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Coconut-Water as Fermentation Medium for Enzyme Production (4): Time-Profiled Control of Batch 5L Stirred Tank Fermentation

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ABSTRACT

The use of coconut-water as a cheap fermentation medium for the production of the enzyme cytochrome p-450 by the yeast Saccharomyces cerevisiae *N.C.Y.C 754 was investigated in a 5L stirred tank fermenter by building state variable models and generating optimum control time-profiles for the control variables temperature (T), pH and agitation rate (RPM) using Pontryagin's Continuous Maximum Principle. The theoretical maximum enzyme yield that can be achieved under optimum time-profiled control of the three variables is 694.87 nmol L^{-1} in 30.5 hours of fermentation. Except for T, the continuous optimum time-profiles of the variables do not differ significantly from that of set point control. Experiments conducted with the three variables controlled along these time-profiles gave an average yield of 633.70 nmol L^{-1} in 28 hours of fermentation.*

ABSTRAK

Kegunaan air kelapa sebagai medium murah fermentasi untuk penghasilan enzim sitokrom p-450 oleh yis Saccharomyces cerevisiae *N.C.Y.C. 754 telah dikaji dalam fermenter tangki teraduk sesekumpul dengan membina peraga-peraga pembolehubah-pembolehubah keadaan dan menjanakan profail-masa optimum untuk pembolehubah-pembolehubah kawalan suhu (T), pH dan kadar putaran pendesak (RPM) menggunakan Prinsip Maksimum Berterusan Pontryagin. Hasil isipadu enzim maksimum teori yang boleh dicapai di bawah kawalan profail-masa optimum ketiga-tiga pembolehubah itu ialah 694.87 nmol L^{-1} dalam masa fermentasi 30.5 jam. Kecuali untuk T, didapati profail-masa optimum berterusan pembolehubah-pembolehubah kawalan tidak berbeza dengan bererti dari kawalan titik set. Ujikaji-ujikaji yang dijalankan dengan tiga pembolehubah kawalan itu dikawal pada profail-masa optimum masing-masing memberi hasil isipadu enzim purata 633.70 nmol L^{-1} dalam masa fermentasi 28 jam.*

INTRODUCTION

We have previously reported our work [1] on the optimisation of a batch 5L stirred tank fermentation for the production of the enzyme cytochrome p-450 in the yeast *Saccharomyces cerevisiae* N.C.Y.C. 754 grown in coconut- water based medium using set point controls on the variables temperature (T), pH and agitation rate (RPM). To further improve the yield, work has now been

done on the optimisation of this fermentation process by applying the Continuous Maximum Principle [8,16] to generate optimum time-profiles of the control variables T, pH and RPM and using external control on the fermenter by a personal computer via the software SISKA (Caidmark Sdn. Bhd. Malaysia)[12] to maintain the control variables T, pH and RPM along their respective optimum time-profiles in time.

Using time-profiled control optimisations improvements were obtained in all published work [4, 7, 9, 11, 17] compared to the constant set-point versions. However, it was not always clear whether the constant set-point versions have themselves been optimised. It could therefore happen that optimisation of the time-profiles would produce no greater an improvement than optimisation of the constant set-point conditions alone. Since the data used for the generation of the optimum time-profiles of the control variables in our work come from the constant set point control optimisation [1], this work serves as a useful comparison between the two modes of control.

The Continuous Maximum Principle [8,16] is in theory also capable of solving problems involving any number of variables [5]. In fermentation process control, the method has never been applied to more than two variables. Thus this work also serves as a useful test whether the application of the Continuous Maximum Principle [8,16] on three control variables will be successful in the face of experimental and modelling errors involved.

