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Ethanol fermentation of mixed-sugars using a two-phase, fed-batch process: method to minimize D-glucose repression of *Candida shehatae* D-xylose fermentations

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Candida shehatae **cells pre-grown on D-xylose simultaneously consumed mixtures of D-xylose and D-glucose, under both non-growing (anoxic) and actively growing conditions (aerobic), to produce ethanol. The rate of D-glucose consumption was independent of the D-xylose concentration for cells induced on D-xylose. However, the D-xylose consumption rate was approximately three times lower than the D-glucose consumption rate at a 50% D-glucose: 50% D-xylose mixture. Repression was not observed (substrate utilization rates were approximately equal) when the percentage of D-glucose and D-xylose was changed to 22% and 78%, respectively. In fermentations with actively growing cells (50% glucose and D-xylose), ethanol yields from D-xylose increased, the % D-xylose utilized increased, and the xylitol yield was significantly reduced in the presence of D-glucose, compared to anoxic fermentations ($Y_{ETOH,xylose}$ = 0.2–0.40 g g^{-1}, 75–100%, and $Y_{xylitol}$ = 0–0.2 g g^{-1} compared to $Y_{ETOH,xylose}$ = 0.15 g g^{-1}, 56%, $Y_{xylitol}$ = 0.51 g g^{-1}, respectively). To increase ethanol levels and reduce process time, fed-batch fermentations were performed in a single stage reactor employing two phases: (1) rapid aerobic growth on D-xylose (μ = 0.32 h^{-1}) to high cell densities; (2) D-glucose addition and anaerobic conditions to produce ethanol ($Y_{ETOH,xylose}$ = 0.23 g g^{-1}). The process generated high cell densities, 2×10^9 cells ml^{-1}, and produced 45–50 g L^{-1} ethanol within 50 h from a mixture of D-glucose and D-xylose (compared to 30 g L^{-1} in 80 h in the best batch process). The two-phase process minimized loss of cell viability, increased D-xylose utilization, reduced process time, and increased final ethanol levels compared to the batch process.**

Keywords: D-xylose; mixed-sugars; fermentation; fed-batch; viability; *Candida shehatae*

Introduction

Biomass in the form of agricultural and wood residues, is primarily composed of three biopolymers: hemicellulose, cellulose and lignin. The hemicellulose and cellulose biopolymers can be hydrolyzed using a mineral acid to produce a hydrolyzate mainly consisting of d-glucose and d-xylose. If the hydrolyzate is to be fermented to ethanol, a microbial process that can utilize both carbohydrates in a single stage has the potential to reduce manufacturing costs and increase product yields [4]. However, only some yeasts are capable of fermenting d-xylose. At present, *Candida shehatae* and *Pichia stipitis*, are the preferred microorganisms to ferment d-xylose [12]. These yeasts preferentially utilize d-glucose in the presence of d-xylose and d-glucose mixtures [1]. Following d-glucose consumption, a lag occurs prior to d-xylose utilization, as d-xylose-metabolizing enzymes are synthesized. This increases fermentation time and lowers ethanol yield, since more of the substrate is funneled into cell growth and enzyme production.

Induction of d-xylose metabolism before inoculation into a mixed-sugar environment induces simultaneous utilization of mixed-sugars and alleviates the lag period [5]. However, mixed-sugar fermentations performed under anoxic conditions resulted in low ethanol levels (30 g L^{-1}), long fermentation times (82 h), low ethanol yields from d-xylose (0.02–0.22 g g^{-1}), incomplete d-xylose utilization (50–77%), and a significant reduction in cell viability [5].

Methods have been described to improve d-xylose and mixed-sugar fermentation by genetically engineering *Zymomonas mobilis* [13] but GEMs in general require a rich medium, are slow growing, may not be stable, and presently produce low levels of ethanol. Alternatively, processes using a genetically stable, rapid growing, d-xylose fermenting yeast, such as *C. shehatae*, may yield improvements by environmentally manipulating yeast metabolism (eg, preinduction on d-xylose). The objectives of this research were to determine if simultaneous fermentation of d-xylose and d-glucose could be performed using actively growing cells, since fermentations with actively growing cells reduce xylitol by-product formation and increase ethanol yields. An additional objective was to determine if two-phase, fed-batch fermentations (aerobic metabolism followed by anaerobiosis) would increase ethanol levels from mixtures of d-xylose and d-glucose, potentially by prolonging cell viability and increasing volumetric ethanol productivity (ie, increase cell densities).

