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Section: Review

Oxidative stress in fungal fermentation processes: the roles of alternative respiration

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Abstract

Filamentous fungi are arguably the most industrially important group of microorganisms. Production processes involving these simple eukaryotes are often highly aerobic in nature, which implies these cultures are routinely subject to oxidative stress. Despite this, little is known about how filamentous fungi cope with high levels of oxidative stress as experienced in fermenter systems. More surprisingly, much of our knowledge of oxidative stress responses in fungi comes from environmental or medical studies. Here, the current understanding of oxidative stress effects and cellular responses in filamentous fungi is critically discussed. In particular the role of alternative respiration is evaluated, and the contributions of the alternative oxidase and alternative dehydrogenases in defence against oxidative stress, and their profound influence on fungal metabolism is critically examined. Finally, the importance of further research which would underpin a less empirical approach to optimising fungal strains for the fermenter environment is emphasised.

Introduction

Fungi, which consist of both the yeasts and the filamentous fungi, are among the most widely used microorganisms in traditional and advanced bio-industries. In the past decade, novel yeast strains and filamentous fungi have been progressively invading the field of bio-pharmaceutical

protein expression dominated by *Escherichia coli* and mammalian cell expression systems (Sodoyer 2004).

In terms of synthesis of heterologous proteins, filamentous fungi have a complex post-translational protein modification system, unlike the simpler bacterial systems which tend to be used for production of non-glycosylated proteins (Schmidt 2004). The fungal species preferred for recombinant protein expression are those widely employed in traditional processes for manufacturing enzymes, acids and antibiotics, and these include species from a few key genera including *Aspergillus*, *Penicillium*, *Acremonium*, *Fusarium* and *Trichoderma*. Since filamentous fungi typically obtain the nutrients they require by secretion of digestive enzymes and subsequent absorption of the digested materials, the innate protein productivity and secretion potential of these filamentous fungal hosts are often far superior to other expression systems. However, even some yeasts, in particular *Saccharomyces cerevisiae* and *Pichia pastoris*, have been used successfully to express a multitude of recombinant proteins, such as Fab (fragment antibody) and ScFv (single chain variable fragment) therapeutic antibodies (Schoonooghe et al. 2009; Yamawaki et al. 2007) and protein vaccines (Avril et al. 2009), since they possess many of the bioprocessing advantages of *E. coli*. For example, short doubling times and readily manipulated genomes, but also have the additional benefits of eukaryotes such as improved protein folding and many post-translational modifications (Durocher & Butler 2009; Yurimoto & Sakai 2009). Furthermore, filamentous fungi and yeasts are more robust than mammalian cells and can produce some enzymes in excess of 100g/l in fermenters (Cherry 2003) and are easily adapted to fermentation processes in simple and cost-effective culture media (Gasser and Mattanovich 2007; Durocher & Butler 2009). Even though filamentous fungi and yeasts have now been used industrially for decades, and are now used for production of some of our most

complex and potent drugs, they are still not adequately characterized at the physiological and genetic level, particularly in terms of protein modification and secretion metabolism (Durocher & Butler 2009) and especially in relation to their physiological responses to the stressful growing environment in bioreactors (Li et al. 2009 ; Bai et al. 2003a).

The term “stress” is widely used, and is somewhat controversial, but it is also very difficult to define in objective terms. “Oxidative stress” is a very frequently encountered term, so at the outset this term should be clearly defined. Oxidative stress can be simply defined as a state where there is a relative imbalance within cells between generation of reactive oxygen species (ROS) and the intracellular anti-oxidant defences in favour of the former (Li et al. 2009). Most fungal processes are highly aerobic in nature, and thus sufficient O₂ supply and the maintenance of O₂ transfer rate in the bioreactor is always a challenge during production processes, and especially in those processes involving high cell density. As a solution, the use of O₂-enriched air is frequently applied in many industrial fungal processes (Bai et al. 2003a). However, O₂ can be a two-sided sword for microbial cells inside bioreactors, mainly due to the generation of ROS, which is of particular relevance to the cultivation of industrial fungi. A clear understanding of how fungal cells cope with the challenges of toxic ROS is, thus, critical for improved insight into fungal physiology. To a large extent, this is consistent with the regulatory view that we should seek to better understand our bio-manufacturing processes. Unfortunately our knowledge on many aspects of oxidative stress in fungal processes is still deficient in many areas. To fully exploit the potential of filamentous fungi and yeast, their physiology and particularly the mechanisms of defensive responses to such a highly aerobic growing environment has to be investigated and clarified. Recent advances in this important field are discussed here, with an emphasis on the implications of the findings of these studies to fungal bio-processing.

