

Polyol accumulation by *Aspergillus oryzae* at low water activity in solid-state fermentation

George J. G. Ruijter,^{1,2†} Jaap Visser^{1‡} and Arjen Rinzema^{1,3}

Correspondence
George J. G. Ruijter
g.j.g.ruijter@lumc.nl

¹Wageningen Centre for Food Sciences, PO Box 557, 6700AN Wageningen, The Netherlands

²Wageningen University, Laboratory of Microbiology, Dreijenlaan 2, 6703HA Wageningen, The Netherlands

³Wageningen University, Food and Bioprocess Engineering Group, PO Box 8129, 6700EV Wageningen, The Netherlands

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Polyol accumulation and metabolism were examined in *Aspergillus oryzae* cultured on whole wheat grains or on wheat dough as a model for solid-state culture. In solid-state fermentation (SSF), water activity (a_w) is typically low resulting in osmotic stress. In addition to a high level of mannitol, which is always present in the cells, *A. oryzae* accumulated high concentrations of glycerol, erythritol and arabitol at relatively low a_w (0.96–0.97) in SSF. Accumulation of such a mixture of polyols is rather unusual and might be typical for SSF. *A. oryzae* mycelium accumulating various polyols at low a_w contained at least four distinct polyol dehydrogenases with highest activities toward glycerol, erythritol, D-arabitol and mannitol. NADP⁺-dependent glycerol dehydrogenase activity correlated very well with glycerol accumulation. A similar correlation was observed for erythritol and NADP⁺-erythritol dehydrogenase suggesting that NADP⁺-dependent glycerol and erythritol dehydrogenases are involved in biosynthesis of glycerol and erythritol, respectively, and that these enzymes are induced by osmotic stress.

INTRODUCTION

Fungal fermentations are typically performed in liquid culture medium (submerged fermentation; SmF) or on a solid substrate, such as cereals or beans (solid-state fermentation; SSF). SmF is usually the production method of choice in the West, whereas SSF is much more used in Asia. SSF can be superior with respect to productivity or yield of certain products and, in addition, fungi may produce a different spectrum of products in SSF (Pandey *et al.*, 2000; Robinson *et al.*, 2001). The reasons for this are not fully understood, but it is likely to be a consequence of the different physiology of the fungi on a solid substrate compared to submerged cultivation. Fungal physiology in SSF is not well characterized, but an understanding of physiology is required to explore the possibilities for controlling or directing product formation. Pioneering work has been reported by Ishida *et al.* (1998) with regard to the factors that induce the SSF-specific glucoamylase gene, *gluA*, in *Aspergillus oryzae*. Among other factors, low water activity (a_w) induced *gluA* (Ishida *et al.*, 1998). It is a

well known fact that a_w has a profound influence on growth and metabolism (for example, see Pandey *et al.*, 1994). Low a_w , i.e. below 1, is characteristic of SSF; the substrate is moist, but there is no water phase as in SmF. Although a_w may still be close to 1 at the start of a fermentation, it will probably decrease during the process as a result of (i) water loss due to evaporative cooling which is required to remove heat generated by metabolism (Nagel *et al.*, 2001), and/or (ii) hydrolysis of polysaccharides and protein resulting in accumulation of sugars and amino acids.

At low a_w , micro-organisms try to prevent water loss from the cells by accumulating compatible solutes, such as ions, amino acids or polyols. Fungi generally accumulate polyols such as glycerol, erythritol, arabitol or mannitol. Very high levels, up to several molar, can be accumulated in the cells, but polyols are also secreted to the environment (Witteveen & Visser, 1995; Gutierrez-Rojas *et al.*, 1995). For SmF, polyol accumulation has been described for a number of species, such as glycerol production by *Aspergillus niger* (Witteveen & Visser, 1995) and *Aspergillus wentii* (El-Kady *et al.*, 1994), glycerol and erythritol production by *Aspergillus nidulans* (Beever & Laracy, 1986; Redkar *et al.*, 1995), and production of a mixture of polyols by *Aspergillus repens* (Kelavkar & Chhatpar, 1993) and *Aspergillus flavus* (Mellon *et al.*, 2002). Much less is known about polyol production at low a_w in SSF. *A. niger* cultured at low a_w on Amberlite as an inert support produced glycerol and erythritol (Gutierrez-Rojas

†Present address: Metabolic Diseases Laboratory, Centre for Human and Clinical Genetics, Leiden University Medical Centre, PO Box 9600, 2300RC Leiden, The Netherlands.