Materials and methods

A strain of *Candida shehatae* (ATCC 22984) was obtained from the American Type Culture Collection (Rockville, MD, USA) and was maintained on slants of yeast extract,

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malt extract medium containing 2% agar (Difco Laboratories, Detroit, MI, USA). Long-term stock cultures were maintained at 4°C, and subcultured at 1- to 2-month intervals.

A rich medium, consisting of yeast extract (3 g L^{-1}), $(NH_4)_2SO_4$ (5 g L^{-1}) was used in anoxic fermentations [5]. Medium used in the anoxic fermentation experiments was buffered at pH 6.0 with 2.5 g L^{-1} of KH_2PO_4 and 5 g L^{-1} of K_2HPO_4. Seed cultures for the anoxic fermentations were incubated on either d-xylose or d-glucose, depending on the experiment [5]. Anoxic fermentations were performed in 500-ml Bellco spinner flasks (250-ml working volume) at high initial cell densities ($2.6–3.5 \times 10^8$ cells ml^{-1}, 250 rpm, 30°C [5]).

A chemically defined medium, consisting of mineral salts, trace elements, and vitamins, was used in both the batch and fed-batch mixed-sugar fermentations performed with actively growing cells [6]. Mixed-sugar fermentations with actively growing cells were performed in 500-ml Erlenmeyer flasks (250-ml working volume) at lower initial cell densities (1×10^7 cells ml^{-1}, 30°C and 250 rpm). Cells used in these fermentations were pre-grown on d-xylose [6]. Two mixed-sugar fermentations were performed with 50% each of d-glucose and d-xylose, but with different initial total sugar concentrations of 40 and 73 g L^{-1}. A pure d-xylose fermentation was also performed with an initial xylose concentration of 76 g L^{-1}. Each flask was equipped with a cotton plug, and liquid samples were withdrawn from the flasks using sterile 8-in long needles (20 gauge) attached to 5-ml syringes. The initial pH in each reactor was 6.25 ± 0.03 and the fermentations were performed in duplicate.

To initiate fed-batch fermentations, cells from a seed culture, washed with media, were inoculated into duplicate benchtop fermentors (Multigen Bench-Top Culture Apparatus, Model F-2000; New Brunswick Scientific Co, Edison, NJ, USA) with a working volume of 1 L. The initial d-xylose concentration ranged between 100–120 g L^{-1} and the temperature was controlled at 30°C. The initial pH ranged between 5 and 6 and was allowed to decline until the pH reached 4.5; it was then maintained at pH 4.5 by the controlled addition of 6 N NaOH. Aeration was 1.5–2.0 L min^{-1} and the agitation rate was 700–800 rpm. At the stationary growth phase, aeration was terminated, nitrogen gas was sparged through the reactor for 3–5 min, and the agitation was reduced to 350 rpm. Due to the high cell density, high oxygen utilization rate, and lack of aeration, anoxic conditions were maintained within the reactor (dissolved oxygen equal to zero). This was confirmed via measurement with a dissolved oxygen probe (Galvanic DO probe; New Brunswick Scientific Co, Edison, NJ, USA). Following this change to anoxic conditions, the reactors were fed 90 ml of a concentrated d-glucose solution (50%, w/v) to achieve a d-glucose concentration of approximately 40–45 g L^{-1}.

Viable cell counts were determined by methylene blue-staining [9]. Total cell counts were obtained using a hemacytometer (Bright-Line by American Optical, Buffalo, NY, USA) and a particle counter (Elzone Model 80XY Particle Counter, Particle Data, Elmhurst, IL, USA) [7]. A methylene blue viability index was calculated as the number of unstained cells divided by the total number of cells. d-Xylose, d-glucose, xylitol, and ethanol concentrations were determined by HPLC [5].

