Bioprocess Engineering 22 (2000) 175–180 © Springer-Verlag 2000

Multi-step feeding systems for lactic acid production by simultaneous saccharification and fermentation of processed wood

A. B. Moldes, J. L. Alonso, J. C. Parajó

Abstract *Eucalyptus globulus* wood samples were delignified in acetic acid media and swelled with NaOH solutions in a further stage. Solid residues from treatments were used as substrates for lactic acid production by Simultaneous Saccharification and Fermentation (SSF) in media containing *Trichoderma reesei* cellulases and *Lactobacillus delbrueckii* cells. The improvements in the overall process derived from adding fresh enzymes and/or substrate during the SSF process were assessed. In order to obtain comparative data on the efficiency of substrate utilization, enzymatic hydrolysis runs (in absence of microorganisms) were also carried out. Lactic acid concentrations in the range 48–62 g/l were obtained in SSF experiments. The solid residues after SSF (made up of microbial biomass and the non-hydrolyzed fraction of substrate) were characterized for measuring their potential as feed additives.

1 Introduction

Eucalyptus globulus wood is a renewable resource for the chemical industry with increasing importance in the North-West country of Spain. Fast growth and high cellulose content are main advantages of *Eucalyptus* wood as a raw material.

The enzymatic hydrolysis of the cellulosic fraction of *Eucalyptus* wood has been previously studied by our research group as a procedure for making fermentation media that can be used for obtaining a variety of end-products. This kind of technology is particularly interesting because of its selectivity and low environmental impact [1], but the enzymatic hydrolysis of native lignocellulosics proceeds with slow kinetics and reduced yields. In order to improve both factors, the raw materials have to be pretreated for altering their physico-chemical features, particularly the accessibility of enzymes to cellulose and the crystallinity of this polymer. Two-stage processing of wood (delignification in HCl-acetic acid-water media followed by swelling with alkaline solutions) has been successfully employed for obtaining cellulosic substrates highly susceptible to enzymatic hydrolysis [2, 3]. After supplementation with nutrients, enzymatic hydrolysates of processed wood can be used as fermentation media for producing a variety of commercial products.

Lactic acid is an interesting end-product for this type of processes owing to several reasons, including: (i) comparatively high market price, (ii) growing demand, (iii) high fermentation yield (theoretical product yield in homolactic fermentations, 1 g lactic acid/g consumed glucose) and (iv) wide range of applications. Lactic acid is forecast to become a commodity chemical [4, 5].

Most part of the lactic acid produced by biotechnological methods is used in the food and feed industries. Because of its ability for lowering pH, lactic acid is widely used for extending the life of milk, meat, eggs and seafood, whereas ammonium lactate is a better nitrogen supplement for feed than other non-proteic nitrogen sources [6, 7].

Lactic acid production from lignocellulosic materials has been successfully carried out in two sequential steps of enzymatic hydrolysis and hydrolysate fermentation, an operational method that allowed high productivities and yields [8]. As an alternative strategy, SSF technology needs only a single reaction vessel for both hydrolysis and fermentation and it is applicable for lactic acid production from processed lignocellulose since (i) little inhibition is caused by the fermentation product on cellulases, and (ii) the operational conditions (pH and temperature) for hydrolysis and fermentation are compatible [9]. On the other hand, SSF overcomes the inhibition caused by cellobiose and glucose on cellulases. The first study on the SSF production of lactic acid from cellulosic raw materials was reported by Abe and Takagi [10], and since then several articles have been reported on this subject [4, 11, 12]. If the enzymatic complex used for saccharification is deficitary in β-glucosidase, the cellobiose generated as an intermediate reaction product can be used by bacteria as an additional carbon source for lactic acid production [4, 11]. This fact avoids the β-glucosidase supplementation of cellulases, an experimental procedure that has been widely employed in the enzymatic hydrolysis of cellulose.

As the cost of enzymes is a main factor affecting the economic features of lignocellulose hydrolysis [9, 13–16], special attention must be devoted to improve the ratio between the amount of sugar generated and the enzymatic activity required for this purpose. Recovery and recycling of enzymes [14, 16] or utilization of enzymes until exhaustion of their activity [17] are strategies that have been applied in this field.

