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Recombinant bacterial hemoglobin alters metabolism of *Aspergillus niger*

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ABSTRACT

The filamentous fungus *Aspergillus niger* is used extensively for the production of enzymes and organic acids. A major problem in industrial fermentations with this fungus is to ensure sufficient supply of oxygen required for respiratory metabolism of the fungus. In case of oxygen limitation, the fungus will produce various by-products like organic acids and polyols. In order to circumvent this problem we here study the effects of the expression of a bacterial hemoglobin protein on the metabolism of *A. niger*. We integrated the *vgb* gene from *Vitreoscilla* sp. into the genome at the *pyrA* locus behind the strong *gpdA* promoter from *Aspergillus nidulans*. Analysis of secreted metabolites, oxygen uptake, CO_2 evolution and biomass formation points towards a relief of stress in the mutant expressing VHB when it is exposed to oxygen limitation. Our findings therefore point to an interesting strategy to attenuate unwanted side effects resulting from oxygen limitation during industrial fermentations with *A. niger*.

1. Introduction

Aspergillus niger is widely exploited as a cell factory in different biotechnological production processes. Its applications range from the production of pharmaceutical compounds and industrial enzymes to primary metabolites such as citric acid, and several production processes have been classified as "General Regarded As Safe" (Schuster et al., 2002). However, one of the challenges, often encountered in these industrial fermentation processes, is the high viscosity of the fermentation medium, particularly at high biomass concentrations. This leads to several problems with respect to mixing and oxygen transfer and it is therefore valuable to identify new strategies that can improve the process performance by reducing for example the impact of oxygen limitation. One strategy that could possibly provide these results is the expression of an oxygen-carrying protein in the host cell, as it has been suggested previously (Khosla and Bailey, 1988). Subsequently, it has been demonstrated that the expression of the *vgb* gene, which encodes a hemoglobin protein (VHB) from the bacteria *Vitreoscilla* sp., improves many different biotechnological processes in a whole range of different prokaryotic and eukaryotic organisms (Frey and Kallio, 2003). Two recent examples are the production of beta-galactosidase by *Enterobacter aerogenes* (Khleifat et al., 2006), and the production of alpha-amylase by the yeast *Schwanniomyces occidentalis* (Suthar and Chattoo, 2006).

Beneficial effects for the production of itaconic acid in *Aspergillus terreus* presumably due to the expression of VHB (Lin et al., 2004), as well as improvement of growth and enzyme production of *Aspergillus oryzae* on solid substrates, which are suggested to result from the overexpression of hemoglobin domains (te Biesebeke et al., 2006), have been described. However, none of these studies provide any detailed insight into the metabolic changes caused by these genetic manipulations.

This study was therefore designed to study the impact on the metabolism of *A. niger* when VHB is expressed intracellularly, and to investigate if this approach of metabolic engineering (Bailey et al., 2002) might be fruitful for the production processes that utilize this versatile biotechnological work horse.

2. Material and methods

2.1. Strain, media, growth conditions

The strains used in this study were *A. niger* A733 (also called N402) [*cspA*1] and *A. niger* A742 [*cspA*1; *pyrA*5], and both were obtained from the Fungal Genetics Stock Center. The media for plates and shake-flasks were prepared as described by Cove (1966).

Concerning batch cultivations, the medium composition, fermentation set up and cultivation conditions have been previously described by Diano et al. (2006). However, in the present study the ammonium source used was 12 g/L of NH_4Cl, the carbon source was 110 g/L of monohydrate glucose, the working volume was 4.5 L and the stirring was set to 700 rpm. Briefly, the same amount of spores of each strain was used for the

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inoculation of in-house built 5 L glass fermenters, and the aeration in the bioreactors was set to 0.2 vvm in order to achieve oxygen limitation after an initial exponential growth phase. The amount of oxygen during the fermentation was followed with an oxygen-sensitive electrode and this was used to determine the onset of the oxygen-limited growth phase. Samples were taken in duplicates every 4 h as described by Diano et al. (2006).

2.2. Strain construction

Standard molecular techniques were carried out according to Sambrook and Russell (2001). Genomic DNA was isolated from overnight shake-flask cultures (150 rpm, 30 °C) with 100 mL of minimal medium supplemented with 2.44 g/L of uridine and 1.12 g/L of uracil. The mycelium was harvested by filtration, frozen in liquid nitrogen, crushed with a mortar and pestle, and approximately 200 mg were transferred to 750 μL of breaking buffer (50 mM Tris–HCl pH 7.5, 20 mM EDTA). After vortexing, 25 μL of 20% sodium dodecyl sulfate was added and then incubated at 65 °C for 30 min. This was followed by the addition of 225 μL of 5 M potassium acetate and further incubation for 30 min on ice. After centrifugation for 10 min at 10,000*g* the supernatant was transferred into a fresh tube, 2 volumes of 96% ethanol was added and followed by another centrifugation for 10 min at 15,000*g*. The pellet was washed with 70% ethanol and dried for 5 min at room temperature after the ethanol was removed. The so obtained DNA was dissolved in 200 μL of sterile distilled water.

