THE INFLUENCE OF TEMPERATURE, pH AND AERATION ON THE ETHANOLIC FERMENTATION OF GLUCOSE BY *Pachysolen tannophilus*

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The ethanolic fermentation of 25 g l^{-1} solutions of glucose in batch cultures of *Pachysolen tannophilus* has been studied experimentally in terms of three environmental variables: initial pH from 1·5 to 6·5, temperatures of 25°C, 30°C and 35°C and aeration values of $Q = 0{\cdot}150$ v/v/min, $Q = 0{\cdot}075$ v/v/min and $Q = 0$ v/v/min (i.e. with air entering through the stirring vortex alone). Using the values for the concentrations of biomass, residual glucose and ethanol produced at intervals throughout the experiments, the maximum specific rates of growth, glucose consumption and ethanol production, together with the biomass and ethanol yields, have been calculated. The most favourable conditions for ethanol production are an initial pH of about 3, temperature of 30°C and $Q = 0$ v/v/min. Under these conditions the ethanol yield is approximately 0·36 (g ethanol) (g glucose)$^{-1}$ and the maximum specific production rate is 1·2 (g ethanol) (g biomass)$^{-1}$ h^{-1}.

Both primary and residual biomass will in the near future be important sources of foods, animal fodder, fertilizers, biomolecules and gaseous and liquid fuels[1-3]. Residual biomass ought to be the first to be taken advantage of, as this would at the same time take care of at least one aspect of environmental pollution.

One way of making use of lignocellulose residues is to subject them, after suitable pretreatment, to acid or enzymatic hydrolysis and thus obtain solutions made up largely of glucose and xylose. These solutions can then be fermented to get ethanol, which is valuable as a fuel or as a raw material in the organic chemical industry[4].

One problem attached to this enterprise is that many of the microorganisms in present-day use convert only hexoses into ethanol, thus wasting a significant part of the products of hydrolysis, as xylose may account for between 10% and 30% of the carbohydrates obtained[5]. Many yeasts are in fact known to consume pentoses oxidatively, but without producing ethanol, while a few are capable of producing an ethanolic fermentation of xylulose, a catabolite of xylose. It has also been suggested that when the oxygen tension is lowered the catabolism of the xylose is in-

hibited somewhat and xylulose is thus not formed[6]. Recently, however, *Pachysolen tannophilus* has been identified as a yeast capable of converting xylose directly to ethanol in aerobic conditions[7,8], yielding approximately 0·27 g of ethanol per g of xylose at 30°C (ref.[8]).

As a first step in a study into the possible uses of *Pachysolen tannophilus* we have initiated fermentations of glucose using this yeast, and analysed the influence of certain environmental variables, such as the pH, temperature and aeration conditions.

EXPERIMENTAL

All the experiments were carried out at laboratory scale in a batch-culture unit with three magnetically stirred fermentors, which has been described elsewhere[9].

The growth medium composition in $g\,l^{-1}$ was: $MgSO_4$, 1; KPO_4H_2, 2; $(NH_4)_2SO_4$, 3; peptone, 3·6; yeast extract, 4 and glucose, 25. This medium was sterilized by filtration through 0·2 μm cellulose nitrate, as was also the air used. Inocula were prepared by growing for 48—60 h at 30°C in a culture medium containing 3 $g\,l^{-1}$ of malt extract, 3 $g\,l^{-1}$ of yeast extract, 5 $g\,l^{-1}$ of peptone and 10 $g\,l^{-1}$ of glucose, solidified with 20 $g\,l^{-1}$ of agar-agar.

The cellular concentration (x) was measured indirectly through the absorbance of the suspension at a wavelength of 620 nm, having previously obtained an absorbance versus dry weight calibration line.

The concentrations of the residual glucose ($g\,l^{-1}$) and of the ethanol produced ($g\,l^{-1}$) were determined, after suitable dilution, by using the glucose oxidase[10] and alcohol dehydrogenase[11] enzymatic methods, respectively.

