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ORIGINAL PAPER

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Factorial designs: an efficient approach to choosing the main factors influencing growth and hydrolase production by *Tetrahymena thermophila*

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Abstract A fractional factorial design with eight trials was applied to select and model the effects of major factors, individually and in combination, on improving *Tetrahymena thermophila* growth and enzyme production. Regulated pH at 6.8 and olive oil at 0.5% (v/v) showed positive effects on fermentation, and increased cell growth parameters including generation time and maximal population formation. Lipase and protease production were also improved by these factors and were favoured by cultivation of *Tetrahymena* in darkness. This statistical experiment offers a beneficial and rapid screening procedure to select the most effective combination of factors influencing fermentation processes.

Keywords *Tetrahymena thermophila* · Statistical experimental design · Fermentation · Enzyme production

Introduction

Advances in fundamental and applied research require the constant introduction of novel biotechnological tools and useful model micro-organisms. Among the ciliated protozoa, *Tetrahymena thermophila* has been successfully cultivated with industrial media [3, 11] and under conditions [9] yielding a potential source of enzymes and metabolites [15]. The release of large quantities of hydrolases to the extracellular medium could also improve milk production [21]. Moreover, these protozoa are a model for development and growth, cell cycle and nutritional studies [22].

Unicellular organisms such as *Tetrahymena* produce metabolites that are functionally similar to mammalian hormones. Many of these hormones directly influence physiological processes [4, 12]. While melatonin synthesis by *Tetrahymena* is decreased in the presence of light, *Tetrahymena* growth seems to be relatively insensitive to light levels [12]. To date, there have been no reports on the combined influence of hormones, light, and culture conditions on hydrolytic enzyme production by *T. thermophila*.

To study biomass and hydrolytic enzyme production (lipases and proteases) by *T. thermophila*, we tested the combinatory effect of (1) the biogenic amine melatonin, (2) the biogenic amine serotonin, (3) the amine precursor tryptophan, (4) light, (5) olive oil, (6) regulated pH at 6.8, and (7) regulated dissolved oxygen at 50% on *Tetrahymena* cultures.

Studying one variable at a time might be appropriate in some situations, but fails to consider the combined effects of multiple factors involved in physiological processes. During fermentation, where operational variables interact and influence their respective effects on response, it is essential that the experimental method accounts for these interactions so that a set of optimal research conditions can be determined. Factorial designs fulfill this requirement. Empirical models and statistical analysis are extremely important in elucidating basic mechanisms underlying complex processes, thus providing better control and understanding of the model micro-organism. We have chosen fractional factorial design 2^{7-3} to quantify the individual and major interactive effects of the seven factors described.

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Material and methods

Strains and cultivation

Investigations were carried out with *T. thermophila* BIII, obtained from the Carlsberg Institute, Copenhagen. Batch fermentations were carried out in 21 fermentors with turbine impellers (Biolafitte, Saint Germain en Laye, France) equipped with digital control units. The temperature was kept constant at 28°C. The stirrer speed was limited to 300 rpm (linear speed 0.7 m/s) to avoid cellular damage. The aeration rate was maintained at 1 vvm. Mye medium [19] 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) organic antifoam (Sigma, St Quentin Fallavier, France; cat no. A6426) were autoclaved for 25 min at 120°C. Inocula (10 vol%) were maintained as exponentially growing cultures to obtain ~10,000 cells/ml in the fresh medium. Cells were counted electronically (Coulter counter Z1, Beckman Coulter France, Roissy CDG, France).

Enzyme assay

Cells were discarded from the medium at the end of exponential phase, by centrifugation at 8,000 *g* for 20 min at 0°C.