For a given media with fixed enzymatic activity, the maximization of the product concentration is an indirect

Received: 8 March 1999

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method for improving the efficiency of enzyme utilization. High concentrations of hydrolysis products have been obtained using packed-bed, column reactors [14], but additional problems occur when using stirred reactors, owing to limitations in mixing and mass transfer. Moritz and Duff [17] proposed to avoid this kind of problems by using substrate concentrations below 10% w/v. Multi-step feeding is an interesting way for overcoming the inconveniences derived from using high solid concentrations in stirred reactors. Doran and Ingram [1] applied this method to the SSF production of ethanol and improved the results obtained when the overall amount of substrate was added at the beginning of the process. The same idea can be applied to the production of lactic acid, with the advantage that the cellulase and/or microorganism inhibition by this compound is only significant in concentrations above 60–80 g/l [9, 12, 18]. This approach, in combination with Alamine extraction, has been assessed recently [4].

This work deals with the SSF production of lactic acid from processed wood using multi-step feeding. In order to obtain comparative data, enzymatic hydrolysis runs were carried out under similar operational conditions. On the basis of the time courses of enzymatic hydrolysis and SSF experiments, the influence of the operational conditions on the lactic acid concentration and enzyme utilization are discussed.

2 Materials and methods

2.1 Raw material

Eucalyptus globulus wood chips, obtained from local industries, were milled, screened to select the fraction of particles with a size between 0.2 and 0.5 mm, homogenized to ensure identical composition among the various aliquots taken from the wood lot, air dried, and stored.

2.2 Chemical processing of wood

Wood samples were sequentially delignified in acetic acid-water-HCl media and swelled in aqueous NaOH under the operational conditions listed in Table 1, which were selected on the basis of previous studies by authors [19, 20]. Solid residues from treatments were assayed for hemicelluloses, cellulose and lignin by means of a quantitative hydrolysis with 72% sulphuric acid [21]. Sugars in hydrolysates were determined by HPLC using a ION-300 column and RI detection (mobile phase, H_2SO_4 0.003 M).

2.3 Enzymatic hydrolysis

Commercial cellulase concentrates ("Celluclast" from Novo, Denmark) were used in all the experiments. The cellulase activity of concentrates was measured by the Filter Paper Activity test (FPA) according to Mandels et al. [22], and expressed as Filter Paper Units (F.P.U.)/ml. In selected experiments, the hydrolysis media were supplemented with β-glucosidase from *Aspergillus niger* ("Novozym" from Novo, Denmark). The β-glucosidase activity was measured in International Units [18]. Enzymatic solutions were diluted and buffered (pH = 4.85) to reach the desired enzyme activities. The operational conditions used for the enzymatic hydrolysis were: temperature, 45 °C; liquor/solid ratio, 30 g/g, orbital agitation (150 rpm). At given reaction times, samples were withdrawn from the reaction media, heated, centrifuged, filtered through 0.45 mμ membranes and analyzed by HPLC for glucose and cellobiose using the same method described above.

Table 1. Operational conditions used in experiments

a) Delignification stage
- Composition of medium: 95% acetic acid, 4.8% water, 0.2% HCl
- Normal boiling temperature
- Liquor/solid ratio: 10 g/g
- Duration: 1 h

b) Swelling stage
- Composition of medium: 10% NaOH
- Temperature: 130 °C
- Liquor/solid ratio: 10 g/g
- Duration: 2 h

c) Enzymatic hydrolysis
- Concentration of citrate buffer: 0.05 N
- pH of citrate buffer: 4.85
- Temperature: 45 °C
- Orbital agitation (150 rpm)
- Initial solid charge: 1 g/30 g initial liquor
- Amount of fresh solid added in subsequent feeding: 1 g/30 g initial liquor
- Duration: up to 110 h
- Initial cellulase loading: 28 FPU/g substrate
- Initial β-glucosidase loading: 13 IU/FPU (only in selected experiments)
- Amount of fresh enzymes added in subsequent feeding: 28 FPU/g initial substrate

d) Simultaneous saccharification and fermentation
- Nutrients: Yeast extract (5 g/l), peptone (10 g/l), sodium acetate (5 g/l), sodium citrate (2 g/l), K_2HPO_4 (2 g/l), Tween 80 (1 mL/l), $MgSO_4 \cdot 7H_2O$ (0.58 g/l), $MnSO_4 \cdot H_2O$ (0.12 g/l), $FeSO_4 \cdot 7H_2O$ (0.05 g/l)
- Temperature: 45 °C
- pH = 4.85
- Solid concentration and cellulase loading: as in enzymatic hydrolysis experiments
- Duration: up to 125 h
- (Inoculum volume)/(fermentation volume) ratio: 1/10

