IDENTIFICATION AND CHARACTERISATION OF SPOILAGE MOULDS IN CHOCOLATE CONFECTIONERY AND PATULIN-PRODUCING MOULDS IN APPLES

Nikki DE CLERCQ

Promotors:

Prof. dr. ir. Bruno De Meulenaer

Prof. dr. ir. Frank Devlieghere

Dr. ir. Els Van Coillie

Dr. ir. Geertrui Vlaemynck

Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) of Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad van Doctor in de Bio-ingenieurswetenschappen

Members reading committee:

Prof. dr. ir. Monica Höfte, Faculty of Bioscience Engineering, Ghent University, Belgium

Dr. Simbarashe Samapundo, Faculty of Bioscience Engineering, Ghent University, Belgium

Dr. François Van Hove, Mycothèque de l'Université catholique de Louvain (MUCL), Belgium

Dr. Olivier Puel, French National Institute for Agricultural Research (INRA), France

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Chairman: Prof. dr. ir. Patrick Van Damme, Faculty of Biosciences, Ghent University

Prof. dr. ir. Bruno De Meulenaer, Faculty of Bioscience Engineering, Ghent University

Prof. dr. ir. Frank Devlieghere, Faculty of Bioscience Engineering, Ghent University

Dr. ir. Els Van Coillie, Institute for Agricultural and Fisheries Research (ILVO)

Dr. ir. Geertrui Vlaemynck, Institute for Agricultural and Fisheries Research (ILVO)

Prof. dr. ir. Monica Höfte, Faculty of Bioscience Engineering, Ghent University

Dr. Simbarashe Samapundo, Faculty of Bioscience Engineering, Ghent University

Dr. François Van Hove, Mycothèque de l'Université catholique de Louvain (MUCL)

Dr. Olivier Puel, French National Institute for Agricultural Research (INRA)

Dean: **Prof. dr. ir. Marc VAN MEIRVENNE**

Rector: **Prof. dr. Anne DE PAEPE**

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Identificatie en karakterisering van schimmels die bederf veroorzaken van zoetwaren en van schimmels die patuline produceren in appels

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Abbreviation list

ABBREVIATION LIST

0–9

6MSA	6-methyl salicylic acid
6MSAS	6-methyl salicylic acid synthase
6msas	Gene enconding 6-methyl salicylic acid synthase

A

ABC	ATP binding cassette
ACN	Acetonitrile
AcOEt	Ethyl acetate
AFTOL	Assembling the Fungal Tree of Life
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
APAM	Apple Puree Agar Medium
ATCC	American Type Culture Collection
AU	Arbitrary units
a_w	Water activity

B

BCCM	Belgian Coordinated Collections of Microorganisms

C

C_{18}	Octadecyl
CA	Controlled atmosphere
CAST	Council for Agricultural Science and Technology
CBS	Centraalbureau voor Schimmelcultures
CCα	Decision limit
cDNA	Complementary DNA
CFU	Colony forming units
Choprabisco	Royal Belgian Association of the Biscuit, Chocolate, Pralines and Confectionery
CO_2	Carbon dioxide
CoA	Coenzyme A
C_q	Quantification cycle
CREA	Creatine Agar
CV	Coefficient of variation
CY20S	Czapek Yeast autolysate 20% Sucrose agar
CYA	Czapek Yeast Autolysate agar

D

Da	Dalton
DALY	Disability-adjusted life years
DG18	Dichloran 18% Glycerol agar
DG18I	DG18 supplemented with Iprodione
DG18T	DG18 supplemented with Triton X-301

DNA	Deoxyribonucleic acid

E

EC	European Commission

F

FAO	Food and Agriculture Organization
FASFC	Federal Agency for the Safety of the Food Chain
FC	Fungi Collection
FDA	US Food and Drug Administration

G

g	Gravitational constant
G25N	25% Glycerol Nitrate agar
GC-MS	Gas chromatography-mass spectrometry
GHP	Good hygiene practices
GMP	Good manufacturing practices
G/NG	Growth/no growth
gpd	Gene encoding glyceraldehyde-3-phosphate dehydrogenase

H

HACCP	Hazard analysis - critical control point
HEPA	High Efficiency Particulate Air
HTS	High-throughput sequencing
HLB	Hydrophillic lipophilic balance
HMF	5-hydroxymethyl furfural
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet
HVAC	Heating, Ventilation and Air Conditioning

I

iao	Gene encoding isoamyl alcohol oxidase
IARC	International Agency for Research on Cancer
ICFM	International Commission on Food Mycology
ICMSF	International Commission on Microbiological Specifications for Foods
ICN	*International Code of Nomenclature for algae, fungi, and plants*
ICPA	International Commission of *Penicillium* and *Aspergillus*
idh	Gene encoding isoepoxydon dehydrogenase
IMF	Intermediate moisture food
ISO	International Organization for Standardization
ITS	Internal transcribed spacer

J

JECFA	Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives

L

LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification

M

M20	Malt extract 20% sucrose agar
M40	Malt extract 40% sucrose agar
MEA	Malt Extract Agar
MIQE	Minimum Information for publication of Quantitative real-time PCR Experiments
mRNA	Messenger RNA
MSA	Malt Salt Agar
MUCL	Mycothèque de l'Université catholique de Louvain
MY5-12	Malt extract Yeast extract 5% salt 12% glucose agar
MY20G	Malt extract Yeast extract 20% Glucose agar
MY40G	Malt extract Yeast extract 40% Glucose agar
MY50G	Malt extract Yeast extract 50% Glucose agar
MY60G	Malt extract Yeast extract 60% Glucose agar
MY70GF	Malt extract Yeast extract 70% Glucose Fructose agar
m/z	Mass-to-charge ratio

N

N_2	Nitrogen
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium carbonate
Na_2SO_4	Anhydrous sodium sulfate
NOEL	No-observed effect level

O

O_2	Oxygen

P

PA	Phosphatidic acid
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDB	Potato dextrose broth agar
PE	Phosphatidylethanolamine
peg	Gene encoding polygalacturonase
pH	Potential of hydrogen
PI	Phosphatidylinositol
PKS	Polyketide synthase
PL	Phospholipid
PMTDI	Provisional maximum tolerable daily intake
PS	Phosphatidylserine

Q

qPCR	Quantitative real-time PCR
QRAM	Quantitative risk assessment model
QuEChERS	Quick, easy, cheap, effective, rugged and safe

R

R^2	Correlation coefficient
R_A	Average recovery
RAPD	Random amplification of polymorphic DNA
RCS	Reuter centrifugal air sampler
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal RNA
r_s	Spearman's rank correlation
RSD	Relative standard deviation
RSD_r	Repeatability or intraday precision
RSD_R	Reproducibility or interday precision
RT	Reverse transcription

S

SCOOP	Scientific cooperation
SD	Standard deviation
SNP	Single-nucleotide polymorphism
Spp.	Species (plural)
SPE	Solid-phase extraction
SSU rRNA	Small subunit rRNA

T

TWA	Tap Water Agar

U

U	Expanded measurement uncertainty
UPGMA	Unweighted pair group method with arithmetic average

V

V8	Vegetable 8 juice agar

W

WHO	World Heatlh Organization
w/v	Weight/volume
w/w	Weight/weight

Y

YEP	Yeast Extract Peptone agar
YES	Yeast Extract Sucrose agar

General introduction and objectives

GENERAL INTRODUCTION AND OBJECTIVES

Moulds are able to grow on different kinds of food and feed commodities, such as grains, meat products, dairy products, fruit and vegetables, nuts, etc. Their presence is very often undesirable as mould growth is in many cases associated with spoilage (e.g. off-flavours and discolouration) and in certain cases with mycotoxin contamination. Both mould and mycotoxin contamination poses a major obstacle in addressing sustainable food and nutritional security, and in increasing international trade. Food spoilage due to fungal growth negatively affects the microbial stability and consequently shelf life of products. Moreover, it may lead to great economic and environmental losses because of disposal of contaminated food and feed. In addition to these losses, mycotoxins also imply substantial risks for humans and animals due to their adverse health effects including birth defects, liver and kidney disease.

Despite efforts to control or avoid contamination by moulds and their mycotoxins, they remain to occur frequently in food commodities widespread. Chocolate confectionery and apples are two important Belgian food products, each characterised by their own fungal problems and related economic implications. The correct identification and physiology of those fungi of relevance for specific food products, and the mechanisms involved in regulating fungal growth and mycotoxin biosynthesis, are very complex and specific, and still remain to be elucidated. Therefore, prevention and control strategies can only be successful when the identity and characteristics of the associated mycobiota are well known. Hence, the **main goal of this PhD thesis** was detection, identification and characterisation of the mycobiota associated with chocolate confectionery and apples. Within this goal, focus was put on optimizing and developing methodologies through the application of a multidisciplinary research approach, i.e. a combination of conventional, molecular and/or chemotaxonomical analysis.