Oxidative stress is the unavoidable consequence of an aerobic lifestyle. We, as biotechnologists, operate fungal bioproduction processes with very high O₂ transfer rates, and occasionally O₂ enrichment in order to achieve high productivities. Thus, as an industry we take for granted a set of conditions which impose, at some process time points, intense oxidative stress on the fungal production and expression systems we employ. Despite this, it is astounding that we have spent little time or effort trying to understand clearly how the fungi we use in bioreactors deal with the conditions we impose. This leaves us in the realm of empiricism in process definition and operation. Much of what knowledge we possess about fungal defence against oxidative stress comes not from fermenter studies, but from environmental and medical mycology, and often uses relatively convenient chemical stressors, e.g. simple addition of exogenous hydrogen peroxide to cultures, to simulate actual oxygen induced stress (as in fermenters), thus, the relevance to aerobic fermentations of fungi is questionable. Our first need is, therefore, for more carefully thought-out studies focused upon fungal bioproduction systems and the process consequences of oxidative stress.

General responses to oxidative stress in fungi

There is some evidence suggesting that oxidative stress imposes significant effects on fungal cultures in bioproduction systems, in terms of morphology, growth rate, metabolism, and protein secretion. This review will briefly summarise the findings of such studies and then discuss an increasingly important but generally neglected area, i.e. the role of alternative respiratory

enzymes in oxidative stress. For more comprehensive discussions, readers are encouraged to refer to a recent review by our group (Li et al. 2009).

The effects of oxidative stress on fungal morphology

Filamentous fungi exhibit two extreme types of morphology in submerged culture, pelleted and filamentous forms. Gassing batch cultures of *A. niger* with O₂-enriched air decreases the size of hyphal elements and increases the proportion of active cytoplasm, but hyphal adhesion occurs at the highest level of O₂ enrichment (50% v/v) (Wongwicharn et al. 1999). Similar results have been found where O₂ enrichment results in branched and shorter hyphae (Kreiner et al. 2003; Bai et al. 2004). Addition of exogenous H₂O₂ leads to a decrease of the hyphal growth unit (Kreiner et al. 2003). In yeast, a study showed morphological changes from unicellular to pseudomycelium in the yeast *Ustilago maydis* when treated with a pro-oxidant compound, octyl gallate (Sierra-Campos et al. 2009). Substantial evidence therefore exists to indicate that morphological change may be a direct consequence of oxidatively stressful conditions in the fungi.

The effects of oxidative stress on fungal growth rates and metabolism

Oxidative stress can be provoked by addition of exogenous chemical stressors (simulated) or by highly aerobic conditions within a fermenter system, for example, by oxygen enrichment of the gas phase. Oxidative stress generally exerts a negative impact on fungal growth. This was shown by the prolonged lag phase noted in cultures transferred from shake flasks to a highly aerated and well mixed fermenter system (O'Donnell et al. 2007), slow growth rates, low substrate

consumption rates (O'Donnell et al. 2007; Li et al. 2008a), low protein content (Bai et al. 2004) and low ATP content (Li et al. 2008b) in oxidatively-stressed fungal cells. One possible explanation for many of these observations is the engagement of alternative respiration, one of whose putative roles is to diminish ROS production, but at the expense of ATP generation due to their non-proton-pumping nature. Another possible explanation is the specific inhibition of key metabolic enzymes by ROS. Two frequently studied enzymes that are vulnerable to ROS inactivation are glyceraldehyde-3-phosphate dehydrogenase (Li et al. 2008a) and aconitase (Murakami and Yoshino 1997). Both these enzymes are essential for primary metabolism in fungi. Our results indicate that the decreased glucose uptake rate in *A. niger* correlates closely with decreased glyceraldehyde-3-phosphate dehydrogenase activity when cells are treated with H₂O₂, but that the decreased ammonium uptake rate is due to decreased ATP and NADPH availability rather than oxidative inactivation of the ammonium assimilatory enzymes in cells (Li et al. 2008a).

