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Influence of Cultivation Conditions on the Production of Cellulolytic Enzymes with *Trichoderma reesei* Rutgers C30 in Aqueous Two-Phase Systems

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ABSTRACT

Cellulolytic enzyme production in aqueous two-phase systems with *Trichoderma reesei* Rutgers C30 has been investigated. The influence of different phase systems, as well as addition of media components and substrate on enzyme production have been studied.

Extractive enzyme production in fed-batch cultivations was performed in a phase system composed of PEG 8000 5%–Dextran T500 7% with 1% Solka-Floc BW 200 as substrate. The cellulolytic enzyme system was intermittently withdrawn with the top phase. Addition of media components every 24 h and cellulose every 72 h gave an average enzyme activity in the withdrawn top phase of 2.2 FPU/mL during 170 h cultivation. The corresponding productivity was 18 FPU/l·h. The productivity was increased to 24 FPU/l·h when media components and cellulose were added every 72 h. The average enzyme concentration was then 1.6 FPU/mL. The results are discussed in relation to methods for cellulolytic enzyme production involving immobilization and cell recycling.

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

INTRODUCTION

Cellulose can be hydrolyzed to glucose in the presence of acid or by using cellulolytic enzymes. The use of enzyme is advantageous for this conversion as they work under more moderate conditions and form no byproducts. However, the enzyme costs have so far prohibited the development of an economic process for the bioconversion of cellulose to glucose. It has been estimated that the cost for the enzymes constitutes approx 60% of the total process cost (*1*). One way of reducing the enzyme costs is to improve the enzyme production.

The most commonly used microorganism for cellulolytic enzyme production is the fungus *Trichoderma reesei.* In a recent review, the influence of strain improvement, choice of substrate, substrate concentration, and cultivation conditions on cellulolytic enzyme production has been summarized (*2*). Strain improvement in *Trichoderma reesei* has increased the enzyme concentration, productivity, and yield by a factor of three (*3*). Strain improvement has also resulted in strains resistent to carbon catabolic repression (*4–6*). Enzyme concentration, productivity and yield can be further increased by using solid cellulosic substrate rather than a soluble substrate (*7*). By changing culture conditions from batch cultivation to fed-batch cultivation, the enzyme concentration can be further increased by a factor of three and the productivity by a factor of four (*8,9*). Continuous cultivation, on the other hand, increases the productivity by a factor of two compared to batch culture but results in low enzyme concentrations (*9*). In the present study, we have investigated the use of aqueous two-phase systems for improved production of cellulolytic enzymes with *T. reesei* Rutgers C30. The influence of different phase systems, the addition of media components and the addition of substrate on cellulolytic enzyme production was studied. An aqueous two-phase system can be obtained when two different water-soluble polymers are mixed, e.g., polyethylene glycol and dextran. These systems have the great advantage of being biocompatible due to the high water content in both phases (*10*). Aqueous two-phase systems have been used for separation of cells, cell-organelles, and macromolecules (*10,11*), and in bioconversions (*12*). They have been used in the enzymatic hydrolysis of cellulose (*13*), and it has earlier been demonstrated that *T. reesei* Rutgers C30 produces cellulolytic enzymes in these systems (*14*). The cells are recycled in one of the phases and solid substrates can be used. With *Bacillus subtilis* (*15*) and *Aspergillus phoenicis* (*16*), an enhanced enzyme production was observed in aqueous two-phase systems.

MATERIALS AND METHODS

Microorganism

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

Medium

The cultivation medium used was Vogel's medium *N* (*17*) 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 used as carbon source at an initial concentration of 10 g/L in all cultivations.

Cellulolytic Enzyme System

In the partition studies NOVO Cellulclast 2.0 L (76 FPU/g) was used. In the studies of the enzyme production in the presence of enzyme, NOVO Celluclast L (56 FPU/g) was used. Both were generous gifts from NOVO A/S (Bagsvaerd, Denmark). The enzymes are preparations from *Trichoderma* species.

Enzyme Assay

The filter paper activity (FPU) was determined according to Mandels et al. (*18*) 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 and was not allowed to exceed 0.2.

