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Dynamics of Cellulase Production by Glucose Grown Cultures of *Trichoderma reesei* Rut-C30 as a Response to Addition of Cellulose

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Abstract

An economic process for the enzymatic hydrolysis of cellulose would allow utilization of cellulosic biomass for the production of easily fermentable low-cost sugars. New and more efficient fermentation processes are emerging to convert this biologic currency to a variety of commodity products with a special emphasis on fuel ethanol production. Since the cost of cellulase production currently accounts for a large fraction of the estimated total production costs of bioethanol, a significantly less expensive process for cellulase enzyme production is needed. It will most likely be desirable to obtain cellulase production on different carbon sources—including both polymeric carbohydrates and monosaccharides. The relation between enzyme production and growth profile of the microorganism is key for designing such processes. We conducted a careful characterization of growth and cellulase production by the soft-rot fungus *Trichoderma reesei*. Glucose-grown cultures of *T. reesei* Rut-C30 were subjected to pulse additions of Solka-floc (delignified pine pulp), and the response was monitored in terms of CO_2 evolution and increased enzyme activity. There was an immediate and unexpectedly strong CO_2 evolution at the point of Solka-floc addition. The time profiles of induction of cellulase activity, cellulose degradation, and CO_2 evolution are analyzed and discussed herein.

Index Entries: *Trichoderma reesei*; fermentation; cellulase; growth characterization; cellulose hydrolysis.

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Introduction

The accelerating accumulation of CO_2 and other greenhouse gases in the atmosphere may lead to adverse climate changes that would seriously endanger the sensitive ecologic balance of Earth *(1)*. Energy shortages in the world coupled with environmental considerations have directed applied research toward the development of novel processes to produce renewable fuels with a special emphasis on fuel ethanol production from cellulosic materials *(2)*. Even though CO_2 is released during the bioprocess of fuel ethanol production and also during its combustion, the CO_2 is reutilized to grow new biomass, replacing that harvested for ethanol production. As a result, the net produced CO_2 is small in comparison with that released by the utilization of fossil fuels, thus reducing the hazards of a global climate change *(1,3)*.

The potential for using cellulosic materials to produce fermentable sugars for biotechnological processes—including bioethanol production—is enormous *(4,5)*. Ethanol production from cellulose comprises hydrolysis of cellulosic raw materials to sugars and the subsequent anaerobic fermentation of sugar compounds by yeast to produce ethanol. Although enzymatic hydrolysis is superior, in several aspects, to acid hydrolysis, its economic realization is highly hindered by the presently too high production cost of cellulose-degrading enzymes.

Cellulases are inducible enzymes, which are synthesized by many microorganisms during their growth on cellulosic materials. Example microorganisms known to produce cellulases include bacterial species of *Clostridium* and *Bacillus* and species of filamentous fungi from *Penicillium*, *Aspergillus*, and *Trichoderma (6)*. Complete enzymatic degradation of native cellulose requires the synergistic action of three general types of cellulolytic enzymes, traditionally classified as endoglucanases, cellobiohydrolases, and β-glucosidases *(7)*. Endoglucanases preferentially hydrolyze the amorphous regions of the fibrils by randomly cleaving β-glucosidic bonds; cellobiohydrolases are exoglucanases releasing cellobiose, the repeat unit of cellulose from the chain ends; while β-glucosidases complete the degradation process by hydrolyzing cellobiose and other cellodextrins with a low degree of polymerization to glucose units. The high level of synergy among cellulase enzymes results from their different, but complementary, mode of action. This synergy increases the degree of hydrolysis by more than twofold over that achieved with individual enzymes *(8)*.

Because of its ability to produce and secrete the complete set of cellulolytic enzymes, thus making it particularly potent in hydrolyzing the cellulose polymer to glucose monomers, the soft-rot fungus *Trichoderma*, in particular *T. reesei* has been the focus of cellulase research for decades *(8)*. The preferred substrates used by most researchers for cellulase production are pure celluloses such as Avicel, Solka-floc, and cotton *(9)*. Cellulase production by *Trichoderma* is controlled by a complex metabolic regulation *(10–12)*. Cellulose acts (indirectly) as an inducer for the production of cellulases. Expression of cellulases is furthermore subject to repres-

sion by the end product of the hydrolysis—glucose. Cellulose-derived inducers, sophorose being the most potent, are likely to provide an effective induction during cultivation on cellulose, but the concentration of the end product, glucose, may negatively affect cellulase production. Glucose concentration is determined by the dynamic balance between the rates of glucose generation (by cellulose hydrolysis) and consumption (by microbial uptake). At low concentrations of cellulase and/or cellulose, glucose generation may be too slow to meet the need of active cell growth and function. On the other hand, cellulase synthesis can be halted by glucose repression when glucose generation is faster than its consumption. Glucose repression of enzyme expression is an obvious target for strain improvement. Many of the high-producing strains of *T. reesei* that have been isolated have also been shown to be partly glucose derepressed. This is the case for, e.g., the strain *T. reesei* Rut-C30 *(6)*, which is used in the present study.

