Process Biochemistry 63 (2017) 169–176

Contents lists available at ScienceDirect

Process Biochemistry

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

Itaconic acid production from wheat chaff by *Aspergillus terreus*

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ARTICLE INFO

Keywords:
Aspergillus terreus
Itaconic acid
Lignocellulose
Wheat chaff
Alkaline pretreatment

ABSTRACT

Biotechnologically produced itaconic acid is an important building block for the chemical industry and still based on pure carbon sources, detoxified molasses or starch hydrolysates. Changing these first generation feedstocks to alternative renewable resources of a second generation implies new challenges for the cultivation process of the industrial itaconic acid producer *Aspergillus terreus*, which is known to be very sensitive towards impurities. To select a suitable pretreatment method of a second generation feedstock, the influences of different hydrolysate components, like monosaccharides and sugar degradation products, were tested. Particular the impact of those components on itaconic acid yield, productivity, titer and morphology was investigated in detail. Wheat chaff was used as lignocellulosic biomass, which is an agricultural residue. An alkaline pretreatment method with sodium hydroxide at room temperature and a subsequent enzymatic saccharification at pH 4.8 at 50 °C with 10 FPU/$g_{Biomass}$ Biogazyme 2x proved to be very suitable for a subsequent biotechnological production of itaconic acid. A purification by a cation exchanger of the wheat chaff hydrolysate resulted in a final titer of 27.7 g/L itaconic acid with a yield of 0.41 g/$g_{total\ sugar}$.

1. Introduction

Biotechnologically produced itaconic acid is an important building block for the chemical industry and replaces petrochemical-based monomers like acrylic or methacrylic acid for polyesters [1,2]. The unsaturated dicarboxylic acid is industrially produced with the filamentous fungus *Aspergillus terreus* [3], whereby glucose and sucrose give the highest yields [4]. With pure glucose, final titers of up to 160 g/L itaconic acid can be reached [5–7]. If crude molasses or other complex substrates are employed, purification by ion exchanger or ferrocyanide is needed, because *A. terreus* is very sensitive to impurities [8–11]. Especially a manganese concentration > 3 ppb influences the morphology of *A. terreus* negatively and leads to dispersed hyphae with a decreased itaconic acid yield [6]. High yields of 55–59% can only be achieved with small and frayed pellets, lower rates exhibits by loose mycelium or compact pellets [51]. Moreover, starch-based hydrolysates have been successfully used for itaconic acid production [12–15].

The usage of lignocellulose based feedstocks is significantly more difficult, due to the complex structure of the substrate consisting of cellulose, hemicellulose and lignin [16]. Particularly so far unused and low-cost agricultural feedstocks, like wheat chaff, which are not in competition with food and feed production, provide alternative renewable resources for itaconic acid production. Wheat chaff accrues 1–2 t/ha [17] and consists of 32% cellulose, 24% hemicellulose and 16% lignin as main components [18]. To convert cellulose and hemicellulose into fermentable sugars, a pretreatment and a hydrolysis, preferable an enzymatic one, is necessary [19].

Different pretreatment methods are described in literature, which are reviewed by Hendriks and Zeeman [20] or Agbor et al. [21]. An ideal pretreatment method disrupts the lignocellulose structure, makes it accessible for enzymes and avoids the formation of sugar degradation products or other inhibiting components for the subsequent hydrolysis and fermentation [21]. Inhibitors like weak acids, furan derivates or phenolic components can occur due to harsh condition, caused by high temperatures or low pH-values [22]. These inhibitors have to be actively removed for itaconic acid production [10,23,24] or the tolerance of *A. terreus* needs to be increased by mutagenesis [14,25]. Thereby enzymatic hydrolysis and fermentation can be performed as a separated hydrolysis and fermentation (SHF) or a simultaneous saccharification and fermentation (SSF) [26]. Systematic studies, which identify and quantify the inhibitory components in hydrolysates for itaconic acid production, are lacking in literature [27].

