Bioresource Technology 250 (2018) 429–438

Contents lists available at ScienceDirect

Bioresource Technology

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

Complex effect of lignocellulosic biomass pretreatment with 1-butyl-3-methylimidazolium chloride ionic liquid on various aspects of ethanol and fumaric acid production by immobilized cells within SSF

Anna S. Dotsenko[a,*], Gleb S. Dotsenko[b,1], Olga V. Senko[b], Nikolay A. Stepanov[b], Ilya V. Lyagin[b], Elena N. Efremenko[b], Alexander V. Gusakov[a,b], Ivan N. Zorov[a,b], Ekaterina A. Rubtsova[a]

[a] Federal Research Centre "Fundamentals of Biotechnology", Russian Academy of Sciences, Moscow 119071, Russia
[b] Department of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119991, Russia

ARTICLE INFO

Keywords:
Imidazolium-based ionic liquid [Bmim]Cl
Pretreatment of biomass
Simultaneous enzymatic saccharification and microbial fermentation (SSF)
Immobilized cells
Cryogel of polyvinyl alcohol

ABSTRACT

The pretreatment of softwood and hardwood samples (spruce and hornbeam wood) with 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) was undertaken for further simultaneous enzymatic saccharification of renewable non-food lignocellulosic biomass and microbial fermentation of obtained sugars to ethanol and fumaric acid. A multienzyme cocktail based on cellulases and yeast or fungus cells producing ethanol and fumaric acid were the main objects of [Bmim]Cl influence studies. A complex effect of lignocellulosic biomass pretreatment with [Bmim]Cl on various aspects of the process (both action of cellulases and microbial conversion of hydrolysates to target products) was revealed. Positive effects of the pretreatment with [Bmim]Cl included decreasing the lignin content in the biomass, and increasing the effectiveness of enzymatic hydrolysis and microbial transformation of pretreated biomass. Immobilized cells of both yeasts and fungi possessed improved productive characteristics in the biotransformation of biomass pretreated with [Bmim]Cl to ethanol and fumaric acid.

1. Introduction

Transforming non-food lignocellulosic feedstocks into a variety of commercially important products, such as alcohols and organic acids, is one of the urgent tasks of biotechnology based on renewable resources (Saini et al., 2015). The biomass pretreatment process is a necessary stage of processing lignocellulosic materials because of their recalcitrant structure. The purpose of pretreatment process is to carry out a partial destruction of intermolecular bonds between the components of biomass, making polysaccharides more reactive towards enzymes during the hydrolysis stage followed by transformation of sugars obtained into alcohols and organic acids.

Pretreatment of lignocellulosic biomass with ionic liquids (ILs) appears to be an effective method of changing the supramolecular structure of polysaccharides and improving the effectiveness of their subsequent processing. Imidazolium-based ILs are among the most effective and widely discussed reagents used for pretreatment (Zavrel et al., 2009). However, ILs remaining in the biomass after the pretreatment procedure can influence enzyme activity and microbial productivity. The immobilization of cells can positively impact the tolerance of microorganisms towards various toxic compounds. The resistance of different cells in the immobilized state to the influence of ILs is interesting, but it was not studied previously.

Applicability of the pretreatment method requires maintaining the cellulase activity and microbial productivity at a high level while increasing the biotransformation effectiveness in general, for example, by the use of simultaneous saccharification and fermentation of renewable resources pretreated with ILs.

In this work, experiments on biotransformation of two types of lignocellulosic biomass were carried out: softwood (spruce wood) and hardwood (hornbeam wood). Both the initial materials and those pretreated with IL [Bmim]Cl were used to produce ethanol and fumaric acid as the final products. The influence of [Bmim]Cl on all stages of multistep biotransformation was investigated, including changes in biomass composition after the pretreatment, changes in cellulase activity during enzymatic hydrolysis of biomass and microbial productivity during the transformation of sugars into the target products. Various cells immobilized in cryogel of polyvinyl alcohol were used for simultaneous enzymatic saccharification and microbial fermentation (SSF) of biomass pretreated with IL. Additional data on the effect of two

* Corresponding author.
E-mail address: ansdotsenko@gmail.com (A.S. Dotsenko).
[1] Present address: Department of Molecular Sciences, Swedish University of Agricultural Sciences, P.O. Box 7015, SE-750 07 Uppsala, Sweden.

https://doi.org/10.1016/j.biortech.2017.11.064
Received 3 October 2017; Received in revised form 21 November 2017; Accepted 22 November 2017
Available online 23 November 2017
0960-8524/

other imidazolium-based ILs ([Hmim]Cl and [Omim]Cl) on cellulase activity are also presented.

2. Materials and methods

2.1. Ionic liquids

Imidazolium-based ILs, that is, 1-butyl-3-methylimidazolium chloride ([Bmim]Cl), 1-hexyl-3-methylimidazolium chloride ([Hmim]Cl) and 1-octyl-3-methylimidazolium chloride ([Omim]Cl) were purchased from Sigma-Aldrich (USA) and used as supplied.

2.2. Enzymes

Cellulases of *Trichoderma viride* were supplied from Sigma-Aldrich (USA).

2.3. Microorganisms

Fungi *Mucor circinelloides* F 1627, *Aspergillus terreus* F 728, *Rhizopus oryzae* F1032 were supplied from the Russian National Collection of Industrial microorganisms [http://eng.genetika.ru/service-offer/vkpm/] and thermotolerant yeasts *Saccharomyces cerevisiae* T2 was supplied from the collection of Institute of chemistry VAST (Hanoi, Vietnam).

S. cerevisiae cells were used in free and immobilized forms, other microorganisms were used in immobilized forms. Immobilization of cells in the polyvinyl alcohol cryogel was described previously (Efremenko et al., 2010; Efremenko et al., 2013; Efremenko et al., 2008).

