Bioresource Technology 101 (2010) 2755–2761

Contents lists available at ScienceDirect

Bioresource Technology

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

Second-generation bioethanol from eucalypt sulphite spent liquor

Ana M.R.B. Xavier *, Mariana F. Correia, Susana R. Pereira, Dmitry V. Evtuguin

CICECO, Departamento de Química, Universidade de Aveiro, Campus Universitário de Santiago, P-3810-193 Aveiro, Portugal

ARTICLE INFO

Article history:
Received 4 September 2009
Received in revised form 13 November 2009
Accepted 19 November 2009
Available online 31 December 2009

Keywords:
Second-generation bioethanol
Hardwood sulphite spent liquor
Pichia stipitis
Pentoses fermentation

ABSTRACT

The spent liquor from acidic sulphite pulping of *Eucalyptus globulus* (HSSL) is the side product from sulphite pulp production and besides sulphonated lignin contains sugars from degraded hemicelluloses, mainly pentoses. *Pichia stipitis* fermentation of these sugars for bioethanol production was the primary goal of this work. The increasing of HSSL proportion in fermentation media affected negatively the ethanol yield. Thus with 20% of HSSL (v/v) attained maximum ethanol yield was 0.15 g of ethanol by g of sugar consumed ($g_e\ g_s^{-1}$) and with 60% (v/v) only 0.08 $g_e\ g_s^{-1}$. Biological removal of acetic acid from HSSL improved fermentation though the complete removal of acetic acid and polyphenolics (including sulphonated species) by treatment with ion-exchange resins was required for highly successful bioethanol production. Accordingly, the fermentative metabolic pathway of *P. stipitis* has been promoted allowing fair ethanol productivity and yield ($Yp/s = 0.49\ g_e\ g_s^{-1}$) at relatively low maximum of cell growth rate ($\mu_{max} = 0.21\ h^{-1}$).

1. Introduction

Lignocellulosic materials are the main source of renewable biomass possessing a target potential for fuel to heat and power production and also for chemicals or materials feedstocks. There is an increasing industrial interest for replacing petrochemical based products by bio-based ones in order to attain a sustainable economy (Sanchez and Cardona, 2008; Yang, 2007). The rising of oil prices and the limited capacity of oil sources demand the search for renewable sources of energy and also for new technologies able to the production of biofuels in a profitable way (Cardona and Sanchez, 2007). A great focus is being developed to achieve new and non cost-intensive technologies for bioconversion of lignocellulosic renewable resources into biofuels (Lucia, 2008).

Fossil fuels are being replaced by biofuels in different countries as it happens with Brazil bioethanol (Luo et al., 2009). New methodologies for biofuels production, like biodiesel, have been developed in the last years. The European Union sets a goal that each country should achieve 10% biofuel usage of all traffic fuel until 2020 (Zhang, 2008). However this strategy was reviewed, since the so-called "first generation" biofuels were essentially produced from seeds or grains that usually enter in the human or animal food chains. The use of crops for biofuels production contributed for the rise of prices of food all over the world, resulting in social disturbance in many countries. More research in this area is mandatory and for this reason, there is an increasing search for biotechnological generation of energy and sources of energy, including production of biofuels from non-food crops or from vegetable wastes.

Sulphite spent liquors (SSLs) are the side product from acidic sulphide wood pulping and are usually burned, after its concentration to ca. 60% dry solids concentration, for the energy and chemicals regeneration (Casey, 1980). The chemical composition of SSL depends of wood species used for the pulping and this information is essential regarding eventual SSL utilization for different purposes (Bjorsvik and Liguori, 2002; Hoyt and Goheen, 1971; Marques et al., 2009; Plank, 2004). Organic matter of SSLs obtained from acidic sulphite pulping of softwood (SSSL) consists essentially of water-soluble lignosulphonates (sulphonated lignin) and hexoses arisen from hydrolysis of glucomannan (Sjöström, 1993). These liquors are industrially bioprocessed to some extent for ethanol using *Saccharomyces* spp. and/or single cell protein production (Hoyt and Goheen, 1971). SSLs obtained from sulphite pulping of hardwoods (HSSL) did not find some significant applications for biofuels till now.