2.4 Simultaneous saccharification and fermentation

SSF media were supplemented with the nutrients listed in Table 1. After sterilization and enzyme addition (performed as in the enzymatic hydrolysis assays), SSF experiments were started by inoculating with *Lactobacillus delbrueckii* NRRL B-445 cells (obtained from the Northern Regional Research Center, USDA, Peoria, Illinois). The microorganisms used as inocula were proliferated at 41.5 °C in shaker cultures (150 rpm) during 15 hours. SSF experiments were performed at 45 °C in a Biostat B batch fermentor in which the pH was controlled to 4.85 by addition of 4 M NaOH. At given fermentation times, samples were withdrawn from the media and centrifuged. Supernatants were assayed for lactic acid, glucose and cellobiose

by HPLC as above, whereas pellets were washed and used for determination of polysaccharide and lignin (by quantitative saccharification) and total nitrogen (by the Kjeldahl method). Total nitrogen was converted into microbial biomass by comparison with the total nitrogen content of *Lactobacillus* cells grown on glucose-containing media.

3
Results and discussion

3.1
Chemical processing of wood

Wood processing in acetic acid-HCl-water media causes both delignification and hemicellulose hydrolysis. This kind of technology is interesting because (i) the cellulose percent of the solid residue is markedly increased in relation to that of the raw material, and (ii) valuable by-products (such as sulphur-free lignin, hemicellulosic sugars and furfural) are produced. However, delignified wood samples behave as poor hydrolysis substrates owing to their unfavourable physicochemical properties. A subsequent swelling stage in media containing NaOH provided solid residues containing 91% cellulose, 3.9% Hemicellulose and 3.0% lignin with high susceptibility towards enzymatic saccharification, which were used as hydrolysis substrates for further experimentation.

3.2
Enzymatic hydrolysis in media containing cellulases and β-glucosidase

In order to establish a basis of comparison for evaluating the efficiency of enzymatic hydrolysis and SSF in terms of substrate consumption and enzyme utilization, experiments combining addition of fresh substrate (in the same amount used at the beginning of the reaction) and/or fresh cellulase addition (in the same concentration used at the beginning of the reaction) were performed. Figure 1 shows the experimental data as well as the details on the substrate and enzymes added during reaction. Since the media were supplemented with β-glucosidase (see Table 1), glucose was the only reaction product obtained in significant amounts.

The data corresponding to Experiment 1 (Fig. 1a) show that 24 g glucose/l (corresponding to 74% of substrate conversion) were reached after 10 h, this reaction time being selected for adding fresh substrate. The mean volumetric rate of glucose generation during this period (2.4 g/l · h) proved the suitability of processed wood samples as hydrolysis substrates. For reaction times longer than 10 h, the reaction rate was enhanced immediately after the addition of fresh substrate, but the mean volumetric glucose productivities decreased with time, as it was expected on the basis of information from literature [17, 23]. The enhanced kinetics was in relation with the higher substrate concentration (and, particularly, with the presence of amorphous, easily hydrolysable cellulose in the fresh substrate) and also with the presence of free enzyme. In this way, there is evidence that enzymes were released from the hydrolysis substrate and remained in solution after reaching a given conversion [24, 25]. The reaction time necessary for increasing the glucose concentration from 24 to 48 g/l was 30 hours, i.e., 3 times more than the one required for reaching 24 g glucose/l in the reaction medium. This kinetic behaviour can be explained on the basis of losses of enzymatic activity (for example, thermal or shear-related inactivation and/or irreversible adsorption) or glucose inhibition. After 100 hours, the glucose concentration (57 g/l) corresponded to a remarkable substrate conversion (about 90%).

