Bioresource Technology 209 (2016) 297–304

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Identifying inhibitory effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms using a rapid small-scale screening method

Edwin C. van der Pol [a,b,*], Evelien Vaessen [b], Ruud A. Weusthuis [b], Gerrit Eggink [a,b]

[a] Food and Biobased Research, Wageningen University and Research Centre, PO Box 17, 6700 AA Wageningen, Netherlands
[b] Bioprocess Engineering, Wageningen University and Research Centre, PO Box 16, 6700 AA Wageningen, Netherlands

## HIGHLIGHTS

- Effect of lignocellulosic by-products on growth of lactic acid bacteria was evaluated.
- A large inter species variation of inhibitory effects was observed.
- Effects of single and combined by-products were identified by small-scale screening.
- Furfural was identified as key inhibitor in acid pretreated lignocellulose.
- Synergy between acids/phenols inhibited growth in alkaline treated lignocellulose.

## ARTICLE INFO

*Article history:*
Received 2 January 2016
Received in revised form 4 March 2016
Accepted 5 March 2016
Available online 11 March 2016

*Keywords:*
Inhibition
Lactic acid
Bacteria
Lignocellulosic by-products
Screening

## ABSTRACT

Sugars obtained from pretreated lignocellulose are interesting as substrate for the production of lactic acid in fermentation processes. However, by-products formed during pretreatment of lignocellulose can inhibit microbial growth. In this study, a small-scale rapid screening method was used to identify inhibitory effects of single and combined by-products on growth of lactic acid producing micro-organisms. The small-scale screening was performed in 48-well plates using 5 bacterial species and 12 by-products. Large differences were observed in inhibitory effects of by-products between different species. Predictions can be made for growth behaviour of different micro-organisms on acid pretreated or alkaline pretreated bagasse substrates using data from the small-scale screening. Both individual and combined inhibition effects were shown to be important parameters to predict growth. Synergy between coumaric acid, formic acid and acetic acid is a key inhibitory parameter in alkaline pretreated lignocellulose, while furfural is a key inhibitor in acid pretreated lignocellulose.



## 1. Introduction

Poly-lactic acid (PLA) is a renewable alternative for petrochemically derived plastics such as polyethylene (PE) and polystyrene (PS) (Garlotta, 2001). Lignocellulose is an interesting renewable carbon source, which can be used as feedstock in lactic acid producing fermentation processes (Sreenath et al., 2001; van der Pol et al., 2014).

Thermo-chemical pretreatment and enzymatic hydrolysis are required to depolymerize lignocellulose to fermentable monomeric sugars. Conditions used during chemical pretreatment are often severe, using temperatures up to 200 °C combinated with the presence of chemicals such as sulphuric acid or sodium hydroxide (Hendriks and Zeeman, 2009). Although chemical pretreatment is an efficient method to increase accessibility of lignocellulosic sugars, unwanted by-products can be formed (Rivera et al., 2007; van der Pol et al., 2015). These by-products can be divided in three main categories: furans, phenols and organic acids. Furans such as furfural, 5-hydroxymethylfurfural (HMF) and furoic acid are formed when monomeric sugars are exposed to high temperatures in an acidic environment (Kabel et al., 2007). Phenols, ferulates and coumarins are formed when lignin is degraded, or when the cross-links between hemicellulose and lignin are broken (Hatfield et al., 1999; Boerjan et al., 2003). Several organic acids are present in the hemicellulosic structure that are released when

* Corresponding author at: Bioprocess Engineering, Wageningen University and Research Centre, PO Box 16, 6700 AA Wageningen, Netherlands. Tel.: +31 317 485096.
*E-mail address:* edwin.vanderpol@wur.nl (E.C. van der Pol).

http://dx.doi.org/10.1016/j.biortech.2016.03.037
0960-8524/

hemicellulose is depolymerized, e.g. acetic acid (Sun et al., 2004). Organic acids can be also formed when furans are dehydrated to levulinic acid and formic acid. Furthermore, oxidation of sugars can lead to the formation of formic acid and acetic acid during alkaline pretreatment in the presence of oxygen (Klinke et al., 2003).

The type and quantity of by-products found in pretreated lignocellulose is mainly influenced by the pretreatment method and/or the lignocellulose composition of the crop species (van der Pol et al., 2014).

By-products can have an inhibitory effect on micro-organisms in fermentation processes, negatively effecting overall process performance. Currently, most studies on toxicity of by-products focus on ethanol producing yeast, using a limited amount of by-products in only one or a few concentrations (Delgenes et al., 1996; Larsson et al., 2000; Klinke et al., 2003). Combinations of different by-products can have synergistic inhibitory effects on growth of yeast strains (Taherzadeh et al., 1999; Oliva et al., 2004).

The toxicity of by-products on lactic acid producing strains has been investigated to a limited extent (Bischoff et al., 2010; Guo et al., 2010). More information is required to assess the suitability of lactic acid producing strains for the conversion of pretreated lignocellulosic hydrolysates. A complete evaluation is however not possible, since it requires toxicity analysis of a dozen by-products, at several concentrations, in several combinations on a number of strains, requiring billions of experiments.

In this research, a rapid screening method was used to identify inhibition effects of 12 lignocellulosic by-products, both individually and combined. Inhibition effects of individual by-products on the growth of 5 different lactic acid producing strains were evaluated. This screening was used to identify differences between microbial strains in their initial response towards the presence of by-products. Two parameters were determined during this screening. At first, concentrations were determined, where by-products become inhibitory for the micro-organism. Furthermore, when all results for individual inhibition were combined, an overall sensitivity of micro-organisms towards by-products can be determined. Based on the overall sensitivity, 3 lactic acid producing strains, showing the highest potential in the individual screening, were assessed for synergistic inhibitory effect between combinations of by-products. The concentrations used for this synergy experiment were determined in the individual screening, and are the concentrations at which the by-products become inhibitory.

## 2. Methods

### 2.1. Chemicals

All chemicals were ordered at Sigma–Aldrich (St. Louis, USA), and had a purity of at least 98% with the exception of formic acid, which was 95% pure. Medium components such as peptone, glucose, yeast extract and BIS-Tris were ordered at Duchefa (The Netherlands). Pre-mixed MRS medium was ordered at Merck chemicals (Germany).