Results

Anoxic batch fermentations

Cell growth did not occur in the mixed-sugar fermentations performed under anoxic conditions and the total cell concentration remained constant at $2.5–3.6 \times 10^8$ cells ml^{-1}, over the course of the fermentation. The anoxic conditions and the accumulation of ethanol prevented cell growth and apparently caused a decline in cell viability. Cell viability, as measured by methylene blue staining, declined from 98% to ~84% for cells pre-grown on d-glucose (at 62 h for both sugar combinations), and from 98% to 30–60% for cells pre-grown on d-xylose (at 82 h for both sugar combinations).

Cells grown on d-glucose and inoculated into mixed-sugar fermentations exhibited a sequential substrate utilization pattern. As previously reported, d-xylose was not utilized until d-glucose was exhausted [5]. The initial d-glucose consumption rate was inhibited by d-xylose, since the rate of d-glucose consumption was approximately three times lower when the d-xylose concentration was increased from 65 to 80 g L^{-1} (% d-xylose reduced from 50% to 24%, Table 1). Ethanol and xylitol yields indicated that little ethanol was produced from d-xylose at an initial d-glucose concentration of 65 g L^{-1}. Ethanol yields increased, however, when the d-xylose concentration was increased and the d-glucose concentration decreased (Table 1). An ethanol yield, $Y_{ETOH,glucose}$ of 0.44 g g^{-1} was used in these calculations [10]. Moreover, the ethanol yield from d-glucose, over the course of a mixed-sugar (50% d-glucose) fermentation in which d-glucose was preferentially utilized, was 0.41 g g^{-1}. This datum indicates the validity of using $Y_{ETOH,glucose} = 0.44$ g g^{-1} for the mass balance calculations. The d-xylose utilization rate, although not a function of d-xylose concentration, was significantly lower than for cells pre-grown on d-xylose (Table 1). The percentage of d-xylose utilized was also much lower when the cells were pre-grown on d-glucose (Table 1).

Cells grown on d-xylose and inoculated into the anoxic, mixed-sugar fermentations, simultaneously utilized d-glucose and d-xylose, as previously reported [5]. However, the initial d-xylose consumption rate was appreciably inhibited by d-glucose (Table 1). When the d-glucose concentration was increased from 25 to 60 g L^{-1}, the initial d-xylose consumption rate decreased by a factor of three (% glucose increased from 22% to 50%, Table 1). The rate of d-glucose consumption followed zero-order kinetics and was independent of d-glucose and d-xylose concentrations (Table 1). Although, ethanol and xylitol were formed during the period of simultaneous sugar utilization, mass balance calculations indicate that most of the ethanol formed during this period was produced from d-glucose (Table 1). The data also indicate that xylitol was the predominant fermentation product from d-xylose. Once d-glucose had been exhausted, d-xylose fermentation continued, with insignificant ethanol production for the 50% d-glucose fermentation (Table 1). However, ethanol yields calculated for the

Table 1 Parameters for mixed-sugar, anoxic fermentations performed with cells pre-grown on d-xylose or d-glucose

Parameter	Method of induction				
	Glucose : xylose (g L^{-1})				
	in d-glucose-grown		in d-xylose-grown		
	25 : 80	65 : 65	25 : 90	60 : 60	0 : 110
$Y_{ETOH,xylose}$	0.27 ± 0.18^a	0.0 ± 0.0	0.073 ± 0.013^b (0.24 ± 0.02)^c	0.1 ± 0.1^b (0.15 ± 0.02)^c	0.33 ± 0.05
$Y_{ETOH,glucose}$	0.44^e	0.41 ± 0.05	0.44^e	0.44^e	0.0
$Y_{xylitol}$	0.43 ± 0.07^a	0.45 ± 0.03^a	0.51 ± 0.015^b (0.36 ± 0.06)^c	0.34 ± 0.05^b (0.51 ± 0.07)^c	0.34 ± 0.04
$ETOH_{max}$	16.4 ± 2 (62)^d	25 ± 0.6 (62)^d	27 ± 0.71(82)^d	29 ± 3 (82)^d	26 ± 1 (85)^d
% Xylose utilized^e	29 ± 6	22 ± 4.4	77 ± 2	56.2 ± 3.5	73.2 ± 4.0
$Q_{glucose} \times 10^{12}$ g cells^{-1} h^{-1}	4.1 ± 0.4^f	11.6 ± 1.3^f	9.4 ± 1.7^f	11.6 ± 1.7^f	0.0 ± 0.0
$Q_{xylose} \times 10^{12}$ g cells^{-1} h^{-1}	2.2 ± 0.9^g	1.3 ± 1.23^g	11 ± 1.4^f	3.7 ± 1.9^f	7.4 ± 1.0