Protease activity

Global proteolytic activity was measured spectrophotometrically using haemoglobin as the substrate and with modifications of the procedure of Beynon and Bond [1]. The buffer used in 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 4,500 *g* for 20 min and tyrosine was assayed as described previously [17]. One unit of proteolytic activity corresponds to the amount of enzyme required for the release of 1 μmol tyrosine per minute. Results were expressed as milliunits per millilitre (mU/ml)

Global lipase activity

The substrate was a solution (v/v) of gum arabic (50 mg/ml) and olive oil (Sigma; cat no. O1500). The buffer for the reaction was 0.1 M Tris buffer, pH 8. Samples (cells in medium) were sonicated for 30 s at 100 W. Substrate (5 ml) was mixed with 2 ml buffer and added to enzyme solution (3 ml). After incubation at 37°C for 3 h, the reaction was stopped with 10 ml of an ethanol:acetone solution (1:1 v/v). Fatty acids released were measured using the SIGMA titrimetric procedure based on the method of Tietz and Fiereck [20]. One lipase unit was defined as the amount of enzyme releasing 1 μmol fatty acid per min at 37°C.

Table 1 Factor levels of the seven chosen factors

Factors	Level (−)	Level (+)
Supplemental tryptophan	Without	0.7 g/l
pH	No regulation	Regulation at 6.8 with HCl
Olive oil	Without	0.5% (v/v)
Melatonin	Without	10^{-6} mol/l
Serotonin	Without	10^{-6} mol/l
Dissolved oxygen	Without	Regulation at 50% minimum
Light	Darkness	24 h/day

Statistical analysis

Choice of factors

Previous work on *Tetrahymena* growth justified the choice of nine factors influencing generation time, maximum cell concentration and hydrolase production (protease, lipase), but only seven of these factors were tested by a two-level factorial design [2]. Aeration and agitation were not tested because their influence on growth and protease production has been optimised previously [7].

Fractional factorial design

A 2^{7-3} fractional factorial design was chosen with the same seven factors at two levels being evaluated in 16 experimental runs. Factor levels are represented in Table 1, where the two levels of each variable were identified by either a minus (−) or a plus (+) sign. Each trial was run with a mixture of these factors.

This design had a resolution IV without aliases between main effects, or between a principle effect and two factor combinations, but with aliases for combinations between two or more factors [18].

The fractional factorial experimental design was based on the first order model:

$$\begin{aligned}\textbf{Response} = {} & b_0 + b_1(\text{Tryp}) + b_2(\text{Sero}) + b_3(\text{Mela}) + b_4(O_2) + b_5(\text{Light}) + b_6(\text{pH}) + b_7(\text{Oil}) \\ & + b_{14}(\text{Tryp} \times O_2) + b_{13}(\text{Trp} \times \text{Mela}) + b_{15}(\text{Trp} \times \text{Light}) + b_{17}(\text{Trp} \times \text{Oil}) + b_{24}(\text{Sero} \times O_2) \\ & + b_{25}(\text{Sero} \times \text{Light}) + b_{26}(\text{Sero} \times \text{pH}) + b_{36}(\text{Mela} \times \text{pH}) + b_{37}(\text{Mela} \times \text{Oil}) + b_{56}(\text{Light} \times \text{pH}) \\ & + b_{57}(\text{Light} \times \text{Oil}) + b_{74}(\text{Oil} \times O_2)\end{aligned}$$

where the response is growth or enzyme activities, b_0 is the model intercept, and b_I is the linear coefficient. Only the two factor combinations were included in this model. Preliminary analysis of variance (ANOVA) for the full model was performed (data not shown). The variables whose confidence levels were higher than 90% were considered to significantly influence growth or enzyme production and were included in the model. The reduced models containing only the major effects and the factor combinations that affected the responses were adjusted and evaluated by a second analysis of variance. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2.

Results and discussion

Quantification of the effects of the seven factors individually and in combination: fractional factorial design

The results of the 16 experimental runs are presented in Table 2. In these experiments, variables with confidence levels greater than 90% were considered as significant (Table 3). Neglecting the terms that were insignificant, the model accounts for generation time, maximal population, and protease and lipase production, giving equations written as:

Table 2 Fractional factorial design and results. *Tryp* Tryptophan, *sero* serotonin, *mela* melatonin, *GT* generation time, *max pop* maximal population