Polymers

Polyethylene glycol (PEG) 4000 (Serva, Heidelberg, FRG) has a mol wt in the range of 3000–3700 and PEG 8000 (Union Carbide, Danbury, CT) has a mol wt in the range of 7000–9000. Aquaphase PPT (Perstorp Biolytica, Lund, Sweden) has a mol wt of $35 \cdot 10^3$, whereas fractionated Dextran T500 (Pharmacia Biotechnology AB, Uppsala, Sweden) has a mol wt of $500 \cdot 10^3$. Crude dextran ($Mw > 5 \cdot 10^6$ (*19*); a generous gift from Pharmacia AB, Staffanstorp, Sweden) was used unhydrolyzed, and hydrolyzed. The hydrolysis was performed according to Kroner et al. (*19*) with HCl, 20 min hydrolyzing time, in order to obtain a viscosity similar to Dextran T500. The viscosity was estimated by measuring the time taken for the liquid level to drop from 2 to 1 mL in a 2 mL pipe. A hydrolyzed crude dextran solution of 10% (w/w) contained approx 1.2% reducing sugars, as determined by the DNS-method (*20*)with glucose as standard.

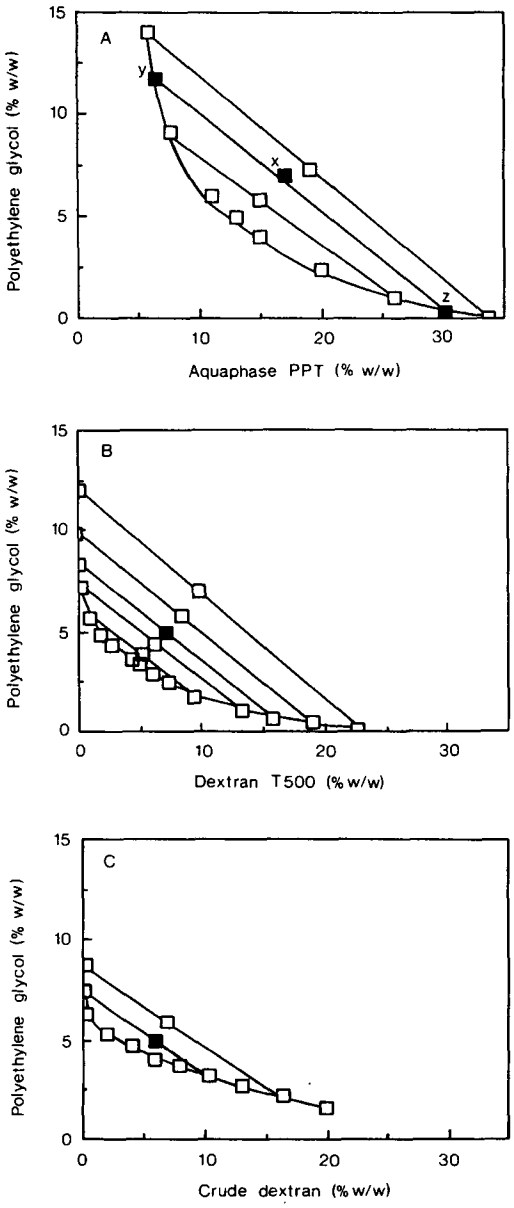

Fig. 1. Phase diagrams at +20°C for the systems: A. polyethylene glycol (PEG) 8000–Aquaphase PPT–water; B. PEG 8000–Dextran T500–water, from Ref. 10; C. PEG 4000–crude dextran–water. Point X = total composition of the phase system; point Y = top phase composition; point Z = bottom phase composition. The tie line = Y–Z. The line Y–X = bottom phase volume. The line X–Z = top phase volume. The binodial curves were experimentally obtained.

Aqueous Two-Phase System

The composition of an aqueous two-phase system can be illustrated in a phase diagram. In Fig. 1 A, B, and C phase diagrams of polyethylene glycol (PEG) 8000–Aquaphase PPT, PEG 8000–Dextran T500, PEG 4000–crude dextran are shown. Experimental binodial and tie lines were obtained according to Albertsson (*10*). Phase separation occurs at polymer concentrations above the binodial curve. An aqueous two-phase system, with a total polymer composition represented by point X in Fig. 1A, has a top phase composition represented by point Y and a bottom phase composition represented by point Z. The tie line can be used to estimate the vol ratio between the phases where the length Y–X represents the bottom phase vol and X–Z the top phase volume (*10*).