### 2.2. Micro-organisms

*Lactobacillus casei* DSM 20011, *Lactobacillus delbrueckii* DSM 20073, *Lactococcus lactis* DSM 20481, *Bacillus coagulans* DSM 2314 and *Bacillus smithii* DSM 4216 were obtained as freeze dried stocks from the German collection of micro-organisms and cell cultures (DSMZ, Germany). Strain selection criteria used to choose these 5 strains were maximum productivities of lactic acid of at least 2 gram per litre per hour, capable of producing of optically pure lactic acid, and converting glucose to lactic acid with a yield of at least 80% on a weight basis (Akerberg et al., 1998; Hofvendahl et al., 1999; González-vara et al., 1999; Michelson et al., 2006; Maas et al., 2008). Cells were suspended for 30 min in 5 ml PYPD medium, consisting of 5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose and 10 g/l BIS-Tris. After 30 min pre-incubation, cells were transferred to 50 ml anaerobic flasks containing 45 ml fresh PYPD medium, sealed with a rubber cap, and incubated in these flasks for 16 h to an optical density at 660 nm of around 2. After addition of 15% v/v glycerol, cells were stored in 1.5 ml aliquots in cryovials at −80 °C until used.

### 2.3. Media and solutions

*L. delbrueckii* was grown on MRS medium (de Man et al., 1960). All other strains were grown on PYPD medium. Both media were autoclaved for 20 min at 121 °C prior to use.

All pure lignocellulosic by-products were dissolved in milliQ water. Stock solutions to test individual inhibitory effects were made in the following concentrations; phenols were dissolved in concentrations of 5 g/l, furans in concentrations of 10 g/l and small organic acids in 20 g/l. Stock solutions used to test synergistic effects had the following concentrations: Ferulic acid and coumaric acid 6 g/l, other phenols and furans 10 g/l, small organic acids 40 g/l. Acidic chemicals were pH adjusted to pH 6 using 4 M KOH prior to use. All stock solutions were heated for 1 h at 85 °C instead of being autoclaved to prevent thermal degradation. Heat treatment is required to prevent contamination of cultures by bacteriophages (Atamer et al., 2009).

### 2.4. Experiments in 48-well plates: cultivation and analysis

Cultivation was performed in Costar 48-well plates (Corning, New York, USA) with a working volume of 1 ml. 500 µl of 2× concentrated sterile PYPD medium was added to each well. Stocks solutions of pure lignocellulosic by-products were added to obtain the right concentration of by-product in each well, and sterile milliQ water was added to reach a final working volume of 1 ml.

Cultivations were performed in a Bactron II anaerobic chamber (Sheldon, Oregon, USA) for 16–18 h, using pressurised gas with 4% v/v $H_2$, 15% v/v $CO_2$ and 81% v/v $N_2$ to create an anaerobic chamber. To keep evaporation in the wells within 10% v/v, humidity was increased by placing beakers with water in the incubation room of the anaerobic chamber. The 4 corner wells experienced more than 10% v/v evaporation, and were therefore not used in the experiment. Each 48-well plate contained 4 negative control wells (PYPD without micro-organism), and 4 reference wells (PYPD with micro-organisms and without lignocellulosic by-products). The micro-organisms in the 48-well plates were incubated at temperature optima for the micro-organism based on literature (González-vara et al., 1999; Michelson et al., 2006; Akerberg et al., 1998; Hofvendahl et al., 1999; Maas et al., 2008) and cultivation times based on when the reference wells reached an OD660 of 1. The following cultivation conditions were chosen: *L. casei* was grown at 37 °C for 18 h, *L. delbrueckii* was grown at 45 °C for 16 h, *L. lactis* was grown at 30 °C for 17 h, *B. coagulans* was grown at 50 °C for 16 h, and *B. smithii* was grown at 50 °C for 17 h. Each concentration of lignocellulosic by-product was tested in triplicate, thus 12 different experiments can be performed per 48-well plate. Average standard deviations observed between triplicate experiments were 7%.

Inoculation occurred with 0.5% v/v cell stock coming directly from defrozen −80 °C cryovials, acquiring an initial cell optical density at 660 nm (OD660) around 0.01. The OD660 of the 48-well plates was measured using a plate reader (Tecan, Switzerland) before and after fermentation. Relative growth was obtained with the following formula:

$$\text{Relative growth}\,(\%) = \frac{(A_{\text{ferm},t=\text{end}} - A_{\text{ferm},t=\text{end}})}{(A_{\text{ref},t=\text{end}} - A_{\text{ref},t=\text{end}})} \times 100\%$$

where $A$ is OD660, $t$ = end is time at the end of the cultivation, $t = 0$ is time at the start of the cultivation, ref is the average of four reference wells, and ferm is the average of triplicate wells with a certain concentration of a pure lignocellulosic by-product.

### 2.5. Anaerobic flasks: cultivation and analysis

Experiments were performed in 60 ml glass anaerobic flasks with a working volume of 50 ml. The flasks were sealed off by a rubber stopper, which was kept in place with an aluminium crimp cap. The cultivation conditions in the flask experiments were similar to the 48-well plates with regard to temperature, medium and inoculum size.

All glass flasks and rubber caps were autoclaved at 121 °C for 20 min prior to the cultivation. Simultaneously, a 2× concentrated medium and milliQ water was autoclaved. The pure lignocellulose by-product stock solutions were heated at 85 °C for 1 h.

In Table 1, 4 mixtures used to simulate alkaline or acid pretreated lignocellulose, alk-75, alk-150, acid-75 and acid-150, are shown. Every 50 ml anaerobic flask was filled with 25 ml of 2× concentrated PYPD medium containing, 25 ml of 2× concentrated alk-75, alk-150, acid-75 or acid-150 mixture, and inoculated with 250 µl of freezer stock (0.5 v/v).

Sampling was performed through the rubber stopper using sterile syringes and needles. Of every sample of 1.5 ml, 1 ml was used for determining the OD660 using a Ultrospec 200 spectrophotometer (Pharmacia Biotech, Sweden) and 0.5 ml was used to determine lactic acid concentrations using a Waters 717 HPLC system equipped with a Shodex RS-PAK KC-811 column as previously described (van der Pol et al., 2015).

## 3. Results and discussion

### 3.1. Experimental set up

To determine the individual and combined effects of 12 by-products in 6 different concentrations in triplicate for 5 strains, billions of fermentation experiments are required. Clearly, this asks for a simplified approach.