‡Present address: FGT Consultancy, PO Box 396, 6700AJ Wageningen, The Netherlands.

Abbreviations: a_w , water activity; SSF, solid-state fermentation.

et al., 1995), whereas *Aspergillus ochraceus* accumulated a mixture of polyols on agar media (Ramos *et al.*, 1999).

The metabolic pathways for polyol biosynthesis in fungi have only been elucidated in a few cases. In general, biosynthesis of a polyol from the corresponding phosphorylated sugar requires two steps, reduction and dephosphorylation, which may take place in either order. *Saccharomyces cerevisiae* produces glycerol from dihydroxyacetone phosphate via glycerol 3-phosphate using NAD⁺-dependent glycerol-3-phosphate dehydrogenase (Gpd1p) and glycerol-3-phosphate phosphatase (Gpp2p) (Albertyn *et al.*, 1994; Norbeck *et al.*, 1996). Recently, it has been shown that *A. nidulans* utilizes a different route for glycerol biosynthesis, involving NADP⁺-dependent glycerol dehydrogenase and not glycerol-3-phosphate dehydrogenase (de Vries *et al.*, 2003; Fillinger *et al.*, 2001). In *A. niger*, mannitol biosynthesis from fructose 6-phosphate is accomplished by mannitol-1-phosphate dehydrogenase and mannitol-1-phosphate phosphatase (Ruijter *et al.*, 2003).

In this study, we have investigated the composition of the polyol pools in *A. oryzae* during SSF and the metabolic pathways involved in the biosynthesis of these polyols.

METHODS

Strain, culture media and growth conditions. *A. oryzae* CBS 570.65 (ATCC 16868) was obtained from the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands). For preparation of conidiospores, *A. oryzae* was grown for 3–4 days at 30 °C on potato dextrose agar containing 1 M NaCl. Spores were harvested with a solution containing 0.05% (w/v) Tween 80. The spore suspension was filtered through sterile glass wool to remove mycelium; spores (10⁷ ml⁻¹) were then stored in the presence of 20% (v/v) glycerol at -70 °C.

A single batch of wheat grains (Blok, Woerden, The Netherlands) was used in all experiments. Two types of solid media were used, wheat dough and whole grains. Wheat dough was prepared essentially as described by Nagel *et al.* (2001) with either 0.7 or 1 kg water (kg dry mass)⁻¹. The initial *a_w* of the doughs was 0.981 ± 0.002 and 0.991 ± 0.003 for 0.7 and 1 kg water (kg dry mass)⁻¹, respectively. Discs of the wheat dough (Nagel *et al.*, 2001) were put into Petri dishes (60 mm) and inoculated by spreading 2.5 × 10³ spores over the surface. Twenty Petri dishes were incubated without lids in a closed container, which was placed in a temperature-controlled cabinet at 35 °C. The container was flushed with 350 ml sterile air min⁻¹. The inlet air was humidified with water in the case of wheat dough with initial *a_w* 0.991 or with a 0.6 M NaCl solution for dough with initial *a_w* 0.981. Mycelium was cultured for 48 h.

