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Effect of temperature and pH on lipid accumulation by *Trichoderma reesei*

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Summary. The fungal micro-organism *Trichoderma reesei* was grown in batch culture with excess glucose at pH values between 2.7 and 4.5 and temperatures between 25° C and 35° C. A maximum lipid concentration of 16.90% of the cell dry weight was achieved at pH 3.2 and a temperature of 27° C. Lipid concentration was shown to be correlated with a calculated maximum specific growth rate (μ_{mc}) and the maximum lipid value occurred at $\mu_{mc} = 0.10\ h^{-1}$. Fatty acid analysis was carried out and found to change with changing pH and temperature. Palmitic (16:0) acid and unusually high proportions of stearic acid (18:0) were commonly present. A conversion of fatty acids to palmitoleic acid (16:1) occurred following an unidentified nutrient limitation other than nitrogen depletion after 70 h of culture.

Introduction

Most of the reported work on enhanced microbial fat production has been involved in the study of oleaginous yeasts (Botham and Ratledge 1979), even though they constitute a comparatively small part of the total of oleaginous species. Screening procedures have led, possibly, to many potentially oleaginous organisms being disregarded and hence, for example, little work has been carried out using many of the fungal classes (Ratledge 1982). Culture conditions for many organisms affect both the total lipid content and composition, with higher temperatures favouring the more saturated lipids (Summer et al. 1969; Mumma et al. 1969; Taneja et al. 1979). The possibility that any fungal organism might be persuaded to accumulate lipids under appropriate culture conditions was investigated by Brown et al. (1988). That work suggested that pH might have a significant effect and it was for that reason that this more detailed investigation of environmental factors was undertaken.

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Materials and methods

Microorganism. The microorganism used in this work was *Trichoderma reesei*, formerly *viride* (Simmons 1977) QM 9123.

Culture media. The culture medium consisted (in $kg \cdot m^{-3}$) of: glucose, 80.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.4; urea, 0.3 and 1 cm^3 per dm^3 of a trace element solution containing (in $kg \cdot m^{-3}$): $MnSO_4 \cdot 4H_2O$, 2.06; $FeSO_4 \cdot 7H_2O$, 5.0; $CoCl_2 \cdot 6H_2O$, 3.66 and $ZnSO_4 \cdot 7H_2O$, 1.4. The pH of the medium was adjusted to 5.3 before autoclaving at 1 bar for 15 min in order to minimise salt precipitation, by adding 0.8 cm^3 of 12.5 M HCl. The medium was transferred aseptically to a sterile fermentor.

The inoculum for the fermentor was grown in three 500-cm^3 Erlenmeyer flasks each containing 100 cm^3 inoculated medium and was incubated for 2-3 days.

The fermentor. The fermentor was a 22-cm diameter stirred batch culture reactor with a working volume of 8 l. It was equipped with automatic controls for the environmental conditions of temperature, pH, and stirrer speed. Sterilisation of the fermentor and associated pipework was carried out in situ using steam at a pressure of 5 psi for 8 h. The aeration rate was held constant at 1 vvm and foaming was prevented by the manual addition of antifoam (polypropylene glycol 2000) as needed.

Experimental procedures. Batch fermentations were carried out using the single medium described above. Following the sterilisation and cooling of the medium, it's pH value was adjusted to 6.5 prior to inoculation. As the culture started to grow, the pH was allowed to fall until it reached the desired experimental value, at which it was then controlled by the addition of NaOH solution. For each experiment, samples were removed at convenient intervals and determinations were made of cell dry weight, total lipid content and medium glucose and ammonia nitrogen concentrations. In addition, the dissolved oxygen (DO) tension was recorded.

Culture dry weight. Each sample of culture fluid was filtered on a Buchner apparatus using a Whatman no. 1 filter paper previously dried to constant weight in an oven at 90° C. The mycelium was washed with 100 cm^3 distilled water and then the filter paper and mycelium were similarly dried on a watch glass to constant weight. The dry weight was determined by subtraction.

Total fats and fatty acid composition. The samples from the fermentor were filtered using Whatman no. 1 filter paper. The mycelium was washed with 100 cm^3 distilled water and then dried at 90° C for 24 h. The dried cells were ground in a pestle and mortar to a particle size of < 500 µm. Some of the dried cell powder was then placed in a previously dried and weighed Soxhlet thimble and further dried in a desiccator for 24 h. The thimble was then weighed, leached with petroleum ether (b.p. 40–60° C) for 24 h, the solvent evaporated and the thimble desiccated and then reweighed. The weight difference was the total fats removed from the sample.