Partition of Cellulolytic Enzymes

The cellulolytic enzyme system from NOVO Cellulclast 2.0 L was partitioned in four different aqueous two-phase systems; PEG 8000 7%–Aquaphase PPT 17%, PEG 8000 5%–Dextran T500 7%, PEG 8000 5%–hydrolyzed crude dextran 7%, and PEG 4000 5%–crude dextran 6%. The vol ratio between the top and bottom phases was approx 1:1 in all four phase systems. The partition coefficient, K, for an enzyme in a phase system is defined as: the enzyme concentration in the top phase divided by the enzyme concentration in the bottom phase. The K-value for the cellulolytic enzyme system was calculated from the filter paper activity (FPU/mL). The partition studies were performed at room temperature with media components present but in the absence of cellulose. The phase systems were mixed, then allowed to settle for 30–60 min. Samples were taken from both top and bottom phases.

Cultivation of *T. reesei*

The cultivations were carried out in 1 L Erlenmeyer flasks (with a cultivation vol of 100 mL) maintained in a shaking water bath at +25°C. pH was not controlled.

The influence of phase systems on cellulolytic enzyme production with *T. reesei* Rutgers C30 was studied in the four different aqueous two-phase systems used in the partition studies. The enzyme production, measured as filter paper activity, was followed. A regular medium without phase components was used as reference. Shaking was stopped every 24 h, the cultures transferred to measuring cylinders and allowed to settle for 30–60 min. The phase systems separated into a clear top phase and a bottom phase containing the cellulose and the fungus. In the regular medium, the solids settled to the bottom of the cylinder, leaving a layer of

clear solution above them. The vol of top phase and the clear solution above the solids were measured, withdrawn, and replaced with new top phase and sterile water, respectively. Samples were taken and stored frozen for future analysis of filter paper activity. The concentration of enzyme in the top phase after addition of new top phase was calculated from the partition coefficient and the phase vol. The new enzyme concentration in the regular medium was also calculated. The mixing was then resumed and the continuation of the enzyme production was followed.

The influence of media and cellulose concentration was studied in a PEG 8000 5%–Dextran T500 7% system and a regular medium. Every 24 h, the culture in the regular medium was centrifuged and the liquid vol withdrawn. In the phase system, the top phase was withdrawn after separation for 30–60 min. The withdrawn vol were replaced with an equal vol of medium and top phase, respectively. In the case of both the regular medium and one of the phase system cultures, media components of original concentration were added with the new vol every 24 h. Cellulose of original concentration was added with the new vol every 72 h. In the other phase system culture, both media components and cellulose were added every 72 h.

The Influence of Enzyme Concentration

The influence of enzyme concentration on cellulolytic enzyme production was studied by the addition of enzyme to shake flask cultivations. Addition was made after 24 h cultivation time. The enzyme solution was filtered sterile before addition. Enzyme was added to a final concentration of 1 and 2 FPU/mL, respectively. The enzyme production was compared with a reference cultivation maintained without addition of enzyme. Samples were taken every 24 h and stored frozen for future analysis of filter paper activity.

RESULTS AND DISCUSSION

Partition of Cellulolytic Enzymes, Substrate, and Fungus

In an earlier study aiming at recycling the cellulolytic enzymes in enzymatic cellulose hydrolysis with an aqueous two-phase system the partition of the enzymes in different phase systems was investigated (*21*). As the cellulose substrate partitions to the bottom phase and the produced glucose is withdrawn with the top phase, the aim was to obtain as low a partition coefficient, K, for the cellulolytic enzymes as possible. It was found that a low mol wt bottom phase polymer reduced the K value. The presence of cellulose further reduced K.

Table 1
Partition of Cellulolytic Enzyme System
from NOVO Celluclast 2.0 L in Aqueous Two-Phase Systems[a]

Aqueous two-phase system	Molecular weight of the bottom phase polymer	Partition coefficient (K)
PEG 8000 5% - Aquaphase PPT 17%	$3.5 \cdot 10^4$	0.83
PEG 8000 5% - Dextran T500 7%	$5.0 \cdot 10^5$	1.41
PEG 8000 5% - Hydrolyzed crude dextran 7%	$< 5 \cdot 10^6$	1.60
PEG 4000 5% - Crude dextran 6%	$\approx 5 \cdot 10^6$	3.56

[a]The enzyme activity was measured as filter paper activity. The partition coefficient (K) is defined as: K = the enzyme concentration in the top phase divided by the enzyme concentration in the bottom phase. The polymer concentration of the phase systems is given as % (w/w) of the total weight of the phase system.

