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Optimization of temperature-glycerol -pH conditions for fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninurn)* anticoagulant peptide (AcAP-5) production by *Pichia pastoris*

ABSTRACT

This study was undertaken to determine the optimum pH, temperature and glycerol feed rate for the production of recombinant hookworm (Ancylostoma caninum) anticoagulant peptide (rAcAP-5) by Pichia pastoris using response surface methodology (RSM). A central composite design was used as an experimental design for allocation of treatment combinations in three blocks. The variables selected for study were pH, temperature and glycerol feed rate. pH was the most important variable affecting yield, specific yield and specific activity of rAcAP-5. Glycerol feed rate had a significant effect on the specific activity of rAcAP-5 (% of total secreted protein) while temperature did not have a significant effect on the responses. The data showed a trend that gave maximum responses and there was no blocking effect on the responses. The RSM formulated three second order polynomial empirical models relating to the responses. From these models it was possible to determine the optimum conditions variables for maximum yield of rAcAP-5 (1.2g I-1), the maximum specific yield of rAcAP-5 (11.5 mg g -1dry cell) and the maximum specific activity of rAcAP-5 (96% of total secreted protein).

Keywords: *Pichia pastoris;* recombinant fed-batch fermentation; *Ancylostoma caninum* anticoagulant peptide (rAcAP-5); response surface methodology

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Introduction

The methylotrophic yeast, *Pichia pastoris*, has been developed as a host for high level heterologous gene expression using the methanol-controlled alcohol oxidase (AOX1) promoter. *P. pastoris* has the potential for high expression levels, efficient secretion of product and growth to very high cell densities.[1] Regulation of heterologous gene expression by methanol has been shown to be simple, easy to scale-up, and cost-effective for industrial fermentation.[2]

The genome of *P. pastoris* contains two different AOX genes.[3] Recombinant *P. pastoris* strains are routinely obtained by integrating foreign DNA into one of two AOX genes or into the HIS4 (histidinol dehydrogenase) gene by homologous recombination.[2] If the desired gene is inserted into a site other then AOX1 locus, a mut$^+$ strain (*m*ethanol *ut*ilization) is generated that is fully capable of utilizing methanol. Insertion of the desired gene into the AOX1 locus results in a mut^s strain which grows slowly on methanol (μ = 0.01–0.04 h^{-1}) since the lower levels of alcohol oxidase provided from AOX2 gene are growth limiting. The disruption of both AOX1 and AOX2 results in a mut$^-$ strain that lack alcohol oxidase activity and thus can not grow on methanol as a sole carbon source.[2]

For the production of heterologous proteins by fermentation, a two-stage, high cell density (HCD) scheme is normally employed. In the first stage, the recombinant yeast is cultured in a defined salts medium on a non-fermentable carbon source, such as glycerol. Upon depletion of glycerol, the second phase is initiated by adding methanol, which induces protein production. A third phase (transition phase) may also be included between the growth and production phase, in which the culture is primed for induction by feeding glycerol at growth-limiting rates.[4] Chiruvolu *et al.*[5] found that addition of a transition phase reduced the total induction time of 15 h for the mut$^+$ strain. In the production phase, the concentration of methanol in the fermentor is crucial for the mut$^+$ strain which is very sensitive to methanol.[6,7]

Over 30 heterologous proteins of commercial interest have been successfully produced with *P. pastoris*. *P. pastoris* grown to HCD produced intracellularly 12 g l^{-1} of tetanus toxin fragment C which compromised 27% of total cell protein.[8] Tick anticoagulant peptide (TAP) was expressed in a mut$^+$ strain and 1.7 g l^{-1} of rTAP was produced.[9] The same gene expressed in *S. cerevisiae* yielded 200–250 mg l^{-1} of rTAP.[10,11] High-level expression of human tumor necrosis factor,[12] pertactin (P69),[13] human insulin-like growth factor,[14] kunitz protease inhibitor,[15] α-amylase,[16] interferon-τ,[17] mouse epidermal growth factor,[18] aprotinin,[19] bovine lysozyme precursor,[20] invertase,[1] HIV-1 envelope protein[21] have also been reported in *P. pastoris*.