The ether was evaporated and the residue was saponified by adding 2 cm^3 of 10% (w/v) KOH in ethanol and extracted with ether. This procedure was repeated and the second extract was dried over anhydrous sodium sulphate. The methyl esters were formed, after further removal of the ether, by reaction with 5 cm^3 of $BF_3 \cdot MeOH$ in a boiling water bath for 3 min. After cooling, two 1 cm^3 aliquots of chloroform were used to extract the methyl esters, again dried over anhydrous sodium sulphate.

The methyl esters were indentified using gas liquid chromatography, separated in a column containing 20% diethylene glycol succinate on WH-P Chromosorb. Peaks were identified using standard samples as described by Gunstone (1967).

Glucose and nitrogen analysis. Filtered samples of culture were analysed for glucose as reducing sugar using a Technicon Auto-Analyzer (Technicon Instruments Corporation, New York, USA) and for nitrogen using a Kjel-Foss Automatic Analyzer 16210 (A/S N. Foss Electric, Hilleroed, Denmark).

Dissolved oxygen monitor. An electrode of the galvanic type (Borkowski and Johnson 1967) was used to measure the dissolved oxygen tension. The current output was fed to a six-channel Xactaline recorder (Ether, Stevenage, UK) via a 1 kohm potentiometer.

Results and discussion

A number of experimental fermentations were carried out at different temperatures and pH values to determine the maximum fat content. Results are discussed in terms of the rates of metabolism and some data on fatty acid analysis are presented.

Fermentation profiles

A detailed analysis of a typical fermentation is shown for run H6 (pH 3.0, temperature 27° C) in Fig. 1. After a short lag, growth occurred quite rapidly until approximately 25 h when nitrogen limitation was established. The DO value increased sharply as the growth gave way to fat storage. The fat content of the cells increased steadily until 70 h, reaching 15.00% (w/w), at which point some new limitation occurred, affecting the fat production and indicated by changes in DO, nitrogen and cell concentration. After that moment, the glucose concentration continued to fall and the cell concentration continued to rise until the end of the experiment at approximately 160 h. It is possible that a further unidentified storage material was formed from 90 h onwards.

Lipid formation

The effect of pH 2.7, 3.0 and 4.5, at a temperature of 30° C, on the lipid profiles is shown in Fig. 2a. Each run

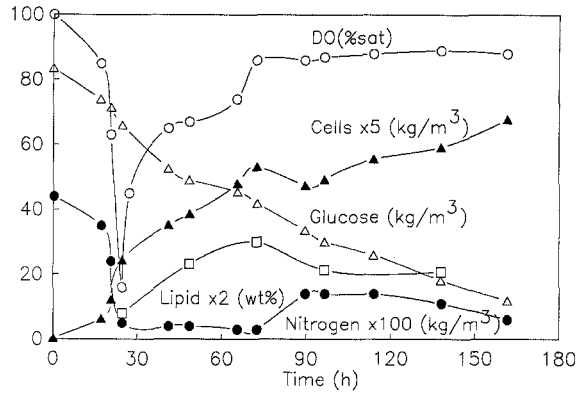

Fig. 1. Details of fermentation profile for run H6 (27° C and pH 3.0): DO, dissolved oxygen