The majority of the experiments to determine the variations in biomass, glucose and ethano concentrations versus time were repeated on two different occasions, starting at different times of day, so as to cover the nocturnal hours, and the agreements between both were completely acceptable. Furthemore, all the analyses were made in duplicate.

RESULTS

We carried out three series of experiments. In the first, the influence of pH was studied by varying this parameter from 1·5 to 6·5, while the temperature was kept constant at 30°C and the air flow at 0·075 v/v/min. In the second series temperatures of 25°C and 35°C were tried, while the other two parameters were fixed at pH_i 3·5 and aeration at 0·075 v/v/min. Finally, the effects of changes in aeration, 0 and 0·15 v/v/min, were checked against constant temperature $T = 30$°C and pH_i 3·5, conditions, with $Q = 0$ v/v/min, the aeration entering only through the stirring vortex, which would be somewhat attenuated anyway by the simultaneous release of carbon dioxide.

The variations in the concentrations of residual glucose and ethanol produced versus time, for all the experiments, are shown in Tables I to III.

Maximum Specific Growth Rate

During the time in which a microorganism is growing exponentially it is usual to employ

$$\ln (x/x_0) = a + \mu_m \tau , \qquad (1)$$

so that an intercept not equal to nought appears in the equation, as the use of x_0, the initial biomass concentration of the discontinuous experiment, instead of x_1,

TABLE I

Residual glucose and ethanol produced according to different initial pH values, $T = 30°C$, $Q = 0{\cdot}075$ v/v/min

				pH_i 1·5					
τ, h	0·0	21·5	25·5						
s, g l^{-1}	25·0	24·8	23·2						
				pH_i 2·5					
τ, h	0·0	3·5	5·5	21·5	25·5	29·5	46·0		
s, g l^{-1}	24·0	23·6	22·9	12·1	1·2	0·06	0·00		
E, g l^{-1}	—	—	0·01	4·3	7·1	—	7·3		
				pH_i 3·5					
τ, h	0·0	5·0	11·0	15·0	19·0	20·5	24·0	30·5	40·0
s, g l^{-1}	25·0	23·2	22·4	18·5	7·1	2·2	0·00		
E, g l^{-1}	—	0·02	0·24	1·8	5·7	—	7·1	8·8	8·2
				pH_i 4·5					
τ, h	0·0	5·0	11·0	15·0	19·0	20·5	24·0	40·0	
s, g l^{-1}	25·0	—	23·0	18·8	7·5	0·9	0·00		
E, g l^{-1}	—	0·01	0·07	1·3	6·2	—	7·5	7·8	
				pH_i 5·5					
τ, h	0·0	5·5	11·0	15·0	19·0	20·5	24·0	30·5	40·0
s, g l^{-1}	25·0	23·7	22·6	19·4	8·5	1·2	0·00		
E, g l^{-1}	—	0·01	0·2	1·6	5·3	—	7·5	9·2	8·3
				pH_i 6·5					
τ, h	0·0	4·5	6·5	21·5	24·0	25·5	28·0	30·0	
s, g l^{-1}	25·0	24·7	24·7	13·2	6·9	3·8	3·6	0·03	
E, g l^{-1}	—	—	0·01	3·2	—	7·8			

the biomass concentration at the begining of the exponential phase, includes the lag phase when the microorganism is adapting itself to the medium, and consequently growing very slowly. If this phase were to be totally neglible x_I would tend towards x_0, and thus the value of a would be nought.

