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Yeast Adaptation on Softwood Prehydrolysate

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ABSTRACT

Several strains and genera of yeast, including *Saccharomyces cerevisiae* D_5A, *Pachysolen tannophilus*, *S. cerevisiae* K-1, *Brettanomyces custersii*, *Candida shehatae*, and *Candida acidothermophilum*, are screened for growth on dilute acid-pretreated softwood prehydrolysate. Selected softwood species found in forest underbrush of the western United States, which contain predominantly hexosan hemicellulose, were studied. This phase of the work emphasized debarked Douglas fir. The two best initial isolates were gradually selected for improved growth by adaptation to increasing prehydrolysate concentrations in batch culture, with due consideration of nutrient requirements. Microaerophilic conditions were evaluated to encourage tolerance of pretreatment hydrolysate, as well as ethanol product. Adaptation and simultaneous saccharification and fermentation (SSF) results are used to illustrate improved performance with an adapted strain, compared to the wild type.

Index Entries: Yeast; adaptation; softwood; ethanol; fermentation.

INTRODUCTION

Effort to produce renewable alternative sources of transportation fuels from biomass have resulted in considerable progress in the conversion of hardwood and agricultural waste into ethanol. Added yields are expected if the pentosan hemicellulose, in addition to the cellulose, is effectively fermented to ethanol. Except for sulfite waste liquor, reports of the conversion of softwood (SW) materials have been limited. Furthermore, the hemicellulose for most SWs studied is primarily hexosan, composed mainly of mannose with smaller amounts of glucose and galactose, as well as some pentoses. Traditional *Saccharomyces cerevisiae* yeast cultures ferment these

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hexoses to ethanol very well, and are expected to produce high ethanol yields if they can tolerate low concentrations of countless toxins present in prehydrolysates generated from SW. Prehydrolysates from SW may be more toxic than those from hardwood biomass sources, because Sws usually contain more extractives and often more bark than do hardwoods. Potential toxic substances include biomass components themselves, particularly extractives such as terpenes, aldehydes, and polyhydroxy aromatics. Other sources of toxins are prehydrolysis products and degradation products including acetic acid from acetylated sugars, furfural, and hydroxymethyl furfural, the initial degradation products from pentose and hexose sugars, respectively, and oligomers formed by reaction of the furfurals with sugars. Degradation of coniferous lignin yields complex guaiacyl propyl units. Corrosion products from equipment also can be toxic, or the metallic ions can behave as catalysts to produce additional products. Fortunately most of the toxins in well-prepared prehydrolysate are present at less than one g/L, and only a very few, such as furfural, are present at a few g/L. However, over time, yeast can adapt themselves to tolerate many of these substances in the presence of glucose sugar. These toxins prevent or greatly reduce growth and ethanol production. The authors intend to test the capability of yeast to gradually adapt to their environment and encourage them to eventually grow and produce very near stoichiometric ethanol, while the unadapted (wild-type) yeast can do neither.

Traditional wastes, such as SW trimmings and sawdust, are available sources of SW biomass. Recently, however, SW forest underbrush of selected regions of western United States has been harvested in an effort to prevent extremely hot forest fires which can destroy all vegetation, along with the trees. Serious land erosion or floods can follow. The authors intend to test the capability of yeasts to gradually adapt to their environment and encourage them to eventually grow and produce very near stoichiometric ethanol, when the unadapted (wild-type) yeast could do neither.

OBJECTIVES

Fermentation processes are usually both capitals and operating-cost-intensive. The authors' ultimate objectives are to determine whether these costs can be significantly reduced by demonstrating improved yeast fermentation performance through adaptation of selected yeast cultures. Specifically, high fermentation yield, process efficiency, and volumetric productivity in the presence of real prehydrolysates, while supplying minimal nutritional requirements is wanted. The high yield and efficiency ensures good conversion of saccharides to ethanol with a minimum of by products. The high productivity and low nutrient charge minimizes the fermentor size (and cost) and keeps cost of supplies down, respectively. Another objective factor is the concentration of the broth or slurry used. Higher concentrations minimize the fermenter size for a given product

capacity. But perhaps what is more important, they also increase the potential final ethanol concentration in the spent beer, significantly improving the economics of ethanol recovery. Consequently, one of the first direct benefits of culture adaptation to higher prehydrolysate slurry concentrations, and the objective of this phase of the work, is to achieve significantly improved ethanol production at desired elevated slurry prehydrolysate concentrations, in which an unadapted strain does poorly, or cannot survive at all.