Ancylostoma caninum anticoagulant peptide

It has been known for almost a century that hookworms produce at least one anticoagulant agent, although its nature was not known. Hotez and Pritchard[22] found that hookworms produce a substance which prevents aggregation of platelets in the test tube. Capello *et al.*[23] later isolated an 8.7-kDa peptide from *Ancylostoma caninum* (AcAP) that displayed anticoagulant activity. The researchers purified and measured the properties of AcAP on blood coagulation factor Xa and found that AcAP bound to free factor Xa and factor Xa in a prothrombinase complex. AcAP prolonged clotting time more than either hirudin or tick anticoagulant peptide.

Recently, an entire family of anticoagulant peptides have been described from *A. caninum* (AcAP-5, AcAP-6 and AcAPc-2).[24] These small proteins of 75–84 amino acids have now been expressed in *P. pastoris* and recovered as active secreted proteins. In this report we have devised a three-stage fed-batch fermentation process for the production of recombinant hookworm (*A. caninum*) anticoagulant peptide (rAcAP-5) in *P. pastoris*. The fermentation was optimized by determining the effect of pH, temperature and glycerol feed rate on the production of rAcAP-5.

Material and methods

Organism

A *Pichia pastoris* mut$^+$ strain expressing rAcAP-5 was provided by Corvas International Inc., San Diego, CA. The AcAP-5 cDNA was cloned into the *P. pastoris expression* plasmid pYAM&SP8 as described by Stanssens *et al.*[24]. A HIS$^+$, mut$^+$ transformant of strain GS115 was chosen and characterized for AcAP-5 expression using fXa inhibitory activity.[9] A single copy of the AcAP-5 gene was integrated into the recombinant *Pichia* host genome at the His4 locus as determined by Southern blot analysis[9] using both the AcAP-5 cDNA and *HIS4* gene as hybridization probes. rAcAP-5 was secreted into the fermentation broth.

Cultivation

The strain was maintained on minimal dextrose agar (MD)[25] plates at 4°C and was kept fresh by transferring onto MD plates every month. The fermentation inoculum was prepared by inoculating 10 ml of minimal glycerol medium (MGY)[25] in a test tube with a loopful of culture from a single colony. The culture was grown overnight at 200 rpm in the rotary shaker at 30°C. The test tube was used to inoculate a 250-ml shake flask containing 50 ml of buffered yeast nitrogen base (YNB)[25] medium supplemented with 2% glycerol and incubated at the same condition. The 50-ml culture was used to inoculate a 1.5-l working volume fermentor containing 700 ml of FM22[9] medium.

Fermentor system

Three 1.5-l working volume multigen fermentors (New Brunswick Scientific Co., Inc., NBS) were used in this study. The fermentors were equipped with dissolved oxygen, pH (Valley Instrument Company, INC, Exton, PA) and temperature control. Adding pure oxygen to the air accommodated the elevated oxygen demand of the high-density culture. The Biocommand fermentation software (New Brunswick Scientific Co., Inc., NBS) was used to record pH and DO.

FM22 medium containing, 4% glycerol as a carbon source, was used as fermentation starting media. One milliliter of a PTM1[8] trace salts solution was added and the pH was adjusted to 5.0 with 10 M KOH. The pH was controlled at 5.0 by the addition of 30% (v/v) NH_4OH during entire fermentation. The batch phase lasted 20–24 h.

Upon exhaustion of glycerol, indicated by a sharp increase in dissolved oxygen, glycerol fed-batch phase was started by adding 50% of glycerol solution at a desired fed rate. Temperature was maintained at 30°C. During the glycerol fed-batch phase, stirring speed was increased to 800 rpm and maintained for the duration of the fermentation. After the 4-h glycerol fed-batch phase, a methanol feed [100% methanol + 4 ml of PTM1 salt solution + 1 ml of biotin solution (200 mg l^{-1}) was started at 3 ml l^{-1} h^{-1} based on

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the initial volume of the fermentor. Acclimation of culture to methanol took 2–5 h. If the induction pH was < 5 the pH was allowed to drop and then maintained by base addition. The dissolved oxygen of the fermentation was maintained between 25 and 60% of saturation by manually adjusting the air flow rates. At this stage, temperature was set to the desired value. Once the culture was acclimated to the methanol, the feed was increased to 4 ml l^{-1} h^{-1} for 4 h. The methanol feed was gradually increased until a feed rate of 9 ml l^{-1} was reached. The methanol induction phase lasted for 84 h with samples taken at 12-h intervals.

