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

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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.

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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

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http://dx.doi.org/10.1016/j.biortech.2016.03.037
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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:

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