When only individual effects of by-products would be studied at 6 different concentrations in triplicate for 5 strains, 1080 experiments would still need to be performed. Preparing and sampling over 1000 anaerobic flasks will require extensive labour. As a solution, a rapid screening to determine initial growth in 48-well plates can be performed. Effects of individual by-products for 5 different strains in triplicate can be identified by only using 30 48-well plates. Therefore, this method can be seen as a suitable first step in the screening of inhibitory effects of by-products. The concentrations at which individual by-products become inhibitory for a micro-organism were determined during the first screening. These concentrations can be used in synergy experiments, where inhibitory effects between combinations of by-products can be found using only 5.5 48-well plates per strain.

Determination of lactic acid concentrations may be difficult in the screening experiments. Some by-products can interfere with enzyme assays used to determine lactic acid. Moreover, determining lactic acid for thousands of samples by other methods such as HPLC will require too much time. For ethanol producing micro-organisms, a correlation between by-product inhibition on growth and on ethanol production was observed (van der Pol et al., 2014). A similar correlation between inhibition of growth and inhibition of lactic acid production was found for *B. coagulans* DSM 2314 and *B. smithii* DSM 4216 in this research (data not shown). Therefore, only determining growth during a screening experiment may provide a good indication of the overall process performance. Analysis of optical density in 48-well plates is simple and can be operated at high throughput rates. The effect of by-products on lactic acid producing micro-organisms was therefore determined by assessing growth rates using OD660 measurements.

**Table 1**
Mixtures of by-products as used in 50 ml cultivation. The composition of the mixtures resemble alkaline and acid pretreated sugarcane bagasse lignocellulose, in either 75 g/l sugar equivalent (alkali-75 and acid-75) or 150 g/l sugar equivalent (alkali-150 and acid-150) as measured previously (van der Pol et al., 2015). The concentrations shown are the final concentrations present in the 50 ml flasks.

| Lignocellulose by-product | Concentration in mixture (g/l) | | | |
|---|---|---|---|---|
| | Alkali-75 | Alkali-150 | Acid-75 | Acid-150 |
| Acetic acid | 6.75 | 13.5 | 2.34 | 4.68 |
| Glycolic acid | 2.49 | 4.98 | 1.61 | 3.21 |
| Levulinic acid | 1.11 | 2.22 | 1.55 | 3.09 |
| Formic acid | 1.24 | 2.48 | 0.214 | 0.428 |
| Furfural | 0 | 0 | 1.22 | 2.45 |
| HMF | 0 | 0 | 0.142 | 0.284 |
| Coumaric acid | 0.354 | 0.708 | 0.215 | 0.431 |
| Ferulic acid | 0.018 | 0.036 | 0.031 | 0.062 |
| 4-hydroxybenzaldehyde | 0.081 | 0.162 | 0.030 | 0.060 |
| Vanillin | 0.021 | 0.042 | 0.051 | 0.102 |
| Syringaldehyde | 0.023 | 0.045 | 0.031 | 0.062 |

### 3.2. Growth of lactic acid producing micro-organisms in presence of single by-products

In total, 12 different by-products were tested in the screening experiment. Six of the lignocellulosic by-products tested were phenolics, of which one was a phenolic ketone, two were phenolic acids, and three were phenolic aldehydes. Although syringaldehyde, vanillin and 4-hydroxybenzaldehyde are very similar in structure, their inhibitory potential differed significantly (Table 2). 4-Hydroxybenzaldehyde was observed to be more inhibitory than vanillin and syringaldehyde. The average growth inhibition for the 5 lactic acid producing micro-organisms in the presence of 1 g/l 4-hydroxybenzaldehyde, vanillin and syringaldehyde was 60%, 34% and 11% respectively. A higher toxicity of 4-hydroxybenzaldehyde in comparison to vanillin and syringaldehyde has also been previously observed for ethanol producing yeast (Delgenes et al., 1996; Zaldivar and Ingram, 1999; Oliva et al., 2003), and for lactic acid producing bacteria (Bischoff et al., 2010).

Large differences between micro-organisms were observed for the inhibitory effect of phenolic aldehydes. Where *L. delbrueckii* was significantly inhibited by vanillin concentrations of 0.75 g/l, *L. lactis* was still growing at vanillin concentrations of 2.5 g/l. Overall, *L. lactis* was least inhibited by phenolic aldehydes. Although phenolic aldehydes can severely inhibit cell growth, their individual presence in most pretreated lignocellulosic feedstocks is below 0.2 g/l (van der Pol et al., 2014, 2015). At these concentrations, phenolic aldehydes are not likely to induce significant inhibition as individual compound (Table 2).

Phenolic acids such as coumaric acid and ferulic acid were significantly inhibiting growth of most lactic acid producing micro-organisms in concentrations above 1 g/l, only *L. casei* is not fully inhibited at a phenolic acid concentration of 2.5 g/l. Coumaric acid can be present in pretreated lignocellulose in concentrations up to 0.75 g/l, which can cause significant inhibition of growth for *L. lactis* and *B. smithii* as individual compound (Table 2) (van der Pol et al., 2014, 2015).

The concentration at which furans becomes an inhibitor for growth differs significantly between the tested strains. The differences may be related to growth temperature, where strains grown at a higher growth temperature of 50 °C (*B. coagulans* and *B.*

**Table 2**
Growth of *L. casei*, *L delbrueckii*, *L. lactis*, *B. coagulans* and *B. smithii* in 48-well plates in the presence of 6 concentrations of 12 lignocellulosic by-products. The percentage shown is the relative growth compared to a fully-grown reference culture in the absence of lignocellulosic by-products.