MODELLING

Optimisation results based on the Continuous Maximum Principle are often highly dependent on the models used to describe the process [18]. Where the exact process mechanisms are known, as in the case of gluconic acid fermentation [16] the process can be modelled very accurately, subject to experimental error. Where the process mechanisms are not fully understood, rationally-based models cannot be developed; and only empirical models are used, as in the case of penicillin fermentations [5,6] and erythromycin fermentations [4]. The biosynthesis of the enzyme cytochrome p-450 by the yeast *S. cerevisiae* is a similar case. The reactions leading to the formation of the enzyme are not fully understood and the ways in which the environmental conditions affect these reactions are far from clear. As such, exact stoichiometric models depicting the reactions are not available and empirical models based on observed macro behaviour have to be used.

THE MODELLING OF BIOMASS PRODUCTION

Although the Monod Model has been shown to work well with pure culture in defined medium [2], in cultures with a complex medium such as the case presently, it has inherent limitations [18] in that the concept of a limiting substrate may not hold. The Logistic Model [13] is similiar to the Monod Model in many respects but different in that it assumes that growth limitation may be caused by other causes besides substrate limitation. It also leaves out the limiting substrate from the model, thus allowing the model to be fitted to the data of biomass on its own, without having to incorporate the limiting substrate data. Therefore it was more suitable for our purpose and was chosen. The Logistic Model can be stated as follows:

$$\frac{dy_1}{dt} = k_1 y_1 - \frac{k_1 y_1^2}{k_2} \quad (1)$$

where y_1 is the biomass concentration (gL^{-1})
k_1 is the parameter representing the growth rate (h^{-1})
k_2 is the final biomass concentration (gL^{-1})
t is time (h)

The model does not allow for a lag phase, but this did not matter because our data do not show a lag phase anyway.

THE MODELLING OF CYTOCHROME P-450 BIOSYNTHESIS

Before we can develop a model to describe the biosynthesis of cytochrome p-450 in yeast, certain assumptions will have to be made. The first assumption is that each yeast cell at any particular point in time contains the same amount of cytochrome p-450 as every other cell in the fermentation broth. The second assumption is that the cyctochrome p-450 level as determined by the CO-reduction method [15] represents the nett amount resulting from synthesis and loss. The third assumption is that at any particular point in time all yeast cells are equally capable of and are producing cytochrome p-450 at the same rate. Therefore, at any particular point in time the rate of production of cytochrome p-450 in the fermentation broth is proportional to the amount of yeast present,

$$\frac{dy_2}{dt} = k_3 y_1 \quad (2)$$

where y_2 is the concentration of cytochrome p-450 (nmol L^{-1})
k_3 is the proportionality parameter.

The fourth assumption is that the denaturation or loss of cytochrome p-450 takes place in each of the yeast cells at the same rate. In experiments with yeast microsomal fraction held in polyethylene glycol (PEG) buffer at constant temperatures, the loss of cytochrome p-450 fits first order kinetics (Sadler, A.M.; unpublished results). If we make a fifth assumption that in the yeast microsomal fraction that was used in these experiments cytochrome p-450 was not being formed at the same time as it was being lost, and that in the whole yeast cells the same mechanism of destruction was in operation, then we can incorporate a first order loss term to equation (2), giving

$$\frac{dy_2}{dt} = k_3 y_1 - k_4 y_2 \quad (3)$$

where k_4 is the rate of denaturation of cytochrome p-450 (h^{-1}).

TREATMENT OF RESULTS

The experimental plan of the Rotatable Composite Design [3] is given in Table 1 and the levels of the variables used in the experiments are given in Table 2. The results of the experimental runs are given in Table 3 (Biomass) and Table 4 (Cytochrome p-450).

The models that have been developed were fitted to the experimental data of the fermentation runs. The fitting was done by computer by numerically integrating the differential equations using the Runge-Kutta 4th Order Numerical Integration Method [10] and minimising the sum of squared errors between the predicted data and the actual data using the Simplex Method [14]. The values of the parameters of the models which give them the best fit on the data for each fermentation run are given in Table 5.

