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Production of D(−)-lactic acid from cellulose by simultaneous saccharification and fermentation using *Lactobacillus coryniformis* subsp. *torquens*

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Abstract

D(−)-Lactic acid was produced from cellulose by simultaneous saccharification and fermentation (SSF) in media containing cellulolytic enzymes and *Lactobacillus coryniformis* subsp. *torquens* ATCC 25600 at 39 °C and pH 5.4, yielding 0.89 g D(−)-lactic acid g^{-1} cellulose at a mean volumetric productivity of 0.5 g l^{-1} h^{-1}. No L(+)-lactic acid was found in the medium.

Introduction

Besides its traditional applications in the food and pharmaceutical industries, lactic acid is used in the manufacture of polymers such as polylactic acid (PLA). The manufacture of PLA can be carried out from D(−)- or L(+)-lactic acid or from a racemic mixture. When PLA is produced from a pure isomer, the product is crystalline and a better stability than the amorphous polymers obtained from a racemic mixture (Hofvendahl & Hahn-Hägerdal 1997). Opposed to chemical synthesis of lactic acid (which leads to a racemic mixture), the fermentative production of a given isomer can be achieved. Lactic acid can be produced from waste cellulosic substrates such as newspaper or cardboard (Schmidt & Padukone 1997). In enzymatic hydrolysis-fermentation processes, both cellulose saccharification by cellulolytic enzymes and fermentation of released sugars by lactic acid bacteria can be carried out in a single step (according to the SSF technology).

Lactobacillus coryniformis subsp. *torquens* is a homofermentative, D(−)-lactic acid producing microorganism. To our knowledge, no studies exist on the selective production of D(−)-lactic acid by SSF of cellulosic substrates.

This work deals with the SSF production of D(−)-lactic acid from a model cellulosic substrate. In a preliminary set of experiments, operational conditions suitable for both enzymatic hydrolysis of cellulose and lactic acid fermentation were identified. In further experiments, the SSF production of D(−)-lactic acid was assessed.

Materials and methods

Raw material and enzymes

Pieces (1 × 1 cm) of filter paper (containing 69% w/w cellulose, 16.8% w/w hemicelluloses and 11% w/w Klason lignin) were used as substrate. Enzyme concentrates (‘Celluclast’ cellulases and ‘Novozym’ β-glucosidase) were used for SSF experiments.

Microorganism and culture media

Lactobacillus coryniformis subsp. *torquens* ATCC 25600 was grown in a medium containing 50 g glucose

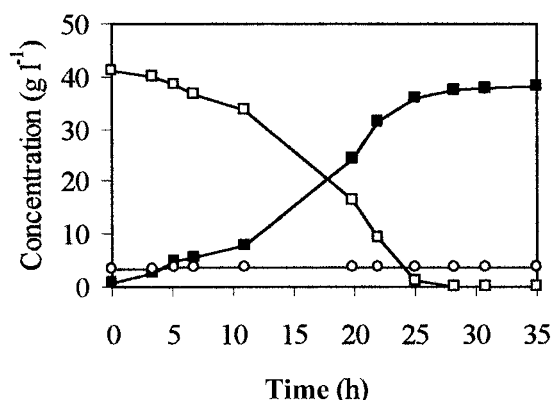
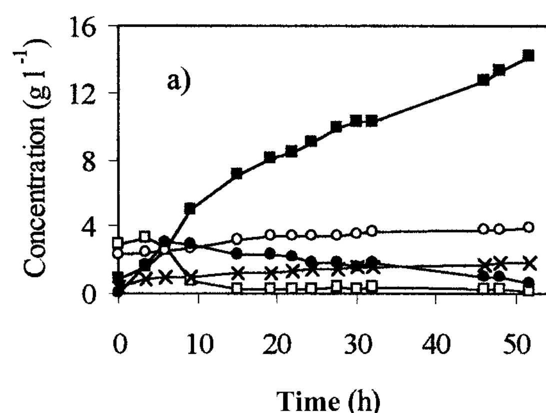


Fig. 1. Time course of lactic acid, acetic acid and glucose concentrations in the batch fermentation of glucose-containing media (39 °C, pH 5.4). (Lactic acid, ■; glucose, □; acetic acid, ○).