The major organic components of HSSLs are lignosulphonates (LS) and pentoses arisen from hydrolysis of glucuronoxylan, that also could be used to produce the second-generation biofuel, i.e. bioethanol (Nigam, 2001). Using of HSSL as the raw material to produce added value products fits well to the biorefinery concept invoked to decrease the dependence from fossil resources and to improve the economic sustainability of pulp mills. Moreover, HSSL as a substrate for second-generation biofuels, has an important advantage over the agro-forestry wastes. These wastes require complex chemical or enzymatic hydrolysis, which are one of the process bottle-necks. On contrary, in HSSL the part of lignocellulosic components, namely hemicelluloses, have already suffered a

* Corresponding author. Tel.: +351 234933282; fax: +351 234370084.
E-mail address: abx@ua.pt (A.M.R.B. Xavier).

0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.11.092

hydrolysis step, releasing most of the sugars that could be directly converted into bioethanol. The bioprocessing of HSSL is much more difficult than that of industrially explored SSSL containing essentially hexoses. The main reasons are related to the selection of suitable microorganisms capable to convert selectively pentoses to ethanol and allowing its sustainable production in the presence of concomitant inhibitors in HSSL (formic and acetic acids, furfural, levulinic acid, polyphenolics, etc.). The economically feasible HSSL pre-treatment/purification is another technical problem (Schneider, 1996). Unlike to SSSL, the bioprocessing of HSSL to produce ethanol is much less investigated.

Among different microorganisms, the yeast *Pichia stipitis* has the particular capacity to consume pentoses besides hexoses (Chandel et al., 2009; Nigam, 2001; Sanchez and Cardona, 2008; Schneider, 1996). This yeast is one of the fasters to consume xylose that present in substrate at relatively high concentration and simultaneously attains one of the highest ethanol conversion rates (Alexander et al., 1987). Despite of sensitivity to most of referred inhibitors in HSSL, *P. stipitis* showed promising results on ethanol production while applying the appropriate trial for substrate purification (Chandel et al., 2009; Nigam, 2001; Schneider, 1996).

The present study aimed to evaluate the possibility of second-generation bioethanol production from HSSL using *P. stipitis*. HSSL from acidic sulphite pulping of *Eucalyptus globulus* was examined as pentose-containing substrate for the first time. Eucalypt species is rather common raw material for the production of bleached sulphite eucalypt pulp in South Europe and South Africa, which annual sale market exceed of 1 million tons. Different methodologies of HSSL ennoblement have been tested aiming to promote *P. stipitis* fermentative metabolic pathway: (1) fermentation after previous biological deacidification with yeasts and (2) total purification of substrate from organic acids and polyphenolic compounds by ion-exchange resins treatment. The effect of HSSL ennoblement on the ethanol productivity and yield has been evaluated.

2. Methods

2.1. HSSL supply and pre-treatment

Industrial HSSL from magnesium based acidic sulphite pulping of *E. globulus* was supplied by Caima-Industria de Celulose SA (Constância, Portugal). Pre-evaporated HSSL was collected from inlet evaporator in a set of multiple-effect evaporators to avoid the presence of free SO_2 in liquor. A pre-treatment of HSSL consisted in pH adjustment to 7.0 with NH_4OH followed by aeration with compressed air ($2\,h\,l^{-1}$). The precipitated colloids were filtered off using a 1.0 μm glass microfiber filter (Ahlstrom, grade 131) and the final pH was 5.5.

2.2. Microorganisms and maintenance

P. stipitis NRRL-7124 was generously supplied by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA. The lyophilized preparation was transferred to YM liquid broth and then to YM agar plates with: 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 20 g agar per litre of distilled water. Deacidificant yeasts were supplied by Portuguese Yeast Culture Collection on agar tubes: *Candida tropicalis 3097*, *Candida utilis 2541*, *Kluyveromyces marxianus 3886T*, *Zygosaccharomyces baillii 5167T*, *S. cerevisiae 4072* and *P. anomala 4121T*. All cultures were grown at 28 ± 0.5 °C and maintained at 4 °C on YM agar slants. All media and material were sterilized by autoclaving at 120 °C during 20 min except vitamins solution that has been ultrafiltrated with a sterile 0.2 μm membrane of cellulose acetate.