| Lignocellulose by product (g/l) | Relative growth relation to reference in % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *L. casei* (%) | *Stdev* (%) | *L. delbrueckii* (%) | *Stdev* (%) | *L. lactis* (%) | *Stdev* (%) | *B. coagulans* (%) | *Stdev* (%) | *B. smithii* (%) | *Stdev* (%) |
| *Ferulic acid* | | | | | | | | | | |
| 0.1 | 100 | 7 | 101 | 19 | 103 | 6 | 102 | 7 | 89 | 15 |
| 0.25 | 100 | 2 | 108 | 9 | 109 | 13 | 121 | 20 | 87 | 16 |
| 0.5 | 97 | 4 | 101 | 13 | 92 | 6 | 126 | 10 | 67 | 23 |
| 0.75 | 93 | 3 | 85 | 7 | 100 | 4 | 117 | 12 | 27 | 2 |
| 1 | 82 | 4 | 15 | 4 | 75 | 2 | 109 | 16 | 19 | 8 |
| 2.5 | 42 | 5 | 2 | 2 | 7 | 1 | 18 | 4 | −3 | 2 |
| *Coumaric acid* | | | | | | | | | | |
| 0.1 | 104 | 1 | 108 | 15 | 109 | 8 | 110 | 6 | 97 | 8 |
| 0.25 | 109 | 5 | 106 | 19 | 114 | 8 | 123 | 9 | 95 | 13 |
| 0.5 | 105 | 6 | 91 | 10 | 110 | 11 | 127 | 12 | 78 | 5 |
| 0.75 | 96 | 5 | 77 | 8 | 52 | 1 | 115 | 9 | 46 | 8 |
| 1 | 91 | 7 | 66 | 8 | 22 | 2 | 116 | 7 | 26 | 8 |
| 2.5 | 24 | 8 | 5 | 1 | 2 | 3 | 0 | 1 | 5 | 7 |
| *4-H.benzaldehyde* | | | | | | | | | | |
| 0.1 | 94 | 13 | 95 | 22 | 115 | 8 | 106 | 6 | 104 | 8 |
| 0.25 | 93 | 1 | 76 | 9 | 104 | 8 | 114 | 15 | 100 | 7 |
| 0.5 | 73 | 8 | 44 | 14 | 111 | 6 | 98 | 12 | 79 | 9 |
| 0.75 | 68 | 3 | 18 | 14 | 118 | 18 | 7 | 0 | 34 | 8 |
| 1 | 55 | 7 | 7 | 3 | 129 | 10 | 0 | 0 | 7 | 5 |
| 2.5 | 1 | 4 | 3 | 1 | 1 | 1 | −1 | 0 | 9 | 8 |
| *Vanillin* | | | | | | | | | | |
| 0.1 | 107 | 18 | 107 | 5 | 118 | 4 | 116 | 2 | 97 | 4 |
| 0.25 | 107 | 11 | 109 | 18 | 117 | 0 | 117 | 11 | 96 | 6 |
| 0.5 | 98 | 9 | 68 | 1 | 121 | 5 | 113 | 9 | 89 | 9 |
| 0.75 | 84 | 12 | 21 | 5 | 118 | 5 | 101 | 5 | 68 | 14 |
| 1 | 64 | 2 | 8 | 1 | 121 | 1 | 94 | 4 | 43 | 13 |
| 2.5 | 11 | 1 | 4 | 1 | 69 | 5 | 27 | 2 | 6 | 0 |
| *Syringaldehyde* | | | | | | | | | | |
| 0.1 | 97 | 9 | 111 | 10 | 107 | 4 | 99 | 17 | 103 | 12 |
| 0.25 | 103 | 7 | 111 | 7 | 101 | 9 | 101 | 3 | 106 | 7 |
| 0.5 | 94 | 2 | 104 | 11 | 99 | 10 | 93 | 11 | 104 | 6 |
| 0.75 | 89 | 5 | 91 | 4 | 99 | 4 | 88 | 8 | 100 | 5 |
| 1 | 85 | 2 | 85 | 14 | 97 | 9 | 81 | 4 | 99 | 3 |
| 2.5 | 57 | 12 | 49 | 12 | 74 | 5 | 57 | 7 | 24 | 5 |
| *Acetosyringone* | | | | | | | | | | |
| 0.1 | 95 | 2 | 99 | 5 | 107 | 8 | 108 | 4 | 105 | 1 |
| 0.25 | 97 | 7 | 107 | 8 | 111 | 3 | 101 | 14 | 103 | 1 |
| 0.5 | 87 | 6 | 111 | 12 | 107 | 5 | 105 | 20 | 102 | 3 |
| 0.75 | 83 | 8 | 105 | 11 | 105 | 8 | 108 | 7 | 97 | 6 |
| 1 | 71 | 1 | 94 | 11 | 110 | 9 | 109 | 4 | 92 | 7 |
| 2.5 | 40 | 7 | 62 | 9 | 77 | 4 | 103 | 4 | 16 | 3 |
| *5-HMF* | | | | | | | | | | |
| 0.25 | 95 | 3 | 102 | 17 | 105 | 13* | 115 | 2 | 89 | 2 |
| 0.5 | 96 | 5 | 109 | 26 | 103 | 25* | 115 | 7 | 84 | 3 |
| 0.75 | 93 | 11 | 93 | 18 | 89 | 7* | 118 | 16 | 70 | 2 |
| 1 | 81 | 6 | 96 | 14 | 91 | 22* | 111 | 12 | 48 | 5 |
| 2.5 | 41 | 3 | 12 | 6 | 39 | 13* | 29 | 11 | −1 | 7 |
| 5 | 7 | 3 | 5 | 6 | 4 | 5* | 3 | 28 | 1 | 3 |
| *Furfural* | | | | | | | | | | |
| 0.25 | 102 | 3 | 115 | 21 | 115 | 33* | 103 | 8 | 106 | 6 |
| 0.5 | 99 | 3 | 110 | 19 | 113 | 22* | 94 | 11 | 107 | 3 |
| 0.75 | 97 | 3 | 103 | 6 | 94 | 2* | 20 | 7 | 102 | 2 |
| 1 | 84 | 6 | 72 | 22 | 80 | 8* | 7 | 2 | 47 | 3 |
| 2.5 | 57 | 7 | 6 | 3 | 37 | 29* | −1 | 0 | 1 | 7 |
| 5 | 11 | 4 | 4 | 1 | 7 | 6* | 0 | 1 | 2 | 0 |
| *Formic acid* | | | | | | | | | | |
| 0.5 | 105 | 4 | 133 | 3 | 103 | 7 | 92 | 2 | 99 | 10 |
| 1 | 92 | 1 | 129 | 12 | 100 | 2 | 94 | 5 | 95 | 11 |
| 2.5 | 88 | 3 | 101 | 5 | 90 | 8 | 71 | 3 | 77 | 12 |
| 5 | 75 | 4 | 86 | 5 | 75 | 7 | 65 | 2 | 31 | 8 |
| 7.5 | 68 | 6 | 74 | 5 | 71 | 3 | 57 | 2 | 5 | 3 |
| 10 | 51 | 2 | 68 | 10 | 60 | 3 | 49 | 2 | 5 | 2 |
| *Acetic acid* | | | | | | | | | | |
| 0.5 | 101 | 9 | 109 | 5 | 124 | 20 | 117 | 9 | 104 | 9 |
| 1 | 102 | 7 | 97 | 7 | 132 | 33 | 107 | 17 | 100 | 8 |
| 2.5 | 103 | 6 | 97 | 9 | 124 | 19 | 109 | 5 | 86 | 8 |
| 5 | 106 | 6 | 89 | 15 | 122 | 2 | 91 | 8 | 55 | 6 |