In the present study, the aim was to recycle the enzyme producing fungus in one of the phases, and to withdraw the cellulolytic enzymes from the other phase. The fungus partitions completely to the bottom phase, therefore the aim was to obtain a high partition coefficient for the enzymes. The partition coefficient was investigated in the absence of cellulose, as a result of the fact that during the enzyme production the cellulose substrate is consumed (Table 1). The phase system composed of PEG 8000 7%–Aquaphase PPT 17% gave the lowest K-value, 0.8, and the system composed of PEG 4000 5%–crude dextran 6% gave the highest K-value, 3.6. In agreement with our previous results, the partition coefficient increases with increasing mol wt of the bottom phase polymer (*21*). This was also found in another study that showed that high mol wt bottom phase polymers partition proteins to the top phase (*22*).

Influence of Phase Systems on Extractive Enzyme Production

Cellulolytic enzyme production was compared in the four aqueous two-phase systems (Fig. 2 and Table 2). No media components or cellulose were added and the enzyme containing top phase was intermittently withdrawn during the cultivations. These cultivations were compared with a cultivation in a regular medium where the clear solution above the

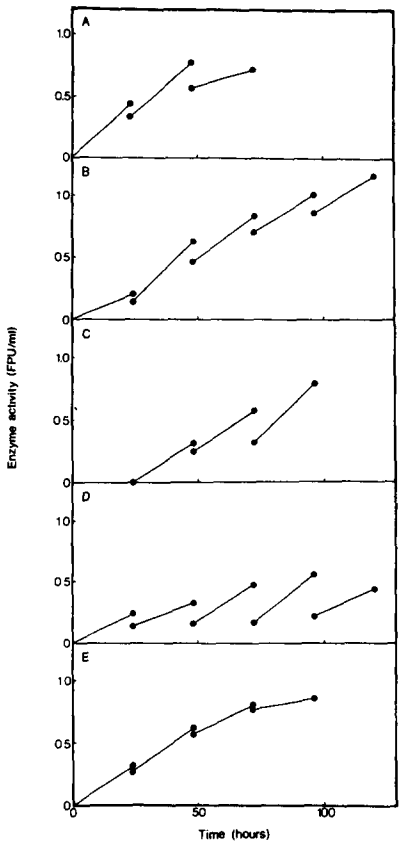

Fig. 2. The influence of phase system on extractive cellulolytic enzyme production. Carbon source: 10 g/L Solka-Floc BW 200. No addition of media components or cellulose. A. phase system composed of PEG 8000 5%–Aquaphase PPT 17%; B. PEG 8000 5%–Dextran T500 7%; C. PEG 8000 5%–hydrolyzed crude dextran 7%; D. PEG 4000 5%–crude dextran 6%; E. regular medium without phase components.

solids was intermittently withdrawn. The withdrawn enzyme activities, the productivities and the yields have been calculated from the withdrawn vol, the partition coefficients, and the enzyme concentrations (Table 2).

The highest enzyme concentration (Fig. 2, Table 2) was obtained in the PEG 8000–Dextran T500 system, 1.2 FPU/mL, and the lowest in the PEG 4000–crude dextran system, 0.6 FPU/mL. In the reference system 0.9 FPU/mL was observed. In the PEG 8000–Aquaphase PPT phase system, the rate of cellulolytic enzyme production is rapid in the beginning, however, the cultivation was terminated after 72 h following fungal sporulation. In the PEG 8000–hydrolyzed crude dextran system the enzyme production was delayed until 24 h after inoculation.

Table 2
Cellulolytic Enzyme Production in Four Different Aqueous Two-Phase Systems with *Trichoderma reesei* Rutgers C30. Substrate: 10 g/L Solka-Floc BW 200. The Individual Top Phase Volumes Are Shown in Brackets.

Aqueous two-phase system	Withdrawn volume (ml)	Cultivation time (hours)	Withdrawn enzyme activity (FPU)	Productivity[c] (FPU/l · h)	Yield (withdrawn, FPU/g cellulose)	Total Yield (FPU/g cellulose)	Maximum enzyme activity (FPU/ml)
PEG 8000 7% -	(28,30,44 ml)						
Aquaphase PPT 17%	102	72	67	9.3	67	115[a]	0.72
PEG 8000 5% -	(24,20,12,15,16 ml)						
Dextran T500 7%	87	126	62	4.9	62	131[a]	1.2
PEG 8000 5% - Hydrolyzed crude	(15,34,49 ml)						
dextran 7%	98	96	62	6.5	62	88[a]	0.79
PEG 4000 5% -	(18,23,34,30,26 ml)						
Crude dextran 6%	131	126	56	4.5	56	66[a]	0.56
Regular medium	(13,7,4,6 ml) 30	96	17	2.0	17	97[b]	0.86

[a] Withdrawn enzyme activity + residual activity in the bottom phase at the end of the cultivation, calculated from the partition coefficient and the volumes.
[b] Withdrawn enzyme activity + residual activity at the end of the culviation.
[c] Calculated from the enzyme activity in the withdrawn top phase.