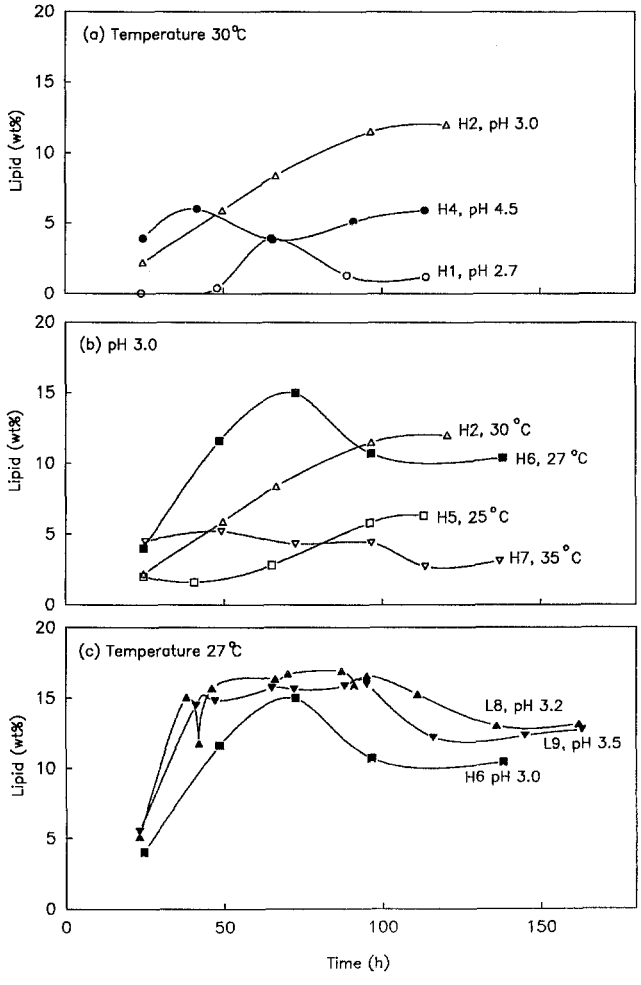

Fig. 2. Experimental results of lipid profiles obtained at 30° C (**a**), pH 3.0 (**b**) and 27° C (**c**)

was started at pH 6.5 and the initial growth reduced the pH to the chosen control value. The highest value of lipid was obtained at pH 3.0, so that the effect of temperatures 25° C, 30° C and 35° C are compared at this pH in Fig. 2b. The third group of experiments in Fig. 2c compares the effect of pH 3.0, 3.2, and 3.5 at a temperature of 27° C.

Maximum lipid production

The maximum value of the lipid concentration inside the cells for each experiment shown in Fig. 2 is listed in Table 1, together with the time at which it occurred and the maximum cell concentration at that time. In addition to the results from the eight experiments described above, there are included values for runs H3 and H13 (Hasan 1980) and runs L10–L12 (Lepe-Casillas 1980). These runs give additional information at different combinations of temperatures and pH. By simple inspection, it can be seen that the highest lipid concentration of 16.90% (w/w) was achieved in run L8 at 27° C and pH 3.2.

The optimum environmental conditions for lipid biosynthesis of 27° C and pH 3.2 are not the conditions that are optimum for growth. A correlation of the maximum specific growth rate (μ_m) for this organism as a function of temperature and pH, expressed as hydrogen ion concentration [H^+] ($kg \cdot m^{-3}$), is reported by Brown (1988) from the work by Zainudeen (1974) to be:

$$\mu_m = 10^{12}(1.44 - 355[H^+])e^{\frac{-74816}{RT}} - 10^{27}(1.80 - 625[H^+])e^{\frac{-166067}{RT}} \quad (1)$$

where R = the gas constant (8.314 $kJ \cdot kmol^{-1} \cdot h^{-1}$), T = temperature (K).

Using Eq. 1, the highest μ_m for pH 3.2 would be at a temperature of 37.5° C. It is thus possible that lipid biosynthesis or more specifically the production of enzmes associated with lipid biosynthesis is repressed by high rates of metabolism.

Although it is appreciated that lipid formation occurs primarily as a storage process after growth has ceased under nitrogen limitation, it is suggested here that environmental factors might influence rates of lipid biosynthesis to the same extent as they influence specific growth rates. Thus, values of μ_m were calculated from Eq. 1 (μ_{mc}) for all the combinations of temperature and pH values listed in Table 1 and are included in that table. The maximum lipid concentrations were then plotted as Fig. 3 versus this calculated μ_m (μ_{mc}) unifying the effects of temperature and pH and being proportional to their effects on the rate of lipid biosynthesis. A good correlation of the temperature and pH effects resulted with the optimum conditions occurring at $\mu_{mc} = 0.10\ h^{-1}$ in comparison with possible values of $\mu_{mc} = 0.17\ h^{-1}$ for the high rates of growth of this organism. At a temperature of 27° C, $\mu_{mc} = 0.10\ h^{-1}$ would correspond to a value of pH 3.27 from Eq. 1, close to that observed above. The shape of this correlation suggests that as the rate of metabolism is reduced, the rate of lipid biosynthesis increases until it reaches a maximum. This effect is characteristic of a metabolic repression control of enzyme biosynthesis and is reminiscent of a similar effect of dilution rate on lipid concentration in the continuous culture of *Candida* 107 described by Ratledge (1978) from data in Gill et al. (1977). At lower μ_{mc} values, the lipid concentration falls off very steeply. This is possibly due to a considerable reduction in the generation of the energy needed to drive the biosynthetic process.