Figures 1 to 4, show the natural logarithm values of the adimensional biomass concentration, $\ln x/x_0$ vs time, for the three series of experiments carried out. In Fig. 1 it can be seen clearly that there is quite a short lag phase, followed by an exponential one, and finally by another stationary phase when all the glucose has been assimilated. The growth curves for the three initial pH values shown practically coincide. In the experiments where the initial pH was outside the range 3·5 to 5·5

TABLE II

Residual glucose and ethanol produced according to different temperatures, pH_i 3·5, Q = = 0·075 v/v/min

					T = 25°C						
τ, h	0·0	7·0	8·5	15·5	17·0	19·5	22·5	27·5	28·0	41·0	49·5
s, g l^{-1}	25·0	—	24·9	13·4	11·8	—	—	—	3·7	0·00	
E, g l^{-1}	—	0·01	—	0·3	1·7	3·5	3·7	7·7	—	7·9	6·4
					T = 35°C						
τ, h	0·0	4·0	6·0	8·0	10·0	11·5	15·0	22·5	25·0	31·0	47·0
s, g l^{-1}	25·0	24·7	—	24·4	—	23·1	19·4	0·04			
E, g l^{-1}	—	—	0·01	—	0·2	—	1·5	8·3	8·7	8·1	7·6

TABLE III

Residual glucose and ethanol produced according to aeration conditions, pH_i 3·5, T = 30°C

					Q = 0 v/v/min						
τ, h	0·0	6·0	8·0	10·0	11·5	15·0	22·0	25·0	28·0	31·0	34·0
s, g l^{-1}	25·0	—	24·1	—	23·0	18·0	—	0·00			
E, g l^{-1}	—	0·01	—	0·3	0·5	1·7	7·7	9·2	8·8	9·1	9·2
					Q = 0·15 v/v/min						
τ, h	0·0	4·0	6·0	8·0	10·0	11·5	15·0	22·5	25·0	28·0	31·0
s, g l^{-1}	25·0	24·99	—	24·8	—	22·4	19·1	0·1	0·00		
E, g l^{-1}	—	—	0·01	—	0·1	—	1·5	8·6	8·4	7·3	5·9