The next task was to formulate a mathematical relationship between each of these parameters and the three control variables T, pH and RPM. Previous workers dealing with two control variables namely T and pH [4, 16] have used two approaches. Rai and Constantinides [16] described the parameters as function of T with Arrhenius-type equations with linear interpolation between pH values. Cheruy and Durand [4] used non-linear regression functions for each parameter and used the Student-Fisher test to check whether each regression term was significant, taking into account the dispersions of the measurements, and then eliminating the insignificant terms to simplify the functions. We chose to follow the approach used by Cheruy and Durand [4] because with our three variables a non-linear regression will be easier to handle than the three-dimensional linear interpolation that would result if we follow the approach of Rai and Contantinides [16]. The non linear regression has also the advantage that it will take into account the interaction between the control variables. With 3 variables the regression based on a quadratic approximation has the form:

$$k_i = a_{oi} + a_{1i}x_1 + a_{2i}x_2 + a_{3i}x_3 + a_{11i}x_1^2 + a_{22i}x_2^2 + a_{33i}x_3^2 + a_{12i}x_1x_2 + a_{13i}x_1x_3 + a_{23i}x_2x_3 \quad (4)$$

where x_1, x_2, x_3 are the levels of variables temperature, pH and agitation rate respectively. The subscript i refers to a specific parameter.

The parameters of the two models were submitted to this treatment. The values of the regression coefficients for these parameters are given in Table 6.

THE CONTINUOUS MAXIMUM PRINCIPLE

The performance equations of a process have the form

$$\frac{dy_i}{dt} = f_i(y_1(t), y_2(t), \ldots y_m(t); x_1(t), \ldots x_n(t)), \quad t_0 \le t \le t_f \quad (5)$$

where $y_i(t)$ is a state variable that represents the state of the process at time t.

$x(t)$ is a control variable that represent the decision at time t.

TABLE 1. The Experimental Plan of the Rotatable Composite Design Based on 2^3 Factorial

No.	x_1 T	x_2 pH	x_3 RPM
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

TABLE 2. Levels of Variables Used in the Rotatable Composite Design

Variable	$\alpha = -1.68$	$\alpha = -1$	$\alpha = 0$	$\alpha = 1$	$\alpha = 1.68$	Unit
T	23.8	25.0	27.0	29.0	30.4	℃
pH	4.61	4.80	5.10	5.40	5.59	pH unit
RPM	168	200	250	300	332	rpm

TABLE 3. Biomass Data (in gL^{-1}) for Experimental Runs in the Rotatable Composite Design

	Time (hr)							
No.	0	4	8	12	16	20	24	28
1	1.05	5.06	8.46	10.84	13.42	13.78	13.55	13.86
2	1.05	6.61	8.30	10.47	12.90	13.70	13.45	
3	1.05	5.59	9.40	12.08	14.68	15.55	14.54	
4	1.05	6.30	9.62	13.83	14.89	15.24	14.96	
5	1.05	6.78	9.93	12.13	14.47	15.23	15.35	15.24
6	1.05	5.80	10.21	13.48	14.20	14.44	14.75	14.80
7	1.05	5.98	8.88	10.63	13.04	13.36	12.75	
8	1.05	6.11	9.69	12.18	13.51	13.95	14.15	
9	1.05	5.43	9.12	11.25	13.16	13.39	13.24	

TABLE 3 Continue

No	0	4	8	12	16	20	24	28
10	1.05	6.55	11.42	12.81	13.54	13.78	13.95	
11	1.05	6.33	10.92	13.07	14.55	14.73	14.39	
12	1.05	4.28	9.69	12.95	13.98	13.55	13.23	
13	1.05	5.99	11.49	13.96	14.75	14.94	14.81	
14	1.05	6.47	11.95	13.07	15.65	16.15	15.47	
15	1.05	5.83	9.65	12.29	13.45	13.58	13.47	
16	1.05	7.98	10.13	13.15	15.19	15.37	14.77	
17	1.05	5.47	8.96	12.16	14.57	14.61	14.33	
18	1.05	6.04	10.30	13.50	15.36	15.02	15.41	
19	1.05	6.63	10.39	13.68	15.05	14.08	15.10	
20	1.05	6.78	8.90	13.89	15.24	15.45	15.08	