Table 1. Maximum lipid concentration

Run. no.	T (°C)	pH	Max. lipid conc (wt.%)	Time (h)	Max.x. ($kg \cdot m^{-3}$)	μ_{mc} (h^{-1})
H1	30	2.7	3.90	65.0	3.91	0.080
H2	30	3.0	12.00	120.5	10.62	0.110
H4	30	4.5	5.90	113.5	7.20	0.139
H5	25	3.0	6.30	113.5	7.97	0.074
H6	27	3.0	15.00	73.0	10.64	0.088
H7	35	3.0	4.50	25.0	4.66	0.142
L8	27	3.2	16.90	87.0	9.15	0.097
L9	27	3.5	16.03	95.0	10.63	0.105
H3[a]	30	4.2	8.00	90.0	13.41	0.138
H13[a]	35	4.0	2.20	72.0	6.76	0.169
L10[b]	25	3.2	9.91	63.0	7.28	0.083
L11[b]	25	3.5	8.74	65.0	10.28	0.090
L12[b]	25	4.0	7.11	98.0	15.10	0.094

Max.x is maximum cell (dry wt) concentration; μ_{mc}, calculated maximum specific growth rate

[a] Hasan (1980)

[b] Lepe-Casillas (1980)

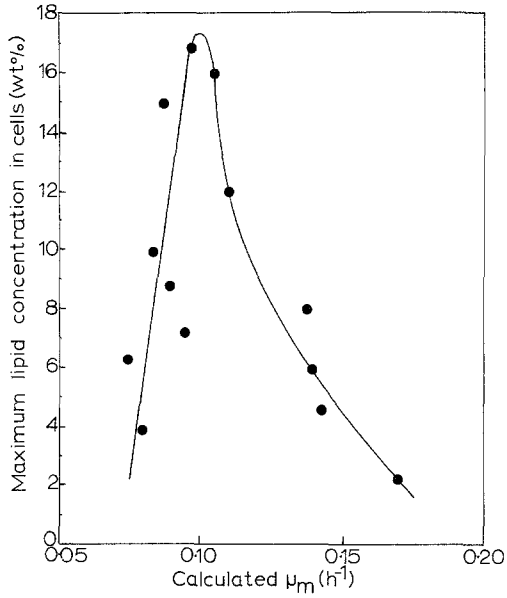

Fig. 3. Correlation of maximum lipid concentration with calculated maximum specific growth rate (μ_{mc})

Analysis of fatty acids

Detailed fatty acid analysis was carried out for runs H1, H2, H4, H5 and H6 at time intervals of approximately 24 h. The results are listed in Table 2 in increasing values of μ_{mc}. Except for a small number of samples, the values of the wt% of lauric (12:0) myristic (14:0) and arachidic (20:0) acids were always less than 1.0 and so were not included in the table. Included in Table 2 is the appropriate cell concentration and the associated lipid concentration.

In common with other fungi (Boulton and Ratledge 1985) there was the general pattern of notable quanti-

Table 2. Weight fraction (%) of fatty acid in lipid material

Run no.	μ_{mc} (h^{-1})	Time (h)	x ($kg \cdot m^{-3}$)	Lipid (%)	16:0 (%)	16:1 (%)	18:0 (%)	18:1 (%)	18:2 (%)	18:3 (%)
H5	0.074	25	1.31	2.00	9.3	—	71.3	—	1.1	0.7
		41	4.38	1.60	10.4	—	30.2	57.3	1.1	—
		65	6.68	2.80	52.6	—	45.5	—	1.0	—
		96	7.54	5.80	33.1	—	24.8	—	0.1	0.1
		113	7.97	6.30	10.4	—	60.3	0.9	0.9	0.1
H1	0.080	24	3.27	0.03	21.5	—	1.7	60.0	11.4	—
		48	3.73	0.40	88.3	1.9	—	—	6.4	0.5
		65	3.91	3.90	66.2	—	23.7	—	0.7	—
		89	3.40	1.30	68.1	15.9	2.4	4.7	—	0.1
		114	3.26	1.20	52.9	5.8	34.8	—	5.3	—
H6	0.088	25	4.89	4.00	28.2	—	7.4	20.5	43.1	—
		49	7.75	11.60	22.9	—	17.2	25.2	30.7	2.7
		73	10.64	15.00	23.3	5.4	19.4	20.0	26.5	1.6
		97	9.84	10.70	0.7	40.5	—	12.4	27.7	—
		138	11.84	10.40	—	76.4	13.0	5.2	2.6	0.5
H2	0.110	25	5.93	2.20	17.4	0.8	10.8	13.9	37.3	—
		50	8.42	5.90	38.3	—	23.5	27.5	8.4	0.1
		67	9.41	8.40	41.3	—	26.5	25.6	4.5	0.1
		97	10.63	11.50	35.3	—	23.8	34.6	4.0	—
		121	10.62	12.00	41.3	—	22.8	27.0	6.9	—
H4	0.139	25	5.56	3.90	6.5	—	42.2	40.1	10.9	—
		42	8.00	6.00	5.9	—	22.6	63.3	7.9	—
		66	7.10	3.80	18.0	—	30.3	0.3	49.0	—
		91	6.69	5.10	40.1	—	—	—	29.3	1.3
		114	7.20	5.90	9.0	—	—	59.9	30.6	—

