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Aspergillus oryzae in solid-state and submerged fermentations Progress report on a multi-disciplinary project

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Abstract

We report the progress of a multi-disciplinary research project on solid-state fermentation (SSF) of the filamentous fungus *Aspergillus oryzae*. The molecular and physiological aspects of the fungus in submerged fermentation (SmF) and SSF are compared and we observe a number of differences correlated with the different growth conditions. First, the aerial hyphae which occur only in SSFs are mainly responsible for oxygen uptake. Second, SSF is characterised by gradients in temperature, water activity and nutrient concentration, and inside the hyphae different polyols are accumulating. Third, pelleted growth in SmF and mycelial growth in SSF show different gene expression and protein secretion patterns. With this approach we aim to expand our knowledge of mechanisms of fungal growth on solid substrates and to exploit the biotechnological applications.

Keywords: Solid state; Submerged fermentation; Morphology; Polyol; Amylase; Protease; Genome-wide expression analysis; *Aspergillus oryzae*

1. Introduction

Solid-state fermentations (SSFs) are applied in many traditional food fermentation processes and offer possibilities for improved production of novel as well as existing food products and ingredients [1]. In recent developments, the organisms are used in SSF to produce high yields of pure enzymes which are much more efficiently produced than in submerged fermentations (SmF) [2].

Fungi play a key role in SSF, for their hyphal development allows them to effectively colonise and penetrate the solid substrate [1]. As a consequence of hyphal growth the fungus is confronted with gradients in concentration of substrates and enzymes, the presence of a substrate–air interface, and gradients in water content and temperature [3]. Water is frequently a limiting factor for fungal growth in SSF and heat removal is one of the major problems in large-scale SSF. These problems do not occur with SmF of fungi. In SmF, water is abundantly present and gradients in temperature, oxygen concentration and nutrients are small or absent.

The focus of SSF research has been primarily on process and fermenter design where the organism involved is regarded as a black box [4]. The molecular mechanisms underlying the behaviour of fungi in SSF have not been described in detail. *Aspergillus oryzae* is an obligate aerobic filamentous fungus frequently used in SSF processes. We have initiated a multi-disciplinary study on molecular mechanisms of *A. oryzae* SSF on wheat kernels to under-

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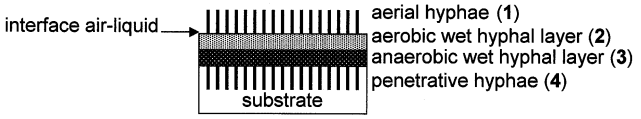

Fig. 1. Conceptual representation of filamentous fungal growth on a solid substrate. The bold numbers correspond to the four layers indicated in the text.

stand the differences observed between SSF and SmF and to improve the application of fungi in biotechnological processes. In this letter we report our progress with SSF-grown *A. oryzae* considering morphology, basic metabolism and gene expression.

2. Fungal growth on a solid substrate

Wheat kernels are an excellent substrate for use in mixed solid-state bioreactors [5]. A drawback of using such a complex substrate is that its composition may vary and its pre-treatment may affect the fermentation. An important goal of pre-treatment is to give the grain an increased water content [5]. In most wheat types difference in respiration could be explained by difference in water binding inside the kernel [6], and for some grain types the pre-treatment may affect water availability, which warrants further studies.