Whole grains were soaked in excess water for 4 h at 50 °C. Following sieving, the grains were sterilized for 1.5 h at 121 °C. Wheat grains were put into a Petri dish (90 mm) in a packed layer and immersed in a sterile solution containing 1.5% (w/v) agar such that the grains were just exposed to the surface of the agar. In order to decrease *a_w*, 1.1 M NaCl was added to the agar solution. Plates were inoculated by spreading 10⁴ spores over the surface. Five plates were incubated in a closed container as described above. Air was humidified with either water or 1.1 M NaCl. Mycelium was cultured for 48 h.

a_w of solid substrates was measured using an Aqualab 3TE at 35 °C. *a_w*

and osmotic pressure are related by $\pi V_m = -RT \ln(a_w)$, where π is the osmotic pressure (Pa), V_m is the molar volume of water (mol m⁻³), $R = 8.314$ is the gas constant (J mol⁻¹ K⁻¹), T is the temperature (K) and a_w is the water activity. Under the conditions used, *a_w* 0.98 corresponds to osmotic pressure -2.9 MPa and *a_w* 0.96 to -5.8 MPa.

Extraction and analysis of polyols. Mycelium growing on solid substrates was rapidly frozen by pouring liquid nitrogen over the mycelium. Frozen mycelium was scraped from the substrate and lyophilized. Polyol extraction and determination were performed as described previously (Witteveen *et al.*, 1994). In short, the method for polyol analysis was HPLC using a Dionex MA1 column combined with amperometric detection. This method will detect most polyols and sugars.

Preparation of cell extracts, enzyme assays and partial purification of polyol dehydrogenases. Frozen mycelium (approx. 0.25 g), obtained as described above, was ground using a micro-dismembrator (B. Braun Biotech) and suspended in 0.5 ml of extraction buffer (50 mM potassium phosphate pH 7.0, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.5 mM EDTA). Following centrifugation for 5 min at 15 000 g, the resulting supernatants were desalted by passage through a 2.5 ml Sephadex G25 column pre-equilibrated with extraction buffer.

Enzyme activities were determined at 30 °C. Polyol dehydrogenase activities were determined in 100 mM glycine pH 9.6 containing 0.5 mM NAD(P)⁺ and 100 mM glycerol, *meso*-erythritol, D-arabitol or D-mannitol. Reductase activities for aldoses, ketoses or their phosphorylated derivatives were assayed in 50 mM triethanolamine pH 7.4 containing 4 mM MgCl₂, 0.2 mM NAD(P)H and one of the following substrates: 50 mM dihydroxyacetone, 50 mM D-erythrose, 50 mM D-ribulose, 50 mM D-xylulose, 50 mM D-ribose, 2 mM dihydroxyacetone phosphate, 1 mM D-ribulose 5-phosphate, 1 mM D-xylulose 5-phosphate or 1 mM D-ribose 5-phosphate. Mannitol-1-phosphate dehydrogenase activity was determined in 50 mM Tris pH 7.0 containing 0.2 mM NADH and 2.5 mM fructose 6-phosphate. Protein concentrations in cell extracts were determined with the Bicinchoninic acid protein kit (Sigma) according to the supplier's instructions using BSA as a standard.

Product analysis after incubation of cell extracts with various sugars was performed as follows. Desalted cell extract was incubated with 50 mM sugar in 50 mM triethanolamine pH 7.6, 2.5 mM MgCl₂, 0.2 mM NADH. After different incubation times, 220 µl samples were taken and the reaction was stopped by addition of 20 µl of 9.1 M HClO₄. Following 30 min incubation on ice, the samples were neutralized by addition of 40 µl of 5 M KOH and 20 µl of 2 M KHCO₃. Precipitates were removed by centrifugation and reaction products were analysed in the supernatant by HPLC as described previously (Witteveen *et al.*, 1994).

Fractionation of polyol dehydrogenases was performed by anion-exchange chromatography. A cell extract was prepared as described above using either 20 mM Tris pH 7.5 or 20 mM Bistris pH 6.5 as a buffer, in both cases containing 1 mM MgCl₂, 5 mM 2-mercaptoethanol and 0.5 mM EDTA. Four millilitres of desalted cell extract was applied to a ResourceQ column (Amersham Biosciences) and, following rinsing with extraction buffer, protein bound to the column was eluted with a linear gradient of 0–0.5 M NaCl in extraction buffer. Polyol dehydrogenase activities were measured in the fractions obtained.

