required the separate handling of growth rate and specific product formation rate. The aim of the analysis was to select the variable for model based optimization. As our model product, human serum albumin is a relatively simple, unglikosylated, extracellulary expressed protein, the proposed production optimum and model may be generally applicable for extracellular recombinant protein formation of *P. pastoris* Mut^S cells, unless the product does not require complex post-translational modifications or shows sensitivity for proteolytic degradation.

P. pastoris GS115 Mut^S expressing human serum albumin (HSA) was used as model strain. HSA is the major protein component of the human plasma with a molecular mass of 66.5 kDa. HSA is naturally produced in the liver and besides maintaining the osmolarity of the blood stream also functions as carrier for small molecules. Albumin for clinical use is produced by fractionation of whole blood, however, the extended demand of this protein (450 t y^{-1} worldwide) implies the necessity of introducing other sources. Since the disulfide structure[28] and physiological features of recombinant HSA derived from *P. pastoris* fermentation has been described as identical to the native molecule,[29,30] this methylotrophic yeast is an outstanding candidate for industrial HSA production if the fermentation parameters are well defined.

Materials and methods

Strain

Pichia pastoris GS115 Mut^S with extracellular expression of human serum albumin (Invitrogen, USA).

Culture conditions

Strain was maintained on YEPD agar slants at +4 °C (20 g L^{-1} glucose (Reanal, Hungary), 20 g L^{-1} bacteriological peptone (Oxoid, USA), 10 g L^{-1} bacto yeast extract (Difco Laboratories, Becton, Dickinson & Co., USA), 20 g L^{-1} agar-agar (Reanal, Hungary)).

Cultivations were conducted in 800 mL working volume bench top Biostat Q fermentors (B. Braun Biotech, Germany) at controlled pH and temperature. 1200 rpm agitation and 1 L min^{-1} aeration were applied. All fermentation parameters except substrate concentration and dry cell mass were registered and controlled by MFCS II data acquisition software of B. Braun Biotech.

Culture medium was prepared as follows: 40 g L^{-1} glycerol, 18.2 g L^{-1} K_2SO_4, 14.9 g L^{-1} $MgSO_4 \cdot 7H_2O$, 4.13 g L^{-1} KOH, 0.93 g L^{-1} $CaSO_4 \cdot 7H_2O$, 26.7 ml L^{-1} cc. ortophosphoric-acid and 4.35 ml L^{-1} of PTM_1 trace element solution. The composition of the trace element solution was:[31] 65.00 g L^{-1} $FeSO_4 \cdot 7H_2O$, 20.00 g L^{-1} $ZnCl_2 \cdot 4H_2O$, 6.00 g L^{-1} $CuSO_4 \cdot 5H_2O$, 1.50 g L^{-1} $MnSO_4 \cdot H_2O$, 0.25 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.10 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.10 g L^{-1} biotin and 0.04 g L^{-1} NaI.

When substrate feed was applied, the nutrient feed consisted of analytical grade pure methanol with 12 ml L^{-1} PTM_1 solution. Steady substrate addition by infusion driver (Infudriver, Kutesz, Hungary) was applied at the rate of 0.66 mL L^{-1} h^{-1}.

The fermentation procedure started with a batch phase on glycerol at pH 5.0 and 29 °C, which was terminated when dissolved oxygen level of the fermentation broth increased indicating the depletion of glycerol from the system. When the dissolved oxygen level increased, the pH and temperature of the fermentation broth was adjusted to the desired levels of the product forming phase along a one hour lasting linear gradient. The pH was set by 25 % NH_4OH and 25 % H_2SO_4 solutions. The subsequent methanol fed phase lasted for 70 h.

Analysis of media components

Biomass concentration was determined from the optical density of the fermentation broth at 600 nm (Pharmacia LKB-Ultrospec Plus, Pharmacia Co., USA). Biomass is expressed as dry cell mass, which was calibrated to the optical density.

Methanol concentration was measured by off line gas chromatography (Laboratorni Pristroje Praha, Chrom4 GC system, Czech R., column – 0.2 % Carbowax 1500 on 80/100 Carbopack C, 6', Supelco, USA; sample volume – 3 μL; mobile phase – nitrogen; column temperature – 70 °C).