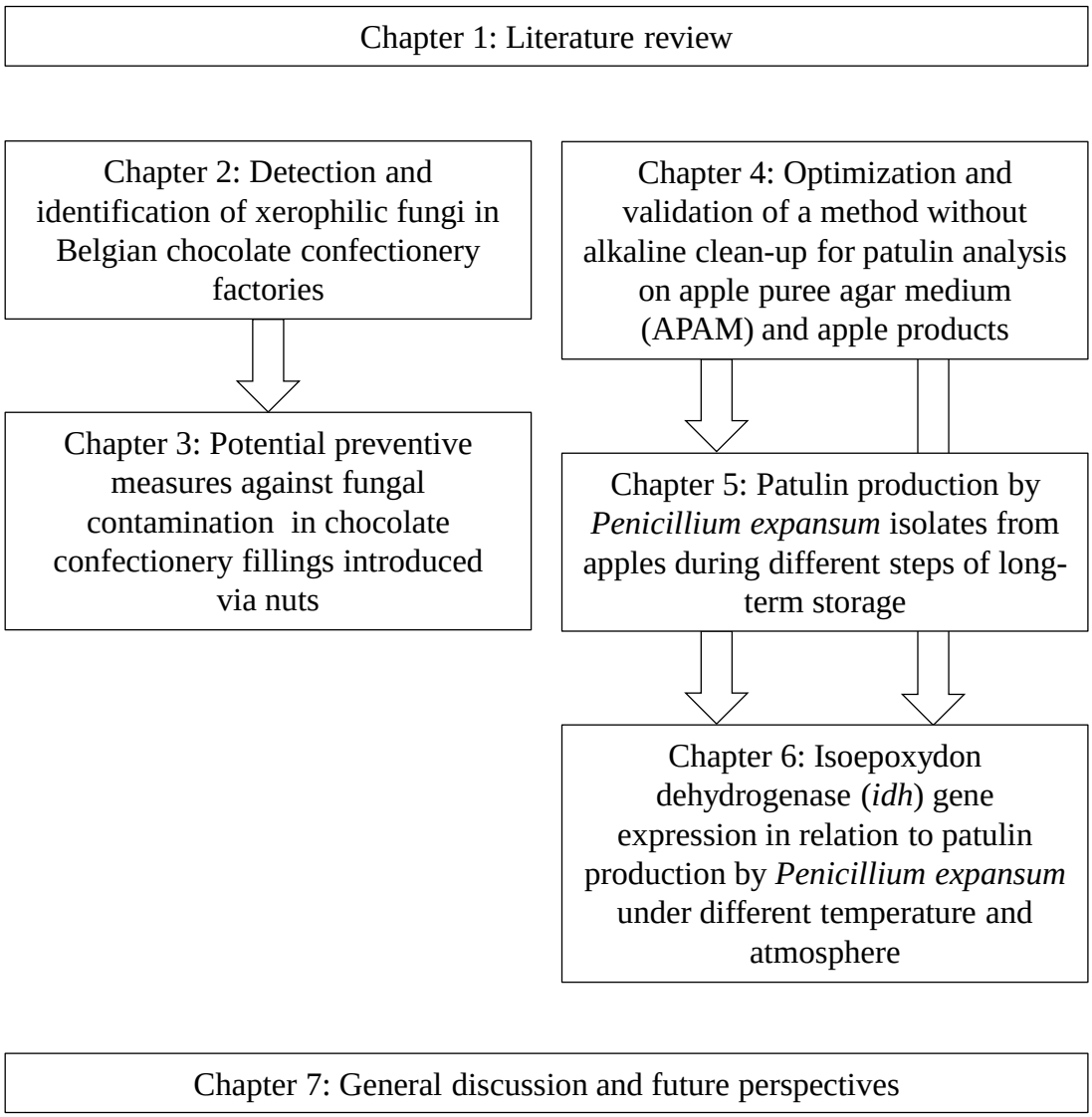
Chocolate confectionery fillings are generally regarded as microbiologically stable. Their stability is largely due to a low water activity (<0.60), which does not allow microbial growth, to the general practice of adding either preservatives or alcohol, or by maintaining a relatively short shelf life. Increased consumer demands for high-quality products containing less sugar, fat and preservatives direct the industry towards the production of innovative formulations (non-alcoholic or "clean label"). In addition, export of these products is of great economic importance to the Belgian food industry and demands sufficiently long shelf life. Changes in composition of chocolate confectionery fillings can impede the stability of these products and may lead to spoilage by xerophilic fungi.

One strategy to prevent early spoilage of sweet intermediate moisture foods is by controlling the growth of spoilage organisms in the final product. Another strategy to address the problem is by controlling the initial sources of contamination. Hence, the first objective of the **first part of this thesis** was to determine the presence and prevalence of fungal spoilage organisms in both the production environment of Belgian chocolate confectionery factories as well as in commonly used ingredients of chocolate confectionery fillings. This information provides insight into potentially important contamination sources and species of fungi that may negatively affect the quality and shelf life of chocolate confectionery products (CHAPTER 2). Based on the obtained results, CHAPTER 3 focused on the importance of nuts as potential source of fungal contamination of confectionery fillings. The application of heat-treatment of walnuts was investigated as a possible preventive measure to reduce initial fungal contamination that may end up in the final product.

Apples, as a seasonal product, need a prolonged shelf life in order to supply the market throughout the year. *Penicillium expansum* is a commonly distributed fungal species in apple orchards in the temperate regions, easily disseminated by different vectors in the orchard and by field equipment at harvest. After harvest, apples are transported to storage rooms of packing houses. During long-term storage, *P. expansum* is the principal cause of blue mould rot, one of the most severe post-harvest diseases of apples worldwide. Spoilage by blue mould rot poses an economic concern to the fresh fruit industry. However, more important is the fact that *P. expansum* is capable of producing the mycotoxin patulin, which may end up in secondary products such as apple juice. Long-term storage of apples at low temperature combined with controlled atmosphere (i.e. reduced O_2 and elevated CO_2) is a commonly applied strategy to extend their shelf life and secure a year-round supply. Baert (2007) demonstrated that this storage step is most decisive for the final patulin contamination of apple juice. Therefore, the **second part of this thesis**, focused further on the effect of temperature and atmosphere on patulin biosynthesis of various *P. expansum* strains. First, a sensitive High Performance Liquid Chromatography-UV (HPLC-UV) method, based on the AOAC Official method 2000.02, was optimized and validated for the high-throughput analysis of patulin in *in vitro* experiments on Apple Puree Agar Medium (APAM) and apple products (CHAPTER 4). This method was used to investigate the extent to which each successive step during long-term storage contributes to patulin production in various *P. expansum* isolates (CHAPTER 5). Finally, a sensitive and reliable molecular technique was developed to quantify the expression of the isoepoxydon dehydrogenase gene (*idh*) involved in the patulin biosynthesis of *P. expansum* (CHAPTER 6).

This newly developed RT-qPCR method was subsequently utilized to determine the influence of temperature and atmosphere on *idh* gene expression. In addition, *idh* gene expression levels were correlated with the patulin production that was quantified simultaneously by means of the earlier developed HPLC-UV method.

A general overview of the PhD thesis is presented below.



Chapter 1

Fungi in foods

CHAPTER 1 FUNGI IN FOODS

1.1 FUNGI

1.1.1 Introduction

The term "Fungi" comprises of organisms known as mushrooms, moulds and yeasts. This doctoral thesis focuses on moulds. Moulds can cause allergies, plant diseases and mycoses. They are able to grow on different kinds of food and feed commodities resulting in spoilage (e.g. off-flavours and discolouration) and possibly mycotoxin contamination. Spoilage typically involves shortened shelf life of food products or disposal of contaminated food and feeds, and mycotoxins imply substantial risks for humans and animals due to their adverse health effects. Each commodity has its own associated mycobiota. Attempts to prevent mould spoilage and mycotoxin contamination can only be successful if these so-called associated mycobiota are known. This thesis focuses on the characterisation of the mycobiota of chocolate confectionery fillings and apples. Before characterising those fungi that are of importance for these foods, it is relevant to start by situating them among other living organisms on Earth.

1.1.2 Classification

During history, living organisms have been classified in a variety of ways based on all different kinds of features, e.g. mode of nutrition (photosynthesis, ingestion and absorption) and cell structure (nucleus). In 1969, Whittaker proposed a five-kingdom classification, with the fungi as one of five kingdoms of living organisms, all with equal taxonomic status. Around the beginning of the 21st century, modern molecular and cladistic (phylogenetic) approaches yielded a wealth of new insights into the evolutionary relationships between organisms. This has led to the classification of living organisms into the Tree of Life, exhibiting seven kingdoms of which two are prokaryotic and five are eukaryotic (Figure 1.1).

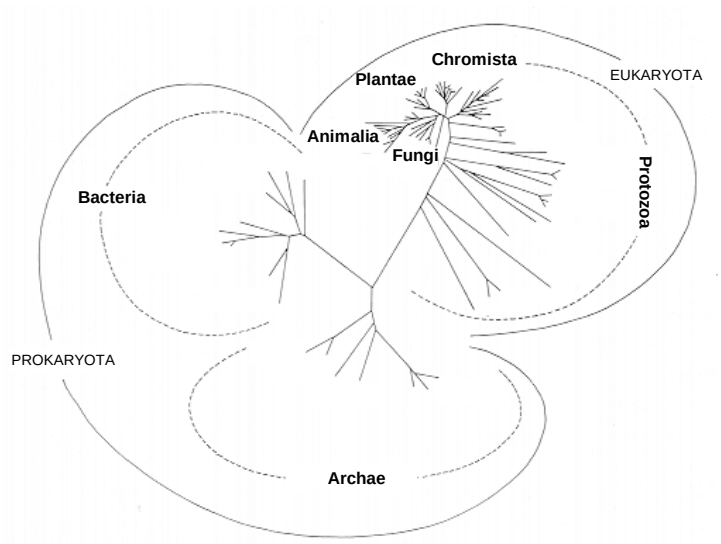


Figure 1.1 Phylogenetic tree indicating the relationship between the two prokaryotic and five eukaryotic kingdoms. The tree is unrooted, involving no assumptions about the point where the common ancestor is situated, but indicating the amount of evolutionary change and pattern of divergence, based on the extent of differences between the small subunit (SSU) ribosomal ribonucleic acid (rRNA) sequences (Carlile et al., 2001).