Number	Tryp	Sero	Xela	O_2	Light	pH	Oil	GT (min)	Max pop (cells/ml)	Protease (mU/ml)	Lipase (U/l)
1	1	1	1	−1	1	−1	−1	128.36	2.18E+06	261.80	0.02
2	−1	1	1	1	−1	1	−1	154.03	2.80E+06	391.80	0.08
3	−1	−1	1	1	1	−1	1	177.73	2.39E+06	209.30	0.09
4	1	−1	−1	1	1	1	−1	126.03	2.66E+06	449.30	0.09
5	−1	1	−1	−1	1	1	1	223.60	2.58E+06	342.30	0.07
6	1	−1	1	−1	−1	1	1	150.68	2.72E+06	538.50	0.12
7	1	1	−1	1	−1	−1	1	135.91	2.29E+06	446.40	0.08
8	−1	−1	−1	−1	−1	−1	−1	154.03	1.70E+06	372.20	0.09
9	1	1	1	1	1	1	1	216.60	2.71E+06	575.54	0.07
10	−1	−1	−1	1	−1	1	1	192.50	3.41E+06	546.12	0.17
11	1	−1	−1	−1	1	−1	1	165.00	1.71E+06	436.98	0.04
12	−1	1	1	−1	−1	−1	1	147.48	2.73E+06	202.78	0.14
13	1	1	−1	−1	−1	1	−1	169.10	2.35E+06	425.05	0.12
14	−1	−1	1	−1	1	1	−1	154.03	2.41E+06	472.80	0.05
15	1	−1	1	1	−1	−1	−1	119.51	1.90E+06	422.49	0.05
16	−1	1	−1	1	1	−1	−1	117.48	2.21E+06	425.47	0.08

Table 3 Regression analysis for generation time, maximal population formation, protease and lipase activity

Parameters		Generation time	Maximal population	Protease activity	Lipase activity
Term	Coefficients				
Intercept	b_0	158.254	2421875	407.43	0.08500
Tryp	b_1	−6.856	−10687	37.08	−0.01125
Sero	b_2	3.316	59375	−23.53	NS
Mela	b_3	NS[a]	58125	−23.05	−0.00750
O_2	b_4	−3.281	124375	25.88	NS
Light	b_5	5.349	−65625	NS	−0.02125
pH	b_6	15.067	283125	60.25	0.01125
Oil	b_7	17.933	145625	NS	0.0125
Tryp × O_2	b_{14}	NS	−49375	NS	NS
Tryp × Mela	b_{13}	NS	NS	28.13	NS
Tryp × Light	b_{15}	2.249	65625	NS	0.00250
Tryp × Oil	b_{17}	NS	NS	NS	−0.00875
Sero × O_2	b_{24}	−2.284	NS	NS	NS
Sero × Light	b_{25}	4.591	NS	NS	NS
Sero × pH	b_{26}	14.196	−154375	NS	NS
Mela × pH	b_{36}	NS	−103125	50.03	NS
Mela × Oil	b_{37}	NS	NS	NS	0.01500
Light × pH	b_{56}	NS	NS	NS	NS
Light × Oil	b_{57}	NS	NS	NS	−0.00875
Oil × O_2	b_{74}	7.778	NS	NS	NS
Model R^2		0.988	0.998	0.900	0.965

[a]Not significant

$$\begin{aligned}\text{Generation time (min)} &= 158.25 - 6.86\ \text{Tryptophan} + 3.32\ \text{Serotonin} - 3.28\ O_2 \\ &\quad +5.35\ \text{Light} + 15.06\ \text{pH} + 17.93\ \text{Oil} + 7.78\ O_2 \times \text{Oil} \\ &\quad +2.25\ \text{Tryptophan} \times \text{Light} - 2.28\ \text{Serotonin} \times O_2 \\ &\quad +4.59\ \text{Serotonin} \times \text{Light} + 14.19\ \text{Serotonin} \times \text{pH}\end{aligned}$$

$$\begin{aligned}\text{Maximal population (cells/ml)} &= 2421875 - 106875\ \text{Tryptophan} + 59375\ \text{Serotonin} \\ &\quad +58125\ \text{Melatonin} + 124375\ O_2 - 65625\ \text{Light} + 283125\ \text{pH} \\ &\quad +145625\ \text{Oil} - 49375\ \text{Tryptophan} \times O_2 + 65625\ \text{Tryptophan} \times \text{Light} \\ &\quad -103125\ \text{Melatonin} \times \text{pH} - 154375\ \text{Serotonin} \times \text{pH}\end{aligned}$$