In order to alleviate oxidative stress that is caused to the cells during bioprocessing, there have been some attempts to generate fungal mutants with increased antioxidant capacity. Such studies have a measure of success, for example, in over-expression of genes coding for antioxidant enzymes such as catalase (Abbott et al. 2009) and thioredoxin (Perez-Torrado et al. 2009), introduction of genes for synthesis of antioxidants such as vitamin C (Branduardi et al. 2007) and astaxanthin (Ukibe et al. 2009) all increase yeast cells' resistance to oxidative stress. Provided the genetic alterations made to these strains do not reduce vigour, or lead to altered proteolysis, such mutants could have clear advantages in industrial fermentation where oxidative stress is encountered.

The effects of oxidative stress on enzyme production in fungi

Oxidative stress, caused by over-oxygenation to bioreactors, adversely affects the production of a recombinant protein from *A. niger* (Wongwicharn et al. 1999; Bai et al. 2004). Thus, mitigating oxidative damage could ultimately increase protein production by fungi. Overexpression of the *Kluyveromyces lactis SOD1 (KISOD1)* gene increased the production of two different heterologous proteins by *K. lactis*, human serum albumin (HSA) and *Arxula adenivorans* glucoamylase (Raimondi et al. 2008). This was thought to be due to the alleviation of the oxidative stress caused by sustained endoplasmic reticulum damage during recombinant protein production. Sustained stress of the endoplasmic reticulum (ER) causes oxidative stress both in yeast and mammalian cells (Harding et al. 2003; Haynes et al. 2004). Overexpression of SOD alleviated oxidative stress, thus promoting recombinant protein production.

Conversely, oxidative stress may contribute to increased enzyme production under certain circumstances, for example, enhanced extracellular laccase activity is found as a part of the response system of white rot fungi, *Trametes versicolor* and *Abortiporus biennis*, to paraquat-caused oxidative stress conditions (Jaszek et al. 2006).

Having briefly outlined the general responses of fungi to oxidative stress, the discussion will now centre upon the central role of the alternative respiratory pathways in oxidative stress.

Alternative respiratory pathways

In the fungi, as in other eukaryotes, the cytochrome respiratory chain consists of four protein complexes: Complex I (NADH:ubiquinone oxidoreductase), Complex II (succinate:ubiquinone

oxidoreductase), Complex III (ubiquinone:cytochrome *c* oxidoreductase), Complex IV (cytochrome *c* oxidase). During electron transfer, energy is conserved by pumping out protons at Complexes I, III, IV across the inner membrane of mitochondria. Each complex has specific inhibitors, such as rotenone and piericidin for complex I, antimycin for complex III and cyanide for complex IV (Joseph-Horne et al. 2000). Complex II does not pump protons due to the low free energy change during respiration.

The alternative respiratory and electron transfer chain

[Figure 1 here]

In addition to Complex I, most plants and many fungi have alternative NADH dehydrogenases which accept electrons from NADH and transfer them to ubiquinone directly (Fig 1). However, these enzymes do not pump protons out of the matrix across the inner mitochondrial membrane, and the free energy between NADH and ubiquinone (73kJ/2e-) is lost as heat (Siedow and Umbach 1995). Unlike Complex I, alternative NADH dehydrogenases are insensitive to rotenone, a fact that is often used to estimate the alternative respiratory activity by inhibiting the cytochrome pathway using rotenone.

In addition to the alternative dehydrogenase, there is also an alternative oxidase (AOX) which transfers electrons from ubiquinol to the final acceptor, O₂, bypassing both Complex III and IV (Fig.1). As with the alternative dehydrogenases, AOX does not translocate protons when electrons flow through this pathway, thus no energy is conserved but is instead again lost as heat (Moore et al. 2002). This enzyme has been found in the inner membrane of mitochondria from

all the higher plants, many algae, fungi, yeast and certain protozoa (Moore et al. 2002 ; Baurain et al. 2003 ; Juarez et al. 2006). It is distinguished from the conventional cytochrome pathway by its insensitivity to cyanide, which inhibits cytochrome *c* oxidase (complex IV), and sensitivity to salicylhydroxamic acid (SHAM) and *n*-propyl gallate. These characteristics are often used to measure the activity of AOX in intact cells or isolated mitochondria by measuring O₂ uptake rate in the presence of cyanide and/or SHAM.

Regulation of AOX in fungi.