Analytical procedure

Determination of dry weight cell concentration. Turbidity in the fermentation broth was measured with a spectrophotometer (DU-70 Beckman Inc., Fullerton, CA) at 600 nm. The dry weight of the cells was determined from a 1-ml culture sample which was centrifuged at 10,000 rpm for 10 min, the pellet was collected, washed with de-ionized water twice, and dried at 80°C for 24 h. Dry weight cell concentration was estimated from $OD_{600\ nm}$ (10–500 OD) using the following equation;

$$\text{Dry cell weight } (g\ l^{-1}) = 0.2652 * OD_{600\ nm} - 0.2902$$

Ethanol and methanol determination. Ethanol and methanol concentrations were determined with a Hewlett Packard 5880A gas chromatography using a column packed with chromosorb w(AW) [80/100; 10% carbowax 20M-TPA + 0.1% H_3PO_4 (Supelco, Belefonte, PA)]. Nitrogen, at 18 ml min^{-1}, was used as a carrier gas and *N*-propanol was used as an internal standard. The flame ionization detector and injector were maintained at 350 and 250°C, respectively. The column oven temperature was constant at 80°C.

Determination of protein concentration. Three different variables were measured in the fermentation broth; total protein, biologically active rAcAP-5 and biomass concentration. This resulted in three different responses, rAcAP-5 concentration in the fermentation broth (g l^{-1}), rAcAP-5 produced per gram dry cell mass (mg g^{-1} dcw) and percentage of total secreted protein. Total protein in the fermentation broth was quantified using the BioRad protein assay (BioRad, Richmond, CA). Concentration of biologically active rAcAP-5 in the fermentation broth was determined by the standard assay for protrombin (PT) time as described by Cappello *et al.*[23]

Total rAcAP-5 was determined by HPLC using a 4.6 × 150-mm, 5-μm column Vydak C18 as described by Stanssens *et al.*[24]. Flow rate was 1 ml mm^{-1}. Samples were also analyzed by 15%-SDS-PAGE under non-reducing conditions[26] and visualized by coomassie blue staining.

Response surface methodology

Response surface methodology (RSM) is a group of techniques used to evaluate relationships between one or more measured responses and a number of quantitative independent variables that may have important effects on the measured responses.[27] It requires fewer treatments than equivalent factorial designs to evaluate how the independent variables affect the measured response.[28] This procedure was used to understand the overall effect of pH, temperature and glycerol feeding rate on the production of AcAP-5 by *P. pastoris*. Since this study was limited to pH, temperature and glycerol feeding rate, all other parameters influencing the yield, specific yield and specific activity of AcAP-5 were fixed at constant values as described above.

A central composite design for three factors at five levels each in three blocks was conducted to evaluate the effect of factors on the production of rAcAP-5, as described by Myers.[29] The three factors were pH, temperature during protein production (induction) phase and glycerol feed rate during the fed-batch phase. The responses were yield (g l^{-1}), specific yield (g g^{-1} dcw) and specific activity (percentage of total secreted protein) of rAcAP-5. Specific yield was chosen as the response to determine how high cell density and yield correlated in fed-batch fermentation of *P. pastoris*.

The actual values, which were chosen from preliminary studies, and corresponding coded values of the three independent variables are given in *Table 1*. The independent coded values were $-\alpha$ (-1.633, lowest level), -1, 0 (middle point), 1 and α (1.633, highest level).

The entire experimental setup was a central composite design, with the axial portion being in one block (fermentor A), and the factorial portion being divided into two blocks (fermentor B and C). Block sizes were 8, 6 and 6, respectively, with a total of 20 fermentations.