For the evaluation of product yield on substrate, the methanol consumption was calculated as follows. The total amount of methanol addition (m_{add}) was determined electronically by the additive counter of the infusion driver. The change in the total alcohol content of the fermentation broth was calculated from the methanol mass concentration of samples (γ_{ti}, $\gamma_{t(i-1)}$) multiplied by the volume of the fermentation broth (V_{ti}, $V_{t(i-1)}$). The amount of methanol which evaporated with the aeration (m_{ev}) was determined according to our earlier model.[24] Finally, the sum of the amount of methanol added and the change in the total alcohol content of the fermentation broth between two sample points decreased by the amount of evaporated alcohol was considered to be the value of the substrate consumed (m_{cons}):

$$m_{cons} = m_{add} - (\gamma_{ti} \cdot V_i - \gamma_{t(i-1)} \cdot V_{t(i-1)}) - m_{ev} \quad (1)$$

The HSA concentration was determined from the cell free supernatant of fermentation samples by gradient SDS gel electrophoresis (Pharmacia Phast System, Phast Gel Gradient 8–25 gel [Amersham-Pharmacia, Sweden], crystallized HSA V standard [Sigma, USA], silver staining, evaluated by Kodak Digital Science 1D Image Analysis Software).

Parameter fit

Statistica for Windows 6.0 (StatSoft Inc., USA) was used for statistical data analysis while SigmaPlot 7.0 (SPSS Inc., USA) was applied for fitting of functions. The Marquardt-Levenberg algorithm was used to determine the model coefficients.[32]

Results and discussion

Experimental design

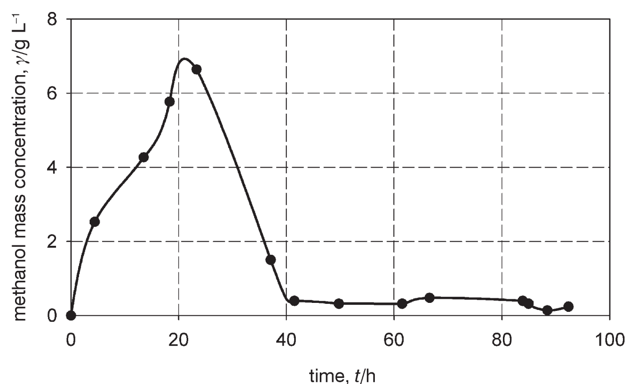
Linear orthogonal design with a centre point in the range of pH 3.2, 5.2 and T = 23, 29 °C was used to create a wide range of circumstances in the product formation period of recombinant *Pichia* fermentations in order to examine, whether, *P. pastoris* is capable to utilise methanol and to form recombinant product in significant quantities at low pH values (Table 1.). Since product formation was not detectable below pH 4.2 and the final recombinant protein concentration seemed to increase towards higher pH and lower temperature values, the experimental design was expanded with a second order full orthogonal design in the range of pH 5.2, 7.2 and T = 23, 17 °C.

The steady methanol feed in the product forming period of the fermentations ensured the initiation of the Aox enzyme and of the recombinant protein production. After a transient period of 25 – 40 h, the adequate substrate addition strategy ensured a

Table 1

Experiment No.	pH	T / °C	γ_{HSA} / mg L^{-1}	μ_x / g g^{-1} h^{-1}	J_{HSA} / mg L^{-1} h^{-1}	μ_p / mg g^{-1} h^{-1}
1	3.2	29	0	0.0037	0.0	0.000
2	3.2	23	0	0.0017	0.0	0.000
3	5.2	29	394	0.0085	5.5	0.126
4	5.2	23	609	0.0080	8.5	0.313
5	4.2	26	0	0.0036	0.0	0.000
6	5.2	20	604	0.0062	7.2	0.350
7	5.2	17	561	0.0080	8.1	0.296
8	6.2	23	650	0.0071	9.4	0.226
9	6.2	20	831	0.0090	11.5	0.292
10	6.2	17	591	0.0068	8.3	0.231
11	7.2	23	319	0.0113	4.2	0.128
12	7.2	20	410	0.0079	5.8	0.151
13	7.2	17	213	0.0074	2.5	0.083
14	5.7e	20	894	0.0123	11.9	0.379