Expression of AOX genes is constitutive

In *Magnaporthe grisea* (Yukioka et al. 1998), *Neurospora crassa* (Tanton et al. 2003) and *A. niger* (Hattori et al. 2009), AOX genes were constitutively transcribed at a low basal level even though the protein and enzyme activity were not detectable. The observation that non-induced wild-type cultures occasionally contained similar amounts of *aod-1* mRNA to induced cultures but revealed no detectable AOX in mitochondria of *N. crassa* suggested translational control is also indispensable for the regulation of gene expression (Tanton et al. 2003).

AOX is not stimulated by pyruvate but by purine nucleotides

In contrast to plants, the fungal AOX is not stimulated by pyruvate, but by purine nucleotide monophosphate and diphosphates like AMP, ADP, dAMP and GMP. Evidence for this has been found in a wide range of yeasts and fungi, such as *P. stipitis*, *N. crassa* (Umbach and Siedow 2000), *U. aylis* (Juarez et al. 2004), *U. maydis* (Sierra-Campos et al. 2009), *Tapesia acufiformis*

(Joseph-Horne et al. 2000) and in the yeasts *Hansenula anomala* (Sakajo et al. 1997) and *Yarrowia lipolytica* (Medentsev et al. 2004).

AOX is activated in the presence of cytochrome respiration inhibitors

Numerous studies have shown that AOX is activated when antimycin (an inhibitor of Complex III in the main respiratory chain), is added to fungal cultures (Minagawa et al. 1992; Uribe and Khachatourians 2008; Sierra-Campos et al. 2009). This property indicates that AOX overflows electrons when the cytochrome respiratory chain is inhibited or saturated. In the pathogenic fungus, *U. maydis*, AOX is thought to increase the metabolic flexibility of the cell, a role that might be valuable for an organism exposed to variations in temperature, nutrient source and availability, and biotic or abiotic factors that limit the activity of the cytochrome pathway (Juarez et al. 2006).

The role of AOX in oxidative stress

It is generally accepted that mitochondria are the site for the generation of ROS in fungi due to electron leakage during the process of aerobic respiration (Li et al. 2009). One of the putative roles of the fungal alternative respiratory pathways may be to diminish ROS generation due to the non-proton pumping nature of many of the components of this pathway. There is some evidence to support this hypothesis. In *M. grisea*, for example, active oxygen species are thought to be signal mediators to activate transcription of the AOX gene (Yukioka et al. 1998), and the superoxide anion ($O_2^{\cdot-}$) is involved in the induction of cyanide-resistant-respiration (CRR) in the yeast *H. anomala* (Minagawa et al. 1992). The presence of H_2O_2 and menadione

induces CRR in *Pichia membranifaciens* and *Debaryomyces hansenii* (Veiga et al. 2003). Karrafa et al. (2001) reported a negative correlation between CRR and intracellular peroxide levels in *A. chrysogenum*, supporting the protective role of alternative respiration against oxidative damage in cells. The effects of two ROS-generating agents, paraquat and H₂O₂ were investigated in 12 fungal species, the results of which indicated exposure of fungal spores or mycelia to both agents stimulated CRR (Angelova et al. 2005). In *A. fumigatus*, AOX activity and mRNA expression were both induced with menadione or paraquat, suggesting an important role of AOX under oxidative stress conditions (Magnani et al. 2007). A recent study by our group showed that oxidative stress in batch cultures of *A. niger* caused by over-oxygenation led to a general decrease of ATP content in the cells (Li et al. 2008b) which corroborates the engagement of alternative respiration under such conditions proposed by Bai et al. (2003b).

Though the role of AOX in oxidative stress has been extensively researched, most of the work is based on “simulated” oxidative stress events, that is using oxidants that are highly pro-oxidative to simulate an oxidative environment. Valuable as those studies undoubtedly are, the unambiguous role (or roles) of AOX in aerobic fungal fermentation processes is still elusive. To address this, a comparison between a wild type and a mutant that is deficient in AOX cultivated under fermentation conditions that are known to induce oxidative stress would be very helpful.