x, cell (dry wt) concentration

ties of palmitic (16:0), oleic (18:1) and linoleic (18:2) acids and almost no production of linolenic acid (18:3). In contrast, however, is the important presence of stearic acid (18:0) in higher percentages than is normally observed in either fungi or yeasts.

It is possible to identify changes in the fatty acid analysis with respect both to time and μ_{mc}. As the culture process proceeded with time there was some lipid production during cell growth, lipid accumulation after nitrogen limitation and some lipid modifications after approximately 70 h. Changes in fatty acid composition as a function of time are most clearly evident for the runs with the μ_{mc} less than optimal. Support for the changes recorded for run H6 can be found in Fig. 1. The results indicate only minor changes in palmitic acid (16:0) and oleic acid (18:1), a gradual rise in stearic acid (18:0) and fall in linoleic acid (18:2) until 73 h when the maximum lipid content was reached. From this point, the various disturbances seen in Fig. 1 were associated with the loss of 16:0, 18:0, and 18:1, apparently converted into palmitoleic acid (16:1). At approximately 95 h, 18:2 declined, contributing further to 16:1. A similar tendency can be seen for run H1, but with the formation of lower quantities of 16:1.

In contrast, fatty acid data from run H2 at $\mu_{mc} = 0.110\ h^{-1}$ and thus slightly above the optimum of $\mu_{mc} = 0.10\ h^{-1}$, has some notable differences from run H6. The total lipid content rose smoothly throughout the run, shown in Fig. 2a, reaching a maximum value of 12.00% after 121 h. This run is characterised by producing no 16:1 and 18:3, a constant low value of 18:2 and a relatively constant composition of 16:0, 18:0 and 18:1. Slight changes at 97 h, equivalent to a cell concentration of 10.63 $kg \cdot m^{-3}$ might be caused by the same possible nutrient limitation that was encountered at 73 h (cell concentration 10.64 $kg \cdot m^{-3}$) in run H6.

It is apparent that the fatty acid composition has the general characteristics of reported values for fungi. However, the fatty acid composition can change as a function of time which, in a batch process, is probably due to changes in nutrient limitations. Some nutrient limitation other than nitrogen is indicated by the results and it's effect on the fatty acid composition will occur at different times depending upon the overall rates of metabolism dictated by the environmental factors of pH and temperature.

In conclusion, batch cultures of *T. reesei* were shown to accumulate lipids to levels affected by both pH and temperature. A maximum lipid concentration of 16.90% of the cell dry weight was achieved at pH 3.2 and a temperature of 27° C. The effects of both pH and temperature were unified through their effects on the growth rate and a maximum lipid concentration was found to be correlated by with μ_{mc}. The optimum value of μ_{mc} was found to be 0.10 h^{-1}. The possibility of further nutrient limitation after nitrogen depletion was indicated in some runs.

Fatty acid analysis of the lipids indicated that the main components were palmitic acid (16:0) and the 18-type components. The percentage of stearic acid (18:0)

was higher than normally observed in fungi. A particularly high conversion of components to palmitoleic acid (16:1) occurred under the second unknown nutrient limitation. Work is continuing to determine the nutrient limitation other than nitrogen and to confirm the correlation between the rate of lipid biosynthesis and the rate of metabolism.

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