Primers for the PCR amplification of the *pyrA* locus were designed (PpyrA_fw 5′-GGAACTGCCTTTGCAGGTGTGGC-3′; PpyrA_re 5′-CACCCTATAATAGCCTGCAGGATC-3′) to identify the specific mutation that leads to the pyrimidine auxotrophy in this strain, and after subcloning the resulting PCR product from genomic DNA of the *A. niger* strains A733 and A742 into pGEM-T (Promega, USA), resulting in the plasmids pGEM-T_pyrA and pGEM-T_pyrA5, and subsequent sequencing of the inserts was carried out by MWG Biotech (Germany).

For the construction of the plasmid pANvHb, the *vgb* gene was amplified by PCR from the plasmid pV6h that was obtained from Dr. Bülow (Lund University, Sweden), using the primers Pvhb_NcoI_fw (5′-AGTACCCATGGATGTTAGACCAGCAAACCAT-3′) and Pvhb_BamHI_re (5′-GCGGATCCTTATTCAACCGCTTGAGCG-3′). The resulting PCR fragment was digested with *Bam*HI and *Nco*I and cloned into the plasmid pMH-C (Bautista et al., 2000), from which the *creA* antisense fragment has been removed by digestion with *Bam*HI and *Nco*I and subsequently purified from an agarose gel. A *Eco*RI–*Not*I fragment of the *pyrA* gene (cut out from pGEM-T_pyrA), which can complement the pyrimidine auxotrophy, if it is integrated at the *pyrA5* locus, was blunt ended and ligated to the *Hind*III digested and blunt ended pANvHb to give the plasmid pANvHb_pyrA.

This plasmid was transformed into *A. niger* A742 by the method described by Nielsen et al. (2006), and in a parallel transformation the plasmid pGEM-T_pyrA was also transformed in the same strain. Transformants were recovered and selected on solid minimal medium.

2.3. Metabolite analysis

Quantification of substrate and products was carried out as detailed by Diano et al. (2006). Malic acid was quantified using the same HPLC set up as described previously (Diano et al., 2006) but using 2 mM H_2SO_4 as eluent. Trehalose, xylitol and acetate were measured, but data on these compounds will not be mentioned further, as very low amount of trehalose was measured (corresponding to <5 mg/L) and no xylitol and acetate were detected.

3. Results

3.1. Strain construction

In order to integrate the *vgb* gene at a specific locus in the genome of *A. niger*, the mutation that confers the pyrimidine auxotrophy in the strain *A. niger* A742, *pyrA5*, was identified by sequencing the *pyrA* locus of that strain. This revealed a single C to T transition at position 751 (downstream of the translation initiation codon ATG) of the *pyrA* gene that gives rise to a stop codon instead of a glutamine, and thereby presumably results in a truncated and non-functional form of the orotidine-5-phosphate decarboxylase, whose functionality is required for the biosynthesis of pyrimidines.

The gene encoding the bacterial hemoglobin was cloned in a plasmid between the promoter of the *gpdA* gene (Punt et al., 1991), which supposedly should result in constitutive expression of the bacterial hemoglobin, and the terminator of the *trpC* gene from *Aspergillus nidulans*. A fragment of the *pyrA* gene was also inserted into this plasmid that was then named pANvHb_pyrA.

Transformation of *A. niger* A742 with this plasmid resulted in transformants that had lost the auxotrophy for pyrimidines due to the integration of the vector at the *pyrA* locus, and also had the *vgb* gene integrated in the genome. The integration was confirmed by PCR (data not show). The hereby obtained strain was named *A. niger* VHB. The strain that was obtained after transformation with the plasmid pGEM-T_pyrA and subsequent selection on minimal medium was named *A. niger* PYR^+, and was used as reference strain throughout this study.

3.2. Strain characterization

To investigate the impact of the insertion of the bacterial hemoglobin on the physiology of *A. niger* batch fermentations were carried out with *A. niger* VHB and *A. niger* PYR^+ (see Figs. 1 and 2).

During the exponential growth phase, which lasted approximately 13 h for both strains, the two strains had similar maximum specific growth rates (see Table 1). Approximately 31 h after inoculation, the dissolved oxygen tension dropped below 1% for both strains and they entered a linear growth phase. At this point, the secretion of significant amounts of metabolites that could be detected via HPLC analysis of the culture filtrate was observed (see Fig. 1). Glycerol was the only compound that was found to be produced in concentrations up to 1 g/L already before the oxygen was almost exhausted in the cultures.

During the course of the oxygen-limited growth phase, a number of differences could be observed between the *vgb* expressing strain and the reference strain. The average specific growth rate of the *vgb* strain was determined to be 0.016 h^{-1}, whereas the reference strain grew at 0.013 h^{-1}, and this reflects a significant higher growth for the strain that carries the bacterial hemoglobin. Interestingly, the same trend was also found for the specific glucose uptake rate, which shows a 19% increase (see Table 1). Furthermore, the specific glycerol production rate was higher in *A. niger* VHB. On the other hand, a reduction of the production rates of compounds that participate in the tricarboxylic acid (TCA) cycle (succinate, fumerate, citrate) was observed, as well as for the production rates of mannitol and ethanol.

The analysis of the off gas revealed that the specific oxygen uptake rate was increased in the *vgb* strain, but only slightly ($<10\%$), and this was also found for the CO_2 production rate.