[a] γ_{HSA} – final HSA concentration in fermentation broth
[b] μ_x – average specific growth rate in the product formation phase
[c] J_{HSA} – overall recombinant HSA productivity calculated for the substrate limited phase
[d] μ_P – specific product formation rate in the product formation phase
[e] experimental optimum point for productivity



F i g . 1 – *Methanol mass concentration profile during the product formation phase of fed batch fermentations (experiment No.4)*

gradually decreasing 0.3 – 0.5 g L^{-1} methanol concentration (Fig 1.). This limiting substrate level is reported to favour the recombinant protein production.[18] The role of pH and temperature on product formation was evaluated for this substrate limited period of the fermentations. At low pH values, the Aox system could not reach satisfactory high activity in a relatively short period and for this reason the methanol feed exceeded the consumption rate resulting continuously increasing substrate levels in the fermentation broth. HSA formation was not detectable in these experiments.

Nitrogen starvation is reported to raise of proteolytic activity in the fermentation broth.[33] In all cases, the mass balance calculation showed that satisfactory nitrogen supply was ensured and as a result, significant product degradation was detected by SDS PAGE only at 29 °C, pH 5.2.

Significance analysis

Two characteristic variables of physiological importance, ie. specific growth rate and product formation rate and two variables of economic interest ie. volumetric productivity and product yield, were chosen as objective functions for the statistical analysis of the fermentation runs realized on the base of the above experimental designs. Our purpose was to determine whether the economic variables with respect of the pH and temperature are preliminarily determined by the growth of the yeast or by the product formation. If the product yield on methanol or the specific product formation rate is mostly determined by the growth rate in respect of the pH and temperature, a generalized technique at the growth optimum can be accepted regardless of the feature of the heterologous protein. On the other hand, if optimization of growth holds less importance to perform high productivity fermentations, more concern should be on the specific product formation rate and yield, and individual optimization of these functions may be required to design economically feasible fermentations. Our concept was to determine if growth rate or specific product formation shows more similar pH and temperature dependence to the productivity and yield, and this way to chose the variable to be optimized in order to increase heterologous product formation efficiency.

As the methanol concentration was successfully maintained steady in the substrate limited period of the fermentation, the average specific growth rate, the specific product formation rate, the volumetric productivity, and the product yield were considered to be constant at the given pH and temperature preset values. Since the substrate feed was approximately equal to the sum of the evaporation volume loss of the fermentation broth and of the samples taken, the dilution rate had small negative value. The resulted total volume decrease was less than 3 % during the substrate limited period, thus as an affordable simplification, the change of fermentation broth volume was neglected and the mass balances for cells and product were simplified to batch type descriptions. Average specific growth rate was calculated by exponential fit on the dry cell mass using the following considerations:

$$\frac{d\gamma_X}{dt} = \mu_X \cdot \gamma_X ; \qquad \mu_X = \text{constant} \tag{2}$$

$$\mu_X = \frac{\ln \dfrac{\gamma_{X_t}}{\gamma_0}}{\Delta t} \tag{3}$$

Equation 3 was obtained by analytical integration of equation 2 between times 0 and t. Specific product formation rate based on the above simplification was calculated as follows:

$$\frac{d\gamma_P}{dt} = \mu_P \cdot \gamma_X \tag{4}$$

$$\mu_P = \frac{\gamma_P}{\int_0^t \gamma_X \, dt} \tag{5}$$

The time integral of the dry cell mass was calculated by the trapezoid rule of integration. The volumetric productivity and the product yield were calculated on the base of the amount of HSA produced in the methanol limited phase using the mass of methanol consumed for the calculation of the latter.