Temperature control is one of the main problems in large-scale SSF [4,5,7], so it is important to know how temperature affects the fungus. The heat production of the fungus is proportional to its respiration rate [4]. Therefore we measured the respiration rate of *A. oryzae* grown on wheat kernels at different temperatures. Based on this it is clear that the respiration has a temperature optimum between 30 and 35°C. The oxygen uptake rate (OUR) is a reflection of metabolic activity and is also related to heat production. A better understanding of oxygen consumption in fungal SSF might be useful to deal with heat removal problems encountered in SSF. In a conceptual model, we distinguish four layers in the mycelium (Fig. 1). Fig. 2 illustrates *A. oryzae* growing out of a wheat kernel, showing both mycelium on the surface of the kernel as well as abundant aerial mycelium. For *Rhizopus oligosporus* and *Coniothyrium minitans*, we found that the OUR during SSF is limited by diffusion of oxygen in the layer of densely packed fungal hyphae [8,9]. For *A. oryzae*, however, we found that the aerial hyphae contribute up to 75% to the OUR. This was demonstrated by suppressing their formation by covering colonies of *A. oryzae* with a gas-permeable polycarbonate membrane [10]. Measurements with micro-electrodes in the layer of aerial hyphae (1 in Fig. 1) showed no decrease in oxygen concentration, i.e. no diffusion limitation. From these results it can be concluded that cooling problems in SSF with *A. oryzae* or other aerial mycelium-producing fungi will be more pronounced than in fermentations with fungi that produce little aerial mycelium, such as *C. minitans*.

3. Substrate hydrolysis and product formation

Upon penetration of the wheat kernel the fungal hyphae will first come across a variety of polysaccharides in the kernel coat. The core of the kernel consists largely of starch and hydrolysis by fungal amylases results in release of glucose, which is used as the main carbon and energy source. Accumulation of malto-oligosaccharides is investigated to address the question whether these compounds play a role in induction of amylases. Currently, it is believed that growth in the substrate particle is limited by availability of oxygen [11] and that amylase activity is sufficiently high to hydrolyse starch at a higher rate than the rate of utilisation by the fungus. Our data using *A. oryzae* growing on wheat dough are in agreement with this, although glucose accumulation in the substrate varied strongly with culture conditions. For example, the glucose content of wheat dough with an initial water content of 1 kg per kg dry matter increased from an initial 2 g kg^{-1} substrate to 2.7 g kg^{-1} substrate after 48 h of cultivation. On the other hand, in dough with 0.7 kg water per kg dry matter, glucose increased from 2.3 to 16 g per kg substrate in 48 h, which may be related to slower growth and/or higher amylase induction (see later).

In the SSF process, *A. oryzae* encounters nutrient gradients. Oxygen is mainly or only available at the outside of the kernel, whereas carbon substrates are present only inside the kernel. Since aerial hyphae are not in direct contact with the substrate, they should be provided with a carbon and energy source by transport of one or more carbon compounds through the hyphae. Mannitol is found at a very high concentration in mycelium growing on wheat kernels (Fig. 3) and would be a good candidate.

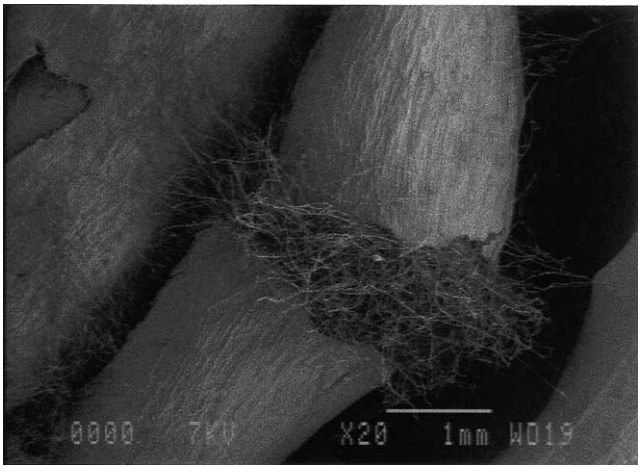

Fig. 2. *A. oryzae* growing out of a wheat kernel. The picture was taken after 24 h of incubation with a low-temperature scanning electron micrograph at 20× amplification. The bar at the bottom represents 1 mm in length.

