Journal of Industrial Microbiology, 9 (1992) 69–72
Elsevier

SIM 00379

Short communication

Some factors influencing the proportion of periplasmic hepatitis B virus pre-S2 antigen in the recombinant yeast *Hansenula polymorpha*

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(Received 18 April 1991; revision received 7 september 1991; accepted 9 September 1991)

Key words: Secretion; Periplasmic pre-S2 antigen; Recombinant protein; Experimental design; Methylotrophic yeast

SUMMARY

A central composite design (CCD) was used to evaluate, for the purpose of future process optimization, the influence of pH, yeast extract and ammonium chloride concentrations on the proportion of periplasmic hepatitis B pre-S2 antigen in the recombinant yeast *Hansenula polymorpha*. Each factor was tested at five levels, and a second order polynomial model for the proportion of periplasmic antigen was fitted to the treatment combinations. pH showed the greatest effect: the proportion of periplasmic antigen was greatly increased at the higher pH levels. At the higher pH levels used, the proportion of periplasmic antigen was enhanced by a high concentration of ammonium chloride. Additional experiments have confirmed both the validity of the selected model and the optimal conditions found. A significant correlation was found between the proportion of periplasmic antigen and the total yield of antigen. These results indicated that it should be possible to modulate the distribution of the pre-S2 antigen between the periplasm and the cytoplasm of the yeast.

INTRODUCTION

The hepatitis B virus pre-S2 antigen (middle surface antigen), described by Blum et al. [1], has been recently produced in significant amounts by a recombinant strain of the methylotrophic yeast *Hansenula polymorpha* under control of the methanol oxidase promoter [5,7]. The antigen was shown to be present as 22-nm particles identical to those found in the blood of patients infected with hepatitis B virus [5,7]. The pre-S2 antigen contains internal signal sequences able to direct the antigen across the cytoplasmic membrane; however, due to their large size, the 22-nm particles remain trapped in the periplasmic space. The periplasmic protein, nevertheless, can be easily released by treating the cells with a mixture of lytic enzymes, which simplifies the recovery and the purification of the antigen. Only a fraction of the total antigen produced is found in the periplasm [7]; the rest remains inside the cells.

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It has been observed that the proportion of periplasmic pre-S2 antigen produced by *H. polymorpha* varied to a certain extent with the physico-chemical conditions used for cultivation and expression. Many factors may be expected to influence the accumulation of a periplasmic protein, and these factors may also interact. A second order response surface technique was employed to efficiently determine the relationship between the proportion of periplasmic pre-S2 antigen (response variable) and three pre-selected experimental factors presumed to affect the response.

MATERIALS AND METHODS

Strain, media and growth conditions

The recombinant *Hansenula polymorpha* strain, the medium and culture conditions used were the same as described previously [5], except for the following modifications: no phosphoric acid was included; pH was adjusted to 4 with KOH and then to the desired value with NaOH; the levels of the three factors chosen for optimization are given in Table 1.

Pre-S2 antigen determination

Samples of the cultures were taken 6 days after inoculation and assayed for both periplasmic and cytoplasmic antigen exactly as described previously [5]. Cells were first treated with a lytic enzyme mixture to release periplasmic antigen then broken using glass beads to recover cytoplasmic antigen. Total antigen yield was the sum of both antigen fractions.

Quantification of the antigen was done as described earlier [5] using an enzyme-linked immunoassay method (ADI Diagnostics, Willowdale, Canada).

Experimental design

pH, yeast extract and ammonium chloride concentrations were selected as experimental factors based on preliminary observations. A second order response surface was presumed as described by the following second order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where Y is the percentage of periplasmic antigen; β_0 is the mean response; β_i, β_{ii}, β_{ij} are the measures of the effects of the variables X_i (independent), X_i^2 (second order), and $X_i X_j$ (interaction), respectively.

To estimate the parameters of the model, a central composite design (CCD) was selected [2,4]. The coded levels used in this design (i.e. -2, -1, 0, $+1$, $+2$) corresponded respectively to the following values: 4.3, 4.8, 5.3, 5.8, 6.3 for pH; 0, 10, 20, 30, 40 for NH_4Cl concentration (g/l); 15, 30, 45, 60, 75 for yeast extract concentration (g/l). Table 1 gives the settings of the design matrix and the measured responses. The experimental data were analyzed by the response surface regression procedure in the statistical analysis system [6].

