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Roles of extracellular lactose hydrolysis in cellulase production by *Trichoderma reesei* Rut C30 using lactose as inducing substrate

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ABSTRACT

Lactose, an inexpensive, soluble substrate, offers reasonably good induction for cellulase production by *Trichoderma reesei*. The fungus does not uptake lactose directly. Lactose is hydrolyzed to extracellular glucose and galactose for subsequent ingestion. The roles of this extracellular hydrolysis step were investigated in this study. Batch and continuous cultures were grown on the following substrates: lactose, lactose–glycerol mixtures, glucose, galactose, and glucose–galactose mixtures. Cell growth, substrate consumption, lactose hydrolysis, and lactase and cellulase production were followed and modeled. Cells grew much faster on glucose than on galactose, but with comparable cell yields. Glucose (at >0.3 g/L) repressed the galactose consumption. Cellulase synthesis was growth-independent while lactase synthesis was growth-dependent, except at $D < \sim 0.065\ h^{-1}$ where a basal level lactase production was observed. For cellulase production the optimal D was $0.055–0.065\ h^{-1}$ where the enzyme activity and productivity were both near maxima. The model suggested that lactase synthesis was subject to weak galactose repression. As the galactose concentration increased at high D ($>0.1\ h^{-1}$), lactase synthesis became repressed. The insufficient lactase synthesis limited the lactose hydrolysis rate. Extracellular lactose hydrolysis was concluded to be the rate-limiting step for growth of *T. reesei* Rut C30 on lactose.

1. Introduction

Lignocellulose hydrolysis to sugars is a critical first step to many potential usages of biomass, including production of bioethanol and other fermentation products [1]. Cellulase is a group of enzymes capable of hydrolyzing cellulose to glucose [2]. Economic production of cellulase is therefore critically important to biomass utilization. *Trichoderma reesei*, anamorph of the fungus *Hypocrea jecorina*, is commonly used for industrial cellulase production [3–6]. The cellulase expression requires induction [7,8]. While cellulose is the source of natural inducer(s), repeated addition of the solid substrate to bioreactors presents challenges to the maintenance of long-term sterile operation. Lactose is an inexpensive soluble substrate that can induce cellulase production by *T. reesei* [3,9–15]. It is important to improve understanding and modeling of the behaviors of *T. reesei* fermentation that uses lactose as the inducing substrate (i.e., as both the substrate to support cell growth and other metabolic activities and as the inducer for cellulase synthesis).

Despite repeated efforts, activity of either a lactose permease (to transport lactose into the cells) or an intracellular lactase (to hydrolyze lactose into sugars for catabolism) could not be detected in *T. reesei* [11]. Lactose metabolism in the fungus was therefore believed to be initiated by extracellular hydrolysis to glucose and galactose [11]. *T. reesei* is known to produce extracellular lactase (β-galactosidase, EC 3.2.1.108), capable of lactose hydrolysis [16]. How lactose induces cellulase expression remains uncertain. A study showed that galactokinase (encoded by the gene *gal1*) was essential for transcription of *cbh1* and *cbh2* (genes for important exoglucanase components of cellulase) when the fungus was grown on lactose [17]. As galactokinase is induced by galactose, it was thought that lactose might exert the cellulase-inducing ability via the galactose formed from lactose hydrolysis. The implication however was not supported by further investigations: galactose was found not to induce cellulase production in batch culture [17] and, in continuous cultures, the endoglucanase activity was detected only at the lowest dilution rate studied ($D = 0.015\ h^{-1}$) and the activity was very low [18]. Nonetheless, there are increasingly more observations suggesting the important roles played by the pathways of galactose catabolism, particularly the activities of galactokinase (GAL1) and xylose reductase (XYL1) for formation of the intermediates D-galactose-1-phosphate and galactitol [11,17–21].

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Table 1
Batch and continuous culture systems investigated, including the carbon substrates used.