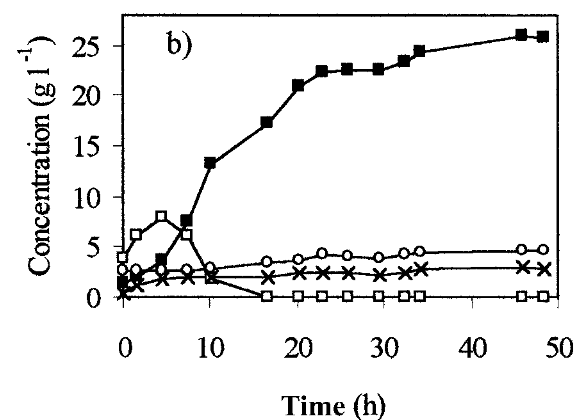


Fig. 2. Time course of glucose, cellobiose, hemicellulosic sugars, lactic acid and acetic acid concentrations in SSF experiments performed at 39 °C and pH 5.4. (a) Without external cellobiase supplementation; (b) with external cellobiase supplementation. Liquor to solid ratio = 30 g liquid g^{-1} filter paper, cellulase to solid ratio = 28 FPU g^{-1} filter paper and β-glucosidase to solid ratio = 360 IU g^{-1} filter paper (only in experiment b). (Lactic acid, ■; glucose, □; acetic acid, ○; cellobiose, ●; hemicellulosic sugars, ×.)

l^{-1}, 4 g yeast extract l^{-1}, 8 g meat extract l^{-1}, 10 g peptone proteose l^{-1}, 5 g sodium acetate l^{-1}, 2 g tri-ammonium citrate l^{-1}, 2 g K_2HPO_4 l^{-1}, 0.2 g $MgSO_4$ l^{-1}, 0.05 g $MnSO_4$ l^{-1} and 1 g polysorbate 80 l^{-1}. The microorganisms used as inocula were proliferated at 37 °C in shaker cultures (150 rpm) for 15 h.

Fermentation and SSF experiments

Fermentation and SSF runs were carried out under selected conditions in a Biostat B fermenter. In SSF experiments glucose was replaced by filter paper at a liquor to solid ratio of 30 g liquid g^{-1} solid. After addition of nutrients, the media were sterilised, enzymes (only in SSF experiments) were added, and *Lactobacillus coryniformis* subsp. *torquens* ATCC 25600 cells were inoculated. All the SSF experiments were performed for 48 h in media containing 28 filter paper units (FPU) g^{-1} filter paper and 360 IU β-glucosidase g^{-1} filter paper.

The pH of fermentation media was automatically controlled by addition of 4 M NaOH. At given fermentation times, samples were withdrawn from the media and centrifuged. The concentrations of glucose, hemicellulosic sugars, total lactic acid and acetic acid were measured by HPLC in the supernatants (see below).

Analytical methods

The composition of the raw material and the concentrations of glucose, cellobiose, hemicellulosic sugars, total lactic acid and acetic acid in fermentation media were determined as reported previously (Moldes *et al.* 2000). L(+)-Lactic acid concentrations were measured enzymatically using the Test-Combination of Boehringer (Mannheim, Germany).

Results and discussion

Determination of operational conditions suitable for SSF

In the first stage of this work, the time course of fermentations was assessed under a variety of conditions: favourable conditions for the fermentation stage were 37 °C and pH 6; for the hydrolysis stage, 45 °C

and pH 4.8; and for both hydrolysis and fermentation, 41 °C and pH 5.4.

Operating at 37 °C and pH 6 (data not shown), lactic acid was produced at high productivity (Q_{Pmax} = 3.3 g l^{-1} h^{-1} and Q_{Pmean} = 1.32 g l^{-1} h^{-1} after 25 h) at a product yield ($Y_{P/S}$) of 0.82 g lactic acid g^{-1} of consumed glucose. No L(+)-lactic acid was detected at the end of the fermentation.