Alternative NADH dehydrogenases

In addition to the alternative oxidase (AOX), many filamentous fungi contain alternative NADH:ubiquinone oxidoreductase (NADH dehydrogenase) enzymes. These alternative NADH

dehydrogenase enzymes do not contain characteristic Fe-S clusters and are insensitive to rotenone or amytal (Kerscher 2000). Likewise, the alternative NADH dehydrogenases do not contain FMN, instead incorporating flavin adenine dinucleotide (FAD) as their sole prosthetic group (Prömper 1993), and are, like AOX, unable to translocate protons (Videira & Duarte 2002). As a result of the non-proton pumping nature of these alternative enzymes, fungi must avoid pairing alternative NADH dehydrogenases with the alternative oxidase, as this would result in complete dissolution of the proton gradient necessary for ATP synthesis (Joseph-Horne et al. 2001). Therefore, alternative NADH dehydrogenases must be used synergistically with Complex III and IV, whereas AOX must only be used alongside Complex I, if the cell is to maintain sufficient ATP synthesis to drive all cellular processes (Joseph-Horne et al. 2001). Therefore, no filamentous fungus has been shown to contain an electron transport chain in which AOX is present but Complex I is absent. In many filamentous fungi there are three alternative NADH dehydrogenases, two of which are oriented towards the intermembrane space and hence oxidise cytosolic NADH/NADPH (“external” enzymes), and another which faces the mitochondrial matrix and oxidises internal NADH (“internal” enzyme) (Prömper et al. 1993; Videira & Duarte 2002).

Thus far, three alternative dehydrogenase enzymes have been characterised in *N. crassa*, two external and one internal. These are designated NDE1, NDE2, and NDI1, respectively. NDE1 is calcium-dependent and can oxidise NADPH in addition to NADH, as can NDE2, whereas NDI1 can oxidise internal NADH alone (Duarte et al. 2003; Carneiro et al. 2004). Of these, NDI1 is undoubtedly the most important protein complex, since inactivation of the *ndi1* gene, which

encodes the enzyme, has been shown to result in complete inhibition of respiratory activity in the presence of rotenone (Duarte et al. 2003).

There is marked contrast between the alternative NADH dehydrogenases of filamentous fungi and those of yeast, although some homology is conserved. The yeast *Yarrowia lipolytica*, for instance, contains only one alternative NADH dehydrogenase, in contrast with the numerous alternative respiratory enzymes of filamentous fungi such as *N. crassa*. This enzyme is designated NDH2, and is encoded by the YLNDH2 gene. It is oriented towards the external face of the mitochondrial inner membrane, and oxidises cytosolic NADH or NADPH (Kerscher et al. 1999). Conversely, *S. cerevisiae* contains no Complex I, instead relying on three alternative NADH:ubiquinone oxidoreductases, SCNDE1, SCNDE2, and SCNDI1, the first two of which are external and the latter internal (Kerscher 2000). No specific inhibitor of the alternative NADH dehydrogenases of fungi has yet been reported, although unspecific inhibitors include flavone, platanetin (6-dimethylallyl-3,5,7,8-tetrahydroxyflavone) (Kerscher 2000), and 7-iodoacridone 4-carboxylic acid (IACA) (Roberts et al. 1996). Polymyxin B and nanaomycin A also inhibit alternative NADH dehydrogenases in bacteria (Mogi et al. 2009), which provides further scope for investigation of these compounds against fungal NADH dehydrogenases with which the bacterial dehydrogenases share a degree of similarity (Duarte 2003).

Although at present the exact physiological role of alternative NADH dehydrogenases in filamentous fungi is obscure, numerous researchers have postulated that they may be involved in prevention of, or perhaps even contribute to, reactive oxygen species generation. As regards the prevention of oxidative stress, Complex I and Complex III are the major sites of ROS generation by the mitochondrial ETC, accounting for some 20% and 80% of total O_2^- production, respectively (Turrens et al. 1985; Sigler et al. 1999). Therefore, in cultures experiencing

oxidatively stressful conditions, for example O₂ enrichment, it would be expected that levels of ROS would rise due to an increased rate of respiration. However, Bai et al. (2003b) described a significantly decreased O₂⁻ generation in cultures of *A. niger* in O₂-enriched air, and therefore proposed that this may be due to enhanced activity of an alternative NADH dehydrogenase enzyme, which could act in place of Complex I. Upon studying the total and rotenone-insensitive respiration of these cultures, those sparged with O₂-enriched air had markedly higher ratios of rotenone-insensitive:total respiration (RR/TR). The researchers therefore concluded that under oxidatively challenging conditions, filamentous fungi manipulate electron transport to favour flux through the alternative respiratory pathway, thus reducing ROS generation, a theory which has likewise been proposed for other organisms (Li & Trush 1998; Guerrero-Castillo et al. 2009). Seo et al. (2006) demonstrated that transduction of human neuroblastoma cells with the gene encoding the internal alternative NADH dehydrogenase of *S. cerevisiae* significantly reduced the generation of superoxide radicals within the cell. Inhibition of fungal NDI1 with IACA also led to a significant increase in superoxide production by the culture, most likely as a result of the high rate of electron leakage from Complex I (O'Donnell 2007).

