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Biosaccharification of cellulosic biomass in immiscible solvent–water mixtures

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Abstract

The enzymatic hydrolysis of wheat straw was carried out in bi-phasic media prepared with acetate esters and Na-acetate buffer. The volume percentage of the organic chemicals was 75%. The biomass was pretreated in a steam explosion plant at 217°C and for 3 min. A cellulase complex from commercial source was utilised and the experiments were run at 45°C and at constant enzyme to biomass weight ratio (0.06). Biomass loadings ranged from 6.25 to 100 g per litre of reactor. The amount of glucose formed per litre of reactor and hour and the glucose yield (grams of product per gram of biomass) were close to the values attained in pure buffer. The glucose concentration in the aqueous phase was in bi-phasic media much higher than in pure buffer and reached the value of 146 g $l^{-1}_{H_2O}$ during 72 h of saccharification. The results were poorly dependent on the physical–chemical properties of the solvents. Nevertheless, butyl acetate could be slightly preferred to propyl and i-amyl acetate. The use of bi-phasic media did not require stirring rate higher than in pure buffer. The presence of acetate ester traces did not alter markedly the production of ethanol in the fermentation stage, but determined the extension of the lag phase.

Keywords: Lignocellulosic biomass; Enzymatic hydrolysis; Bi-phasic media; Acetate esters; Fermentation

1. Introduction

Lignocellulosic biomasses are widely considered as an important source for the production of sugar streams that can be fermented to ethanol and other organic chemicals. The pretreatment of the biomass for the fractionation of the three main components (cellulose, hemicellulose and lignin), the hydrolysis of the cellulose and the hemicellulose, and the fermentation of pentoses and glucose to ethanol has been under study for many years. The economic analysis of the process has been made and, even though differences exist between the situation in Europe and in USA–Canada, the prospect for an industrial development in the future is good [1–4].

A present limitation is the low concentration of sugar in the hydrolysis reactor adopting the separate saccharification and fermentation (SHF) process. This is partially determined by glucose inhibition on enzyme activity that lowers the productivity. Brilliant results have been attained with the simultaneous

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saccharification and fermentation (SSF) process converting the formed glucose to ethanol [5–7]. However, the high water content of the wet biomass and the water required for assuring good rheological properties of the reaction medium do not allow to overcome the problem of a small final product concentration.

The aim of this study was to investigate the possibility of replacing an aliquot of the water with an immiscible organic solvent, at least that necessary to assure stirring of the system. The use of non-purely aqueous systems for cellulose hydrolysis was reported in the literature. The reaction yield was improved, but the sugar concentration in the system minimally increased or did not at all [8–10]. By contrast, saccharification in the presence of organic solvents could reach this goal because of the favoured partition of glucose in the water phase.

Results of previous investigations in our laboratory proved that acetate esters were suitable chemicals for use in this bioconversion as far as the stability and the activity of the cellulase components are concerned [11–13]. During this study, the presence of acetate esters in the hydrolysis medium and in the fermentation broth was studied separately in order to achieve clear information on each stage of the bioprocess. Wheat straw was used as model because of its abundance as agricultural waste all around the world. The biomass was pretreated in a steam-explosion plant being this pretreatment one of the less expensive and most effective for performing the enzymatic hydrolysis [14].

2. Materials and methods

2.1. Enzymes and hydrolysis conditions

The cellulase (SAFIZYM C80L) was from SAFISIS (France). Total protein concentration, 271 g l^{-1}, was determined with the Lowry method, using crystalline bovine serum albumin as standard [15]. The enzymatic units per milligram of protein, at 45°C in 50 mM Na-acetate buffer pH 4.8, were: 0.42 for β-glucosidase tested with 10 mM cellobiose; 6.02 for endo-glucanase with 0.25% w/v CMC; 0.29 for exo-glucanase with 0.25% w/v Avicel; 0.53 for xylanase with 0.25% w/v xylan; 0.31 for FP-ase with Whatman 1 (1×6 cm) in 1.5 ml. One cellulase unit was defined as the amount of enzyme that causes the formation of 1 μM of reducing sugar per minute at the above conditions.

The hydrolysis of wheat straw was carried out in stirred batch reactor (350 ml) and thermostated at 45°C. The product formation was monitored during 72 h of biosaccharification either in pure buffer, 50 mM Na-acetate pH 4.8, or in bi-phasic media, 25% v/v buffer and 75% v/v acetate esters. The enzyme to biomass ratio was always 0.06 g g_b^{-1}.