The use of central composite design allows the dependent variables to be expressed as a polynomial model of the form:

$$\begin{aligned} y_i = {} & \mu_i + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 \\ & + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 \\ & + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \end{aligned} \quad (1)$$

where

y_i = responses (yield, specific yield, specific activity);
μ_i = intercept for fermentor or block i ($i = 1, 2, 3$);
X = factors (X_1 = pH, X_2 = temperature, X_3 = glycerol feed rate);
β = partial regression coefficient; and
ε = residual error.

This model was fit to data generated from the experiment using RSREG procedure of SAS/STAT.[30] The fitted model [Eq. (1)] was evaluated for each response variable based upon the multiple correlation coefficients (R^2), regression parameter significance (P value) and tested lack of fit. A response surface graph was plotted from the equations.

Results and discussion

The three-factor central composite design and the values of the responses are shown in *Table 1*. The lack of fit for each of the three models was insignificant meaning the model form in Eq. (1) was adequate. The estimated partial regression coefficients for the three quadratic models, R^2 values and the results of the significance tests on the coefficients are given in *Table 2*. The pH had the largest effect ($P < 0.05$) on yield, specific yield and specific activity of rAcAP-5. Glycerol feed rate affected only specific activity ($P < 0.05$). Tem-

Table 1 The 3-factor central composite design coded values, factor levels and the values of the responses

Exp. No.	Coded values			Factors			Responses		
	X_{pH}	X_T	X_{gly}	pH	T	Gly	Yield[a]	Specific yield[b]	Specific activity[c]
B1	1	1	1	6.2	30.5	20	0.547	5.06	36.35
B2	1	−1	−1	6.2	25.5	5	0.643	6.05	21.30
B5	−1	1	−1	3.8	30.5	5	0.112	1.19	15.96
B3	−1	−1	1	3.8	25.5	20	0.272	3.96	43.60
B6	0	0	0	5	28	12.5	0.442	3.52	32.74
B4	0	0	0	5	28	12.5	0.108	2.02	19.91
C2	1	1	−1	6.2	30.5	5	0.752	7.50	79.16
C1	1	−1	1	6.2	25.5	20	0.615	5.12	23.06
C4	−1	1	1	3.8	30.5	20	0.063	0.77	18.57
C6	−1	−1	−1	3.8	25.5	5	0.144	1.25	11.08
C3	0	0	0	5	28	12.5	0.341	3.96	25.29
C5	0	0	0	5	28	12.5	0.315	3.20	46.93
A1	$-\alpha$	0	0	3	28	12.5	0.637	5.89	59.65
A3	α	0	0	7	28	12.5	1.029	9.98	89.93
A8	0	$-\alpha$	0	5	24	12.5	0.248	1.85	25.58
A5	0	α	0	5	32	12.5	0.008	0.37	4.21
A4	0	0	$-\alpha$	5	28	0	0.024	0.70	3.72
A6	0	0	α	5	28	25	0.037	0.38	8.22
A2	0	0	0	5	28	12.5	0.638	5.74	65.31
A7	0	0	0	5	28	12.5	0.375	3.90	42.27

$\alpha = 1.633$
[a]rAcAP-5 g l^{-1}
[b]rAcAP-5 mg g^{-1} dcw
[c]% of total secreted protein

perature did not have a significant effect on the measured responses. Also interactions between variables were found to be insignificant. The models developed for yield, specific yield and specific activity of AcAP-5 seemed adequate with satisfactory R^2 values (0.854, 0.894, 0.853, respectively). There were no significant fermentor effects on the responses.

Effect of pH on rAcAP-5 production

P. pastoris is capable of growing over a broad pH range, thus AcAP-5 expression was evaluated from pH 3 to 7. Although this range of pH has little or no effect on the growth rate,[31] it can have an effect on secreted recombinant proteins since proteases activity in the fermentation medium is affected by pH.[2,14] Changing the pH of the medium from 5, the optimum for growth,[31] is a tactic used for optimization studies.[2,14,18,32,33] In addition, optimum pH for shake flask culture may not necessarily translate into optimum conditions for fermentation as Zhu *et al.* found.[32] They determined the optimum pH for production of α-glucosidase in shake flask culture was 4.5, but during fermentation the optimum was 6.5.