TABLE 4. Cytochrome p-450 Data (in nmol L^{-1}) for Experimental Runs in the Rotatable Composite Design

	Time (hr)							
No.	0	4	8	12	16	20	24	28
1	22.0	109.9	219.8	373.6	417.6	481.5	584.4	
2	22.0	153.8	329.7	395.6	428.6	489.0	549.4	549.4
3	22.0	109.9	197.8	340.7	428.6	516.5	593.4	
4	22.0	131.9	285.7	428.6	450.6	472.5	560.4	681.3
5	22.0	175.8	351.6	494.5	527.5	561.5	571.4	
6	22.0	109.9	241.8	435.4	490.4	516.5	582.4	
7	22.0	131.9	241.8	395.6	489.0	582.4	483.5	
8	22.0	105.9	105.9	263.7	425.4	571.4	549.4	
9	22.0	95.4	241.8	395.6	445.5	483.5	549.4	
10	22.0	145.4	360.4	471.4	571.4	640.4	703.3	
11	22.0	87.9	285.7	373.6	439.6	545.4	593.4	
12	22.0	140.4	164.8	384.6	458.2	538.5	604.4	
13	22.0	100.4	197.8	351.6	405.4	461.5	516.5	439.6
14	22.0	109.9	235.5	472.5	525.5	549.3	659.3	703.3
15	22.0	145.8	285.7	560.4	590.4	604.4	637.4	
16	22.0	155.8	301.6	395.6	525.8	637.4	703.3	
17	22.0	153.8	263.7	351.6	495.8	615.4	692.3	
18	22.0	175.8	329.7	439.5	505.5	560.4	615.4	648.4
19	22.0	153.8	307.7	461.5	560.4	637.4	725.3	
20	22.0	153.8	307.7	461.5	560.4	637.4	725.3	

$$\frac{dz_i}{dt} = \frac{\partial H}{\partial y_i} = -\sum_{j=1}^{m} z_j \frac{\partial f_j}{\partial y_i}, \quad i = 1,2 \text{ K K } m \tag{10}$$

$$z_i(t_f) = c_i, \qquad i = 1,2 \text{ K K } m \tag{11}$$

where z_i is an element of the vector matrix Z(t)
c_i is an element of the scalar matrix C

The optimal decision vector function X(t) which makes M an extremum is the decision factor function X(t) which renders the Hamiltonian function H an extremum for every t, $t_o \leq t \leq t_f$. Therefore the optimum value of a control variable at any point in time is given by

$$\frac{\partial H}{\partial x_j} = 0, \quad j = 1,2 \text{ K n} \tag{12}$$

when x_j lies in the interior of the region $x_j(t)$, or
H = maximum (13)
when x_j lies at the boundary of the constraint.

CALCULATION OF THE OPTIMUM PROFILES

In this work the batch fermentation time for the optimised version was fixed at 32 hours. The initial concentration of biomass was fixed at 1.05 gL^{-1} by fixing the inoculum size. The initial concentration of cytochrome p-450 was taken to be constant at 22.00 nmol L^{-1}, which is the average of the values observed in each of the inoculum preparation for the experimental fermentation runs. The objective function M is the final concentration of cytochrome p-450. The following equations were submitted for optimisation using the Continuous Maximum Principle:

1. State Equations

Biomass Equation $$\frac{dy_1}{dt} = k_1 y_1 - \frac{k_1 y_1^2}{k_2} \tag{1}$$

Cytochrome p – 450 Equation $$\frac{dy_2}{dt} = k_3 y_1 - k_4 y_2 \tag{3}$$

2. Adjoint Equations

$$\frac{dz_1}{dt} = -z_1 k_1 + 2z_1 \frac{k_1 y_1}{k_2} - z_2 k_3, \quad z_1(t_f) = 0.0 \tag{14}$$