2.2. Fermentation conditions

Fermentation was carried out at 37°C in 300-ml flasks on a rotary shaker (220 rpm), incubator G25-KC from New Brunswick Scientific (USA). Broth volume (100 ml) was prepared with 2.5 g l^{-1} yeast extract from Oxoid (England), 0.25 g l^{-1} $(NH_4)_2$-HPO_4 and 0.025 g l^{-1} $MgSO_4 \cdot 7H_20$ from Aldrich Chemie (Germany). The pH was controlled at 4.8 with 50 mM Na-acetate buffer. Sterile commercial glucose (60 g l^{-1}) was added to the broth and the fermentation was started with the addition of 3 g l^{-1} *Saccharomyces cerevisiae* (Baker yeast, Type I) from Sigma (USA).

2.3. Biomass pretreatment

Wheat straw, 0.5 kg, was exploded with steam in the batch plant operated by ENEA at the Research Centre of Trisaia (Italy). The temperature and the time of treatment were 217°C and 3 min, respectively. This operational condition determines a severity factor, log R_0, equal to 3.94, according to the relationship, $R_0 = t \cdot \exp[(T - 100)/14.75]$ reported in Ref. [16], where t is the time (min) and T is the temperature (°C). The weight percentage of the lignocellulosic components in the pretreated biomass was: cellulose 65%, hemicellulose 11.5%, lignin 18.2%.

2.4. Solvents

n-Butyl acetate and i-amyl acetate were from Baker Chemicals (The Netherlands), *n*-propyl acetate was from Alfa Products (USA). All the chemi-

cals were analytical grade and used without further purification. The physical–chemical properties of the acetate esters are quoted in Table 1, where P is the solvent polarity defined as the ratio of solvent solubility in water and octanol, and T_b is the boiling temperature.

2.5. Product determination

Aliquots were withdrawn from the hydrolysis reactor and boiling for 20 min stopped the reaction. The samples were then centrifuged at 3000 rpm and at room temperature for 5 min to remove biomass. In the two-phase experiments, the water and the solvent were easily separated because of the difference in density. The water phase was recovered from the organic phase supernatant and the total concentration of reducing sugars was measured with the Nelson method [17]. The glucose formed during the enzymatic hydrolysis was quantified using the GOD-Perid kit from Boehringer (Germany), modified with the addition of 10 mM δ-gluconolactone, a specific inhibitor of the β-glucosidase that contaminates the glucose oxidase of the assay kit. Calibration curves were produced in both determinations using glucose as standard. The absorbance was measured spectrophotometrically (Perkin Elmer, USA, Spectrophotometer model Lambda 2) at 520 nm for reducing sugar and at 436 nm for glucose.

By contrast, the ethanol formed during the fermentation and the residual glucose were determined at the HPLC (Perkin Elmer, USA, liquid chromatograph Series 2) equipped with the refractive index detector (Perkin Elmer mod. LC25) and the Supercolgel Ca column (300×7.8 mm) from Supelco (USA) thermostated at 70°C in the column heater (Biorad, USA). The flow rate of the mobile phase (distilled and degassed water) was 0.4 ml min^{-1}. The sample (1 ml) was withdrawn from the fermentation broth and centrifuged at 3000 rpm. Aliquot of the supernatant (100 μl) was deproteinised with addition of 1 ml of $HClO_4$ solution (3.1 ml of $HClO_4$ at 65%, diluted with 100 ml of bidistilled water). A second centrifugation step at $11{,}000 \times g$ for 10 min at 15°C was adopted for the preparation of the sample to be injected in the column. All the determinations were always made at least in duplicate to ensure reliability.

Table 1
Physical properties of the acetate esters under study

	Solubility in H_2O (wt.%)	Log P	T_b (°C)	Density (g ml^{-1})
Propyl acetate	1.6	1.2	102	0.886
Butyl acetate	0.7	1.7	125	0.882
i-Amyl acetate	0.3	2.2	142	0.876

3. Results and discussion

3.1. Time course of biomass hydrolysis

Media containing pure buffer, 50 mM Na-acetate buffer pH 4.8, or acetate ester and buffer were investigated for the enzymatic hydrolysis of the steam-exploded wheat straw. The standard conditions for the comparison of the glucose yield were selected on the basis of previous studies. The results, reported in [12,18], showed that biosaccharification of the wheat straw, chemically pretreated in a two-step process with diluted H_2SO_4 and NaOH, can attain in these bi-phasic systems yields that are even higher than in pure buffer. Both glucose production and selectivity depended on the volumetric ratio of the liquid phases and the optimum solvent percentage was 75% v/v.