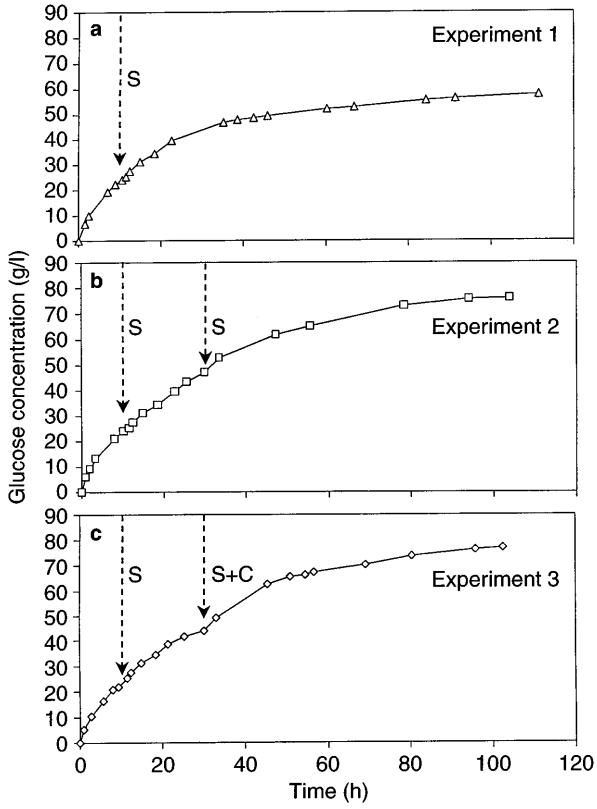

Fig. 1. Time course of enzymatic hydrolysis experiments in media containing cellulases and β-glucosidase with addition of fresh substrate and/or cellulases. Nomenclature: S = substrate addition; S + C = addition of substrate and cellulases

The effects derived from performing two additions of fresh substrate (after 10 and 30 hours) were explored in the Experiment 2 (Fig. 1b). Substrate addition resulted in enhanced reaction rates, but the glucose concentration after 100 hours (75.5 g/l) showed that the substrate conversion dropped to 84%. It can be noted that the ratio (glucose generated)/(enzyme utilized) increased in this case by 33% respect to Experiment 1, with increased both efficiency in enzyme utilization and volumetric productivity.

Finally, a new assay (Experiment 3) was performed to assess the possibility of improving the results of Experiment 2 by adding fresh enzymes after 30 hours. The experimental results shown in Fig. 1c proved that this strategy did not increase the glucose concentration, confirming the occurrence of enzyme saturation.

3.3
Enzymatic hydrolysis in media containing cellulases

Experiments 4, 5 and 6 of Fig. 2 show the time courses of glucose and cellobiose concentrations corresponding to

media containing cellulases as sole enzymatic complex. A comparison of these data with those of Fig. 1 provides a direct evaluation of the negative effect of cellobiose (a disacharide with strong inhibitory effect) on the overall saccharification process. On the other hand, Figs. 1 and 2 show extreme situations with respect to substrate utilization and cellobiose concentration, allowing the establishment of variation ranges for these variables that are expected to include the corresponding values of SSF experiments (see below).

Owing to the comparative deficit of the *Trichoderma reesei* enzymatic complex in β-glucosidase activity, the lack of supplementation of the media with *Aspergillus niger* β-glucosidase resulted in both decreased substrate conversion and reduced overall productivity in comparison with the experiments of Fig. 1. Under the experimental conditions of the experiments of Fig. 2, the cellobiose concentration (up to 14 g/l at prolonged reaction times) exerted a significant inhibition on cellulases and it seems to be a limiting factor. Because of this, the percents of substrate conversion achieved after 100 hours (45.1% in Experiment 5, 62.7% in Experiment 4 and 58% in Experiment 6) were lower than the correspondent experiments of Fig. 1.

Similarly to the previous cases, substrate addition resulted in enhanced reaction rates. Experiments 4 and 5 (Figs. 2a and 2b) followed a similar behaviour, leading to the same glucose concentration after 100 hours of reaction independently from the overall amount of substrate added. The most interesting feature of Experiment 6 (Fig. 2c) was the significant increase in the reaction rate observed after the addition of fresh cellulase, which can be explained by the presence of additional β-glucosidase activity on the fresh enzyme.

