ELSEVIER

Process Biochemistry 43 (2008) 238–243

Process Biochemistry

www.elsevier.com/locate/procbio

Oxygen enrichment effects on protein oxidation, proteolytic activity and the energy status of submerged batch cultures of *Aspergillus niger* B1-D

Qiang Li, Linda M. Harvey, Brian McNeil *

Strathclyde Fermentation Centre, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, United Kingdom

Received 24 September 2007; received in revised form 15 November 2007; accepted 28 November 2007

Abstract

Oxygen enrichment (sparging bioreactors with oxygen-enriched air) ameliorates oxygen limitation in batch cultures of *Aspergillus niger* B1-D, and although nutrient utilization rates for both the carbon source and the nitrogen source are higher in oxygen-enriched cultures, excess oxygen does not enhance specific growth rate, instead the ''extra'' nutrient consumption is associated with defensive measures. Commencement of oxygen enrichment in early exponential phase leads to a transient rise in proteins showing oxidative damage (carbonylation), and to induction of enhanced proteolytic activity, which points to the antioxidant defense being temporarily overwhelmed, and that *A. niger* adapts to this oxidative environment by enhancing intracellular proteolytic activity to degrade damaged proteins which might otherwise accumulate under these conditions. Also the energy status of *A. niger*, reflected by intracellular ATP content, is found to be altered upon the commencement of oxygen enrichment and then reaches much lower levels than in the control. The reasons underlying this are discussed in the context of what is known regarding alternative respiration in industrial fungi.

Keywords: Oxygen enrichment; *Aspergillus niger*; Fermentation; Protein carbonyl; Proteolytic activity; ATP

1. Introduction

Oxygen plays a central role in the submerged cultures of fungal fermentation. As the final electron acceptor along the respiratory chain, oxygen is vital to the process of energy generation in all aerobic organisms. However, the leaky nature of the electron transfer chain in mitochondria may result in incomplete reduction of oxygen, giving rise to superoxide anion radicals, which may in turn initiate a cascade of other reactive oxygen species (ROS) [1]. The subsequent imbalance between ROS production, and the antioxidant defenses in micro-organisms, generally described as oxidative stress, leads to a series of deleterious effects upon growth, nutrient uptake and intracellular metabolism.

One particular aspect of cytotoxicity imposed by ROS is its oxidative modification to intracellular proteins [2]. Proteins may undergo a series of oxidative modifications, depending on the nature of the oxidants and the level of exposure. A general indicator, and commonly used biomarker for protein oxidation are protein carbonyls, which are introduced to amino acid residues, especially proline, arginine, lysine and threonine, by oxidation [3]. Compared to other oxidative modifications, carbonyls are relatively difficult to induce, and in contrast to, for example, methionine sulfoxide and cysteine disulfide formation, carbonylation is irreversible and unrepairable, thus, modified proteins can only be eliminated by protein degradation [4]. It is generally proposed that in eukaryotes, mildly oxidized proteins are selectively degraded by the 20S proteasome, a large multi-catalytic proteinase complex, in an ATP-independent manner [5,6]. However, extensively oxidized proteins tend to aggregate or covalently cross-link each other, and escape proteolysis.

Oxygen enrichment, in which oxygen-enriched air is sparged into submerged cultures of micro-organism, is a potentially useful route to relieving oxygen limitation effects and has been widely employed, for example, oxygen enrichment has been used for high cell density cultivation of *Escherichia coli* [7], and overproduction of heterologous protein in *Aspergillus niger* [8]. But more detailed studies

* Corresponding author at: Strathclyde Fermentation Centre, Strathclyde Institute of Pharmacy and Biomedical Sciences, Royal College Building, University of Strathclyde, 204 George Street, Glasgow G1 1XW, United Kingdom. Tel.: +44 141 5484379; fax: +44 141 5534124.

E-mail address: b.mcneil@strath.ac.uk (B. McNeil).