**Table 2** (*continued*)

| Lignocellulose by product (g/l) | Relative growth relation to reference in % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *L. casei* (%) | *Stdev* (%) | *L. delbrueckii* (%) | *Stdev* (%) | *L. lactis* (%) | *Stdev* (%) | *B. coagulans* (%) | *Stdev* (%) | *B. smithii* (%) | *Stdev* (%) |
| 7.5 | 99 | 6 | 102 | 9 | 108 | 7 | 73 | 6 | 19 | 9 |
| 10 | 77 | 20 | 115 | 3 | 74 | 6 | 74 | 3 | −1 | 0 |
| *Glycolic acid* | | | | | | | | | | |
| 0.5 | 71 | 11* | 108 | 3 | 102 | 10 | 100 | 6 | 77 | 4 |
| 1 | 133 | 40* | 105 | 3 | 85 | 13 | 96 | 7 | 76 | 2 |
| 2.5 | 92 | 38* | 104 | 0 | 83 | 3 | 79 | 7 | 66 | 7 |
| 5 | 44 | 11* | 95 | 3 | 84 | 3 | 53 | 7 | 16 | 10 |
| 7.5 | 24 | 16* | 126 | 8 | 71 | 2 | 34 | 0 | 7 | 5 |
| 10 | 12 | 6* | 109 | 4 | 70 | 4 | 35 | 2 | 8 | 8 |
| *Levulinic acid* | | | | | | | | | | |
| 0.5 | 103 | 11 | 87 | 10 | 117 | 16 | 104 | 7 | 110 | 4 |
| 1 | 108 | 10 | 86 | 8 | 125 | 4 | 111 | 10 | 108 | 3 |
| 2.5 | 92 | 3 | 83 | 11 | 129 | 22 | 116 | 9 | 108 | 2 |
| 5 | 93 | 8 | 67 | 9 | 108 | 19 | 109 | 6 | 104 | 4 |
| 7.5 | 88 | 2 | 94 | 7 | 117 | 12 | 100 | 1 | 92 | 4 |
| 10 | 89 | 16 | 98 | 21 | 74 | 11 | 91 | 4 | 52 | 4 |

* Glycolic acids resulting in cell clumps in *L. casei* cultures, and furfural and HMF caused cell clumping for *L. lactis*, making the measurements less accurate. Stdev = standard deviation of triplicate experiments.

*smithii*) or 45 °C (*L. delbrueckii*) show a stronger growth inhibition in the presence of furans. In previous studies, furfural toxicity was linked to the formation of reactive oxygen species (ROS). A high temperature accelerates the formation of ROS (Feron et al., 1991; Allen et al., 2010). Strains growing at lower temperatures are therefore less exposed to ROS, thus it can be theorised that they may therefore tolerate higher concentrations of furans. In contrast to what was found in previous experiments with ethanol producing yeast strains (Delgenes et al., 1996; Oliva et al., 2003), most micro-organisms tested in this research did not show a significant difference in inhibitory effects between furfural and 5-HMF. *B. coagulans* was an exception, this micro-organism was less growth-inhibited by 5-HMF.

Levulinic acid is the least inhibitory by-product out of 12 different by-products tested in this study. At a concentration of 10 g/l, only *B. smithii* is significantly growth inhibited. Acetic acid results in a minor growth inhibition at a concentration of 10 g/l. Acetic acid has previously been described as only a minor growth inhibitor for *B. coagulans* (Walton et al., 2010). Only *B. smithii* was significantly growth inhibited at acetic acid concentrations of 5 g/l. Formic acid can result in significant growth inhibition, and has the most inhibitory effect of all organic acids tested. All strains tested show a significant inhibition of growth at formic acid concentrations of 7.5 g/l. Overall, *L. casei*, *L. delbrueckii*, *L. lactis* and *B. coagulans* show a similar response to different small organic acids when present as individual compound, while *B. smithii* is more sensitive towards these organic acids.

*L. casei* DSM 20011, *L. lactis* DSM 20481 and *B. coagulans* DSM 2314 are in general less inhibited in growth by individual lignocellulosic by-products. *L. delbrueckii DSM 20073* shows significant inhibition towards vanillin and 4-hydroxybenzaldehyde at a concentration of 0.5 g/l, and towards furans at concentrations exceeding 1 g/l. *B. smithii DSM 4216* is significantly inhibited by organic acids at low concentrations, and was also growth inhibited at relatively low concentrations of phenolic acids and phenolic aldehydes.

### 3.3. Growth of lactic acid producing micro-organisms in presence of combinations of by-products

Screening for the effects of individual by-products on the growth of different lactic acid producing bacteria showed that *L. lactis*, *B. coagulans* and *L. casei* were overall least sensitive towards by-products. To further reduce the amount of experiments required, only the most promising strains based on individual by-product screening were evaluated. During individual screening, concentrations were determined where the by-product started to become inhibitory for the micro-organism. Only these concentrations were used in combined by-products screening, further reducing the amount of experiments required.

Combinations of the phenolic aldehydes vanillin, syringaldehyde and p-hydroxybenzaldehyde resulted in a large synergistic growth inhibition effect for all micro-organisms tested (Table 3). The large combined growth inhibition of these compounds may be explained by their similarity. The only difference between the phenolic aldehydes is the number of methyl ether groups on the benzene ring. Combined growth inhibition between the phenolic aldehydes has been observed previously for ethanol producing yeast (Oliva et al., 2004). A large synergistic growth inhibition effect between phenolic aldehydes and phenolic acids was observed for *B. coagulans* and *L. lactis*, while synergy between phenolic aldehydes and phenolic acids was not observed for *L. casei* (Table 3). The observed synergistic growth inhibition effect show that only identifying individual inhibition effects is not sufficient to determine growth of micro-organisms on lignocellulose-derived substrates.

