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Process Biochemistry 36 (2001) 1199–1204

PROCESS BIOCHEMISTRY

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Ethanol production from alfalfa fiber fractions by saccharification and fermentation*

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Received 5 September 2000; accepted 27 February 2001

Abstract

This work describes ethanol production from alfalfa fiber using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) with and without liquid hot water (LHW) pretreatment. *Candida shehatae* FPL-702 produced 5 and 6.4 g/l ethanol with a yield of 0.25 and 0.16 g ethanol/g sugar respectively by SHF and SSF from alfalfa fiber without pretreatment. With LHW pretreatment using SSF, *C. shehatae* FPL-702 produced 18.0 g/l ethanol, a yield of 0.45 g ethanol/g sugar from cellulosic solids or 'raffinate'. Using SHF, it produced 9.6 g/l ethanol, a yield of 0.47 g ethanol/g sugar from raffinate. However, the soluble extract fraction containing hemicelluloses was poorly fermented in both SHF and SSF due to the presence of inhibitors. Addition of dilute acid during LHW pretreatment of alfalfa fiber resulted in fractions that were poorly saccharified and fermented. These results show that unpretreated alfalfa fiber produced a lower ethanol yield. Although LHW pretreatment can increase ethanol production from raffinate fiber fractions, it does not increase production from the hemicellulosic and pectin fractions. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Alfalfa; LHW pretreatment; Enzymes; SSF; SHF; Ethanol

1.Introduction

Alfalfa herbage fibers mainly consist of celluloses, hemicelluloses, lignin, and small amounts of pectin and proteins [1]. Alfalfa biomass has potential biotechnological importance in the production of low fiber, juice-derived co-products such as particulate (chloroplastic) protein concentrates, soluble protein concentrates, carotenoids, vitamins, minerals, growth factors, pharmaceutical agents, cosmetic products, and transgenic enzymes [2–5].

The alfalfa fiber fraction from wet fractionation was pretreated with liquid hot water (LHW) pretreatment (220°C for 2 min) for extraction and separation of hemicelluloses and insoluble cellulosic fractions [6–10]. Enzymic saccharification of alfalfa fiber, with and without LHW pretreatment, gave high yields of pentoses and hexoses [10].

There is a great deal of economic interest in utilizing and converting the sugars from lignocellulosics to ethanol and other chemicals [11-15]. Of the various xylose-fermenting yeasts, Candida shehatae has showed greater ethanol production than *Pichia stipitis;* this was due to increased uptake of xylose, glucose, mannose, and galactose [16,17]. In the present work, ethanol production was studied From alfalfa Fractions before and after LHW pretreatment by separate hydrolysis and fermen-

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PII: S0032-9592(01)00162-5

tation (SHF) and simultaneous saccharification and fermentation (SSF) using *C. shehatae.*

2. Materials and methods

2.1. Alfalfa fiber

Alfalfa *(Medicago sativa)* fiber was obtained by wet fractionation followed by LHW pretreatment at 220°C for 2 min, recovering the fibrous insoluble residue rich in celluloses 'raffinate' and liquid 'extract' rich in hemicelluloses as described in our earlier report [10]. In some experiments, 0.07% sulphuric acid was added during LHW pretreatment.

2.2. Enzymes

Commercial enzyme preparations of pectinase (SP-249) was procured from Novo (Franklinton, NC). Cellulase (Multifect B) was procured from Genencore (Rochester, NY). The enzyme activity was estimated on pectin, and filter paper, and expressed as filter paper units (FPU) and polygalacturonase units (PGU) as described by Sreenath et al. (1999).

2.3. Enzymatic saccharification of alfalfa fractions

Please see Table 1 and reference [10] for saccharification details. The samples were heat inactivated and centrifuged at 16 000 × *g* for 10 min. Sugar analysis was performed in the clear samples by high performance liquid chromatography (HPLC).

