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Semicontinuous Production of Cellulolytic Enzymes with *Trichoderma reesei* Rutgers C30 in an Aqueous Two-Phase System

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ABSTRACT

Cellulolytic enzyme production was studied in an aqueous two-phase system, PEG 8000 5%–Dextran 7%, with *Trichoderma reesei* Rutgers C30 in a 7L fermentor. In batch cultivations, an average of 2.5 filter paper units (FPU)/mL were obtained in the top phase. In cultivations in regular media without polymers, the same enzyme concentration was obtained. The enzyme yield was 205 FPU/g cellulose in the phase system, and 259 FPU/g cellulose in the regular medium. An extractive fed-batch cultivation was maintained in the aqueous two-phase system for 360 h. The enzyme containing top phase was withdrawn after phase separation. New cellulose substrate and nutrients were added with the new top phase. The enzyme extraction was started after 120 h of cultivation, and was repeated every 72 h. The total substrate concentration was 40 g/L. A maximum enzyme concentration of 4.8 FPU/mL was obtained in the withdrawn cell-free top phase. The enzyme yield was 148 FPU/g cellulose.

Index Entries: Cellulolytic enzymes; cellulose production; aqueous two-phase systems; *Trichoderma reesei*.

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INTRODUCTION

The enzymatic hydrolysis of lignocellulose to glucose on a large scale is dependent on the supply of efficient and less expensive cellulolytic enzymes. The most frequently used microorganisms for cellulolytic enzyme production are *Trichoderma reesei* strains. Strain improvement in *T. reesei* has resulted in an enhanced enzyme production and an increased productivty and yield (*1*). By changing the culture conditions from batch to fed-batch cultivation, the enzyme concentration and productivity have also been further increased (*2–7*).

Continuous cellulolytic enzyme production is often performed in continuously stirred tank reactors (CSTR) in one or two stages (*8*). Different immobilization techniques have also been applied (*9–11*). A third approach, using an aqueous two-phase system, has previously been described (*12,13*). Such an aqueous two-phase system can be obtained by mixing two solutions of water-soluble polymers. Aqueous two-phase systems are biocompatible because of their high water content (*14*) and, when applied in enzyme production, they make it possible to use a solid substrate (e.g., cellulose), and at the same time obtain the produced enzymes in a cell-free stream. This substitutes for a cell-separation step.

Cellulolytic enzyme production with *T. reesei* Rutgers C30 in aqueous two-phase systems have earlier been studied in shake flask cultivations (*13*). In the present investigation, cellulolytic enzymes were produced with *T. reesei* Rutgers C30 in the phase system, PEG 8000 5%–Dextran T500 7%, that was the best in the earlier work. In shake flask cultures, the enzyme yield and productivity were lower in a phase system compared with in a regular medium, probably because of reduced oxygen transfer rate in the phase system. The oxygen transfer rate can be improved in fermentor cultivations, however, subjecting the mycelium to shear forces as a result of the agitation caused by the impeller may influence the enzyme production. In order to further increase the cellulolytic enzyme concentration, an extractive fed-batch cultivation was performed in the above phase system.

MATERIALS AND METHODS

Microorganism

Trichoderma reesei Rutgers C30 NRRL 11460 was obtained from the Northern Regional Research Center, Peoria, IL. The cultures were maintained on PDA (potato-dextrose-agar) slants.

Medium

Vogel's mineral salts medium N (*15*) was used in the cultivations with the addition of proteose peptone 1.0 g/L and Tween 80 0.15 mL/L. Solka-

floc BW 200 (a generous gift from James River Corp., Berlin, NH) was the carbon source at an initial concentration of 10 g/L in all cultivations.

Cultivation Conditions

Cultivations were in a 7 L fermentor (Electrolux Fermentation, Getinge, Sweden) with a working vol of 4 L. The temperature was +28°C, and the pH was kept at 5.0 with 2 *M* NH_4OH and 0.1 *M* H_2SO_4. The air flow was 10 L/min. The stirring rate was between 250 and 350 rpm. A 20% silicone antifoam agent (BDH Chemicals Ltd, Poole, UK) was used when needed. Samples were stored frozen and then centrifuged before being analyzed for filter paper activity. The fermentor cultures were inoculated with a 10% (400 mL) inoculum cultivated for approx 65 h in shake flask on 10 g/L Solka-Floc BW 200 in Vogel's medium.