$$\frac{dz_2}{dt} = z_2 k_4, \qquad z_2(t_f) = 1.0 \tag{15}$$

3. The Hamiltonian Function

$$H = z_1 k_1 y_1 - z_1 \frac{k_1}{k_2} y_1^2 + z_2 k_3 y_1 - z_2 k_4 y_2 \tag{16}$$

$$\frac{\partial H}{\partial x_j} = z_1 y_1 \left(\frac{\partial k_i}{\partial x_j}\right) - z_1 \left(\frac{\partial k_1}{\partial x_j}\right) + z_1 y_1^2 \left(\frac{k_1}{k_2^2}\right)\left(\frac{\partial k_2}{\partial x_j}\right) + z_2 y_1 \left(\frac{\partial k_3}{\partial x_j}\right) - z_2 y_2 \left(\frac{\partial k_4}{\partial x_j}\right) \tag{17}$$

4. The Quadratic Relationship Between the Parameters k_i and the Control Variables x_1, x_2, x_3.

$$k_i = a_{oi} + a_{1i}x_1 + a_{2i}x_2 + a_{3i}x_3 + a_{11i}x_1^2 + a_{22_i}x_2^2 + a_{33i}x_3^2 + a_{12i}x_1x_2 + a_{13i}x_1x_3 + a_{23i}x_2x_3 \tag{4}$$

$$\frac{\partial k_i}{\partial x_1} = a_{1i} + 2a_{11i}x_1 + a_{12i}x_2 + a_{13i}x_3 \tag{18}$$

$$\frac{\partial k_i}{\partial x_2} = a_{2i} + 2a_{22i}x_2 + a_{12i}x_1 + a_{23i}x_3 \tag{19}$$

$$\frac{\partial k_i}{\partial x_3} = a_{3i} + 2a_{33i}x_3 + a_{13i}x_1 + a_{23i}x_2 \tag{20}$$

A program to calculate the optimum time-profiles of the control variables was written in fortran based on a flow-chart adapted from Cheruy and Durand [4] and shown in Figure 1.

As can be seen from the flow chart, the iteration will only stop when all the points in the numerical integration along the time axis have either reached

$$\frac{\partial H}{\partial x_1} = \frac{\partial H}{\partial x_2} = \frac{\partial H}{\partial x_3} = 0 \tag{21}$$

or they have hit a constraint with

$$H = \text{maximum} \tag{13}$$

In practice, two difficulties arose. The first concerns the size of the step in the steepest ascent of the Hamiltonian. As long as the step size has a non-zero value, no matter how small, there is the chance that the point represented by equation (21) will be missed by the step, as it may be situated between successive step points. This will cause the iteration to oscillate between the two points indefinitely. The second concerns the value of the error tolerance within which the oscillation will stop. This error should be estimated based on the errors in the predicted values of the parameters and variables

involved in the calculation of equation (13) or equation (21). The problem is that the difference between the actual values of these parameters and variables and their predicted values would not be the same everywhere along the quadratic response surface [2] of the parameters and along the time axis respectively and consequently equation (21) will not be subjected to the same error everywhere in the region considered in the optimisation. If we take the r.m.s. error in the prediction of these parameters and variables and

