Journal of Industrial Microbiology & Biotechnology (1998) 20, 339–343

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Simultaneous utilization of glucose and D-xylose by *Candida shehatae* in a chemostat

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The kinetics of biomass formation, D-xylose utilization, and mixed substrate utilization were determined in a chemostat using the yeast *Candida shehatae*. The maximum growth rate of *C. shehatae* grown aerobically on D-xylose was 0.42 h^{-1} and the Monod constant, K_s, was 0.06 g L^{-1}. The biomass yield, $Y_{\{X/S\}}$, ranged from 0.40 to 0.50 g g^{-1} over a dilution rate range of 0.2–0.3 h^{-1}, when *C. shehatae* was grown on pure D-xylose. Mixtures of D-xylose and glucose (~1 : 1) were simultaneously utilized over a dilution rate from 0.15 to 0.35 h^{-1} at pH 3.5 and 4.5, but pH 3.5 reduced μ_{max} and reduced the dilution rate range over which D-xylose was utilized in the presence of glucose. At pH 4.5, μ_{max} was not reduced with the mixed sugar feed and the overall or lumped K_s value was not significantly increased (0.058 g L^{-1} *vs* 0.06 g L^{-1}), when compared to a pure D-xylose feed. Kinetic data indicate that *C. shehatae* is an excellent candidate for chemostat production of value added products from renewable carbon sources, since simultaneous mixed substrate utilization was observed over a wide range of growth rates on a 1 : 1 mixture of glucose and D-xylose.

Keywords: chemostat; *Candida shehatae*; mixed sugars; D-xylose; Monod kinetics; pH

Introduction

Much research has centered on producing ethanol from d-xylose and mixtures of d-glucose and d-xylose [5,15]. However, d-xylose-fermenting yeast produce ethanol at economically unattractive rates and yields. The microbial synthesis of high value compounds from renewable feedstocks containing d-xylose may be an economical alternative to ethanol. Products of aerobic metabolism, such as individual cells, protein, lipids, and enzymes would be ideal candidates for production from d-xylose-utilizing yeast, such as *Candida* strains, since most grow rapidly under aerobic conditions. *Candida* strains are also reported to have commercial potential as biological control agents [17]. A chemostat or continuous stirred-tank reactor (CSTR) is best suited for the production of biomass (cells, proteins and enzymes) under carbon-limited conditions. Synthesis of such products from a mixed sugar feed would require simultaneous utilization of the glucose and d-xylose over a wide range of dilution rates; glucose should not repress the utilization of the other sugars.

The yeast, *Candida shehatae*, has been shown to simultaneously ferment d-xylose and glucose to ethanol, under anaerobic conditions [11], indicating that *C. shehatae* is not severely repressed by glucose and may be an ideal candidate for chemostat cultivation on mixed sugar feeds. Chemostat studies on pure d-xylose have been performed with *C. shehatae* [1], but to date mixed sugar chemostat studies using *C. shehatae* have not been reported in the literature. This paper reports the results for continuous production of *C. shehatae* in a chemostat from a mixed sugar feed. Data indicate that *C. shehatae* is not severely repressed by glucose and can simultaneously utilize a mixture of sugars over a wide range of dilution rates.

Materials and methods

Medium

A chemically defined medium was used in both the seed cultures and the chemostat cultivation [12] and contained 0.2% (v/v) antifoam (Antifoam A; Sigma, St Louis, MO, USA). The inlet feed at pH 4.5 was prepared in the following manner. A 13.452-L sugar solution was prepared and autoclaved in a 20-L Pyrex carboy. After the sugar solution had cooled, 350 ml of a 40× trace element solution, 56 ml of a 250 × vitamin solution and 100 ml of 35 × $(NH_4)_2SO_4$ solution were added through a 0.2-μm filter and septum. The inlet d-xylose concentration was 5.6 g L^{-1} in the experiments which involved pure d-xylose in a chemostat. In experiments in which mixed sugar was used, the feed contained 2.8 g L^{-1} of d-xylose and 2.7 g L^{-1} of d-glucose (1 : 0.96). In experiments at pH 3.5, the pH was controlled by adding 10 g L^{-1} of KH_2PO_4 and 10 g L^{-1} of K_2HPO_4, the inlet d-xylose and glucose concentrations were 2.0 g L^{-1} and 1.8 g L^{-1} respectively (1: 0.9), and the pH of the feed was 6.0. All other conditions were the same as the experiments performed at pH 4.5.