2.4. Fermentation strains

C. shehatae FPL-702 [16] and FPL-049 [12] were employed in this work. These strains were preserved in 15% glycerol at −80°C. The strains were grown on YEPX agar containing 1.0% yeast extract, 2.0% peptone, 2.0% xylose, and 2.0% agar for 48 h at 32°C.

2.5. Preparation of inoculum

The yeast inoculum was prepared in fermentation medium containing 1.7 g/l filter-sterilized yeast nitrogen base (YNB) (Difco, Detroit, Michigan) plus 2.27 g/l urea, and 6.56 g/l peptone as nitrogen sources (2 × nitrogen) and 0.01 g/l zinc sulphate. The inoculum consisted of cells cultivated on YEPX agar plates for 2 days. The liquid cultures had initial densities 1–2 g/l.

2.6. Fermentation (SHF and SSF)

For fermentation details refer to Table 1. The flasks were incubated under identical conditions. Periodically, 1.5 ml samples were taken and centrifuged at 16 000 × *g* for 3–4 min and clear samples were saved for various analyses. The unfermented residue was dried and dry weight was determined. For determining the best method of cooking of cellulosic residue prior to SSF ethanol production, the raffinate suspension (10%) was

Table 1
Materials and methods — saccharification and fermentation

Substrate	SHF		SSF[c]
	Saccharification[a]	Fermentation[b]	
Untreated alfalfa fiber	Substrate: 5 g Water: 48 ml Enzyme: 2 ml (25 FPU cellulase, 800 PGU pectinase)	Substrate: 20 ml saccharified solution Water: 25 ml Inoculum: 5 ml (1–2g/l cells)	Substrate: 5g Water: 43 ml Enzyme: 2 ml (25 FPU cellulase, 800 PGU pectinase) Inoculum: 5 ml (1–2g/l cells)
Raffinate from LHW	Substrate: 5 g Water: 48 ml Enzyme: 2 ml (50 FPU cellulase)	Substrate: 17 ml saccharified solution Water: 28 ml Inoculum: 5 ml (1–2g/l cells)	Substrate: 5g Water: 43 ml Enzyme: 2 ml (50 FPU cellulase) Inoculum: 5 ml (1–2g/l cells)
Extract from LHW (1.8% dry matter)	Substrate: 49 ml Enzyme: 1 ml (12.5 FPU cellulase, 400 PGU pectinase)	Substrate: 45 ml of 5–6 fold concentrated saccharified solution Inoculum: 5 ml (1–2g/l cells)	Substrate: 44 ml Enzyme: 1 ml (12.5 FPU cellulase, 400 PGU pectinase) Inoculum: 5 ml (1–2g/l cells)

[a] Saccharification was carried out in 125 ml Erlenmeyer flasks at 50°C, 100 rpm and pH 5.4 during 96 h. During saccharification, untreated alfalfa gave 50 g/l reducing sugars, raffinate gave 59 g/l reducing sugars and extract released 6 g/l sugars.
[b] Fermentation was carried out in 125 ml Erlenmeyer flasks at 30°C. 100 rpm and pH 5.4 during 40 h.
[c] Simultaneous saccharification and fermentation was carried out in 125 Erlenmeyer flasks at 30°C, 100 rpm and pH 5.4 during 96 h.

autoclaved for 20 min at 121°C, 15 lb. psi or microwaved for 2 min or steam treated for 10 min at 100°C or oven dryed at 100°C for 3 h.

2.7. Treatments of saccharified alfalfa extract

Activated charcoal, 0.2 g, was added to 5 g saccharified extract at 20°C and the solutions were filtered. In a separate trial, Dowex 1-X2 (Biorad, USA) (200–400 mesh in bicarbonate form, pH 7.0), 0.5 g, was added to 5 g of extract at 20°C for 5–10 min. The solution was centrifuged at 16000 × g for 3–4 min. Similarly the concentrated extract was treated twice with the Dowex. In another trial, fresh cells of *C. shehatae* FPL-702, 0.2 g, were added to 5 g extract and shaken at 180 rpm at 30°C for 4–5 h, followed by centrifuging at 16000 × *g* for 3–4min. The clear sample was heat-treated in a steam bath for 3 min to arrest microbial growth. The concentrated extract, 1.7 ml, was treated with 0.4 g of concentrated cells under identical conditions.