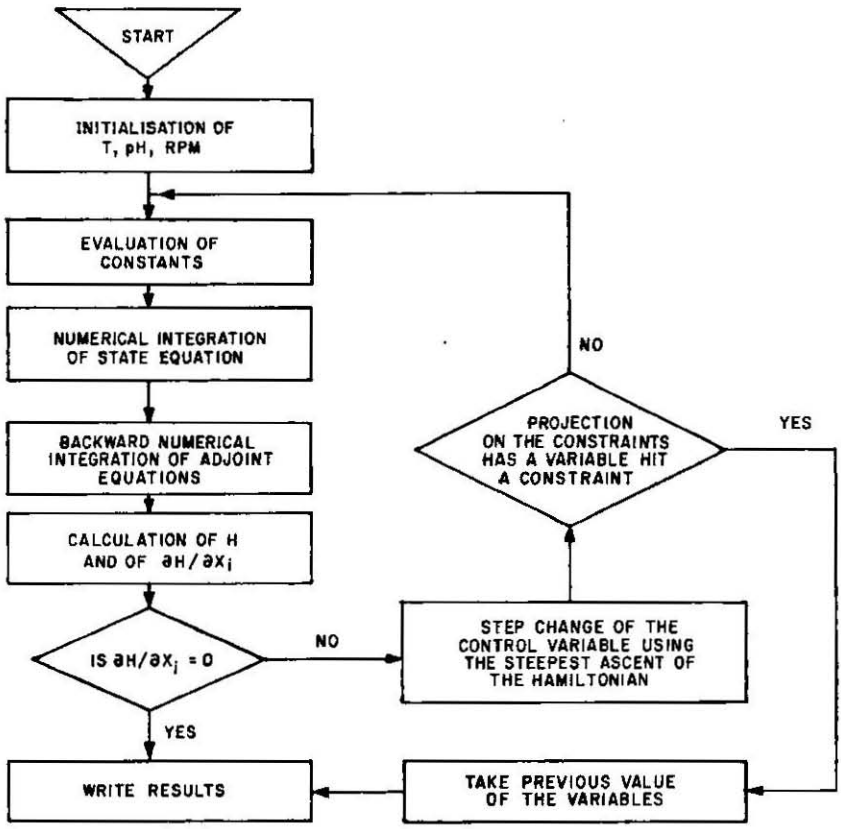

FIGURE 1. Flowchart for the Continuous Maximum Principle Program

use it to calculate the error tolerance of equation (21), the iteration will stop at some points along the time axis even when the maximum H at these points has not been reached because the error there is less than the r.m.s value. On the other hand, at other points where the errors are bigger than the r.m.s value the iteration will not stop at all. For this reason if we increase the error tolerance until the program converges to a solution for every point along the time axis, the error tolerance which first allows the program to do so is the biggest error in the prediction of equation (21), thus rendering the more accurate determination of the optimum profile of other points which have smaller errors than that value unachieveable. To avoid

this disadvantage, the error tolerance is set to zero, the step change factor is set to a very small value and the program is run repeatedly with the maximum number of iterations increased each time until a further increase causes no significant increase in the predicted final yield of cytochrome p-450 compared to the experimental error. With a step change factor of 0.001 and 200 iterations, a final cytochrome p-450 yield of 694.87 nmol L^{-1} is predicted. Increasing the iteration to 1000 causes no significant increase in the predicted final yield compared to the experimental error. The generated optimum time-profile for T is given in Figure 2, that for pH is given in Figure 3 and that for RPM is shown in Figure 4. These optimum time-profiles were used in the experiments.

MATERIALS AND METHODS

Inoculum preparation, medium preparation and inoculation procedure were the same as in the earlier work [1]. The optimum time-profiles for the control variables T, pH and RPM were stored in the software SISKA. In this software an increase in the value of a control variable is represented by a ramp change between two successive data points. The fermenter filled with medium was first started and its controls switched to external to enable the computer control to take over. Initially the system was a little unstable with