The total vol withdrawn from the four different phase systems were in the range of 87–131 mL, however, the individuall withdrawn vol varied between 12 and 49 mL of a total vol of 100 mL. The composition of the phase systems were chosen in order to give a vol ratio of 1:1. It has previously been found that the vol ratio of an aqueous two-phase system is influenced by the amount of cell material (*23*). Visual observation of the four different phase systems gave the impression that the fungus caused an expansion of the bottom phase. This could be a result of growth as well as expansion of the mycelium as such.

Only one quarter of the amount of enzyme could be withdrawn from the regular medium compared with the phase system. This was caused by the vol of solution available for extraction being very small. The total amount of enzyme withdrawn was approx the same in all four phase systems.

In order to compare the enzyme production in the phase systems, the total yield of enzyme was compared based on the different partition coefficients (Table 2). Compared with the reference system, more enzyme is produced in the PEG 8000–Aquaphase and PEG 8000–Dextran T500 systems, whereas in the PEG 8000–hydrolyzed crude dextran and PEG 4000–crude dextran systems less enzyme is produced.

The improved enzyme production in the PEG 8000–Dextran T500 system could be due to increased growth of the fungus as indicated by the increasingly smaller top phase vol observed (Table 2). This does not, however, explain the increased enzyme production in the PEG 8000–Aquaphase system. Unfortunately, the underlying theory of improved microbial enzyme production in aqueous two-phase systems is presently not well understood, e.g., *Aspergillus phoenicis* was found to produce more β-glucosidase in a PEG 1550–Dextran T2000 system when compared with a reference cultivation (*16*). On the other hand, for α-amylase production with *Bacillus subtilis* in aqueous two-phase systems it was found that the top phase component, PEG 600, exclusively enhanced enzyme production (*24*). This was, however, not generally true for *Bacillus* sp. (*25*).

One possibility for improved cellulolytic enzyme production with *T. reesei* Rutgers C30 in aqueous two-phase systems is the repeated withdrawal of an enzyme containing top phase that could stimulate enzyme production by reducing the enzyme concentration. Such a case was investigated for concentrations up to 2 FPU/mL (Fig. 3). Taking into account experimental error, it was concluded that these concentrations did not influence the production of cellulolytic enzymes with *T. reesei* Rutgers C30.

Based on these results, PEG 8000–Dextran T500 was chosen for further studies. In batch culture the enzyme production in this phase system was compared with a regular medium (Fig. 4). Fungi are known to produce a large number of hydrolytic enzymes and the possibility exists that an

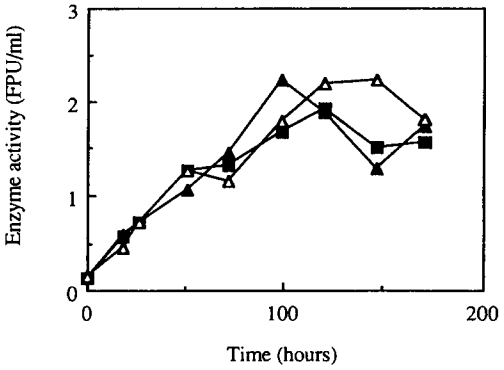

Fig. 3. The influence of enzyme concentration on the cellulolytic enzyme production with *Trichoderma reesei* Rutgers C30. Cellulolytic enzymes, NOVO Celluclast L, were added after 24 hours cultivation. Addition of enzyme to a final concentration of 1 FPU/mL (▲); addition of enzyme to a final concentration of 2 FPU/mL (△); no addition (■).