For all micro-organisms, combinations of formic acid and other by-products resulted in a large synergistic growth inhibition effect (Table 3). For *L. lactis*, acetic acid also showed a large synergistic inhibition effect with other by-products, while for *L. casei* and *B. coagulans* most combinations involving acetic acid did not result in synergistic growth inhibition. Therefore, while a similar inhibition for all strains may be expected when looking at toxicity of acetic acid as individual compound, the inhibitory effect of acetic acid can be significantly different between micro-organisms in mixtures of by-products.

*B. coagulans* showed a diverse response towards combinations of by-products. Cultivation of this strain in the presence of combinations of inhibitors, for instance including by-products such as 5-HMF and furfural, do not show additive growth inhibition effects towards any by-products except p-hydroxybenzaldehyde. However, other combinations of by-products can result in a full inhibition in cell growth. For *L. casei*, no combination of by-products was found which fully inhibited cell growth, but nearly all combinations showed a minor synergistic effect. A large number of combinations of by-products caused synergistic growth inhibition for *L. lactis*.

**Table 3**
Synergistic inhibition effects of lignocellulosic by-products on growth of *L. casei*, *L. lactis* and *B. coagulans*, observed in 48-well plates. Percentage shown is relative growth compared to a fully-grown reference culture in the absence lignocellulosic by-products.

| | | 5-HMF (%) | Furfural (%) | Glycolic acid (%) | Levulinic acid (%) | Acetic acid (%) | Formic acid (%) | Ferulic acid (%) | Coumaric acid (%) | 4-Hydroxy-benzaldehyde (%) | Syringaldehyde (%) | Vanillin (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. lactis* | | | | | | | | | | | | |
| Lignocellulosic by-product (g/l) | | 1.5 | 1 | 5 | 10 | 10 | 7.5 | 1 | 0.5 | 1.5 | 2.5 | 2.5 |
| 5-HMF | 1.5 | – | 76 | 90 | 84 | 32 | 47 | 46 | 83 | 32 | 37 | 25 |
| Furfural | 1 | 76 | – | 88 | 76 | 35 | 73 | 42 | 98 | 29 | 61 | 31 |
| Glycolic acid | 5 | 90 | 88 | – | 100 | 44 | 69 | 21 | 100 | 62 | 66 | 40 |
| Levulinic acid | 10 | 84 | 76 | 100 | – | 3 | 16 | 39 | 111 | 17 | 27 | 10 |
| Acetic acid | 10 | 32 | 35 | 44 | 3 | – | 3 | 10 | 36 | 4 | 5 | 4 |
| Formic acid | 7.5 | 47 | 73 | 69 | 16 | 3 | – | 36 | 74 | 11 | 17 | 8 |
| Ferulic acid | 1 | 46 | 42 | 21 | 39 | 10 | 36 | – | 13 | 11 | 25 | 18 |
| Coumaric acid | 0.5 | 83 | 98 | 100 | 111 | 36 | 74 | 13 | – | 32 | 24 | 29 |
| 4-H.benzaldehyde | 1.5 | 32 | 29 | 62 | 17 | 4 | 11 | 11 | 32 | – | 4 | 2 |
| Syringaldehyde | 2.5 | 37 | 61 | 66 | 27 | 5 | 17 | 25 | 24 | 4 | – | 4 |
| Vanillin | 2.5 | 25 | 31 | 40 | 10 | 4 | 8 | 18 | 29 | 2 | 4 | – |
| *B. coagulans* | | | | | | | | | | | | |
| Lignocellulosic by-product (g/l) | | 1.5 | 0.5 | 4 | 10 | 10 | 4 | 1.5 | 1.5 | 0.5 | 1.5 | 1.5 |
| 5-HMF | 1.5 | – | 90 | 70 | 96 | 84 | 69 | 91 | 79 | 2 | 96 | 80 |
| Furfural | 0.5 | 90 | – | 60 | 89 | 90 | 46 | 80 | 17 | 7 | 82 | 77 |
| Glycolic acid | 4 | 70 | 60 | – | 71 | 71 | 45 | 113 | 92 | 73 | 87 | 85 |
| Levulinic acid | 10 | 96 | 89 | 71 | – | 45 | 54 | 58 | 106 | 85 | 80 | 94 |
| Acetic acid | 10 | 84 | 90 | 71 | 45 | – | 53 | 20 | 99 | 49 | 84 | 107 |
| Formic acid | 4 | 69 | 46 | 45 | 54 | 53 | – | 68 | 29 | 41 | 63 | 63 |
| Ferulic acid | 1. | 91 | 80 | 113 | 58 | 20 | 68 | – | 14 | 0 | 24 | 11 |
| Coumaric acid | 1.5 | 79 | 50 | 92 | 106 | 99 | 29 | 14 | – | 2 | 19 | 27 |
| 4-H.benzaldehyde | 0.5 | 2 | 7 | 73 | 85 | 49 | 41 | 0 | 2 | – | 5 | 0 |
| Syringaldehyde | 1.5 | 96 | 82 | 87 | 80 | 84 | 63 | 24 | 19 | 5 | – | 4 |
| Vanillin | 1.5 | 80 | 77 | 85 | 94 | 107 | 63 | 11 | 27 | 0 | 4 | – |
| *L. casei* | | | | | | | | | | | | |
| Lignocellulosic by-product (g/l) | | 1.5 | 1.5 | 4 | 10 | 10 | 7.5 | 1.5 | 1.5 | 0.75 | 1.5 | 1 g/l |
| 5-HMF | 1.5 | – | 55 | 72 | 90 | 75 | 46 | 68 | 63 | 60 | 54 | 80 |
| Furfural | 1.5 | 55 | – | 61 | 54 | 67 | 33 | 61 | 65 | 49 | 49 | 71 |
| Glycolic acid | 4 | 72 | 61 | – | 71 | 80 | 14 | 58 | 66 | 33 | 49 | 42 |
| Levulinic acid | 10 | 90 | 54 | 71 | – | 69 | 43 | 65 | 68 | 69 | 80 | 74 |
| Acetic acid | 10 | 75 | 67 | 80 | 69 | – | 44 | 54 | 49 | 52 | 66 | 61 |
| Formic acid | 7.5 | 46 | 33 | 14 | 43 | 44 | – | 29 | 40 | 9 | 30 | 19 |
| Ferulic acid | 1.5 | 68 | 61 | 58 | 65 | 54 | 29 | – | 63 | 58 | 48 | 63 |
| Coumaric acid | 1.5 | 63 | 65 | 66 | 68 | 49 | 40 | 63 | – | 55 | 48 | 52 |
| 4-H.benzaldehyde | 0.75 | 60 | 49 | 33 | 69 | 52 | 9 | 58 | 55 | – | 29 | 34 |
| Syringaldehyde | 1.5 | 54 | 49 | 49 | 80 | 66 | 30 | 48 | 48 | 29 | – | 34 |
| Vanillin | 1 | 80 | 71 | 42 | 74 | 61 | 19 | 63 | 52 | 34 | 34 | – |