Northern analysis. Powdered mycelium was prepared as described above. Total RNA was extracted using TRIzol (Life Technologies) according to the supplier's instructions. RNA (5 µg) was incubated with 3.3 µl of 6 M glyoxal, 10 µl DMSO and 2 µl of 0.1 M sodium phosphate buffer pH 7 in a total volume of 20 µl for 1 h at 50 °C to denature the RNA. Following electrophoresis in a 1.5% agarose gel

containing 10 mM sodium phosphate pH 7, RNA was transferred to nylon membranes (Hybond-N; Amersham Pharmacia Biotech) by capillary blotting in $10 \times$ SSC. Hybridization was performed at 42°C in a solution containing 50% (v/v) formamide, 0.9 M NaCl, 90 mM sodium citrate tribasic dihydrate (citric acid), 0.2% (w/v) ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 0.1% (w/v) SDS, 10% (w/v) dextran sulphate and 100 μg single-stranded herring sperm DNA ml^{-1} . The following probes were used: a *gldB* cDNA from *A. nidulans* (de Vries *et al.*, 2003) and a 0.7 kbp *EcoRI* fragment of the 18S rRNA subunit (Melchers *et al.*, 1994) as a loading control. Blots were washed with 0.6 M NaCl, 60 mM citric acid, 0.5% (w/v) SDS at 56°C (*gldB*) or with 30 mM NaCl, 3 mM citric acid, 0.5% (w/v) SDS at 65°C (18S).

RESULTS

A. oryzae accumulates a mixture of polyols at low a_w in SSF

Two different systems were used to culture *A. oryzae* on solid-state substrate, wheat grains and wheat dough slices. Wheat grains are a realistic substrate, but since it is difficult to get sufficient mycelium from loose grains, the kernels were embedded in agar. A disadvantage of the use of agar is that the a_w had to be adjusted by addition of a solute. Secondly, we used cultures on wheat dough slices as a model for surface growth on grains. In this system the a_w was adjusted in a more realistic way, i.e. by changing the quantity of water in the dough. A number of different polyols accumulated in *A. oryzae* mycelium grown for 48 h on solid substrate (Table 1). Similar results were obtained for the two different culture systems. Mannitol was always present at a high concentration. It accounted for 13–17% of the cell dry weight. A high intracellular concentration of mannitol in *A. oryzae* was reported by Horikoshi *et al.* (1965). At relatively high a_w (0.98–1.00), small quantities of

glycerol, erythritol, arabitol and trehalose were detected. At decreased a_w (0.96–0.97), much higher levels of glycerol, erythritol and arabitol accumulated in the cells, whereas less trehalose was produced (Table 1). The following common polyols were not detected: threitol, galactitol and sorbitol. Traces of xylitol and ribitol were occasionally observed [$<5 \mu\text{mol} (\text{g dry wt})^{-1}$], but no correlation with a_w was noticed.

The specific activity of several polyol dehydrogenases is increased in mycelium growing at low a_w

For each polyol accumulated at low a_w , a number of enzyme activities possibly involved in its biosynthesis were assayed in mycelium samples from the cultures also used for polyol analysis. Most of the polyol dehydrogenase activities tested were specific for NADP^+ , i.e. no reaction was found with NAD^+ . The only exception was D-arabitol dehydrogenase, which was NAD^+ -specific. NADP^+ -dependent D-arabitol dehydrogenase activity was not detected. In contrast, all polyol-phosphate dehydrogenase activities detectable in the mycelium were NAD^+ -specific. The following activities were not detected in any sample with either NADH or NADPH as a cofactor: D-erythrose-4-phosphate reductase, D-ribulose-5-phosphate reductase, D-ribose-5-phosphate reductase and D-xylulose-5-phosphate reductase.