Aqueous Two-Phase System

Preparation of the aqueous two-phase system has been described by Albertsson (*14*). Polyethylene glycol (PEG) 8000 (Union Carbide, Danbury, CT) with a mol wt in the range of 7000–9000 and Dextran T500 (Pharmacia Biotechnology AB, Uppsala, Sweden) with a mol wt of $500 \cdot 10^3$ were used in the aqueous two-phase system. A phase diagram is presented in Ref. 13. The polymer concentration in the phase system used in fermentor cultivations were calculated on a wt/vol basis. The dilution of the phase system by titration, in order to keep a constant pH, was compensated for by addition of PEG and dextran from sterile stock solutions of 40 and 20%, respectively. In this phase system, the partition coefficient for the cellulolytic enzyme system is 1.4, measured as filter paper activity (*13*). The phase vol ratio was 1:1 at the start of the fermentation.

Enzyme Assay

The filter paper activity (FPU) was determined according to Mandels et al. (*16*) with the following modifications: 0.05 *M* Na-acetate buffer pH 4.8 was used, the samples were boiled for 10 min, and the absorbance was read at 640 nm. Samples were diluted if the absorbance exceed 0.2.

RESULTS AND DISCUSSION

T. reesei Rut C30 cultivated in a regular medium gave an average of 2.5 FPU/mL after 72 h (Fig. 1). After 115 h, a rapid decrease in enzyme concentration was observed. The enzyme production was found to be more rapid in the fermentor than with shake flask cultures (*13*).

In the aqueous two-phase system, an average enzyme concentration of 2.5 FPU/mL was observed in the top phase after 115 h (Fig. 2). The conditions for the cultivation in the phase system were the same as in the

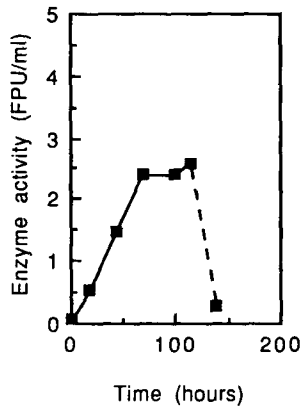

Fig. 1. Celluloytic enzyme production with *Trichoderma reesei* Rutgers C30 in a regular medium. Substrate: 10 g/L Solka-Floc BW 200. Working vol: 4 L. Temperature = +28°C; pH = 5.0; air flow = 10 L/min; agitation: 250 rpm.

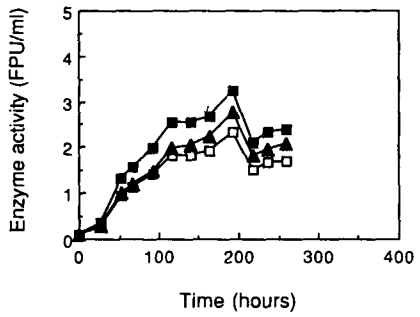

Fig. 2. Cellulolytic enzyme production with *T. reesei* Rut C30 in an aqueous two-phase system composed of PEG 8000 5%-Dextran T500 7%. (■) enzyme concentration in the top phase, (□) enzyme concentration in the bottom phase, (▲) total enzyme concentration. Same culture conditions as in Fig. 1, except that the agitation was changed from 250 to 350 rpm after 48 h.

regular medium except that after 48 h, the stirring was increased to 350 rpm. In Fig. 2, the enzyme concentration in the bottom phase and the total concentration in the phase system have been calculated from the partition coefficient and the changes in the vol ratio. The estimated total enzyme concentration in the phase system reaches a plateau around 2.0 FPU/mL, compared with 2.5 FPU/mL in the regular medium.