Despite the protective properties of alternative NADH dehydrogenases against oxidative stress, further investigation in *A. niger* has shown that when these enzymes are enhanced in their activities, intracellular ATP concentrations and cellular viability are decreased (O'Donnell et al. 2007). This was particularly true under conditions of high (50%) oxygen enrichment, which led primarily to a significant decrease in intracellular ATP concentrations, with a concomitant decrease in both intracellular and secreted protein concentrations and decreased respiratory quotients (O'Donnell 2007). Additionally, inhibition of the alternative NADH dehydrogenases by

IACA significantly increased the intracellular ATP concentrations, both in control and O₂-enriched batch cultures (O'Donnell 2007). Other investigators have also claimed such alternative NADH dehydrogenase enzymes actually increase ROS production. Fang & Beattie (2003) demonstrated that inhibition of alternative NADH dehydrogenases in yeast (*S. cerevisiae*) led to a decrease in ROS production by the culture. The apparent contradiction could well be due to the differing responses of yeasts and filamentous fungi to oxidative stress. (Li 2009).

Since 2005, the genomes have been available for *A. nidulans*, *A. fumigatus* and *A. oryzae* (Jones 2007), with the genome for *A. niger* being determined by DSM (The Netherlands) and made publicly available in 2007 (Pel et al. 2007). The public availability of these genomes brings with it further opportunity to understand the functions of each of the alternative respiratory enzymes much more deeply, and in particular how the genes controlling these currently abstruse proteins are regulated in response to changing environments.

Genome analysis of *Aspergillus*; alternative respiration pathways

Analysis of the fully sequenced genomes of *Aspergillus* species (www.aspergillusgenomes.org) for alternative respiratory pathways shows the presence of homologous genes encoding multiple pathways similar to those used by *N. crassa* (Duarte et al. 2003). BlastP searching of the genomes predicts the presence of at least two AoxA (Pfam PF01786; Finn et al. 2010) enzymes in each fully sequenced *Aspergillus* genome (9 species: *A. nidulans*, *A. niger*, *A. terreus*, *A. oryzae*, *A. flavus*, *A. clavatus*, *A. fumigatus*) providing alternative oxidase activity. Alignment of the conserved AoxA domain exhibits high levels of amino acid identity. Analysis of the genomes for alternative NADH dehydrogenases encoded by *ndeAB* and *ndiA* also revealed high levels of

conservation and genome synteny across the Aspergilli (Fig. 2). The *ndeAB* NADH dehydrogenases are contained within a large family of class I and class II oxidoreductases (Pfam PF07992), which exhibit a characteristic NADH binding domain within a larger FAD binding motif. The alternative NADH dehydrogenases in *Aspergillus* encoded by *ndiA* are also highly conserved throughout the genus with high levels of genome synteny and high levels of amino acid identity (Pfam PF01512). The conservation of these alternative respiration pathways both at the sequence and genome level indicate the fundamental role these enzymes play in the growth of *Aspergillus* in the natural environment in response to oxidative stress and provide useful roles in growth during industrial bioprocesses.

Insert Figure 2 here

Conclusion

The fungi are at the heart of modern biotechnology. Although most fungal bioprocesses are highly aerobic, our understanding of fungal stress responses to high levels of O₂ is far from complete. One major area needing research is the role of the alternative respiratory pathway in such conditions. It is apparent that the enzymes of this pathway (AOX and NADH dehydrogenases) are profoundly affected by oxidative stress. This in turn may lead to minimisation of ROS effects, but at the expense of major metabolic changes in fungal cultures, including decreased ATP supply and enhanced heat evolution. Our knowledge of how fungi regulate the relative flux through each pathway is minimal. Aside from the pressing need to understand more about this neglected area of fungal metabolism in our fermenters, this is a

potentially rewarding target for metabolic engineering of fungal strains optimised for the fermenter environment.

