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Research Article

The Influence of Concentrations of Carbon Dioxide and Residual Oxygen on the Growth of Meat Spoilage Moulds

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Abstract

This study examined the effect of different concentrations of Carbon Dioxide (CO_2) and residual Oxygen (O_2) on the growth of specific spoilage moulds (*Penicillium solitum, Penicillium nordicum* and *Aspergillus proliferans*) isolated from dried, cured meat products. The objective was to assess whether residual oxygen concentrations commonly found in vacuum or modified atmosphere packed products allow for the growth of typical spoilage moulds and whether the addition of CO_2 would inhibit this growth. The three mould species were examined by plate assays. Results showed that even residual O_2 concentration of 0.05% allowed for growth of *Penicillium solitum* and *Penicillium nordicum* while *Aspergillus proliferans* grew at 0.25% O_2. The incorporation of an O_2 absorber in the package completely inhibited the growth of all three species. The addition of CO_2 in the packages significantly decreased the growth of all three species, although higher concentrations were needed to inhibit the growth of *P solitum* (80%) than for *P. nordicum* (60%) and *A. proliferans* (40-50%). In conclusion, this study illustrates the importance of controlling residual O_2 concentrations to reduce the risk of mould growth as well as the possibility to inhibit mould growth using CO_2 as a protective packaging gas.

Keywords: *Aspergillus*; CO_2; Low O_2 concentrations; *Penicillium*

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Introduction

Moulds and yeasts are one of the main causes for microbiological decay of food. Each year, the food industry has to recall or destroy vast amounts of food because of mould contamination. The meat industry in particular regularly experiences problems with the growth of *Penicillium* spp. and *Aspergillus* spp. on their products, especially products that require prolonged storage during production, like dried cured meats [1-6]. In order to reduce the losses due to mould contamination, regular cleaning and disinfection routines are implemented at production sites to prevent mould from establishing and subsequently spreading in the production environments. However, as moulds are easily spread through the air, including ventilations systems, the complete eradication of moulds from the production environments has proven to be practically impossible. The second measure is to prevent residual moulds on the products from growing in the packages. Several packaging strategies for meat are employed, from vacuum-packaging to Modified Atmosphere Packaging (MAP) containing Nitrogen (N_2), Oxygen (O_2) and Carbon Dioxide (CO_2) at various concentrations [7]. Utilizing very low levels of residual O_2 and high levels of CO_2 are suggested as a part of a hurdle technology to prevent fungal spoilage [8].

The main advantage of vacuum-packaging and packaging with pure N_2 gas is that the absence of O_2 will prevent aerobic organisms from growing [9]. However, it is very difficult to remove all O_2 during packaging, and often small O_2 residues from leakages or poor film barrier properties remain in the packages or in the product which can be utilized by moulds [10]. It is known that very small amounts of O_2 are needed in order for moulds to grow, but during growth the residual O_2 is consumed by the organisms [10]. One aim of this study is to investigate the growth of problem moulds from dry cured meat at low residual O_2 levels (<0.5%) in an N_2 atmosphere.

The second aim of the study is to evaluate the addition of CO_2 gas at various concentrations to the same mould species. It is known from several studies that CO_2 inhibits microbial growth, however, various mould species tolerate various levels of CO_2 [11-13]. One challenge with adding CO_2 is the fact that CO_2 is readily absorbed by the product, leading to package deformation after packaging. One way to prevent this is to use flexible packaging, like bags, or to partly add a filler gas, like N_2. By testing various CO_2/N_2 ratios and evaluating their effect on mould growth, the goal is to find an optimal packaging gas mixture.

Materials and Methods

Strains

Three fungal isolates were used in the experiments, strains of *Penicillium solitum*, *Penicillium nordicum* and *Aspergillus proliferans*. All strains were isolated from air samples from production sites for dry-cured meat products and selected as they represented main spoilage organisms at these sites [6] and identified by a polyphasic

approach using various growth media [14] and partial sequencing of the ITS and β-tubulin gene as described in Schirmer et al. [6].