3.4 Simultaneous saccharification and fermentation with multi-step feeding

Based on the previous experience of our research group on the SSF production of lactic acid from lignocellulosic materials with a single substrate charge at the beginning of the process [11], three SSF assays (Experiments 7 to 9 of Fig. 3) were performed with fresh substrate and/or enzyme additions. It can be noted that the operational procedure followed in Experiments 7 to 9 was the same than the one used in the sets of experiments of Figs. 1 and 2.

Figures 3a, 3b and 3c show the accumulation of sugars at the beginning of the reaction as a general feature. A related behaviour has been observed in other works dealing with the SSF production of lactic acid [4, 11].

In Experiment 7 (Fig. 3a), addition of fresh substrate after 10 hours of reaction led to a lactic acid concentration of 47.6 g/l, with substrate conversion higher than 75%. The

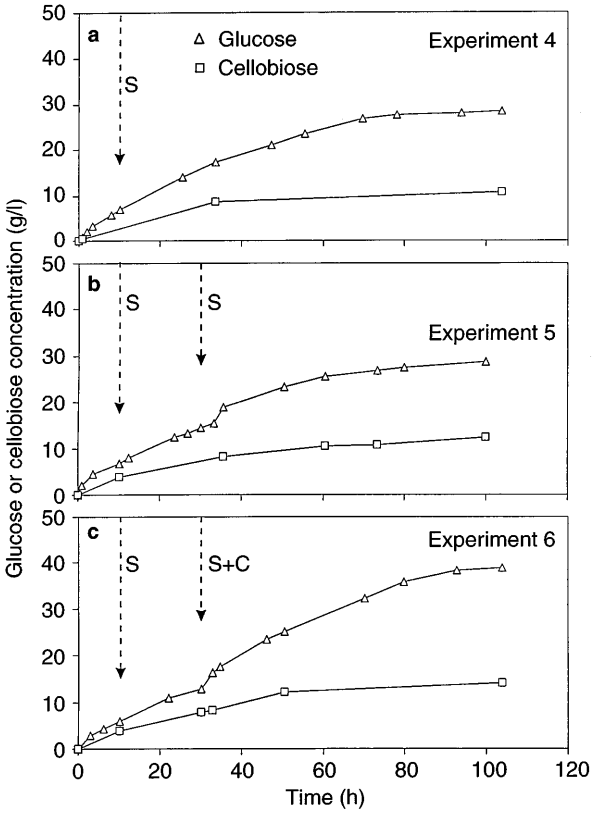

Fig. 2. Time course of enzymatic hydrolysis experiments in media containing cellulases as sole enzymatic complex with addition of fresh substrate and/or cellulases. Nomenclature: S = substrate addition; S + C = addition of substrate and cellulases

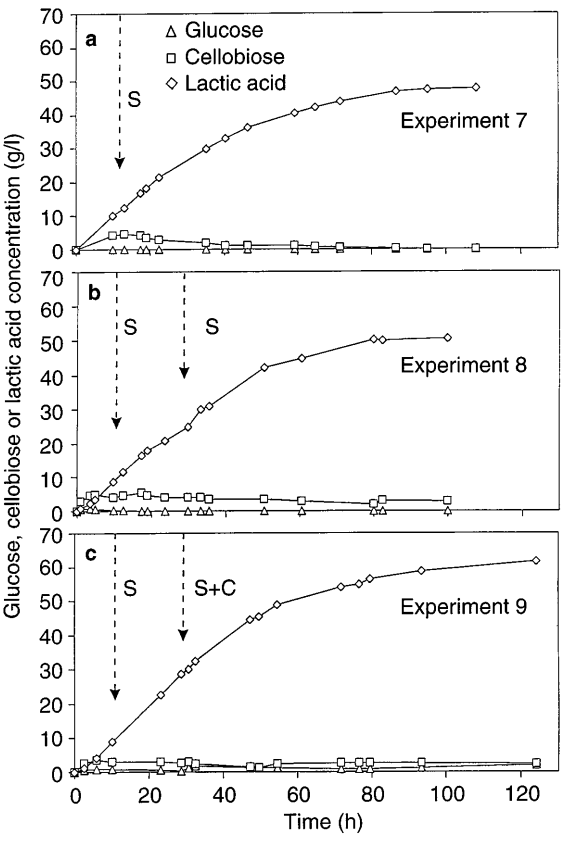

Fig. 3. Time course of SSF experiments (media containing cellulases and microorganisms) with addition of fresh substrate and/or cellulases. Nomenclature: S = substrate addition; S + C = addition of substrate and cellulases