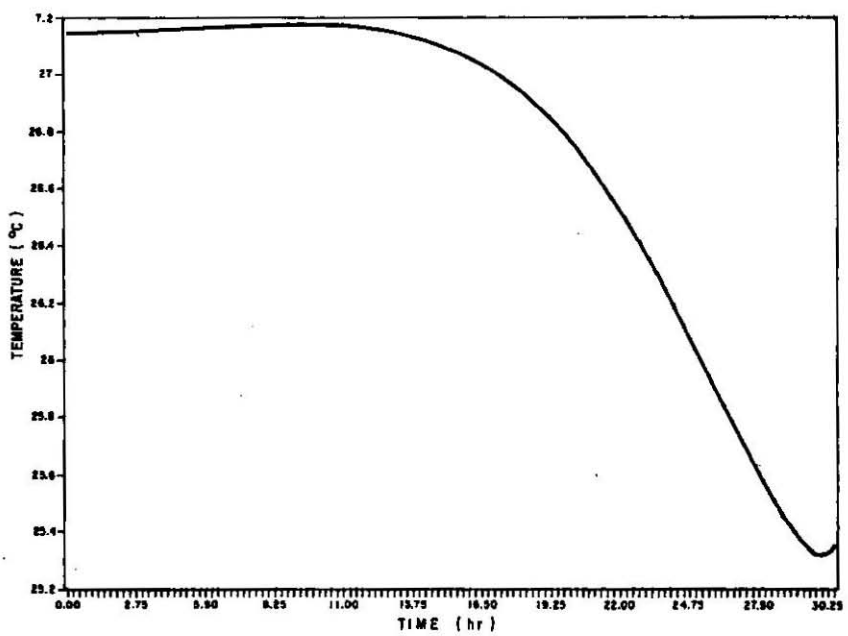

FIGURE 2. The Optimum Time-Profile for Temperature

the controllers forcing the fermenter to the specified control profiles from the respective original values of the variables T, pH and RPM. Once the system was stable the fermentation was inoculated and left to run its course.

