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Optimization of fermentation conditions for thermostable cellulase production by *Thielavia terrestris*

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SUMMARY

Of the eighteen different carbon sources, solka floc was optimal for the induction of cellulases by the thermophilic fungus *Thielavia terrestris*. The temperature optimum for growth was between 44–52°C. The effect of initial and controlled pH on fungal growth and cellulase production was investigated and the results obtained showed that the maximum volumetric productivity (6.07 I.U./l per h) of filter paper activity was achieved when the pH was controlled at 4.5–5.0.

INTRODUCTION

Two of the major limitations encountered in the utilization of cellulases produced by mesophilic fungi are their relatively poor temperature stability characteristics and low rates of cellulose hydrolysis [9]. Thermophilic microorganisms, on the other hand, elaborate enzymes that are, in general, more active at high temperatures and more thermostable than those produced by their mesophilic counterparts [26]. In addition, there is a direct, though not absolute, correlation between temperature optima for growth and thermostability of the enzyme in question. Furthermore, since thermophilic cellulolytic organisms grow at elevated temperatures they may offer faster growth rates when compared to mesophiles [7]. Thus, thermophilic cellulolytic microorganisms present themselves as promising new sources of cellulases which possess superior thermal stability characteristics at elevated temperatures which in turn may facilitate higher rates of saccharification [8]. Other advantages of using thermophilic systems have been listed in Table 1 and discussed in detail elsewhere [13].

Cellulases derived from some strains of *Thielavia terrestris* have been reported to be exceedingly thermostable. For instance, Skinner and Tokuyama [21] reported that 20% of the original enzyme activity still remained when the culture filtrate was exposed to temperatures of 100°C for a period of 3 h. In an earlier publication we also reported that cellulases derived from a different strain of *T. ter-*

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Table 1

Advantages of thermophilic systems

1.	Rapid kinetics
2.	Reduced cooling and energy requirements
3.	Enhanced mass tansfer of non-gaseous reactants
4.	Reduced risk of contamination
5.	Increased cost effectiveness due to potential enzyme reuse
6.	Enzyme purification possible at room temperature
7.	Higher resistance to chemical denaturing agents
8.	Facilitates removal of volatile products and inhibitors
9.	Reduced energy requirements for drying of microorganisms
10.	Lower oxygen solubility favours anaerobiosis
11.	Extreme thermophiles are usually non-pathogenic

restris (ATCC 26917) showed excellent thermal stability characteristics [12]. In our study, 90% of the original filter paper activity (FPase) still remained when the culture filtrate was exposed to temperatures of 50°C for a period of 40 h, and at 60°C, 30% of the original activity was retained even after exposure for 10 h. These desirable stability characteristics prompted us to further investigate the production, properties and applications of both cellulases and xylanases derived from *T. terrestris* [11]. In this paper we report the optimization of fermentation conditions for cellulase production.

MATERIALS AND METHODS

Microorganism and growth medium. *Thielavia terrestris* (ATCC 26917) was obtained from American Type Culture Collection. Stock cultures of the organism were stored in culture tubes as frozen spore suspensions in a 1% w/v solka floc-mineral salts medium.

Growing cultures were started by inoculating a 250 ml shake flask containing 50 ml sterile 1% w/v solka floc-mineral salts medium (pH 4.0), with a culture tube containing 2 ml of a thawed spore suspension. The shake flask was incubated at 48°C, 150 rpm for 48 h and 5 ml of this culture was used to inoculate fresh sterile media. Growing cultures were maintained in this manner on a continual basis by transferring 5 ml aliquots into fresh medium every 48 h. To avoid culture degeneration, new shake flask cultures were initiated bi-weekly from frozen suspensions.

The 1% w/v solka floc-mineral salts medium used for the maintenance and growth of *T. terrestris* was identical to that used earlier [14].

Measurement of enzyme activity. In this study, the culture filtrate represented the enzyme source, and, therefore, all enzyme activities, i.e. endoglucanase (CMCase), exoglucanase (C_1A), FPase and β-glucosidase were determined using the culture filtrate. All assays were performed in duplicate as described by Margaritis and Merchant [12].