RESULTS AND DISCUSSION

Using the selected CCD, 20 different treatment combinations, involving variations of pH and of the concentration of NH_4Cl and yeast extract, were tested in shake-flask experiments in order to measure their influence on the proportion of periplasmic antigen and on total antigen yields.

The experimental results in Table 1 were used to estimate the parameters of the second order polynomial model (see MATERIALS AND METHODS) in order to predict and quantify the effects of the three chosen factors on the proportion of periplasmic antigen. The statistics presented in Table 2 indicate that the selected model is likely to be adequate for describing the relationship between the proportions of periplasmic antigen and the experimental factors used within the experimental range: indeed, 93.6% of the total variation is explained by the model (R^2 in Table 2), and the F statistic for testing overall regression was significant [3]. Furthermore, from the *t*-test values in Table 2, it can be concluded that pH is likely to be the most significant parameter (parameters β_1 and β_{11}) followed by the second order term for ammonium chloride (β_{22}) and yeast extract (β_{33}) concentrations.

TABLE 1

Central composite design and response measurements

Run no.	pH level	NH_4Cl level (g/l)	Yeast extract level (g/l)	Percentage of periplasmic antigen	Total yield (ng/ml)
1	4.8	10	30	6.88	111.9
2	5.8	10	30	56.4	337.2
3	4.8	30	30	8.7	131
4	5.8	30	30	63.8	351.8
5	4.8	10	60	5.19	119.5
6	5.8	10	60	38.1	423.5
7	4.8	30	60	11.3	149.9
8	5.8	30	60	62.1	419.4
9	4.3	20	45	3.25	199.8
10	6.3	20	45	62.5	492.9
11	5.3	0	45	33.0	211.6
12	5.3	40	45	35.2	252.9
13	5.3	20	15	31.2	232.4
14	5.3	20	75	20.4	305.2
15	5.3	20	45	12.8	335
16	5.3	20	45	14.1	361
17	5.3	20	45	10.8	284.7
18	5.3	20	45	11.6	260.7
19	5.3	20	45	12.6	210
20	5.3	20	45	12.0	241.2

The model indicates that high pH levels, within the range of the model, greatly increased the proportion of periplasmic antigen (Figs. 1 and 2). Due to precipitation problems, however, the medium used cannot be brought to a pH level higher than 6.3. Yeast extract concentration exerts an overall small influence on the proportion of periplasmic antigen (see Fig. 2 and significance results in Table 2), whether at low or high pH levels: this effect becomes more pronounced at low pH values. One possible explanation is that yeast extract may protect the periplasmic antigen from proteolytic degradation at the lower pH values; a similar suggestion has been made to explain the effect of organic nitrogen sources [8]. Furthermore, ammonium chloride also had an effect on the proportion of periplasmic antigen at all pH values, which became more pronounced at higher pH (Fig. 1 and Table 2). In all cases, the proportion of periplasmic antigen reached a minimum value at the intermediate concentration (coded level 0) of ammonium chloride.

TABLE 2

Parameter estimates and statistics

Initial experiment				Verification experiment		
Parameter	Estimate	*t*-test	Significance level	Estimate	*t*-test	Significance level
β_0	13.2	4.6	0.001*	18.9	7.4	0.000*
β_1	19.2	10.6	0.0001*	18.8	14.3	0.000*
β_2	2.7	1.5	0.1626	1.7	1.3	0.208
β_3	−2.5	−1.4	0.1921	−2.85	−2.2	0.044*
β_{11}	5.6	3.9	0.003*	4.27	4.1	0.0008*
β_{12}	2.9	1.2	0.278	3.55	1.6	0.137
β_{22}	5.9	4.1	0.002*	3.0	2.9	0.010*
β_{13}	−2.6	−1.01	0.335	−1.8	−0.8	0.439
β_{23}	2.5	1.02	0.336	1.55	0.68	0.504
β_{33}	3.8	2.7	0.024*	4.25	4.1	0.0008*
F-test	16.35 (significant with 9, 10 df)			26.74 (significant 9, 16 df)		
R^2	0.9364			0.9377 (indicative only)		

* Lack of fit *F*-test: 2.99 (not sign., 5, 11 df).

