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Influence of wheat type and pretreatment on fungal growth in solid-state fermentation

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Abstract

The respiration kinetics of *Aspergillus oryzae* on different varieties of whole wheat kernels were studied. Six wheat varieties were pretreated in two different ways. Five of the six substrates fermented similarly and independently of the pretreatment method. However, pretreatment affected fermentation of one variety of soft wheat (Apollo). T_2 ^{1}H-NMR imaging of the water inside the kernels showed a change in water binding inside the kernels when a different pretreatment strategy was used. Differences in free sugar or amino acid content or in kernel stiffness were not significant.

Introduction

Solid-state fermentation (SSF) is defined as the fermentation of solid substrates in the absence of free flowing water. This principle has been used in traditional food fermentation processes for centuries, especially in Asian countries, where products like tempe and sake are produced using SSF. Recently this technique gained interest in western countries due to its potential in solid-waste treatment (Barrios-Gonzalez *et al.* 1993), the production of secondary metabolites, the production of biopesticides (Ooijkaas *et al.* 1998, Oostra *et al.* 2000) and the production of novel foods (Sardjono *et al.* 1998). These new applications call for a deeper understanding of the fermentation process because little is known about the microbial physiology and metabolism under SSF conditions and process engineering aspects of SSF. For our research on fermentation kinetics, physiology and gene expression of *Aspergillus oryzae* in SSF, we want to use a representative model substrate. Therefore, we have compared the respiration kinetics of *Aspergillus oryzae* ATCC 16868 on several varieties of wheat. We used two pretreatment strategies for all types of wheat. Whole kernels were used since it has recently been shown that these are an excellent substrate for use in mixed solid-state bioreactors (Nagel *et al.* 2000).

In previous studies, little attention has been paid to the effects of the choice of substrate and substrate pretreatment on fermentation. Dorta *et al.* (1994) and Liu *et al.* (1999) compared the differences in water retention of related substrates such as rice, rice bran and rice husk. Many researchers studied the influence of water activity on fermentation of one type of substrate (Gervais 1988a, b, Huang 1985, Liu *et al.* 1999). Finally, comparisons have been made between different types of legumes and seeds (Hachmeister *et al.* 1993, Sardjono *et al.* 1998). However, no research has focused on different varieties of the same legume or grain. Differences in growth due to pretreatment of the substrate have so far only been reported with regard to cooking procedures (Hachmeister *et al.* 1993, Chay *et al.* 1986) but not with regard to soaking methods.

Materials and methods

Microorganism

Aspergillus oryzae ATCC 16868 was cultivated on potato/dextrose/agar at 25 °C for 7 days. Spore suspension ($\pm$ 3.7 $\times$ 10^7 spores ml^{-1}) was harvested using sterile water and was stored at -80 °C until it was used for inoculation. Sterile glycerol (10% v/v) was added as a cryo-protectant.

Substrate, pretreatment and fermentation

Six types of wheat of commercial origin were used: two hard varieties (Ritmo and Arnaut), and three soft varieties (Apollo, Minaret (two batches) and D1 304). All wheat was purchased from ACM, Meppel, the Netherlands, and was stored in closed plastic containers at 10 °C. Two pretreatment procedures were applied: the kernels were soaked in distilled water at 50 °C (4 h) or 20 °C (16 h). Excess water was drained off after soaking. The soaked kernels were sterilized at 121 °C for 1 h and were allowed to cool down to room temperature. The total amount of water present in the kernels after pretreatment was 45% (w/w). Inoculation was carried out with 1 ml spore suspension per 100 g of soaked wheat ($\pm$ 6.2 $\times$ 10^5 spores g^{-1} dry wt). The mixture, which was contained in 1 l medium flasks, was then put on a roller bank for 1 h to ensure an even distribution of spores. The inoculated material was divided into 20 g portions in sterile Petri dishes (diameter 90 mm). Fermentation was carried out in a climatic incubator (VEA-Instruments, Houten, the Netherlands) at 25 °C and at a relative humidity of 98%, using procedures described previously (Smits *et al.* 1996). Samples were taken by randomly removing three Petri dishes from the incubator. Removed dishes were replaced with empty dishes to keep the local airstreams in the incubator constant.

