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Research article

Simultaneously saccharification and fermentation approach as a tool for enhanced fossil fuels biodesulfurization

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ABSTRACT

Biodesulfurization can be a complementary technology to the hydrodesulfurization, the commonly physical-chemical process used for sulfur removal from crude oil. The desulfurizing bacterium *Gordonia alkanivorans* strain 1B as a fructophilic microorganism requires fructose as C-source. In this context, the main goal of this work was the optimization of a simultaneous saccharification and fermentation (SSF) approach using the *Zygosaccharomyces bailii* strain Talf1 crude enzymes with invertase activity and sucrose as a cheaper fructose-rich commercial C-source (50% fructose) towards dibenzothiophene (DBT) desulfurization by strain 1B. The determination of optimal conditions, for both sucrose hydrolysis and DBT desulfurization was carried out through two sequential experimental uniform designs according to the Doehlert distribution for two factors: pH (5.5–7.5) and temperature (28–38 °C), with the enzyme load of 1.16 U/g/L; and enzyme load (0–4 U/g/L) and temperature (28–38 °C), with pH at 7.5. Based on 2-hydroxybiphenyl production, the analysis of the response surfaces obtained pointed out for pH 7.5, 32 °C and 1.8 U/g/L as optimal conditions. Further optimized SSF of sucrose during the DBT desulfurization process permitted to attain a 4-fold enhanced biodesulfurization. This study opens a new focus of research through the exploitation of sustainable low cost sucrose-rich feedstocks towards a more economical viable bioprocess scale-up.

1. Introduction

Air pollution and acid rain are caused by sulfur dioxide emissions from fossil fuel combustion, which is increasing with the modern way of life, hence environmental protection should be ensured to achieve a sustainable development. Moreover, gaseous chemical compounds of sulfur constitute a major health hazard when present in the air (Kampa and Castanas, 2008; Mohebali and Ball, 2008; Murphy et al., 1992; Pope et al., 2002).

Gasoline, diesel and non-transportation fuels account for 75–80% of the total oil refinery products (Babich and Moulijn, 2003), hence the countries find the reductions of sulfur concentration in fuels as the most effective way to decrease the amount of SO_2 emitted into the air and limit its hazard effects (Mohebali et al., 2008). Thus, in response to the increasing concerns with environmental and health effects of SO_x molecules, legislation that requires the reduction of sulfur levels in fossil fuels has become increasingly stricter.

Currently, the hydrodesulfurization (HDS) process has been the industry solution to reduce the sulfur levels on crude oil and its derivatives, using high temperatures and pressures in the presence of molecular hydrogen and complex metal catalysts. However, HDS is not very effective in removing heterocyclic sulfur compounds, such as dibenzothiophene (DBT) and its alkylated derivatives, which can account for up to 70% of the sulfur in petroleum (Alves et al., 2015; Borgne and Quintero, 2003), hence requiring harsher conditions to meet the strict EU sulfur regulations (from 500 to <10 ppm). In fact, this deeper desulfurization, performed with even higher temperatures and pressures, results in an increase of the carbon footprint (>CO_2 release), a rise of production costs associated to the higher energy requirements and, sometimes, in a loss of octane value (Khedkar and Shanker, 2015; Mohebali and Ball, 2008; Srivastava, 2012). In this context and moreover with the increasing worldwide interest towards the production of ultra-low sulfur fuels (ULSF), other more efficient and less expensive desulfurization technologies capable of removing HDS recalcitrant organosulfur compounds, such as oxidative desulfurization, extractive

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desulfurization, adsorptive desulfurization and biodesulfurization (BDS), are emerging (Adegunlola et al., 2012; Javadi and de Klerk, 2012; Srivastava, 2012; Wang et al., 2011).

In the last decades, BDS has won wide attention due to its green processing of fossil fuel towards "zero sulfur" products. In fact, recent studies have demonstrated that coupling BDS with conventional HDS can ensure the cutting down of huge investment incurred in HDS petroleum refineries to generate ultra-low sulfur diesel (Alves et al., 2015; Bandyopadhyay et al., 2013; Kilbane, 2006; Labana et al., 2005; Mukhopadhyaya et al., 2006). BDS is based on the use of microorganisms for the selective removal of sulfur even from the most recalcitrant compounds, at mild operation conditions (atmospheric pressure and moderate temperature), making it energetically cheaper and more eco-friendly, since it will produce lesser greenhouse gases (Monticello, 2000). Nevertheless, this bioprocess has still a few limitations, such as the cost of the culture medium for the biocatalysts production. Thus, in order to reduce the production costs, it is important to search for cheaper carbon sources which can contribute to produce less expensive microbial biomass. Due to their low cost, the utilization of agro-industrial residues as carbon sources is one of the most followed strategies in biotechnology processes (Alves et al., 2008; Alves and Paixão, 2014a; Silva et al., 2013, 2015).