[a]Calculated after d-glucose exhaustion.
[b]Calculated during the period of simultaneous d-xylose and d-glucose utilization.
[c]Calculated over the entire mixed-sugar fermentation.
[d]Total fermentation time (h) and time at which maximum ethanol level was measured.
[e]The percentage of d-xylose utilized was measured at 62 h.
[f]Initial utilization rates measured over the first 10 h.
[g]Calculated after d-glucose exhaustion at 65 : 65 (glucose : xylose, g L^{-1}) and when the d-glucose concentration reached 15 g L^{-1} in the 25 : 80 (glucose : xylose, g L^{-1}) fermentation.
[e]Yield value taken from Ligthelm *et al* [10].

entire fermentation period indicated that additional ethanol was formed from d-xylose in the 25% d-glucose mixture (Table 1).

Batch fermentations with actively growing cells

Cell growth occurred in the aerobic batch fermentations (μ = 0.2 h^{-1}) and was independent of the sugar concentration (Figure 1). d-Glucose did not completely repress d-xylose utilization, since both d-glucose and d-xylose were simultaneously consumed during the fermentations (Figure 2). Although both sugars were simultaneously utilized, the rate of d-xylose consumption was inhibited by d-glucose. In both mixed-sugar fermentations, the initial d-xylose consumption rate was 3–3.5 times lower than the d-glucose consumption rate. The initial d-xylose consumption rate, within the first 24 h, in the pure d-xylose fermentation was about 1.6 times greater than the rate in the mixed-sugar fermentations (Table 2). The rate of d-glucose utilization was apparently first-order, independent of both d-glucose and d-xylose concentrations (Table 2).

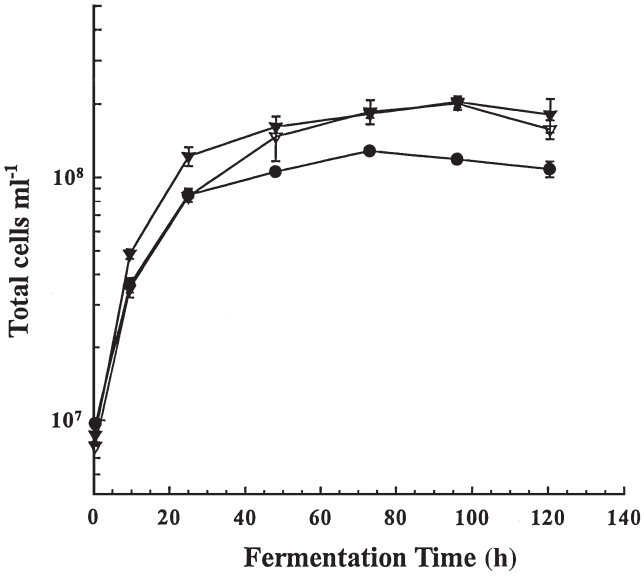

Figure 1 Growth of *C. shehatae* on pure d-xylose (76 g L^{-1}, ∇) and on a 50% mixture of d-xylose and d-glucose (S_{total} = 40 g L^{-1}, ● and S_{total} = 73 g L^{-1}, ▼), under aerobic conditions (inocula pre-grown on d-xylose).

Ethanol was produced in all fermentations as the cells entered the stationary phase (Figure 2). Xylitol, however, was produced at a later time in both the pure d-xylose fermentation and in the mixed-sugar fermentation at the higher initial d-xylose concentration (37 g L^{-1}; Figure 2). Xylitol was not detected in the mixed-sugar fermentation with an initial d-xylose concentration of 20 g L^{-1} (Figure 2).