Evaluation of carbon sources for cellulase induction. A total of 18 different carbon sources (listed in Table 2) were chosen as test substrates on the basis of literature data and availability. All sources were tested in duplicate as follows: a 50 ml aliquot of standard mineral salts medium was added to a 250 ml shake flask containing the appropriate carbon source. The pH of the resulting mixture was adjusted to 4.0 with 2 M sulphuric acid and the flasks autoclaved for 20 min at 121°C and 15 lb/in^2. Upon cooling, the flasks were inoculated with 1 ml spore suspensions of *T. terrestris* and incubated in an orbital shaker (45°C, 200 rpm) for 24 h. The culture filtrate obtained represented the enzyme source. The pH of this filtrate was adjusted to 5.0 with 0.1 M acetate buffer, preserved with 0.01% (w/v) merthiolate and analyzed for various enzyme activities.

Effect of varying temperature on cellulase production. A total of ten shake flasks (250 ml) each containing sterile 50 ml standard solka floc (1% w/v) -mineral salts medium (pH 4.0) were inoculated with 1 ml spore suspensions of *T. terrestris.* The flasks were incubated in an orbital shaker (200 rpm) at either 36, 40, 44, 48 or 52°C for a period of 24 h. The culture filtrate obtained was treated and analyzed for various enzyme activities as described earlier.

Effect of varying pH. The effect of initial pH on fungal growth and cellulase production was studied in a 14 l stirred tank bioreactor (New Brunswick Scientific Co., NJ). In this study, the pH of the fer-

mentation medium was set at the desired value (pH 3.0, 3.5, 4.0, 5.0, 6.0 or 7.0) immediately after inoculation with a 10% (v/v), 24 h mycelial suspension. The pH was monitored during the course of fermentation and was found to increase as a function of time when the initial pH was 3.0. The temperature was maintained at 48°C, whereas the aeration and agitation rates were set at 1 v.v.m. and 100 rpm, respectively. 50-ml samples were withdrawn periodically and analyzed for FPase (a measure of overall cellulase activity) and cellular protein content for the estimation of fungal growth.

The controlled pH study was carried out as above, except that the pH of the medium was adjusted to the desired value (4.0, 4.5, 5.0, 5.5 or 6.0) immediately after inoculation and controlled at that value (± 0.1) during the entire course of fermentation by the automatic addition of 2 M sulphuric acid.

Estimation of biomass. During the course of fermentation, 50-ml samples were periodically withdrawn from the 14.0 l stirred tank bioreactor. The solids (mycelia and residual solka floc) were separated from the culture broth by filtration using glass fibre filter paper (Whatman G7/7). The retained solids were washed with three 100-ml aliquots of cold acetate buffer (0.05 M, pH 5.0). The wet washed sample was scraped off the paper and suspended in 50 ml of 1.0 M NaOH. The resulting mixture was homogenized for 5 min in a Virtis tissue homogenizer, mixed for 2 h in an orbital shaker (150 rpm, 45°C) and finally filtered. The filtrate represented solubilized cellular protein and the protein content of the filtrate was measured by the method of Lowry et al. [10] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Evaluation of carbon sources

The ability of various substrates to induce the synthesis of cellulases by *T. terrestris* is shown in Table 2. These results indicate that both the quality

Table 2

Evaluation of various carbon sources for cellulase induction in *Thielavia terrestris*

Carbon source and concentration	Final pH	CMCase (I.U./ml)	C_1 (units/ml)	FPase (I.U./ml)	β-Glucosidase (I.U./ml)
Fodder beet juice*, 1% v/v	6.4	4.7	0.1	0.04	ND
Potato starch*, 1% w/v	7.0	5.3	ND	ND	0.28
Jerusalem artichoke stalks, 1% w/v	6.1	6.0	ND	0.021	0.02
Pulp and paper waste, 1% w/v	6.3	5.0	0.2	0.041	0.087
Newsprint, 1% w/v	5.8	10.5	0.24	0.026	0.36
Sawdust, 1% w/v	6.3	6.0	ND	0.038	0.11
Carboxy methyl cellulose*, 0.5% w/v	5.7	7.6	ND	ND	0.042
Solka floc, 1% w/v	7.35	10.96	0.26	0.045	0.40
Filter paper, 1% w/v	6.5	12.90	0.40	0.06	0.35
Absorbent cotton, 1% w/v	6.5	5.0	0.19	0.03	0.35
Glycerol*, 1% v/v	5.4	5.3	0.10	0.02	0.02
Microcrystalline cellulose, 1% w/v	6.95	8.6	0.185	0.065	0.42
Cheese whey*, 1% v/v	8.7	9.3	ND	0.01	0.13
Corn steep liquor, 0.5% v/v	6.8	5.7	0.35	0.05	0.37
Wheat straw, 1% w/v	7.35	15.45	0.72	0.08	0.22
Barley straw, 1% w/v	6.65	5.7	0.2	0.024	0.135
Cellobiose*, 0.25% w/v	6.8	ND	0.14	0.01	0.22
Sucrose*, 0.25% w/v	7.6	3.8	ND	0.02	0.06

ND, not detectable.