In Table 1, the calculated enzyme productivities and enzyme yields for the batch cultivations in the regular medium and in the phase system have been summarized. In the phase system, the productivity has been calculated for the top phase concentration as this is the recoverable enzyme. The productivities are 35 FPU/L·h in the regular medium, and 22

Table 1
Batch Cultivation of *Trichoderma reesei* Rutgers C30 in a Regular Medium and in an Aqueous Two-Phase System Composed of PEG 8000 5%–Dextran T500 7%. Substrate Concentration: 10 g/L Solka-Floc BW 200

Cultivation conditions	Maximum enzyme concentration (FPU/ml)	Productivity (FPU/l·h)	Total yield (FPU/g cell.)
Batch	2.5	35[a]	250
Batch PEG 8000 5%-Dextran T 500 7%	2.5[c]	22[b]	205

a) calculated at 72 h
b) calculated at 115 h
c) in the top phase

[a]Calculated at 72 h.
[b]Calculated at 115 h.
[c]In the top phase.

FPU/L·h in the phase system. The yield has been calculated from the total enzyme concentration in the phase system as it gives an indication of the effect of the polymers on the enzyme production of the fungus. In the regular medium, 250 FPU/g cellulose were produced, compared with 205 FPU/g cellulose in the phase system.

In shake flasks, it was observed that the cellulolytic enzyme production was reduced when *T. reesei* Rut C30 was cultivated in aqueous two-phase systems (*13*). This might result from a limitation in the oxygen transfer rate imposed by the increased viscosity of the polymers. Fermentor cultivations offer the possibility to improve the oxygen transfer rate. The present fermentor cultivations show, however, that the enzyme production with *T. reesei* Rut C30 does not reach the same level in an aqueous two-phase system as in a regular medium. Possibly, the oxygen transfer rate of the fermentor in the present study is not high enough to overcome the viscosity of the polymers and/or the phase systems may otherwise exert an inhibitory effect on cellulolytic enzyme production with *T. reesei* Rut C30.

In previous investigations, enhanced α-amylase production with *Bacillus* sp. was observed only in one particular phase system with one

Bacillus subtilis strain (*17–19*). With other *Bacillus* sp. and other phase systems, enzyme production was inhibited, which indicates that enzyme production in aqueous two-phase systems is influenced by specific interactions between the polymers and the microorganism (*20*).

When *T. reesei* Rut C30 was cultivated in aqueous two-phase systems in shake flasks, the vol ratio of the phases changed so that a considerable increase of the bottom phase was observed (*13*). This is most probably a result of the expansion of the mycelium, the degree of which is influenced by the composition of the phase system. An increase of the bottom phase vol was also observed during the first 72 h of the fermentor cultivation in the aqueous two-phase system; vol ratios were seen to change from 1:1 to 1:9 after 72 h. This time was equivalent to the growth phase as indicated by the titration curve (*21*). Contrary to the shake flask cultivations, the bottom phase then decreased again to revert finally to 1:1 after approx 120 h. The mechanism for the contraction of the fungi-holding bottom phase in fermentor cultivations is presently not understood.

Despite the fact that the enzyme production in the aqueous two-phase system was reduced compared with the regular medium, the phase system concept offers certain advantages, namely, a potentially higher enzyme concentration in a recoverable cell-free enzyme stream. Improving enzyme production in the aqueous two-phase system was therefore attempted.

In a recent review, the effect of different parameters on cellulolytic enzyme production were summarized (*22*). Among them, fed-batch cultivation has proven to be one of the most efficient ways to obtain high cellulolytic enzyme concentrations (*2–7*).

The cellulolytic enzyme production in a fed-batch cultivation with *T. reesei* Rut C30 in a regular medium is shown in Fig. 3. The cultivation conditions were the same as in the batch cultivation, except that the impeller speed was 350 rpm. The initial substrate concentration was 10 g/L. In addition to this, 120 g was added in 3×40 g portions, equivalent to a total concentration of 40 g/L. Substrate was added when the enzyme concentration had ceased to increase, that occurred after approx 120 h. After each substrate addition, the fungus grew for approx 48 h, as indicated by the titration curve. After the second addition of substrate, the enzyme concentration reached a maximum of 4.5 FPU/mL. Then, after the third substrate addition, the enzyme concentration decreased rapidly.