2.3. Solid fermentations in Petri plates

Specific solid media with pH indicators based on work described by Schüler (1998) have been used. These media were prepared from different solutions as described below:

(i) *Base medium (%w/v):* $(NH_4)_2SO_4$ 0.5; KH_2PO_4 0.5; $MgSO_4{\cdot}7H_2O$ 0.05; $CaCl_2{\cdot}2H_2O$ 0.013 and agar 2.0.
(ii) *Vitamins solution (%w/v):* biotin 0.001; calcium pantothenate 0.08; myo-inositol 4.0; niacin 0.16; pyridoxine hydrochloride 0.16; thiamine hydrochloride 0.16.
(iii) *Oligoelements A solution (%w/v):* H_3BO_3 1.0; KI 0.2; $Na_2MoO_4{\cdot}2H_2O$ 0.4. Oligoelements B solution (%w/v): $CuSO_4{\cdot}5H_2O$ 0.08; $FeCl_3{\cdot}6H_2O$ 0.4; $MnSO_4{\cdot}4H_2O$ 0.8; $ZnSO_4{\cdot}7H_2O$ 0.8; HCl (10^{-3} N) 0.8 (v/v).
(iv) *Indicators (%w/v):* bromocresol green (pK = 4.7) 0.005; bromocresol purple (pk = 6.3) 0.005.
(v) *Carbon sources solutions (%w/v):* glucose 0.15; xylose 2.0; acetic acid 0.6 or 0.8.

All referred solutions were prepared separately and sterilized at 120 °C during 20 min. To mix all components, solutions were heated at 50–60 °C and pH adjusted to 4.0–4.5 or to 5.5–6.0. The final solutions obtained were 4 different agar media: bromocresol green (pH 4.0–4.5) with 6.0 and 8.0 $g\,l^{-1}$ acetic acid and bromocresol purple (pH 5.5–6.0) with 6.0 and 8.0 $g\,l^{-1}$ acetic acid. The growth on selective media was registered every day with a digital camera Olympus 4MP, C4040ZOOM model.

2.4. Liquid fermentations in Erlenmeyer flasks

All liquid fermentation solutions were prepared with HSSL and contained supplementary medium (SM, $g\,l^{-1}$: yeast extract 2.5; $(NH_4)_2HPO_4$ 2.0; $(NH_4)_2SO_4$ 1.0 and $MgSO_4{\cdot}7H_2O$ 0.5); sugars solution and the inoculum in 500 ml Erlenmeyer flasks containing 250 ml of working volume in an incubator operating at 28 °C and 180 rpm.

To assess the inhibition with acetic acid, a synthetic medium (60% v/v) with sugars concentrations similar to those in HSSL was prepared ($g\,l^{-1}$: xylose 27.0; glucose 6.0; arabinose 6.0) and mixed with SM (40% v/v) in the presence/absence of acetic acid ($10.0\,g\,l^{-1}$).

The inoculum was prepared with SM and glucose (1%) and incubated during 15 h before being transferred to fermentations in a proportion of 20% v/v.

Fermentations with increasing HSSL concentrations (0%, 20%, 40% and 60% (v/v)) were supplemented with SM. Additionally to sugars concentration in HSSL, fermentation media were always completed to a final concentration of $2.0\,g\,l^{-1}$ glucose and $20.0\,g\,l^{-1}$ xylose.

Deacidification liquid fermentations were made with 60% (v/v) HSSL solution with the supplements mentioned above.

The sugars fraction of HSSL, isolated using ion-exchange resins device, as described below, was 4-fold concentrated on a vacuum rotor evaporator at 60 °C. Fermentation with *P. stipitis* was accomplished using concentrated sugars fraction (75%, v/v), SM (15%, v/v) and inoculum (10%, v/v).

2.5. Purification of HSSL employing ion-exchange resins

Two consecutive treatments have been carried out using columns packed with cation-exchange (Dowex 50Wx2, mesh 100–200, H^+ form) and with anion-exchange (Amberlite IRA-93, mesh 20–50, OH^- form) resins. In a normal run, after resins activation with 7% HCl (cation-exchange resin) or with 5% NaOH (anion-exchange resin), HSSL was passed firstly through the column with