The objective of the current work was to characterize carefully the dynamics of cellulase production and metabolic activity following cellulose addition in a batch cultivation of the strain *T. reesei* Rut-C30. Cells were initially grown on glucose as the carbon source, and after its depletion, cellulose was added. Since it is difficult to follow the growth directly after addition of a solid substrate, on-line measurements of CO_2 evolution were used to follow the metabolic activity of the cells. Frequent samples were also taken to measure enzyme activity and sugar concentrations.

Materials and Methods

Chemicals

All chemicals were of analytical grade and obtained from Sigma (St. Louis, MO) with the exception of bacto agar, yeast extract and peptone which were obtained from Merck (Darmstadt, Germany), and D-glucose, which was obtained from VWR. Solka-floc, a delignified pine pulp serving as the cellulosic substrate, was obtained from Fiber Sales & Development (Urbana, OH). For the preparation of solutions and media, distilled water was used.

Stock Culture

The mutant cellulase-producing strain of *T. reesei* Rut-C30 (ATCC 56765) used was obtained from the American Type Culture Collection. The culture was maintained on malt agar slants containing 20 g/L of bacto agar, 20 g/L of malt extract, 1 g/L of peptone, and 5 g/L of glucose, with regular subculturing at 30°C.

Preparation of Inoculum

Conidia were harvested from 30-d-old stock cultures, by adding 5 mL of sterile distilled water to the agar slant and then resuspending the conidia.

The spore concentration in the conidial suspension was determined by counting with a Bürker Counting Chamber. To prepare the inoculum, 1 mL of spore suspension (approx 4×10^7 spores/mL) was inoculated into a 300-mL Erlenmeyer flask containing 75 mL of growth medium similar to the basic nutrient medium of Mandels and Weber (13) with the exception that urea was omitted, a double amount of $(NH_4)_2SO_4$ was included, and the peptone content was elevated by 20%. This medium (later referred to as medium with a single set of nutrients) contained 10 g/L of glucose as the sole carbon source, which was fed separately in the form of a thick solution to the salt medium after sterilization. The initial pH of the sterilized medium was adjusted to 5.0 by adding sterile 2 *M* H_2SO_4 before inoculation, and no pH control was applied during the cultivation run. The inoculum was incubated on a rotary shaker with an agitation rate of 200 rpm at 30°C for 3 d and then was used to inoculate the fermentor.

Fermentation Experiments

Fermentations were performed in a 3-L stirred-tank laboratory fermentor (Biostat A-DCU300; B. Braun Biotech International GmbH, Germany) with a working volume of 2 L. The bioreactor was equipped with a pH electrode and a polarographic oxygen electrode (Mettler-Toledo). To 1950 mL of sterilized (121°C, 20 min) growth medium containing a double set of nutrients, 50 mL of inoculum was added; thus, the volume of the inoculum was made up to 2.5% (v/v) of the total broth volume. Fermentations were performed at 28°C with an agitation rate of 600 rpm and an aeration rate of 500 mL/min (0.25 vvm) at atmospheric pressure. A rather low airflow was used to avoid excessive foaming; however, the dissolved oxygen tension was always above 15% of the saturation value. The initial pH was adjusted to and further controlled at 5.0 by the automatic addition of 2 *M* H_2SO_4 and 2 *M* NaOH. Foaming was controlled by the manual addition of filter-sterilized antifoam agent (Sigma Antifoam 289). Fermentation was continued until the glucose was completely depleted, and then pulse addition of Solka-floc (10 g/L) was applied by adding a thick suspension of 20 g of Solka-floc in a calculated volume of distilled water, thus filling the fermentor to the original volume of 2 L. Nutrients required to support the growth on the second batch of carbon source (i.e., Solka-floc) were supplied by including a double set of nutrients in the original batch in order to avoid any coincidences of response signals that may occur from adding any component along the Solka-floc. The outlet gas composition was continuously monitored using a gas analyzer (Tandem dual gas sensor; Adaptive Biosystems, Leagrave, England). Measurement values from the gas analyzer as well as electrode signals were logged to a computer every 10 min.