EXPERIMENTAL METHODS, MATERIALS, AND MICROORGANISMS

Freshly cut Douglas fir tree trunks that measured 3–8 in. in diameter were debarked, chipped (65 HP Brush Bandit, Foremost, Remiss, MI), and shear-milled (Mitts Merrily, Sagging, MI) through a 9-m*M* screen. The milled wood was then soaked in 60°C acidified water, using 0.35% (w/w) sulfuric acid, for 4 h. The wood particles were allowed to drain and air-dry to about 40–50% (w/w) solids, and then were prehydrolyzed in a batch digester *(1)*. The resulting semisolid prehydrolysate contained ~20–30% (w/w) dry solids.

Our culturing goals were to minimize the requirement for prehydrolysate and retain or recycle all yeast cells from the prehydrolysate, while maximizing the number of generation times accumulated. Achieving the latter two goals improves the probability of obtaining and keeping desirable spontaneous mutants. Therefore, small batch cultures were used. In late log phase, or early stationary phase, cultures to be transferred were split into succeeding cultures. Initially, to facilitate culture-handling, sampling, and analysis, prehydrolysate filtrate was used. It was sterile-filtered before use. To maximize the number of generations the adapting cultures underwent in a limited time, full-strength (~25% w/w) prehydrolysate filtrate was diluted only slightly, using nutrients and inocula, to growth permissive strengths. These final concentrations of diluted liquors are referred to as the equivalent solids (ES) concentration, which is the solids concentration (dry basis) from which they were prepared. This helped in measuring progress of adaptation. This reference also permits direct estimation of the maximum ethanol concentration possible (at maximum theoretical yield and efficiency) from a given total equivalent saccharide concentration in whole prehydrolysate slurry. At the time of dilution, supplements were added and figured into the dilution.

Prehydrolysate Media

A simulated Douglas fir acid prehydrolysate was also used by mixing the five main sugars of Douglas fir hemicellulose, all of research-grade purity. Typically, they were mixed to simulate hydrolyzed hemicellulose, supplemented with significant cellulose hydrolysis. The synthetic, simulated Douglas fir acid prehydrolysate, under these conditions, consisted

of 5% (w/v) glucose, 2.5% (w/v) xylose, 0.5% (w/v) arabinose, 4.5% (w/v) mannose, 0.5% (w/v) galactose, 1% (w/v) yeast extract, and 2% peptone (latter replaced by 1.5% [w/v] ammonium sulfate), allowing room for a 10% (w/v) inoculum. These sugars were filter-sterilized, except xylose, which was autoclaved. Yeast extract and peptone were autoclaved; the ammonium sulfate was filter-sterilized. Chemicals were purchased from Baker (Windsor, UK) or Sigma (St. Louis, MO); yeast extract and peptone from Difco, (Detroit, MI). Oxygen absorption rates, to provide microaerophilic conditions of at least 0.075 m*M* oxygen/L/min, as determined by sulfite oxidation, were provided by controlling liquid volume to flask volume, baffling, and shaker (Model 4000 NBS, Edison, NJ) speed. They were grown at either 30°C, or, for culture 6, at 38°C, and at 150 rpm shaker speed.

Adaptation media were prepared by adding 1.5% (w/v) corn-steep liquor (CSL), 0.5% (w/v) yeast extract, and 1.5% (w/v) ammonium sulfate to debarked Douglas fir hydrolysate filtrate, then adjusting to pH 5.5 ±0.1 with calcium hydroxide (~6 g). CSL (Grain Processing, Muscatine, IA), at a 50% solids (w/v), was sterile-filtered after centrifuging and prefiltering, or was cross-flow-filtered.