Sigma-restricted parametrization and backward stepwise decomposition of second-order statistical models (equation (6) shows the general form of them) were used to evaluate the effects of pH and

temperature, both, on the whole experimental design set as well as on the narrowed range where significant product formation was detected.[34]

$$z = z_0 + z_1\,\mathrm{pH} + z_2\,\mathrm{pH}^2 + z_3\,T + z_4\,T^2 + z_5\,\mathrm{pH},T \qquad (6)$$

For the whole experimental set, both, sigma-restricted parametrization and decomposition of the second-order model showed that only pH had significant influence on all the variables and temperature held minor importance. Furthermore, only the linear term had significant effect on the specific growth rate whereas the quadratic form of pH influenced, both, the specific product formation rate and the volumetric productivity (Table 2). In a secondary analysis run, when only those experimental data were involved in the significance analysis where product formation occurred (pH 5.2, 7.2; T = 17, 29 °C), specific growth rate and product yield proved to be independent on pH and temperature, while specific product formation rate showed dependency on the linear form of pH, and the linear and quadratic form of temperature. Since product yield does not show significant variance in the experimental range, the increase in productivity must be parallel with the change of substrate consumption rate. Examining the specific rates in the function of methanol concentration of $\mathrm{Mut^S}$ type recombinant *P. pastoris*, d'Anjou could not set up a direct correlation between the specific growth rate and the substrate consumption.[32] Similarly, the specific product formation seems to correlate with the methanol consumption rate, and is independent of specific growth rate in $\mathrm{Mut^S}$ type cells, in respect of pH and temperature. Volumetric productivity remained influenced only by the quadratic form of pH (Table 3). As productivity and specific product formation rate both are strongly influenced by the pH, while specific growth rate is independent of it, the productivity seems to be mainly determined by the product formation in respect of pH and temperature. The stepwise decomposition of the second-order statistical model for the specific product formation rate resulted in the parameters showed in Table 4. The calculated optimum points for the full experimental range and the producing range are pH 4.44, 20.6 °C and pH 5.41, 18.9 °C, respectively.

Summarizing the results of the statistical analysis, it became evident that productivity of recombinant protein formation by *Pichia pastoris* $\mathrm{Mut^S}$ cells is mainly determined by the specific product formation rate and not by the growth rate of cells in respect of pH and temperature. This statement is supported by the fact that cell growth is independent of pH and T in the range of recombinant product formation, whereas productivity and specific product formation rate are both dependent on pH. Also, the pH values for maximal specific product formation and volumetric productivity are close (5.4 and 5.7, respectively), suggesting that the former determines the latter. In order to test if we can create a more precise description of specific product formation rate to use in optimization process, we built a non statistical mathematical model, which is based upon well known and generally accepted formulas describing $[\mathrm{H^+}]$ and temperature dependence of specific rates.

Modelling the specific product formation rate

We defined a function to characterise the specific product formation rate as a product of three separate terms each describing an important distinguishable feature (equation 7). The first term is a constant, $\mu_{\mathrm{P_0}}$, characteristic on the given heterologous protein. When statistical analysis were performed with the method described above applying $[\mathrm{H^+}]$ instead of pH, both, linear and quadratic functions of $[\mathrm{H^+}]$ and temperature correlated with the specific growth rate, whereas the linear cross effects were negligible (data not shown). These results suggested the application of substrate inhibition kinetics (or Michaelis pH function) to describe the influence of $[\mathrm{H^+}]$ on the specific product formation rate in a second term.[35]

Although, the influence of temperature proved to hold less importance for the whole experimental range, the decrease of product formation towards higher temperature values is generally accepted and moreover temperature had significant effect on product formation according to the statistical analysis on the product forming experiments. On the bases of reaction kinetics and practical considerations, we investigated the influence of linear, quadratic, logarithmic, and exponential temperature functions on the specific product formation rate. Since the exponential function of temperature showed the strongest correlation with the target function, we incorporated two Arrhenius formulas in the model as its third term, describing the overall effect of temperature-dependent processes enhancing or hindering product formation. The final complex formula showing specific product formation rate dependencies is given in equation (7).

$$\mu_{\mathrm{P}} = \mu_{\mathrm{P_0}} \cdot \frac{K_1 \cdot [\mathrm{H^+}]}{K_1 \cdot K_2 + K_1 \cdot [\mathrm{H^+}] + [\mathrm{H^+}]^2} \cdot \left\{ a \cdot \exp\left(\frac{-\Delta G_1}{R\,T}\right) - b \cdot \exp\left(\frac{-\Delta G_2}{R\,T}\right) \right\} \qquad (7)$$

The parameters fitted to the experimental results are shown in Table 4. The equation predicts a

Table 2 – *Analysis of central composit experiment for μ_X, J_{HSA}, μ_P and $Y_{P/S}$: ANOVA (Analysis of Variance) table for the whole experimental range of pH 3.2, 7.2; T 17, 29 °C*

