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Ninety six well microtiter plate as microbioreactors for production of itaconic acid by six *Aspergillus terreus* strains☆

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ABSTRACT

Itaconic acid (IA) is a building block platform chemical that is currently produced industrially from glucose by fermentation with *Aspergillus terreus*. However, lignocellulosic biomass has the potential to serve as low cost source of sugars for production of IA. Previously, 100 *A. terreus* strains were evaluated for production of IA from pentose sugars in shake-flasks. Six selected strains were then investigated for IA production in shake-flasks. But none of the strains grew and produced IA using biomass hydrolyzates. In order to study the factors inhibiting fungal growth and IA production, we have evaluated these six strains for sugar utilization and IA production from glucose, xylose, arabinose, mixed sugars, and both dilute acid and liquid hot water pretreated wheat straw hydrolyzates in microtiter plate (MTP) microbioreactors at 100 µL scale. The results clearly indicate that MTP is very useful as a convenient, reliable and affordable platform to investigate the reasons for inhibition of growth and IA production by the *A. terreus* strains and should greatly aid in strain development and optimization of IA production by the fungal strains.

1. Introduction

In recent years, shaken cultivation of microorganisms in microtiter plate (MTP) has received increased attention as an alternative to shake flask experiments (Funke et al., 2009; Sohoni et al., 2012; Kosa et al., 2017). Shaken MTP is easy to operate with savings of time, labor, medium and other costs especially for optimizing multiple process parameters for cultivation of microbes in volumes ranging from µL to mL scale (Hevekerl et al., 2014; Long et al., 2014; Shin et al., 2017).

Itaconic acid (IA) is an unsaturated 5- carbon dicarboxilic acid which is formed via decarboxylation of *cis*-aconitic acid by the enzyme *cis*-aconitate decarboxylase (Winskill, 1983; Bonnarme et al., 1995; Okabe et al., 2009). It is produced from glucose by *Aspergillus terreus* as a secondary metabolite. IA has broad applications as a platform chemical for the manufacture of fiber, paint, cleaning products, herbicides, and in the pharmaceutical industry (Saha, 2017; Robert and Friebel, 2016). Polyitaconic acid, a water-soluble polymer has been suggested as an attractive replacement for the petroleum-based polyacrylic acid, with wide range of applications, including super-absorbents, anti-scaling agents in water treatments, co-builders in detergents and dispersants for minerals in coatings (Willke and Vorlop, 2001; Choi et al., 2015). However, the production cost of IA needs to be lowered in order for its widespread applications to become a reality. In 2004, U.S. Department of Energy identified IA as one of the top 12 value-added chemicals that have the potential to be produced from lignocellulose based feedstocks (Werphy and Peterson, 2004). However, there is very little literature available on the use of pentose sugars (xylose, arabinose) for its production (Kautola et al., 1985; Kautola, 1990) and even less on the use of lignocelluloses biomass hydrolyzates (Krull et al., 2017).

Recently, we evaluated 100 *A. terreus* strains for IA production from pentose sugars in shake-flasks and found that 20 strains produced IA in promising quantities from xylose and arabinose and performed time course studies on IA production using the top six selected strains (Saha et al., 2017a). It was observed that none of these strains were able to grow on dilute acid pretreated and enzymatically hydrolyzed wheat straw hydrolyzate prepared by the procedure described previously (Saha et al., 2011). In order to determine the factors responsible for inhibition of growth and production of IA by *A. terreus* strains, we were interested in developing a 96-well MTP based fermentation method for IA production. In the current study, the production of IA by the six *A. terreus* strains in 96-well MTP from glucose, xylose, arabinose and their mixture as well as both dilute acid and liquid hot water pretreated and enzymatically saccharified wheat straw hydrolyzates at 100 µL scale is

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reported. Finally, in an effort to better understand the source of inhibition, the effects of certain metals and inhibitory compounds on growth and IA production were also explored using this culture format.

2. Materials and methods

2.1. Fungal strains and inoculum preparation

Five *A. terreus* strains (NRRL 1960, 1961, 1962, 1972 and 66,125) were supplied by ARS Culture Collection, Peoria, IL. One strain (DSM 23081) was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). The stock cultures were maintained on Czapek-Dox (Merck) agar plates. The conidiospores were collected from the 7-day culture on Czapek-Dox-agar plates incubated at 30 °C by shaving and extracting spores with sterile deionized water containing 0.04% (v/v) Tween 80. The spore suspension was diluted with sterile deionized water to a final concentration of 1×10^6 spores per mL for inoculation. To improve consistency of inoculums, the volume of spores introduced was always 20 μL of the 100 μL fermentation.