Cultivation conditions

Cells from a seed culture [12] were used to inoculate a Multigen-2000 or a Bioflow IIc (New Brunswick Scientific, Edison, NJ, USA). After inoculation, the fermentor was operated in a batch mode for 5–8 h before it was switched to a continuous mode. The feed was pumped to the bottom of the reactor just below the impeller (Multigen-2000). A

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Received 21 August 1997; accepted 28 May 1998

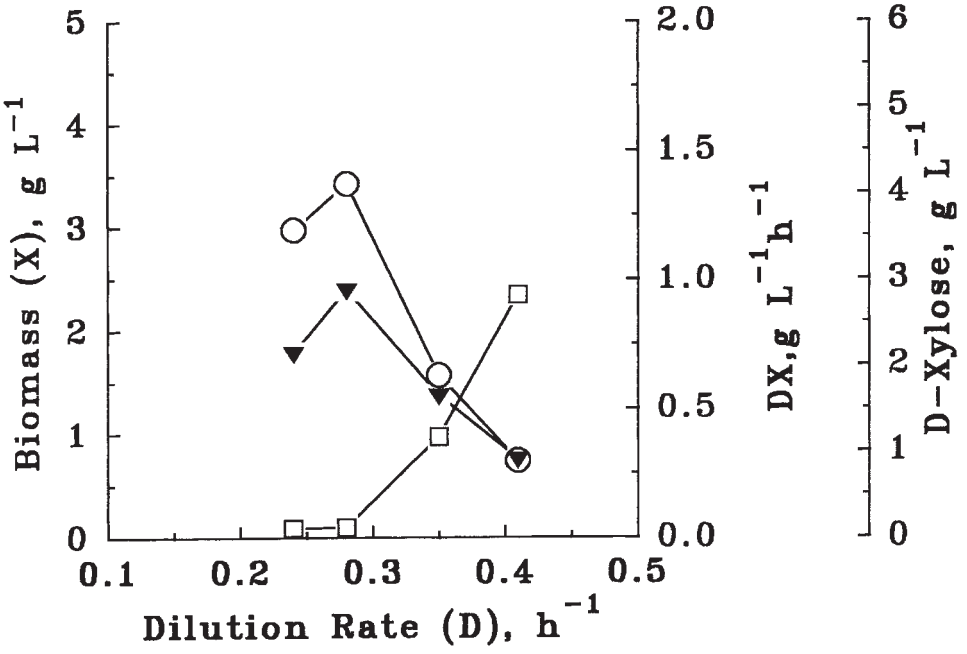

Figure 1 Chemostat cultivation of *C. shehatae* (biomass, ○) on pure d-xylose (□) at pH 4.5 (productivity or dilution rate, D × biomass, X; ▼).

draw tube was set at the top of the liquid level and an outlet pump was set at a much higher flow rate in order to maintain a constant reactor volume of 1200 ml. Temperature and pH were controlled at 30°C and 4.5. Dissolved oxygen was monitored using a galvanic oxygen probe (New Brunswick Model 900 series) and a dissolved oxygen analyzer (New Brunswick Model DO-40). Agitation was varied between 600–1000 rpm and air was sparged at 1.5–2 L min^{-1} to maintain the dissolved oxygen level above 50% saturation. When the dilution rate was changed, six or more reactor volumes were allowed to pass through until a steady state was achieved and triplicate samples were taken from the reactor for analysis (a sample was also taken from the outlet to confirm a well mixed reactor). Samples were taken in triplicate from the feed to verify that the inlet sugar concentration did not deviate significantly from batch to batch.

Chemostat cultivation at pH 3.5 was performed in the New Brunswick Bioflow IIc. The feed was pumped in at the top of the reactor and then pumped from the bottom through a J-shaped draw tube just below the impeller. The reactor volume was maintained at 1000 ml. All other conditions were the same as in the Multigen.