Furthermore, another part of the present investigation was carried out with steam-exploded wheat straw and the importance of the rheology of the system was confirmed [19]. High biomass loading in the reactor tended to depress the glucose production per gram of biomass while the space–time yield (gram of glucose formed per litre of reactor volume and hour) was improved. The enzyme to biomass ratio also significantly affected the process yield. The value of 0.06 g g_b^{-1} was a good compromise between the desired reduction of enzyme consumption and the need of performing the hydrolysis at a sufficiently fast rate.

At first, the time course of wheat straw saccharification was investigated at 45°C with a constant amount of biomass in the aqueous phase, 100 g_b $l_{H_2O}^{-1}$. This value corresponds to 100 g per litre of reactor (l_R) in the one-phase system and to 25 g_b l_R^{-1} in the two-phase system (75% v/v of acetate ester).

The reactor was stirred with a magnetic bar rotating at 250 rpm. The solubility of glucose and water being very negligible in the organic phase, the volumes of both phases can be assumed constant during the bioprocess. The glucose concentration in the water phase only was used in the material balance of product formation. The glucose formed per unit weight of biomass (g g_b^{-1}) in the different media was plotted in Fig. 1 vs. hydrolysis time. The volumetric percentage of the organic phase was always 75% v/v. The cellulose in the biomass being the 65% w/w on the average, the glucose yield was between 86% and 92% of the highest possible value. An almost complete biosaccharification was attained after 72 h of incubation. The curves were quite superimposable at high hydrolysis time, but the differences in glucose yield were in the range of 28% at short hydrolysis times (up to 24 h). Butyl acetate was only just the most effective between the acetate esters and was used as immiscible solvent for the subsequent investigation in bi-phasic media.

3.2. Space–time yield and glucose concentration

The optimisation of biomass hydrolysis would require an high space–time yield, as in all the processes, together with a high glucose concentration ($\approx$ 150 g l^{-1}) as needed for the effective fermentation to ethanol. However, operations at biomass loading higher than 100 g l_R^{-1} are not feasible because of severe fluidodynamic limitations and this would determine levels of glucose concentration at most of 50 and 60 g l_R^{-1} in the hydrolysis reactor. Therefore, a large percentage (75%) of the aqueous phase was replaced with the solvent selected in order to assure insolubility of glucose and sufficient liquid for mixing. Four experiments were run in duplicate loading the bioreactor with 25 g l_R^{-1} or with 100 g l_R^{-1}. In the bi-phasic medium, these biomass-loading values determined conditions of 100 and 400 g of biomass per litre of aqueous phase, respectively. The lowest level (25 g l_R^{-1}) was selected in order to work in the bi-phasic system at the highest biomass to buffer ratio (100 g $l_{H_2O}^{-1}$) investigated in the single-phase system while the highest level (100 g l_R^{-1}) was limited by the system rheology.

The space–time yield (g h^{-1} l_R^{-1}) and the glucose concentration in the aqueous phase (g $l_{H_2O}^{-1}$) are quoted in Table 2. In reactors operating at the same biomass loading (g l_R^{-1}), the space–time yield in bi-phasic media was always lower than in pure buffer but the glucose concentration in the aqueous phase significantly increased. This latter attained, after 72 h