Analysis

Water activity was measured using an electric hydrometer (Type EK 84/3H/63T, sensor type BSK-4, Novasina, Pfafficon, Switzerland).

Carbon dioxide production and oxygen consumption were measured in a setup designed by Smits *et al* (1998). The set-up consisted of a measurement chamber, a magnetic stirrer inside the chamber, a tube pump (Masterflex 7014, Cole Parmer, Chicago, IL), a paramagnetic O_2 analyzer (Servomex series 1100, the Netherlands) and an infrared CO_2 analyzer (Servomex Series 1400, the Netherlands). The respiration measurement took 30 min. All three Petri dishes were measured simultaneously.

Total carbohydrate was measured using a method derived from Dubois *et al.* (1956). In test tubes, 500 μl sample suspension and 50 μl 20% phenol were mixed. Thousand μl conc. H_2SO_4 was injected in the center of the sample. After mixing and cooling, the optical density was measured at 490 nm.

Total terminal amino acids were measured using a trinitrobenzenesulphonic acid method (TNBS). A 12 μl sample was mixed with 83 μl distilled water and 260 μl 0.1 M phosphate buffer (pH 8). After 30 s, 3 μl TNBS (5% TNBS in methanol) and 93 μl distilled water were added. After 6 min, the extinction was measured at 340 nm. Calibration was carried out with a glycine solution (100 mg l^{-1} phosphate buffer (0.01 M)).

Kernel stiffness was measured in a piston type pressure cell with a built-in texture analyzer. Pressure was exerted on the kernels with an aluminum weight that pressed down on a plate of 4 mm thickness. The pressure sensor was fitted in the center of the lower plate. Water distribution was tested using Nuclear Magnetic Resonance (NMR) relaxation time (T_2) imaging, which makes it possible to visualize water fractions with different binding capacities in solid particles. Experiments were carried out on a MARAN Pulsed NMR Spectrometer, Resonance Instruments Ltd., Witney, UK. Settings were as follows: (Larmor) frequency 30 MHz, pulse sequence CPMG, Tau 100 μs, number of echoes 1500, relaxation delay 2 s, number of scans 32. Measurements were performed on a single wheat kernel at room temperature. Six kernels of each pretreatment were tested.

Results and discussion

The six varieties of wheat were pretreated in two different ways, which resulted in the same water activity (0.99) and water content (0.90 $\pm$ 0.02 kg H_2O kg^{-1} dry wt) for all samples.

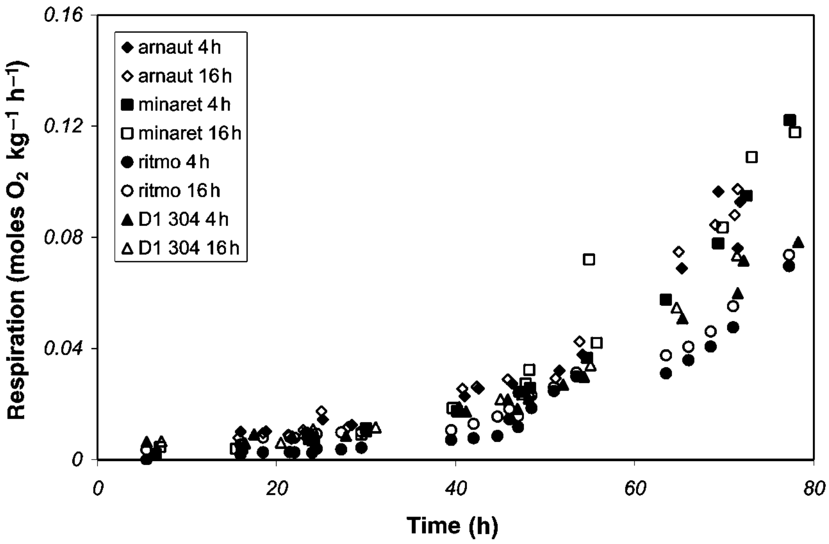

Fig. 1. Respiration rate of *A. oryzae* growing on several types of wheat at 25 °C. The wheat was pretreated in two different ways for all types. Closed symbols indicate wheat soaked 4 h at 50 °C; open symbols indicate wheat soaked 16 h at 20 °C.

