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Effects of ozone exposure on the xerophilic fungus, *Eurotium amstelodami* IS-SAB-01, isolated from naan bread ☆

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ABSTRACT

Xerophilic moulds cause contamination and spoilage of low moisture foods. This study examined the effect of ozone fumigation on growth of a *Eurotium* species isolated from naan bread. Two ozone treatments were used — a low-level long-term exposure (0.4 μmol/mol for 21 days) and high-level short-term exposure (300 μmol/mol for 5 to 120 min). For the low level exposure the combination of different media sucrose concentrations (0, 5, 10 and 20% w/v) with ozone treatment was also assessed. The growth of the isolate was found to be sensitive to low-level ozone fumigation depending on the media sucrose concentration and duration of the exposure. Low-level ozone exposure significantly ($p<0.05$) reduced the number of asexual spores formed in media with no added sucrose, an effect not observed in media with higher sucrose levels. Electron microscope observations of colonies indicated that ozone exposed cultures produced lower numbers of cleistothecia. High-level ozone exposure for short durations reduced spore viability although 100% reduction in viability was achieved only after 120 min exposure. This work demonstrates that ozone may be used to reduce spore production in *Eurotium* but that the ozone effect can be mediated by sucrose levels in the growth medium.

1. Introduction

Xerophilic moulds are common food spoilage organisms of low moisture food such as bakery products (Abellana et al., 1999; Hocking and Pitt, 1980; Samson, 1989; Vega et al., 1998), dried fruit (Pitt and Christian, 1968) and grain flours (Beuchat and Hwang, 1996). *Eurotium*, a well known xerophilic fungal genus, can account for 1 to 5% loss of bakery products along with *Aspergillus* and *Penicillium* depending on the season, type of product and method of processing (Malkki and Rauha, 1978). Xerophilic organisms are well known to be tolerant to several external stresses such as desiccation (Vaamonde and Fernández, 1993), salt/sugar concentrations, pH (Guynot et al., 2002), osmotic pressure (Christensen et al., 1965; Garg and Yadav, 2007) and heat (Splittstoesser et al., 1989). Therefore preventing spoilage caused by these organisms poses a major challenge in the food industry.

Increased awareness of the harmful effects caused by use of pesticides in food has led to interests in cleaner residue free technologies (Eom, 1994; Schafer and Kegley, 2002; Wilson and Otsuki, 2004). Ozone is well known as a strong oxidising agent and can cause elevation of reactive oxygen species in living cells leading to oxidative stress in the cells. This effect has been harnessed for preservation of food, especially fresh produce (Barth et al., 1995; Liew and Prange, 1994; Tzortzakis et al., 2008). Recent work has been carried out to explore the efficiency of ozone treatment in preservation of low moisture foods (Al-Ahmadi et al., 2009; Najafi and Khodaparast, 2009). Nevertheless very little is known on the direct effect of ozone fumigation on fungal survival and development (Antony-Babu and Singleton, 2009; Tzortzakis et al., 2008) and to our knowledge no work has examined the effect of ozone exposure on xerophilic fungi. The work presented in this paper aimed to explore the effects of ozone on *Eurotium amstelodami* IS-SAB-01 isolated from naan bread. The potential use of ozone fumigation in food storage facilities was assessed using a low-level long term (21 days) exposure study on media containing different sucrose levels, whereas the ability of ozone to kill the fungus was examined using high level ozone exposure for short periods (0 to 120 min).

2. Materials and methods

2.1. Source of isolate

The fungal isolate used in this study was obtained from mouldy naan breads (after the normal shelf-life period). The surface of naan bread was scraped using a sterile inoculation loop and the fungal propagules transferred onto the surface of Rose-Bengal agar (Fluka, UK) plate. This isolation media was used initially to isolate a wide variety of spoilage moulds. The plates were incubated at 28 °C for 21 days. An isolate which was morphologically identified as *Eurotium* sp. was used in this study.

☆ GenBank accession number related to the paper: GU808560.

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2.2. Confirmation of taxa

The isolate was characterised based on microscopic morphology as well as 28S rRNA gene sequence data. The micromorphology of the isolate was studied by viewing lacto-phenol cotton blue wet mount preparations prepared using cover-slips from slide cultures as recommended by Harris (1986). The micromophological features observed with light microscope were confirmed using a Hitachi S2400 Environmental Scanning Electron Microscope in Electron Microscopy Services, Newcastle University. Genomic DNA of the isolate was extracted by re-suspending a loop of fungal biomass in 0.5 ml of 10% (w/v) sodium dodecyl sulphate and heating at 100 °C for 5 min. The biomass debris was removed by centrifugation at 15,000×*g* and the supernatant containing the DNA further purified by ethanol precipitation. PCR amplification was carried out using UniF (GCATATCAATAAGCGGAGGAAAAG) and UniR (GGTCCGTGTTTCAAGACG) primers specific for the 28S rRNA gene (Haynes et al., 1995). The oligonucleotide DNA fragment obtained was sequenced using ABI 3730 by Geneius Laboratories Ltd., Newcastle, United Kingdom. The sequence data thus obtained was submitted to the BLAST tool on the NCBI GenBank database and the taxa established based on the maximum identity score obtained.