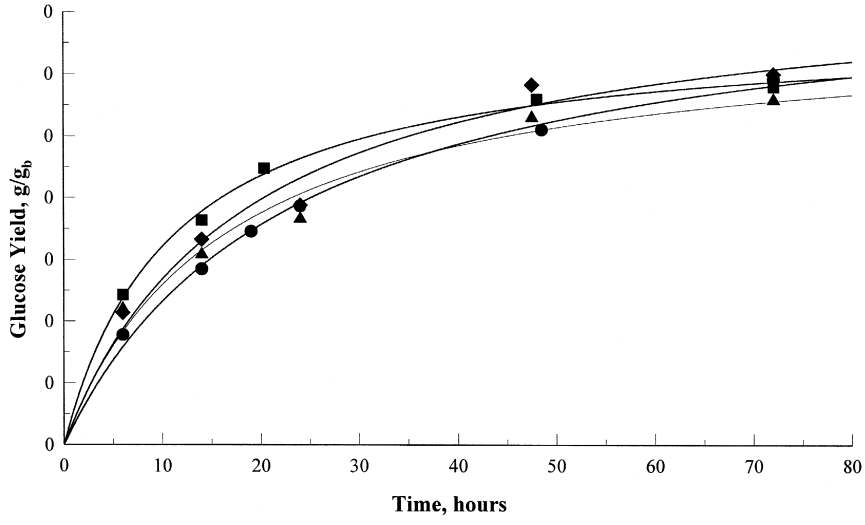

Fig. 1. Time course hydrolysis of wheat straw (100 g_b $l_{H_2O}^{-1}$) in different media. Temperature = 45°C, stirring = 250 rpm, enzyme to biomass ratio = 0.06 g g_b^{-1}. Mono-phasic medium: (●) 50 mM Na-acetate buffer, pH 4.8. Bi-phasic media contain buffer and 75% v/v of organic phase: (♦) propyl acetate; (■) butyl acetate; (▲) i-amyl acetate.

Table 2
Saccharification of wheat straw as function of biomass loading, medium and time

Time (h)	Medium	Biomass loading ($g_b\ l_R^{-1}$)	Space–time yield ($g\ h^{-1}\ l_R^{-1}$)	Glucose concentration ($g\ l_{H_2O}^{-1}$)
24	Buffer	25	0.49	11.86
	Two-phase	25	0.43	41.19
	Buffer	100	1.32	31.70
	Two-phase	100	0.81	77.46
48	Buffer	25	0.31	14.79
	Two-phase	25	0.29	56.52
	Buffer	100	0.97	46.52
	Two-phase	100	0.55	105.14
72	Buffer	25	0.21	15.00
	Two-phase	25	0.20	58.62
	Buffer	100	0.77	55.49
	Two-phase	100	0.51	146.17

of reaction using 100 g l_R^{-1} of wheat straw, a value (146.17 g $l_{H_2O}^{-1}$) much higher than that in pure buffer (55.49 g $l_{H_2O}^{-1}$). This implies that the largest part (75% by volume) of the water in the bioreactor can be conveniently replaced with an immiscible solvent such as butyl acetate. Furthermore, the comparison of the results achieved carrying out the biosaccharification at the same loading of wheat straw in the aqueous phase, i.e. 100 g l_R^{-1} in pure buffer and 25 g l_R^{-1} in bi-phasic medium, indicates that the glucose concentration in the aqueous phase remained enlarged. Both these findings are particularly important since the hydrolysis rate of cellulosic substrate is strongly curtailed by glucose inhibition [20,21] and, therefore, a higher glucose concentration in the aqueous phase, where the enzyme is confined, should depress the space–time yield. By contrast, the largest amount of glucose formed is considered a clear indication that the solvent does not depress the enzyme activity and a confirmation that a good rheology of the medium is also important.

3.3. Effect of biomass loading and hydrolysis time on wheat straw saccharification

The amount of glucose produced per unit weight of biomass was investigated in reactors operating up to 72 h varying the amount of wheat straw in the system. For a complete comparison of the biosaccharification effectiveness in the two-media, the glucose formation was referred to the biomass charged either per unit reactor volume (l_R) or per unit volume of the aqueous phase (l_{H_2O}). In the single-phase bioreactor, runs were performed with biomass loading from 6.25 to 100 g l_R^{-1} that are also equal to the same amounts per litre of aqueous phase. In the two-phase bioreactor, the biomass loading was varied from 25 to 100 g l_R^{-1} that gives values ranging from 6.25 to 25 g per litre of aqueous phase. The amount of enzyme was the same in all the experiments and equal to 0.06 g per gram of biomass.