SSF slurry was prepared with pretreated debarked Douglas fir. The SW was digested at 215°C for 100 s, after being soaked with 0.35% (w/v) sulfuric acid. The final cake was pH-adjusted with calcium hydroxide to pH 5.4 ±0.1, blended, and added to 250-mL shake flasks. After autoclaving, each flask was supplemented with filter-sterilized nutrient solution to provide 1.5% (w/v) filter-sterilized CSL, 0.5% (w/v) yeast extract, and 1.5% (w/v) ammonium sulfate. Filter-sterilized Iogen cellulase enzyme (Ottawa, Ontario, Canada) was added at a concentration of 36 IFPU/g cellulose just before inoculation. The usual ratio of enzyme IFPU/g cellulose was increased from 25 to 36, because of prehydrolysate inhibition of cellulase enzyme. Cultures were inoculated with either an adapted or wild-type strain of *S. cerevisiae* D_5A at proportional concentrations. The adapted strain, growing on prehydrolysate, required 3× the broth volume to yield the same amount of cells (dry wt basis) as did the wild-type growing on synthetic prehydrolysate. To achieve 3× the concentration, one-third was added with spent media; the other two-thirds was centrifuged and resuspended in the fresh liquor of each flask. Cultures were incubated at 30°C, and sampled daily for 7 d.

Chemical Analyses

Ethanol was analyzed with a YSI instrument (Yellow Springs, OH), or HP Series II gas chromatograph (GC) using a Porapak Q column (HP, Palo Alto, CA). Dilutions were made to stay below the YSI standard value of 1.03 g ethanol/L, or 2.5 g glucose/L. An HPLC (HP # 1090 with refractive index detector) was used for hexose concentrations, sampled at selected intervals. During adaptation, absorbances were read with a Genesys 5

Spectronic (Milton Roy, Rochester, NY) spectrophotometer at 600 n*M*. Dry wt were obtained by carefully washing 10 mL of culture once with 10 mL of sterile nanopure water. A final volume of 5 mL was weighed and dried at 105°C overnight. Viability of the cultures, obtained at sampling intervals, was determined by adding Wolford viable stain (*2*) to the culture, at concentrations of 3:1 for liquid cultures and 9:1 for slurry cultures, since the solids consume a lot of stain. Viable cells were able to keep the stain from entering, and appeared colorless. Expired cells took up the stain and appeared purple or blue. Cells were counted using a Pettroff Hauser hemocytometer.

Microorganisms

The yeast strains included *Brettanomyces custersii*, an NREL culture available as #34447 from the American Type Culture Collection (ATCC Rockville, MD). *S. cerevisiae* (D_5A) and (K-1) are NREL strains genetically derived from Commercial Red Star brewers yeast, and from Lallemand brewers yeast, respectively. *Candida shehatae* was obtained from ATCC, # 22984, and *Candida acidothermophilum* from ATCC, # 20381. *Pachysolen tannophilus* was obtained as NRRL Y-2460, from the US Dept. of Agriculture (USDA), National Center for Agricultural Utilization Research, formerly the Northern Regional Research Laboratory.

Wild-type cultures were plated on YPD: 1% (w/v) yeast extract (Y), 2% (w/v) peptone (P), 2% (w/v) dextrose (D), and 2% (w/v) agar; or RMG: 1% (w/v) yeast extract, 0.2% (w/v) monobasic potassium hydrogen phosphate, 2% (w/v) glucose, and 2% (w/v) agar. Adapted strains were plated on prehydrolysate plates 15% (w/v) filter-sterilized prehydrolysate, 1.5% (w/v) agar. All sugars were sterilized separately.

RESULTS AND DISCUSSION

Six species of yeast, which were preserved frozen in glycerol or sucrose, were grown on YPD broth and plated on YPD agar for short-term supply. Baseline performances of the yeast were initially determined by growing them in a simulated Douglas fir acid prehydrolysate that contained YP at concentrations as in YPD. During this time, they were acclimated to grow in medium formulated with 1.5% ammonium sulfate, in place of peptone, and then yeast extract reduced to one-half strength and supplemented with 1.5% (v/v) CSL, in preparation for leaving out the yeast extract entirely.