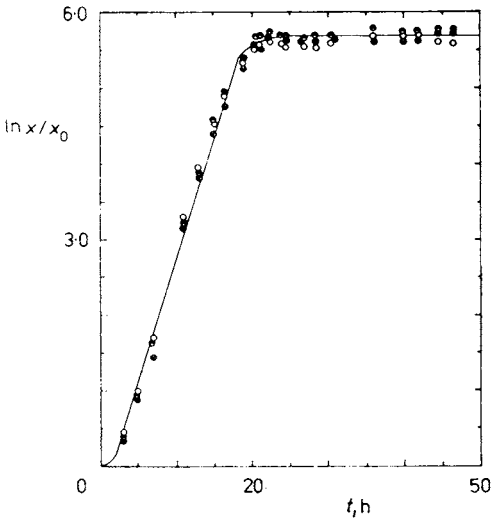

FIG. 1

Growth curves for initial pH: ○ 3·5, ◓ 4·5, ⊗ 5·5

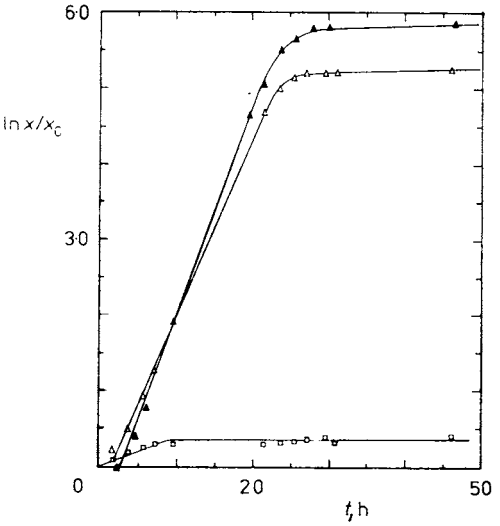

FIG. 2

Growth curves for pH values: □ 1·5, △ 2·5, ▲ 6·5

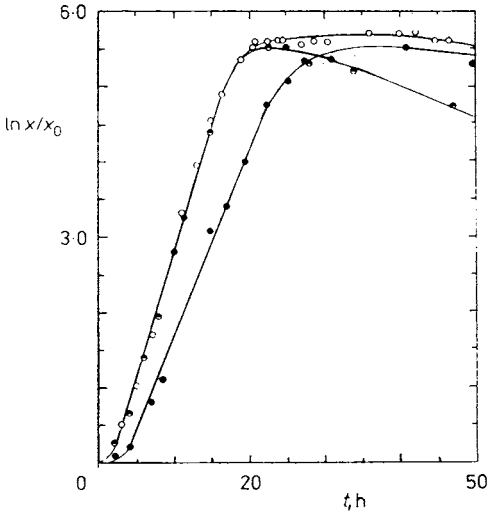

FIG. 3

Growth curves for various temperatures (°C): ● 25, ○ 30, ◓ 35

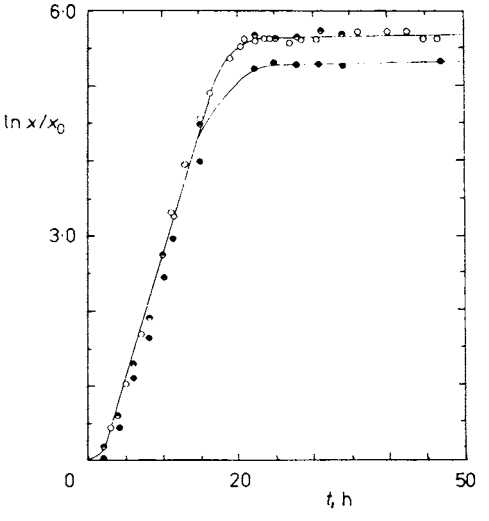

FIG. 4

Growth curves for air-flow rates 0, 0·075 and 0·150 v/v/min; ● 0, ○ 0·075, ◓ 0·150

the slope for the exponential growth is not quite so steep (Fig. 2). In Fig. 3 a similar trend for the exponential-phase growth slope at 25°C is also to be seen. The lag phase is more significant than in Fig. 1 and it is also noteworthy that at 35°C there is a reduction in the biomass concentration once the glucose has been consumed. Finally, the graphs in Fig. 4 indicate that the aeration conditions experimented with did not have any really significant effect, although when the air was supplied through the vortex alone the lag phase was longer and there was also a reduction in biomass concentration at the final stationary phase.

By means of least-square adjustments to Eq. (*1*) of the values obtained for the exponential phase in each experiment we have been able to calculate the maximum specific growth rates, μ_m, which are set out in Tables IV, V and VI. It can be seen that the value $0{\cdot}33\ h^{-1}$ remains constant in the initial pH range 3·5 to 5·5, and diminishes outside these bounds; this confirms that *Pachysolen tannophilus* is acidophilic[7], a fact which may well favour its use, as the celular functions of the majority of possible contaminants are considerably inhibited in the lower pH range. It is also evident that μ_m is lower at 25°C than at 30°C and 35°C, where it remains constant, and that an increase in aeration brings a concommitant, if only slight, upward trend in this value.

Maximum Specific Glucose Consumption Rate

In order to determine the specific glucose consumption rate, q_s, it is necessary to evaluate the glucose consumption rate by volume unit, $-ds/d\tau$, each time, in order to later divide by the biomass concentration. We tried various different empirical equations for an analytical calculation of the derivate and an acceptable reproduction of the s values (mean absolute error $0{\cdot}9\ g\ l^{-1}$) was arrived at with the equation

$$s = s_0 \alpha^{-\tau^\beta} \tag{2}$$

which fulfils the condition that $s \to s_0$ when $\tau \to 0$ and can be linearized in the following way,

$$\ln [\ln (s_0/s)] = \ln (\ln \alpha) + \beta \ln \tau\,. \tag{3}$$

As an example, a representation of Eq. (*3*) applied to the experiment where $Q = 0{\cdot}150$ v/v/min is shown in Fig. 5*b*. It is possible thus to obtain the parameters α and β, and so by using Eq. (*2*), to predict the variations in s vs τ (Fig. 5*a*).