1359-5113/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2007.11.016

indicate that oxygen enrichment of fungal cultures induced oxidative stress, as shown by both a burst of superoxide anion production, and induction of antioxidant (defensive) enzyme activities [9–11]. The potential oxidative damage to endogenous proteins caused by oxygen enrichment, which may have profound effects on cellular activities, has not been examined. At the most fundamental level, such oxidized proteins need to be either repaired or degraded, otherwise accumulation of these proteins may cause toxic effects in the cells [5]. Intracellular proteolytic degradation has been shown to be an effective pathway of removing oxidatively modified proteins, and, for that reason, intracellular proteases have been proposed as ''secondary antioxidant defenses'' in micro-organisms [12,13]. However, intracellular proteolytic activity following oxidative stress induced by oxygen enrichment has not yet been elucidated. In the present work, we cultivated a filamentous fungus, *A. niger* B1-D, in a 15-L bioreactor to investigate a gap in our current knowledge on the effects of oxygen enrichment on the extent of protein oxidation and consequent proteolytic degradation. Intracellular carbonyl content was used as a biomarker for protein oxidation, and non-specific proteolytic activity was measured accordingly. The energy status, estimated by the ATP content, was also monitored. Our results provide new information on the development of protein carbonylation and proteolytic activity in batch cultures of *A. niger* in response to oxidative stress imposed by oxygen.

2. Materials and methods

2.1. Strain and cultivation

A recombinant filamentous fungus, *A. niger* B1-D [14] has been used in present work. *Aspergillus* complete medium was used for batch cultivation [11]. The composition of the medium is 30 g soluble starch, 5 g NH_4Cl, 20 mL salt solution, 10 mL vitamin solution, and water to 1 L. Salt solution: KCl, 26 g; $MgSO_4{\cdot}7H_2O$, 26 g; KH_2PO_4, 76 g; trace-element solution, 50 mL; and water to 1 L. Trace-element solution: $Na_2B_4O_7{\cdot}10H_2O$, 40 mg; $CuSO_4{\cdot}5H_2O$, 400 mg; $FePO_4{\cdot}2H_2O$, 800 mg; $MnSO_4{\cdot}2H_2O$, 800 mg; $Na_2MoO_4{\cdot}2H_2O$, 800 mg; $ZnSO_4{\cdot}7H_2O$, 8 g; and water to 1 L. Vitamin solution: *p*-aminobenzoic acid, 20 mg; thiamine hydrochloride, 50 mg; biotin, 10 μg; nicotinic acid, 100 mg; calcium D-pantothenic acid, 200 mg; pyridoxine monohydrochloride, 50 mg; riboflavin, 100 mg; and water to 1 L.

All fermentations were carried out in batch mode using a 15-L (total volume) stainless steel bioreactor (BIOSTAT C.-DCU, B. Braun Biotech International, Switzerland) with a working volume of 10 L. The fermenter was inoculated with 4.0% of 48-h-old shake flask culture grown at 25 °C and 200 rpm. The pH was kept at 4.0 by automatic titration addition (2 M NaOH and 1 M H_2SO_4). The temperature was kept at 25 °C throughout runs. The agitation rate was set at 400 rpm. The air-flow rate of control runs was controlled at 1.0 volume of air per volume of culture per minute (vvm). When oxygen-enriched air was supplied, total gas flow rate was controlled at 1.0 vvm, but the percentage of oxygen in total gas flow (v/v) was set at 25%, 50% and 75% in early exponential phase (at 24 h). Real time values of pH, dissolved oxygen tension (DOT), agitation speed, temperature, air-flow rate, and oxygen percentage during fermentations were recorded automatically by software, MFCS DA (Sartorius, UK).

2.2. Biochemical assays

Biomass was estimated according to the method described by Bai et al. [11]. 5 mL of fungal culture was withdrawn and filtered through a 4.25-cm diameter GF/C filter, which has a particle retention size of approximately 1.2 μm (Whatman Ltd., UK). The filter cake was washed twice with 5 mL distilled water, dried for 20 min in a microwave oven (650 W) on medium-low power, and cooled in a desiccator before weighing. All the assays were carried out in triplicate.