Upon a decrease in a_w , NADP^+ -dependent glycerol dehydrogenase activity increased (Table 2). In contrast, NAD^+ -dependent glycerol-3-phosphate dehydrogenase activity was lower in mycelium cultured on wheat grains at low a_w , whereas it did not vary with changes in a_w in the case of mycelium cultured on wheat dough. Likewise, NADP^+ -dependent erythritol dehydrogenase (erythrose reductase) activity was increased in mycelium cultured at low a_w (Table 2). NAD^+ -dependent D-arabitol dehydrogenase activity did not vary in wheat-grain-cultured mycelium, but decreased with a reduction in a_w in mycelium cultured on wheat dough. L-Arabitol dehydrogenase activity was not detected and it was therefore assumed that the arabitol found in the cells was D-arabitol. The precursor of the D-arabitol produced by D-arabitol dehydrogenase could be either D-ribulose or D-xylulose. Incubation of cell extract with D-ribulose in the presence of NADH resulted in production of ribitol, whereas incubation with D-xylulose produced arabitol and xylitol.

NADP^+ -mannitol dehydrogenase and NAD^+ -mannitol-1-phosphate dehydrogenase activities were detected, but since the mannitol content of the cells did not vary with a_w , no correlation of enzyme activity with mannitol accumulation could be established.

Partial purification of polyol dehydrogenases from a cell extract was performed by anion-exchange chromatography to find out which activities could be separated and hence were attributable to distinct enzymes. Four enzymes bearing

Table 1. Intracellular polyol concentrations in *A. oryzae* cultured on wheat grains or wheat dough at different water activities

Mycelium was cultured for 48 h. Data are means with SD from three independent determinations for wheat grains and four independent determinations for wheat dough.

Polyol	Polyol concentration [$\mu\text{mol} (\text{g dry wt})^{-1}$] during growth on:			
	Wheat grains		Wheat dough	
	a_w 1.00*	a_w 0.97	a_w 0.98	a_w 0.96
Glycerol	32 \pm 7	531 \pm 19	47 \pm 45	533 \pm 142
Erythritol	17 \pm 2	606 \pm 15	80 \pm 82	469 \pm 117
Arabitol	14 \pm 24	314 \pm 98	73 \pm 89	262 \pm 69
Mannitol	829 \pm 129	912 \pm 203	740 \pm 137	902 \pm 134
Trehalose	108 \pm 14	Not detected	22 \pm 9	17 \pm 10

* a_w , Water activity at the time of harvest. Under the conditions used, a_w 0.98 corresponds to osmotic pressure -2.9 MPa and a_w 0.96 to -5.8 MPa .

Table 2. Enzyme activities related to polyol biosynthesis in *A. oryzae* cultured on wheat grains or wheat dough at different water activities

Mycelium was cultured for 48 h. Data are means with SD from three independent determinations for wheat grains and six independent determinations for wheat dough.

Enzyme activity	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹] during growth on:			
	Wheat grains		Wheat dough	
	<i>a_w</i> 1.00*	<i>a_w</i> 0.97	<i>a_w</i> 0.98	<i>a_w</i> 0.96
Glycerol dehydrogenase (NADP ⁺)	259 ± 10	517 ± 8†	582 ± 170	1034 ± 142†
Glycerol-3-phosphate dehydrogenase (NAD ⁺)	28 ± 1	14 ± 1†	11 ± 8	12 ± 5
Erythritol dehydrogenase (NADP ⁺)	268 ± 36	558 ± 22†	472 ± 121	804 ± 157†
D-Arabitol dehydrogenase (NAD ⁺)	27 ± 1	25 ± 6	31 ± 1	17 ± 8†
Mannitol dehydrogenase (NADP ⁺)	7 ± 4	30 ± 1†	48 ± 12	91 ± 19†
Mannitol-1-phosphate dehydrogenase (NAD ⁺)	35 ± 10	34 ± 1	186 ± 118	68 ± 23†