The glucose produced after 24, 48 and 72 h of hydrolysis was calculated from the volume of the water phase and the sugar concentration. These values were then related to the amount of pretreated wheat straw (g_b) in the reactor and plotted in Fig. 2. In all the experiments, glucose yield ($g\ g_b^{-1}$) depended on the hydrolysis time and reached an asymptotic value after 72 h of hydrolysis. The glucose yield was almost unaffected by the biomass loading in the reactor ($g_b\ l_R^{-1}$) in all the investigated range with the only exception of the runs at 24 h. In both media, glucose yield tended to decrease moderately with the biomass loading and to increase slightly with the saccharification time from 48 to 72 h. The differences in the two media are so small to exclude the possibility of selecting a preferred system. By contrast, the dependence of the glucose yield on the biomass loading in the aqueous phase ($g_b\ l_{H_2O}^{-1}$) was more manifested (see Fig. 3). In most conditions, glucose yield in the bi-phasic system was larger than

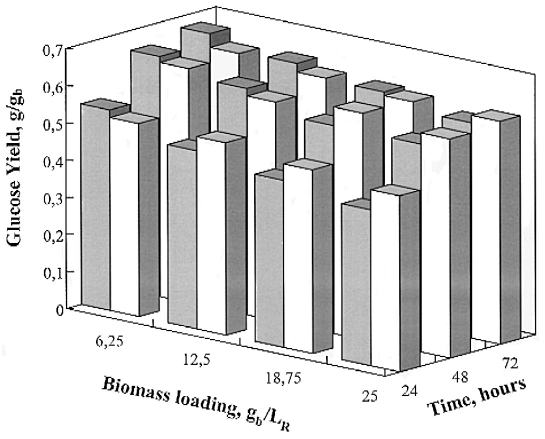

Fig. 2. Glucose yield as function of both biomass loading in the reactor and hydrolysis time. Temperature = 45°C, stirring = 250 rpm, enzyme to biomass ratio = 0.06 g g_b^{-1}. Open bar: 50 mM Na-acetate buffer, pH 4.8; shaded bar: 75% v/v butyl acetate in buffer.

in the single-phase system. The amount of glucose produced per unit weight of biomass decreased with the biomass loading and increased with hydrolysis time. The dependence on the process time was particularly evident in operations at 100 g $l_{H_2O}^{-1}$. The inspection of the bioreactor during the early hours of hydrolysis showed that agitation occurred with difficulty because most of the water in the system was necessary for soaking the wheat straw. This determined unavoidable unhomogeneity of the medium that altered the course of the enzymatic reaction.

3.4. Effect of stirring on glucose yield

Stirring is a prerequisite also for the hydrolysis in pure aqueous medium in order to assure a good contact between the enzyme and biomass. In fact, the adsorption of the biocatalyst on the biomass being one of the steps of the saccharification mechanism, biomass sedimentation in the reactor would depress the bioconversion yield. When the hydrolysis in immiscible solvent–water mixtures takes place, stirring is also necessary to create a homogeneous dispersion of the two phases in the reactor. However, it is also known that the enzymes of the cellulase complex are inactivated by both surface tension forces at the cellulose surface and shear forces in the liquid phase [22–25]. Therefore, experiments in bi-phasic medium were run at three values of agitation, 250, 500, and 750 rpm, looking for the optimum compromise between beneficial and unfavourable effects induced by the stirring on the rate of biosaccharification.

Fig. 4 clarifies the importance of stirring on glucose yield (g g_b^{-1}) at different hydrolysis times. A higher mixing was slightly positive only at short operation time (24 h). No differences in bioprocess performance were observed after 48 and 72 h of enzymatic hydrolysis. Therefore, 250 rpm was considered as the optimum bar rotation speed in order to reduce the demand of mechanical energy without depressing the amount of glucose produced since the bioprocess would be carried out at least 48 h for reaching high glucose yields.

3.5. Biomass hydrolysis in water shortage

Wheat straw needs water four times its dry weight for soaking up. Therefore, in bioreactor operating at the highest biomass loading, i.e. 100 g dry weight per litre of reactor, at least 400 ml of aqueous phase should be added to the organic solvent. The final weight percent of glucose in the aqueous phase would be at most 15–16% considering 60–65% cellulose content in the biomass.