The aim of this study was to depict a process configuration for itaconic acid production from lignocellulose without complex purification, detoxification or mutagenesis of *A. terreus*. To estimate the influence of different hydrolysate components on the itaconic acid production with the fungus *A. terreus*, the utilization of different types of monosaccharides; and the influence of sugar degradation products and enzyme formulation, were examined individually. Based on these findings a suitable pretreatment method was selected and a SHF and

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http://dx.doi.org/10.1016/j.procbio.2017.08.010
Received 11 June 2017; Received in revised form 2 August 2017; Accepted 14 August 2017
Available online 19 August 2017
1359-5113/

SSF were carried out.

2. Material and methods

2.1. Wheat chaff

Wheat chaff of the species Elixer was collected in Adenstedt (Germany) in 2014 and characterised by the method of the National Renewable Energy Laboratory [28]. 1 g wheat chaff harvested in 2014 consisted of 0.37 g glucose, 0.19 g xylose, 0.03 g arabinose, 0.01 g galactose and 0.01 g rhamnose, 0.23 g acid insoluble residues, and had a dry weight of 0.92 g.

2.2. Alkaline pretreatment of wheat chaff

Wheat chaff was ground with a knife mill (Grindomix GM 200, Retsch, Germany) and afterwards with an ultra-centrifugal mill (ZM 200, Retsch, Germany) to a final particle size ≤0.75 mm at 18,000 rpm. For alkaline pretreatment 60 g of the milled wheat chaff was mixed on a rotary shaker at 60 rpm at room temperature with 540 mL of a 0.25 M sodium hydroxide solution in a 1 L-flask for 3 days. The pretreated wheat chaff was separated by centrifugation at 4600*g* for 30 min and washed with 540 mL water. This washing step was repeated four times to remove acetic acid. The wheat chaff was dried at 45 °C for 2 days.

2.3. Enzymatic hydrolysis

The enzyme mixture Biogazyme 2x was provided by ASA Spezialenzyme (Wolfenbüttel, Germany). Enzyme activities were determined based on Ghose [29] at optimum temperature of 50 °C and a pH of 4.8 for the cellulase. The protein content was measured by a Bradford protein assay [30].

The hydrolysis was done with 10 FPU/$g_{Biomass}$ and a solid content of 10% (w/v) pretreated wheat chaff in water. 250 mL shake flasks with working volume of 100 mL were used at 50 °C and 120 rpm for 5 days. The pH was adjusted to pH 4.8 with 0.5 M H_2SO_4.

2.4. Evaporation and purification of hydrolysate

To separate the wheat chaff residual from the hydrolysate, it was centrifuged for 30 min at 4600*g*. The solid was discarded and the supernatant, the hydrolysate, was stored openly in a drying oven at 105 °C for 4 h. The volume of the hydrolysate was halved by the evaporation of water and consequently the total sugar concentration was doubled. Due to this evaporation step, the enzymes and other proteins of the hydrolysate denatured. Crude wheat chaff hydrolysate was obtained by removing the precipitated solid by centrifugation at 4600*g* for 30 min.

To reduce the cation concentration of the crude wheat chaff hydrolysate, it was passed through a column (450 mm × 25 mm) containing a cation exchange resin (Dowex 50 W-X8, 100/200-mesh, Sigma-Aldrich, USA). The pH-value of the detoxicated hydrolysate dropped down to pH 1. The pH-value of the detoxicated hydrolysate was adjusted to pH 3.1 with 4 M NaOH and sterile filtered.

2.5. Microorganism

The filamentous fungus *Aspergillus terreus* DSM 23081 was used. It is a self-isolated strain and is available at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was stored in the form of spores at −80 °C as 50% (v/v) glycerol stock culture.

