Industrial Crops & Products 122 (2018) 114–118

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Short communication

Exploring strategies for the use of glycerol in the production of cellulases and xylanases, and the use of these enzymes in the hydrolysis of lignocellulosic biomass

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ARTICLE INFO

Keywords:
Biorefineries
Bioreactor
Second-generation ethanol
Biofuel
Lignocellulosic biomass

ABSTRACT

Considering the growing importance of biofuels in Brazil and throughout the world, and taking into account that glycerol is a surplus byproduct of biodiesel production, the present study aimed to evaluate the production of cellulases and xylanases by *Penicilium echinulatum*, mutant strain S1M29, using different concentrations of glycerol as a carbon source in combination or not with cellulose. Experimental use of 50% glycerol and 50% cellulose showed promising FPA activity, however, this was only approximately 50% of that obtained by previous studies employing only cellulose as a carbon source. More relevant was the fact that the enzyme broth prepared with 50% glycerol and 50% cellulose proved to be efficient in the hydrolysis of lignocellulosic biomasses, especially for elephant grass pretreated by steam explosion.

1. Introduction

With increasing production of biodiesel in Brazil, due to the mandatory requirement of the addition of 2% by volume of biodiesel to diesel oil in 2008 and the subsequent increase to 8% in early 2017, another new problem has emerged—unrefined glycerol. Unrefined glycerol is a byproduct of a transesterification phase of the predominant biodiesel production process (Leoneti et al., 2012; MME, 2017).

Taking into account that the biodiesel market is expected to produce 45,291 million liters of biodiesel in 2020, which will generate about 10% (w/w) glycerol as the main byproduct (Altenergymag.com, 2010), numerous papers have recently been published on the direct utilization of glycerol from biodiesel production. Among the alternative uses of the crude glycerol produced from biodiesel production, the use as animal feedstuff, feedstocks for chemicals (e.g., 1,3-propanediol, citric acid, hydrogen and other lower molecule fuels, poly (hydroxyalkanoates), lipids) and a few other potential applications stand out. Such other applications include use as a high-boiling-point organic solvent to enhance enzymatic hydrolysis of lignocellulosic biomass, a green solvent for organic reactions (Yang et al., 2012), and as a carbon source for microbial growth, a strategy for the production of enzymes such as cellulases (Delabona et al., 2016; Schneider et al., 2016).

Cellulases are an enzymatic complex consisting of endoglucanases, cellobiohydrolases and β-glucosidase, which act synergistically in the deconstruction of cellulose (Martins et al., 2008). Efficient lignocellulosic biomass hydrolysis requires the cooperative action of cellulases, hemicelulases, pectinases and ligninases, as well as accessory enzymes, such as esterases, and proteins, like swollenins (Glass et al., 2013). *Penicillium echinulatum* mutant strain S1M29 is known to be an excellent secretor of cellulases, with cellulose being recognized as the best inducer of the enzymatic complex of cellulases (Reis et al., 2013). However, in a proteomic study with *P. echinulatum* S1M29, Schneider et al. (2016) found that glycerol, a simple carbon source for rapid assimilation, is associated with the expression of some enzymes related to the degradation of lignocellulosic biomass, such as β-glucosidases.

Therefore, the aim of the present work was to evaluate the production of cellulases and xylanases by *P. echinulatum* S1M29 in a stirred-tank bioreactor using different concentrations of pure and residual glycerol (from biodiesel production), in replacement of commercial cellulose. In addition, this study aimed to evaluate the potential of enzymatic broth formulated with glycerol in the hydrolysis of sugar cane bagasse and elephant grass.

2. Material and methods

2.1. Strains

The present study used *P. echinulatum* strain S1M29, which was

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https://doi.org/10.1016/j.indcrop.2018.05.067
Received 24 March 2018; Received in revised form 14 May 2018; Accepted 25 May 2018

obtained from mutant strain 9A02S1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSM 18942) after several steps of mutagenesis (Dillon et al., 2011).

2.2. Medium and cultivation conditions

A total of seven batch cultivations were carried in a stirred-tank bioreactor (New Brunswick, BioFlo®/CelliGen®115). First, pre-inoculation was performed in 500-mL Erlenmeyer flasks, each containing 100 mL of production medium. The flasks were inoculated with a conidial suspension (1×10^5 conidia/mL) and maintained at 28 °C under reciprocal agitation at 180 rpm for 48 h. The composition of the culture medium of the pre-inoculation flasks was the same as that of the bioreactor, for each condition tested, as will be described in sequence.

The production medium of all experiments consisted of 10 g/L of carbon source, 1 g/L of crude yeast extract (Prodex®), 1 g/L of Tween 80®, 5 mL of mineral solution (20 × concentration) and 500 mL of pre-inoculum. The mineral solution, described by Mandels and Reese (1957), was comprised of 14 g/L ($(NH_4)SO_4$), 10 g/L KH_2PO_4, 6 g/L $MgSO_4{\cdot}7H_2O$, 6 g/L $CO(NH_2)_2$, 6 g/L $CaCl_2$, 0.1 g/L $FeSO_4{\cdot}7H_2O$, 0.0312 g/L $MnSO_4{\cdot}H_2O$, 0.028 g/L $ZnSO_4.7H_2O$, and 0.04 g/L $CoCl_2{\cdot}6H_2O$. The carbon sources used in these experiments were the commercial cellulose *Celuflok E*®, glycerol P.A. (Vetec − Sigma Aldrich®) and residual glycerol, a byproduct of a biodiesel plant in South Brazil.