The pH was the most significant factor effecting

Table 2 Parameter estimates for polynomial regression model

Statistic	Yield	Specific yield	Specific activity
Fermenter A	−6.038	−61.981	−672.495
pH	−1.380	−15.184*	−176.388*
Temperature	0.634	6.553	69.518
Glycerol	0.134	1.693	24.430*
pH × pH	0.127**	1.209**	9.056**
Temperature × pH	0.012	0.198	3.803
Temperature × temperature	−0.012	−0.130	−1.461
Glycerol × pH	−0.004	−0.079	−1.058
Glycerol × temperature	−0.002	−0.030	−0.497
Glycerol × glycerol	−0.002*	−0.018*	−0.209*
Fermenter A vs. B	0.005	−0.087	3.948
Fermenter A vs. C	−0.011	0.025	−4.825
R^2	0.854	0.894	0.853

*Significant at 0.05 level
**Significant at 0.01 level

rAcAP-5 production with a minimum saddle point between 3.5 and 4.5 (*Figure 1*). The low response value between pH 3.5 and 4.5 is due to the susceptibility of rAcAP-5 to proteolytic degradation at this pH range as the presence of host proteases in *Pichia* fermentation is well documented.[2,14,18,33,34] The pH effected all the response variables in a same manner with a maximum response at pH 7, which was unexpected since the pH range typically considered for *P. pastoris* is 2.8–6.5.[33] It was our intent to exceed this range so as to see a maximum, this did not occur. It is anticipated that protein production would drop off as the pH continued to increase since *P. pastoris* has a growth range of pH from 3 to 7.[31] Based on this study, optimum protein expression should occur between 6.8 and 7.0 to avoid proteolytic degradation.

Effect of temperature on rAcAP-5 production

Most *Pichia* fermentation are run at an optimum temperature of 30°C[31] although the effect of temperature on recombinant protein production in *P. pastoris* has not been documented. It was stated that temperature above 32°C could be detrimental to protein expression and could lead to cell death.[25] In our experimental design, the temperature ranged from 24 to 32°C. From the model it appeared that temperatures above 30°C were not appropriate for rAcAP-5 production, *Figure 1*. It is well known that elevated temperature result in cell death, which will intern result in cell lysis and higher protease activity in fermentation media. Since rAcAP-5 was secreted to fermentation medium, it is expected that cell lysis would decrease recombinant protein production. Temperatures of between 24 and 30°C were optimum for rAcAP-5 yield (*Figure 1*).

Effect of glycerol feed rate on rAcAP-5 production

High glycerol feed rates or prolonged feedings during the glycerol fed-batch phase will result in high cell densities in the fermentor. High cell-density fermentation is generally desirable since the concentration of secreted protein in the medium will most often increase proportionately to cell density.[34] However, this is not always the case. Ando *et al.*[35] found that as cell density increased prior to induction phase, antibody production decreased in *P. pastoris*.

Effect of glycerol feed rate on the rAcAP-5 yield is shown in *Figure 1*. Interestingly, higher glycerol feed rates before the induction phase reduced the responses (Run A6). Higher glycerol feed rates during the fed-batch phase resulted in accumulation of over 100 mg l^{-1} ethanol, which is a strong repressor for AOX1 promoter, in runs A6, B1, B3, C1 and C4 at the end of glycerol fed-batch phase. No ethanol was detected in all trials after six h of induction. It is anticipated that ethanol was assimilated first before methanol metabolism and protein expression could occur, since ethanol strongly represses methanol-utilizing enzymes at very low concentration levels.[6] The glycerol fed-batch phase (transition phase) is important for priming the cells for the induction phase, which can increase overall productivity.[5] Chiruvolu *et al.*,[5] in mut$^+$ strain, was able to reduce the total fermentation time by 15 h for