2.3. Effect of ozone on fungal growth with different sucrose concentrations

The *Eurotium* isolate was grown in malt extract broth for 7 days at 28 °C and 160 rpm. The growth media contained sterile steel springs to prevent fungal pellet formation. At the end of the incubation period the biomass was harvested by centrifugation at 15,000×*g*, washed using sterile distilled water and recovered by repeated centrifugation. The clean biomass suspension was adjusted to an OD_{600nm} of 0.7 (2×10^2 cfu/ml). Aliquots of 50 µl from the biomass suspension were added to the centre of malt extract agar plates with 4 different concentrations of sucrose (0%, 5%, 10% and 20% w/v). One set of plates (in replicates of 5) were incubated in an ozone rich chamber (0.4 µmol/mol ozone) and the other set in an otherwise identical ozone-free clean air incubator. The fumigation chambers used in this experiment have been described earlier (Antony-Babu and Singleton, 2009). To describe briefly the fumigation equipment consisted of two chambers ventilated with charcoal -filtered air. The ozone fumigation chamber was flushed with ozone generated by electric discharge from pure oxygen (model SGA01 Pacific Ozone Technology Inc., Brentwood, CA, USA) and the control chamber was ventilated with "clean-air" (Tzortzakis et al., 2007a). The ozone monitors used in this study were serviced weekly, and calibrated monthly against a Dasibi 1008PC unit (in turn calibrated against National Physics Laboratory standards, every 6 months). The plates were incubated in the chambers at 26 °C for 21 days.

2.3.1. Impact of ozone fumigation on growth rate

The diameter of the fungal colonies was recorded every day for 21 days. The increase in colony diameter was equated to radial growth rate of the *Eurotium* sp. Radial growth was estimated as a mean of the five recordings and was plotted against time to evaluate the increase in growth.

2.3.2. Impact of ozone fumigation on conidia production

At the end of the 21 day incubation, 1 cm^2 (1 cm×1 cm) agar plugs from the edge of the colonies (to ensure the effect noted was on the biomass produced during growth under ozone) were removed and shaken separately at 250 rpm for 18 h in an orbital shaker in 50 ml universal vials with 0.1% sodium dodecyl sulphate. This procedure aided in releasing the conidia from the fungi into suspension. The numbers of spores in suspension were counted using an "Assistenet" Neubauer-improved bright-line hemacytometer (Karl Hecht, Germany) under 40× magnification in replicates of 5 and the final spore numbers denoted as an average of all the 5 values.

2.3.3. Visual analysis of effect of ozone on Eurotium by environmental scanning electron microscopy

An agar plug (1 cm^2) was removed from the plates after the 21 day incubation period, but from the centre of the colony rather than the edge. This experiment was carried out to study the effect of ozone on fungal maturation and spore production, so the fungal mass examined was taken from the centre of the colony where the maturation is most likely to be the greatest. The agar plugs were viewed directly using a Hitachi S2400 Environmental Scanning Electron Microscope.

2.4. Effect of high level ozone treatment on viability of conidia

The surface of fungal colonies grown on malt extract agar with 20% (w/v) sucrose was scraped using a sterile inoculation loop and the spores thus harvested were resuspended in 0.1% (w/v) sodium dodecyl sulphate. The resultant suspension was filtered through sterile cotton wool to remove hyphal fragments. Conidial suspensions (checked to consist of at least 99% conidia by microscopy) were prepared in 0.1% sodium dodecyl sulphate from cultures grown on malt extract agar with 20% sucrose. Spore suspension (100 µL) was transferred onto a sterile glass slide. The inoculated glass side was placed in a sterile Petri dish and incubated overnight at 37 °C to remove moisture. The dried slides were fumigated with ozone free air and 300 µmol/mol ozone for 0, 5, 15, 60 and 120 min in triplicate. A full description of the high level ozone chamber has been provided earlier (Antony-Babu and Singleton, 2009), but briefly the set up consisted of a cylindrical stainless steel container fitted with valves. The inlet valve was connected to an ozone generator (model SGA01 Pacific Ozone Technology Inc., Brentwood, CA, USA), with ozone produced from atmospheric air. The ozone level was monitored at the outlet using an ozone monitor (Model 450 Nema, API, San Diego, CA, USA). After the fumigation, the slides were placed in 20 ml of 0.1% SDS and shaken overnight in an orbital shaker at 100 rpm to facilitate resuspension of spores. The resulting spore suspensions were plated (100 µl) on malt extract agar with 20% sucrose and incubated at 28 °C for 5 days. The numbers of colonies obtained were recorded and equated to the numbers of spores that survived the fumigation.

2.5. Statistical data analysis

The data on the colony diameters, spore numbers and colony forming units were statistically analysed for analysis of variance (ANOVA) on Minitab 15. Individual differences between means were assessed using least significant difference calculations. The significances of the data points were checked by rejecting the null hypothesis in cases where the *p* value was less than 0.05.

3. Results

3.1. Confirmation of taxa

The *Eurotium* isolate was slow growing on Rose-Bengal agar showing little growth even after 7 days of incubation in comparison to the other isolates which were identified as *Penicillium* sp. The micromorphology of the isolate showed distinctive aspergillus-like conidiation. Along with the asexual spores comparatively larger cleistothecia and ascospores with conspicuous equatorial furrows were also observed. The 28S rRNA gene sequence analysis showed maximum identity (99.1%) with *E. amstelodami*. The GenBank accession number for the isolate studied here is GU808560.

3.2. Impact of ozone on growth rates and influence of sucrose

The increase in colony diameters of *Eurotium* when cultured on different sucrose concentrations (0%, 5%, 10% and 20%) in malt extract agar after 10 days of incubation under either control (no ozone) or