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SHORT COMMUNICATIONS

Modeling *Tetrahymena thermophila* growth and protease production

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Oxygen concentrations stimulated growth (maximum number of cells) and protease secretion by *Tetrahymena thermophila*. Agitation and aeration conditions for growth and protease secretion were optimised by a central composite design. The best optimised combination was a stirrer speed of 338 rpm and an aeration of 1 vvm. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 58–61.

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Introduction

Protozoa have been little used as sources of commercially produced metabolites. *Tetrahymena*, which is fairly easy to mutagenize, is one of the protozoa presenting a high biotechnical potential for enzyme production [12]. It is also a model for growth, cell cycle and nutritional studies [16]. These protozoa can be cultivated on a large scale [9] on medium containing industrial by-products [6].

Tetrahymena secreted large amounts of lysosomal hydrolases into the growth medium, in particular proteases [1]. The secreted enzymes were glycosylated like those produced by mammalian cells, and unlike those produced by yeast and bacteria.

Optimisation of fermentation conditions are a prerequisite to improve process economics in metabolite production. This work reports the effects of dissolved oxygen concentration, agitation and aeration on the growth and on a model of secreted hydrolase production: protease, in batch fermentation.

To optimise and model *Tetrahymena* growth and protease production, full or fractional factorial designs can be used to measure the response to several parameters which vary simultaneously as well as to determine the interactions between them through a limited number of trials [5,7,8]. The central composite design method was chosen and a polynomial model is used to quantify the relationship between the values of three measurable response variables (generation time, maximal population and protease production) and a combination of experimental factors presumed to affect the response (agitation and aeration).

Materials and methods

Strain and cultivation

Investigations were carried out with *Tetrahymena thermophila* BIII, originally obtained from the Carlsberg Institute, Copenhagen, Denmark. Batch fermentations were carried out in 2-l fermentors with turbine impellers (Biolafitte, Saint Germain en Laye, France) and equipped with digital control units. The temperature was kept constant at 28°C. Dissolved oxygen tension and pH were not regulated, except if mentioned. The chosen aeration and agitation are given in the results section. Mye medium [14] was composed of 1% (w/v) yeast extract and 1% (w/v) skimmed milk. Fermentors containing 1 l medium supplied with 0.001% (v/v) antifoam (Sigma, ref. A6426, Quentin Fallavie, France) were autoclaved for 25 min at 120°C. Inocula (10 vol.%) were exponentially growing cultures to obtain 10^4 cells/ml in the fresh medium. Cells were counted electronically (Coulter counter Z1, Beckman Coulter France, Roissy COG, France).

Enzyme assay

Cells were removed from the medium at the end of the exponential phase by centrifugation at 16°C for 30 min at $300\times g$ (to avoid damaging cells). The proteolytic activity in the cell-free supernatant was measured spectrophotometrically using hemoglobin as substrate (19.8 g/l) with modifications of the procedure of Beynon and Bond [3]. The buffer of the reaction was 0.2 M acetic acid–sodium acetate, pH 4.5. Substrate (1 ml) was added to 0.2 ml cell-free supernatant. After incubation at 55°C for 20 min, the reaction was stopped by adding 2 ml trichloroacetic acid (5% v/v). After 30 min, precipitates were removed by centrifugation at $4500\times g$ for 20 min and tyrosine was assayed as described previously [13]. One proteolytic unit corresponds to the amount of enzyme required for the release of 1 μmol of tyrosine per minute. Results were expressed as milliproteolytic unit per ml (mU/ml).

Statistical analysis

To optimise and model *Tetrahymena* growth and protease production, we chose the central composite design, a widely used method. This method was adopted to assess the combined effect of agitation and aeration on generation time, maximal population and production of proteases. Levels of the two

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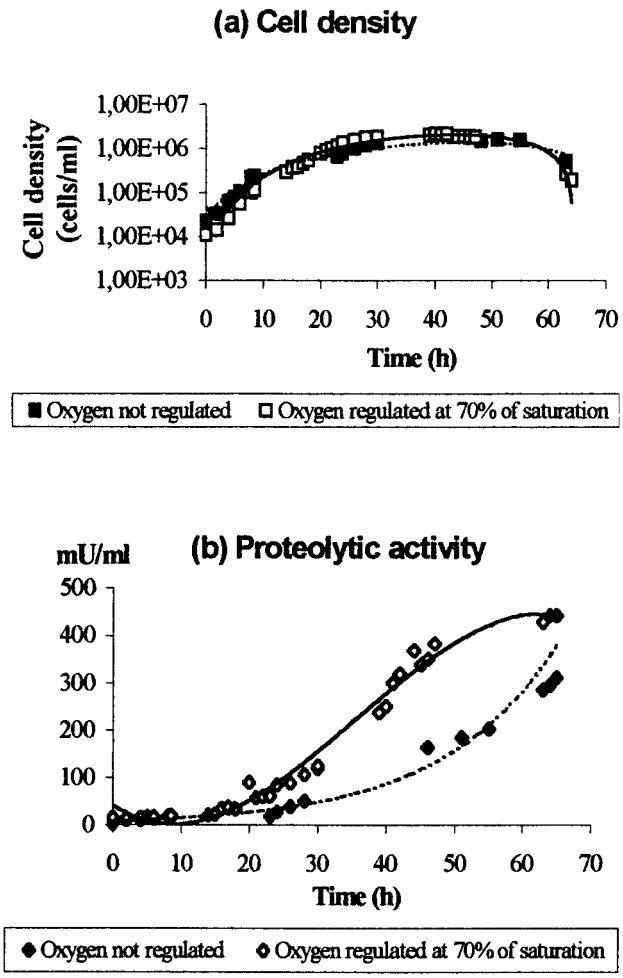