maximum cellobiose concentration (4.5 g/l) was reached near the reaction time at which the substrate addition was performed. The most important finding was that almost no glucose was found in the medium, whereas cellobiose was also depleted at the end of the run. This means that hydrolysis was the limiting step in the overall hydrolysis-fermentation process, an interesting fact because the concentration of the undissociated form of the lactic acid at the end of experiment (calculated taking into account the pKa and the pH of the medium) was 4.9 g/l, a concentration level at which significant inhibition of microorganisms can be expected [26]. The mean volumetric productivity of lactic acid in this assay (0.47 g/l · h) was in the range of the results reported by Schmidt and Padukone [12], who reached 0.54 g/l · h in the bioconversion of waste paper into lactic acid.

The most remarkable finding of Experiment 8 was the increase in the volumetric rate of lactic acid generation after the last addition of substrate (see Fig. 3b). However, the final concentration of lactic acid was 51 g/l, a little improvement in relation to the previous case that resulted in decreased substrate consumption. Glucose was completely depleted after 12 hours, but the presence of cellobiose in the fermentation media suggested that further improvements could be reached in a medium enriched with β-glucosidase.

Using a combination of fresh substrate and enzyme addition (see Experiment 9 of Fig. 3c), the final concentration of lactic acid increased up to 62 g/l after 124 hours. The most remarkable aspect in this case was the presence of little glucose concentrations in the medium, suggesting that the inhibition of microorganisms by lactic acid began to play a measurable role in the overall process.

The solid residue produced in Experiments 7 to 9 is made up of unconverted substrate and microbial biomass, showing potential as a feed additive. The selective hydrolysis of the cellulosic fraction during SSF led to increased lignin contents of the solid residues (in the range 9.4–18.3%, in comparison with the 3.0% lignin determined for the raw material). Increased lignin content, presence of crystalline cellulose, enzyme inactivation and/or rheological properties of the media are possible factors explaining the incomplete conversion of substrates. On the other hand, the concentrations of microbial biomass in the SSF media varied in the range 9.6–11.6 g/l, showing a fairly constant interrelationship with the concentration of lactic acid (5–5.5 g lactic acid/g biomass)

In conclusion, the multi-step feeding SSF production of lactic acid presents a intermediate situation (in terms of substrate consumption and final product concentration) between extreme situations corresponding to enzymatic hydrolysis media containing cellulases with or without supplementation with additional β-glucosidase. For the enzyme loading used in this work, multi-step substrate addition allowed improvements in the final concentration of lactic acid, but simultaneous reductions in the substrate conversion were also observed. The experimental data provide information useful for assessing the best choice on an economic basis. The solid residues from SSF contained substantial amounts of microbial biomass and unconverted cellulose, their lignin contents confirming that they could be used as a nitrogen- and fiber-containing feed supplement.