μ_X	Sum of squares	Degree of freedom	Mean square	F	p
pH	**5.3392E–05**	**1**	**5.3392E–05**	**27.2602**	**0.0012**
pH^2	1.2521E–07	1	1.2521E–07	0.0639	0.8077
T	4.1710E–06	1	4.1710E–06	2.1296	0.1879
T^2	2.8532E–06	1	2.8532E–06	1.4568	0.2666
$pH \cdot T$	3.8706E–06	1	3.8706E–06	1.9762	0.2026
Error	1.3710E–05	7	1.9586E–06		
Total sum of squares	7.8691E–05	12			

J_P	Sum of squares	Degree of freedom	Mean square	F	p
pH	23.9618	1	23.9618	3.5083	0.1032
pH^2	**40.0295**	**1**	**40.0295**	**5.8609**	**0.0460**
T	4.2924	1	4.2924	0.6285	0.4539
T^2	2.2744	1	2.2744	0.3330	0.5810
$pH \cdot T$	0.0731	1	0.0731	0.0107	0.9205
Error	47.8097	7	6.8300		
Total sum of squares	179.2108	12			

μ_P	Sum of squares	Degree of freedom	Mean square	F	p
pH	6.1159E–03	1	6.1159E–03	1.0085	0.3487
pH^2	**4.6112E–02**	**1**	**4.6112E–02**	**7.6036**	**0.0282**
T	1.8313E–02	1	1.8313E–02	3.0196	0.1258
T^2	3.4704E–03	1	3.4704E–03	0.5722	0.4741
$pH \cdot T$	3.7714E–05	1	3.7714E–05	0.0062	0.9394
Error	0.0425E–02	7	6.0646E–03		
Total sum of squares	0.1888	12			

$Y_{P/S}$	Sum of squares	Degree of freedom	Mean square	F	p
pH	32.857	1	32.857	2.7655	0.1403
pH^2	**69.826**	**1**	**69.826**	**5.8772**	**0.0458**
T	21.108	1	21.108	1.7766	0.2243
T^2	2.884	1	2.8841	0.2427	0.6373
$pH \cdot T$	0.1382	1	0.1382	0.0116	0.9171
Error	83.166	7	11.881		
Total sum of squares	335.63	12			

Table 3 – *Analysis of central composit experiment for μ_X, J_{HSA}, μ_P and $Y_{P/S}$: ANOVA table for the producing range of pH 5.2, 7.2; T = 17, 29 °C*

μ_X	Sum of squares	Degree of freedom	Mean square	F	p
pH	7.6607E–06	1	7.6607E–06	4.3772	0.1046
pH^2	4.8896E–07	1	4.8896E–07	0.2794	0.6251
T	6.8304E–06	1	6.8304E–06	3.9027	0.1194
T^2	9.9915E–07	1	9.9915E–07	0.5709	0.4920
pH · T	4.2527E–06	1	4.2527E–06	2.4299	0.1940
Error	7.0007E–06	4	1.7502E–06		
Total sum of squares	1.7996E–05	9			

J_P	Sum of squares	Degree of freedom	Mean square	F	p
pH	7.6873	1	7.6873	4.0650	0.1140
pH^2	**26.6766**	**1**	**26.6766**	**14.1064**	**0.0198**
T	0.5810	1	0.5810	0.3072	0.6089
T^2	3.7665	1	3.7665	1.9917	0.2310
pH · T	0.1273	1	0.1273	0.0673	0.8081
Error	7.5644	4	1.8911		
Total sum of squares	62.8800	9			

μ_P	Sum of squares	Degree of freedom	Mean square	F	p
pH	**2.4156E–02**	**1**	**2.4156E–02**	**51.3430**	**0.0020**
pH^2	1.6320E–03	1	1.6320E–03	3.4686	0.1360
T	**8.9165E–03**	**1**	**8.9165E–03**	**18.9517**	**0.0121**
T^2	**1.1959E–02**	**1**	**1.1959E–02**	**25.4193**	**0.0073**
pH · T	3.1001E–05	1	3.1001E–05	0.0659	0.8101
Error	1.8819E–03	4	4.7049E–04		
Total sum of squares	7.7494E–02	9			