$$\begin{aligned}\text{Protease activity (mU/ml)} &= 407.43 + 37.08\ \text{Tryptophan} - 23.53\ \text{Serotonin} \\ &\quad -23.05\ \text{Melatonin} + 25.88\ O_2 + 60.25\ \text{pH} \\ &\quad +28.13\ \text{Tryptophan} \times \text{Melatonin} + 50.03\ \text{Melatonin} \times \text{pH}\end{aligned}$$

$$\begin{aligned}\text{Lipase activity (U/l)} &= 0.08500 - 0.01125\ \text{Tryptophan} - 0.00750\ \text{Melatonin} \\ &\quad -0.02125\ \text{Light} + 0.01125\ \text{pH} + 0.01250\ \text{Oil} \\ &\quad +0.00250\ \text{Tryptophan} \times \text{Light} + 0.0150\ \text{Melatonin} \times \text{Oil} \\ &\quad -0.00875\ \text{Tryptophan} \times \text{Oil} - 0.005\ \text{Light} \times \text{pH} - 0.00875\ \text{Light} \times \text{Oil}\end{aligned}$$

These regressions of linear models were statistically significant with $P < 0.02$. The range of R^2 values was 0.90–0.99 indicating that these models (major effects and combinations) explained over 90% of the total data variability.

These factorial models include the major effects of factors individually and in combination. A significant combination of factors influencing generation time was analysed by calculating mean values of responses at high and low levels of the two given factors. To decrease generation time, serotonin can be added without regulated pH. Two of the most effective factor combinations were studied for maximal population formation. These two—regulated pH without adding serotonin or melatonin—were found to stimulate cell production. For protease production, the combination of added melatonin and regulated pH showed a positive synergistic effect over the melatonin and pH factors individually. In the same way, the combination of added melatonin with added oil was the most significant interaction and increased lipase production. Conversely, the presence of light had a negative effect and decreased lipase activity.

The main positive effects for growth of *Tetrahymena* were observed with regulated pH at 6.8, regulated dissolved oxygen at 50%, and the presence of oil. To produce hydrolytic enzymes, regulated pH was the most important factor in improving protease and lipase activities. Specifically, the presence of olive oil stimulated lipase production and the presence of tryptophan stimulated protease production. Light must be present at a low level and it was best to cultivate *Tetrahymena* in darkness.

Our results are consistent with previous studies. Addition of unsaturated fatty acids or phospholipids is known to stimulate *T. thermophila* growth [16], and lipase production from a wide variety of micro-organisms is improved by the addition of oil [8, 14].

Hoffman and Cleffman [10] noticed that higher dissolved oxygen concentrations in the medium did not significantly improve cell density. However, our results showed an improvement of biomass when oxygen was regulated at 50%. In the same way, aeration and agitation influenced growth and protease production, and have been previously optimised [7].

Csaba et al. [6] showed that serotonin supplementation stimulated cell division. It is generally accepted that serotonin increases division rates of some protozoa. Our results agree and indicate that serotonin is not the most important factor among the factors analysed. Our results also demonstrate negative effects of serotonin on enzyme production. In contrast to previous studies [5], we noticed that serotonin did not stimulate lipase activity.

Growth suppression by melatonin has been observed with *Tetrahymena pyriformis* [12]. However, in our results, melatonin had a small positive effect on maximal *T. thermophila* population formation. Leclercq et al. [13] reported that melatonin and serotonin in combination had negative effects, particularly for enzyme production. When the combination of melatonin and regulated pH was examined, it was shown that maximal population formation is improved with regulated pH without addition of melatonin. The combination of melatonin and oil added to cultures and the combination of added melatonin and regulated pH were included in the models for lipase and protease production, respectively, and revealed that these combined factors enhanced lipase or protease activities.

This fractional factorial design with resolution IV included the effects of some two-way interactions and permitted the design of a modelling equation for predicting growth of, and enzyme production by, *T. thermophila*.

The next step in the improvement of *Tetrahymena* enzyme production is to apply an optimisation design using response surface methodology. The variables of significant negative effects will not be included in this future optimisation experiment.

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