### *3.4. Growth of lactic acid producing bacteria in mixtures of by-products resembling acid or alkaline pretreated lignocellulose*

The behaviour of the lactic acid producing micro-organisms in mixtures of many different by-products was determined. To see whether growth can be predicted in these mixtures, and whether key inhibitors can be identified using data from the small-scale screening, *L. lactis*, *L. casei*, *B. coagulans* and *B. smithii* have been cultivated at 50 ml scale with simulated alkaline or acid pretreated substrates at two different concentrations (Fig. 1). The lower alkali-75 and acid-75 mixtures resemble the amount of by-products which are found in lignocellulosic substrates containing 75 g/l of lignocellulosic sugar. The alkali-150 and acid-150 mixtures resemble the amount of by-products which are found in lignocellulosic substrates containing 150 g/l of lignocellulosic sugars (Table 1). Fig. 1 shows growth of the four strains on the four media in comparison to a reference grown without any by-products.

Alkaline pretreated material contains high concentrations of organic acids, with acetic acid, glycolic, levulinic, formic acid and coumaric acid being present in a concentration of 6.8, 2.5, 1.1, 1.2 and 0.35 g/l respectively in alkali-75. In the small-scale screening, *B. smithii* showed significant growth inhibition at a concentration of 7.5 g/l of acetic acid, with acetic acid alone causing a significant inhibition of growth. *B. smithii* was significantly growth inhibited in the presence of alkaline-75 mixture, this could be largely contributed to the presence of acetic acid.

Large synergistic growth inhibition effects between different acids, and between acetic acid and phenolic acids and aldehydes were seen for *L. lactis* during small-scale screening. None of the individual by-products was present at a concentration in which it could cause individual growth inhibition. However, the combination of acetic acid with formic acid, glycolic acid, coumaric acid and phenolics causes major growth inhibition for *L. lactis* on the alkali-75 mixture.

For *B. coagulans* and *L. casei*, none of the individual by-products were present in a concentration that can significantly inhibit growth in the alkali-75 mixture. Acetic acid, glycolic, levulinic, formic acid and coumaric acid did not show large synergistic growth inhibition for these micro-organisms in the small-scale screening. As a result, only a small inhibition was observed for *B. coagulans* and *L. casei* when grown on the alkali-75 mixture.

In alkali-150, *L. casei* was expected to be strongly, but not fully, growth inhibited based on the small-scale screening. Strong growth inhibition was indeed observed for *L. casei*, but some growth was still observed. Growth of *B. coagulans* on the alkali-150 mixture was the only experiment not accurately predicted using data from the small-scale screening. Rapid screening predicted that *B. coagulans* would to be able to grow on the

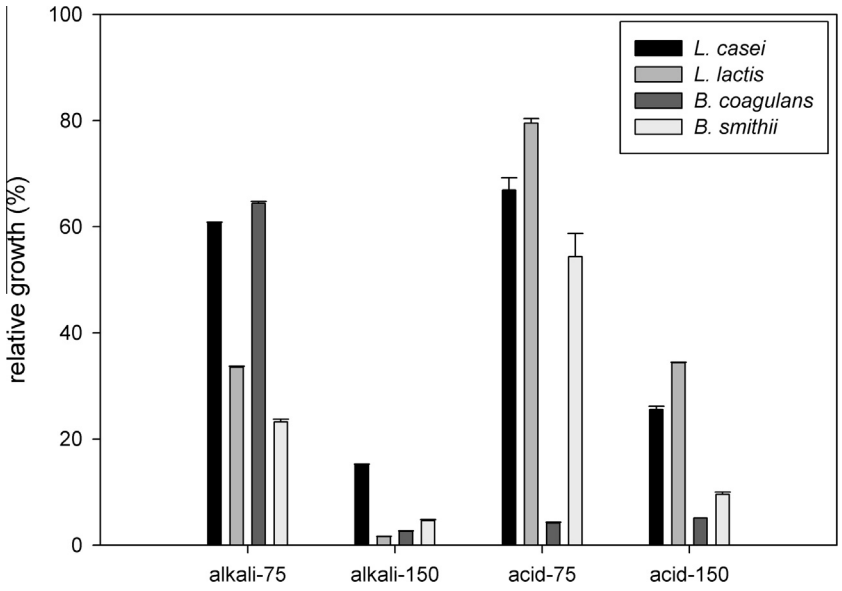


**Fig. 1.** Relative growth of *L. casei*, *L. lactis*, *B. coagulans* and *B. smithii* in 50 ml anaerobic flasks in presence of alkaline or acid pretreated mixture at 75 or 150 g/l sugar equivalent (Table 1), Relative growth rate is determined by comparing the growth in mixtures to a reference grown anaerobic flask grown without by-products.

alkali-150 mixture, although strongly inhibited. However, in the shake flask experiment it was observed that *B. coagulans* was fully inhibited by the alkali-150 mixture.

In acid pretreated lignocellulose, one by-product found in larger quantities is furfural, with a concentration of 1.2 g/l of furfural being present in the acid-75 mixture. Small-scale screening of individual by-products showed that 1.2 g/l of furfural is fully inhibiting growth of *B. coagulans*, and significantly inhibiting *B. smithii*. On the other hand, *L. casei* and *L. lactis* were only mildly inhibited by the acid-75 mixture. All other by-products in acid-75 were present in concentrations that did not result in individual growth inhibition for these micro-organisms, and furfural did not show large combined growth inhibition with most other by-products. Growth for all strains observed on the acid-75 mixture is therefore very similar to growth observed in small-scale experiments for furfural concentrations of 1.2 g/l.

Only *L. casei* and *L. lactis* were not fully growth inhibited in acid-150 mixtures, containing 2.5 g/l of furfural. The relative growth rates found for each strain in acid-150 mixtures are very similar to the growth rates observed during the small-scale screening in the presence of 2.5 g/l furfural as individual by-product. Therefore, it can be concluded that furfural is the key inhibitor in acid-pretreated lignocellulose.