The Fungi are recognised as one of the five eukaryotic kingdoms, the others being the Animalia (animals), Chromista (roughly taken the algae), Plantae (plants) and Protozoa (mainly phagotrophic, unicellular organisms) (Carlile et al., 2001; Whittaker, 1969). However, this delineation is not always as clear as one should think as the fungi, studied by mycologists, can be found in three different kingdoms: the slime moulds are assembled within the kingdom Protozoa and the water moulds (Oomycetes) are common microfungi that belong to the kingdom Chromista. The kingdom Fungi consists exclusively of fungi that are hyphal or clearly related to hyphal species.

Ainsworth and Bisby 's Dictionary of the Fungi (9^{th} edition) as well as numerous phylogenetic studies of the fungal kingdom employing multi-gene sequencing, and a large collaborative analysis of the Assembling the Fungal Tree of Life (AFTOL) project gave rise to a first attempt of a higher-level phylogenetic classification that described seven phyla within the kingdom Fungi: Ascomycota, Basidiomycota, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota and Microsporidia (Hibbett et al., 2007; Kirk et al., 2008; Lutzoni et al., 2004; Schussler et al., 2001).

1.1.3 Nomenclature

Unlike most organisms, fungi can produce sexual spores (meiospores) and asexual spores (mitospores), which can be referred to as the teleomorph (sexual phase) and anamorph (asexual phase) of the fungus, respectively. Mitosporic fungi, solely known for the formation of asexual spores, were formerly classified as Deuteromycetes or Fungi Imperfecti. The whole fungus or the holomorph is defined as "*a fungus in its all meiotic or mitotic, sexual and asexual potential expressions of morphological nature and covers the unknown and known morphs of the fungus*". Not surprisingly, the manifestation of more than one type of reproductive morphology by one single entity has been a major challenge for taxonomists in naming and classifying them. As a result, the teleomorph of a whole fungus has been traditionally classified and named separately from their anamorphs. Consequently, a whole fungus finds itself in two classification and nomenclature systems against the principle of natural classification (Hennebert, 2003; Hennebert and Weresub, 1977; Kirk et al., 2008; Seifert et al., 2003). Hence, *Penicillium* and *Aspergillus* species that had not yet been found to produce a sexual state could keep their *Penicillium* and *Aspergillus* names, because of a special nomenclatural "exception" in the *International Code of Nomenclature for algae, fungi, and plants* (ICN) (Art. 59) that allowed to use two names for a specific fungal species, one for the anamorph and one for the teleomorph. Despite this, it was recommended to use the sexual name for the whole fungus, whenever a sexual state had been found. For this reason many species in *Penicillium* were renamed *Eupenicillium* or *Talaromyces* and many *Aspergillus* species were renamed *Chaetosartorya, Emericella, Eurotium, Fennellia, Hemicarpenteles, Hemisartorya, Neocarpenteles, Neopetromyces, Neosartorya, Petromyces, Saitoa, Sclerocleista,* or *Warcupiella* (Frisvad, 2015). As this was an exception to one of the basic principles of the ICN (according to which a taxon circumscribed in a particular way can have only one correct name (Principle IV)), it has become increasingly anomalous to have separate names for the anamorph and the teleomorph phases of a single fungal species. Since July 2011, mycologists have increasingly supported the concept of one name for one fungus (McNeill et al., 2005; McNeill and Turland, 2011). The International Commission of *Penicillium* and *Aspergillus* (ICPA) has decided to keep *Penicillium*, *Talaromyces* and *Aspergillus* as monophyletic clades, i.e. as big genera accomodating species from other genera instead of using several smaller genera (for example *Penicillium* includes among others *Eupenicillium* and *Aspergillus* includes among others *Eurotium*).

Proposed lists of accepted species in the genera *Penicillium, Talaromyces* and *Aspergillus* have been reported by Hubka et al. (2013), Samson et al. (2014), Visagie et al. (2014) and Yilmaz et al. (2014).

Throughout this thesis, all organisms studied are filamentous microfungi corresponding to the kingdom Fungi, and will further on be mentioned as fungi or moulds. The nomenclature of *Eurotium* has been used throughout this thesis but the newly proposed *Aspergillus* names of the fungal isolates that were identified in CHAPTER 2 have been included in Table 2.3.

1.1.4 Diversity – prevalence

Based on the Dictionary of the Fungi and the addition of Microsporidia as a fungal group, a total of about 99,000 species of known and described fungi has been reported today (Kirk et al., 2008; Lee et al., 2010). Of those, the total number of most commonly occurring species in food and indoor environments is estimated around 175 (Samson et al., 2010). In 1991, an important paper provided an estimation of 1.5 million species of fungi, based on a 6:1 ratio of known fungi to vascular plant species in regions where both were considered to be well-studied (Hawksworth, 1991). However, the estimation is considered to be conservative, as different regions remain understudied and results from molecular methods were not included. More recent estimates, based on data acquired from high-throughput environmental sequencing, have predicted as many as 5.1 million species of fungi (Hawksworth, 2001; O'Brien et al., 2005; Taylor et al., 2010).

The large gap between known and estimated numbers of fungi has led to the rise of important questions such as "which regions of the Earth harbor fungal diversity?". Fungi are able to grow in almost all habitats on Earth (soil and air of tropical to cold regions, desserts, fresh and marine waters, etc.), bypassed only by some bacteria in their ability to withstand certain extremes in temperature, water activity (a_w), and carbon source (Blackwell, 2011).

Although difficulty of isolation and failure to apply molecular methods may contribute to lower numbers of species in certain groups, there is no doubt that the Ascomycota and Basidiomycota make up the vast majority of fungal diversity. The phylum of the Ascomycota comprises around 75% of all currently known fungi including almost all xerophilic fungi (e.g. genus *Eurotium*) and the species *Penicillium expansum*, that are of interest for chocolate confectionery and apples, respectively.

1.1.5 Growth and reproduction of Ascomycota

With the exception of yeasts, fungi are multicellular. Each cell consists of a nucleus and cell organelles. Typically, fungi possess a rigid cell wall containing chitin as well as glucan, chitosan and other components. The cells are long, narrow and cylindrical and make up a network of branching tubes or hyphae called the mycelium. Yeasts grow by budding or fission and hyphae grow apically and branch laterally. Most yeasts and filamentous Ascomycota are haploid, but some species, *Saccharomyces cerevisiae* for example, can also be diploid. The Ascomycota can make spores sexually (ascospores or meiospores) and asexually (conidia or mitospores).

A schematic representation of the reproductive cycle of the *Eurotiales*, comprising most xerophiles and *P. expansum*, is given in Figure 1.2. (Pitt and Hocking, 2009; Samson et al., 2010; Taylor et al., 2006).

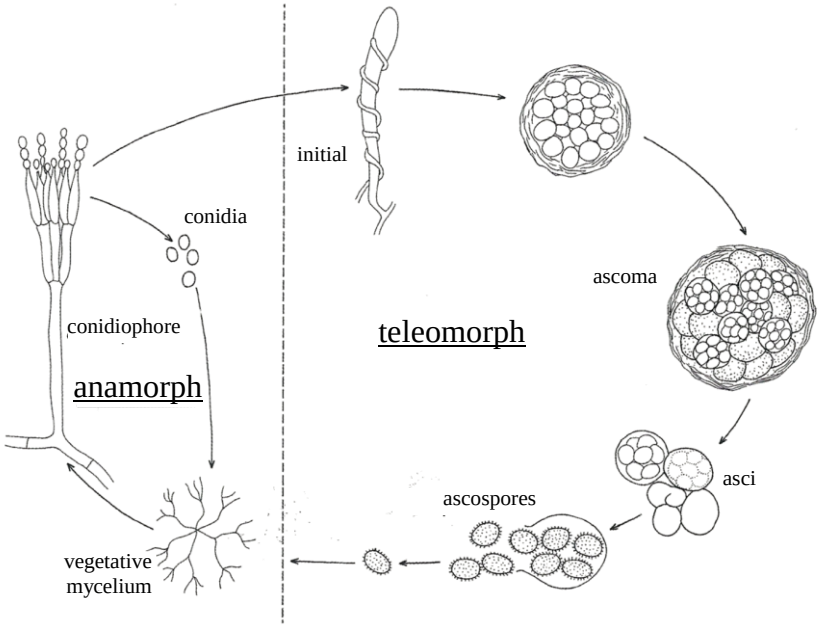


Figure 1.2 Life cycle in the Eurotiales, demonstrating teleomorph and anamorph relation (Samson et al., 2010). The Eurotiales is one of the 68 orders belonging to the phylum Ascomycota and consists of the genera *Penicillium* and *Aspergillus* of which the teleomorphs are termed *Eupenicillium/Talaromyces* and *Emericella/Eurotium/Neosartorya*, respectively (Kirk et al., 2008).

During growth, fungi utilize substrates ranging from simple sugars to cellulose, lignin, pectins and others. They secrete extracellular enzymes allowing them to degrade the substrate and to absorb the resulting, soluble nutrients. Fungal growth is however influenced by a variety of intrinsic (e.g. nutrients, a_w, pH), extrinsic (e.g. temperature, gas composition) and implicit factors (e.g. accompanying flora) (Samson et al., 2004a). The mechanisms involved in regulating fungal growth seem to be very complex and may not easily be generalized but are most probably species-, media- and concentration-dependent (Yigit and Korukluoghlu, 2007). Within the scope of this dissertation, factors influencing growth of xerophilic fungi and *P. expansum* and its patulin production will be described in more detail in paragraph 1.2 and 1.3, respectively.