**a_w*, Water activity at the time of harvest.

†Significantly different from the corresponding sample with higher *a_w* (two-tailed Student's *t*-test with *P* < 0.05).

highest activities with mannitol, glycerol, erythritol and D-arabitol, respectively, were well separated (Fig. 1). The major part of the mannitol dehydrogenase activity (70%) did not bind to the anion-exchange column and eluted in the first two fractions, whereas glycerol dehydrogenase bound weakly. The glycerol dehydrogenase co-eluted with erythritol dehydrogenase activity, but a distinct erythritol dehydrogenase was eluted upon increasing the ionic strength of the buffer. Approximately 30% of the erythritol

dehydrogenase activity was catalysed by the glycerol dehydrogenase enzyme. Finally, D-arabitol dehydrogenase activity eluted after the erythritol dehydrogenase. The erythritol and arabitol dehydrogenase activities were not completely separated, but the first fractions containing erythritol dehydrogenase activity (fractions 10–12) did not contain arabitol dehydrogenase activity while the opposite was observed for the last arabitol dehydrogenase fractions (18–20). Since the erythritol dehydrogenase and the arabitol dehydrogenase have different cofactor specificities (NADP⁺ and NAD⁺, respectively) it is not likely that these activities are contained in one enzyme.

Northern analysis was performed on RNA samples obtained from mycelium also used in the polyol and enzyme analyses. Using the *A. nidulans gldB* gene, encoding the single NADP⁺-glycerol dehydrogenase in this organism, as a probe it was observed that expression of the *A. oryzae gldB* homologue was 1.5-fold higher in mycelium growing on wheat grains at low *a_w* and twofold higher in mycelium growing on wheat dough at low *a_w* (Fig. 2).

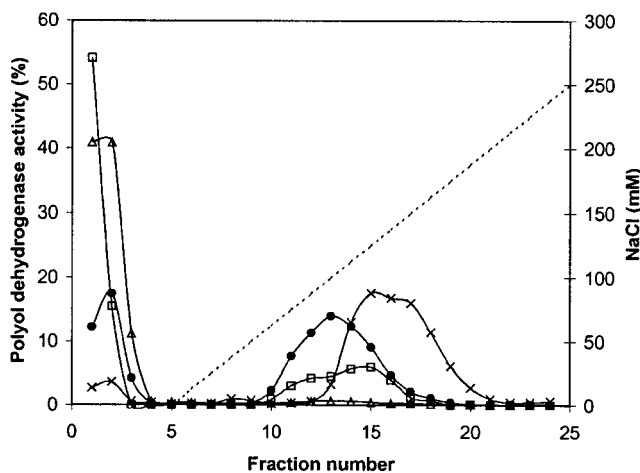


Fig. 1. Fractionation of polyol dehydrogenases by anion-exchange chromatography. Chromatography was performed at pH 7.5 as described in Methods. For each polyol the percentage of the total dehydrogenase activity was plotted against the fraction number. Δ , Glycerol dehydrogenase activity; \bullet , erythritol dehydrogenase activity; \times , D-arabitol dehydrogenase activity; \square , mannitol dehydrogenase activity. The dashed line represents the NaCl gradient employed with the concentration given on the right axis.

DISCUSSION

In this study, we demonstrate accumulation of a mixture of polyol compounds, i.e. glycerol, erythritol, arabitol and mannitol, by *A. oryzae*, cultured at low *a_w* on solid-state substrate. Accumulation of polyols on solid substrate has been investigated primarily using synthetic agar media (e.g. Pascual *et al.*, 2003; Ramos *et al.*, 1999). A disadvantage of the use of agar is that *a_w* has to be adjusted because of a solute. We have used a realistic SSF substrate, i.e. wheat, in our investigation. Concurrent accumulation of comparable quantities of several polyols in response to low *a_w* has been described in only a few reports (Kelavkar & Chhatpar, 1993; Mellon *et al.*, 2002; Ramos *et al.*, 1999). Usually only accumulation of one or, in some cases, two polyols is

