

## 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<sub>2</sub>) and residual Oxygen (O<sub>2</sub>) 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<sub>2</sub> would inhibit this growth. The three mould species were examined by plate assays. Results showed that even residual O<sub>2</sub> concentration of 0.05% allowed for growth of *Penicillium solitum* and *Penicillium nordicum* while *Aspergillus proliferans* grew at 0.25% O<sub>2</sub>. The incorporation of an O<sub>2</sub> absorber in the package completely inhibited the growth of all three species. The addition of CO<sub>2</sub> 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<sub>2</sub> concentrations to reduce the risk of mould growth as well as the possibility to inhibit mould growth using CO<sub>2</sub> as a protective packaging gas.

Keywords: *Aspergillus*; CO<sub>2</sub>; Low O<sub>2</sub> 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<sub>2</sub>), Oxygen (O<sub>2</sub>) and Carbon Dioxide (CO<sub>2</sub>) at various concentrations [7]. Utilizing very low levels of residual O<sub>2</sub> and high levels of CO<sub>2</sub> 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<sub>2</sub> gas is that the absence of O<sub>2</sub> will prevent aerobic organisms from growing [9]. However, it is very difficult to remove all O<sub>2</sub> during packaging, and often small O<sub>2</sub> 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<sub>2</sub> are needed in order for moulds to grow, but during growth the residual O<sub>2</sub> 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<sub>2</sub> levels (<0.5%) in an N<sub>2</sub> atmosphere.

The second aim of the study is to evaluate the addition of CO<sub>2</sub> gas at various concentrations to the same mould species. It is known from several studies that CO<sub>2</sub> inhibits microbial growth, however, various mould species tolerate various levels of CO<sub>2</sub> [11-13]. One challenge with adding CO<sub>2</sub> is the fact that CO<sub>2</sub> 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<sub>2</sub>. By testing various CO<sub>2</sub>/N<sub>2</sub> 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  $\beta$ -tubulin gene as described in Schirmer et al. [6].

For the plate assays, spore solutions ( $10^6$  CFUml<sup>-1</sup>) were prepared in 20% glycerol solution. For each solution, three spots of 1 $\mu$ l 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 25°C for 24h before packaging.

### Packaging and gas measurement

The study consisted of two experiments: one with concentrations of residual O<sub>2</sub> between 0 and 0.5% and one with concentrations of CO<sub>2</sub> 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<sup>3</sup>m<sup>-2</sup>24h<sup>-1</sup> 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<sub>2</sub> or CO<sub>2</sub> or blends of CO<sub>2</sub>/N<sub>2</sub> (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<sub>2</sub>/N<sub>2</sub> gases with <0.1% residual O<sub>2</sub>, 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<sub>2</sub> 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<sub>2</sub> was absent in the packages. All packages in the CO<sub>2</sub> experiments initially contained residual oxygen of 0.5 ( $\pm$ 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<sub>2</sub> were carried out twice, with two plates for each strain and each CO<sub>2</sub> concentration. Samples for determination of gas were taken daily or at termination of experiments.

The concentrations of O<sub>2</sub> and CO<sub>2</sub> 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<sub>2</sub>; however, spore formation was reduced at all O<sub>2</sub> 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<sub>2</sub>.

### Oxygen consumption

Results showed that mould colony size increased as long as there was O<sub>2</sub> remaining in the package, but growth decreased and stopped when O<sub>2</sub> was consumed (Figure 3).