Experiments were run in water shortage operating with 100 g l_R^{-1} of dry biomass in the bi-phasic

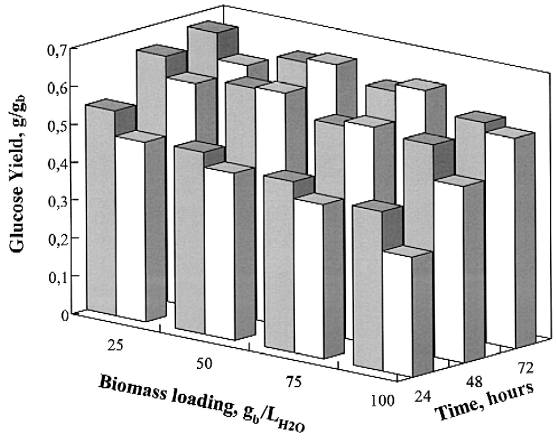

Fig. 3. Glucose yield as function of both biomass loading in the aqueous phase and hydrolysis time. Temperature = 45°C, stirring = 250 rpm, enzyme to biomass ratio = 0.06 g g_b^{-1}. Open bar: 50 mM Na-acetate buffer, pH 4.8; shaded bar: 75% v/v butyl acetate in buffer.

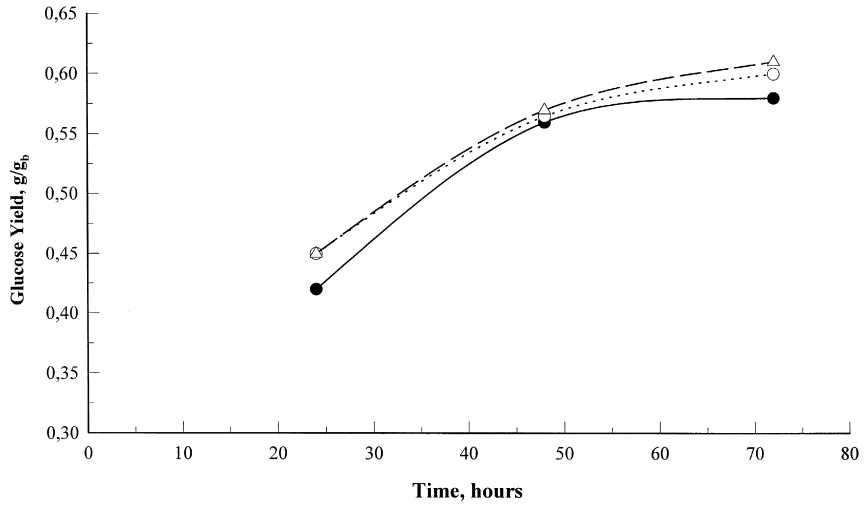

Fig. 4. Glucose yield as function of both hydrolysis time and stirring. Temperature = 45°C, biomass loading = 100 $g_b\ l^{-1}_{H_2O}$, enzyme to biomass ratio = 0.06 g g_b^{-1}, bi-phasic medium with 75% v/v butyl acetate in buffer. Stirring: (●) 250 rpm, (○) 500 rpm, (△) 750 rpm.

medium and with only 250 ml of aqueous phase (50 mM Na-acetate buffer, pH 4.8) per litre of reactor. The glucose formed per litre of buffer and per gram of biomass was determined up to 72 h of hydrolysis. The results plotted in Fig. 5 show that the biosaccharification can be carried out also under conditions of water shortage. The glucose content in the water phase (left-hand axis) reached a very important level ($\approx$ 145 g $l^{-1}_{H_20}$) even though the glucose yield (right-hand axis) was lower ($\approx$ 0.37 g g_b^{-1}) than in the presence of abundant water ($\approx$ 0.60 g g_b^{-1}).

The shape of the curves indicated that after 72 h, the hydrolysis was still in progress while it was almost completed at the conditions discussed in the