Sampling

A sterile syringe was used to collect samples via the sampling tube driven to the very bottom of the fermentor. Prior to sample withdrawal,

3 mL of culture broth was taken and discharged in order to wash the remains away from the sampling tube. When taking the sample a few milliliters of fermentation broth was taken aseptically from the vessel and with the exception of an aliquot used for optical density (OD) measurement, the sample was immediately subjected to phase separation (4000 rpm, 10 min). The supernatant collected was assayed for reducing sugar content, glucose concentration, and cellulase activity.

Optical Density

OD was read at 660 nm (OD_{660}) against distilled water *(14)*.

Determination of Reducing Sugar Content

Total reducing sugar was determined by the dinitrosalicylic acid (DNS) method *(15)* using D-glucose as a standard. Appropriately diluted samples were made up to 1.5 mL with distilled water, and 3 mL of DNS reagent was added. The color obtained after boiling the mixture for 5 min and then diluting with 16 mL of distilled water was evaluated by reading the absorbance at 550 nm. Total reducing sugar generated during the assay was estimated as glucose equivalents.

Determination of Glucose

Samples were analyzed for glucose by high-performance liquid chromatography using an Aminex HPX-87H column at 65°C. The mobile phase was 5 m*M* H_2SO_4 at a flow rate of 0.5 mL/min. In addition, glucose concentrations in some samples were determined by an enzymatic glucose test (Boehringer Mannheim GmbH, Mannheim, Germany).

Enzyme Assay

Filter paper activity, which describes the overall cellulolytic activity of an enzyme preparation, was determined by the method of Mandels et al. *(16)*. A 1 × 6 cm strip of Whatman no.1 filter paper (Hillsboro, OR), which equals 50 mg of cellulose, served as the substrate and was added to the sample solution containing 0.5 mL of appropriate diluted enzyme (supernatant of culture broth) and 1.0 mL of 0.05 *M* citrate buffer (pH 4.8). After 60 min of incubation at 50°C, the hydrolysis was terminated by the addition of 3 mL of DNS solution, and the mixture was further assayed for reducing sugar content by the DNS method. One international filter paper unit (FPU) was defined as the amount of enzyme that releases 1 μmol of glucose/min under the assay conditions. Activities were reported as FPU/milliliter.

Hydrolysis Experiments

To be able to study cellulose (Solka-floc) degradation without microbial conversion of the sugars formed, separate hydrolysis experiments were carried out in stirred flasks without cells. The conditions in these experiments were the same as during the corresponding fermentation

(i.e., the same medium composition, pH 5.0, 28°C, 600 rpm). The medium composition was set according to the assumption that at the moment of Solka-floc addition, the fermentation broth would be depleted for the glucose originally present. The medium for hydrolysis experiments thus contained a single set of nutrients, 10 g/L of Solka-floc as the substrate, and added enzyme giving a desired activity. Two sources of enzyme were used: either Celluclast, a commercially available fungal cellulase preparation (a kind gift from Novozymes, Denmark) or a home-produced cellulase enzyme prepared by collecting the supernatants of Solka-floc grown in shake-flask cultures of *T. reesei* Rut-C30 (Mandels medium, 28°C, pH 5.0, 4 d) by centrifugation (5600*g*, 10 min). The enzyme-to-substrate ratio was adjusted to mimic two chosen specific points in the batch cultivation on cellulose: the point of cellulose addition and the point at which the CO_2 evolution peaked. Experiments were run in duplicate.

Results

T. reesei Rut C-30 was grown aerobically in batch cultures in two stages, using glucose in an initial phase to produce cell mass, and thereafter adding cellulose (in the form of Solka-floc) as described before. The dynamics of cellular activity and produced cellulases following the addition of Solka-floc was monitored by on-line measurements of CO_2 evolution and sampling for determination of enzyme activity and sugar concentrations.

Batch Cultivation on Glucose

A two-phase aerobic batch culture is shown in Fig. 1. The initial exponential growth phase on glucose lasted until about 26 h. The specific growth rate (determined from the logarithm of the CO_2 evolution rate [CER]) was 0.22 h^{-1}. After 25.4 h, DNS measurements of reducing sugars indicated that the glucose was exhausted from the medium (Fig. 2). Although the CO_2 evolution rapidly decreased, there was a residual CO_2 evolution, which only gradually decreased to zero. This was accompanied by a reduction in the OD (Fig. 2), indicating degradation of biomass. The measured cellulase activity remained constant after the depletion of glucose at a level of about 0.3 FPU/mL.