Analytical methods

Periodically, 5-ml samples were aseptically collected from inside the reactor and from the effluent, and immediately placed on ice. From an aliquot of this sample the total cells per ml were determined using an Elzone by Particle Data, Inc (Elmhurst, IL, USA) [12]. The total cell count from the Elzone was verified using a hemacytometer (American Optical, Buffalo, NY, USA). Plate counts were used periodically to check for contamination. The remaining sample was then centrifuged and the supernatant medium decanted and stored in glass vials at 0°C. d-Xylose and glucose were determined by HPLC as previously reported [11].

Samples were taken periodically at both the low and high dilution rates, the dry cell weight was determined, and correlated with the total cell count from the Elzone. Dry cell weights were also determined from batch fermentations on d-xylose and glucose. All of these data were averaged to obtain a constant; one gram of dry cell weight was found to contain $10.5 \pm 1.5 \times 10^{10}$ cells for *C. shehatae* cells grown on d-xylose or glucose. This constant was used to convert the total cell count data (cells L^{-1}) into g of dry biomass per L, to facilitate comparison of the results to those in the literature.

Results

Although chemostat cultivation of *C. shehatae* has been performed [1], d-xylose-limited kinetic data have not been reported (μ_{max}, maximum specific growth rate and K_s, value of rate-limiting substrate at which the specific growth rate is half its maximum value). In experiments with pure d-xylose, the dilution rate was initially set at 0.24 h^{-1} and slowly increased. The biomass level remained relatively constant and the outlet d-xylose concentration close to zero, until the dilution rate was increased beyond 0.28 h^{-1}. The biomass level decreased and the d-xylose concentration increased in the outlet stream beyond this point (Figure 1).

Monod type kinetics were assumed and used to fit the steady-state experimental data [2,3]. The pure d-xylose kinetic parameters, μ_{max} and K_s, were estimated by plotting S/D *vs* S (S, steady-state substrate concentration and D, dilution rate) to give a straight line with a slope equal to $1/\mu_{max}$ and an intercept of k_s/μ_{max}. The maximum specific growth rate, μ_{max}, was also estimated from batch cultures for d-xylose and glucose [13]. The error in K_s calculated from the S/D *vs* S plot was large, because the intercept was very close to zero ((a) of Figure 2 and Table 1). Alternatively, K_s was estimated using a non-linear curve fit of the Monod equation using SigmaPlot (SPSS, Chicago, IL,

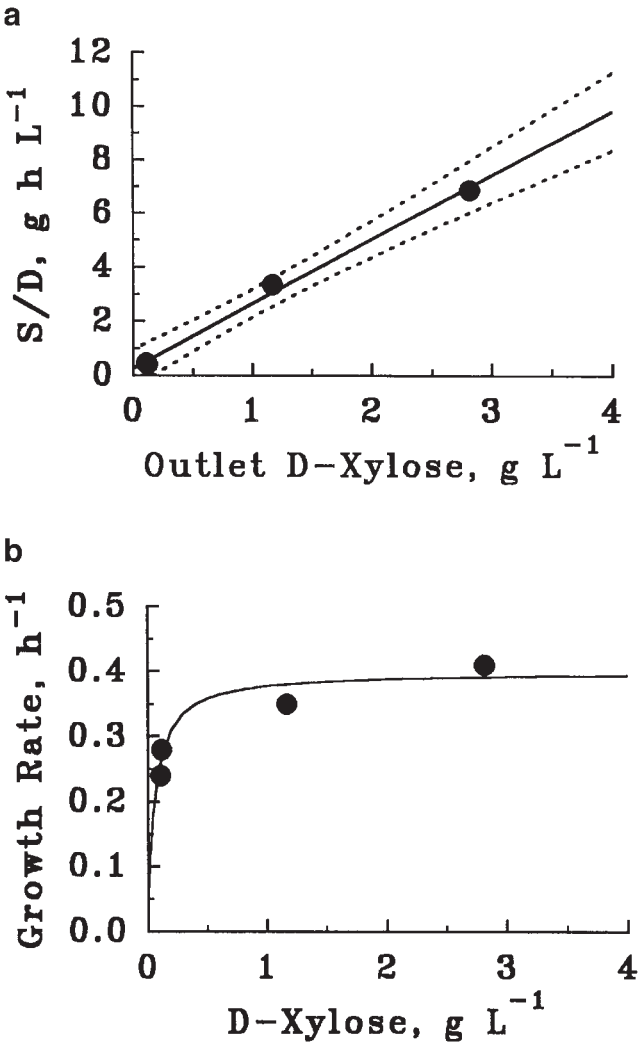

Figure 2 Estimation of kinetic parameters for chemostat cultivation of *C. shehatae* on a pure d-xylose feed at pH 4.5. Part (a) shows a plot of S/D *vs* the steady-state substrate concentration (S) and (b) shows a non-linear curve fit of the Monod equation.