An extractive fed-batch fermentation with the same conditions as in the regular medium was performed in the phase system (Fig. 4). The top phase was withdrawn when the enzyme concentration had ceased to increase, after approx 120 h. The stirring was stopped and the phases were left to separate for one hour. The extraction was repeated approx every 72 h. A vol of 1.0 L was withdrawn on each occasion. Media components of original concentrations and new substrate were added with the new top phase. The average enzyme concentration in the withdrawn top phase

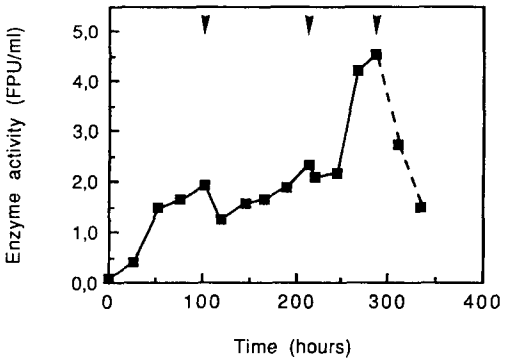

Fig. 3. Fed-batch cultivation in a regular medium with *T. reesei* Rut C30. (►) addition of 40 g Solka-Floc BW 200. Same culture conditions as in Fig. 1, but with an agitation of 350 rpm.

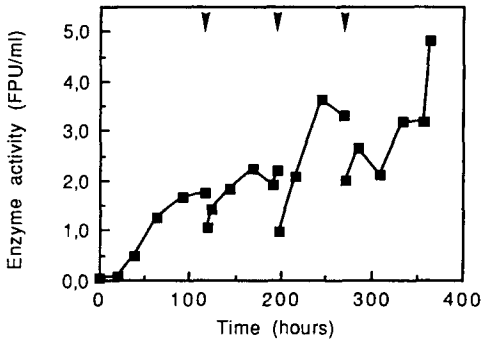

Fig. 4. Extractive fed-batch cultivation in PEG 8000 5%–Dextran T500 7% with *T. reesei* Rut C30. (►) addition of 40 g Solka-Floc BW 200 and change of top phase. Same culture conditions as in Fig. 3.

was 3.0 FPU/mL. The semi-continuous cultivation was maintained for 360 h with increasing enzyme concentration in the top phase. The highest enzyme concentration obtained in the top phase is about the same as in the regular medium, 4.8 FPU/mL and 4.5 FPU/mL, respectively. The enzyme concentration was approx doubled in the fed-batch cultivations, both in the phase system and in the regular medium, compared with batch cultivations (Figs. 1 and 2).

In Table 2, the productivities and yields have been calculated. The productivity in the phase system was 75% of the productivity in the regular medium, 12, compared with 16 FPU/L·h (Table 2), which is a reduction by a factor of two, compared with the batch cultivations (Table 1). The enzyme yield was approx the same in the regular medium and in the phase system, 151 FPU/g cellulose and 148 FPU/g cellulose, respectively. This is again lower than the yields in the batch cultures.

Table 2
Extractive Fed-Batch Cultivations for Cellulolytic Enzyme Production with *Trichoderma reesei* Rutgers C30 in a Phase System, PEG 8000 5%–Dextran T500 7%, Compared with a Fed-Batch Cultivation in a Regular Medium

Cultivation conditions	Enzyme activity (FPU/ml)	Total withdrawn enzyme activity (FPU)	Withdrawn volume (liter)	Productivity (FPU/l·h)	Yield (withdrawn FPU/g cellulose)	Total yield (FPU/g cellulose)	Total added cellulose (gram)
Fed-batch	4.5 (max.)			16[c]		151[c]	160
Extractive fed-batch fermentation							
PEG 8000 5%-	4.8 (max.)						
Dextran T 500 7%	3.0 (average)	12030	4.0	12[a]	75	148[b]	160

[a] Calculated between 120–360 h with the formula $P = D \cdot C$.
[b] Withdrawn enzyme activity + remaining activity in the phase system at the end of the cultivation, calculated from the partition coefficient and the volumes.
[c] Calculated between 0–290 h.

Thus, compared with batch cultivation, fed-batch cultivation increased the enzyme concentration, both in the regular medium and in the phase system by a factor of two, at the expense of productivity and yield. Compared with literature reports on cellulolytic enzyme production in fed-batch cultivation, the enzyme concentrations reached in the present investigation are 4–5 lower, the productivities are a power of ten lower, and both the total substrate concentration (40 g/L compared with 100–150 g/L (*3–7*)), and the rate of substrate addition (10 g/L at 72 h interval compared with 20 g/L at 48 h interval (*4,7*)) being lower in the present study.

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