Operation mode		Carbon substrates (g/L)
Batch culture (2–3 repetitions each)		Glucose: 10 Galactose: 10 Glucose: 5 and galactose: 5
Operation mode	**Dilution rate (h^{-1})**	**Carbon substrates (g/L)**
Continuous culture	0.04–0.12 (8 rates)	Lactose: 10
	0.1	Lactose: 1, glycerol: 10
		Lactose: 5, glycerol: 5
		Glycerol: 12.6
	0.11	Lactose: 1, glycerol: 9

There were several previous reports on modeling cellulase production by the lactose-supported *T. reesei* Rut C30 fermentation [9,12,13]. All were based on batch fermentation results. Rather few continuous culture studies were conducted with lactose-based media. For continuous culture of *T. reesei* Rut C30, Bailey and Tahtiharju studied the cellulase production [3]; Castillo et al. measured the lactase activity [16]. Chaudhuri and Sahai reported results (lactase not included) for a different strain, *T. reesei* C5, at few dilution rates [14]. Ryu et al. investigated a two-stage continuous culture system using yet another strain, MCG77 [15]. None investigated the role of lactose hydrolysis or developed models as complete as the one reported in this work.

In this study, *T. reesei* Rut C30 was grown in both batch and continuous culture systems using various substrates: glucose, galactose, lactose, glucose–galactose mixtures, and lactose–glycerol mixtures. The concentrations of these compounds, the activities of resultant lactase and cellulase enzymes, and the cell concentrations were measured. Accordingly, a mechanistic model was set up and the best-fit model parameters obtained to describe the culture behaviors. The model successfully described the hydrolysis of lactose, the lactose-induced synthesis of lactase and cellulase, the substrate consumption, and the cell growth in continuous culture.

2. Materials and methods

2.1. Microorganisms and media

T. reesei Rut C30 (NRRL 11460) was used in this study. The culture was obtained from the Agricultural Research Service Culture Collection, United States Department of Agriculture, in Peoria, IL. The culture was maintained on potato dextrose agar plates. Inocula were grown with potato dextrose broth in shake flasks. The growth medium, modified from that reported by Mandels et al. [22], contained 10 g/L carbon source, 2.0 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 1.0 g/L peptone, 0.3 g/L urea, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.3 g/L $CaCl_2$, 0.2 mL/L Tween 80, 5.0 mg/L $FeSO_4{\cdot}7H_2O$, 2.0 mg/L $CoCl_2$, 1.6 mg/L $MnSO_4{\cdot}H_2O$, and 1.4 mg/L $ZnSO_4{\cdot}7H_2O$. The carbon sources used in different experiments are given in Table 1.

2.2. Batch and continuous cultures

Batch and continuous cultures were made in the 3 L BioFlo 110 Modular Benchtop Fermentor/Bioreactor from New Brunswick Scientific (Edison, NJ). The working medium volume was 1.5 L for batch fermentations and 1.0 L for continuous cultures. Six batch fermentations were carried out in this study. Continuous cultures were investigated at nine dilution rates (D) with a lactose-based medium, and at three Ds with media containing both lactose and glycerol as C sources (Table 1). The cultivation conditions were controlled at temperature: $25 \pm 1\,^{\circ}C$; pH: 5.0 ± 0.2, by addition of 2 N NaOH; dissolved oxygen concentration (DO): about 70% of air saturation, using LabView (National Instrument, Austin, TX) to adjust the influent oxygen–air mixture; and agitation: 500 rpm, with a 6-blade turbine. Olive oil was added as the anti-foaming agent.

Continuous culture at each D was maintained for 6–8 residence times, with periodic measurements of intracellular protein and substrate concentrations to ensure reach of steady state. Samples taken during the steady-state period were further analyzed for glucose and galactose concentrations and cellulase and lactase activities. Feeding and culture removal were done with LabView-controlled pumps (Masterflex L/S, Cole-Parmer, Barrington, IL). For feeding at very low Ds ($\leq 0.05\ h^{-1}$) and for culture removal at all Ds, the pumps were turned on periodically. This periodic pumping design enabled the use of higher pump rates and larger pump tubing (for culture removal) to prevent clogging of the tubing by solids and mycelial cells and to remove the spent culture more representatively. It was observed that if pumped continuously at slow rates or if simply allowed to overflow, the removed broth contained lower cell concentrations, causing artificial cell retention. The periodic culture removal was performed every 10 or 15 min depending on D. The intervals were much shorter than the residence times used: 500–1500 min. A computer model simulation confirmed that such high-frequency "fed-batch" operation gives essentially the same average culture behaviors as the true continuous culture [23].