Figure 1 Influence of dissolved oxygen (regulated or not) on the growth (a) and protease production (b) of *T. thermophila*.

factors (agitation and aeration) were fixed into a region supposed to be optimal according to previous results. Levels of agitation varied from 200 to 400 rpm. Levels of aeration varied from 0.5 to 2.0 vvm. Other levels were deduced from both minimum and maximum. Other details concerning the method can be found in Box and Draper [4] and Box *et al.* [5].

Data obtained were analysed by multiple regression using Minitab [11] software and the following equation was established to fit the data and calculate the optimal combination of agitation and aeration.

$$y = b_0 + b_1(\mathrm{AE}) + b_2(\mathrm{STI}) + b_3(\mathrm{AE})^2 + b_4(\mathrm{STI})^2 + b_5(\mathrm{AE})(\mathrm{STI}) + \varepsilon$$

where y: generation time (min), maximal population (10^6 cells/ml) or proteolytic unit per cell; b_0 to b_5: regression coefficients of the model; AE: aeration in vvm; STI: stirrer speed in rpm; (AE)(STI): interaction term; ε: error term (residual) supposed to be of null expected value and constant variance for hypothesis testing. First partial derivatives were calculated to obtain optimal values.

Results and discussion

Effects of aeration and agitation on growth and protease secretion

Tetrahymena is an aerobic organism and its growth is influenced by oxygen concentration in the medium. Without aeration, growth

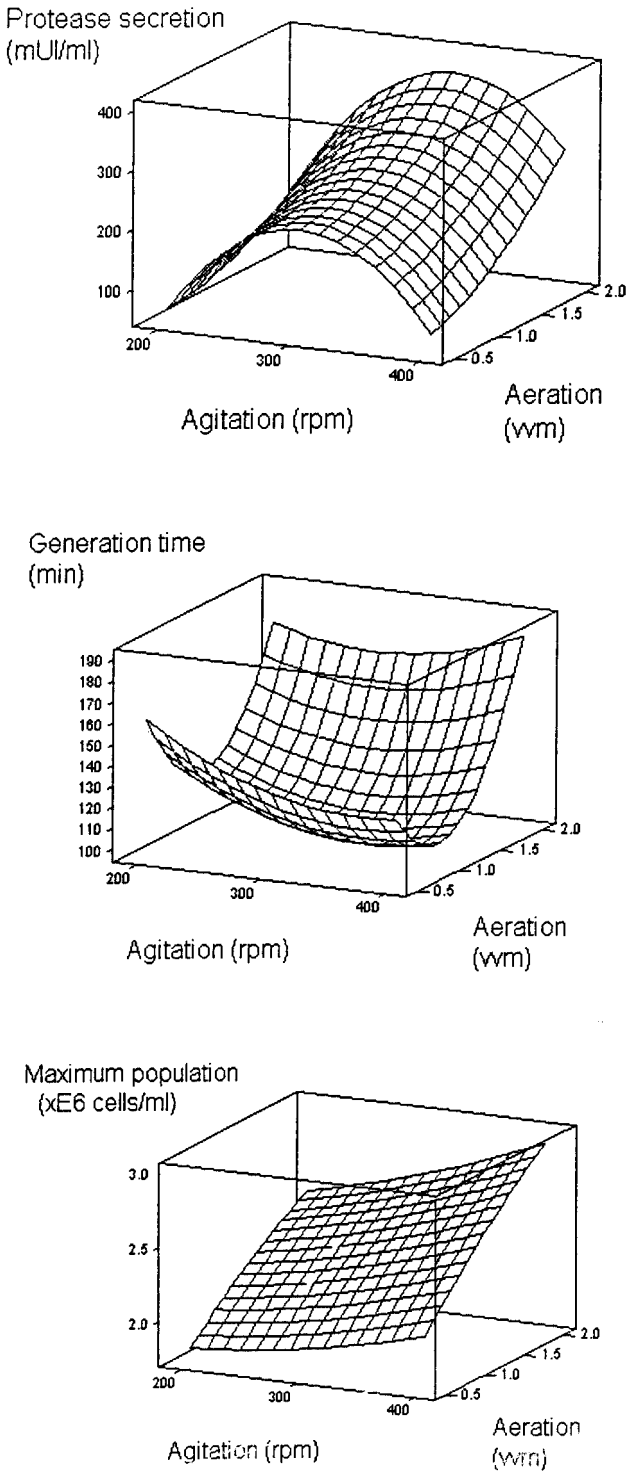

Figure 2 Response surfaces obtained for generation time, maximum population, and secretion of proteases by means of central composite design for each combination of agitation and aeration.