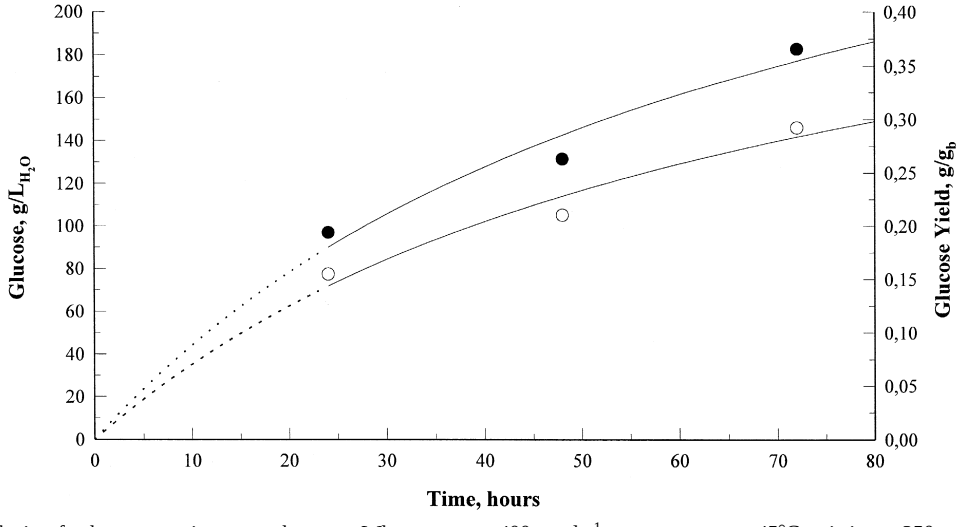

Fig. 5. Hydrolysis of wheat straw in water shortage. Wheat straw = 400 $g_b\ l^{-1}_{H_2O}$, temperature = 45°C, stirring = 250 rpm, enzyme to biomass ratio = 0.06 g g_b^{-1}. Bi-phasic medium with 75% v/v butyl acetate in buffer. (○) glucose concentration in the aqueous phase; (●) glucose yield as function of hydrolysis time.

previous sections. This was attributed to the reduction of the cellulase activity caused by glucose inhibition.

3.6. Fermentation tests

Glucose is the final product of enzymatic hydrolysis of cellulosic substrates, but it is also the intermediate product of the whole process for chemicals and fuels from lignocellulosic biomass. Fermentation of glucose is usually accomplished with yeast and the presence of solvent traces can alter the culture performances. The fermentation to ethanol was studied as model process following the procedure reported in Section 2.2, preparing the broth with the water or the buffer previously saturated with the solvent. A volume of 150 ml of aqueous phase was kept under stirring for 4 h at room temperature in contact with 10 ml of solvents. At the end of this conditioning, stirring was stopped and the aqueous phase was recovered for broth preparation. In such a way, conditions similar to those at the end of the hydrolysis in bi-phasic medium were simulated.

The formation of ethanol and the consumption of glucose were monitored up to the attainment of asymptotic values ($\cong$ 90–100 h). The values of the lag phase (h) and of the ethanol yield ($g_{ethanol}\ g^{-1}_{glucose}$) are quoted in Table 3 and compared with those in pure buffer. The presence of solvents, even in traces (between 0.3 and 1.6 wt.%, see Table 1), caused a 20% average reduction of ethanol yield and a significant prolongation of the lag phase. Only minor differences in both fermentation characteristics were observed using the various solvents. These results did not change in the case of yeast pre-culture in broth prepared with a pure aqueous phase and subsequent transfer of the cells into broths prepared with the conditioned aqueous phase.

Table 3
Fermentation of glucose in media containing traces of acetate esters

Medium	Acetate esters	Lag phase (h)	Ethanol yield (–)
Buffer	–	15	0.50
Water	propyl acetate	≈ 65	0.42
Water	butyl acetate	50–55	0.37
Water	i-amyl acetate	40–45	0.47
Buffer	propyl acetate	≈ 65	0.43
Buffer	butyl acetate	≈ 65	0.39
Buffer	i-amyl acetate	≈ 65	0.42

4. Conclusions

The substitution of a large part (75%) of the water, which is required for the enzymatic hydrolysis of lignocellulosic biomass, with an immiscible organic solvent is technically feasible. The herewith-discussed experiments indicate that acetate esters are suitable chemicals. The process can be carried out even in shortage of the aqueous phase, 250 ml of buffer per 100 g of dry biomass, a value below the requirement ($\cong$ 400 ml) for the complete biomass soaking.

The phase separation is very simple because of the almost complete immiscibility of the two liquids and of the difference in their densities. Glucose is insoluble in the organic phase and is totally retained in the aqueous phase. These physical conditions and the absence of negative effects induced by the solvent on the biosaccharification rate allows to reach glucose concentrations in the buffer close to 15% w/v. This is in the range of optimum values for the fermentation to ethanol.