For the plate assays, spore solutions (10^6 $CFUml^{-1}$) were prepared in 20% glycerol solution. For each solution, three spots of 1µl were inoculated on Malt Extract Agar (MEA, 15 Samson et al.,) or Yeast Extract Agar (YES) [15], for *Penicillium* and *A. proliferans* respectively and incubated in the dark at 25ºC for 24h before packaging.

Packaging and gas measurement

The study consisted of two experiments: one with concentrations of residual O_2 between 0 and 0.5% and one with concentrations of CO_2 between 0 and 80%.

Inoculated agar plates were packaged using pouches of 20x20cm with an ethylene vinyl alcohol laminate of type 3-Seal-Bag M/Pa 72 (Südpack Verpackungen, Ochsenhausen, Germany) with an oxygen permeability of $2.5cm^3m^{-2}24h^{-1}$ at 23°C and 50% relative humidity. The packages of plates were first sealed with air. At one corner, the packages were cut to yield an opening of approximately 5mm and food grade gases of N_2 or CO_2 or blends of CO_2/N_2 (AGA, Oslo, Norway) were flushed from bottles at 1.5 bars into the package for up to 60 seconds to exchange the air with CO_2/N_2 gases with <0.1% residual O_2, before the packages were resealed in the corner. Each pouch was filled with approximately 300ml gas. The gas concentrations in the packages were measured after 5 minutes. To obtain packages with elevated levels of O_2 in the headspace, variable volumes of air were injected into the packages using syringes with needles through self-sealing septas of type 644.029 (Dansensor, Ringsted, Denmark). After adjustments of gas compositions in the headspace, gas concentrations were measured again after 30 minutes.

Oxygen absorbers of type Fresh Pax (Multisorb Filtration Group, Buffalo, USA) were used in experiments where residual O_2 was absent in the packages. All packages in the CO_2 experiments initially contained residual oxygen of 0.5 (±0.1)% to potentially allow for mould growth. The packages were all stored in darkness for 7 days at 20°C. Experiments containing samples with CO_2 were carried out twice, with two plates for each strain and each CO_2 concentration. Samples for determination of gas were taken daily or at termination of experiments.

The concentrations of O_2 and CO_2 in the headspace of the packages were measured with a Dansensor Check mate 3 instrument (Dansensor) by the use of a small vacuum pump and a needle inserted through self-sealing septas (Dansensor).

Results

Residual oxygen

The results showed that even at residual oxygen concentrations of 0.05% mould growth occurred. Both *Penicillium* strains produced sporulating colonies at 0.05% O_2; however, spore formation was reduced at all O_2 concentrations below 0.5% compared to samples stored in air (Figures 1 and 2). *Aspergillus proliferans* did not show any growth below 0.2% O_2.

Oxygen consumption

Results showed that mould colony size increased as long as there was O_2 remaining in the package, but growth decreased and stopped when O_2 was consumed (Figure 3).

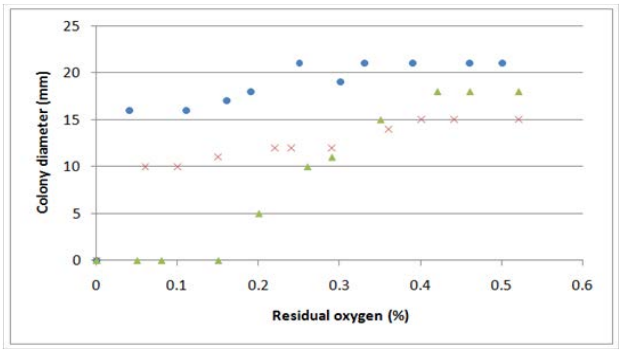

Figure 1: Mould growth after seven days storage depending on initial residual O_2 concentration. *P. solitum* (●), *P. nordicum* (x), *A. proliferans* (Δ)

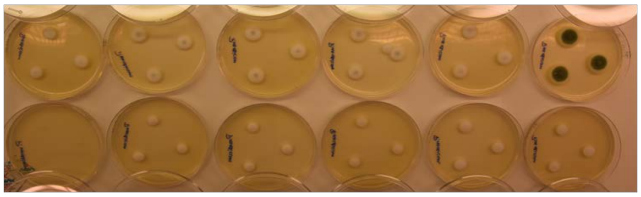

Figure 2: Colony growth of *P. nordicum* at residual O_2 concentrations ranging from 0 (O_2 absorber) to 0.5% and air.