As can be seen from Figure 1, no significant differences were found between Ritmo, Arnaut, Minaret 1 and 2 and D1 304 wheat, irrespective of their pretreatment. This is a comforting result since it shows that the type of wheat and the pretreatment of the wheat do not affect fungal growth for these wheat varieties. However, when *A. oryzae* was grown on Apollo wheat, the different response to the two pretreatments was obvious. This is illustrated in Figure 2. It can be concluded from Figures 1 and 2 that the maximum respiration rate is about the same for all types of wheat except the Apollo type, which has a lower respiration for both pretreatments tested. Apollo wheat that had been soaked for 16 h at 20 °C gave a higher fungal respiration rate than Apollo wheat that had been soaked at 50 °C for 4 h.

To explain the observed effect of pretreatment of Apollo wheat on respiration of the fungus, four aspects were studied:

(1) Effects of bacterial acidification during the soaking process.

(2) The presence of free sugars and amino acids (either present originally or resulting from breakdown of starch and proteins by indigenous amylase or protease activity during the soaking process). The presence of sugars and amino acids might stimulate fungal growth since these products are easier to digest than the polymers.

(3) Differences in kernel stiffness. Fungal penetration might be easier when the kernels are softer; penetration facilitates the access of the fungus to substrates inside the kernels.

(4) Differences in water distribution and water binding in the kernels.

The possible effect of bacterial acidification during the soaking of wheat as described by Hachmeister *et al.* (1993) was checked and discarded. The same decrease of pH was observed for all types of wheat and for both pretreatments (pH decreased from 6.85 to 5.66 ± 0.03).

In order to evaluate the enzymatic breakdown of starch and proteins in Apollo wheat during pretreatment, total reducing sugars and total free amino acids were measured. No significant difference (average deviation 7.2%) in sugar content was found between Apollo grains that were soaked for 4 and 16 h. The measured concentration of free amino acids was low (about 2 μg g^{-1} wheat) for both pretreatments and, again, no significant difference was found.

Kernel stiffness was investigated on single Apollo wheat kernels. Each kernel was crushed with a plunger, and a pressure sensor registered the force needed to deform the kernel. No differences were found between the two pretreatments.

Water diffusion and distribution were investigated using NMR-imaging. We focused on the ratio of the different water fractions present in pretreated wheat and the mobility of these fractions. The experiment

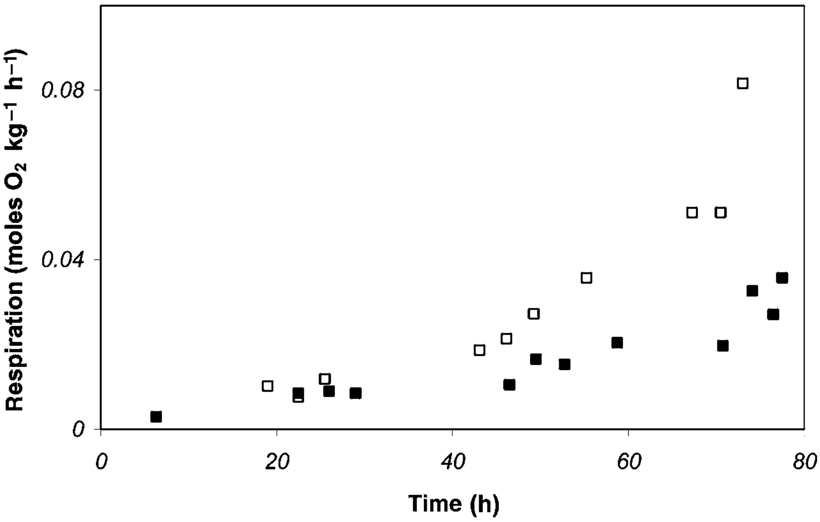

Fig. 2. Respiration rate of *A. oryzae* growing on Apollo wheat (soft) at 25 °C. Closed symbols indicate wheat soaked 4 h at 50 °C; open symbols indicate wheat soaked 16 h at 20 °C.

was carried out with Apollo and Ritmo wheat kernels from both pretreatments. No significant differences were found for the Ritmo kernels.