Ethanol and xylitol yields from d-xylose indicated that ethanol was formed from d-xylose in the mixed-sugar fermentations (Table 2). The maximum ethanol levels produced in fermentations with actively growing cells were lower than in the anoxic fermentations (Tables 1 and 2), because the initial sugar concentrations were lower in fermentations with actively growing cells. However, the average percentage of d-xylose utilized was higher, ethanol yields from d-xylose higher, and the xylitol yields lower with actively growing cells, compared to the anoxic fermentations (Tables 1 and 2, comparing the fermentations at 50% glucose and 50% d-xylose).

Cell viability was also monitored in the fermentations performed with actively growing cells. In the pure d-xylose fermentation and the mixed-sugar fermentation with an

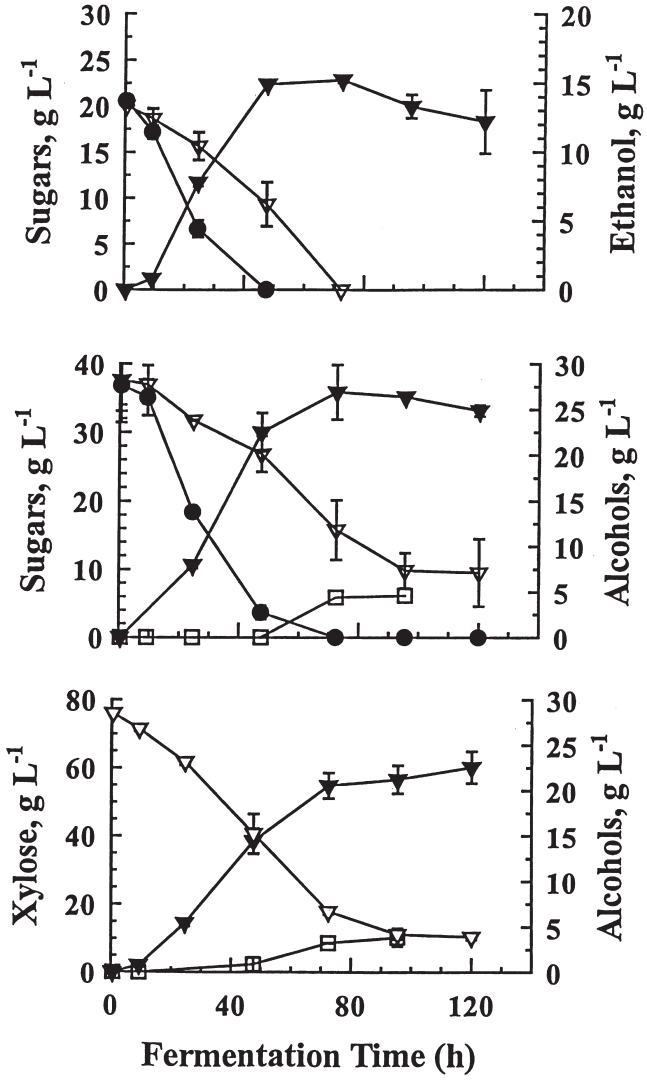

Figure 2 Ethanol (▼) and xylitol (□) production from mixtures of d-glucose (●) and d-xylose (∇) in aerobic fermentations with growing *C. shehatae* cells (corresponding to data in Figure 1).

initial total sugar concentration of 73 g L^{-1}, cell viability was stable from 0–50 h, but declined from 50–120 h. In the mixed-sugar fermentation ($S_{o,total}$ = 73 g L^{-1}), the decline in viability corresponded with depletion of d-glucose or as ethanol reached an inhibitory level (Figures 1 and 3). Further declines in cell viability resulted in incomplete d-xylose utilization at initial sugar concentrations of 73 and 76 g L^{-1} (Figure 2). Cell viability in the mixed-sugar fermentation with an initially lower sugar concentration (40 g L^{-1}) maintained a higher level throughout the fermentations. High cell viability resulted in complete d-glucose and d-xylose utilization, potentially due to lower ethanol levels (Figure 3).