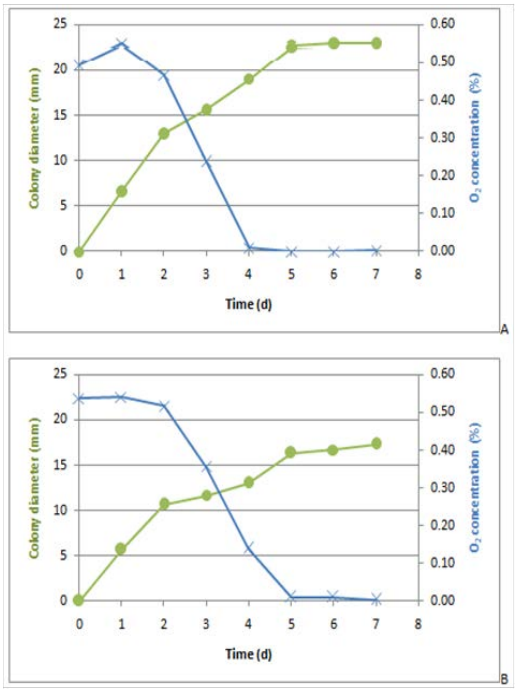

Figure 3: Colony growth (●) of *P. nordicum* (A) and *P. solitum* (B) on agar plates depending on oxygen consumption (x) in N_2 packaging.

Effect of CO_2 on mould growth

Results showed a linear decrease in mould colony size after seven days with increasing initial CO_2 concentrations (Figures 4 and 5).

All three strains were inhibited by CO_2; however, lower concentrations (40-50%) were required to completely inhibit growth of *A. proliferans* compared to *P. nordicum* and *P. solitum* (60 and 80% respectively).

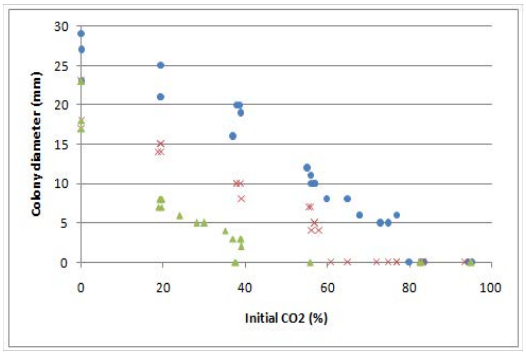

Figure 4: Mould growth after seven days depending on initial CO_2 concentration. *P. solitum* (●), *P. nordicum* (x), *A. proliferans* (Δ)

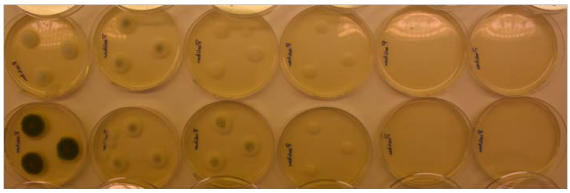

Figure 5: Colony growth of *P. solitum* in air and at CO_2 concentrations ranging from 0% to 100% (all with 0.5% O_2 and N_2 as filling gas).