Second-Stage Batch Cultivation on Cellulose

After 67 h, cellulose in the form of Solka-floc was added as described above. There was an immediate increase in the CER at this point. The increase continued until $t = 73$ h, at which point there was a sharp decrease in CER. This did not coincide with complete depletion of glucose from the medium (Fig. 3). The enzyme activity increased continually up to a value of about 2.6 FPU/mL. This maximum coincided with the depletion of glucose and occurred at about $t = 100$ h. From the integrated area of the CO_2 evolution (Fig. 1), one can estimate that the CO_2 evolved on cellulose was about 80% of the value obtained from glucose.

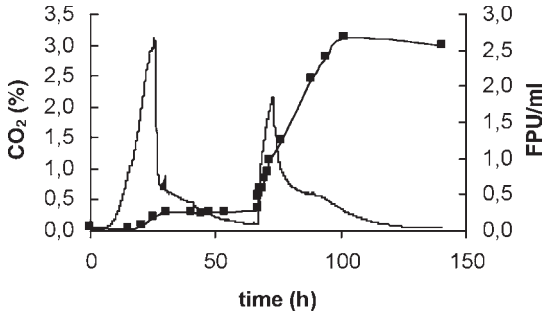

Fig. 1. CO_2 concentration in outlet gas (——) and cellulase activity (■, expressed as FPU/mL) vs time for aerobic batch cultivation of *T. reesei* Rut-C30. The initial growth medium was a Mandels medium with 10 g/L of glucose as the carbon source. At t = 67 h, Solka-floc was added to a concentration of 10 g/L.

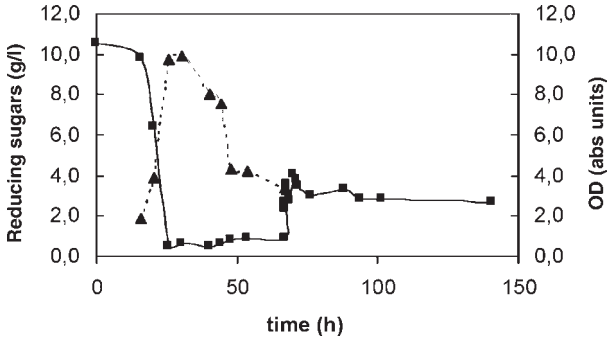

Fig. 2. Reducing sugars (■) determined by DNS method and OD (▲) vs time for aerobic batch cultivation of *T. reesei* Rut-C30. The initial growth medium was a Mandels medium with 10 g/L of glucose as the carbon source. At t = 67 h, Solka Floc was added to a concentration of 10 g/L. (There were no measurements of OD after the addition of cellulose.)

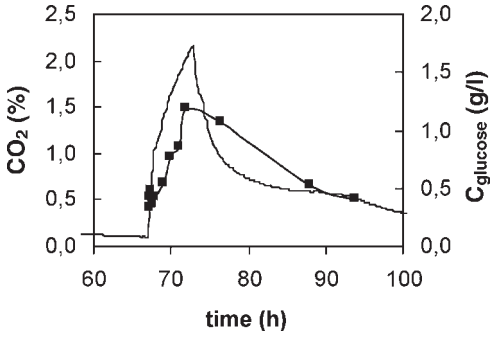

Fig. 3. CO_2 concentration in outlet gas (——) and glucose concentration (■) vs time for second aerobic batch phase in which *T. reesei* Rut-C30 grew on Solka-floc as carbon source.

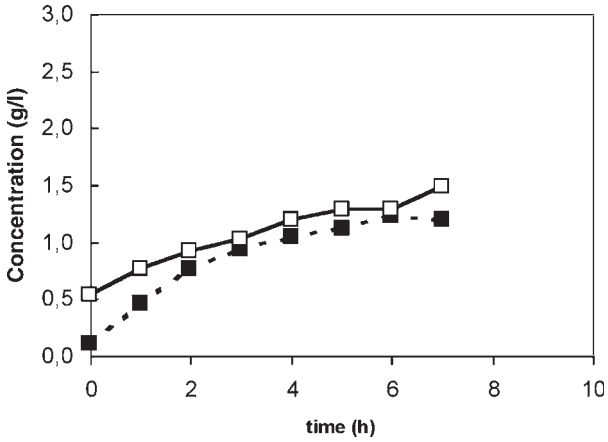

Fig. 4. Concentration of glucose (—□—) and cellobiose (– – ■ – –) during enzymatic hydrolysis of Solka-floc. The enzyme was prepared from a culture of *T. reesei* and the initial enzyme loading corresponded to 27.4 FPU/g substrate. Hydrolysis was carried out at 28°C and pH 5.0.