2.4. Lignocellulosic biomass

Hornbeam and spruce wood, representing the examples of hardwoods and softwoods, were used in the experiments. Samples of dried wood sawdust were milled with an activator-type planetary ball mill for 5 min (centrifugal acceleration, 300 m/s^2; rotation frequency, 1290 rpm) to a particle size of 20–80 μm and oven dried at 100 °C during 2 h.

2.5. ILs influence on cellulase activity and productivity of microorganisms

The effects of ILs on cellulase activity towards different substrates were assayed using 1, 5 and 10 g/L of the [Bmim]Cl, [Hmim]Cl or [Omim]Cl. To estimate the [Bmim]Cl effect on the productivity of microorganisms, the biotransformation of glucose to ethanol was carried out in the presence of 10, 50 and 100 g/L of the IL. Conditions for microorganisms' cultivation were the following: 28 °C and 120 h for an anaerobic cultivation of *S. cerevisiae*, *A. terreus,* and *M. circinelloides*; 33 °C and 48 h for an aerobic cultivation of *R. oryzae*. Media composition was described previously (Efremenko et al., 2013). In the case of *S. cerevisiae*, *A. terreus* and *M. circinelloides*, the concentration of ethanol was measured, while in the case of *R. oryzae* the concentration of ethanol and fumaric acid was determined relative to the cultures without the [Bmim]Cl addition.

2.6. Enzyme activity assays

Enzyme activities were assayed by analyzing reducing sugars (RS) produced in the enzymatic reaction using the method of Nelson-Somogyi (Nelson, 1944). Avicelase activity was assayed with 5 mg/mL Avicel PH-101 (Sigma, St. Louis, MO, USA) as a substrate at pH 5.0 (0.1 M Na-citrate buffer) and 40 °C (Gusakov et al., 2005). Enzyme activity towards carboxymethylcellulose (CMC) (Chimmed, Russia) was measured at pH 5.0 (0.1 M Na-citrate buffer), 50 °C and substrate concentration of 5 mg/mL (Sinitsyna et al., 2003). Enzyme activity towards 1 mM *p*-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma, St. Louis, MO, USA) was determined from the initial rate of *p*-nitrophenol formation at pH 5.0 (0.1 M Na-citrate buffer) and 50 °C (Gusakov et al., 2005). One unit of activity corresponded to the enzyme quantity releasing 1 μmol of reducing sugars or *p*-nitrophenol per minute. The activity assays were carried out in triplicates.

2.7. Ethanol and fumaric acid assays

The concentration of ethanol in the media was measured using gas chromatography (gas chromatograph Shimadzu GC-15A, Japan). The concentration of fumaric acid was determined by an enzymatic method using standard reagents provided by Abcam (UK) according to the method specified in the instructions to the kit (Fumarate Assay Kit (Abcam, USA)). The measurements were carried out in duplicates.

2.8. Pretreatment of lignocellulosic materials

The mass ratio of lignocellulosic materials to [Bmim]Cl was 1:4, i.e., the mass fraction of lignocellulosic materials in the mixtures with [Bmim]Cl was 20%; this ratio was chosen on the basis of literary data analysis (Tan and Lee, 2012; Zhang et al., 2017). The calculated amount of the IL was added to a wood sample, the mixture was blended and incubated at 50, 100 or 150 °C for 0.5, 1 or 2 h (for all temperatures). A temperature higher than 150 °C was not used in the pretreatment procedure because it can cause degradation of polysaccharides and [Bmim]Cl (Fredlake et al., 2004; Hyvarinen et al., 2014; Wendler et al., 2012). After pretreatment, the biomass was washed with distilled water to obtain 95–99% removal of the IL. The presence of IL in water was controlled spectrophotometrically from the absorbance of the solution at the wavelength of 210 nm (Le Rouzo et al., 2007). The pretreated biomass was then dried at 100 °C to a constant weight. Experiments were carried out in triplicates. The standard deviations did not exceed 5% according to the results of subsequent enzymatic hydrolysis.

2.9. Biomass composition analysis

The content of cellulose, acid-insoluble lignin, acid-soluble lignin and furan derivatives in the initial and pretreated lignocellulosic biomass was determined according to TAPPI methods (T203-cm-99, 2004; T222-om-02, 2004; T222-om-88, 2004). The content of hemicelluloses was calculated as the remainder from 100%.

2.10. Enzymatic hydrolysis of lignocellulosic biomass

Experiments on hydrolysis of the initial and pretreated with [Bmim]Cl lignocellulosic biomass were carried out with using *T. viride* cellulase preparation (Sigma-Aldrich, USA). The biomass concentration was 50 g/L, the cellulase loading was 10 mg of total protein per 1 g of dry substrate. The hydrolysis was conducted at pH 5.0 (0.1 M Na-citrate buffer) and temperature 28, 37 or 50 °C. Reduced sugars (RS) and glucose produced in the hydrolysis were determined by the Nelson-Somogyi (Nelson, 1944) and the glucose oxidase method (Photoglucose assay kit, Impact, Moscow, Russia), respectively. Experiments were carried out in triplicates.

2.11. Biotransformation of lignocellulosic biomass into ethanol and fumaric acid by free and immobilized cells

Biotransformation of the initial and pretreated lignocellulosic biomass was carried out using *T. viride* cellulases (under the same conditions as described in the preceding section, except for temperature) and microorganisms in immobilized form (*S. cerevisiae*, *A. terreus, M. circinelloides* and *R. oryzae*). The immobilization of cells was conducted in accordance with previously developed and patented procedures (Efremenko et al., 2010, 2008). The cryogel of polyvinyl alcohol