2.8. Analyses

Cell density was measured at 600 nm. An OD of 1.0 was equivalent to 0.21 mg dry weight of cells/ml [12]. The reducing sugar analysis was estimated using a DNS reagent [18]. Uronic acid was estimated colorimetrically using m-hydroxydiphenyl reagent [19]. Ethanol was estimated by gas chromatography (GC) (Hewlett Packard, USA) using Poropack column with initial and final oven temperatures of 175°C, detector temperature of 275°C, and injector temperature of 225°C. The carrier gas was helium and the detector gas was hydrogen [20]. The sugars and byproducts were separated by HPLC (Hewlett Packard series 1050, USA) with a refractive index (RI) detector using column Aminex Carbohydrate HPX 87C (300 × 7.8 mm) (Biorad, USA) maintained at 85°C [21]. The mobile phase was degassed distilled water at a flow rate of 0.5 ml/min at a pressure of 50–55 bar. The filtered clear sample, 0.5 ml was directly used in HPLC. The organic acids were also determined in HPLC (Shimadzu Liquid chromatograph LC- 10AT) using Biorad Aminex ion exclusion column HPX-87H (300 × 7.8 mm) (Biorad, USA) with Shimadzu refractive index detector RID-6A maintained at 42°C [22]. The mobile phase was 0.01 5 N sulfuric acid with 0.25 mM EDTA free acid at a flow rate of 0.6 ml/min.

3. Results and discussion

3.1. Enzymic saccharification of alfalfa fiber fractions in SHF and SSF

In SHF, unpretreated alfalfa fiber was first hydrolyzed by the enzyme mixture at 50°C and the sugars obtained

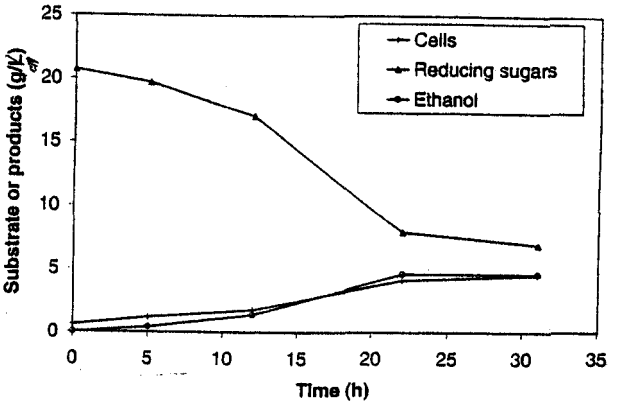

Fig. 1. SHF ethanol production by *Candida shehatae* FPL-702 on enzymatic hydrolysate from alfalfa fiber without LHW pretreatment.

were subsequently fermented with yeast at 31°C. During SSF of unpretreated alfalfa fiber, saccharification and fermentation were carried at 31°C, enabling the yeast to grow. In our earlier work, we reported that 51 g of fermentable sugars were released from 100 g alfalfa fiber at 50°C using a mixture of cellulase and pectinase consisting of 25 FPU and 800 PGU, respectively [10]. At 30°C 100 g/l of unpretreated alfalfa fiber gave 40 g/l fermentable sugars at 90 h after saccharification with the same amounts of cellulase and pectinase. Raffinate yielded 40 g/l fermentable sugars with 50 FPU cellulase alone at 30°C. The extract gave 6 g/l of fermentable sugars from 18 g/l dry matter with mixture of cellulase and pectinase consisting 12.5 FPU and 400 PGU. Hence, the yield of sugars from alfalfa residues during enzymatic saccharification was lower at 30°C than at 50°C [10]. In our previous study, the amount of enzymes for optimum saccharification of alfalfa fiber was reported [10] and the same amounts of enzymes were used in this study.