$Y_{P/S}$	Sum of squares	Degree of freedom	Mean square	F	p
pH	18.9528	1	18.9528	3.2551	0.1455
pH^2	23.1079	1	23.1079	3.9688	0.1172
T	6.6701	1	6.6701	1.1456	0.3448
T^2	8.1729	1	8.1729	1.4037	0.3017
pH · T	0.1045	1	0.1045	0.0179	0.8999
Error	23.2897	4	5.8224		
Total sum of squares	112.026	9			

Table 4 – *Parameters of the model equation*

Statistical model	$\mu_P = z_0 + z_1(\mathrm{pH}) + z_2(\mathrm{pH})^2 + z_3(T) + z_4(T)^2$					
	z_0	z_1	z_2	z_3	z_4	R^2
whole experimental range	–1.5833	0.5153	–0.0476	0.0511	–0.0014	0.7749
range of product formation	–1.5574	0.2540	–0.0286	0.1309	–0.0032	0.9753

Formal kinetic model	$\mu_P = \mu_{P_0} \cdot \frac{K_1 \cdot [H^+]}{K_1 \cdot K_2 + K_1 \cdot [H^+] + [H^+]^2} \cdot \left\{ a \cdot \exp\left(\frac{-\Delta G_1}{RT}\right) - b \cdot \exp\left(\frac{-\Delta G_2}{RT}\right) \right\}$						
ν_P	K_1	K_2	a	$-\Delta H_1/R$	b	$-\Delta H_2/R$	R^2
6.4417 18.4897*	2.1195E–05	2.3009E–07	1.4858	–44.016	3.6512	–72.189	0.9178

*normalized value

maximum specific product formation rate at pH 5.64 and 20.24 °C (Fig 2). The statistical model showed better correlation than equation (7) (R^2 = 0.97 vs. 0.92). The optimum of the statistical model closely coincided with experimental set No. 6 (pH 5.2, 20 °C, Table 1), whereas the optimum of the formal kinetic model (equation (7)) had not been experimentally yet realized. In order to test if the maximum of equation 7 holds practical importance, a fermentation was performed at pH 5.7 and 20 °C. While the formal kinetic model predicted 0.350 mg HSA per g dry mass and hour specific product formation rate among these conditions, the experimentally measured value was 0.379 mg HSA per g dry mass and hour. Since the fermentation at pH 5.7 and 20 °C showed the highest specific product formation value of all evaluated sets, and the predicted product formation rate was close to the measured value, the model of equation 7 can be accepted as an adequate description of *P. pastoris* $\mathrm{Mut^S}$ fermentation, and the calculated pH and temperature optimum points are proved to be realistic. The optimization of specific product formation was performed for the model product HSA, which is a relatively large non-glycosylated protein and did not show significant proteolytic degradation in the experiments. We assume that the derived optimum of the specific product formation rate can be generally accepted for $\mathrm{Mut^S}$ type cells, unless the recombinant product is target of complex post-translational modifications or problems arise at secretion of the protein or the product shows extraordinary sensitivity to proteolytic degradation.

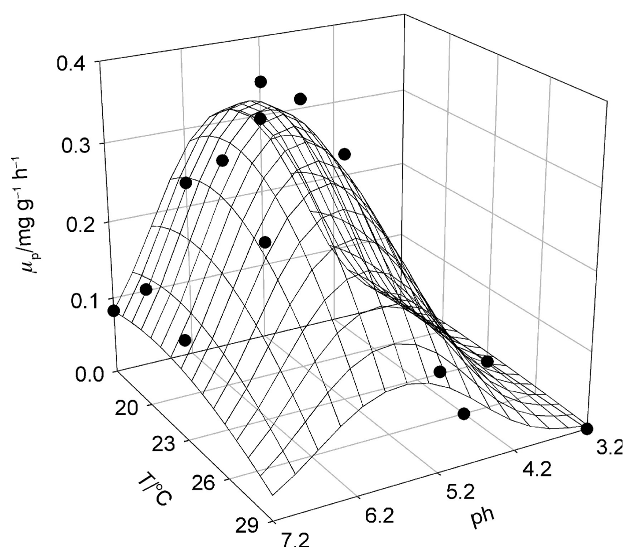


Fig. 2 – *Formal kinetic model (Eq. 7) fitted on the experimental values of specific product formation rate (•)*