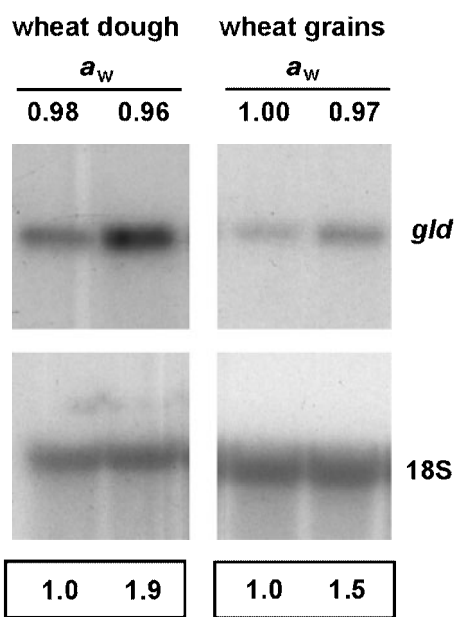


Fig. 2. Expression analysis of glycerol dehydrogenase (*gld*) in *A. oryzae* during growth on wheat grains or wheat dough at different water activities. Detailed culturing conditions are described in Methods. The 18S rRNA was used as a loading control. Numbers underneath the images indicate the relative levels of *gld* transcript corrected for loading differences using the 18S signals. The signal obtained for mycelium growing at high a_w was set at 1.0.

described (e.g. Beever & Laracy, 1986; Dixon *et al.*, 1999; El-Kady *et al.*, 1994; Redkar *et al.*, 1995; Witteveen & Visser, 1995). Why would fungi accumulate just one polyol in one case and a mixture of four in another? The explanation could be a trivial one, i.e. the investigators only looked for one or two polyols. When a variety of polyols is in fact determined and just one or two polyols are observed this may be related to the inability of certain species to produce different polyols; for example, *S. cerevisiae* can only produce glycerol at low a_w . Another explanation might be that the composition of the polyol pool is dependent on growth stage. For example, the composition of the polyol pool accumulated by *A. repens* in liquid cultures changed during growth (Kelavkar & Chhatpar, 1993). Likewise, for *A. oryzae* growing in nitrogen-limited liquid batch culture at a_w 0.98 we observed that during exponential growth glycerol was the major polyol accumulated, whereas the fungus switched to accumulation of erythritol plus some arabitol in the stationary growth phase (G. J. G. Ruijter, unpublished results). Only near exhaustion of the nitrogen source, and hence decreasing growth rate, was a mixture of the three polyols found (G. J. G. Ruijter, unpublished results). Accumulation of a mixture of polyols at low a_w in SSF might therefore be related to relatively low growth rates due to limitation by one or more nutrients.

A. oryzae mycelium that accumulated various polyols at low

a_w contained at least four distinct polyol dehydrogenases: a glycerol dehydrogenase that also had moderate activity toward erythritol, a distinct erythritol dehydrogenase, a D-arabitol dehydrogenase and a mannitol dehydrogenase. The level of NADP⁺-dependent glycerol dehydrogenase was higher at low a_w and this increase in activity correlated very well to glycerol accumulation, suggesting that this enzyme is part of the biosynthetic pathway for glycerol in *A. oryzae*. The function of NADP⁺-dependent glycerol dehydrogenase in glycerol biosynthesis at low a_w is well documented in *A. nidulans* (de Vries *et al.*, 2003). The glycerol biosynthesis pathway in *A. nidulans* and probably also in *A. oryzae* comprises dephosphorylation of dihydroxyacetone phosphate followed by reduction of dihydroxyacetone to glycerol by glycerol dehydrogenase. Induction of glycerol dehydrogenase at low a_w presumably is (part of) the response of *A. oryzae* to osmotic stress in order to be able to produce a larger quantity of polyols to maintain turgor of the cells. However, only a twofold increase in glycerol dehydrogenase activity was observed upon decreasing a_w , while the glycerol concentration in the cells increased 10- to 15-fold. These observations indicate that glycerol biosynthesis is partially controlled by the quantity of glycerol dehydrogenase, but also by other mechanism(s). Possible other control mechanisms are an increase in dihydroxyacetone-phosphate phosphatase activity, regulation of glycerol dehydrogenase activity or better retention of glycerol in the cells. The latter mechanism is well documented in *S. cerevisiae*, where Fps1p controls glycerol accumulation and release (Tamás *et al.*, 1999).