In order to evaluate the influence of glycerol on the production of cellulases, a set of experiments was performed as described in Table 1. The experiments were carried out in a 5-L bioreactor at 28 °C, with sufficient agitation and aeration to maintain a dissolved oxygen concentration of at least 30% for 120 h. Foaming was controlled by the addition of polyglycol antifoam (Fluent Cane 114 Polyglycol®, S. A. Dow Chemical). Samples were collected daily, centrifuged, preserved with sodium azide (0.2 g/L), and refrigerated for subsequent enzymatic analysis. The pH was controlled by the addition of 2 mol/L NaOH or 1 mol/L H_2SO_4 throughout the experiment.

2.3. Determination of cellulose and glycerol content

The residual cellulose content of the samples was determined using the method of Updegraff (1969), which was modified by Ahamed and Vermette (2008), and used 10 mL of culture medium centrifuged at 3000*g* for 20 min. The supernatant was then removed using a pipette. The resulting pellets were resuspended in a mixture (3 mL) of acetic acid and nitric acid (150 mL of 80% acetic acid with 15 mL of pure nitric acid) and boiled for 30 min in a water bath. After cooling and centrifugation at 3000*g* for 20 min, the pellets were washed twice with 10 mL of distilled water on dry filter paper and weighed. The residual pulp was dried at 50 °C for 48 h.

For determining glycerol content, samples were analyzed using high performance liquid chromatography (HPLC, Shimadzu). Samples were filtered through a 0.20-μm nylon membrane and analyzed using an LC-20AD Shimadzu chromatography system, with a DGU-14A mobile phase degasser, a CTO-20A column oven, and a RID-10A detector for the refractive index. The analysis was performed on an Aminex HPX-87H column (Bio-Rad®) at 60 °C, preceded by a Cation-H pre-column and eluted with 5-mmol/L H_2SO_4 mobile phase at a flow rate of 0.6 mL/min.

2.4. Enzymatic assays

Total activity of cellulases (*Filter Paper Activity*, FPA) was measured using Whatman N°. 1 filter paper as a substrate, as described by Camassola and Dillon (2012a, 2012b). Endoglucanase activity was determined according to Ghose (1987), using 2% (w/v) carboxymethyl cellulose in 0.05 mol/L sodium citrate buffer (pH 4.8). The activity of β-glucosidase was measured using 0.4% (w/v) of p-nitrophenyl-β-D-glucopyranoside (Daroit et al., 2008). Activities of the xylanases were determined as described by Bailey et al. (1992), using 1% (w/v) of xylan (from oat spelts). The concentrations of reducing sugars were estimated using the dinitrosalicylic acid method described by Miller (1959).

2.5. Enzymatic hydrolysis and analysis of sugars

Enzymatic hydrolysis was carried out according to Menegol et al. (2014) using sugar cane bagasse (Camassola and Dillon, 2012a, 2012b; Hartmann, 2017) and elephant grass (Scholl et al., 2015a), both *in natura* and pretreated by steam explosion. The sugar cane bagasse was provided by the sugar and ethanol industry through Usina Vale do Rosario (Santa Elisa Vale Conglomerate), Morro Agudo, Sao Paulo (SP), Brazil. Samples of elephant grass were obtained in Nova Petrópolis, RS, Brazil. The sugar cane bagasse was pretreated with steam at 200 °C for 7 min (average pressure of 15 atm), followed by gradual decompression. The elephant grass was also pretreated with steam at 190 °C for 8 min (average pressure of 15 atm), followed by gradual decompression. The sugar cane *in natura* consists of approximately 33.9% cellulose, 15.2% hemicellulose, and 29.1% lignin. The elephant grass *in natura* consists of 35.9% cellulose, 22.4% hemicellulose, and 20.8% lignin. The pretreated sugar cane bagasse consists of approximately 49.8% cellulose, 22.1% hemicellulose, and 23.5% lignin. The pretreated elephant grass consists of approximately 58.5% cellulose, 10.9% hemicellulose, and 21.6% lignin. For enzymatic hydrolysis, an enzymatic broth of *P. echinulatum* mutant strain S1M29 was used. All experiments were carried out in triplicate at 50 °C using an enzyme loading of 15 FPU/g total solids and a substrate concentration of 5% (w/v) in 50-mmol/L pH 4.8 acetate buffer. Reaction aliquots were withdrawn at 24 h of hydrolysis and analyzed by HPLC. Quantification was effected by external standardization based on calibration curves for

Table 1
Experiments performed with *Penicillium echinulatum* S1M29, in bioreactor, varying the concentrations of glycerol and cellulose.

Experiment/Relation of glycerol-cellulose	Glycerol[a] (g/L)	Cellulose[b] (g/L)	Observation
1 (100%–0%)	10	0	Without pH control
2 (0%–100%)	0	10	Without pH control
3 (20%–80%)	2	8	Without pH control
4 (80%–20%)	8	2	Without pH control
5 (50%–50%)	5	5	Without pH control
6 (50%–50%)	5	5	– Medium optimized with 5 g/L of sucrose, 5 g/L of wheat bran and 2 g/L of soybean meal − pH controlled at 6.0 (Reis et al., 2013)[c]
7 (50%–50%)	5	5	– Medium optimized with 5 g/L of sucrose, 5 g/L of wheat bran and 2 g/L of soybean meal – pH controlled at 6.0 (Reis et al., 2013)[c]

[a] In experiments 1–6, glycerol P.A was used. In experiment 7, residual glycerol was used.
[b] The commercial cellulose used in these experiments was *Celuflok E*®.
[c] According to Reis et al. (2013).