To simplify the application of the model for other recombinant proteins, a normalized form of the production rate is proposed as it is given in Table 4. Applying this normalized μ_{P0}, the relative value of specific product formation rate varies between 0 and 1 for different $[H^+]/T$ sets. The normalized equation is capable to predict the rate of specific product formation of a recombinant extracellular protein of *P. pastoris* GS115 $\mathrm{Mut^S}$ at any pH and temperature combination in the range 3.2, 7.2 for pH and 17, 29 °C for temperature by the realization of one fermentation at one single parameter set. This simple calculation of specific product formation rate is useful if a mathematical description of pH and temperature dependence of specific recombinant protein degradation is known. By combining the two rate equations, the most efficient production condition can be calculated with respect to pH and temperature.

Conclusions

The recombinant protein formation of *P. pastoris* Mut^S shows strong pH dependence while the influence of temperature is less explicit. The volumetric productivity of HSA fermentation and the specific product formation rate have the same optima for pH and temperature (T), according to the statistical analysis of a series of fermentation runs, realized upon two consequent experimental designs in constant level substrate limiting conditions. The fact, that neither pH nor temperature has significant effect on the specific growth rate in the range of product formation implies that the volumetric productivity of recombinant *P. pastoris* Mut^S fermentations are mainly determined by the specific product formation rate. This observation supports the idea that, although, the capability of cells to grow extends for more acidic and higher temperature range, the product formation phase of the fermentation should be conducted at more limited conditions. The optimum production parameters are pH 5.7 and 20 °C according to our novel model for specific product formation rate. Both, the experimental results and the statistical model showed that fermentation among acidic conditions in order to avoid product degradation by neutral and alkaline proteases can not be successfully performed as a result of dramatic drop in the specific product formation range.

On the other hand, optimal product formation conditions on methanol do not favour growth on glycerol, mainly due to the low temperature value. As a conclusion, the cell mass formation phase of *P. pastoris* Mut^S fermentation has to be separated from the product formation phase in spite of the common practice. The cell mass production should be performed at the traditional pH 5.0 and 29 °C, whereas pH 5.7 and 20 °C favours the methanol limited recombinant product formation.

The presented empirical model describing the pH and temperature dependence of specific product formation rate is based exclusively on the HSA production of *P. pastoris* Mut^S, although holds greater potential of general use. The normalized model can be used for a series of various products by measuring the specific product formation rate only at one single pH/T set and by modifying only the value of production rate. If the model is further expanded with an additional part describing the protein degradation rate as the function of pH and temperature, the optimal circumstances for a certain recombinant protein can be calculated. This characterization of specific product formation rate can also be attached to former unstructured kinetic models[27,36,37] of *P. pastoris* fermentations in order to expand the description of this widely used expression system.

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List of symbols

m_{cons} – total mass of methanol consumption between two sample points, g

m_{add} – total mass of methanol addition between two sample points, g

γ_{ti} – methanol mass concentration in the sample taken at time i, g/ L^{-1}

V_i – volume of fermentation broth at time i, L

m_{ev} – total of evaporated methanol between two sample points, g

J_{HSA} – overall recombinant HSA productivity calculated for the substrate limited phase, g L^{-1} h^{-1}

γ_X – dry cell mass, g L^{-1}

t – time, h

μ_x – specific growth rate, h^{-1}

γ_P – recombinant product mass concentration, mg L^{-1}

z_{0-5} – parameters of second order statistical model

μ_P – specific product formation rate, $m_{protein} \cdot m^{-1}{}_{DM} \cdot t^{-1}$, mg g^{-1} h^{-1}

μ_{P_0} – protein specific production rate, $m_p \cdot m^{-1}{}_{DM} \cdot t^{-1}$, mg g^{-1} h^{-1}

K_1, K_2 – quantities of Michaelis pH function, mol L^{-1}

a, b – parameters of Arrhenius equations

ΔG_1, ΔG_2 – apparent Gibbs energy, J mol^{-1}

R – Regnault constant, J mol^{-1} K^{-1}

T – temperature, °C

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