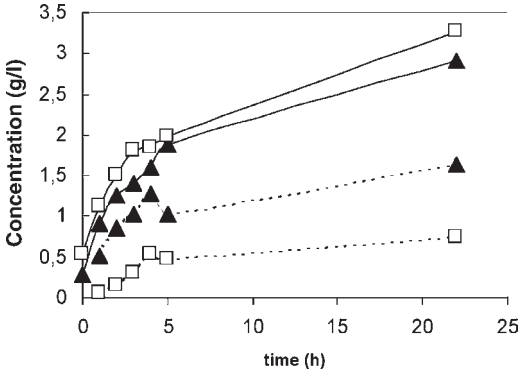

Fig 5. Concentration of glucose (——) and cellobiose (– – –) during enzymatic hydrolysis of Solka-floc. Two different enzymes were used; enzyme was prepared from a culture of *T. reesei* (□) and commercially available Celluclast (▲). Enzyme loading was 91.9 FPU/g substrate. Hydrolysis was carried out at 28°C and pH 5.0.

In Vitro Enzymatic Hydrolysis Rates

The initial, rather rapid increase in CER found in the second stage of two-phase batch cultivation was somewhat unexpected. We decided to compare this value to the initial rates of glucose and cellobiose formation in in vitro enzymatic hydrolysis experiments (Figs. 4 and 5). The enzyme loadings were chosen to represent the actual enzyme-to-substrate ratio relevant to the point of cellulose addition and the point of maximum CER.

For the enzyme solution prepared using *T. reesei*, the approximate formation rate of glucose was in the former case 0.18 g/(L·h) and in the second case 0.5 g/(L·h). The formation rate of cellobiose was 0.33 g/(L·h) in the first case and <0.2 g/(L·h) in the second case. Using Celluclast in a loading revevant to the point of maximum CER, a similar value was found for glucose, but a higher value was found for cellobiose (Fig. 5).

With a typical yield of CO_2 on sugar, Y_{sc}, of 0.4 (C-mol/C-mol), one can estimate that the sugar formation rate would give a CER of 0.018 mol of CO_2/h for a 2-L culture as in Fig. 1. This corresponds to a CO_2 concentration in the outlet gas of 1%. Within 0.5 h after addition of cellulose (Fig. 3), the measured value was in fact 1%, in good agreement with the estimated value. Calculations for the higher enzyme activity (91.9 FPU/mL) indicate that the formed glucose and cellobiose would give a CER of 0.018 mol of CO_2/h, corresponding to a CO_2 concentration in the outlet gas of 1.4%, which is a bit lower than the actual observed value.

Discussion

The final cellulase activity obtained from 10 g/L of cellulose was 2.6 FPU/mL. This is in good agreement with previously reported yields for the strain Rut-C30. For example, Persson et al. *(6)* quote a yield of 233 FPU/g of substrate in batch cultures. There was a steady increase in cellulase activity throughout the cultivation on cellulose in the current work, despite the fact that the free glucose concentration reached a value as high as 1 g/L. However, at that point of maximum glucose concentration, a sharp decrease in CO_2 evolution occurred, and the glucose concentration started to decrease after that point. The reason for this may be depletion of a medium component, or it may also be related to the regulation of enzyme expression. This is supported by the fact that the rate of activity increase changes at that point.

On depletion of glucose in the initial growth phase, there was a rapid decrease in CO_2 evolution, but it did not decrease to zero. Measurements of OD_{660} showed a decrease in biomass, suggesting that the residual CO_2 evolution is the result of endogenous metabolism. A higher volumetric enzyme productivity could therefore potentially be obtained if cellulose had been added earlier, provided that there was no remaining glucose repression effect.

The main point of making a two-stage culture was to enable the study of a pulse addition of cellulose. However, separating an initial biomass formation on glucose (or on other monosaccharides) from cellulase production with cellulose as substrate has advantages also from a process point of view. By using a two-stage process, a basal cellulase activity can be obtained before the addition of cellulose. This level allows the utilization of cellulose to commence rather quickly as shown by the CO_2 evolution. By contrast, a one-phase batch process starting directly from cellulose will initially be very slow owing to a very low hydrolysis rate. As has been pointed out previously, a key question is, to what extent will the cellulase expression be repressed by the glucose liberated in the hydrolysis.

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