Late log-phase cultures on synthetic prehydrolysate were initially divided in half or quarters and yeast incubated after being spun down and reconstituted with actual hydrolysate filtrate back to the original volumes. Growth was monitored by measuring absorbance at 600 n*M*, glucose by (Yellow Springs Instrument, Yellow Springs, OH) YSI, and ethanol by GC or YSI. They were subcultured similarly when the glucose became low

Table 1
Growth of Six Cultures on Prehydrolysate After 48 h

Culture	Equivalent solids concentration	A_{600}
S. cerevisiae, D_5A	**17 b**	**12.10**
S. cerevisiae, D_5A	**21 a**	**11.50**
P. tannophilus	15 b	2.85
P. tannophilus	17 a	3.15
S. cerevisiae, K-1	**17 b**	**12.85**
S. cerevisiae, K-1	**21 a**	**12.00**
B. custersii	15 b	3.40
B. custersii	17 a	3.70
C. shehatea	15 b	2.95
C. shehatea	17 a	2.80
C. acidothermophilum	**15 b**	**3.45**
C. acidothermophilum	**17 a**	**3.90**

Best three cultures are in bold.

or the absorbance approached doubling or quadrupling, depending on whether they were divided into halves or into quarters. However, after splitting, each half or quarter could be reconstituted with a higher concentration prehydrolysate, and then the other half with the same initial hydrolysate concentration. This provided culture backup in the event of loss of the culture at the higher concentration. Carryover of spent, conditioned media was minimized by aseptically centrifuging the cultures, discarding the supernatant, and then resuspending all of the cells in fresh prehydrolysate. This procedure was more toxic (less-conditioned medium was used), and required additional performance from the culture.

Screening for the Most Resistant Cultures

The number of cultures that were continuously maintained, by dropping (and storing) those that adapted more slowly than others. This should not imply that they would not adapt over time, or that, with patience, they would not even be better than those that adapt more quickly; it is simply a matter of time. Cell density (Table 1) for all six yeast strains is measured by the culture absorbance at 600 n*M*, after ~ 2 wk of acclimating the six strains in prehydrolysate filtrate at 15, 17, and 21% ES. The initial absorbance was usually between 2.5 and 3.0; the double-digit numbers are the ES levels. As they were able to develop, they were split into the next higher ES, and a repeat ES. *S. cerevisiae* D_5A and K-1 grew sufficiently well to be transferred into higher and higher ES levels. They did well at 21%, and slightly better at 17% ES; the other strains struggled at 15 and 17% ES.

In this way, we dropped all strains, except for the latter two, shortly after testing them on 15 and 17% ES hydrolysate. The adaptation sequence

was continued with the *S. cerevisiae* strains for 3 wk more, until they were able to tolerate 23% ES, or better. Prehydrolysate concentrations of 23% reduced generation times (doublings) to less than one every 3 d. At that rate, sufficient doublings to maintain reasonable probabilities of getting spontaneous mutants were not obtained. Some progress was made however, because these cultures did better than those kept at lower concentrations (17%), but concentrations that initially would not support growth. Selection at the lower concentrations for less than 2 wk was maintained at which time we compared an adapted culture of *S. cerevisiae* D_5A with an unadapted (wild) strain in the simultaneous saccharification and fermentation (SSF) process.

Strain Comparison in Simultaneous Saccharification and Fermentation

This SSF test was run in duplicate with three levels of whole prehydrolysate slurry at 6.5, 10, and 15% total solids (dry basis), consisting of 2.1, 3.2, and 4.8% cellulose per flask. Table 2 shows the results of the first test. All data are averages of duplicates. The standard deviations for glucose and ethanol values are all less than $+/-5\%$ of the concentrations. An overview of Table 2 shows immediately that the adapted strain in culture sets 3 (10% solids) and 5 (15% solids) took off rapidly in the presence of initial sugar, and quadrupled the ethanol concentration within 1 d, from 0 to 24 h. The wild-type performed similarly, but only at the low 6.5% solids. The wild-type caught up to the adapted strain in 10% solids, at 72 h, but only after a long lag, from 0 to 72 h. At 15% solids, which is near the minimum solids concentration for economic ethanol production, the wild-type produced no ethanol, even in the presence of at least 2% (20 g/L) sugar concentration, as noted by the glucose level, from the very beginning through 168 h. The ethanol volumetric productivity of the adapted strain at 15% solids was at least about 0.41 g/Lh over the first 24 h, including a lag. There was some prehydrolysate inhibition of enzyme, but even at 15% solids, and feedback inhibition from greater than 2% sugar, the enzyme slowly produced about another 1% glucose (column 6). The enzyme levels decreased with solids level, because they were dosed on the basis of units of enzyme/g cellulose. Enzyme inhibition is probably the reason cultures 5 and 3 became sugar (i.e., enzyme rate)-limiting at about 48 h. Cultures 4 almost catch up to 5, albeit about 3 d later and 5% less solids. All adapted strains carried over some hydrolysate with the 10% (v/v) inoculum. Therefore, prehydrolysate inhibition of the enzyme is probably the reason the adapted culture pair became sugar-limiting sooner than the wild-strain pairs. Table 2 shows that the adapted culture viability is very good at 96 h. This shows quite an improvement over that observed for the unadapted yeast under similar low-sugar conditions, as indicated in Table 3. The actual conditions of SSF may not be as severe as sugar