Polyhydroxy compounds (polyols), such as glycerol, mannitol, erythritol and arabitol, can be accumulated in filamentous fungi to a high concentration. The composition of the polyol pool is dependent on the growth condition (e.g. in [12]) and polyols therefore apparently play a role in the adaptation to the environmental conditions. One such condition, low water activity (a_w), is usually encountered in SSF and provokes fungi to accumulate polyols. Glycerol is often reported as the predominant polyol accumulated at low a_w [13], but preliminary results indicate that *A. oryzae* accumulates a mixture of glycerol, erythritol and arabitol at low a_w in SSF (Fig. 3).

Since polyols are also secreted to some extent by the mycelium, they form one class of products of fungal metabolism in SSF. Another class of metabolites is formed by organic acids. *A. oryzae* is not a strong acid producer, but a low quantity of citric acid (1 g per kg substrate) and traces of oxalic acid were found in wheat dough after 72 h cultivation with *A. oryzae*.

Also production of flavour compounds is characteristic for SSF (reviewed in [14]) and may be related to the limited availability of oxygen inside the kernels, resulting in production of 'fermentation' products such as alcohols, aldehyde and keto-compounds. Much research on metabolism and microbial physiology will be necessary to establish the potential of SSF in production of these biotechnologically interesting volatile metabolites.

4. Protein secretion and gene expression

As we plan to study secretion and localisation of amylases and proteases, our first objective was to identify proteins secreted in SSF of *A. oryzae*. In a first approach we isolated secreted proteins from SmF and wheat kernel *A. oryzae* cultures. In a SDS–PAGE analysis it was shown that the pattern of secreted proteins during wheat kernel cultivation is clearly different from that in SmF. The identity of a few proteins was determined by N-terminal sequence analysis after isolation of protein bands from SDS–PAGE gels. A 50-kDa protein from both the SmF and SSF SDS–PAGE gel was identified as α-amylase. Three SSF-specific proteins of 35, 28 and 20 kDa were isolated from the SSF SDS–PAGE gel and the 28-kDa protein could be identified as neutral protease II. We are currently identifying other proteins specifically secreted in wheat kernel SSF by *A. oryzae*.

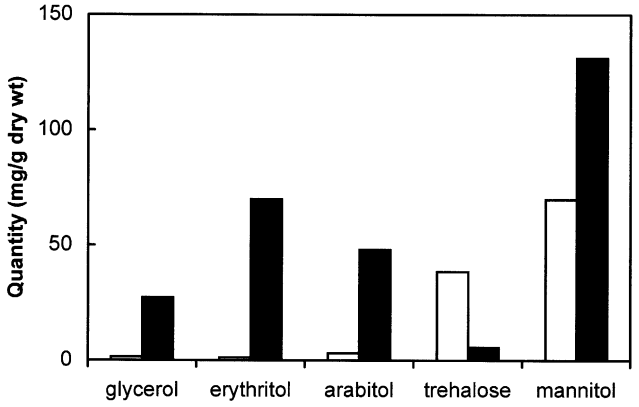

Fig. 3. Intracellular polyol quantities in *A. oryzae* mycelium cultivated on wheat kernels. Mycelium was cultivated for 72 h at water activity 1 (open bars) or water activity 0.97 (filled bars).

For a detailed analysis of secretion and localisation of gene expression of amylases and proteases, various molecular genetic tools such as a gene transfer system [15] and green fluorescent protein-based reporter proteins [16] have been developed for *A. oryzae*.

Recently, it was shown that glucoamylase B (*glaB*) is highly induced during rice-koji making SSF, but not in standard liquid cultivation [3,17]. Expression of *glaB* was also induced by starch or malto-oligosaccharides in surface cultures [3], but the expression levels obtained were lower than the expression levels found in rice-koji making SSFs. In a further study Ishida et al. [18] showed that motifs in the promoter region of *glaB* were responsible for induction by starch, low a_w and high temperature. This result suggests that the physical factors which are supposed to be typical for rice-koji making SSF give rise to the high level of *glaB* induction.