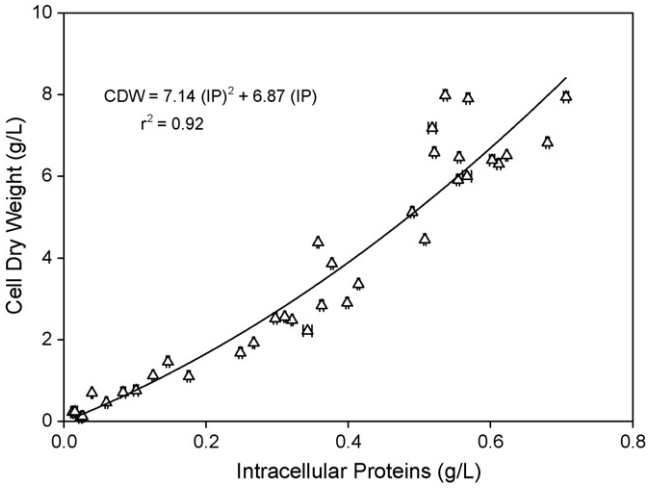

Fig. 1. Correlation between cell dry-weight concentrations and intracellular protein concentrations.

2.3. Sample analyses

The lactose and galactose concentrations were measured with an enzymatic kit (Boehringer Mannheim/R-biopharm 176 303). The kit contained β-galactosidase and β-galactose dehydrogenase; the former hydrolyzes lactose to galactose and glucose and the latter converts galactose and NAD^+ (oxidized form of NAD, nicotinamide adenine dinucleotide) to D-galactonic acid and NADH (reduced form of NAD). The NADH formed was detected at 340 nm and correlated with the galactose or lactose concentration in the sample. Glucose was analyzed using the PGO (peroxidase and glucose oxidase) test kit (Sigma P7119). Cellulase activity was measured as the Filter Paper Unit (FPU) according to the IUPAC procedure [24,25].

Lactase activity was measured as the hydrolytic activity of 10 mM o-nitrophenyl-galactopyranoside (ONPG) (Sigma) according to the reported procedure [16]. It was recognized that lactose, if present at high concentrations in the samples, could be a competitive inhibitor and affect the ONPG hydrolysis. Preliminary study was made to evaluate the effect of presence of different lactose concentrations (3, 6, 12, and 15 mM) on the lactase analysis of *T. reesei* broth samples (collected at the end of batch fermentation). The results fit well with the typical competitive inhibition equation ($r^2 = 0.98$) [26]:

$$v = \frac{v_{max} S_{ONPG}}{K_{ONPG}\left(1 + \frac{L}{K_L^{i,ONPG}}\right) + S_{ONPG}}$$

where v is the observed rate of ONPG hydrolysis, v_{max} is the maximum ONPG hydrolysis rate of the particular sample with ample substrate ONPG concentrations and under no inhibition of lactose, S_{ONPG} (=10 mM) is the ONPG concentration, K_{ONPG} is the Michaelis–Menten constant of ONPG for the *T. reesei* lactase, L is the lactose concentration, and $K_L^{i,ONPG}$ is the competitive inhibition constant of lactose on ONPG hydrolysis by lactase. The best-fit values for K_{ONPG} and $K_L^{i,ONPG}$ were 0.27 mM and 3.8 mM (i.e., 0.08 g/L and 1.3 g/L), respectively. The small Michaelis–Menten constant indicated that *T. reesei* lactase had very high affinity to the chosen substrate for analysis (ONPG). The much larger inhibition constant indicated that the ONPG hydrolysis was not very sensitive to the competitive inhibition by the presence of lactose. The above equation was used to adjust the measured lactase activities using the lactose concentrations present in the particular samples. (It should be noted that the lactose concentrations in the continuous culture samples were very low, 0.07–0.7 mM, except that at the highest dilution rate, i.e., 22 mM at $D = 0.12\ h^{-1}$. The above adjustment had an appreciable effect only for the samples taken at the highest D.)

Many batch fermentation samples were measured for both cell dry-weight (CDW) and intracellular protein (IP) concentrations. These are summarized in Fig. 1. The nonlinear correlation was best-fit with the following equation ($r^2 = 0.92$):

$$CDW = 7.14(IP)^2 + 6.87(IP).$$