1.1.6 Occurrence: economic significance (applications/implications)

In terrestrial ecosystems, plant litter decomposition and mineral nutrient recycling are among the most prominent processes regulated by saprotrophic fungi. Besides their essential role as decomposers, they may form mutualistic symbiotic associations with plants and animals or their mycelium may provide a direct food source to small animals. Fungi are of great economic significance for humans as they have yielded an increasing range of valuable products, including antibiotics (mainly penicillins and cephalosporins), other pharmaceutical drugs (e.g. for controlling cholesterol synthesis, immunosuppressive responses, blood pressure) and agrochemicals (fungicides and plant growth regulators). In addition, they have been largely exploited in the food industry (Carlile et al., 2001; Turk et al., 2006). Noteworthy is the large-scale industrial production of citric acid mainly through the growth of *Aspergillus niger* on molasses, a byproduct of sugar production (Max. et al., 2010). Perhaps one of the best known applications of microfungi in the food industry is in cheese-making, notably *Penicillium camemberti* that is used as a starter culture for the manufacture of camembert cheese and *Penicillium roqueforti* for blue-veined cheeses such as roquefort, stilton, and Danish blue (Kirk et al., 2008).

Next to the economic benefits fungi represent, their presence may also result in great economic losses, as fungi are very important agents of biodeterioration of wooden structures and stored timber, building stones, storage tanks of aviation fuel, etc. (Allsopp et al., 2004; Carlile et al., 2001).

In addition, their presence in food and feed commodities is very often undesirable as they may cause spoilage (e.g. off-flavours and discolouration). Fact is that moulds can grow on the crops in the field (pre-harvest) as well as on harvested and processed feed/food commodities and beverages (post-harvest) of all kinds, e.g. silage, cereals, meat, dairy products, fruit, vegetables, nuts, etc. (Filtenborg et al., 1996). Some fungi are major plant and insect pathogens, e.g. the fungal species *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* are the causal agents of Dutch elm disease across Europe, North America, and Southwest and Central Asia (Brasier and Buck, 2001). In addition, some fungal agents are capable of causing disease in vertebrates. Animals as well as humans may suffer allergies, mycoses and mycotoxicoses. Mycoses, as the result of fungal growth on animal or human hosts, range from being merely inconvenient (e.g. athlete's foot) to life threatening (e.g. invasive aspergilliosis). The ordinary portal of entry is through the pulmonary tract via inhalation of spores from the environment or by unusual growth of a commensal species that is normally resident on human skin or the gastrointestinal tract. Mycotoxicoses are the diseases caused by mycotoxins (e.g. "St Anthony's fire" or ergotism) (Bennett and Klich, 2003; Gravesen, 1979). Defining these mycotoxins is not as straightforward as one would think. In general, they are low-molecular weight natural products produced as secondary metabolites by filamentous fungi and exerting (acute or chronic) toxic effects on humans and animals after exposure (Bennett and Klich, 2003; Hussein and Brasel, 2001; Peraica et al., 1999). Mycotoxins are mostly consumed through contaminated food and feed (CAST, 2003). It has been estimated that 25% of the world's crops, in both developing and developed countries, are contaminated to some extent with mycotoxins (Bryden, 2007; FAO, 2003; Fink-Gremmels, 1999). Exposure to high concentrations of mycotoxins will lead to acute mycotoxicosis, having a rapid onset and an obvious toxic response (in the worst case even death). However, in a normal diet, constant exposure to low levels of several toxins is more likely to occur (Kumar et al., 2008). The corresponding chronic toxicity is characterised by a low-dose exposure over a long time and can result in diseases like cancer and other adverse effects such as irritation of the pulmonary or of the digestive tract, feed refusal, poor feed conversion and nutrient absorption, diminished body-weight gain, increased disease incidence due to immune suppression, reduced vaccination efficacy and disturbance of the endocrine and exocrine system (Coulombe, 1993; Kubena et al., 1998).

1.1.7 Identification

Attempts to prevent mould spoilage and mycotoxin contamination of food/feed can only be successful, if the so-called associated mycobiota, are known. The identity of the species will reveal important characteristics of the spoilage organism. It is however, not easy to define a fungal species. Research on the fungi that can cause food spoilage and the mycotoxins they produce, has to be based on accurate identification. Reliable results are obtained when applying a polyphasic approach, i.e. a combination of morphological, molecular and/or chemotaxonomical analysis.

1.1.7.1 Cultivation

For morphological identification, it is important to start by cultivating each pure isolate on appropriate media in order to achieve typical growth and sporulation patterns. Malt Extract Agar (MEA), V8 juice agar (V8) and Czapek Yeast Autolysate agar (CYA) are recommended as general purpose media. In the case of xerophilic fungi, the media Dichloran 18% Glycerol agar (DG18) or Malt extract Yeast extract 50% Glucose agar (MY50G) can be used. Besides these general purpose media, a variety of selective isolation media exists that may be used to select for more specific groups of fungi or even particular fungal species. It is advised to use at least two different media for identification (Samson et al., 2010). A Petri dish containing a certain medium is in most cases inoculated with a single culture at three points, equidistant from the centre and the edge of the plate and from each other (Pitt and Hocking, 2009). Most fungi can be incubated in the dark at 25°C and identified after 5-10 days. Some fungi, however, demand special incubation conditions to produce typical sporulation. For example, *Phoma* should be incubated in the dark for seven days followed by seven days in alternating darkness/diffuse daylight to show its typical sporulation pattern (Samson et al., 2010).

Within the scope of this doctoral thesis, selection of media and incubation regimes for detection, isolation and identification of xerophilic fungi will be discussed in more detail in paragraph 1.2.1. Cultivation and identification of the fungal species *P. expansum* was conducted using a combination of general and more selective media including MEA, CYA, creatine agar (CREA), and yeast extract sucrose agar (YES), as recommended by Samson et al. (2010). Standard incubation conditions of seven days at 25°C in the dark were applied.

1.1.7.2 Morphological identification

After cultivation, macro- and microscopic identification can be achieved by examining qualitative and quantitative features, which rely on observations of colonial and microscopic morphology, along with tables, keys and textbook descriptions. Examples of qualitative features are colony texture/colour(s) and ornamentation of the conidia, while commonly used quantitative features are for example colony diameter and conidia size (Pitt and Hocking, 2009; Samson et al., 2010). Unfortunately, microscopic identification is time-consuming, requires thorough taxonomic expertise and can be insufficient for separating species within species complexes. Additional constraints can be atypical morphology, failure to sporulate, long incubation periods and the sometimes-subjective nature of this approach (Balajee et al., 2007).

In the course of this thesis, morphological identification was used for classification of the fungal isolates to genus level as shown in the first steps of Figure 1.3. Subsequent identification to species level was conducted by means of molecular analysis (1.1.7.3).

1.1.7.3 Molecular identification

In the past, identification was primarily based on morphologically identified phenotypic characteristics. The constraints associated with phenotype-based methods have led to increasing use of genotype-based methods. Various techniques, such as DNA sequencing and random amplification of polymorphic DNA (RAPD) have been used for species recognition, but DNA sequencing is most frequently used for identification purposes. Nowadays, comparative sequence-based identification strategies can be considered as the "gold standard" for fungal species identification (Summerbell et al., 2005).

The flow chart in Figure 1.3 describes the different steps of molecular identification to species level. Different genes can be used for identification and an important step before starting the analysis is the selection of a good genetic marker. This selection is based on some important criteria such as a) easy to amplify, b) the presence of enough variability to allow species identification with low levels of intraspecies variation, and c) the availability of a reference dataset for comparison of the sequences obtained.