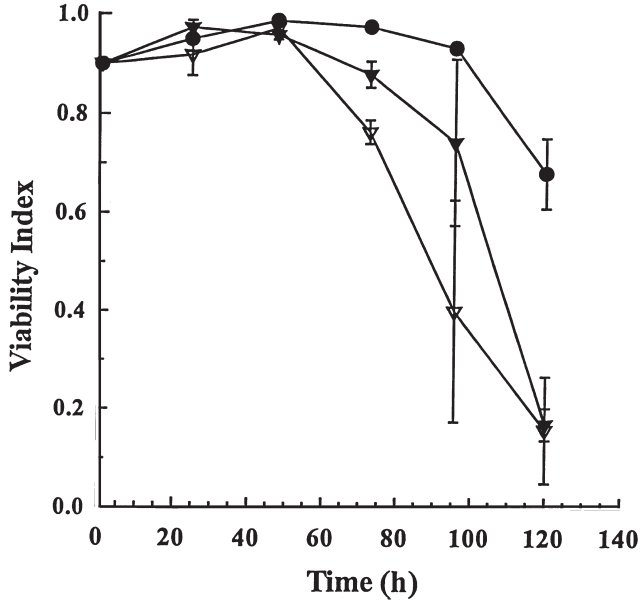

Figure 3 Viability of *C. shehatae* cells, as measured by methylene blue staining, in mixtures of 50% d-xylose and d-glucose ($S_{o,total}$ = 40 g L^{-1}, ● and $S_{o,total}$ = 73 g L^{-1}, ▼) and d-xylose fermentations (∇) in aerobic fermentations with growing *C. shehatae* cells (corresponding to data in Figures 1 and 2).

Table 2 Kinetic parameters for batch fermentations with actively growing cells and fed-batch mixed-sugar fermentations (aerobic/ anaerobic) performed with cells pre-grown on d-xylose

Parameter	Reactor/process			
	Glucose : xylose (g L^{-1}) in aerobic batch fermentations (actively growing cells)			Glucose : xylose (g L^{-1}) in fed-batch fermentations
	20 : 20	37 : 37	0 : 76	50 : 120
$Y_{ETOH,xylose}$	0.18 ± 0.13	0.4 ± 0.004	0.35 ± 0.3	0.30 ± 0.02
$Y_{ETOH,glucose}$	0.44[a]	0.44[a]	0.0 ± 0.0	0.44 ± 0.00
$Y_{xylitol}$	0.0 ± 0.0	0.16	0.06 ± 0.02	0.26 ± 0.00
$ETOH_{max}$	12.2 ± 2.8	25 ± 0.8	23 ± 2.2	47 ± 3.5
% Xylose utilized	100	75.5 ± 13	86 ± 1.42	97.5 ± 0.89
$Q_{glucose} \times 10^{12}$ g cells^{-1} h^{-1}	15.6 ± 0.22	13.1 ± 1.7	0.0 ± 0.0	1.16 ± 0.07
$Q_{xylose} \times 10^{12}$ g cells^{-1} h^{-1}	4.4 ± 1.9	4.3 ± 2.8	7.2 ± 0.64	1.5 ± 0.00

[a]Yield value taken from Ligthelm *et al* [10].

Fed-batch fermentations

Low cell densities of $1–4 \times 10^8$ cells ml^{-1}, in the fermentations with actively growing cells, resulted in maximum ethanol levels of approximately 30 g L^{-1}. It is important to achieve high cell densities in *C. shehatae* fermentations before conditions become anoxic (or oxygen mass transfer becomes rate limiting), since *C. shehatae* does not grow in the absence of oxygen [10]. High cell densities would likely result in higher ethanol concentrations.

In subsequent fermentations, higher cell densities were achieved by growing the cells under highly aerobic conditions on d-xylose ($\mu = 0.3\ h^{-1}$). Cell densities of $1–2 \times 10^9$ cells ml^{-1} were achieved, which represents a tenfold increase over the previous fermentations (Figure 4). Once the stationary phase was reached, d-glucose (50 g L^{-1}) was added to the reactors, which contained a residual d-xylose level of 65 g L^{-1}. As observed in preceding mixed-sugar fermentations, cells grown on d-xylose simultaneously consumed d-xylose and d-glucose. The addition of d-glucose did not affect the rate of d-xylose utilization (Figure 4 and Table 2).