Furthermore, using Eq. (*2*) to evaluate the derivate $ds/d\tau$, it can be deduced that

$$q_s = \frac{s_0 \beta (\ln \alpha)\,(\tau^{\beta-1})\,(\alpha^{-\tau^\beta})}{x} \tag{4}$$

and thus the values of q_s can be determined for the times where the value of x is known. The results for the chosen experiment appear in Fig. 5c. In this figure it can be seen that q_s appears to have a maximum value and that this value occurs in the exponential-growth zone, making it analytically determinable by combining Eqs (*1*)

TABLE IV
Influence of initial pH, $T = 30°C$, $Q = 0{\cdot}075$ v/v/min

pH_i	$Y_{x/s}$	μ_m	q_s	$\mu_m/(Y_{x/s})$	q_E	$(Y_{E/x})\,\mu_m$
1·5	—	0·032	—	—	—	—
2·5	0·087	0·23	3·0	2·6	1·04	0·78
3·5	0·112	0·33	3·2	2·9	0·82	0·92
4·5	0·127	0·33	3·2	2·6	0·88	0·89
5·5	0·132	0·33	3·9	2·5	0·81	0·79
6·5	0·138	0·27	2·5	2·0	0·55	0·63

TABLE V
Influence of temperature, pH_i 3·5, $Q = 0{\cdot}075$ v/v/min

T, °C	$Y_{x/s}$	μ_m	q_s	$\mu_m/(Y_{x/s})$	q_E	$(Y_{E/s})\,\mu_m$
25	0·118	0·25	3·0	2·1	0·64	0·72
30	0·112	0·33	3·2	2·9	0·82	0·92
35	0·121	0·33	3·0	2·7	0·57	0·56

TABLE VI
Influence of aeration conditions, pH_i 3·5, $T = 30°C$

Q	$Y_{x/s}$	μ_m	q_s	$\mu_m/(Y_{x/s})$	q_E	$(Y_{E/x})\,\mu_m$
0·000	0·090	0·31	4·3	3·4	1·19	1·08
0·075	0·112	0·33	3·2	2·9	0·82	0·92
0·150	0·123	0·35	3·7	2·8	0·56	0·51

and (*4*), thus obtaining

$$q_s = \frac{s_0\beta(\ln\alpha)(\tau^{\beta-1})(\alpha-\tau^{\beta})}{x_0 \exp(a + \mu_m\tau)} \qquad (5)$$

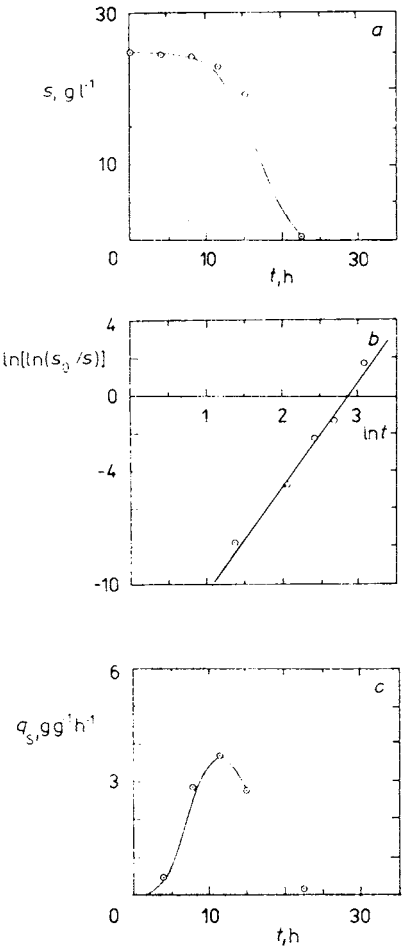

FIG. 5

Time dependence of glucose utilization, $Q = 0{\cdot}15$ v/v/min, pH_i 3·5, $T = 30°C$. *a* variation in glucose concentration versus time (solid line predicted by Eq. (*2*)); *b* graphic representation of Eq. (*3*); *c* variation in q_s versus time (solid line predicted by Eq.(*5*))