Similar to glycerol, accumulation of erythritol correlated to NADP⁺-dependent erythritol dehydrogenase (erythrose reductase) activity. These data suggest that (i) *A. oryzae* developed similar pathways for biosynthesis of glycerol and erythritol, i.e. dephosphorylation of an intermediary metabolite followed by reduction of the resulting aldose/ketose to a polyol, and (ii) that erythritol dehydrogenase is induced at low a_w . A different pathway for erythritol biosynthesis was reported for the lactic acid bacterium *Leuconostoc oenos* (Veiga-da-Cunha *et al.*, 1993). In *L. oenos*, erythrose 4-phosphate is converted via erythritol 4-phosphate to erythritol, which is catalysed by erythrose-4-phosphate reductase and erythritol-4-phosphate phosphatase (Veiga-da-Cunha *et al.*, 1993). We did not detect any erythrose-4-phosphate reductase activity in *A. oryzae*, whereas high erythrose reductase activity was found in erythritol-accumulating mycelium. Erythrose reductases have been reported for *Aureobasidium* species (Tokuoka *et al.*, 1992), but these authors have not reported whether these enzymes are differentially expressed at low a_w .

D-Arabitol production during osmotic stress has been described for a few fungal species, including *Zygosaccharomyces rouxii* (Blakeley & Spencer, 1962), *Magnaporthe grisea* (Dixon *et al.*, 1999), *Aspergillus flavus* (Mellon *et al.*, 2002), *Epicoccum nigrum* (Pascual *et al.*, 2003) and *Cladosporium fulvum* (Clark *et al.*, 2003). *Z. rouxii* and the marine fungus

Dendryphiella salina convert D-xylulose 5-phosphate to D-xylulose which is subsequently reduced to D-arabitol by an NAD⁺-dependent D-arabitol dehydrogenase (Blakeley & Spencer, 1962; Low & Jennings, 1975). In *Candida albicans*, it was first thought that D-arabitol was formed by dephosphorylation of D-ribulose 5-phosphate and subsequent reduction of D-ribulose by an NAD⁺-dependent D-arabitol dehydrogenase (Wong *et al.*, 1993), but this model was later proven incorrect. A *C. albicans* *ard* mutant, lacking NAD⁺-dependent D-arabitol dehydrogenase, failed to grow on minimal D-arabitol and D-arabinose medium, but its D-arabitol production was not affected (Wong *et al.*, 1995). These results showed that NAD⁺-dependent D-arabitol dehydrogenase is involved in D-arabitol utilization, but that it is not required for D-arabitol biosynthesis. Our data are consistent with *A. oryzae* converting D-xylulose into D-arabitol using a pathway as described for *Z. rouxii* and *D. salina*.

In summary, we have shown in this study accumulation of glycerol, erythritol and arabitol by *A. oryzae* at low a_w on solid-state substrate. Accumulation of a mixture of polyols might be typical for SSF due to the specific growth conditions present during growth on a solid substrate. NADP⁺-dependent glycerol and erythritol dehydrogenases were induced at low a_w suggesting that these enzymes are involved in biosynthesis of glycerol and erythritol, respectively, but this needs to be proven by construction of mutants lacking these enzymes.

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