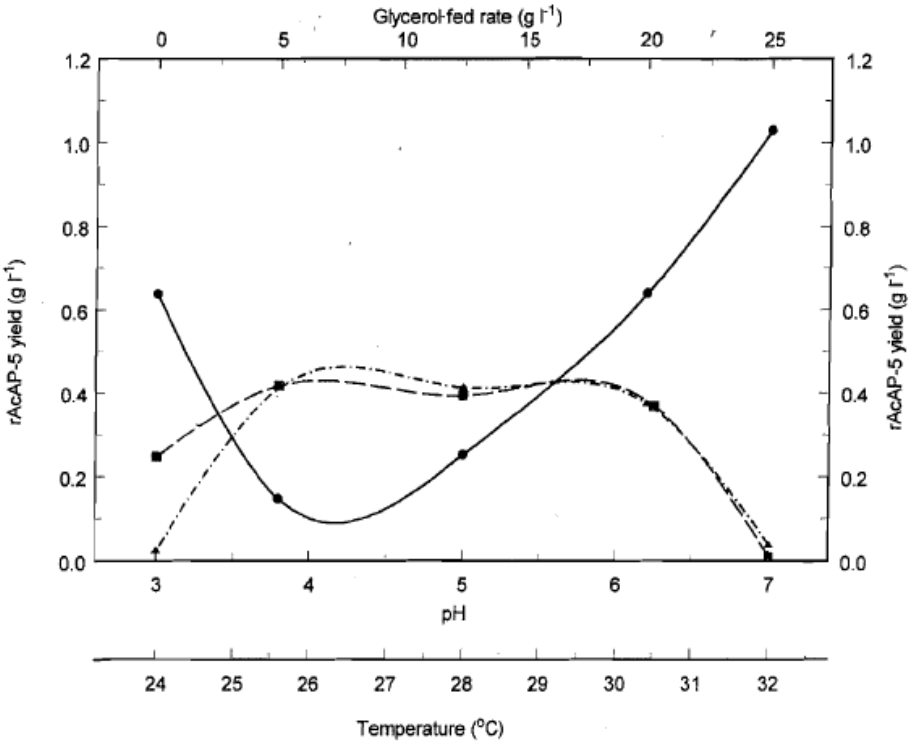

Figure 1 Effect of pH, temperature and glycerol feed rate on rAcAP-5 yield. Yield was determined by bioassay analysis of the fermentation broth after 84-h induction. ●, pH; ■, temperature; ▲, glycerol feed rate

Table 3 Optimum level of variables and predicted responses

	pH	Temperature (°C)	Glycerol (g l^{-1} h^{-1})	Predicted response
Yield (g l^{-1})	7.00	28	12.00	1.2
Specific yield (mg g^{-1} cell)	7.00	28	11.00	11.5
Specific activity (% of total secreted protein)	6.90	29	10.00	96

the same level of protein expression by incorporating a fed-batch phase vs. switching over to methanol right after the batch phase. They also found that a higher glycerol feed rate increased the amount of ethanol produced which decreased the specific activity of β-galactosidase in fed-batch fermentation of mut$^-$ strain of *P. pastoris*.

Chiruvolu *et al.*[5] reported reduced protein production for cells that were starved for carbon prior to induction. In this study it was determined that a 4-h-starvation condition (run A4) resulted in nearly no biologically active protein at the end of the fermentation, even though the cell density increased. It is difficult to speculate as to why no protein was produced, even though growth did occur on methanol.

Optimum conditions

Canonical analysis is a mathematical approach used to examine the overall shape of the response surface and to determine if the estimated optimal response point is a maximum, minimum, or a saddle point.[29] The results from the canonical analysis of the response surface indicated that the stationary points were saddle points. Thus, using canonical analysis with the RIDGE MAX option of the SAS/RSREG[30] procedure, the values of pH, temperature and glycerol were obtained to maximize the responses (*Table 3*).

A three-dimensional response surface was generated to show the interrelationships between pH and glycerol feeding rate using the regression equations in yield keeping temperature constant at 28°C (*Figure 2*). An effect of the factors on rAcAP-5 production is also presented in a two-dimensional graph (*Figure 1*).

Optimum conditions leading to maximum rAcAP-5 yield within the experimental region, as predicted by the model, were pH 7, 28°C and 12.0 g l^{-1} h^{-1} glycerol feed rate (*Table 3*). The conditions correlated well within the experimental data and with the conditions for run A3. Fermentation profiles of A3 is given in *Figure 3A*. Accumulation of rAcAP-5 is seen on SDS-PAGE (*Figure 3B*).