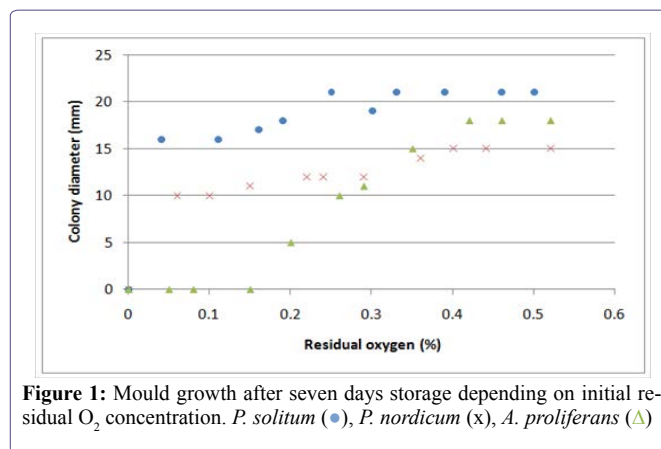


Figure 1: Mould growth after seven days storage depending on initial residual O<sub>2</sub> concentration. *P. solitum* (●), *P. nordicum* (x), *A. proliferans* (Δ)



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

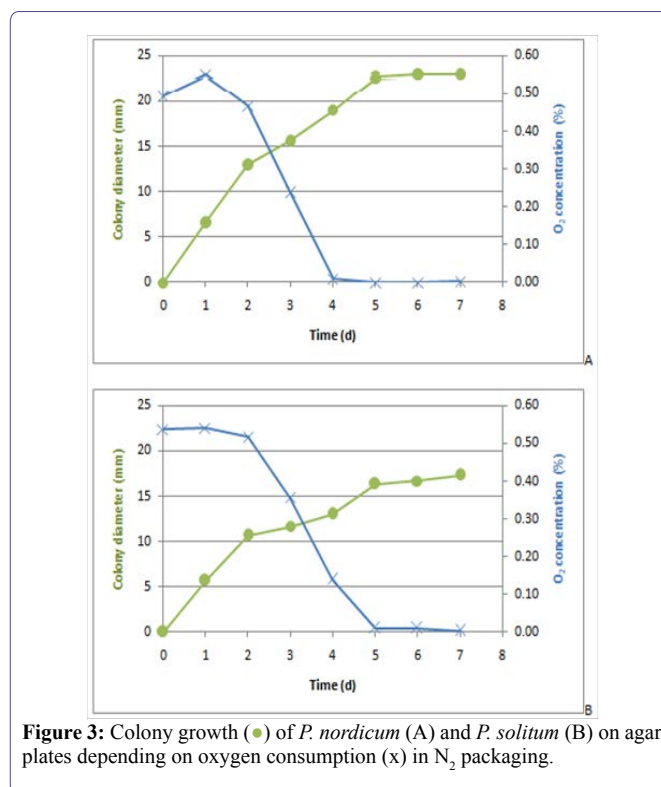
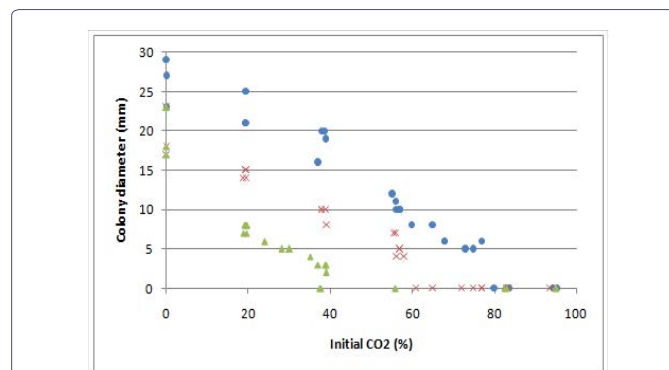


Figure 3: Colony growth (●) of *P. nordicum* (A) and *P. solitum* (B) on agar plates depending on oxygen consumption (x) in N<sub>2</sub> packaging.

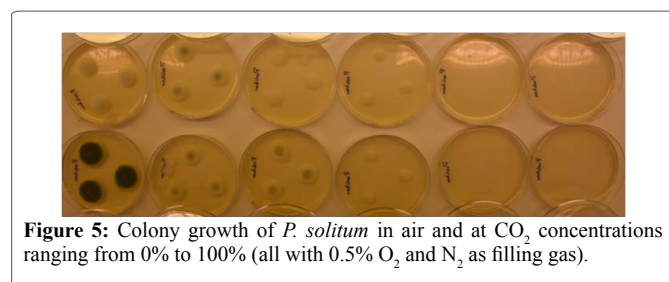
### Effect of CO<sub>2</sub> on mould growth

Results showed a linear decrease in mould colony size after seven days with increasing initial CO<sub>2</sub> concentrations (Figures 4 and 5).