In order to analyse the observed SSF-specific high level of gene expression in more detail, we performed transcription analysis of various genes for *A. oryzae* grown on wheat kernel substrates. In a first approach we isolated mRNA from the fungus cultivated on the wheat kernels for 5 days and in liquid medium containing 1% ground wheat kernels for 2 days. In Northern analysis (Fig. 4), four PCR-amplified gene fragments were used for hybridisation. The *enoA* gene fragment hybridised equally well under both conditions and was used as reference for metabolic activity of the fungus. Sporulation in *Aspergillus* is induced by the zinc-finger transcription factor encoded by the *brlA* gene [19] and the induction of sporulation is regulated at the *brlA* transcription level [20,21]. As expected, the *brlA* gene was transcribed only in SSF and can be used as a positive control for SSF transcription. The *amyA* gene, encoding α-amylase, was transcribed equally well in both cultivation conditions as expected from our protein analyses (Fig. 4). The *glaB* gene is induced only during wheat kernel SSF. From the initial results it is clear that also two protease genes are only expressed in SSF. In additional genome-wide expression-profiling approaches we aim to find new genes specifically expressed in SSF.

5. Conclusion

Many studies on SSF have focussed on process and fermenter design while the organism has been considered as a black box [4]. In this paper, the first experimental

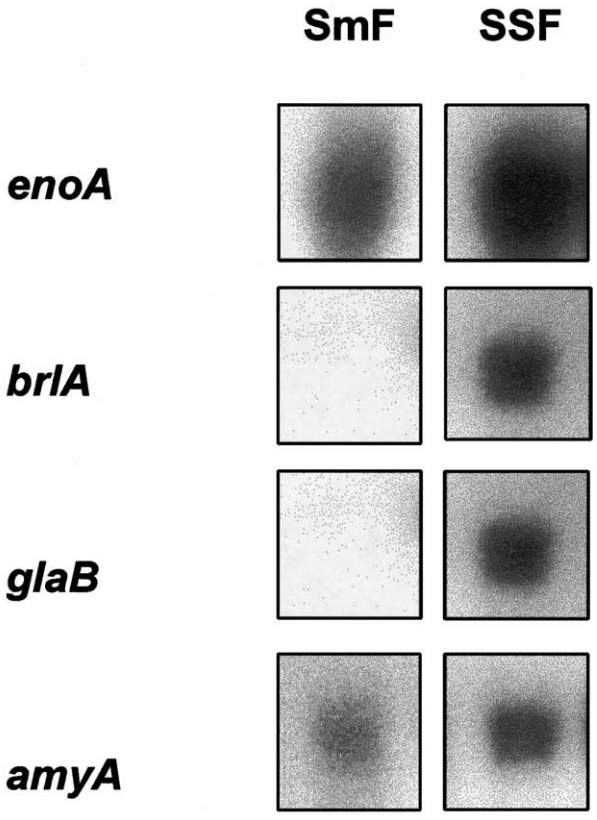

Fig. 4. Gene expression analysis of *A. oryzae* in submerged (SmF) and surface growth (SSF) cultures. The enolase (*enoA*) and amylase (*amyA*) genes are expressed in both conditions whereas the zinc-finger transcription factor (*brlA*) and glucoamylase (*glaB*) genes are only expressed in solid-state cultivation.

results with our model organism *A. oryzae* are described during surface growth on wheat kernels and a comparison is made with submerged growth conditions. During SSF the fungus displays a characteristic growth phenotype and has distinct physiological and molecular characteristics. From our physiological studies, we find that oxygen uptake is mainly performed by the aerial hyphae and we show that different polyols, potential intracellular carbon source transporters, are accumulated inside the hyphae of the fungus during SSF. Our molecular biological studies with *A. oryzae* show that different proteins are produced and genes are differentially transcribed in liquid fermentation vs. SSF. With these approaches we aim at elucidating the mechanisms behind the differences in SSF and SmF and exploring the biotechnological applications.

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