Conclusions

The production of rAcAP-5 using fed-batch fermentation process was accomplished using a central composite design. Second order-polynomial models accurately predicted the yield, specific yield and specific activity of rAcAP-5 as a function of the described factors. The conditions of pH 7, 28°C and 12.0 g l^{-1} h^{-1} glycerol feed rate within the experimental region gave the maximum yield (1.2 g l^{-1}) in the fed-batch fermentation of *P. pastoris* as predicted by the model. A value of 1.03 g l^{-1} was observed at these conditions (Run A3).

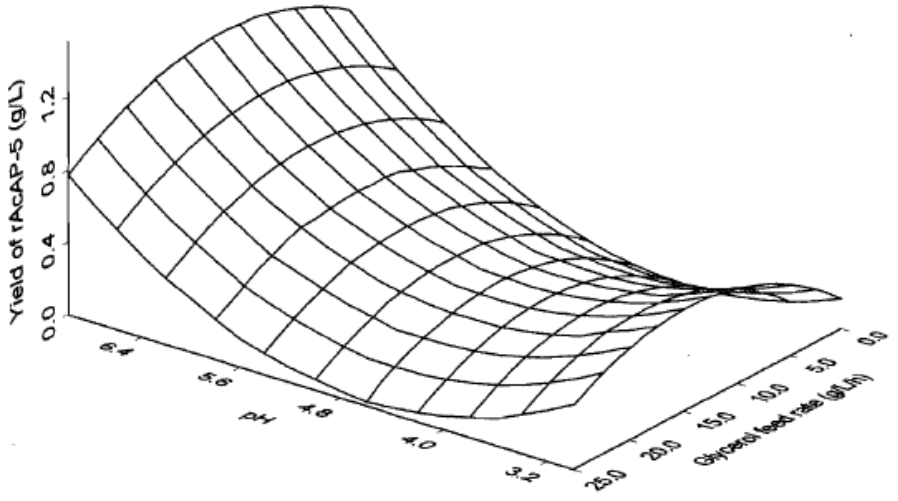

Figure 2 Response surface of rAcAP-5 yield as a function of pH and glycerol feed rate at constant temperature of 28°C

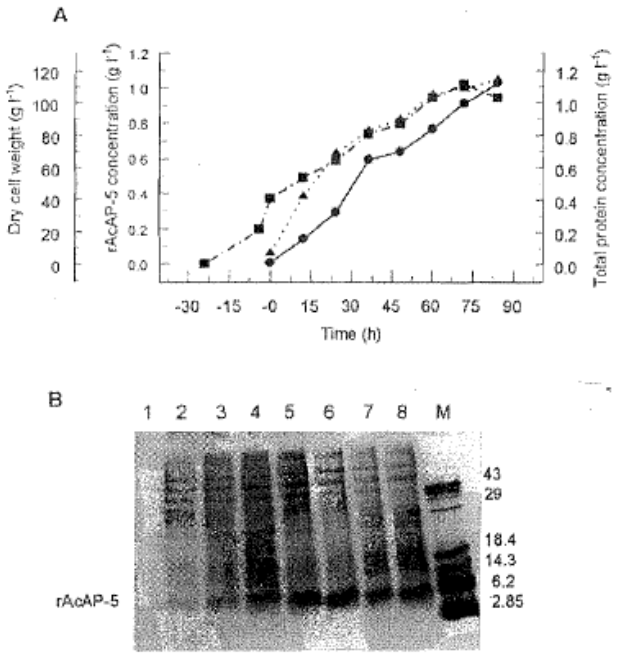

Figure 3 (A) Fermentation profiles of run A3. ●, rAcAP-5 yield; ■, dry cell weight; ▲, total protein. (B) SDS-PAGE of time course sample of run A3. Fermentation conditions are pH 7, 28°C and 12.5 g l^{-1} h^{-1} glycerol feed rate. Lanes 1–8 correspond to time points of induction 0, 12, 24, 36, 48, 60, 72 and 84 h, respectively, each lane containing 10 ml of fermentation broth. Lane M corresponds to the molecular weight markers

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