2.2. Cultivation of A. terreus strains on glucose, xylose and arabinose

The optimized medium composition described by Hevekerl et al. (2014) was used. It contained 0.8 g KH_2PO_4, 3 g NH_4NO_3, 1 g $MgSO_4{\cdot}7H_2O$, 5 g $CaCl_2{\cdot}2\ H_2O$, 1.67 mg $FeCl_3{\cdot}6H_2O$, 8 mg $ZnSO_4{\cdot}7H_2O$, and 15 mg $CuSO_4{\cdot}7H_2O$ per L. Each pentose sugar (xylose, arabinose) along with glucose and their mixture (1:1:1 or 4:3:1) was used at 80 g/L. Sugars and all other components were added from sterile stock solutions. The pH of the medium without $CaCl_2$ was adjusted to 3.1 with 0.5 M $H_2\ SO_4$ before inoculating the spore preparation for each strain. MTP cultivations was performed with 100 μL medium in a 96-well (400 μL) Nunc Edge MTP (ThermoFisher Scientific, Waltham, MA, USA) with sterile deionized water added to the surrounding moat, lidded, and wrapped in parafilm. MTP cultures were incubated in a thermoshaker incubator (MB 100-4A, Allsheng Inst. Co., Hangzhou City, China) at 33 °C and 950 rpm for up to 10 days (Hevekerl et al., 2014). The pH was not controlled during fermentation. For time course studies, six sets of replicate plates were incubated. At a specific time interval, one plate was withdrawn from the plate shaker, centrifuged using a centrifuge (Model 5430R, Eppendorf North America, Hauppauge, NY, USA) with a rotor for MTPs at 2000 rpm for 10 min and the supernatant solutions were analyzed for sugar utilization and IA production. All experiments were performed in triplicate. The sugars and other medium components were obtained from Sigma Chemical Co., St. Louis, MO, USA. Each sugar was dissolved in deionized water and passed through a column (440 × 45 mm) of Dowex 50W-X8 (100/200 mesh) (Bio-Rad Laboratories, Inc., Hercules, CA, USA) cation exchange resin to remove any manganese present (Karaffa et al., 2015).

2.3. Preparation of wheat straw hydrolyzate

The dilute acid pretreated and enzymatically saccharified wheat straw hydrolyzate (DAWSH) was prepared by the procedure described in detail previously (Saha et al., 2011). Briefly, ground wheat straw (WS, 150 g/L) was slurried in 0.75% (v/v) H_2SO_4 and pretreated in rotating stainless steel reactors at 160 °C for 10 min. The enzymatic saccharification of the pretreated WS was performed at pH 5.0 and 45 °C for 72 h using a cocktail of commercial cellulase, β-glucosidase and xylanase preparations (Saha et al., 2017b). The liquid hot water pretreatment of WS (150 g/L) was carried out at 200 °C for 5 min and enzymatic saccharification was performed exactly under the same conditions described.

2.4. Analytical procedures

Sugars (glucose, xylose, arabinose), organic acids (IA, propionic acid), hydroxymethyl furfural (HMF) and furfural were quantified by using high-performance liquid chromatography (HPLC). A Shimadzu Prominence Series (Shimadzu America, Inc., Columbia, MD) HPLC system was used. Two columns (Aminex HPX-87P column, 300 × 7.8 mm with deashing and Carbo-P guard cartridge) and Aminex HPX 87H column, 300 × 7.8 mm with Cation H microguard cartridge) were used for analysis of sugars and organic acids, respectively. The Aminex HPX 87P column was maintained at 85 °C and the sugars were eluted with Milli-Q filtered water (Millipore Corp., Bedford, MA) at a flow rate of 0.6 mL/min. The Aminex HPX 87H column was maintained at 65 °C and the sugars and organic acids were eluted with 5 mM H_2SO_4 prepared using Milli-Q filtered water at a flow rate of 0.5 mL/min. Peaks were detected by refractive index or UV absorption at 210 nm and were identified and quantified by comparison with authentic standards. Propionic acid (1%, w/v) was used as internal standard in order to estimate the liquid lost during the shaken aerobic fermentation at 33 °C. The elemental analysis of DAWSH was performed using a Perkin-Elmer (Waltham, MA) Optima 7000DV Inductively-Coupled Plasma-Optical Emission Spectrometer (ICP-OES) by the procedure described by Bakota et al. (2015). The pH of the medium after fermentation in MTP was visually analyzed by removing 20 μL of centrifuged fermentation broth and placing it in another MTP. Then 20 μL of 0.04% (w/v) thymol blue in water was added. Yellow indicates a pH of 3.1 while red indicates a pH drop to ~2.0 (Fig. 1). Growth in MTP was photographed for qualitative comparison purposes.

2.5. Statistical analysis

IA data were statistically analyzed by using SigmaPlot® 12 (Systat Software, Inc., San Jose, CA, USA). Triplicate fermentations with identical conditions were conducted separately over six MTP batches. A single factor ANOVA was conducted using IA concentration as the measured response to test for significant differences between batches ($p < 0.05$). Standard deviation was < 4% of the average IA concentration across all six batches. Reported values are means ± standard deviations.

3. Results and discussion

3.1. Sugar consumption and itaconic acid production by A. terreus strains from glucose, xylose and arabinose in microtiter plates

The time courses of sugar utilization and IA production from glucose, xylose and arabinose by six *A. terreus* strains were investigated at 100 μL scale in MTP. The data is presented in Fig. 2. As expected from previous shake-flask studies (Saha et al., 2017a), more IA was produced

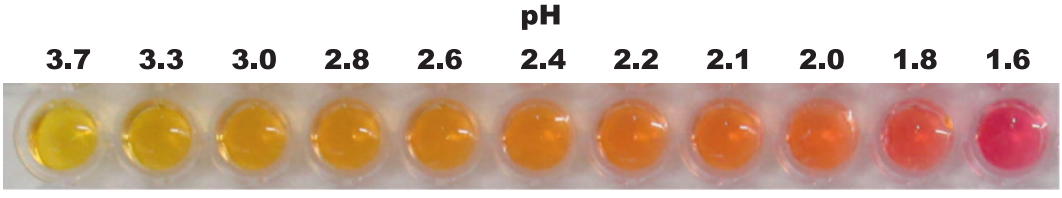

Fig. 1. pH change indication from pH 3.7 to 1.6 using thymol blue as indicator.

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