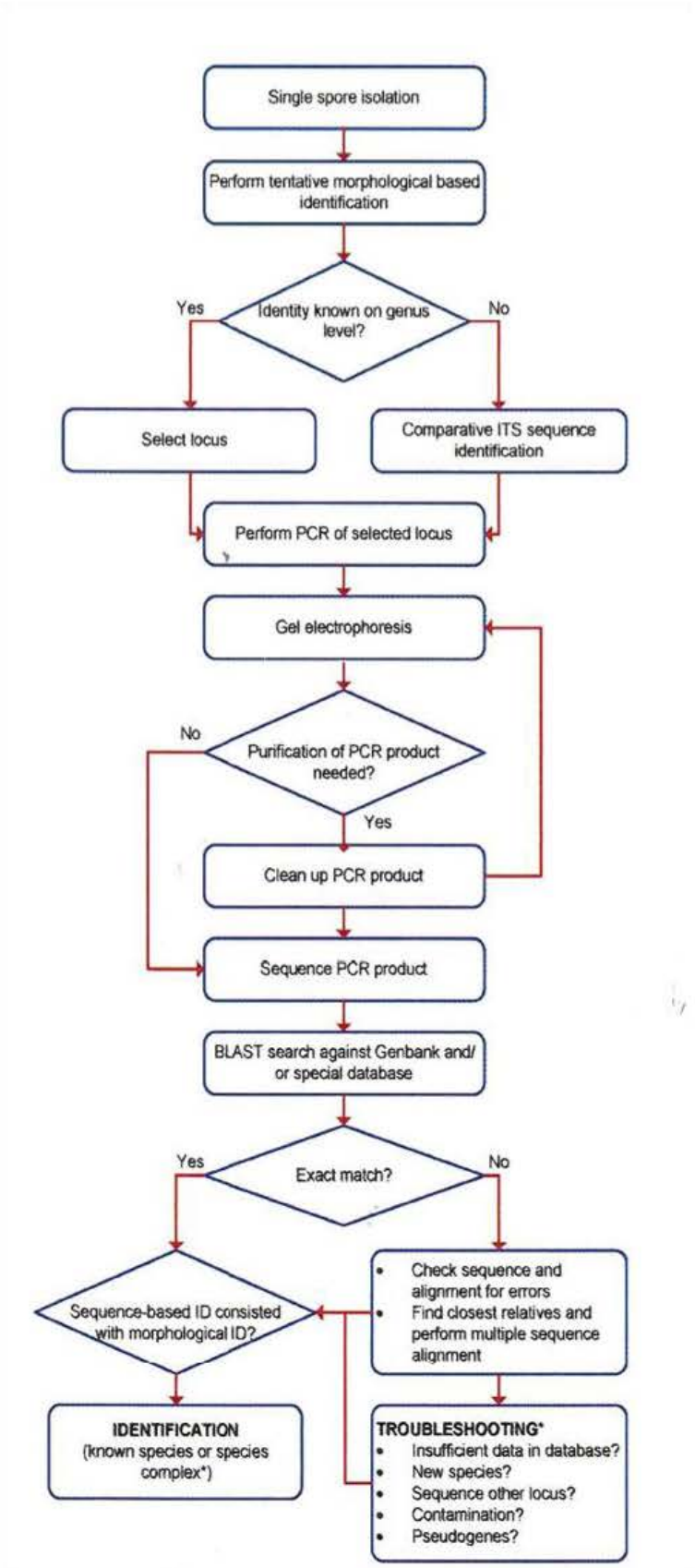


Figure 1.3 Flow chart showing the procedure to prepare and analyse a fungal culture for morphological- and molecular-based identification (Samson et al., 2010).

Sequencing of the Internal Transcribed Spacer (ITS) region along with "housekeeping genes" such as *calmodulin*, *β-tubulin* and elongation factor 1-α is commonly applied for identification of food spoilage and mycotoxigenic fungi in the genera *Aspergillus* (Geiser et al., 2007; Peterson, 2008), *Penicillium* (Peterson, 2004; Peterson, 2006; Serra et al., 2008; Wang and Zhuang, 2007) and *Fusarium* (Leslie et al., 2007; O'Donnell et al., 2004; Scott and Chakraborty, 2006).

There is no locus that can be used for all fungi and selection of a good genetic marker is genus/species dependent. More recently however, the ITS was accepted as the official barcode for fungi. ITS is the most widely sequenced marker for fungi, and universal primers are available (Schoch et al., 2012). As such, it is good practice to include ITS sequences whenever new species need to be identified. Unfortunately, for *Penicillium*, *Aspergillus* and other genera of Ascomycetes, the ITS is not variable enough for distinguishing all closely related species. Because of these limitations, a secondary barcode or identification marker is often needed for identifying isolates to species level. Based on the earlier described criteria for a good genetic marker, *β-tubulin* was proposed as the best option for *Penicillium* and *Talaromyces*, and *calmodulin* for *Aspergillus* (Samson et al., 2014; Visagie et al., 2014; Yilmaz et al., 2014) (Figure 1.3).

Primers designed for amplification of common loci including ITS, *β-tubulin* and *calmodulin* of fungal species are clearly described in literature (de Hoog and Gerrits van den Ende AH, 1998; Glass and Donaldson, 1995; Hong et al., 2006; Jaklitsch et al., 2005; Masclaux et al., 1995; O'Donnell and Cigelnik, 1997; Peterson, 2004; Peterson, 2008; Vilgalys and Sun, 1994; White et al., 1990). An overview of recommended loci and databases for identification of different groups of filamentous fungi is given in Table 1.1.

One of the main disadvantages remains the high number of misidentified strains in public databases, meaning these databases accept all sequences submitted and cannot always verify the taxonomic names attributed to the sequences (Nilsson et al., 2006). This problem can be overcome by using (if present) specific databases and by comparing sequences to type strains (if available) (Table 1.1). In a step towards cleaning up misidentified Genbank sequences, the RefSeq initiative was launched (http://www.ncbi.nlm.nih.gov/refseq/), which contains only verified ITS sequences (Samson et al., 2014; Visagie et al., 2014). However, much care should be given to the result obtained.

Table 1.1 Overview of recommended loci and databases for species identification of filamentous fungi (modification of Samson et al., 2010).

Genus	Databases	Recommended locus	Reference
Aspergillus and teleomorphs	Genbank[1]; CBS website[2]	ITS[3]	Samson et al. (2014)
		Calmodulin	
Penicillium and teleomorphs	Genbank[1]; CBS website[2]	ITS[3]	Visagie et al. (2014)
		β-tubulin	
Talaromyces	Genbank[1]; CBS website[2]	ITS[3]	Yilmaz et al. (2014)
		β-tubulin	
Fusarium	FUSARIUM-ID database (http://isolate.fusariumdb.org/)	ITS	Geiser et al. (2004)
		TEF	
Trichoderma	*Trich*OKEY (http://www.isth.info/)[3]	ITS[3]	Druzhinina et al. (2005)
		EF-1α[4]	Kopchinskiy et al. (2005)
Zygomycetes	*Trich*BLAST (http://www.isth.info/)[3]	ITS	Schwarz et al. (2006)
Alternaria		*Gpd*[5]	Hong et al. (2005)

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi (go to "nucleotide blast" for comparison of nucleotide sequences) and the reference sequence data set for ITS on http://www.ncbi.nlm.nih.gov/refseq/;[2] www.cbs.knaw.nl (go to "databases" and a drop-down menu will appear with several databases);[3] Identification based on ITS sequences sometimes fails to distinguish between closely related species;[4] Glyceraldehyde-3-phosphate dehydrogenase (*gpd*) sequences differentiate only between the *Aspergillus infectoria*-group and the *Aspergillus arborescens*/*Aspergillus tenuissima* group but not between the last two species groups.[5] Elongation factor 1 α.

1.2 XEROPHILIC FUNGI IN SWEET INTERMEDIATE MOISTURE FOOD

1.2.1 Xerophilic fungi

1.2.1.1 Habitats and physiology

Two major types of environment provide habitats for the most xerophilic organisms, namely foods preserved by some form of dehydration or enhanced sugar levels, and hypersaline sites where water availability is limited by a high concentration of salts (usually NaCl) (Grant, 2004). Microorganisms exposed to a low a_w environment all rely on a common strategy for survival, namely the intracellular accumulation of a solute or solutes, known as compatible solutes, to balance the external water activity (a_w), thus preventing the mass movement of water out of the cell (Hocking, 1988). Glycerol has been shown to be the major compatible solute accumulated internally by xerophilic fungi in response to decreased a_w (Beuchat and Hocking, 1990; Brown, 1978).

Mannitol, arabitol, xylitol, dulcitol, and sorbitol may also be accumulated but are usually regarded as storage products of carbohydrate metabolism rather than compatible solutes (Beuchat and Hocking, 1990). Some xerophilic moulds may grow equally well on substrates containing high concentrations of sugars or sodium chloride, while others prefer specific solutes. *Wallemia sebi* for example can grow on a wide range of solutes, while *Xeromyces bisporus* and *Chrysosporium fastidium* grow best in high-sugar environments, and *Polypaecilum pisce* and *Basipetospora halophila* prefer high-salt media (Andrews and Pitt, 1987; Beuchat and Pitt, 1990; Luard and Griffin, 1981; Pitt and Hocking, 1977; Wheeler and Hocking, 1988). Scott (1957) reported that *Eurotium amstelodami* grew 50% faster at its optimal a_w (0.96) when a_w was controlled by glucose rather than magnesium chloride, sodium chloride or glycerol. A similar effect was shown for *Eurotium chevalieri* (Pitt and Hocking, 1977). Next to the presence of particular solutes, the type of carbon source can also exert additional effects on the growth of fungi. Whereas bacteria are more likely to spoil proteinaceous foods, fungal metabolism is best suited to substrates high in carbohydrates. Most common mould species appear to be able to assimilate almost any food-derived carbon source. However, some xerophiles have been shown to be more demanding. Ormerod (1967) for example reported that growth of *W. sebi* was strongly stimulated by the amino acid proline.

Besides the presence of nutrients, one of the most important factors for determining fungal growth is a_w. Water activity is defined as the ratio of water vapour pressure of the food substrate to the vapour pressure of pure water at the same temperature (Jay, 2000):

$$a_w = p/p_o,$$

where p = vapour pressure of the solution and p_o = vapour pressure of water. The a_w of pure water is 1.00 and the a_w of a completely dehydrated food is 0.00.

Water activity can be defined as a measure of the unbound water in the food product, which is available for microorganisms to grow. Table 1.2 lists the approximate a_w values of some common food categories and the approximate minimum a_w values for the growth of different microorganisms relevant to food. It must be emphasized that these are approximate values because solutes can vary in their ability to inhibit microorganisms at the same a_w value. On the a_w scale, growth of microorganisms exists over the range of >0.99 to 0.60. Most bacterial pathogens are controlled at water activities well above 0.86 and only *S. aureus* can grow and produce toxin below a_w 0.90 (Mossel et al., 1995). In most fresh, high a_w foods, fungi do not grow well due to competition from bacteria. In food products, which have conditions such as low a_w, especially moulds and some yeasts may proliferate (Kirk et al., 2008). Filamentous fungi belonging to the phylum Ascomycota comprise most of the organisms capable of growth below 0.90 a_w and those capable of growth below 0.85 a_w are the earlier described xerophilic fungi (Table 1.2).

