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Continuous aryl alcohol oxidase production under growth-limited conditions using a trickle bed reactor

Oscar Pardo-Planas[a], Hasan K. Atiyeh[a], Rolf A. Prade[b], Michael Müller[c], Mark R. Wilkins[a,*]

[a] Department of Biosystems and Agricultural Engineering, 111 Agriculture Hall, Oklahoma State University, Stillwater, OK 74078, USA
[b] Department of Microbiology and Molecular Genetics, 307 Life Sciences East, Oklahoma State University, Stillwater, OK 74078, USA
[c] Prüf-und-Forschungsinstitut Pirmasens, Marie-Curie-Straße 19, 66953 Pirmasens, Germany

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ABSTRACT

An *A. nidulans* strain with a pyridoxine marker was used for continuous production of aryl alcohol oxidase (AAO) in a trickle bed reactor (TBR). Modified medium with reduced zinc, no copper, and 5 g/L ascorbic acid that reduced melanin production and increased AAO productivity under growth limited conditions was used. Two air flow rates, 0.11 L/min (0.1 vvm) and 1.1 L/min (1.0 vvm) were tested. More melanin formation and reduced protein productivity were observed with air flow rate of 1.1 L/min. Three random packings were used as support for the fungus inside the TBR column, two of which were hydrophobic and one which was hydrophilic, and three different dilution rates were tested. The use of GEA BCN 030 hydrophobic packing resulted in greater AAO yield and productivity than the other packings. Increasing dilution rates favored melanin formation and citric, lactic and succinic acid accumulation, which decreased AAO yield and productivity.

1. Introduction

Industrial enzyme production is currently carried out using submerged fermentation (SmF) technology, in which microorganisms are grown in large agitated vessels containing liquid media (Couto and Toca-Herrera, 2007). SmF allows good process control, continuous production, and is well established as a commercial scale technology. Disadvantages include limitation of mass transfer due to increased apparent viscosity paired to mycelia growth, shear stress to microorganisms, and a challenging product recovery from the microorganism-broth mixture (Couto and Toca-Herrera, 2007; Krishna, 2005). Solid state fermentation (SSF) can overcome these disadvantages by growing the microorganisms in an environment with little or no free water. In addition, SSF presents lower capital and operational costs due to lower volume reactors and water and energy demands. However, there are challenges associated with control of process conditions such as heat, moisture and aeration, and the absence of continuous production methods (Couto and Toca-Herrera, 2007; Krishna et al., 2005).

Another approach uses a trickle bed reactor (TBR) that combines characteristics of both SmF and SSF. In TBRs, there is free liquid media but in lower amounts than in SmF and the energy requirement is lower because no agitation is needed: media is pumped to the top of the column and it trickles down the bed where the microorganism grows. Similar to SSF, the cells grow on solid surfaces as a biofilm, which are considered a more natural environment that results in higher resistance to antimicrobial agents, shear stress and environmental changes (Couto, 2007). TBR technology, contrary to SSF, does permit continuous production, and the product stream is a cell-free broth, which reduces the complexity of recovery processes. Unfortunately, the aggressive growth of fungal strains that are often used for enzyme production causes clogging issues in the TBR system. A solution to this problem is the inclusion of an auxotrophic marker in the fungal genotype to control vegetative growth. This technology was demonstrated using an *A. nidulans* cell factory with a pyridoxine marker in a TBR with lava rocks as the solid bed support for production of xylanase (Müller et al., 2015). When pyridoxine was not included in the media recipe, the cell factory was unable to grow and clogging issues were prevented (Müller et al., 2015). On the negative side, continued pyridoxine deprivation caused a pH drop, increased melanin production, and reduction of enzyme yields. Periodic additions of pyridoxine mitigated these issues (Müller et al., 2014). Two other studies used a similar *A. nidulans* cell factory with a pyridoxine marker for production of a different enzyme, an aryl alcohol oxidase (AAO) (Pardo-Planas et al., 2017a; Pardo-Planas et al., 2017b). In these studies, pyridoxine was continuously present in the medium at very low levels to limit growth without halting enzyme production, eliminating the need for periodic pyridoxine replenishment. This AAO-producing strain, however, resulted in much higher melanin production when cultured in a TBR than the xylanase-

* Corresponding author at: Industrial Agricultural Products Center, University of Nebraska–Lincoln, 211 L.W. Chase Hall, Lincoln, NE 68583, USA.
E-mail address: mwilkins3@unl.edu (M.R. Wilkins).