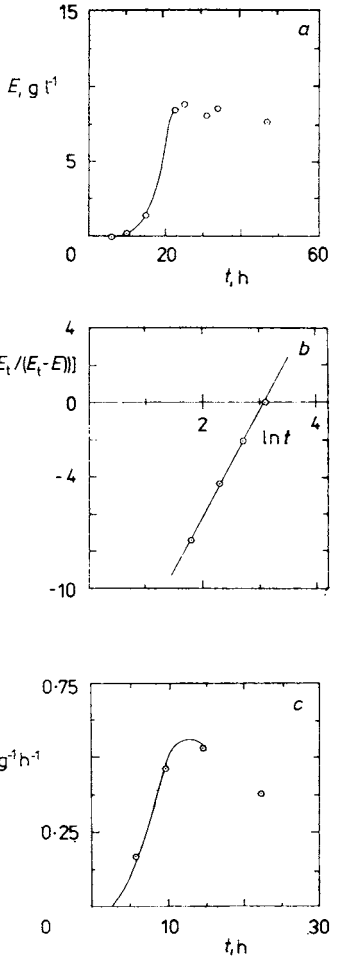

FIG. 6

Time dependence of ethanol formation, $Q = 0{\cdot}075$ v/v/min, $pH_i = 3{\cdot}5$, $T = 35°C$. *a* variation in ethanol concentration versus time (solid line predicted by Eq. (*7*)); *b* graphic representation of Eq. (*9*); *c* variation in q_E versus time (solid line predicted by Eq. (*11*))

in which an application of the stationary point condition leads to

$$\mu_m \tau_{max,s} + \beta(\ln \alpha)\, \tau^{\beta}_{max,s} = \beta - 1 \,. \tag{6}$$

By applying an iterative method, this equation allows us to obtain, the time at which q_s is at its maximum, $\tau_{max,s}$, and consequently, on substituting this time value into Eq. (5), to arrive at the q_s^{max} values (see Tables IV, V and VI). A comparison of the q_s values deriving from Eq. (4) with those obtained by using Eq. (5) (solid line), for the exponential growth zone, appears in Fig. 5c.

Specific Maximum Ethanol Production Rate

Just as described in the section above, in order to directly determine the specific ethanol production rates q_E, we chose to use an empirical model which allowed us to adjust the ethanol concentration versus time, although, as this concentration decreases during the final stationary phase due to the fact that *Pachysolen tannophilus* assimilates ethanol in the absence of glucose[12], we only took the phase when E was on the increase into account. The equation that gave the closest reproduction (mean absolute error 0·15 g l^{-1}) is expressed by

$$E_T/(E_T - E) = A^{\tau^B} \,, \tag{7}$$

where E_T represents the maximum concentration possible if the ethanol yield were the theoretical one,

$$E_T = s_0(2M_E/M_s) \,. \tag{8}$$

Equation (7) can then be linearized in an manner analogous to that described in the last section, giving

$$\ln\left[\ln\left(E_T/(E_T - E)\right)\right] = \ln(\ln A) + B \ln \tau \tag{9}$$

and once the parameters A and B have been calculated q_E can be arrived at by

$$q_E = \frac{E_T B(\ln A)\, \tau^{B-1} A^{-\tau^B}}{x} \tag{10}$$

which, in the exponential-growth phase, would be applied in the form of

$$q_E = \frac{E_T B(\ln A)\,(\tau^{B-1})\,(A^{-\tau^B})}{x_0 \exp(a + \mu_m \tau)} \,. \tag{11}$$

Finally, the time at which q_E is at its maximum, $\tau_{max,E}$, can be determined iteratively by using

$$\mu_m \tau_{max,E} + B(\ln A)(\tau_{max,E})^B = B - 1 . \qquad (12)$$

As an example, a representation of Eq. (*12*) applied to the experiment at a temperature of 35°C, is shown in Fig. 6*b*. A comparison between the experimental values of E and those calculated by using Eq. (*7*) can be seen in Fig. 6*a*, while in Fig. 6*c* appear the values arrived at through Eq. (*10*) together with those calculated for the exponential-growth zone using Eq. (*11*) (solid line). In this way we have been able to obtain the values of q_E^{max} set out in Tables IV, V and VI.