In this study, the desulfurizing microorganism used was the *Gordonia alkanivorans* strain 1B, which metabolizes DBT and its alkyl-DBTs through the 4S pathway (Alves et al., 2005) and, moreover, it is a fructophilic bacterium (Alves and Paixão, 2014b). Hence, the optimization of BDS process using strain 1B requires the availability of fructose to the microorganism. Thus, cheaper alternative carbon sources rich in fructose are the preferred. Sucrose-rich carbon sources, such as molasses could present a very interesting applicability in BDS if sucrose, a disaccharide of glucose and fructose linked via an ether bond between C1 on the glucosyl unit and C2 on the fructosyl unit ($\alpha1\rightarrow\beta2$ bond), could be prior hydrolyzed in its component monosaccharides. This can be achieved either by acid hydrolysis, which requires high temperatures and addition of acids, or by enzymatic hydrolysis using invertases. Invertase (β-fructofuranoside fructohydrolase EC 3.2.1.26) catalyzes the liberation of β-D-fructofuranose from the non-reducing terminus of the β-D-fructofuranosides such as sucrose (Reddy and Maley, 1996).

The enzymes with invertase activity were produced by a novel yeast strain inulinase-producer isolated at LNEG and identified as *Zygosaccharomyces bailii* strain Talf1 (Arez et al., 2014; Paixão et al., 2013). It is well known that invertase activity is associated with inulinase activity (Bonciu and Bahrim, 2011).

Therefore, the main goal of this work was the optimization of DBT desulfurization by *G. alkanivorans* strain 1B using commercial sucrose as carbon source in the presence of enzymes with invertase activity. This SSF approach has several advantages, being the most important the avoidance of end-product inhibition and the decrease in overall operation costs and time of process, as inoculation is not dependent on the prior hydrolysis process (Wingren et al., 2003). However, this process has the disadvantage of optimal pH and temperature for hydrolysis usually differ from those for microbial growth, being crucial to find optimal conditions for the overall biotechnological process. In this context, the determination of optimal conditions for the simultaneous sucrose hydrolysis by Talf1 enzymatic extract and DBT desulfurization by *G. alkanivorans* strain 1B, were carried out using two sequential experimental designs (ED) for two factors (ED1: pH and temperature; and ED2: enzyme load and temperature).

2. Materials and methods

2.1. Chemicals

DBT (99%) was obtained from Acros Organics, 2-hydroxybiphenyl (2-HBP) was from Sigma and dimethylformamide (DMF) was from Riedel de Haën. DBT stock solution is prepared by dissolving 150 mM DBT in DMF. All other reagents were of the highest grade commercially available.

2.2. Microorganisms

In this study, two different microorganisms isolated at LNEG (Portugal) were used: the yeast *Zygosaccharomyces bailii* strain Talf1 (Paixão et al., 2013) and the bacterium *Gordonia alkanivorans* strain 1B (Alves et al., 2005). The *Z. bailii* strain Talf1 is maintained on Yeast Malt Agar (YMA, Sigma) slants at 4 °C and sub-cultured monthly for laboratory routine. *G. alkanivorans* strain 1B is maintained on Tryptic Soya Agar (TSA, Merck) slants at 4 °C and sub-cultured monthly for laboratory routine. Both microorganisms are also maintained at −20 °C by addition of 30% (v/v) glycerol to previously grown cultures in the appropriate culture medium, namely, yeast malt broth (YMB, Sigma) for the yeast; and the sulfur free mineral (SFM) medium, containing 1.22 g/L NH_4Cl, 2.5 g/L KH_2PO_4, 2.5 g/L $Na_2HPO_4{\cdot}2H_2O$ and 0.17 g/L $MgCl_2{\cdot}6H_2O$, supplemented with 0.5 mL/L of a sulfur-free trace elements solution (TES; Alves and Paixão, 2014b), 5 g/L of a selected C-source and 150 μM DBT as S-source for strain 1B.

2.3. Experimental design methodology

A surface response methodology (SRM), based on the Doehlert distribution for two factors (Doehlert, 1970), was used towards two experimental designs (ED1 and ED2). In ED1, the explanatory variables or factors studied and the respective experimental domains tested were: temperature (28–38 °C) and pH (5.5–7.5), maintaining an enzyme load of 1.16 U/g/L (units of invertase activity per g/L of sucrose). In ED2, the factors and the respective experimental domains tested were: temperature (28–38 °C) and enzyme load (0–4 U/g/L of sucrose), maintaining the initial pH at 7.5. For each ED, fourteen experiments (7 conditions in duplicate) were carried out. The responses studied in both EDs were: 2-HBP production and sucrose concentration, at 24 h and 48 h. The relationships between factors and responses were evaluated from surface response results obtained from the fitness of a second order polynomial model to the data set: $Yi = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{12}X_{12} + \beta_{11}X_1^2 + \beta_{22}X_2^2$ where: Yi is the response from experiment i; β are parameters of the polynomial model; X is the experimental factor level (Silva et al., 2013, 2015).

2.4. Enzymatic extract with invertase activity

The crude extract with invertase activity, used for the sucrose hydrolysis in a SSF approach during the DBT desulfurization by strain 1B BDS process, was produced by growing the *Zygosaccharomyces bailii* strain Talf1 in yeast malt broth (YMB, Sigma) supplemented with 25% Jerusalem artichoke juice for 7 days at 25 °C and 150 rpm. The enzymatic extract was previously dialyzed using a dialysis membrane with a cut-off of 10 kDa overnight to remove sulfur sources that can inhibit the desulfurization process. The initial invertase activity of this dialyzed extract was determined as 91.3 U/mL, at the optimal conditions (pH 5.5 and 50 °C), according to Arez et al. (2014).