Table 1.2 Water activity (a_w) of food products in relation to approximate minima for growth of different microorganisms as reported in literature (adapted from ICMSF (1996) and Pitt and Hocking (2009)).

a_w	Foods	Bacteria	Yeasts	Moulds
>0.99	Milk, fruit, vegetables,fresh meat and fish			
0.95-0.99	Cooked ham, smoked salmon	Gram-negative bacteria (e.g. *Escherichia coli*)	Basidiomycota	Basidiomycota, most soil fungi
0.90-0.95	Bread	Gram-positive bacteria (e.g. *Listeria monocytogenes*)	Most Ascomycota	*Mucor, Fusarium*
0.85-0.90	Dry salami	*Staphylococcus aureus*	*Zygosaccharomyces rouxii* (salt)	*Rhizopus, Cladosporium*
0.60-0.85	Dried fruit, grains, confectionery, salt fish, honey, fruit cakes, soy sauce, chocolate fillings, jams	Salt lake halophiles	*Zygosaccharomyces bailii, Z. rouxii* (sugar)	xerophilic *Penicillia* and *Aspergilli, Eurotium, Chrysosporium, W. sebi, X. bisporus*
0.20-0.60	Dry pasta, spices			
0.10-0.25	Freeze-dried products			

Higher a_w values are generally required for spore formation rather than spore germination. Furthermore, minimum a_w levels for growth are lower than those required for mycotoxin production. Minimum a_w for mycotoxin production is 0.80, with the majority not producing mycotoxins at a_w<0.85 (Beuchat et al., 2013). Xerophilic fungi exhibit no special temperature requirements for growth and can commonly grow over a broad pH range of 3-8 (Pitt and Hocking, 2009). Most filamentous xerophiles grow best in the 22 to 25°C range and the optimal pH for growth is around 6.5 to 6.8 (Beuchat and Hocking, 1990).

1.2.1.2 Terminology

Xerophilic fungi are distinguished by their ability to grow under conditions of reduced a_w, i.e. to complete their life cycles on substrates that have been dried or concentrated, in the presence of high levels of soluble solids such as salts or sugars. Authors have discussed the use of various terminologies such as "halophile" (capable of growth in high salt concentrations), "osmophile" (capable of growth at high osmotic pressure), "xerophile" (capable of growth at low a_w), and "xerotolerant" (tolerance of low a_w) for fungi growing at low a_w (Pitt and Hocking, 2009). A quite comprehensive and practical working definition of Pitt (1975) describes a xerophile as a fungus capable of growth, under at least one set of conditions, at a a_w below 0.85. Moderately xerophilic fungi are than defined as fungi that are capable of growth below a_w 0.85 but that do not require this, nor is it there optimal condition. Xerophilic species of the genera *Penicillium*, *Aspergillus* and *Eurotium*, the species *W. sebi*, and a few others belong to this group. Strictly xerophilic fungi are those that require low a_w for growth and grow poorly on media in which the controlling solute is something other than sugars. *X. bisporus*, *C. fastidium*, *Chrysosporium farinicola*, *Chrysosporium inops*, *Chrysosporium xerophilum*, *Eremascus albus* and *Eremascus fertilis*, and the halophilic xerophiles *Pol. pisce* and *B. halophila* are in this group (Beuchat and Hocking, 1990; Pitt and Hocking, 2009).

1.2.1.3 Laboratory growth media

As a rule of thumb, the laboratory growth media chosen for detection, enumeration and isolation of xerophilic fungi should reflect the characteristics of the food or food-processing environment to be analysed, or the species suspected to be present. Glucose, sucrose, or glycerol supplemented media should be used for analysis of high-sugar products, while for high-salt foods, the use of media containing sodium chloride, perhaps in combination with a sugar, is more suitable.

In general, media used for the isolation and enumeration of fungi in food products and other substrates have traditionally been of high a_w values (0.99). Although such media are satisfactory for enumeration and isolation of fungi from fresh foods such as fruit, vegetables, dairy products and meat, they are inadequate for sampling the fungal flora of (semi)-dried foods (Beuchat, 1992). Hocking and Pitt (1980) were the first to develop Dichloran 18% Glycerol agar (DG18; a_w 0.95), a medium for the enumeration and isolation of xerophilic fungi from intermediate moisture food (IMF) products such as stored grains, nuts, flour, spices, dried fruits, and dried meat and fish products. It has been shown that this medium supports growth of the common *Aspergillus*, *Penicillium* and *Fusarium* species, as well as most yeasts, and many other common foodborne and airborne fungi. So, on the one hand, DG18 has been described as a general purpose isolation and enumeration medium for samples of high a_w, i.e. a_w>0.90. On the other hand, it is also recommended for the detection and enumeration of moderately xerophilic species in samples with a_w<0.90 and in dry food processing environments. Extreme xerophiles grow slowly on DG18 and are quickly overgrown by rapidly spreading xerophiles such as common *Eurotium* species. An effective medium, suitable for all except halophilic species, and recommended for foods with a_w<0.70, is Malt extract Yeast extract 50% Glucose agar (MY50G; a_w 0.89) (ISO 21527-2, 2008; Pitt and Hocking, 2009; Samson et al., 2010). Other commonly used media for isolating and enumerating xerophiles are: Malt extract Yeast extract containing 20% (MY20G; a_w 0.97), 40% (MY40G; a_w 0.93) or 60% (MY60G; a_w 0.85) Glucose, Malt extract Yeast extract 70% Glucose Fructose (MY70GF; a_w 0.76), Malt extract Yeast extract 5% salt 12% glucose (MY5-12; a_w 0.95). Other sugar-supplemented agars include malt extract agar containing 50% sucrose, used for analysing jams and jellies, fruit concentrates, and dried fruits. Czapek Yeast autolysate agar supplemented with 20% Sucrose (CY20S) enhances the probability of detecting xerophiles, particularly *Eurotium* species, in dry cheeses, grains, cured meats, and baked goods.

Malt Salt Agar (MSA) was developed in the 1940's for enumerating moulds in flour (Beuchat and Hocking, 1990). A comparison of DG18, which is typically supplemented with chloramphenicol, to DG18 supplemented with Triton X-301 (DG18T) and DG18 supplemented with Iprodione (DG18I) for enumerating xerophiles in wheat flour, proposed DG18T to be superior to DG18 and DG18I (Beuchat and Hwang, 1996).

1.2.1.4 Detection, enumeration and isolation techniques

In the food industry, fungal spoilage of raw materials or final products may occur and is often connected to the development of a specially adapted mycobiota in the factory environment. In order to obtain a complete picture, one should combine mycological analysis of raw materials and final products with analysis of surface and air samples originating from the indoor environment of production and storage areas.

As well as special media, extreme xerophiles require special techniques for isolation. Direct plating of low a_w commodities provides a good means of estimating the extent of xerophilic fungal infection (Mislivec and Bruce, 1977). In most situations, food particles should be surface disinfected before plating in order to effectively measure the inherent mycological quality. However, surface disinfection should be omitted where surface contaminants become part of the downstream mycobiota, for example, in nuts intended for confectionery fillings. Visible fungal growth on food or industrial or building materials can be examined directly under the microscope. Whether the fungi are detected directly on a food or surface substrate, or as mixed cultures on isolation media after plating, pure cultures of each isolate should be prepared by streak-inoculation on specific media as described in § 1.2.1.3. Sampling of surfaces can be conducted in a variety of ways, e.g. by means of a mycometer test, sellotape preparations, direct plating, contact plate sampling and swab sampling. Air sampling of the indoor environment may be performed both volumetric (quantitative) as well as non-volumetric (qualitative). The sedimentation-, settle- or gravity-plate method is a non-volumetric air sampling technique in which petri dishes with appropriate media are exposed to the air for a given amount of time. Volumetric air sampling is conducted by using air sampler devices, e.g. Andersen, Reuter Centrifugal air Sampler (RCS) and MAS100, that impact a defined quantity of air onto a strip or plate containing appropriate growth media (Pitt and Hocking, 2009; Samson et al., 2010)

After sampling, plates and strips should be incubated upright and the standard incubation conditions as specified by the International Commission on Food Mycology (ICFM) are 25°C for 5 days in darkness. It is recommended to continue incubation up till 2 to 3 weeks to allow germination and growth of slow growing fungi such as the extreme xerophile *X. bisporus*.

An example of analysing the xerophilic fungal flora on walnut samples is presented in Figure 1.4.

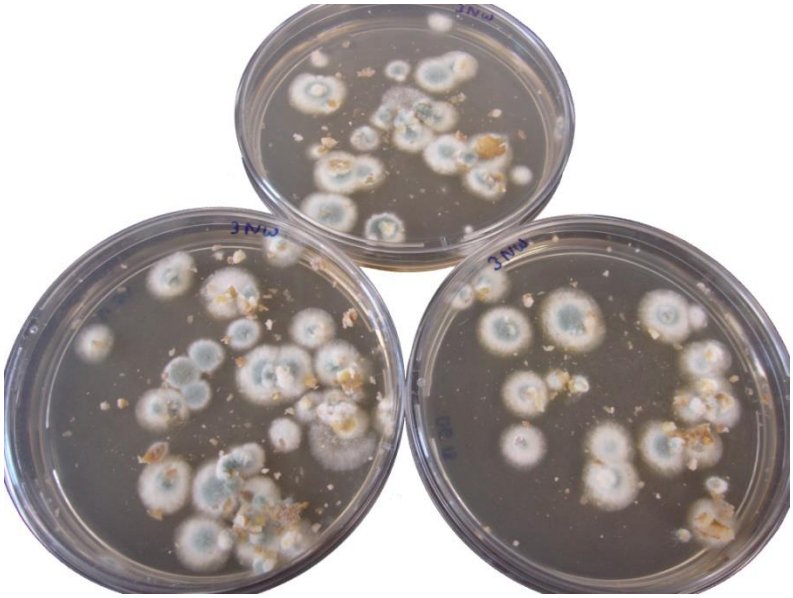


Figure 1.4 Detection and enumeration of fungal species on walnuts by direct plating of 1 g crushed walnuts on three petri plates containing Dichloran 18% Glycerol agar (DG18) medium followed by incubation for 5 days at 25°C (obtained during the course of this thesis).