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Fig 1 The respiratory chain for filamentous fungi. The arrows indicate the flow of electrons. UQ, ubiquinone/ubiquinol; NDE1, external alternative NAD(P)H dehydrogenase 1; NDE2, external alternative NAD(P)H dehydrogenase 2; NDI1, internal alternative NADH dehydrogenase 1; AOX, alternative oxidase; Cyt *c*, Cytochrome *c*; I, II, III, IV, complex I, II, III, IV. (Joseph-Horne et al. 2000; Duarte et al. 2003; Carneiro et al. 2004).

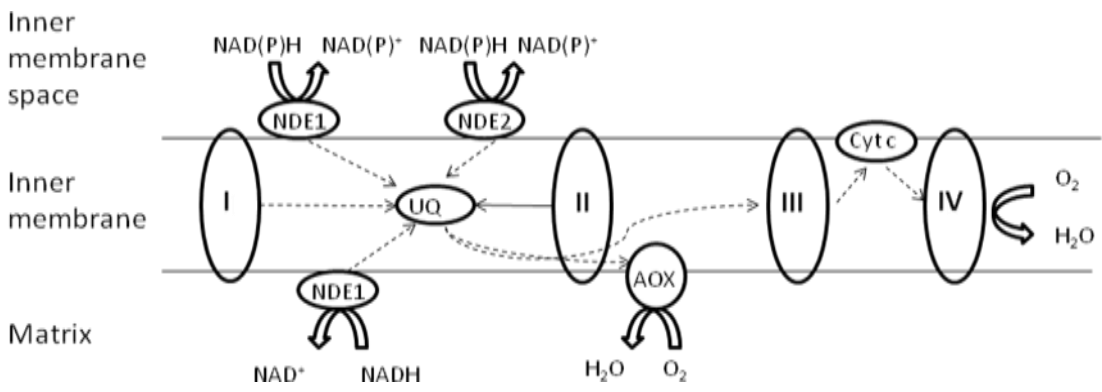
Fig. 2 Genome Analysis of alternative respiratory pathways.

A: Schematic representation of syntenous regions surrounding *aoxA* (AN2099; *A. nidulans*, *A. niger*, *A. flavus*, *A. clavatus*, *A. fumigatus*), *ndeA* (AN7500; *A. nidulans*, *A. niger*, *A. terreus*, *A. oryzae*, *A. flavus*, *A. clavatus*, *A. fumigatus*) and *ndiA* (AN5629; *A. nidulans*, *A. niger*, *A. terreus*, *A. oryzae*, *A. flavus*, *A. clavatus*, *A. fumigatus*) based on annotation at www.aspergillusgenome.org.

B: A ClustalW alignment (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) of AoxA homologues from various *Aspergillus* genomes compared with that of *Penicillium chrysogenum* indicating the high levels of homology in the PF01786 domain across the filamentous fungi (Uniprot accession numbers follow the species names due to the presence of two homologues in each genome).

C: A ClustalW alignment of NdeA homologues from four *Aspergillus* genomes indicating high levels of homology in the PF07992 domain.

D: A ClustalW alignment of NdiA homologues from four *Aspergillus* genomes indicating high levels of homology in the PF01512 domain.



A_nidulans_NdeA 1 NSGATLADSPFANDALRRRIGLLSRRLGSGSRNSGSRNAICLANVYHRRKFPQCLASRAFF
A_niger_NdeA 1 NSGALLRSRPFPMALSTLLSRRTYSSSRSPAGSLAERDRFPHSTATAOCLASRAFF
A_oryzae_NdeA 1 NSGTLRSPFOTALRRRIGSLGALSSSRGSRPLATSRAPFPCQIYFQCLASRAFF
A_fumigatus_NdeA 1 NSGALLASRSPALRRRIGSLGALSSSRGSRPLATSRAPFPCQIYFQCLASRAFF

A_nidulans_NdeA 61 ESHTSVRENSTALSEHGFPARFRIDNITVWNPAYCOFFIIMSRAAVVAFFIYDATTYRE
A_niger_NdeA 60 NSQYVRENSTLTPKASSTPRSSFRMAQPRKAYCOFFIYMSGAUVVAFIYDATTYRE
A_oryzae_NdeA 60 HSHSRRENSTALSRGSRRCRSPSRMAATKSPAYCOFFIYMSGAUVVAFIYDATTYRE
A_fumigatus_NdeA 61 SASLSVRYESTLKDPMASNAKSRALNITVRESFAYCOFFIYMSGAUVVAFIYDATTYRE