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producing strain (Pardo-Planas et al., 2017a). High melanin production negatively affected enzyme production due to lower substrate availability and increased operational problems related to melanin deposition in the column and tubing. Melanin formation with *A. nidulans* is regulated by the enzyme tyrosinase (Held and Kutzner, 1990). Reducing the concentration of trace elements (especially copper and zinc) in the medium, as well as adding ascorbic acid as a tyrosinase inhibitor, reduced melanin formation and had a positive effect of enzyme production (Pardo-Planas et al., 2017a).

Several factors can affect the performance of TBRs in bioprocessing, some of which are specific to this type of reactor configuration, such as the selection of packing or liquid recirculation rate, and others that are common to any bioreactor operation, such as dilution rate. The selection of the type of solid support in the packed bed aims to obtain a high surface area to allow growth of the microorganism and proper contact between liquid and gas phases, sufficient bed porosity, low cost and durability, and low pressure drops (Ranade et al., 2011; Tchobanoglous and Burton, 1991). The packed bed can be comprised of random rings or can be a continuous structure often referred to as structured packing. Random packing rings are usually less expensive, but offer higher pressure drops and lower mass transfer efficiencies than structured packing (Seader ad Henley, 2006). An important challenge of TBRs is to achieve a homogenous liquid distribution across the bed, ensuring proper wetting of the biocatalyst and avoiding channeling. The liquid recirculation rate can be crucial for reactions in which mass transfer is limiting, such as conversion of gas substrates by microorganisms (Devarapalli et al., 2016). A study that evaluated the gas-liquid mass transfer in reactors for syngas fermentation concluded that the mass transfer in a TBR decreased with higher recirculation rates due to an increase in liquid hold-ups (Orgill et al., 2013). Dilution rates, on another hand, are important for any bioreactor configuration because they control the overall retention time of the liquid media in the system. Higher dilution rates lead to higher growth rates in systems without growth limitations due to the increased availability of substrate, and potentially higher productivities until the kinetic limitations of the culture are reached. A previous study using an *A. nidulans* cell factory with a pyridoxine marker for xylanase production investigated the effect of recirculation rate on the performance of a TBR (Müller et al., 2015). The authors concluded that lowering the recirculation rate had a positive effect on xylanase production due to increased contact time between the liquid media and the fungus (Müller et al., 2015). However, the effects of packing selection and dilution rate on the process were not investigated.

In previous work, the effect of pyridoxine on AAO production (Pardo-Planas et al., 2017b) and methods to prevent melanin production by an *A. nidulans* cell factory (Pardo-Planas et al., 2017a) were investigated. The objective of the present study was to investigate the effect of the operational parameters dilution rate, packing, and air flow rate for a stable aryl alcohol oxidase (AAO) production process in a TBR system using a growth-limited culture of the same *A. nidulans* cell factory investigated previously. AAO is an enzyme with potential application in lignin degradation (Pardo-Planas et al., 2017a; Pardo-Planas et al., 2017b). Parameters determined in previous work were used in the present study (Pardo-Planas et al., 2017a; Pardo-Planas et al., 2017b).