Table 2
Adapted vs Wild *S. cerevisiae* D_5A SSF Performance on Prehydrolysate

Time (h)	Analyte (g/L)	6.50% Solids		10.00% Solids		15.00% Solids	
		Adapted culture 1	Wild culture 2	Adapted culture 3	Wild culture 4	Adapted culture 5	Wild culture 6
0	EtOH	3.60	5.20	3.65	5.65	3.80	6.00
	Glucose	10.85	9.15	14.45	15.15	23.90	23.50
t-24	EtOH	9.60	13.20	12.90	7.90	13.20	6.25
	Glucose	0.47	0.12	3.50	11.30	16.30	25.25
t-48	EtOH	11.00	14.65	15.65	11.40	20.50	6.45
	Glucose	0.26	0.14	0.77	7.22	4.00	26.85
t-72	EtOH	10.15	14.35	16.15	16.55	19.90	6.15
	Glucose	1.56	0.37	2.97	1.36	7.09	31.03
t-96	EtOH	10.05	12.30	16.35	17.80	22.15	5.05
	Glucose	1.51	0.32	2.80	0.67	5.99	32.42
	Viability, %	88	83.00	74.00		98.00	72.00
t-120	EtOH	9.30	12.25	15.90	20.20	22.60	5.70
	Glucose	1.49	0.31	2.53	0.58	5.27	34.98
t-144	EtOH	7.75	10.85	14.40	20.40	22.55	5.30
	Glucose	0.14	0.06	0.23	0.17	0.81	33.80
t-168	EtOH						
	Glucose	0.15	0.06	0.25	0.14	0.77	36.60

% Solids = % insoluble solids of whole prehydrolysate, dry basis.

Table 3
Yeast Viability at Low Sugar Concentration

Expt.	Yeast	Viability	Notes
1 a,b	*S. cerevisiae* D_5A	2.3, 4.0	Wolford Stain
2 a,b	*P. tannophilus*	< 10/mL	On plates
3 a,b	*S. cerevisiae* K-1	50.7, 54.9	Wolford Stain
4 a,b	*B. custersii*	~ 0.005%	On plates
5 a,b	*C. shehatae*	55.0, 64.7	Wolford Stain
6 a,b	*C. acidothermophilum*	< 10/mL	On plates