All three strains were inhibited by CO<sub>2</sub>; 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<sub>2</sub> concentration. *P. solitum* (●), *P. nordicum* (x), *A. proliferans* (Δ)



**Figure 5:** Colony growth of *P. solitum* in air and at CO<sub>2</sub> concentrations ranging from 0% to 100% (all with 0.5% O<sub>2</sub> and N<sub>2</sub> as filling gas).

## Discussion

The results showed that the residual O<sub>2</sub> concentrations that are to be expected in products with vacuum-packaging or packaging in N<sub>2</sub> (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<sub>2</sub>, *P. nordicum* and *P. solitum* grew at O<sub>2</sub> concentrations of 0.05%, indicating that vacuum- or N<sub>2</sub>-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<sub>2</sub>. However, few studies have shown the effect on growth rates at various levels of O<sub>2</sub> below 0.5%. One study [17], showed that O<sub>2</sub> levels below 0.05% were required to inhibit the growth of *Aspergillus fischerianus* in fruit puree while another study [18], reported that various mould types could grow at as low as 0.01% O<sub>2</sub>. Smith et al. [19], showed that 0.4% O<sub>2</sub> were required for mould growth in CO<sub>2</sub>/N<sub>2</sub> atmospheres.

In the present study it was observed that mould growth and sporulation at O<sub>2</sub> concentrations below 0.5% were delayed compared to growth in air; however, visible *Penicillium* growth occurred at all O<sub>2</sub> concentrations except 0% and sporulation could be observed at 0.2-0.4% O<sub>2</sub> after 7 days of storage. Residual O<sub>2</sub> was efficiently removed using an O<sub>2</sub> absorber and no growth was observed when an absorber was incorporated in the package. O<sub>2</sub> absorbers have proven useful for modified atmosphere packaged products. They are, however, less

useful in vacuum-packed products. O<sub>2</sub> can be located at specific pockets in the package and absorbers may not be able to scavenge all available O<sub>2</sub>. 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<sub>2</sub> level need to be lower than achievable by most commercial vacuum or N<sub>2</sub> packaging alone.

When viewing the results it must be considered that absolute amount of O<sub>2</sub> 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<sub>2</sub> amount. As results showed that mould growth prevailed until all residual O<sub>2</sub> was consumed, the total amount of O<sub>2</sub> in large packages (as for instance in 1 or 2kg bags of *Pinnekjøtt* (dry cured mutton ribs)) with a residual O<sub>2</sub> concentration of 0.5% may be substantial. As mould growth may appear unevenly distributed in the packages, the amount of O<sub>2</sub> may allow for substantial mould growth on parts of the product.

Previous studies have shown that elevated CO<sub>2</sub> concentrations are generally much more efficient in controlling fungal growth than oxygen depletion and that a combination of high CO<sub>2</sub> and low O<sub>2</sub> concentrations is effective in inhibiting both fungal growth and mycotoxin production [20-23]. In the present study, adding CO<sub>2</sub> to the packages significantly decreased mould growth and sporulation. Sixty and 80% CO<sub>2</sub> 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<sub>2</sub> concentrations [22,23].

As CO<sub>2</sub> dissolves into the product, the use of high CO<sub>2</sub> 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<sub>2</sub> depletion and CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> concentrations. However, results show that even the addition of low levels of CO<sub>2</sub> 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<sub>2</sub> concentration to 0% by using an oxygen absorber and adding sufficient

levels of CO<sub>2</sub> to the packages significantly reduced the growth of undesired mould species, while conventional N<sub>2</sub> packaging or vacuum packaging alone may not be enough to prevent mould growth on dry cured meat products.

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