Discussion

The results showed that the residual O_2 concentrations that are to be expected in products with vacuum-packaging or packaging in N_2 (approximately 0.5%) are sufficient to allow growth of undesired mould species on agar rich in nutrients. While the growth of *A. proliferans* was inhibited at 0.2% O_2, *P. nordicum* and *P. solitum* grew at O_2 concentrations of 0.05%, indicating that vacuum- or N_2-packaging alone is not sufficient to prevent mould growth. Hocking [16], previously found that many field and spoilage fungi including *Aspergillus* and *Penicillium* species are able to grow in atmospheres containing <1% O_2. However, few studies have shown the effect on growth rates at various levels of O_2 below 0.5% .One study [17], showed that O_2 levels below 0.05% were required to inhibit the growth of *Aspergillus fischeranius* in fruit puree while another study [18], reported that various mould types could grow at as low as 0.01% O_2. Smith et al. [19], showed that 0.4% O_2 were required for mould growth in CO_2/N_2 atmospheres.

In the present study it was observed that mould growth and sporulation at O_2 concentrations below 0.5% were delayed compared to growth in air; however, visible *Penicillium* growth occurred at all O_2 concentrations except 0% and sporulation could be observed at 0.2-0.4% O_2 after 7 days of storage. Residual O_2 was efficiently removed using an O_2 absorber and no growth was observed when an absorber was incorporated in the package. O_2 absorbers have proven useful for modified atmosphere packaged products. They are, however, less useful in vacuum-packed products. O_2 can be located at specific pockets in the package and absorbers may not be able to scavenge all available O_2. Also, the addition of an absorber presents an additional cost and is dependent on both consumer acceptance and the practical incorporation of the absorber in the package. Still, mould growth on dry cured meat is unacceptable for the consumer and the results show that in order to inhibit the growth of the spoilage mould the O_2 level need to be lower than achievable by most commercial vacuum or N_2 packaging alone.

When viewing the results it must be considered that absolute amount of O_2 in the packages is dependent on the total package volume. In the presented work, the total amount of gas in the packages was approximately 300ml; however, the volume was not measured for each package, thereby allowing for minor variations in O_2 amount. As results showed that mould growth prevailed until all residual O_2 was consumed, the total amount of O_2 in large packages (as for instance in 1 or 2kg bags of *Pinnekjøtt* (dry cured mutton ribs)) with a residual O_2 concentration of 0.5% may be substantial. As mould growth may appear unevenly distributed in the packages, the amount of O_2 may allow for substantial mould growth on parts of the product.

Previous studies have shown that elevated CO_2 concentrations are generally much more efficient in controlling fungal growth than oxygen depletion and that a combination of high CO_2 and low O_2 concentrations is effective in inhibiting both fungal growth and mycotoxin production [20-23]. In the present study, adding CO_2 to the packages significantly decreased mould growth and sporulation. Sixty and 80% CO_2 were required to completely inhibit the growth of *Penicillium nordicum* and *P. solitum* respectively, while lower concentrations (40-50%) were sufficient to inhibit the growth of *A. proliferans*. This is in accordance with earlier studies that showed similar inhibiting concentrations and also highlights that different mould species are able to grow at different CO_2 concentrations [22,23].

As CO_2 dissolves into the product, the use of high CO_2 concentrations (>60%) may be unsuitable for use in rigid tray packages with top films, as it may lead to package deformation. It may, however, be suitable for flexible bags.

Our study investigated the growth of three different, yet commonly found mould species from dried cured meat. Results showed that various species react differently to O_2 depletion and CO_2 exposure. This highlights the importance of customizing packaging methods to specific products and monitoring the mycobiota on the products. If new mould species with higher CO_2 tolerances are introduced to the product, mould growth may appear on previously safe products.

In the present study, the three mould types were examined on agar plates with almost ideal growth conditions with abundant access to important nutrients. The results remain to be tested on the meat products and as product properties like water content and nutritional value are of importance for mould growth, analyses have to be carried out for each product in order to determine exact threshold limits for CO_2 concentrations. However, results show that even the addition of low levels of CO_2 will significantly reduce the growth and sporulation of problem moulds and may hence add additional protection to the product.

In summary, results showed that both reducing the residual O_2 concentration to 0% by using an oxygen absorber and adding sufficient

levels of CO_2 to the packages significantly reduced the growth of undesired mould species, while conventional N_2 packaging or vacuum packaging alone may not be enough to prevent mould growth on dry cured meat products.

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