3.2. Ethanol production from alfalfa fiber by SHF and SSF with and without LHW pretreatment

3.2.1. Unpretreated alfalfa fiber

SHF with *C. shehatae* FPL-702 produced 5 g/l of ethanol from 20 g/l saccharified sugars derived from alfalfa fiber (Fig. 1). The yield obtained in SHF was 0.25 g ethanol/g sugar. SSF gave 6.4 g/l of ethanol with a yield of 0.16 g ethanol/g sugar from alfalfa fiber (Table 2 and Fig. 2). The quantity of sugar produced was ~ 40 g/l from 100 g of alfalfa fiber at 30°C. This is ~40% of the initial material. The expected ethanol production from SSF of alfalfa fiber was ~ 20 g/l ethanol from 100 g fiber. However, the actual ethanol produced was slightly > 6 g/l or ~ 30% of expected production [23]. The reduced ethanol production was

Table 2
Effects of various treatments on ethanol production by *Candida shehatae* FPL–702 from alfalfa residues by simultaneous saccharification and fermentation (SSF)[a]

Time (h)	Untreated residue	Ethanol (g/l)			
		LHW		LHW+0.07% H_2SO_4	
		Raffinate	Extract	Raffinate	Extract
1	0.5±0.1	0.2±0.10	0	0	0
17	3.3±0.1	4.6±0.1	0.6±0.1	0.1±0	0.8±0.1
42	5.7±0.3	11.5±0.1	0.9±0.1	1.3±0.1	1.3±0.1
65	6.4±0.5	15.2±0.3	0.3±0	1.0±0.1	0.1±0
90	6.0±0.4	16.7±0	0	0	0
147	4.9±0.8	17.9±0	0	0	0

[a] See Section 2 for enzymatic hydrolysis and fermentation conditions. For maximum saccharification of untreated residue, cellulosic raffinate and hemicellulosic extract, optimized amounts of enzymes were used as recommended in our previous report [10].

possibly due to inhibition of yeasts. Hence, sugars were still available at the end of SHF and SSF (Figs. 1 and 2). We suspect low amounts of pectic acids and other organic acids released during hydrolysis of pectin and hemicellulose could have inhibited the yeast fermentation.

3.2.2. Raffinate

Cellulase alone was used to hydrolyze the cellulosic raffinate. When saccharified at 30°C with 10 FPU cellulase/g, the raffinate produced 40 g/l reducing sugar of which 35 g/l was glucose. During SSF, ethanol production by *C. shehatae* was 17.9 g/l with a yield of 0.45 g ethanol/g sugar (Table 2). Sugars derived from SSF were completely utilized by the yeast in ethanol fermentation (Fig. 3). The fraction of raffinate degraded in SSF was 40%. At 50°C, with the similar concentrations of cellulase, the sugars generated from raffinate were 64 g/l [10]. A portion of this saccharified raffinate with 20.2 g/l fermentable sugars was taken for SHF medium. Ethanol production by SHF was 9.6 g/l with a yield of 0.47 g ethanol/g sugar. The ethanol yield was slightly higher than that of SSF (Fig. 4). These results suggest that the raffinate resulted from LHW fractionation did not inhibit yeast fermentation.

3.2.3. Extract

Hemicellulose was clearly the major constituent extracted as solubilized liquid during LHW pretreatment [6,7,10]. The extract with ~ 1.8% dry matter contained 1 g/l acetic acid, 0.29 g/l formic acid, and 0.5 g/l uronic acids. About 40% of the extract dry matter were in the form of fine particulates. After saccharification, this soluble fraction generated 7 g/l fermentable sugars. Hence, the extract was concentrated 5–6-fold to increase sugar levels, which also resulted in higher levels of inhibitors. Analysis of the concentrated saccharified extract showed ~ 6 g/l acetic acid, known to inhibit yeast growth. There was no yeast growth and ethanol formation during SHF. The growth of *Candida utilis* and *Pachysolen tannophilus* was completely inhibited by acetic acid concentrations > 1.2 and 1.45 g/l, respectively [24]. Wilson et al. [25] reported that 2.7 g/l acetic acid reduce the utilization of pentoses by *P. stipitis.* The saccharified extract without concentration was poorly fermentable in SSF due to low sugar concentrations. The amount of ethanol produced was negligible in SSF of the extract-sugars with *C. shehatae* (Table 2). The concentrated extract was also not fermentable by SSF due to increased level of inhibitors.