USA; (b) of Figure 2 and Table 1). Additionally, K_s was estimated from the biomass steady-state mass balance equation ($K_s = (\mu_{max} - D)/D \times S$), using the μ_{max} value determined from the S/D *vs* S plots [9]. A lumped μ_{max} and K_s value, based on the total sugar concentration, was calculated in a similar manner for the cultivations involving mixed sugar at pH 3.5 and 4.5, using the above methods. Estimated $K_{s,xylose}$, $K_{s,glucose}$ and μ_{max} values based on individual substrate concentrations in the mixture sugar cultivations were not statistically different from those based on total sugars, except where noted (Table 1). This was due to the fact that steady-state glucose levels were below the detection limit at many dilution rates; this approach to reporting kinetic parameters for mixed substrate cultivation has been used previously [14].

C. shehatae fermentations of ethanol have a reported pH optimum between 4.5 and 6.0 [6,13]. However, most lignocellulosic feedstocks are acidic (eg, acid hydrolyzates and spent sulfite liquors) and must be neutralized before fermentation. Continuous biomass production at low pH would reduce caustic requirements and minimize contamination in non-sterile operations. Experiments with mixed sugars were performed at pH 3.5 for this reason. The initial dilution rate was set at 0.12 h^{-1} and then increased. Biomass levels remained constant until a dilution rate of 0.24 h^{-1} was reached. d-Xylose was utilized in the presence of glucose from a dilution rate of 0.12 h^{-1} to 0.24 h^{-1}, but utilization declined significantly at a dilution rate of 0.30 h^{-1}, as biomass levels declined (Figure 3). Glucose was not detected in the outlet stream over the range of dilution rates tested and the maximum biomass productivity occurred at a dilution rate of 0.24 h^{-1} (Figure 3). *C. shehatae* utilized 100% of glucose and 87.5% of d-xylose at 0.24 h^{-1} and utilized all of the sugars at 0.18 h^{-1} (Figure 3).

An increase in pH from 3.5 to 4.5 significantly impacted sugar utilization patterns. Initially the dilution rate was set at 0.15 h^{-1} and then increased, during which the biomass level remained constant until 0.28 h^{-1}. Both sugars were completely consumed over this range of dilution rates (Figure 4). When the dilution rate was increased beyond 0.28 h^{-1}, the biomass level decreased, d-xylose appeared in the outlet at 0.35 h^{-1}, and glucose appeared in the outlet stream at 0.37 h^{-1} (Figure 4).

Table 1 Kinetic parameters for pure D-xylose and mixed sugar chemostat cultivation of *C. shehatae* (parameters for mixed sugars were based on total substrate concentration, except as noted)

Substrate (pH)	Kinetic parameters		
	μ_{max} (h^{-1})	K_s (g L^{-1})	D_{max}[e] (h^{-1})
d-Xylose (4.5)	0.42 ± 0.04[a] 0.40 ± 0.024[b] 0.41 ± 0.001[c]	0.11 ± 0.14[a] 0.06 ± 0.02[b] 0.11 ± 0.08[d]	0.37
Mixed sugar (4.5)	0.38 ± 0.01[a] 0.37 ± 0.05[b]	0.08 ± 0.046[a] 0.058 ± 0.031[b] 0.076 ± 0.044[d]	0.34
Mixed sugar (3.5)	0.32 ± 0.12[a] 0.34 ± 0.18[b]	0.02 ± 0.05[a] 0.01 ± 0.031[b] 0.076 ± 0.004[d]	0.30
Glucose (4.5)	0.33 ± 0.02[c]	0.025[d,f]	NP