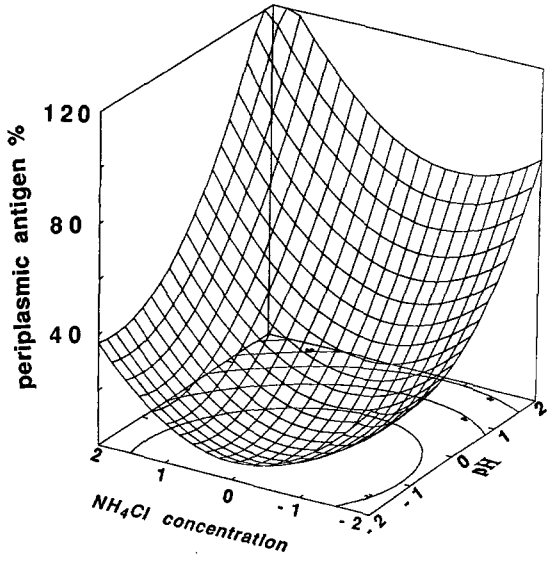

Fig. 1. Influence of pH and of ammonium chloride concentration on the proportion of pre-S2 antigen recovered from the periplasmic fraction of *Hansenula polymorpha*. The response surfaces were generated using a second order polynomial model fitted to data from a central composite design. Yeast extract concentration fixed at the +1 level (Table 1).

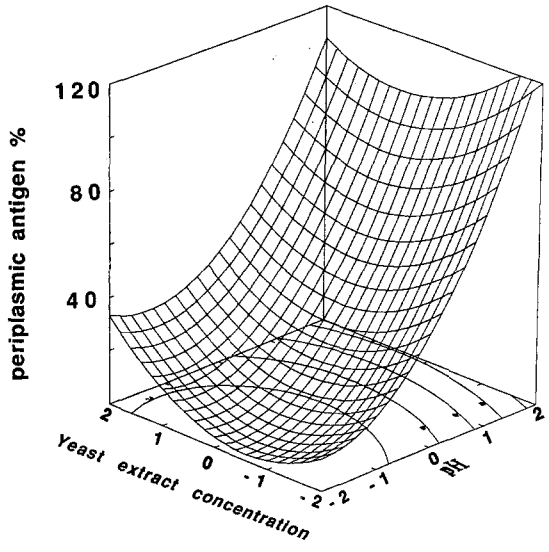

Fig. 2. Influence of pH and yeast extract concentration on the proportion of pre-S2 antigen recovered from the periplasmic fraction of *Hansenula polymorpha*. The response surfaces were generated from a second order polynomial model fitted to data from a central composite design. Ammonium chloride concentration fixed at the +1 level (Table 1).

To confirm the validity of these results, the same experiment was repeated with duplicates at all the axial points (-2, $+2$ levels). These duplicated levels and the six centre points provided a sufficient number of observations for estimating the pure error sum of squares and testing for lack of fit which was not significant at a 5% level [3]. In Table 2 the parameter estimates for both experiments indicate the same response within the limits of experimental error. Furthermore, the best predicted conditions (i.e. NH_4Cl concentration at 40 g/l, yeast extract concentration at 15 g/l, and pH 6.3) were verified in shake flask experiments leading to 80% of the antigen found in the periplasmic space.

A significant correlation was found between the percentage of periplasmic antigen and the total yield of antigen (i.e. Pearson correlation coefficient = 0.82), although a high proportion of periplasmic antigen was not always associated with high yields of antigen (Table 1).

This study establishes, for the first time, a quantitative relationship between culture conditions and the proportion of a secreted heterologous protein in *H. polymorpha*, which will be important in order to facilitate protein recovery. Furthermore, these results provide a rational background for studying the influence of each of these factors, thus allowing a better knowledge of the expression system.

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