1.2.1.5 Identification

As described earlier, a polyphasic approach, i.e. combining morphological, molecular and/or chemotaxonomical analysis, is advised to ensure a correct identification of unknown fungal isolates. Morphological characterization is based on observations of colonial and microscopic morphology, along with tables, keys and textbook descriptions. Pitt and Hocking (2009) present a very useful identification key of food spoilage fungi with a specific section of xerophiles, encompassing the genera *Aspergillus*, *Basipetospora*, *Bettsia*, *Chrysosporium*, *Eremascus*, *Eurotium*, *Polypaecilum*, *Wallemia* and *Xeromyces*. For practical reasons, the authors had given a different and much narrower circumscription to the term xerophile, by which a species was included solely if after seven days at 25°C, colony diameters on 25% Glycerol Nitrate agar (G25N) exceeded those on CYA and MEA. As such, many xerophilic fungi (e.g. *Pencillium brevicompactum*) that meet the definition of Pitt (1975) do not meet this criterion and were consequently not keyed within this particular section. Another equally useful key to identify the most common food and indoor fungi including xerophilic species is presented in Samson et al. (2010). Molecular analysis of xerophilic species has to be conducted as described in § 1.1.7.3.

An example of cultivating a xerophilic isolate for morphological characterization is illustrated in Figure 1.5.

Figure 1.5 Three-point inoculation of a pure culture of *E. repens* isolated from nuts. Colonies grown on Malt Extract Agar (MEA) (left) and Dichloran 18% Glycerol agar (DG18) (right) after 7 days at 25°C (obtained during the course of this thesis).

1.2.2 Two common xerophilic species: *Eurotium repens* and *Penicillium brevicompactum*

1.2.2.1 E. repens

E. repens forms colony diameters of 10-20 mm on both CYA and MEA after 7 days incubation at 25°C. The species is capable of growing over a temperature range between 4 and 40°C, and has an optimal growth at 25-27°C (Panasenko, 1967). Minimum a_w for germination in NaCl and glycerol medium were reported as 0.83 and 0.72, respectively, with optima at 0.95 and 0.91 a_w, respectively (Andrews and Pitt, 1987). There was only a slight influence of pH from 4.0-6.5, with an optimal pH around 4.5-5.5. *E. repens* produces ascospores, also known as 'heat-resistant vehicle', that survive heating to 60-70°C for 10 min. The species is not known to produce any mycotoxins (Pitt and Hocking, 2009). Mycophenolic acid production, by *E. repens* isolated from agricultural and indoor environments, was more recently reported for the first time by Séguin et al. (2014). Mycophenolic acic is an extrolite that has been shown to be a potent pharmaceutical used as an immunosuppressant in organ transplantations (Bentley, 2000). *E. repens* is a very common species and has been isolated from a broad range of food products including spoiled prunes, strawberry puree, cake, nuts, cheese, and other products. It is of common occurrence in stored grains (e.g. maize) and on processed and dried meat or fish (e.g. salami, dried salted fish) (Pitt and Hocking, 2009).

1.2.2.2 P. brevicompactum

After 7 days incubation at 25°C, *P. brevicompactum* reaches colony diameters of 20-30 mm and 12-22 mm on CYA and MEA medium, respectively. The minimum and maximum temperature for growth are -2 and 30°C, respectively, with an optimal growth near 23°C (Mislivec and Tuite, 1970). Minimum a_w for germination and growth is 0.78 at 25°C (Hocking and Pitt, 1979) categorising it as one of the most xerophilic Penicillia. The species is reported to produce a wide range of metabolites (e.g. asperphenamate, Raistrick phenols) including the most toxic compound botryodiploidin. However the frequency of producing isolates and the significance of this mycotoxin is not clear. In addition, the fungus is well known for the production of mycophenolic acid (Frisvad and Samson, 2004). *P. brevicompactum* is very common in the arctic, temperate and subtropical regions, and is widely occurring on a broad range of food products. The species has been found in dried foods (e.g. beans and nuts), on meat products (e.g. hams and salami), dairy products (e.g. cheese and margarine), fruit purée, curry paste, bakery products and in bottled water and tap water.

Moreover, it can behave as a weak pathogen already having caused spoilage of stored apples, mushrooms, cassava, potato, pumpkin, grapes, yams and lychees (Pitt and Hocking, 2009). *P. brevicompactum* also occurs in soil, indoor air, and on water-damaged building materials (Samson et al., 2010).

1.2.3 Sweet intermediate moisture food (IMF)

1.2.3.1 Introduction

The economic importance of sweet intermediate moisture food (IMF), e.g. jams, bakery products and chocolate fillings, in Belgium can be illustrated with data collated from The Royal Belgian Association of the Biscuit, Chocolate, Pralines and Confectionery, abbreviated Choprabisco. Among the different branches of the Belgian food industry, the Biscuit, Chocolate, Pralines and Confectionery branch represents 10.4% of its global turnover, 16.7% of its exports and realises a turnover of 5 billion euro (Choprabisco).

The focus of this thesis is put on fillings of the Belgian chocolate confectionery products "pralines". Typical fillings consist of butter, liquor, sugar, fruit, nuts, marzipan or different kinds of chocolate such as ganache (a mixture of cream and chocolate). These fillings contain between 20 and 50% (w/w) of water and a high amount of soluble compounds, which results in low a_w values of 0.60-0.85. Most microorganisms cannot grow below 0.90 a_w, hence, microbial spoilage of confectionery fillings is mainly caused by specialized xerophilic fungi and osmophilic yeasts (Pitt and Hocking, 2009). Typical spoilage is seen as production of off-flavours, slime formation, gas production that leads to cracking of the chocolates or visible growth on the surface of the liquid fraction (Fleet, 1992; Mossel and Sand, 1968). Chocolate confectionery products are generally regarded as microbiologically stable, largely due to their low a_w or the addition of alcohol or preservatives, which considerably reduces the risk of spoilage and extends shelf life.

Nowadays, increasing consumer demands direct the industry towards high-quality products consisting of less sugar, fat and preservatives (so-called 'clean label', i.e. ingredient list without E-numbers) but maintaining long shelf life. In addition, the use of alcohol to reduce the risk of spoilage is not allowed in some importing countries. Changes in composition of chocolate confectionery fillings and the reduction or elimination of preservatives can impede the stability of these fillings and cause spoilage by growth of osmophilic yeasts and xerophilic fungi.

1.2.3.2 Occurrence of xerophilic fungi

Table 1.3 presents literature data regarding the isolation of xerophilic mould species from sweet IMF products that may be used in the chocolate confectionery industry. Nuts were not included as numerous literature regarding the isolation of a wide range of fungal species belonging to a variety of genera including *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Eurotium*, *Rhizopus* and *Mucor* were clearly described in Pitt and Hocking (2009).

Table 1.3 Reports regarding fungal species isolated from sweet IMF of relevance for chocolate confectionery products.

Sweet confectionery product	Fungal species	Reference
Cake (intermediate moisture)	*Eurotium chevalieri, Eremascus fertilis, Eurotium repens, Eurotium rubrum, Penicillium brevicompactum, Wallemia sebi*	CBS, 2007; Membré et al., 2000; Pitt and Hocking, 2009
Caramel	*W. sebi*	Patriarca et al., 2011
Chocolate (bars, glazing, sauce, confectionery)	*Bettsia alvei, Chrysosporium farinicola, Chrysosporium inops, Chrysosporium xerophilum, E. chevalieri, E. rubrum, W. sebi, X. bisporus*	CBS, 2007; Dallyn and Everton, 1969; Hocking et al., 1994; Kinderlerer, 1997; Larumbe et al., 1991; Pitt, 1966, Pitt and Hocking, 2009; Vytrosova et al., 2002
Chocolate praline fillings (sugar- and cream-based with marzipan, fruit, nuts, spices and/or coffee)	*Aspergillus niger, Aspergillus oryzae, Aspergillus terreus, Aspergillus tubingensis, E. amstelodami, E. repens, P. brevicompactum, Penicillium chrysogenum, Penillium corylophilum*	Marvig et al., 2014
Dried fruits (prunes, vine)	*Aspergillus flavus, A. niger, Aspergillus ochraceus, Aspergillus restrictus, C. farinicola, Chrysosporium fastidium, E. amstelodami, E. chevalieri, E. repens, Eurotium herbariorum, E. rubrum, W. sebi, X. bisporus*	Dallyn and Everton, 1969; Iamanaka et al., 2005; Leong et al., 2011; Pitt, 1966; Pitt and Christian, 1968; Pitt and Hocking, 2009; Valero et al., 2007
Fruit jam	*E. amstelodami, E. rubrum, W. sebi*	Cantoni et al., 2007; Pitt and Hocking, 2009; Vytrosova et al., 2002
Gelatine confectionery	*C. inops, X. bisporus*	Pitt and Hocking, 2009
Sugar solution	*E. amstelodami, E. herbariorum, W. sebi*	Vytrosova et al., 2002
Syrup	*E. rubrum, W. sebi*	Pitt and Hocking, 2009

1.2.3.3 Preservation of IMF

Preservation of IMF is based on the delay or prevention of mould and yeast growth. It must therefore operate through those factors that most effectively influence growth of these spoilage organisms. Except for the addition of preservatives to food products, the most important intrinsic and extrinsic parameters (e.g. a_w, pH and temperature) governing growth of xerophilic fungi have been previously discussed in paragraph 1.2.1.1.