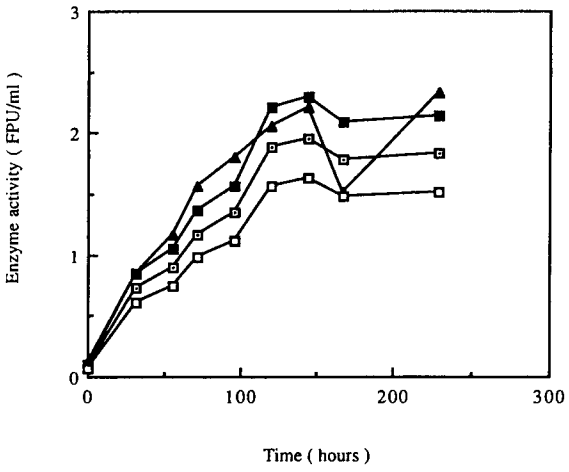

Fig. 4. Cultivation of *Trichoderma reesei* Rutgers C30 in an aqueous two-phase system composed of PEG 8000 5%–Dextran T500 7% (⊡) and in a regular medium without phase components (▲). Cellulose concentration: 10 g/L Solka-Floc BW 200. The cellulolytic enzyme activity was measured as filter paper activity. The enzyme activity measured in the top phase of the phase system (■). The enzyme activity in the bottom phase of the phase system, calculated from the partition coefficient and the phase volumes (□). Total amount of produced cellulolytic enzyme activity in the aqueous two-phase system (⊡) calculated from the enzyme concentration in the top phase and the partition coefficient. No dextran degrading enzymes were detected.

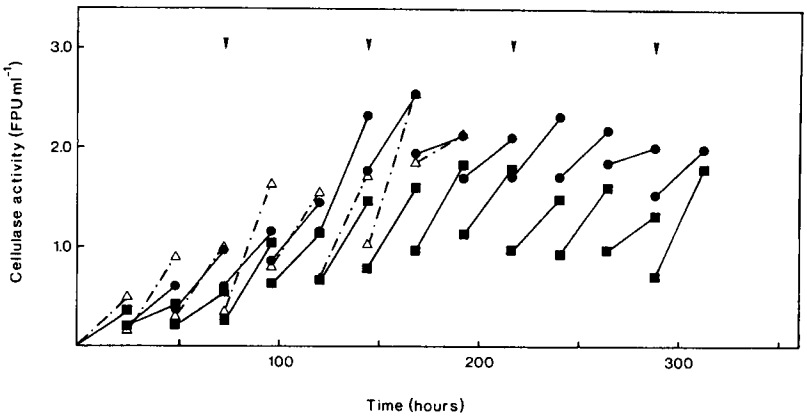

Fig. 5. Extractive cellulolytic enzyme production in an aqueous two-phase system composed of PEG 8000 5%–Dextran T500 7% (■, ●) and in a regular medium (△). Initial cellulose concentation: 10 g/L Solka-Floc BW 200. Cellulose and media components of original concentrations were added. Addition of media components everyday, and cellulose every 72 h (●,△); addition of media components and cellulose every 72 h (■). Addition of cellulose (►).

aqueous two-phase system with a dextran bottom phase would induce the production of dextran degrading enzymes in *T. reesei* Rutgers C30. Earlier observations in our laboratory have shown that certain fungal enzyme preparations contain dextran degrading enzymes, that when used in an aqueous two-phase system cause the two phases to transform into one phase. Over a 240 h cultivation period no such transformation of the phase system was observed (Fig. 4). The total amount of enzyme produced was approx the same as in the regular medium.

Extractive Enzyme Production in Fed-Batch Cultivations

In order to improve enzyme production, media components and cellulose substrate were intermittently added to cultivations in a regular medium and the PEG 8000–Dextran T500 phase system (Fig. 5, Table 3). The previous cultivations had indicated that the fungal growth could be nutrient limited when the top phase was repeatedly withdrawn (Fig. 2), the degree of limitation being related to the vol of the withdrawn top phase. Media components were therefore added every 24 h in one phase system culture and every 72 h in another. In the regular medium, media components were added every 24 h and in all three cultivations cellulose was added every 72 h. This time interval was based on the results from the batch cultures (Fig. 4), where the enzyme production had yet to reach its peak value at 72 h. The fed-batch cultures were followed for 310 h.

Table 3
Extractive Cellulolytic Enzyme Production with *Trichoderma reesei* Rutger C30 in an Aqueous Two-Phase System Composed of PEG 8000 5%–Dextran T 500 7% and in a Regular Medium. Addition of Media Components and Cellulose of Original Concentrations. Original Substrate Concentration: 10 g/L Solka-Floc BW 200. Cultivation Time: 310 h.