[a] μ_{max} and K_s from S/D *vs* S plot.
[b] μ_{max} and K_s from non-linear fit of μ or D *vs* S (Monod equation).
[c] μ_{max} from batch experiments.
[d] $K_s = S(\frac{\mu_{max} - D}{D})$; μ_{max} from S/D *vs* S plot.
[e] $D_{max} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_f}}\right)$ (g L^{-1}), where S_f is the total inlet substrate concentration and K_s was taken from the non-linear fit and μ_{max} was taken from the S/D *vs* S plot.
[f] Determined from the mixed sugar chemostat cultivation data[d] at pH 4.5 using a single dilution rate of 0.3725 h^{-1}, $S_{glucose} = 1.245 \pm 0.0022$ g L^{-1} and $S_{xylose} = 2.5$ g L^{-1}, indicating that glucose was the only rate-limiting substrate.
NP, not performed.

Discussion

Batch cultivation of *C. shehatae* on mixtures of glucose and d-xylose leads to sequential substrate utilization, with glucose consumed first followed by d-xylose [11]. Simul-

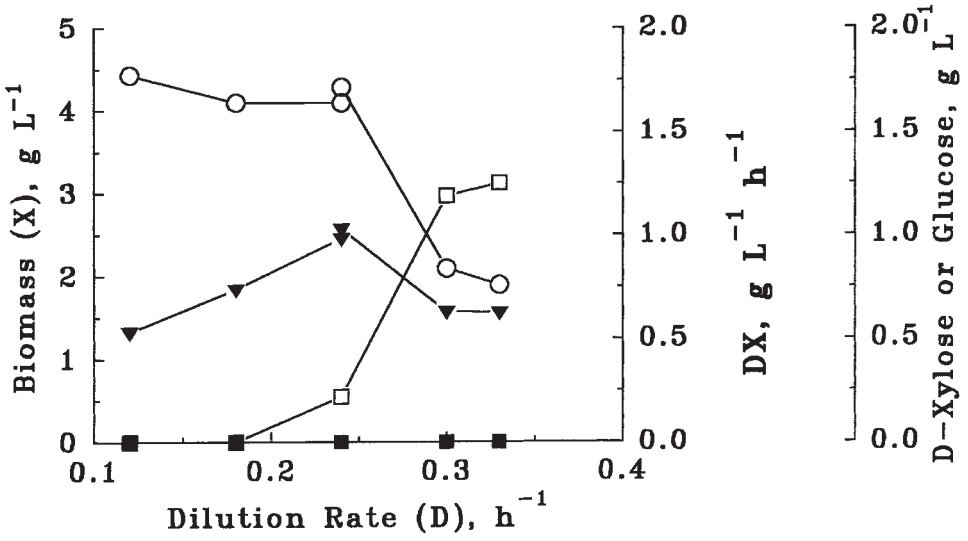

Figure 3 Chemostat cultivation of *C. shehatae* (biomass, ○) on a mixed sugar feed consisting of d-xylose (□) and glucose (■) at pH 3.5 (productivity or DX, ▼).