Various trials to remove inhibitors completely from the extract were unsuccessful. Both charcoal treatment and cell pretreatment (one cycle of cell washing) did not remove acetic acid, whereas anion exchange resin (in bicarbonate form) removed almost 50% of the inhibitors as determined by HPLC of the extract or

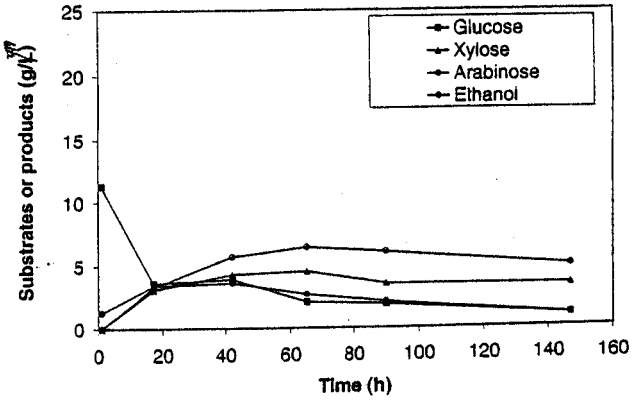

Fig. 2. SSF ethanol production by *C. shehatae* FPL-702 from alfalfa fiber without LHW pretreatment.

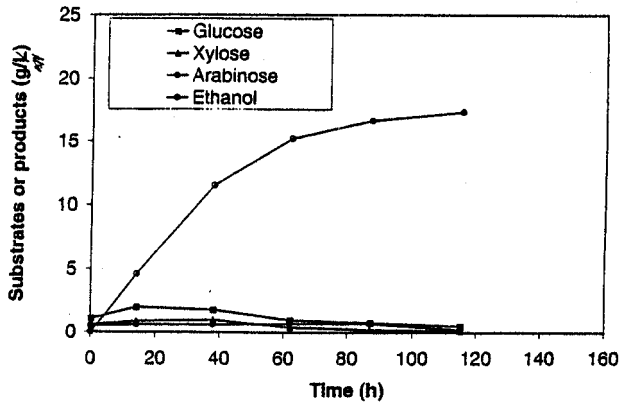

Fig. 3. SSF ethanol production by *C. shehatae* FPL-702 from raffinate after LHW pretreatment.

concentrated extract. However, the resin also adsorbed 20% sugars from the extract (data not shown).

3.3. Effect of various methods of cooking of raffinate on SSF ethanol production

SSF of cellulosic raffinate gave a better yield of ethanol in less time than SHF. The efficiency of ethanol production from raffinate was ascertained by cooking raffinate in various ways prior to SSF. Cellulosic raffinate was cooked by regular autoclaving, microwaving, steaming, and oven drying and tested for ethanol production using SSF. Using C. shehatae FPL-702, steamed or microwaved raffinate produced 19–19.5 g/l ethanol with an yield of 0.475–0.488 g ethanol/g sugar and autoclaved raffinate produced a maximum of 18.6 g/l ethanol with an yield of 0.465 g ethanol/g sugar (Fig. 5). The oven drying was least effective. The residue of raffinate after SSF was ~40–43%. Once again this fermentation demonstrated higher yields of ethanol from alfalfa cellulose resulting from LHW pretreatment.