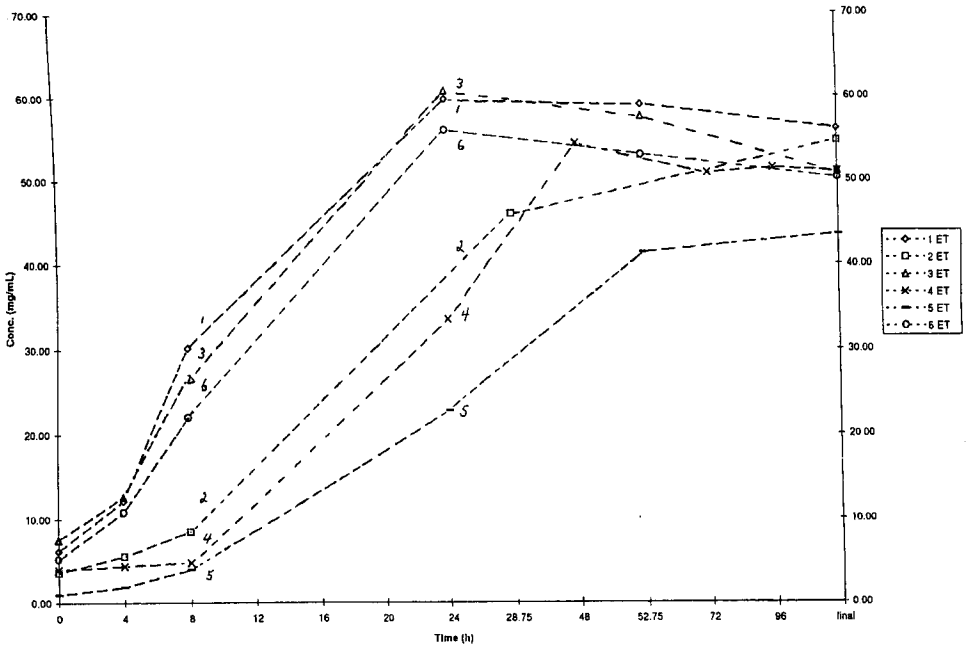

Fig. 1. Ethanol production by six yeast species grown on simulated hydrolysate.

starvation, because even though the sugar concentration is low in SSF, it usually is still being produced, but is consumed by yeast as rapidly as it is generated by the enzyme.

Characterization of Strains on Simulated Prehydrolysate

Characterization of culture performance on simulated prehydrolysate in the absence of inhibitors is expected to indicate the ultimate performance that may be expected from the strains after adaptation to real prehydrolysate containing inhibitors. Figure 1 illustrates ethanol concentrations and productivities achieved by the six cultures grown on rich nitrogen and nutrient sources, using late log-phase inocula. The cultures that per-

Table 4
Ethanol Performance Parameters

Culture	Ethanol concn @ 24 h (%, w/v)	Ethanol concn max (%, w/v)	Ethanol dP/dt max (g/L-h)	Ethanol eff. max (% theor., w/w)	Ethanol yield max (% theor., w/w)
1 *S. cerevisiae* D_5A	6.0	6.0	6.7	89.0	85.1
2 *P. tannophilus*	4.6	5.4	2.2	84.1	78.2
3 *S. cerevisiae* K-1	6.1	6.1	4.5	88.2	86.3
4 *B. custersii*	2.1	5.4	1.1	83.8	77.6
5 *C. shehatae*	2.3	4.3	1.4	66.9	62.2
6 *C. acidothermophilum*	5.6	5.6	4.4	83.1	75.3

Table 5
Performance Data for Evaluated Yeasts Grown on Simulated Hydrolysate

	Xylitol (g/L)	HOLac (g/L)	Gly (g/L)	HOAc (g/L)	Cell mass (%, g/100 mL)	Cell N (%, w/w)	Beer N (%, g/100 mL)	μ (1/h)	t_g (h)
1 *S. cerevisiae*, D_5A	6.03	–	3.31	2.37	0.68	8.19	0.0611	0.26	2.7
2 *P. tannophilus*	2.23	–	–	1.01	0.69			0.104	6.6
3 *S. cerevisiae*, K-1	6.12	0.87	2.88	2.31	0.95	8.62	0.0819	0.23	3.0
4 *B. custersii*	1.17	–	0.45	2.77	1.16			0.066	10.6
5 *C. shehatea*	4.84	–	5.21	0.74	0.70			0.49	1.4
6 *C. acidothermophilum*	1.86	0.20	3.55	0.71	0.47	11.3	0.0530	0.24	2.9

formed the best in actual prehydrolysate 1 and 3 (Table 1), also achieved the highest ethanol concentrations and the highest ethanol productivities (Fig. 1) when cultured on simulated prehydrolysate. The highest values observed are noted (Table 4) when sampled at 4, 8, 24, 48, and 72 h of fermentation. Lower efficiency is usually a result of formation of by products such as xylitol and glycerol (Table 5). Table 5 also reports cell mass produced, maximum specific growth rates observed (or minimum generation times), and cell mass nitrogen as percent (w/w) in dry cell mass, as well as cell nitrogen per culture volume, expressed as nitrogen in beer because of nitrogen contained by the yeast. For example, although culture 6 contained high relative nitrogen concentration in its cell mass, because of relatively low cell mass per culture volume, the nitrogen consumed by the cell mass per volume was actually lower than cultures 1 and 3.

It was also determined that the apparent cell viabilities after 48–72 h incubation in spent beer with low hexose sugar, as shown in Table 3. This may be thought of as simulating conditions during enzyme rate-limiting SSF. In Table 3, culture 1 had relatively low viability, 2.3–4%. However, in Table 2, after prehydrolysate adaptation, the cell viability is very good (96–100%) at (96 h) with low sugar.

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