No fermentative activity was detected at 45 °C and pH 4.8 (the experimental conditions used, for example, in the SSF production of lactic acid with *Lactobacillus delbrueckii*) (Parajó *et al.* 1997).

Operating at 41 °C and pH 5.4, the maximal and mean volumetric productivities decreased (Q_{Pmax} = 1.56 g l^{-1} h^{-1}, Q_{Pmean} = 0.7 g l^{-1} h^{-1}) and glucose was not completely consumed. At 39 °C and the same pH (see Figure 1), Q_{Pmax} reached 3.37 g l^{-1} h^{-1} and Q_{Pmean} decreased up to 1.3 g l^{-1} h^{-1}. A product yield of 0.89 g D(−)-lactic acid g^{-1} consumed glucose was achieved.

According to these results, compromise conditions (39 °C, pH 5.4) were considered in further SSF assays. Similar conditions (pH about 5, and from 37–39 °C) have been reported for the SSF production of ethanol (Hari Krishna & Chowdary 2000, Stenberg *et al.* 2000).

SSF experiments with and without externally added cellobiase

SSF experiments were carried out under the selected conditions (39 °C and pH 5.4) in order to check if low substrate concentrations could result in the production of metabolic byproducts (Hofvendahl & Hahn-Hägerdal 2000). Experiments were performed in media containing either cellulases as sole enzymatic complex or cellulases and externally added cellobiase to assess possible operational advantages.

Figure 2a shows the SSF concentration profiles for media without externally added cellobiase. Cellobiose accumulated in the initial stages of the process and then decreased owing to the action of the cellobiasic activity already present in the cellulolytic enzymes. Significant inhibition effects have been reported at the maximal cellobiase concentration (3.1 g l^{-1}) found in this work (Parajó *et al.* 1997, Moldes *et al.* 1999). In this experiment, Q_{pmean} reached 0.26 g l^{-1} h^{-1} at $Y_{P/S}$ = 0.46 g lactic acid g^{-1} cellulose.

To decrease the inhibition caused by cellobiose, an experiment (see Figure 2b) was carried out at the same temperature, pH and cellulase loading than in the previous case but with externally added cellobiase (to reach 13 IU FPU^{-1}). The product yield now increased up to 0.89 g lactic acid g^{-1} cellulose and Q_{Pmean} reached 0.5 g l^{-1} h^{-1} after 48 h. No L(+)-isomer was produced.

Figures 2a and 2b also show concentration profiles for additional compounds (hemicellulosic sugars, denoted as HS, and acetic acid). The lower HS concentrations achieved in SSF experiments in comparison with enzymatic hydrolysis assays (about 4.5 g l^{-1}) suggests that a part of HS were utilised by the microorganism. This fact could explain the observed increase in acetic acid concentration along the SSF runs (up to 2.1 g l^{-1}), as homofermentative lactic acid bacteria can utilise pentoses by a different metabolic pathway leading to acetic acid generation (Hofvendahl & Hahn-Hagerdahl 2000), a fact that could be enhanced by carbon source limitation (Thomas 2000).

To confirm that the conditions of Figure 2b corresponded to an optimal situation, an experiment with externally added cellobiase was performed at 37 °C and pH 6. In this case, the product yield (0.72 g lactic acid g^{-1} cellulose) and mean volumetric productiviy Q_{Pmean} (0.42 g l^{-1} h^{-1} after 48 h) were lower than in the best case.

In conclusion, D(−)-lactic acid has been obtained by SSF from cellulosic substrates using *Lactobacillus coryniformis* subsp. *torquens*. Under the best operational conditions (39 °C and pH 5.4), not significant metabolic alterations were found, and 0.5 g D(−)-lactic acid l^{-1} h^{-1} were obtained after 48 h at a product yield of 0.89 g D(−)-lactic acid g^{-1} cellulose in culture media free from L(+)-isomer.

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