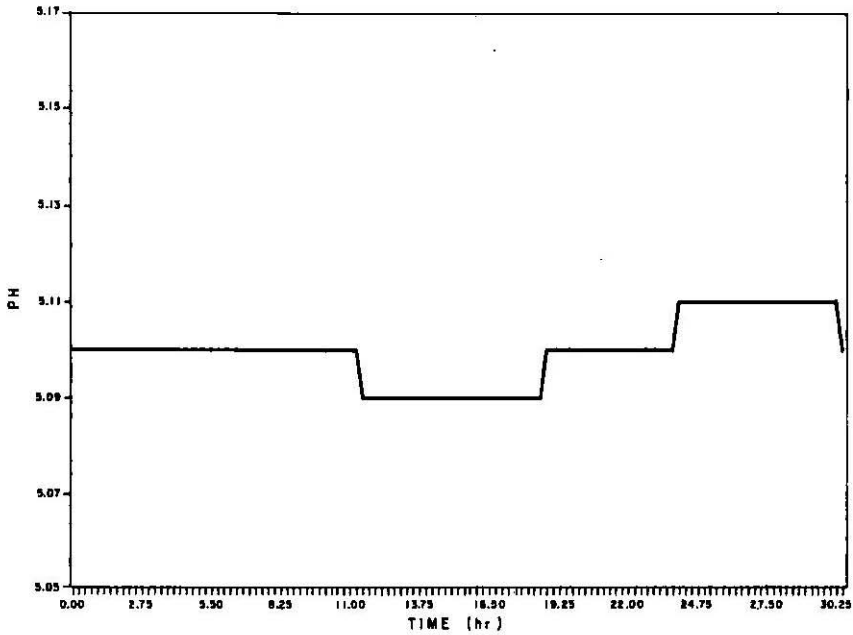

FIGURE 3. The Optimum Time-Profile for pH

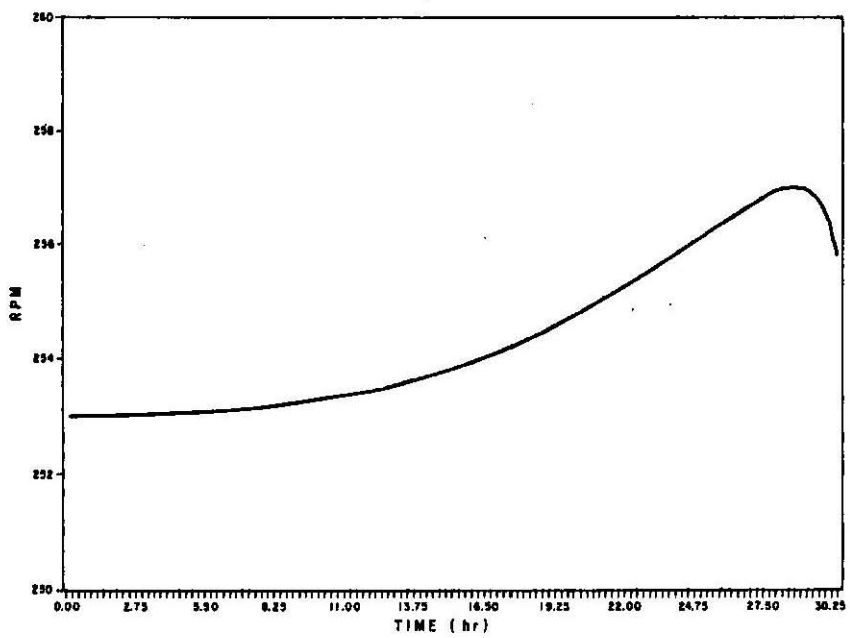

FIGURE 4. The Optimum Time-Profile for RPM

RESULTS

Table 7 shows the enzyme yields of three fermentation runs under time-profiled control of the variables T, pH and RPM.

DISCUSSION AND CONCLUSIONS

The predicted maximum enzyme yield under optimum time-profiled control of T, pH and RPM of 694.87 $nmolL^{-1}$ is only 1.6% higher then the predicted maximum enzyme yield under optimum set-point control of T, pH and RPM of 683.92 $nmolL^{-1}$ [1]. Experimental runs under optimum time-profiled control of T, pH and RPM gave an average enzyme yield of 633.70 $nmolL^{-1}$ at 28 hours (Table 7) while experimental runs under optimum set-point control of T, pH and RPM gave an average enzyme yield of 637.36 $nmolL^{-1}$ [1]. Thus it can be concluded that for this particular type of fermentation, optimum time-profiled control of T, pH and RPM do not give significant improvement in enzyme yield compared to optimum set-point control of T, pH and RPM.

In the optimum time-profiles the variations in pH ($5.09 \leq pH \leq 5.11$) and RPM ($253 \leq RPM \leq 257$) are too small to be significant compared to the experimental and modelling errors. Nevertheless, the variations in T are quite large ($25.3°C \leq T \leq 27.2°C$) and imply that the models and time-profiles optimisation procedures were at least sensitive enough to pick up an optimum time-profile if it exist. Set point control of a variable gives a straight line time-profile. A straight line is a particular form of profile. If through out the fermentation the process favours a particular level of pH or RPM, then straight line profiles are produced and the time-profiled control is reduced to set-point control for those two variables. Yeast biomass growth is maximum at 26.54°C while enzyme production is maximum at 24.48°C [19]. Plots of biomass data (Table 3) and of enzyme data (Table 4) would show that biomass was mainly produced during the first half of the fermentation whilst enzyme was mainly produced during the second half of the fermentation. Thus the T time-profile with a higher value in the first half favouring biomass growth and with a lower value in the second half favouring enzyme production. In contrast, from the same reference the initial pH requirement for biomass growth was pH 5.44 while that for enzyme production was pH 5.25. Thus the near straight-line optimum time-profile of pH can be explained in terms of the near identical pH requirements of the two state variables. There were however no data for the RPM requirements of biomass growth and enzyme production and similar conclusions cannot be derived.

ACKNOWLEDGEMENT

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NOTATION

Symbols	Description	Units
a	Regression coefficient	-
b	Initial condition	(Various)
C	Constant	-
f	Function	-
H	Hamiltonian	-
k_1	Specific growth rate	hr^{-1}
k_2	Final biomass concentration	gL^{-1}
k_3	Proportionality parameter	-
k_4	Rate of denaturation of enzyme	hr^{-1}
M	Objective function	-
RPM	Agitation rate	rpm
T	Temperature	°C
t	time	hr
x	Control variable	(various)
X	Vector function	-
y_1	Biomass concentration	gL^{-1}
y_2	Enzyme concentration	nmol L^{-1}
Y	Vector function	-
Z	Adjoint vector matrix	-
z	Adjoint vector element	-

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