A_nidulans_NdeA 121 SSSAEDIYVSELALNPRRGGPKNLPTAEVLGSDGSEAMCQKDKPRLVILGTWGSIAL
A_niger_NdeA 119 NSSAEDIYVSELALNPRRGGPKNLPTAEVLGSDGSEAMCQKDKPRLVILGTWGSIAL
A_oryzae_NdeA 119 HSSAEDIYVSELALNPRRGGPKNLPTAEVLGSDGSEAMCQKDKPRLVILGTWGSIAL
A_fumigatus_NdeA 121 DSSAEDIYVSELALNPRRGGPKNLPTAEVLGSDGSEAMCQKDKPRLVILGTWGSIAL

A_nidulans_NdeA 181 LRLNPGDYHVTYVSPINYLFTFEMLPSATVGTLLGLSLVEFVRRIVQRVGHFLRGEAV
A_niger_NdeA 179 LRLNPGDYHVTYVSPINYLFTFEMLPSATVGTLLGLSLVEFVRRIVQRVGHFLRGEAV
A_oryzae_NdeA 179 LRLNPGDYHVTYVSPINYLFTFEMLPSATVGTLLGLSLVEFVRRIVQRVGHFLRGEAV
A_fumigatus_NdeA 181 LRLNPGDYHVTYVSPINYLFTFEMLPSATVGTLLGLSLVEFVRRIVQRVGHFLRGEAV

A_nidulans_NdA 60 NSGADLSAMKYGDWERTEDIVLEGHVWLIISELEASQLAGROGAGFFSGLKYSFMSFFDWD
A_niger_NdA 60 NSGADLSAMKYGDWERTEDIVLEGHVWLIISELEASQLAGROGAGFFSGLKYSFMSFFDWD
A_oryzae_NdA 61 NSGADLSAMKYGDWERTEDIVLEGHVWLIISELEASQLAGROGAGFFSGLKYSFMSFFDWD
A_fumigatus_NdA 61 NSGADLSAMKYGDWERTEDIVLEGHVWLIISELEASQLAGROGAGFFSGLKYSFMSFFDWD

A_nidulans_NdA 120 SDPRFRYLWVNADESEPTCCDREIMRSDPQLIDGCLVVGGRAMSANGAAYIYIRGEPTPE
A_niger_NdA 120 SDPRFRYLWVNADESEPTCCDREIMRSDPQLIDGCLVVGGRAMSANGAAYIYIRGEPTPE
A_oryzae_NdA 121 SDPRFRYLWVNADESEPTCCDREIMRSDPQLIDGCLVVGGRAMSANGAAYIYIRGEPTPE
A_fumigatus_NdA 121 SDPRFRYLWVNADESEPTCCDREIMRSDPQLIDGCLVVGGRAMSANGAAYIYIRGEPTPE

A_nidulans_NdA 180 ATVLCRAINAEYRAGLIGENACGGYDFDVIIMRGMAYVCGEETSLEIESIEGKAGKPRD
A_niger_NdA 180 ATVLCRAINAEYRAGLIGENACGGYDFDVIIMRGMAYVCGEETSLEIESIEGKAGKPRD
A_oryzae_NdA 181 ATVLCRAINAEYRAGLIGENACGGYDFDVIIMRGMAYVCGEETSLEIESIEGKAGKPRD
A_fumigatus_NdA 181 ATVLCRAINAEYRAGLIGENACGGYDFDVIIMRGMAYVCGEETSLEIESIEGKAGKPRD

A_nidulans_NdA 240 KPPFFAAVGLGCFSTYVNVETVAVTPTIMRRCASWFSFGRENNAGTELPCIISGVNND
A_niger_NdA 240 KPPFFAAVGLGCFSTYVNVETVAVTPTIMRRCASWFSFGRENNAGTELPCIISGVNND
A_oryzae_NdA 241 KPPFFAAVGLGCFSTYVNVETVAVTPTIMRRCASWFSFGRENNAGTELPCIISGVNND
A_fumigatus_NdA 241 KPPFFAAVGLGCFSTYVNVETVAVTPTIMRRCASWFSFGRENNAGTELPCIISGVNND