## 4. Conclusion

In this study, the effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms were evaluated using a small-scale screening method. Both individual and combined growth inhibition effects between different by-products were studied. Large differences were observed between micro-organisms with respect to the concentration where by-products become inhibitory. Results from the rapid screening method can predict growth in mixtures of different by-products, and can identify inhibitory by-product(s) in the mixture. This rapid screening method can therefore help to select the most appropriate micro-organism for a chosen combination of pretreatment method and lignocellulosic feedstock.

## Acknowledgements

The authors would like to thank BE-Basic, DSM and Corbion, for funding this research. Furthermore, the authors would like to thank Jorrit Knoef for his help with laboratory experiments.

## References

Akerberg, C., Hofvendahl, K., Zacchi, G., Hahn-Hägerdal, B., 1998. Modelling the influence of pH, temperature, glucose and lactic acid concentrations on the kinetics of lactic acid producting by *Lactococcus lactis* ssp. *lactis* ATCC 19435 in whole-weat flour. Appl. Microbiol. Biotechnol. 49, 682–690.

Allen, S., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3 (2), 1–10.

Atamer, Z., Dietrich, J., Müller-Merbach, M., Neve, H., Heller, K.J., Hindrichs, J., 2009. Screening for and characterization of *Lactococcus lactis* bacteriophages with high thermal resistance. Int. Dairy J. 19, 228–235.

Bischoff, K.M., Liu, S., Hughes, S.R., Rich, J.O., 2010. Fermentation of corn fiber hydrolysate to lactic acid by moderate thermophile *Bacillus coagulans*. Biotechnol. Lett. 32, 823–828.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519–546.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130–135.

Delgenes, J.P., Moletta, R., Navarro, J.M., 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*. Enzyme Microb. Technol. 19, 220–225.

Feron, V.J., Til, H.P., de Vrijer, F., Woutersen, R.A., Cassee, F.R., van Bladeren, P.J., 1991. Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. Mutat. Res. Genet. Toxicol. 259, 363–385.

Garlotta, D., 2001. A literature review of poly(lactic acid). J. Polym. Environ. 9, 63–84.

González-Vara, Y.R.A., Vaccari, G., Dosi, E., Trilli, A., Rossi, M., Matteuzzi, D., 1999. Enhanced production of L-(+)-lactic acid in chemostat by *Lactobacillus casei* DSM 20011 using ion-exchange resins and cross-flow filtration in a fully automated pilot plant controlled via NIR. Biotechnol. Bioeng. 67, 147–156.

Guo, W., Jia, W., Li, Y., Chen, S., 2010. Performances of *Lactobacillus brevis* for producing lactic acid from hydrolysate of lignocellulosics. Appl. Biochem. Biotechnol. 161, 124–136.

Hatfield, R.D., Ralph, J., Grabber, J.H., 1999. Cell wall cross-linking by ferulates and diferulates in grasses. J. Sci. Food Agric. 79, 403–407.

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18.

Hofvendahl, K., Akerberg, C., Zacchi, G., Hahn-Hägerdal, B., 1999. Simultaneous enzymatic wheat starch saccharification and fermentation to lactic acid by *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 52, 163–169.

Kabel, M.A., Bos, G., Zeevalking, J., Voragen, A.G.J., Schols, H.A., 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour. Technol. 98, 2034–2042.

Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K., 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast. Biotechnol. Bioeng. 81, 738–747.

Larsson, S., Quintana-Sáinz, A., Reimann, A., Nilvebrant, N.-O., Jönsson, L.J., 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. Appl. Biochem. Biotechnol. 84, 617–632.

Maas, R.H.W., Bakker, R.R., Jansen, M.L.A., Visser, D., de Jong, E., Eggink, G., Weusthuis, R.A., 2008. Lactic acid production from lime treated wheat straw by *Bacillus coagulans*: neutralization of acid by fed-batch addition of alkaline substrate. Appl. Microbiol. Biotechnol. 78, 751–758.

Michelson, T., Kask, K., Jõgi, E., Talpsep, E., Suitso, I., Nurk, A., 2006. L(+)-lactic acid producer *Bacillus coagulans* SIM-7 DSM 14043 and its comparison with *Lactobacillus delbrueckii* spp. *Lactis* DSM 20073. Enzyme Microb. Technol. 39, 861–867.

Oliva, J., Sáez, F., Ballesteros, I., González, A., Negro, M.J., Manzanares, P., Ballesteros, M., 2003. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. Appl. Biochem. Biotechnol. 105, 141–153.

Oliva, J.M., Ballesteros, I., Negro, M.J., Manzanares, P., Cabañas, A., Ballesteros, M., 2004. Effect of binary combinations of selected toxic compounds on growth and fermentation of *Kluyveromyces marxianus*. Biotechnol. Prog. 20, 715–720.

Rivera, O.M.P., Moldes, A.B., Torrado, A.M., Dominguez, J.M., 2007. Lactic acid and biosurfactants production from hydrolyzed distilled grape marc. Process Biochem. 42, 1010–1020.

Sreenath, H.K., Moldes, A.B., Koegel, R.G., Straub, R.J., 2001. Lactic acid production from agriculture residues. Biotechnol. Lett. 23, 179–184.

Sun, J.X., Sun, X.F., Sun, R.C., Su, Y.Q., 2004. Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses. Carbohydr. Polym. 56, 195–204.

Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Lidén, G., 1999. Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 87, 169–174.

Van der Pol, E.C., Bakker, R.R., Baets, P., Eggink, G., 2014. By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. Appl. Microbiol. Biotechnol. 98, 9579–9593.

van der Pol, E.C., Bakker, R.R., van Zeeland, A.N.T., Sanchez Garcia, D., Punt, A., Eggink, G., 2015. Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: differences between autohydrolysis, alkaline and acid pretreatment. Bioresour. Technol. 181, 114–123.

Walton, S.L., Bischoff, K.M., van Heiningen, A.R., van Walsum, G.P., 2010. Production of lactic acid from hemicellulose extracts by *Bacillus coagulans* MXL-9. J. Ind. Microbiol. Biotechnol. 37, 823–830.

Zaldivar, J., Ingram, L.O., 1999. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. Biotechnol. Bioeng. 66, 203–210.