References

1. **Doran, J.B.; Ingram, L.O.:** Fermentation of crystalline cellulose to ethanol by *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes. Biotechnol. Progress 9 (1993) 533–538
2. **Vázquez, D.; Lage, M.A.; Parajó, J.C; Alonso, J.L.:** Empirical assessment on the cellulase digestibility of processed *Eucalyptus* wood. Appl. Biochem. Biotechnol. 37 (1992) 123–139
3. **Parajó, J.C.; Alonso, J.L.; Santos, V.:** Delignification and swelling of *Eucalyptus* wood ahead of the enzymatic hydrolysis of the cellulosic fraction. Process Biochem. 30 (1995) 537–545
4. **Chen, R.; Lee, Y.Y.:** Membrane-mediated extractive fermentation for lactic acid production from cellulosic biomass. Appl. Biochem. Biotechnol. 63–65 (1997) 435–448
5. **Lipinsky E.S.; Sinclair R.G.:** Is lactic acid a commodity chemical? Chem. Eng. Progress 82 (1986) 26–32
6. **Keller, A.K.; Gerhardt, P.:** Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. 17 (1975) 997–1018
7. **Norton, S.; Lacroix, C.; Vuillemard, J.C.:** Kinetic study of continuous whey permeate fermentation by immobilized *Lactobacillus helveticus* for lactic acid production. Enzym. Microb. Technol. 16 (1994) 457–466
8. **Parajó, J.C.; Alonso, J.L.; Santos, V.:** Lactic acid from wood. Process Biochem. 31 (1996) 271–280
9. **Takagi, M.:** Inhibition of cellulase by fermentation products. Biotechnol. Bioeng. 26 (1984) 1506–1507
10. **Abe, S.; Takagi, M.:** Simultaneous saccharification and fermentation of cellulose to lactic acid. Biotechnol. Bioeng. 37 (1991) 93–96
11. **Parajó, J.C.; Alonso, J.L.; Moldes, A.B.:** Production of lactic acid from lignocellulose in a single stage of hydrolysis and fermentation. Food Biotechnol. 11 (1997) 45–58
12. **Schmidt, S.; Padukone, N.:** Production of lactic acid from wastepaper as a cellulosic feedstock. J. Ind. Microbiol. Biotechnol. 18 (1997) 10–14
13. **Vallander, L.; Erikson, K.E.:** Enzymic saccharification of pretreated wheat straw. Biotechnol. Bioeng. 27 (1985) 650–659
14. **González, G.; Caminal, G.; Mas. C.; López-Santín, J.:** Enzymatic hydrolysis of wheat straw: studies on a column reactor with enzyme recycling. Process Biochem. 4 (1989) 62–67
15. **Eklund, R.; Galbe, M.; Zacchi G.:** Recovery of cellulases after hydrolysis by adsorption on steam-pretreated willow. Appl. Biochem. Biotechnol. 34–35 (1992) 105–113
16. **Ramos, L.P.; Saddler, J.N.:** Enzyme recycling during fed-batch hydrolysis of cellulose derived from steam-exploded *Eucalyptus viminalis*. Appl. Biochem. Biotechnol. 45–46 (1994) 193–207
17. **Moritz, J.W.; Duff, S.J.B.:** Simultaneous saccharification and extractive fermentation of cellulosic substrates. Biotechnol. Bioeng. 49 (1996) 504–511
18. **Paquot, M.; Thonart, Ph.:** Hydrolyse enzymatique de la cellulose regenerée. Holzforschung 36 (1982) 177–181
19. **Parajó, J.C.; Alonso, J.L.; Lage, M.A.; Vázquez, D.:** Empirical modeling of *Eucalyptus* wood processing. Bioproc. Eng. 8 (1992) 129–136
20. **Parajó, J.C.; Alonso, J.L.; Vázquez, D.:** On the behaviour of lignin and hemicelluloses during the Acetosolv processing of wood. Biores. Technol. 46 (1993) 233–240
21. **Browning, B.L.:** Methods of Wood Chemistry. In: John Wiley & Sons. New York 1967
22. **Mandels, M.; Andreotti, R.; Roche, C.:** Measurement of saccharifying cellulose. Biotechnol. Bioeng. Symp. 6 (1976) 21–33

23. Herr, D.: Conversion of cellulose to glucose with cellulase of *Trichoderma viride* ITCC-1433. Biotechnol. Bioeng. 22 (1980) 1601–1611

24. Castanon, M.; Wilke, C.R.: Adsorption and recovery of cellulase during hydrolysis of newspaper. Biotechnol. Bioeng. 22 (1980) 1037–1053

25. Lee, S.B.; Kim, I.H.; Ryu, D.D.Y.; Taguchi, H.: Structural properties of cellulose and cellulase reaction mechanism. Biotechnol. Bioeng. 25 (1983) 33–51

26. Yeh, P.L.H.; Bajpai, R.K.; Iannotti, E.L.: An improved kinetic model for lactic acid fermentation. J. Ferment. Bioeng. 71 (1991) 75–77