The higher cell densities generated in these fermentations, not only increased the percentage of d-xylose utilized (Tables 1 and 2), but significantly increased the final ethanol concentrations to 49 g L^{-1} compared to 29 g L^{-1} for anoxic batch fermentations with 60 g L^{-1} of d-glucose and 60 g L^{-1} of d-xylose (Tables 1 and 2). Cell viability was maintained at a level greater than 95% from 0–50 h and decreased to 80% and 70% at 70 and 105 h respectively in duplicate fermentations.

Discussion

Cells aerobically grown on d-glucose did not metabolize d-xylose until d-glucose was completely consumed. A severe lag in d-xylose metabolism was observed in d-glucose-grown cells, even after d-glucose was consumed [5], indicating that d-xylose catabolic enzymes were not induced. Initially, the presence of d-glucose may have inhibited the transport of d-xylose into *C. shehatae* and prevented the synthesis of the d-xylose catabolic enzymes. This would inhibit d-xylose metabolism until d-glucose was completely utilized or below repressive levels. *Candida shehatae* cells grown on d-glucose transport d-xylose by a facilitated transport system [11]. Initially, d-xylose may have been transported into the cells, but was not metabolized because of d-glucose repression. The internal build-up of d-xylose would have then prevented further transport of d-xylose. Hoffer and Dahle [3] showed that d-glucose blocks the entry of d-xylose into the yeast *Rhodotorula gracilis* until all of the d-glucose has been consumed. This same regulatory process may occur in *C. shehatae*.

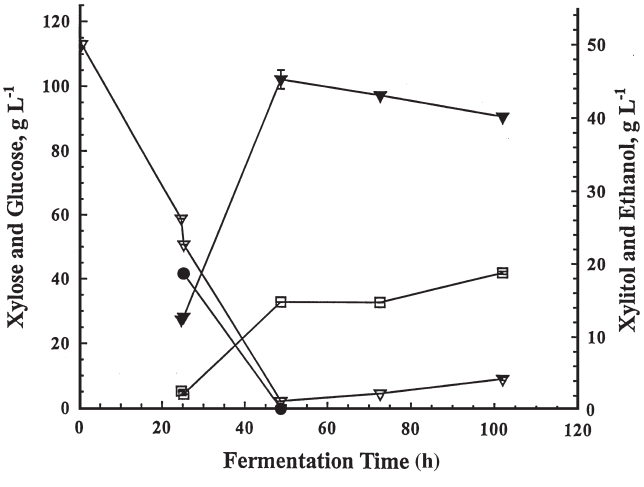

Figure 4 Ethanol (▼) and xylitol (□) production in a two-phase (dissolved oxygen = 0 at 25 h), fed-batch mixed-sugar fermentation from a mixture of d-glucose (●) and d-xylose (∇).

After d-glucose was completely utilized, d-xylose utilization was probably inhibited by the anoxic conditions (ie, oxygen limitation and ethanol inhibition). The anoxic conditions would limit enzymatic synthesis of the d-xylose pathway, as the cells were in the process of switching from d-glucose metabolism. Anoxic conditions inhibit *C. shehatae* growth on both xylose [6] and glucose [10]. Moreover, cell viability declines in anoxic batch xylose fermentations [6]. Ethanol completely inhibits aerobic growth of *C. shehatae* at a concentration of 37.5 g L^{-1} and 30°C [2]. Under oxygen-limited growth conditions, added ethanol of 25 and 50 g L^{-1} completely inhibits growth, xylose utilization, and causes a rapid decline in cell viability [6]. Although significant amounts of xylitol were produced, it does not inhibit cell growth or d-xylose utilization [8].

In cells grown on d-glucose, the rate of d-glucose utilization increased when the d-xylose concentration was decreased from 80 g L^{-1} to 65 g L^{-1}. These data suggest that d-xylose may have competitively inhibited d-glucose transport into the cells pre-grown on d-glucose. d-Glucose and d-xylose are mutual competitors of each others transport system in *C. shehatae* [11].