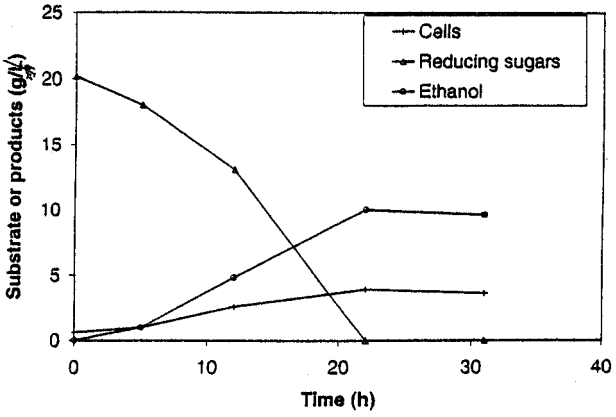

Fig. 4. SHF ethanol production by *C. shehatae* FPL-702 on enzymatic hydrolysate from raffinate after LHW pretreatment.

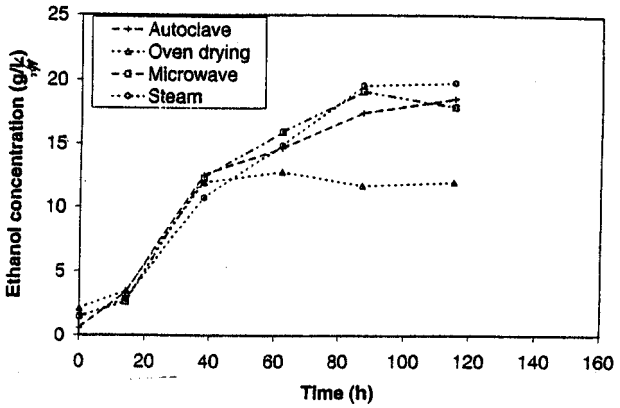

Fig. 5. Influence of various cooking methods on SSF ethanol production by *C. shehatae* FPL-702.

3.4. Ethanol production from mild acid treated LHW fractions of alfalfa fiber

The addition of 0.07% H_2SO_4 during LHW pretreatment lowered the release of sugars by 60% during subsequent enzymatic saccharification of alfalfa fiber [10]. This negative effect was probably due to the seventy of the dilute acid at 220°C in LHW pretreatment. Acid impregnation of lignocellulosic biomass before steam treatment was reported to produce a negative effect on hydrolysis yield [6,26]. Other causes may also underlie the inhibitory effect of mild acid pretreatment on enzyme penetration. Ethanol production rates were significantly lower in raffinate obtained by the mild acid LHW pretreatment (Table 2, Fig. 6). Similarly, hemicellulose fractions failed to show improved fermentation.

4. Conclusions

Ethanol production was not increased from unpretreated alfalfa fiber, and, the hemicellulosic and pectin

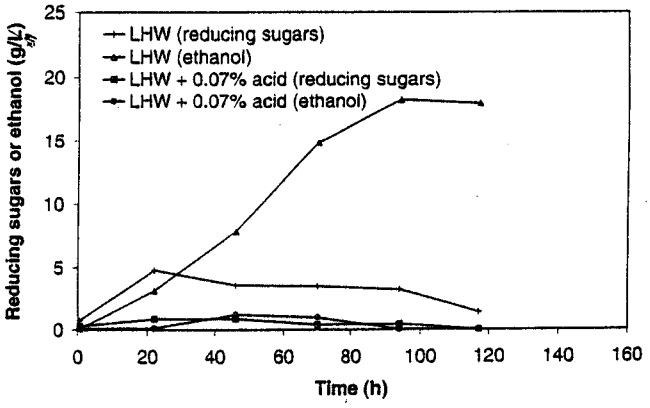

Fig. 6. Effect of mild acid during LHW pretreatment of alfalfa fiber on SSF ethanol production by *C. shehatae* FPL-702.

fractions in the extract. Sugars remaining at the end of these fermentations suggest inhibition of the organism used. However, LHW pretreatment can improve yields of ethanol from the cellulose in alfalfa fiber.

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