Figure 3 shows the relaxation time of the water in Apollo kernels. The two lines are averages of 192 measurements using 6 individual kernels. The NMR spectrum was measured 32 times for each kernel, to rule out fluctuations in the magnetic field. The deviation between the average spectrums of the 6 kernels was 1.5%. The four distinct peaks indicate the presence of four water fractions. The ratio of the four fractions differed for the two pretreatments. When read from left (least mobile fraction) to right, the peak ratios are 0, 1, 75 and 24% for the wheat that was soaked for 4 h at 50 °C and 0, 1, 61 and 38% for the wheat that was soaked for 16 h at 20 °C. The most mobile fraction, situated on the right in the spectrum (with label * in Figure 3), was less mobile in the wheat that was soaked 4 h, which can been seen from the shift of this peak to the left side of the spectrum. The shift indicates that this fraction was bound more firmly in Apollo wheat after the 50 °C pretreatment. This might indicate a lower local water activity for this pretreatment, which might explain the observed difference in fungal growth. 3D imaging can be used to locate the position of this fraction inside the kernel and to get more information on its influence on fungal growth.

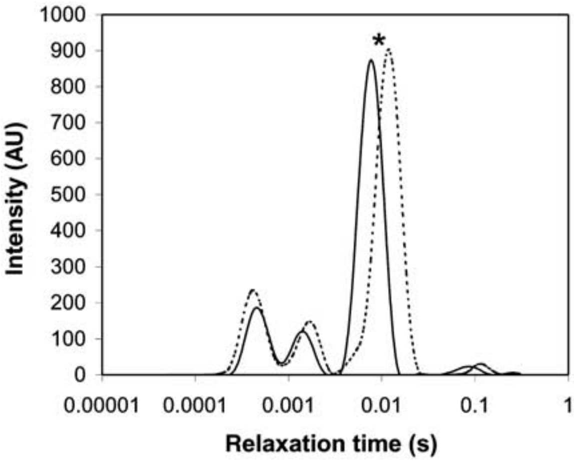

Fig. 3. ^{1}H NMR T_2 response time for Apollo wheat kernels. This figure shows the average response of 6 kernels for each pretreatment. ——: soaked for 4 h at 50 °C; - - - - - -: soaked for 16 h at 20 °C. An important difference can be seen for the most mobile fraction (with label *) in the spectrum. This fraction is shifted to the left for the wheat that was soaked at 50 °C for 4 h, which means it is less mobile than the same fraction in wheat that was soaked at room temperature.

Conclusion

Respiration of *A. oryzae* was studied during solid-state cultivation on six types of wheat that were either soaked for 16 h at 20 °C or for 4 h at 50 °C, prior to sterilization. We found that the wheat variety and pretreatment of wheat can have a considerable effect on the performance of solid-state fermentation.

Respiration rates were comparable for five out of six substrates, independent of the pretreatment method applied. However, for one of the soft wheat types different pretreatment methods caused differences in fungal respiration.

Respiration of *A. oryzae* was slower on this wheat if it had been soaked for 16 h at 20 °C. This difference could not be attributed to differences in free sugar and amino acid content, pH or kernel stiffness after soaking and sterilization. A difference in water mobility was found with NMR, which may be the cause of the difference in fungal respiration rate. One of the water fractions that could be distinguished in wheat kernels had a reduced mobility in wheat that was soaked at 50 °C for 4 h. No direct evidence that this is the cause of the observed decrease of fungal respiration rate is available yet; further research into the relation between water mobility and fungal activity in SSF is needed.

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