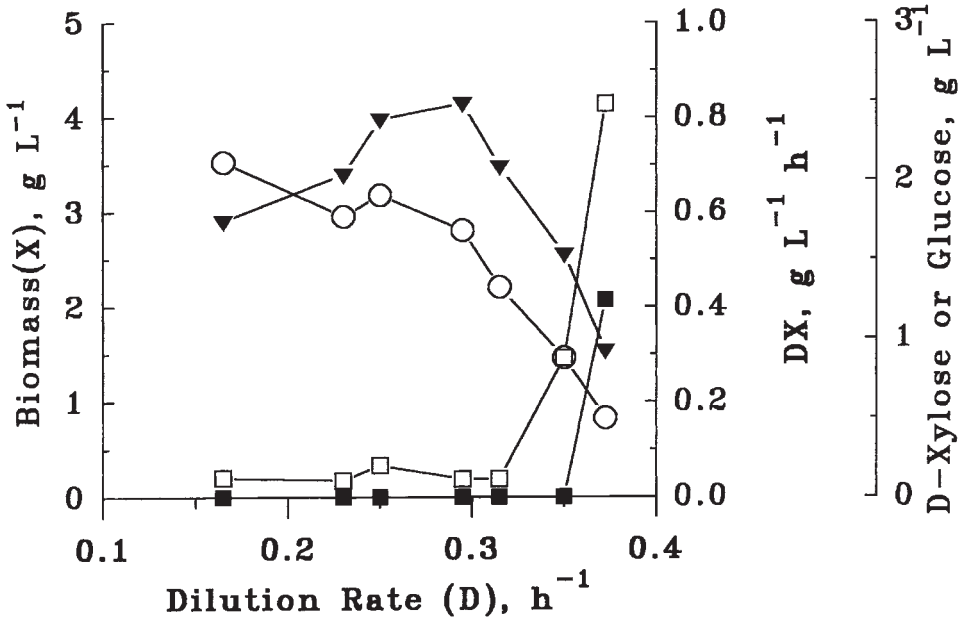

Figure 4 Chemostat cultivation of *C. shehatae* (biomass, ○) on a mixture of d-xylose (□) and glucose (■) at pH 4.5 (productivity or DX, ▼).

taneous utilization can be induced if cells are pre-grown on d-xylose in batch cultures, without typical diauxic growth patterns [11]. Diauxic growth patterns are also observed in chemostat cultivation on mixtures of two growth-limiting substrates [4,7]. At low dilution rates both substrates are utilized. As the dilution rate is increased a point is reached where the biomass levels decrease to a new steady-state level and one of the substrates accumulates in the outlet at its inlet concentration. The substrate that accumulates first is the substrate that supports a lower growth rate.

Diauxic growth and sequential substrate utilization was not apparent in the chemostat cultivation of *C. shehatae* on mixed sugars at pH 4.5. The maximum growth rate of *C. shehatae* was not significantly lower when grown on a mixture of glucose and d-xylose (Table 1). There was no clear transition from d-xylose to glucose utilization and a subsequent change in the biomass levels. d-Xylose did not appear in the outlet until a dilution rate of 0.35 (h^{-1}) was reached and shortly after this point glucose began to appear in the effluent. However, the biomass did not reach a new lower steady-state level, but continued to decline, suggesting that the system was close to the washout point.

At pH 3.5, d-xylose began to appear in the outlet stream at a much lower dilution rate. As the dilution rate was increased the biomass level declined to a new lower level and d-xylose accumulated in the reactor, suggesting pH had a regulatory effect on d-xylose transport and metabolism. It is clear that chemostat cultivation of *C. shehatae* was improved by increasing medium pH. At pH 4.5, biomass levels did not begin to decline until 0.3 h^{-1}, compared to

0.24 h^{-1} at pH 3.5. A longer residence time was required to achieve complete utilization of both d-xylose and glucose at pH 3.5 compared to pH 4.5. Thus D_{max} (the dilution rate at which maximum biomass productivity occurred), declined at pH 3.5, compared to pH 4.5 (Table 1). It is felt that the lower pH caused the apparent diauxic growth and sequential sugar utilization patterns (potentially due to a change in the transport mechanism) and that this change was not due to glucose repression.

Since *C. shehatae* produces poly-unsaturated fatty acids from glucose [16], and can utilize both d-xylose and glucose over a wide range of dilution rates, it would be of industrial interest to see if *C. shehatae* can produce distinct lipids from such a mixture. *Candida curvata* D simultaneously consumed a 50% mixture of d-xylose and glucose to produce unsaturated fatty acids under nitrogen-limited conditions [10]. However, experiments were only conducted at D equal to 0.06 (h^{-1}) and it is not clear over what range of dilution rates d-xylose and glucose were completely utilized.

Candida utilis utilizes spent sulfite liquors to produce single-cell protein [8]. However, only 60% of the reducing sugars were consumed at 0.33 (h^{-1}) and pH 4.5. A Monod constant (K_s) of 10 g L^{-1} was reported for *C. utilis* (based on total sugars). *C. shehatae* consumed essentially 100% of the inlet sugars at a dilution rate of 0.32 (h^{-1}) and had a nominal K_s value of 0.085 g L^{-1} (for pure d-xylose and total mixed sugars). These data indicate that *C. shehatae* has a greater potential for complete utilization of mixed sugars from renewable carbon sources than *C. utilis* and *C. curvata* D and may be an ideal candidate for lipid and single-cell protein production.

Acknowledgements

This research was supported by a grant from the Tennessee Valley Authority, Biomass Research Branch (Muscle Shoals, AL, USA) and the Georgia Tech Foundation.

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