Cells grown aerobically on d-xylose exhibited immediate d-xylose catabolism in the anoxic mixed-sugar fermentations [5]. Pre-growth on d-xylose apparently induced synthesis of the d-xylose catabolic enzymes and prevented d-glucose inhibition. It was also evident that cell growth in the presence of d-glucose did not result in complete repression of d-xylose utilization for cells grown on d-xylose. These data indicate that d-glucose does not completely repress or inhibit enzymes for d-xylose catabolism if cells are pre-incubated on d-xylose, and d-glucose and products of d-glucose metabolism do not completely inactivate d-xylose-catabolizing enzymes. d-Glucose may modulate enzyme activity, possibly by inhibiting d-xylose transport [3,11], but it apparently does not completely inhibit d-xylose metabolism.

Ethanol yields from d-xylose were higher and xylitol yields lower in the mixed-sugar fermentations performed with actively growing cells (S_o = 73 g L^{-1}), compared to the anoxic, mixed-sugar fermentations (Tables 1 and 2). The aerobic conditions apparently allowed for more d-xylose to be metabolized to ethanol and less to xylitol, potentially due to cofactor regeneration [12].

Aerobic growth of *C. shehatae*, immediately before establishment of anoxic conditions, improved d-xylose fermentations and maintained long-term cell viability. Fed-batch fermentations initiated with high cell densities resulted in final ethanol levels of 45 g L^{-1}, compared to

25–30 g L^{-1} achieved in batch fermentations. Higher ethanol levels were attained, potentially due to higher *C. shehatae* cell densities and viability. Addition of d-glucose (4.1–4.5%) to d-xylose-grown cells did not repress d-xylose utilization in the mixed-sugar fermentations. The rates of d-glucose and d-xylose utilization were similar, further demonstrating the value of d-xylose induction. During the initial 50 h of fermentation, cell viability was high in both the batch and fed-batch mixed-sugar fermentations. However, at the end of the fed-batch fermentation (100 h), 70% of the cells were viable compared to approximately 20% viability for the aerobic batch, mixed-sugar fermentation (50% d-xylose and d-glucose, 73 g L^{-1} total sugars, at 85 h). The higher cell viability may have resulted from the reduced time cells spent under anoxic conditions compared to the batch fermentations.

Conclusions

A method to simultaneously ferment d-glucose and d-xylose has been developed using the yeast *C. shehatae*. Cells pre-grown on d-xylose simultaneously fermented d-glucose and d-xylose to ethanol and xylitol, under both anaerobic (non-growth) and aerobic (growth) conditions. The fact that d-glucose does not severely repress d-xylose utilization indicates that *C. shehatae* may be successfully used in batch and fed-batch reactors to produce ethanol from a mixed-sugar stream. In fact, the data presented here compare favorably with the recent report of d-xylose and mixed-sugar fermentations using a genetically engineered strain of a *Z. mobilis* (μ = 0.057 h^{-1} and $Y_{ETOH\text{-}xylose}$ = 0.44 g g^{-1}, $ETOH_{max}$ = 30 g L^{-1} [13]). The simultaneous utilization of mixed-sugars and lack of complete d-glucose repression, indicates that *C. shehatae* may be used for the chemostat production of single cell protein or more value added products such as enzymes.

Mixed-sugar fermentations performed with growing cells increased the percentage of sugars utilized in the fermentations and probably improved ethanol yields, due to the relatively higher oxygen levels and higher cell viability than non-growing cells. It is evident from the cell viability data that long-term exposure of *C. shehatae* cells to anoxic conditions caused a decrease in cell viability. The mechanism responsible for the decline in cell viability is presently unclear. Further research is needed to determine why *C. shehatae* cells cannot grow in the absence of oxygen and to understand the factors responsible for loss of cell viability. Anoxic growth would alleviate the need to perform fermentations under controlled aeration conditions, allow immobilized bed reactors to be used (since rapid cell death occurs under anoxic conditions, an immobilized bed reactor would be impractical), and may improve ethanol tolerance. In the absence of this knowledge, novel reactor designs, such as extractive fermentation, could be combined with the induction step and fed-batch process to improve ethanol levels.

Acknowledgements

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