* soluble carbon source.

and quantity of cellulases produced depends substantially on the type of inducers used. For instance, the highest levels of C_1 and CMCase activities were achieved when the fungus was grown on wheat straw. This finding is especially important because on an industrial scale, the use of 'waste cellulosics' would be more desirable, whereas the use of purified substrates would prove to the prohibitively expensive. Furthermore, Moloney et al. [16] reported that the enzyme produced by a given inducer would be more effective in digesting the same carbon source in the saccharification reactor. Thus, wheat straw could be advantageously utilized as both an inducer and as a cheap substrate for glucose production. Additionally, despite the lower cellulose content of wheat straw (approximately 40% cellulose based on dry weight) when compared to the pure cellulosic substrates such as avicel and solka floc, higher levels of cellulases were produced with wheat straw, suggesting that the latter was a powerful inducer.

The data in Table 2 also show that soluble inducers were generally weaker in their inducive power when compared to insoluble substrates. In addition, different inducers produced enzyme complexes containing varying ratios of CMCase, C_1 and β-glucosidase. This change in ratio of various components of the cellulase complex with change in inducer suggests that the synthesis of individual enzymes is independently regulated. This phenomenon has also been observed by Montenecourt and Eveleigh [17] based on their work with *Trichoderma reesei.*

Canevascini et al. [1] working on cellulase induction by *Sporotrichum thermophile* also reported that the amount of CMCase produced by the organism depended almost exclusively on the inducer used. For instance, highest levels of CMCase were obtained when avicel was used as an inducer, whereas substrates such as reprecipitated avicel and glucose gave significantly lower enzyme yields. In another study, Eriksen and Goksoyr [4] reported that the only carbon sources found to initiate cellulase formation by *Chaetomium thermophile* var. *dissitum* were cellulose and xylan. Furthermore, among the different types of cellulosics tested, cotton gave the highest cellulase activity in the medium, followed by avicel, Whatman CC41 cellulose and filter paper giving the lowest enzyme yield. Moloney et al. [16] also studied cellulase induction by *Talaromyces emersonii* using pure and 'waste' cellulosics. They discovered that avicel and filter paper were superior to other cellulosic substrates.

Thus, these findings and our data strongly suggest that the biosynthesis of cellulases is markedly dependent upon the type of inducer used. Therefore, by conducting a systematic evaluation of various carbon sources, it is possible to screen for highly efficient inducers which can lead to substantial increases in cellulase yield. In our study, wheat straw presented itself as the most economical choice amongst all inducers tested. However, due to variability encountered in the composition of wheat straw with seasonal changes and age of material, it was considered to be unsuitable for optimization studies. Solka floc was selected as an alternative carbon source due to its purity and wide use as a substrate for cellulase induction in numerous research laboratories enabling meaningful comparisons to be made. It was, therefore, used for all subsequent optimization studies.

Effect of temperature

The results obtained from the temperature study are shown in Table 3. These data indicate that *T. terrestris* grows and produces cellulases over a wide range of temperatures. The optimal temperature for cellulase production was between 44 and 52°C depending on the cellulase component. Thus, the maximal yields of CMCase and β-glucosidase were obtained when the fungus was cultivated at 52°C and 48°C, respectively, whereas maximal C_1 and FPase yields were achieved when the fermentation temperature was set at 44–52°C.

The optimal temperature range (44–52°C) for cellulase production by *T. terrestris* in this study, coincides with that of several thermophilic fungi. For instance, *Sporotrichum thermophile* [2], *Chaetomium thermophile* var. *dissitum* [4], *Aspergillus fumigatus* [23], *Myceliopthora thermophila* [20], *Talaromyces emersonii* [6], *Humicola insolens* [24] and *Humicola grisea* var. *thermoidea* [25] all exhibit op-

Table 3

Effect of varying temperature on the final yields of cellulases from *Thielavia terrestris*

Incubation temperature (°C)	CMCase (I.U./ml)	C_1 (units/ml)	β-Glucosidase (I.U./ml)	FPase (I.U./ml)
36	4.1	0.45	0.08	0.03
40	5.4	0.6	0.11	0.080
44	17.0	1.00	0.24	0.11
48	17.50	0.90	0.40	0.10
52	18.30	1.00	0.17	0.11

timal temperatures of between 45 and 50°C. The knowledge of such data is especially important when considering mixed fermentation systems. For instance, two microorganisms each lacking in a complimentary cellulase complex can be cultivated in the same bioreactor to produce a culture filtrate complete in all the cellulase components. However, this may only be possible if both organisms have similar growth requirements.