Operations at the same biomass loading in the reactor give comparable yield of glucose per unit biomass weight in the two systems under study, and the saccharification is almost complete after 48 h. However, the concentration of glucose per milliliter of buffer is much higher in the bi-phasic system because of the substitution of a water aliquot with the solvent. In the case only of water shortage, the glucose yield after 72 h of hydrolysis in bi-phasic media is smaller than the average one in pure buffer because of the higher enzyme inhibition by glucose. Besides, cellulose hydrolysis in the bi-phasic medium is still in progress and higher yield could be attained at longer time.

The presence of the organic phase does not require higher agitation and, hence, the energy consumption for the stirring of the two systems is the same. The solvent can efficiently replace the water needed for a sufficient good rheology of the reaction medium.

The fermentation of glucose in the presence of solvent traces shows a small reduction of the ethanol yield and an undesired extension of the lag phase. Studies are in progress aiming to solve this problem through the use of either other immiscible solvent or of adapted yeast.

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References

[1] M. von Sivers, G. Zacchi, L. Olsson, B. Hahn-Hägerdal, Biotechnol. Prog. 10 (1994) 555.
[2] M. von Sivers, G. Zacchi, Bioresour. Technol. 51 (1995) 43.
[3] C.E. Wyman, Proc. IXth Int. Simp. Alcohol Fuels-ISAF '91, Firenze, 12–15 November 1991, p. 219.
[4] D.J. Gregg, J.N. Saddler, Proc. 2nd European Motor Biofuels Forum, Graz, 22–25 September 1996, p. 55.
[5] W.F. Gauss, S. Suzuki, M. Takagi, US Patent 3.900.944, 9 November 1976.
[6] N.D. Hinman, D.J. Schell, C.J. Riley, P.W. Bergeron, P.J. Walter, Appl. Biochem. Biotechnol. 34/35 (1992) 639.
[7] R. Eklund, G. Zacchi, Enzyme Microb. Technol. 17 (1995) 259.
[8] F. Tjerneld, I. Persson, P. Albertsson, B. Hahn-Hägerdal, Biotechnol. Bioeng. 27 (1995) 1036.
[9] F. Tjerneld, I. Persson, P. Albertsson, B. Hahn-Hägerdal, Biotechnol. Bioeng. 27 (1995) 1044.
[10] F. Tjerneld, I. Persson, J.M. Lech, Biotechnol. Bioeng. 37 (1991) 876.
[11] M. Cantarella, L. Cantarella, F. Alfani, Enzyme Microb. Technol. 13 (1991) 547.
[12] M. Cantarella, L. Cantarella, A. Gallifuoco, F. Alfani, Ann. N. Y. Acad. Sci. 672 (1992) 318.
[13] F. Alfani, M. Cantarella, D. Cifoni, A. Gallifuoco, N. Spreti, L. Cantarella, G. Savelli, Proc. Int. Symp. Environmental Biotechnology, Oostende-Belgium, 21–23 April 1997, p. 401.
[14] J. Weil, P. Westgate, K. Kollmann, M.R. Ladisch, Enzyme Microb. Technol. 16 (1994) 1002.
[15] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[16] D.J. Brash, K.W. Frek, Tappi 48 (1995) 245.
[17] N.A. Nelson, J. Biol. Chem. 153 (1944) 375.
[18] M. Cantarella, L. Bassetti, L. Cantarella, A. Gallifuoco, F. Alfani, Proc. 6th E.C. Conference, Athens, 22–26 April 1991, p. 485.
[19] A. Saporosi, Chem. Eng. Thesis, University of L'Aquila, 1999.
[20] F. Alfani, L. Cantarella, A. Gallifuoco, M. Cantarella, J. Membr. Sci. 52 (1990) 339.
[21] M. Cantarella, A. Gallifuoco, F. Alfani, L. Cantarella, Proc. IXth Int. Symp. Alcohol Fuels-ISAF '91, Firenze, 12–15 November 1991, p. 149.
[22] E.O. Jones, J.M. Lee, Biotechnol. Bioeng. 31 (1988) 35.
[23] M.H. Kim, S.B. Lee, D.D.Y. Ryu, E.T. Reese, Enzyme Microb. Technol. 4 (1982) 99.
[24] P.W. Furcht, H. Silla, Biotechnol. Bioeng. 35 (1990) 630.
[25] L. Vallander, K.E.L. Eriksonn, Biochem. Eng. Biotechnol. 42 (1992) 63.