2.6. Inoculum preparation

Spores of surface cultures were gained from modified Czapek-Dox-Agar plates (30 g/L sucrose, 15 g/L Agar-Agar, 3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.0005 g/L $CaCl_2{\cdot}2H_2O$, 0.0005 g/L $ZnSO_4{\cdot}7H_2O$ and 0.0005 g/L $CuSO_4{\cdot}5H_2O$), which were incubated at 30 °C. After 4 days spores were harvested with a 0.9% (w/v) NaCl solution and the number of spores was determined using a Thoma counting chamber at a Zeiss microscope (Axioplan, Carl Zeiss AG, Germany).

These spores from surface cultures were used for cultivation in a 1.5-L bioreactor at 33 °C and pH 3.1 with a concentration of $1{\cdot}10^6$ spores/mL. The pH was not controlled during the cultivation, after 2 days the pH was shifted to pH 5, by adding a 5 M NaOH manually [5]. Submerse spores were formed, which were separated by a sieve (mesh width 80 µm) from the mycelia after 5 days. Afterwards the suspension was centrifuged at 4600*g* for 30 min, and washed twice with 0.9% (w/v) NaCl solution. The concentration of the spore suspension was determined by a Thoma counting chamber at a Zeiss microscope (Axioplan, Carl Zeiss AG, Germany) and stored at 4 °C. These submerse spores were used for all cultivations.

2.7. Media composition

Pure sugars were obtained by Roth (Germany – D-glucose and L-arabinose), Sigma-Aldrich (USA – D-xylose and L-rhamnose) or ThermoFisher (Germany – D-galactose). The production media contained 100 g/L sugar, 0.1 g/L KH_2PO_4, 3 g/L NH_4NO_3, 1 g/L $MgSO_4{\cdot}7H_2O$, 5 g/L $CaCl_2{\cdot}2H_2O$, 1.67 mg/L $FeCl_3{\cdot}6H_2O$, 8 mg $ZnSO_4{\cdot}7H_2O$ and 15 mg/L $CuSO_4{\cdot}5H_2O$. The media components were p.a. quality and purchased from Merck (Germany), Sigma-Aldrich (USA) or Roth (Germany). Stock solutions of all media components were prepared separately and the pH-value was adjusted to pH 3.1 for all solutions with 0.5 M H_2SO_4, excepting the $CaCl_2$ solution. The solutions were autoclaved for 20 min at 121 °C. The $FeCl_3$ solution was not autoclaved but sterile-filtered.

2.8. Cultivation

The experiments were inoculated with a concentration of $1{\cdot}10^6$ spores/mL and carried out at 33 °C. The initial pH of 3.1 was not corrected during the cultivation.

For small scale experiments, round 96-well microtiter plates (Sarstedt, Germany) were used at 950 rpm (2.5 mm shaking diameter, Kisker, Germany). A working volume of 100 µL was used. The two outer rows of the plate were filled with sterile water and were not used for cultivation. The plate was covered with a lid and sealed with parafilm [31]. After 4 days samples were taken.

Experiments using artificial wheat chaff hydrolysate, SHF and SSF were performed in 250 mL shake flasks with three baffles and a filling volume of 100 mL at 120 rpm (50 mm shaking diameter). Evaporation was compensated by adding sterile water at every sampling [32]. Samples were taken regularly during cultivation. For the SSF a solid content of 10% (w/v) alkaline pretreated wheat chaff and 10 FPU/$g_{Biomass}$ Biogazyme 2x were used.

Cultivations were carried out in three replicates, whereby one replicate was used for microscopic examination.

2.9. Analytical methods

All samples were centrifuged at 21,000*g* for 25 min at 20 °C and the supernatant was used for further analysis. To analyze the concentrations of organic acids, a Shimadzu (Shimadzu Corp., Japan) HPLC with a HPX-87H column (BioRad, Germany) at 40 °C and a refractive index detector and UV detector at 210 nm was used. A 5 mM H_2SO_4 solution at a flow rate of 0.6 mL/min was used as mobile phase.

Monosaccharides were analyzed by a HPAEC-PAD system of Thermo Scientific (IC 5000, Thermo Scientific, USA) with a CaboPac PA20 column at 30 °C (3 × 150 mm, 4.5 µm, Thermo Scientific, USA)