2. Material and methods

2.1. Strain and spore production

An. *A. nidulans* strain was purchased from the Fungal Genetic Stock Center (FGSC, Manhattan, Kansas, USA). The strain, deposited under the identifier A773, includes a marker (*pyroA4*) for suppression of growth of the microorganism when pyridoxine is not supplied externally. This strain was further genetically modified by DNA mediated transformation of a plasmid (pEXPYR) that contains a wild-type *pyrG* complementing gene and a construct that contains the glucoamylase promoter fused to the *MtgloA* AAO open reading frame (including its own signal peptide), as described elsewhere (Segato et al., 2012). *MtgloA* is a gene from *Myceliophthora thermophila* that codes for an AAO. The resulting *A. nidulans* cell factory overexpresses and secretes *M. thermophila* AAO in media containing maltose.

Spores of the *A. nidulans* strain discussed in the previous paragraph were produced by growing the transformed strain on solid medium containing (per L) 9.0 g glucose, 50 mL of 20× Clutterbuck salt solution (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1 mL 1000x trace element solution I (22 g/L $ZnSO_4{\cdot}7H_2O$, 11 g/L H_3BO_3, 5.0 g/L $MnCl_2{\cdot}7H_2O$, 5.0 g/L $FeSO_4{\cdot}7H_2O$, 1.6 g/L $CoCl_2{\cdot}5H_2O$, 1.6 g/L, $CuSO_4{\cdot}5H_2O$, 1.1 g/L $Na_2MoO_4{\cdot}4H_2O$, 50 g/L Na_2-EDTA), 0.001 g pyridoxine, and 15% agar. The pH was adjusted to between 6 and 6.5 using a 6 N NaOH solution. Media was sterilized by autoclaving at 121 °C for 20 min (PRIMUS, Sterilizer Co., Inc., Omaha, NE, USA). Twenty-five µL of spore stock solution were pipetted onto the solid medium and spread homogeneously under aseptic conditions. The plates were then incubated at 37 °C for 48 h until the surface of the solid media was covered by white spores. A control plate with medium lacking pyridoxine was inoculated in a similar manner to verify the stability of the strain. Observation of growth on this control plate served as an indicator of contamination or issues with the engineered strain. The plates with spores were kept refrigerated (4°C) until used.

2.2. Reactor setup

The scheme and a picture of the TBR system is shown in Pardo-Planas et al. (2017a). The reactor was constructed using a 60 cm tall glass column with an internal diameter of 10.5 cm. The inside of the column was filled with an inert packing for the fungus to grow on. Three different random packings were used; two made of high-density polyethylene plastic (HDPE) (GEA 2H Water Technologies, Dusseldorf, Germany) and one made of stainless steel (Sulzer Chemtech, Winterthur, Switzerland). Both hydrophilic and hydrophobic packing was selected to determine if hydrophilicity affected AAO production. Two different hydrophobic packings were suggested for testing by their manufacturer, while only one type of stainless steel packing was available from the suppliers we contacted that would fit our reactor. Non-porous packing was selected to facilitate cleaning and reuse of the material in multiple experiments, something that was not possible in a previous study that used a porous packing material (Müller et al., 2015). Table 1 summarizes the characteristics of each packing; hereafter refered to as packing #1, 2, or 3. The liquid medium was recycled from the sump to the top of the column through a recirculation loop using a peristaltic pump (Masterflex, Cole-Parmer, IL, USA). Four outlets on top of the column distributed the liquid media across the top of the packed bed. Two other pumps were used to add fresh medium and

Table 1
Properties of the three random packings used during this study.

Property	Packing #1	Packing #2	Packing #3
Company	GEA 2H Water Technologies	GEA 2H Water Technologies	Sulzer Chemtech
Model	BCN 030	BCN 020	NeXRing #0.6
Material	HDPE	HDPE	316 Stainless Steel
Hydrophobicity	Hydrophobic	Hydrophobic	Hydrophilic
Surface (m^2/m^3)	320	610	NA[a]
Protected surface (m^2/m^3)	259	400	NA[a]
Length (mm)	30	20	20
Diameter (mm)	30/36[b]	20	21/22

[a] NA: Not available, information was kept confidential by the manufacturer.
[b] The two numbers refer to the different diameters of the two ends of the cylindrical shaped particle.