Culture conditions	Withdrawn volume (ml)	Total withdrawn enzyme activity (FPU)	Maximum enzyme activity (FPU/ml)	Average enzyme activity (FPU/ml)	Productivity [e] (FPU/l · h)	Yield (withdrawn, FPU/g cellulose)	Total Yield (FPU/g cellulose)
Phase system							
Media components							
every day, cellulose				1.2 [a]	12 [a]		
every 72 hours	271	407	2.5 [a]	2.2 [b]	18 [b]	159	203 [d]
Phase system							
Media components							
and cellulose				1.0 [a]	15 [a]		
every 72 hours	445	534	1.8 [a]	1.6 [b]	24 [b]	131	157 [d]
Regular medium							
Media components							
every day, cellulose					28 [a]		
every 72 hours	423	547	2.5 [a]	1.3 [a]	c	244 [a]	244

[a]Measured 0–190 hours; [b]measured between 140–310 hours; [c]terminated at 190 hours
[d]Withdrawn enzyme activity + residual activity in the bottom phase at 312 h, calculated from the partition coefficient and the bottom phase volume.
[e]Calculated from the enzyme activity in the withdrawn top phase.

In the previous comparison, extremely small vol could be withdrawn from the regular medium (Table 2). Therefore, in this comparison, the regular medium culture was centrifuged before the supernatant was withdrawn.

The enzyme concentrations in the withdrawn volumes were approx the same in the cultures that were given media components every 24 h with a maximum of 2.5 FPU/mL (Fig. 5). The filter paper activity was slightly lower in the culture that received media components only every 72 h, indicating a nutrient limitation. The enzyme concentration reached a maximum value of 1.8 FPU/mL. Up to 140 h the enzyme production is higher than the amount withdrawn, thereafter the enzyme production reached a steady-state in the sense that the amount withdrawn and the amount produced balanced. The regular medium cultivation was terminated after 190 h, as a result of fungal sporulation.

The productivity and the yield were calculated from the withdrawn vol and the partition coefficients (Table 3). The yield depends on the withdrawn volumes. When the phase system culture received media components every 24 h, only half the top phase vol could be withdrawn, compared with the culture receiving media components only every 72 h. In the case of the regular medium culture, however, when media components were given every 24 h, no reduction in the withdrawn supernatant vol was observed; on the contrary, 420 mL were withdrawn in only 190 h. This is most probably because this culture was centrifuged which counteracts the expansion of the mycelium.

The productivity obtained in the phase system is only half the productivity obtained in the regular culture (Table 3). The productivities were increased in the phase systems when the productivity was calculated between 140–310 h, however, they were still lower than the productivity obtained in the regular medium.

The total yield based on added cellulose is highest in the regular medium culture. Of the phase system cultures, total yield was highest where media components were added every 24 h rather than 72 h. Again the importance of adding media components in cultivations with intermittent enzyme extraction is emphasized. The fact that the enzyme production in the phase system is lower than in the regular medium might be owing to a decreased oxygen transfer rate imposed by the increased viscosity in the polymer phase system.

In this study a new method for recycling cellulolytic enzyme producing fungi, aqueous two-phase systems, was compared with centrifugation. In Table 4 the present results are compared with the results of other methods for recirculation of *T. reesei* for production of cellulolytic enzymes. Immobilization to solid matrices has been used in several investigations (*26–28*) and recycling by allowing the fungus to settle was used

Table 4
Different Methods for Recirculation of *T. reesei* for Production of Cellulolytic Enzymes

Cultivation conditions	Cellulolytic enzyme concentration (FPU/ml)	Productivity (FPU/l·h)	Yield (FPU/g cellulose)	Ref
immobilization non-woven material	1.15	8	154	26
immobilization stainless steel beads	0.21	32	97	27
immobilization in carrageenan	0.4 (max)	26	252	28
cellrecycling	1.2	30		29
aqueous two-phase system	2.2 (max.2.5)	18[c]	159[a] 203[b]	this work
centrifugation	1.3 (max.2.5)	28	244	this work

[a] Amount of cellulolytic enzymes withdrawn with the top phase.
[b] Total amount of produced cellulolytic enzymes.
[c] Calculated from the enzyme activity in the withdrawn top phase.

in one study (*29*). The average enzyme concentration, the productivity and the yield obtained in an aqueous two-phase system are superior or equal to those obtained when the fungus is immobilized to solid supports. The present study shows that a higher productivity and a higher yield can be obtained when the cellulolytic enzyme production is performed in a regular medium and the fungus recycled by centrifugation. However, the large-scale recycling of filamentous fungi in centrifugal separators has not been investigated and may prove to be difficult. When aqueous two-phase systems are used the separation of the fungus from the enzymes is achieved without the use of centrifugal separators. The enzyme containing top phase can easily be integrated in an enzyme purification scheme. By the addition of a concentrated salt solution to the PEG-phase, a PEG/salt two-phase system is created. The enzymes will be partitioned to the salt-rich phase and the PEG-phase can be recycled (*30,31*). The salt phase can then be desalted by ultrafiltration and a purified and concentrated enzyme solution is obtained.