The filtrate was also collected for starch and ammonia assays. Starch in the filtrate was converted into glucose by glucoamylase (Sigma, UK). Glucose and ammonia concentrations were determined by enzymatic assay kits (r-biopharm, Germany) performed by a robot, COBAS, MIRA (Roche, USA). All the assays were carried out in duplicate.

After the fungal cells were harvested, filtered and washed, the filter cake was re-suspended in the same volume of distilled water. The fungal cells were disrupted by a high-pressure cell disrupter (Model 4000, Constant System Ltd., UK). Cell free extracts were separated from cell debris by centrifugation at 4 °C, 18,407 × *g* for 30 min. The clear supernatant was used to measure intracellular proteolytic activity and protein carbonyl content immediately.

Intracellular proteolytic activities were determined according to the method of van den Hombergh et al. [15]. Each 0.45 mL sample was incubated with 0.05 mL 1% (w/v) BSA (Fraction V, Sigma) in assay buffers (0.1 M sodium phosphate buffer, pH 7.0) at 37 °C. The reaction was terminated after 30 min by adding 0.5 mL 10% (w/v) trichloroacetic acid (TCA). After incubation at 0 °C for 30 min, precipitated proteins were removed by centrifugation at 13,362 × *g* for 5 min, and the optical density of the TCA-soluble fraction was measured at 280 nm. One unit (U) of proteolytic activity was defined as a change of one absorbency unit per hour at 280 nm.

Intracellular protein carbonyl content was measured by a spectrophotometric method [16]. Principally, carbonyl reacts with 2,4-dinitrophenylhydrazine (DNPH), giving rise to protein hydrazones which were quantified by the

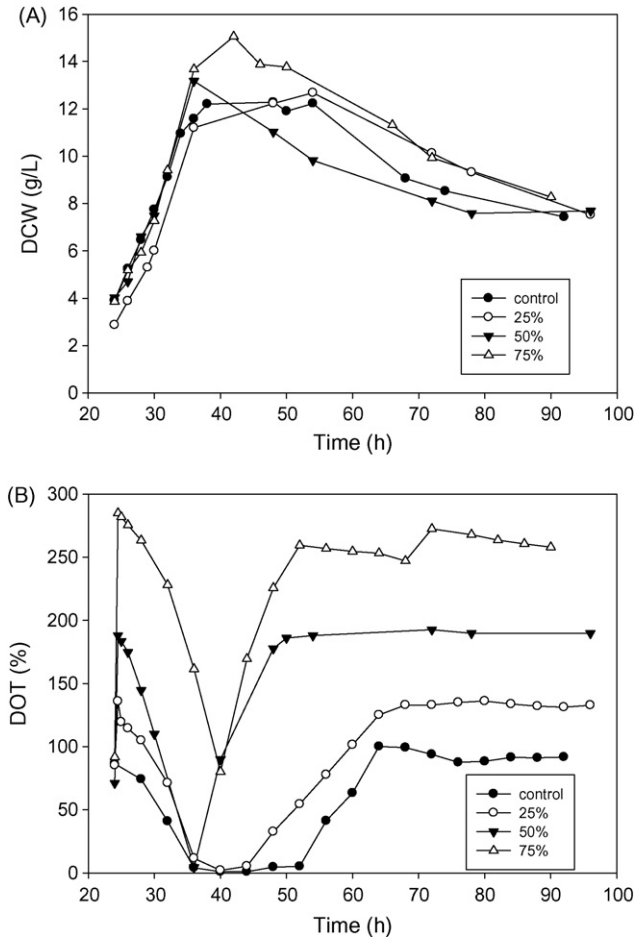

Fig. 1. Effects of gassing batch cultures of *A. niger* B1-D with oxygen-enriched air on DCW (A) and DOT (B). Cultivation conditions: 25 °C, 1 vvm, 400 rpm, pH 4.0 and 10 L. Oxygen enrichment started in early exponential phase (24 h). Control was gassed continuously with air.