Effect of pH

The influence of pH on fungal growth (based on cellular protein content) and cellulase (FPase) production was studied in two phases. The effect of varying the initial pH on the two parameters was investigated and the results obtained are shown in Fig. 1 and 2. These results indicate that the maximum yield of FPase was attained when the initial pH was set at values ranging from 3.5 to 5.0. However, the rate of enzyme production was faster at an initial pH of 4.0 and was, therefore, regarded as optimal for cellulase production in the absence of pH control (Fig. 1). Similarly, the maximum cellular protein concentration was achieved at an initial pH of 4.0 to 5.0 (Fig. 2). The above indicates that in the absence of pH control, an initial pH of 4.0–5.0 is optimal for both fungal growth and cellulase production by *T. terrestris*.

In the second phase of our pH study, the effect of controlled pH on FPase and cellular protein production was examined and the results obtained are shown in Figs. 3 and 4, respectively. These data show that the highest level of FPase was obtained at a controlled pH of 4.5, whereas the maximum cellular protein concentration was obtained at a controlled pH of 4.0 and 5.0.

Since the filter paper test represents the overall measure of cellulolytic activity, the volumetric productivity of FPase was determined at pH values ranging from 3.0 to 7.0 under conditions of both

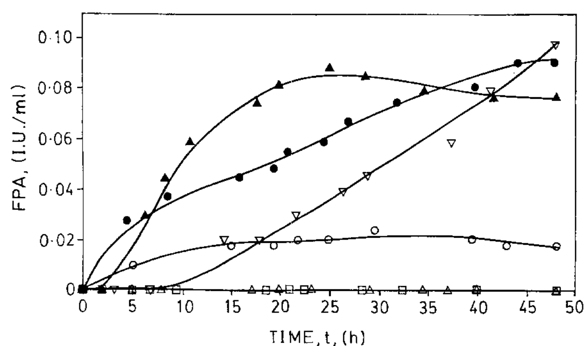

Fig. 1. Effect of varying initial pH on FPase production (△, 3.0; ▽, 3.5; ▲, 4.0; ●, 5.0; ○, 6.0; □, 7.0).

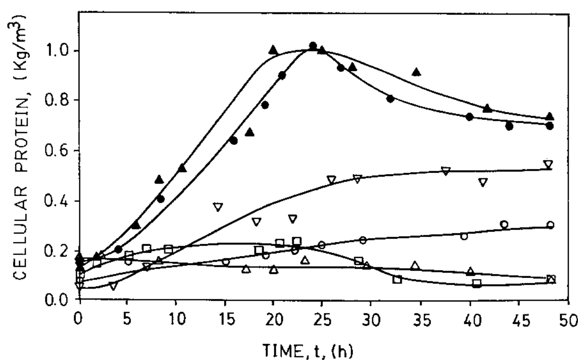

Fig. 2. Effect of varying initial pH on cellular protein production (△, 3.0; ▽, 3.5; ▲, 4.0; ●, 5.0; ○, 6.0; □, 7.0).

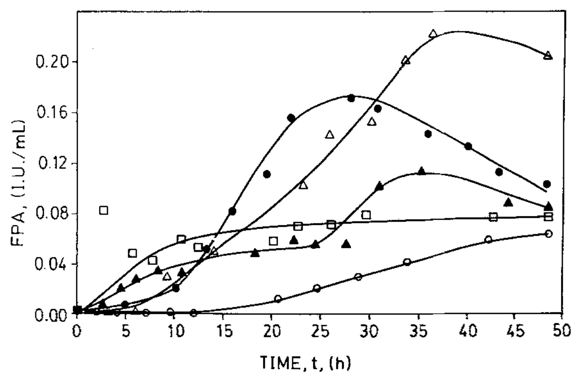

Fig. 3. The effect of controlled pH on FPase production (▲, 4.0; △, 4.5; ●, 5.0; ○, 5,5; □, 6.0).