Biomass Yield

From the dry weight of samples taken during the stationary phase of each experiment, x_{FE}, we have determined the biomass yield expressed as

$$Y_{x/s} = \frac{x_{FE} - x_0 - x_{s0}}{s_0} , \qquad (13)$$

where x_{s0} represents the net biomass production in a control experiment carried out in a culture medium without glucose, which gave a result of 0·0058 g l^{-1}. Although this value may appear to be insignificant compared to the values of x_{FE}, we consider it to be conceptually more correct to take it into account when calculating $Y_{x/s}$.

We also checked to see if the biomass yields were constant throughout the experiments, by comparing the measurements of biomass produced, $x - x_0$, with glucose consumed, $s - s_0$, at specific points of time; an example appears in Fig. 7. The $Y_{x/s}$ values obtained from the slopes, practically coinciding with the yields calculated from Eq. (*13*), are shown in Tables IV, V, and VI. In these tables it can be seen that there is a significant increase in the biomass yield as the intial pH increases and also when the aeration is higher, but that the temperature changes have scarcely any effect.

Ethanol Yield

In order to determine the ethanol yield, $Y_{E/s}$, we compared the ethanol concentration, E, with the quantity of glucose consumed, $s_0 - s$, at any given time. As can be seen in Fig. 8, a straight line results for all the experiments except that with an initial pH of 1·5, in which the assimilation of glucose was very low and the ethanol produced hardly significant. The slope of this line gives an ethanol yield of 0·36 g of ethanol per g of glucose, or 70% of the theoretical yield predicted by Eq. (*8*). This value is lower than that obtained for similar fermentations of glucose with *Saccharomyces cerevisiae*, which comes close to 100% yield[13].

DISCUSSION

Five separate parameters involved in the fermentation of glucose by *Pachysolen tannophilus* to produce ethanol have been evaluated: the maximum specific rates of growth, μ_m, of glucose consumption, q_s^{max}, and of ethanol production, q_E^{max}, together with the ethanol yield, $Y_{E/s}$, and the biomass yield, $Y_{x/s}$.

In order to test the consistency of these values q_s^{max} and q_E^{max} can be worked out in a simplified fashion, based on the fact that there is a linear relationship between $(x - x_0)$ and $(s - s_0)$, as has been shown in the calculation of $Y_{x/s}$, and also between E and $(x - x_0)$. The latter relationship has been found to hold good in all our

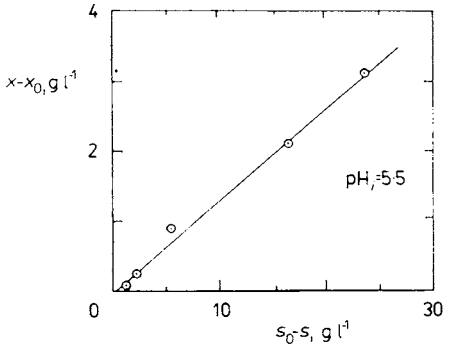

FIG. 7

Biomass produced versus glucose consumed at different times, for the experiment with pH_i 4·5