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REFERENCES

1. Castanon, M. and Wilke, C. R. (1980), *Biotech Bioeng* **22,** 1037.
2. Persson, I., Tjerneld, F., and Hahn-Hägerdal, B. (1989), *Proc. Biochem.*, accepted for publication.
3. Ryu, D. D. T. and Mandels, M. (1980), *Enzyme Microb. Technol* **2,** 91.
4. Montenecourt, B. S. and Eveleigh, D. E. (1979), *Adv. Chem. Ser.* **181,** 289.
5. Shoemaker, S. P., Raymond, J. C., and Bruner, R. (1981), *Basic Life Sci.* (Trends in the Biol. Ferment. Fuels Chem.) **18,** 89.
6. Warzywoda, M., Ferre V., and Pourquie, J. (1983), *Biotech. Bioeng.* **25,** 3005.
7. Mandels, M. (1975), *Biotech. Bioeng. Symp.* No 5, 81.
8. Watson, T. G., Nelligan, I., and Lessing, L. (1984), *Biotechnol. Lett.* **6,** (10), 667.
9. Hendy, N. A., Wilke, C. R., and Blanch, H. W. (1984), *Enzyme Microb. Technol* **6,** 73.
10. Albertsson, P-Å (1986), in *Partition of Cell Particles and Macromolecules,* Third Edition, Wiley and Sons, Inc., New York.
11. Kula, M-R., Kroner, K. H., and Hustedt, H. (1982), *Adv. Biochem. Eng.* **24,** 74.
12. Andersson, E. and Hahn-Hägerdal, B. (1990), *Enzyme Microb. Technol.* **12,** 242.
13. Tjerneld, F., Persson, I., Albertsson, P-Å. and Hahn-Hägerdal, B. (1985), *Biotech. Bioeng.* **27,** 1044.
14. Persson, I., Tjerneld, F., and Hahn-Hägerdal, B. (1984), *Enzyme Microb. Technol.* **6,** 415.
15. Andersson, E., Johansson, A-C., and Hahn-Hägerdal, B. (1985), *Enzyme Microb. Technol.* **7,** 333.
16. Persson, I., Tjerneld, F., and Hahn-Hägerdal, B. (1989), *Biotechnol. Techniques* **3,** 265.
17. Vogel, H.J. (1964), *The American Naturalist* **XCVII,** (903), 435.
18. Mandels, M., Andreotti, R., and Roche, C. (1976), *Biotech. Bioeng.* **6,** 21.
19. Kroner, K. H., Hustedt, H., and Kula M-R. (1982), *Biotech. Bioeng.* **24,** 1015.
20. Miller, G. L., Blum, R., Glennon, W. E., and Burton, A. L. (1960), *Anal. Biochem.* **2,** 127.
21. Tjerneld, F., Persson, I., Albertsson, P-Å., and Hahn-Hägerdal, B. (1985), *Biotech. Bioeng.* **27,** 1036.
22. Albertsson, P-Å., Cajarville, A., Brooks, D. E., and Tjerneld, F. (1987), *Biochim. Biophys. Acta* **926,** 87.
23. Veide, A., Smeds, A. L., and Enfors, S-O. (1983), *Biotech. Bioeng.* **25,** 1789.
24. Ramgren, M., Andersson, E., and Hahn-Hägerdal, B. (1988), *Appl. Microbiol. Biotechnol.* **29,** 337.
25. Andersson, E. and Hahn-Hägerdal, B. (1988), *Appl. Microbiol. Biotechnol.* **29,** 329.

26. Kumakura, M. and Kaetsu, I. (1986), *Biotechnol. Appl. Biochem.* **8,** 195.
27. Webb, C., Fukuda, H., and Atkinson, B. (1986), *Biotech. Bioeng.* **28,** 41.
28. Frein, E. M., Montenecourt, B. S., and Eveleigh, D. E. (1982), *Biotechnol. Lett.* **4,** (5), 287.
29. Ghose, T. K. and Sahai, V. (1979), *Biotech. Bioeng.* **21,** 283.
30. Kula, M.-R., Kroner, K., and Hustedt, H. (1982), *Adv. Biochem. Eng.* **24,** 73.
31. Tjerneld, F., Johansson, G., and Joelsson, M. (1987), *Biotech. Bioeng.* **30,** 809.