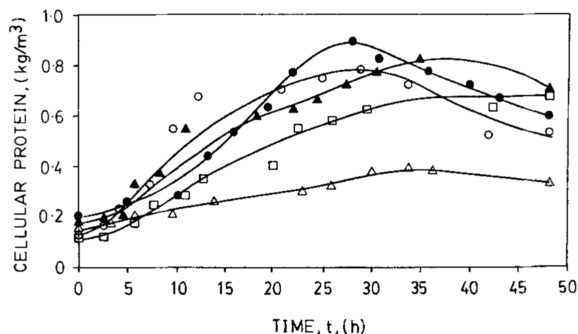

Fig. 4. The effect of controlled pH on cellular protein production (▲, 4.0; △, 4.5; ●, 5.0; ○, 5.5; □, 6.0).

controlled and uncontrolled pH. The volumetric productivity at maximal enzyme yield was calculated according to equation 1.

$$\frac{\text{I.U.}}{\text{l} \cdot \text{h}} = \frac{\text{maximum enzyme activity (I.U/ml)}}{\text{time taken for maximal yield (h)}} \times \frac{1000 \text{ ml}}{1 \text{ l}} \quad (1)$$

A comparison of the volumetric productivity of FPase under initial and controlled pH conditions is summarized in Table 4. These data show that the maximum FPase productivity was attained at an initial pH of 4.0 under uncontrolled conditions, whereas in the presence of pH control, the maximum FPase productivity was achieved at a pH of 4.5 and 5.0. Furthermore, it is readily apparent that the final enzyme yields and the volumetric FPase productivities (Table 4) were consistently higher under conditions of pH control when compared to that in the absence of pH control. It is therefore desirable to control the pH at the optimum level during the course of enzyme production.

Using solka floc as the carbon source and under optimal conditions of controlled temperature (48°C) and pH (controlled pH 5.0) the volumetric productivity of FPase at maximum enzyme yield by *T. terrestris* was calculated to be 6.07 I.U./l per h.

Table 4

Effect of initial and controlled pH on maximal FPase yield and volumetric productivity

Initial pH				Controlled pH		
pH	maximal yield (I.U./ml)	time for maximal yield (h)	volumetric productivity (I.U./l per h)	maximal yield (I.U./ml)	time for maximal yield (h)	volumetric productivity (I.U./l per h)
3.0	0.0	–	0.0	ND	ND	ND
3.5	0.1	48	2.08	ND	ND	ND
4.0	0.09	25	3.60	0.11	35	3.142
4.5	ND	ND	ND	0.22	36.5	6.02
5.0	0.092	44	2.09	0.17	28.0	6.07
5.5	ND	ND	ND	0.06	48.0	1.25
6.0	0.024	29	0.827	0.077	29.5	2.61
7.0	0.0	48	0.0	ND	ND	ND

ND, not determined.

Table 5

Comparison of FPase productivities of various cellulolytic microorganisms based on batch fermentation

a, wild type; b, thermophile; c, mesophile; d, mutant.

Microorganism	Cellulosic substrate and concentration	Maximal FPA (I.U./ml)	FPase productivity (I.U./l per h)	Reference
Sporotrichum thermophile (a,b)	1% w/v wheat straw	0.07 (t = 48 h)	1.46	3
Thermonospora, mutant N-35 (b,d)	1% w/v avicel	0.33 (t = 12 h)	27.5	15
Thermonospora curvata (a,b)	cellulose	0.10 (t = 72 h)	1.39	5
Thermonospora curvata, mutant 7 (b,d)	cellulose	0.18 (t = 72 h)	2.50	5
Thermoctinomyces YX (a,b)	avicel pH 102	0.225 (t = 29 h)	5.10	18
Clostridium thermocellum (a,b)	1% w/v solka floc	0.14 (t = 72 h)	1.94	19
Trichoderma reesi QM 9414 (c,d)	2% w/v solka floc	2.60 (t = 120 h)	21.66	22
Thielavia terrestris ATCC 26917 (a,b)	1% w/v solka floc	0.17 (t = 28 h)	6.07	this work

A comparison of volumetric productivities (based on FPase) of various cellulolytic microorganisms is shown in Table 5. The data in this table clearly show that the volumetric productivity of FPase by *T. terrestris* is superior to all the wild-type microorganisms listed. By optimization of agitation rate, aeration rate, substrate concentration coupled to a well-designed mutation programme, one might anticipate further improvements in the rate and yield of cellulase production by *T. terrestris.*

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