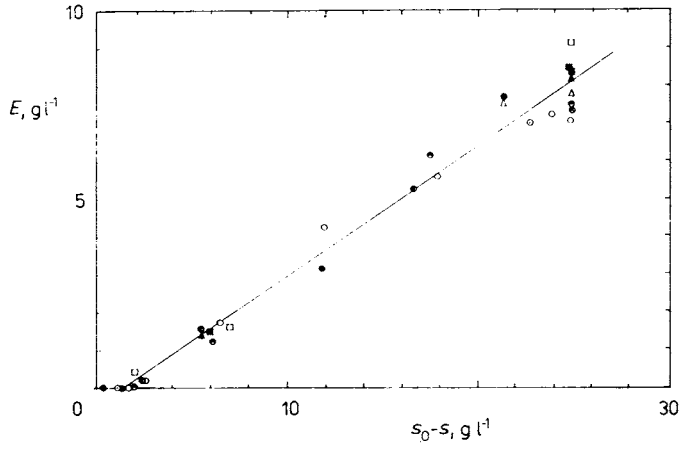

FIG. 8

Ethanol produced versus glucose consumed at different times for all the experiments, except that with pH_i 1·5. pH: ⊙ 2·5; ○ 3·5; ◓ 4·5; ⊗ 5·5; ● 6·5. T, °C: △ 25; ▲ 35. Q, v/v/min: □ 0; ■ 0·15

experiments, and the slope of the resulting graph is designated as $Y_{E/x}$. Thus, q_s^{max} can be obtained by $\mu_m/Y_{x/s}$ and q_E^{max} by $\mu_m Y_{E/x}$.

It can be seen from Tables IV, V and VI, where the values of $q_E^{max} - \mu_m/Y_{x/s}$ and those of $q_s^{max} - \mu_m Y_{E/x}$ are compared, that in general they coincide fairly well, all of which suggests that both the assimilation of glucose and the production of ethanol are closely related to yeast growth.

The most favorable experimental conditions for the ethanolic fermentation of glucose using *Pachysolen tannophilus* were thus: an initial pH of 2·5 to 3·5, and a temperature of 30°C, while sufficient aeration is admitted by the stirring vortex. Under our conditions the maximum specific ethanol production rate was about 1·2 g of ethanol per g of biomass per h and the ethanol yield about 0·36 g of ethanol per g of glucose.

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LIST OF SYMBOLS

A	empirical constant, Eq. (7)
a	intercept, Eq. (1)
B	empirical constant, Eq. (7)
$-ds/d\tau$	glucose consumption rate by volume unit, (g glucose) l^{-1} h^{-1}
E	ethanol concentration, g l^{-1}
E_T	maximum theoretical ethanol production, g l^{-1}
M_E	molecular weight of ethanol
M_s	molecular weight of glucose
Q	air-flow rate, v/v/min
q_E	specific ethanol production rate, (g ethanol) (g biomass)$^{-1}$ h^{-1}
q_s	specific glucose consumption rate, (g glucose) (g biomass)$^{-1}$ h^{-1}
q_E^{max}	maximum specific ethanol production rate, (g ethanol) (g biomass)$^{-1}$ h^{-1}
q_s^{max}	maximum specific glucose consumption rate, (g glucose) (g biomass)$^{-1}$ h^{-1}
s	residual glucose concentration, g l^{-1}
s_0	initial glucose concentration, g l^{-1}
T	temperature, °C
x	biomass concentration, g l^{-1}
x_{FE}	biomass concentration during the stationary phase, when $s = 0$, g l^{-1}
x_{s0}	maximum biomass concentration in a control experiment carried out in the culture medium without glucose, g l^{-1}
x_I	biomass concentration, at the begining of the exponential phase, g l^{-1}
x_0	initial biomass concentration, g l^{-1}
$Y_{x/s}$	average biomass yield, (g biomass) (g glucose)$^{-1}$
$Y_{E/s}$	average ethanol yield, (g ethanol) (g glucose)$^{-1}$
$Y_{E/x}$	slope of the graph of E vs $(x - x_0)$, (g ethanol) (g biomass)$^{-1}$
α	empirical constant, Eq. (2)
β	empirical constant, Eq. (2)
μ_m	maximum specific rate of growth, h^{-1}

τ, t time, h
$\tau_{max,E}$ time corresponding to q_E, h
$\tau_{max,s}$ time corresponding to q_s, h

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