Table 1 Best combinations of agitation and aeration and corresponding optimal generation times and maximal populations

Optimal conditions	Generation time (min), 101	Maximal population (10^6 cells/ml), 2.26
Agitation (rpm)	338	338
Aeration (vvm)	1	1

remained low (maximum cell concentration about 10-fold lower than that in aerated cultures) and little protease production was detected. Dissolved oxygen in the liquid phase was measured with an O_2-electrode. Conditions of agitation and aeration were chosen to avoid damaging cells and to test protease secretion in medium (not global protease production).

Figure 1a and b demonstrate the time course of dissolved oxygen (pO_2) for this experiment. When aeration and stirrer speed were not regulated, they were chosen at 90 litre gas per hour (1 vvm) and 300 rpm, respectively. Up to 10 h fermentation, pO_2 values were constant and then decreased from 10% of saturation. When dissolved oxygen was maintained at 70% of saturation by controlling the air flow and keeping agitation speed constant at 300 rpm, we did not notice a longer stationary phase (Figure 1a). The two types of culture (regulated or not) yielded similar numbers of cell but maximal cell production in regulated cultures (2.1×10^6 cells/ml) was 20% higher than in unregulated cultures (1.7×10^6 cells/ml).

Increased biomass was obtained when oxygen was regulated. In the same way, Berline and Antipa [2] demonstrated that batch cultures of *Tetrahymena* entered stationary phase when they became oxygen limited. This is not in agreement with the results obtained by Hoffman and Cleffman [10] who noticed that greater oxygen concentrations in the medium did not significantly improve cell yield.

Protease production was 66% higher in oxygen cultures which were regulated than in unregulated cultures, although cell numbers were similar. When pO_2 was not regulated high protease production occurred at the end of growth, because a lot of cells lysed (Figure 1b) in contrast to cultures where pO_2 was not regulated and protease was more constantly secreted. Tiedtke *et al.* [15] noticed that Ca^{2+} stimulated secretion of hydrolases (including protease). Our results show that when oxygen is maintained at a sufficient concentration protease secretion is stimulated.

Optimised conditions of aeration and agitation

We modeled only protease secretion in the medium without damaging cells. This could be important in view of possible further process developments such as continuous culture with recycle cells.

Models of response surfaces (Figure 2) obtained with the central composite design were determined using multiple regression analysis. Regression models could be used only between the levels of combined factors chosen for the central composite design. Following are significant regression equations linking generation time (min), maximal population (10^6 cells/ml), secretion of proteases (mU/ml) and agitation (rpm), aeration (vvm).

$$\begin{aligned}\mathrm{GT} = {} & 374.99 - 0.929(\mathrm{STI}) - 219.17(\mathrm{AE}) \\ & + 0.001155(\mathrm{STI})^2 - 81.01(\mathrm{AE})^2 + 0.138(\mathrm{STI})(\mathrm{AE})\end{aligned}$$

$$\begin{aligned}\mathrm{MP} = {} & 1.65 - 0.00157(\mathrm{STI}) + 0.478(\mathrm{AE}) \\ & + 0.00000419(\mathrm{STI})^2 - 0.0817(\mathrm{AE})^2 \\ & + 0.000798(\mathrm{AE})(\mathrm{STI})\end{aligned}$$

$$\begin{aligned}\mathrm{PROT} = {} & -1101 + 8.92(\mathrm{STI}) - 115(\mathrm{AE}) \\ & - 0.0151(\mathrm{STI})^2 + 50.5(\mathrm{AE})^2 + 0.304(\mathrm{AE})(\mathrm{STI})\end{aligned}$$

where GT: generation time; MP: maximum population, PROT: secretion of proteases in medium. With smaller values, the model could not be applied as confirmed by preliminary tests. Stronger values of agitation were limited because cells could be damaged. Significant interaction terms between agitation and aeration were noticed, indicating that the effect of aeration was dependent on agitation value. Optimal values of aeration and agitation (for dissolved oxygen=20% of saturation) were determined and then optimal values of generation time and maximal population were calculated (Table 1).

For maximal population, different best combinations of aeration and agitation could be chosen but for technical reasons, the same conditions as generation time were proposed: agitation 338 rpm and 1 vvm of aeration.

Optimal secretions of proteases were chosen in a confidence region after a simulation with different values of aeration and agitation, i.e. agitation 300–350 rpm and aeration 1–1.5 vvm.

In conclusion, to produce optimal biomass and protease secretion with *Tetrahymena*, dissolved oxygen concentration must be sufficiently maintained in the medium and a stirrer speed of 338 min and aeration of 1 vvm were required. As a next step for optimisation, a process integrating continuous fermentation coupled with tangential microfiltration will be tested to improve growth and protease production by *T. thermophila*.

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