The use of preservatives in food products in Europe is regulated by the Commission Regulation (EU) (2011) No 1129/2011. Traditional weak-organic acids, e.g. sorbic- and benzoic acids, or their salts are among the most commonly used food preservatives. They are allowed in concentrations of up to 1500 ppm in confectionery products. However, the suitability and efficacy of these weak-acid preservatives is not always as straightforward as one may think. Some limitations to be considered are: odour, solubility, adverse health effects, pH-dependent efficacy and sub-optimal concentration risk. Several publications have linked traditional preservatives (e.g. sorbic- and propionic acid) with adverse health effects e.g. suppression of the immune system (Maier et al., 2010; Murr et al., 2005; Winkler et al., 2006). In addition, the use of sub-optimal levels of weak-acid preservatives in the control of mould growth has been studied in bakery products (Guynot et al., 2002; Guynot et al., 2005; Marín et al., 2002). Marín et al. (2002) for example studied the hurdle technology approach to prevent fungal growth of common contaminants of bakery products including isolates belonging to the genera *Eurotium*, *Aspergillus* and *Penicillium*. Several levels (0.003%, 0.03% and 0.3%) of calcium propionate, potassium sorbate and sodium benzoate were assayed on a model agar system in a full-factorial experimental design in which the other factors assayed were pH (4.5, 6 and 7.5) and a_w (0.80, 085, 0.90 and 0.95). Potassium sorbate was found to be the more suitable preservative to be used in combination with the common levels of pH and a_w in Spanish bakery products. However, sub-optimal concentrations (0.003% and sometimes 0.03%) led to an enhancement of fungal growth, and none of the preservatives had a significant inhibitory effect at neutral pH. It has to be noted that 0.15% is the maximum allowed concentration of these weak-acid preservatives in confectionery products. Moreover, the effective levels of preservatives might differ considerable in between different food products and in between the food product itself and the simulation medium used for the *in vitro* study.

Besides traditional preservatives, 'natural' antifungal components such as alcohol, other 'natural' weak organic acids (e.g. acetic- and lactic acid) and essential oils can be applied to extend the shelf life of confectionery products. In Commission Regulation No 1129/2011, maximum levels for both acetic acid and lactic acid are reported as "quantum satis" meaning you can add the amount needed, which emphasizes their more harmless character. There is no specific legislation on the use of ethanol, except for the labeling of beverages (Commission Regulation (EC), 2000). However, earlier research reported the rejection of cake and bread containing more than 2% alcohol on the basis of flavour and/or odour (Seiler, 1978). Moreover, the use of alcohol in chocolate confectionery products is restricted or not allowed in some importing countries. The applicability of acetic acid is rather restrained due to the sharp taste and smell of the acid (Smith et al., 2004). The use of lactic acid, which has a milder taste, may imply some risks on food spoilage because, under some conditions, growth stimulation instead of inhibition was noted for the osmotolerant yeast *Zygosaccharomyces bailii* (Vermeulen et al., 2008). The use of natural extracts (e.g. cinnamon, oregano and garlic) can be promising only when combined with the right type of confectionery filling as their particular flavour may be a major drawback. Over the last years, the use of additives in food products has been more and more criticized, due to the possible adverse health effects (e.g. suppression of the immune system), pH-dependent efficacy and sub-optimal concentration risk, and negative consumer perception towards food additives in general.

An option to enhance preservative effect and meet consumer demands in reducing the use of preservatives is the application of the hurdle concept or the combined preservation method. The principle of this technique is that combining multiple hurdles or stress factors may act synergistically through interference with the homeostasis of microbial cells (Gould, 1996; Leistner, 2000).

Predictive modeling is a very useful quantitative tool to predict the effect of a combination of factors on the growth of particular spoilage organisms. Predictive growth/no growth (G/NG) models have been developed for *Zygosaccharamyces rouxii* in sweet IMFs such as fillings of chocolate confectionery products. *Z. rouxii* is one of the few and most notorious yeast species causing spoilage of high sugar products (Fleet, 1992; Martorell et al., 2005). Vermeulen et al. (2012) developed a model including the factors a_w (0.76-0.88), pH (5.0-6.2), ethanol (0-14.5% (w/w) in water phase), sorbic acid (1500 ppm), acetic acid (0-3.5% (w/w) in water phase) and temperature (8-25°C). Microbial stability of sweet IMF could not be guaranteed by simply lowering pH and a_w.

Even with the addition of ethanol and acetic acid, it was impossible to prevent growth of *Z. rouxxi* at the highest pH (6.2) and a_w (0.88) tested. The model was further extended by incorporating the factors storage and temperature. The authors determined that storage of sweet IMFs at refrigerated temperatures is beneficial for their microbial stability. However, this also has consequences on the product itself (texture, taste, mouth feeling, etc.) and on the energy requirements in the process chain of these products (Vermeulen et al., 2015). Several IMFs, such as fruit-based chocolate confectionery fillings and jams, have lower a_w and pH values than included in the previous models. Therefore, Marvig et al. (2015) developed additional G/NG models covering acidic sweet IMFs incorporating the factors a_w (0.65-0.80), pH (2.5-4.0) and ethanol (0-14.5% (w/w) in water phase). The influence of ethanol and a_w was most pronounced during the first 30 and 60 days of incubation, respectively, and the effect of pH was almost negligible in the tested range. Besides spoilage by *Z. rouxii*, *W. sebi* and *Eurotium herbariorum* are two important xerophilic moulds capable of spoiling sugar-rich products (Deschuyffeleer et al., 2011; Samson et al., 2010). G/NG models were built including the factors a_w (0.75-0.90), pH (5.0-6.2) and ethanol concentration (0 and 5% (w/w) in water phase). Growth could only be inhibited for a prolonged time (>3 months) if an ethanol concentration of 5% was present. Based on these predictive models, it seems difficult to guarantee the microbial stability of sweet IMF products over a prolonged time without the addition of alcohol or preservatives.

One strategy to prevent early spoilage of sweet IMFs is by assuming that post-contamination cannot be excluded and that the growth of spoilage organisms can only be controlled in the final product. However, another strategy to address the problem is by controlling the initial sources of contamination. Studies concerning mould spoilage of bakery products suggest two possible sources of contamination: 1) exposure to airborne fungal spores that may originate directly from the air or that originate from dry ingredients and spread throughout the processing environment; or 2) direct introduction of fungal spores via ingredients such as sugars and nuts (Legan, 1993; Ooraikul et al., 1987; Ueda and Kuwabara, 1982). Quite some research has been performed on fungal spoilage of bakery products but little is known about the fungal environment of chocolate confectionery factories.

1.3 *P. EXPANSUM* AND PATULIN IN FOOD

1.3.1 *P. expansum*

P. expansum was one of the first *Penicillium* species to be described. Today, it is one of 58 species belonging to the genus *Penicillium* subgenus *Penicillium*, which is in turn classified within the family of the Trichocomaceae, order Eurotiales, subclass Eurotiomycetidae, class Eurotiomycetes, phylum Ascomycota (Frisvad and Samson, 2004; Kirk et al., 2008).

1.3.1.1 Morphology

P. expansum colonies on Czapek Yeast Autolysate (CYA) medium are fast growing at 25°C, reaching 26-50 mm diameter after seven days (Figure 1.6 A). The upper side of the colony is blue-green to green while the reverse appears cream to yellow with a brown center. The colonies often exhibit a broad white margin and the presence of hyaline to light yellow exudate droplets is quite common. *P. expansum* is a typically fasciculate species that can sporulate heavily. From a microscopic point of view, they are distinguished by their brush-like ter- to quarterverticillate conidiophores (Figure 1.6 C). The stipe is usually smooth-walled, in some strains occasionally rough-walled, terminating in more or less cylindrical metulaes, each bearing five to eight flask-shaped phialides. Conidia, deriving from these phialide structures, are around 3-3.5 × 2.5-3 µm in size, and mostly smooth-walled, ellipsoidal shaped (Frisvad and Samson, 2004).

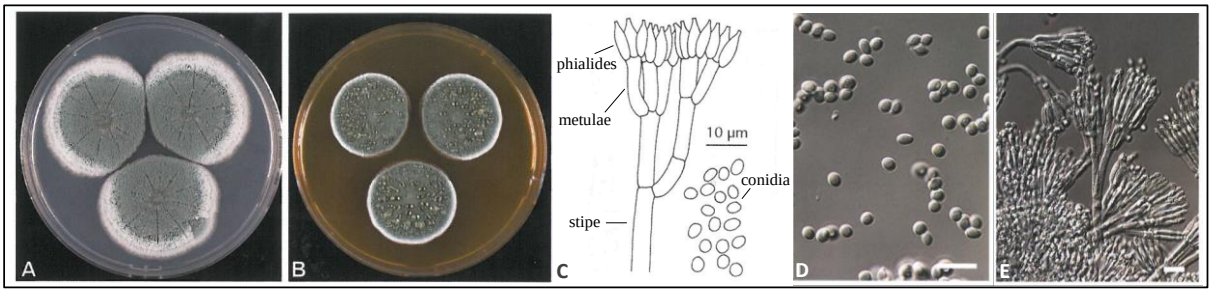


Figure 1.6 *P. expansum*. A-B) Colonies grown at 25°C for seven days on (A) CYA and (B) MEA. C-E) Conidiophores and conidia (adapted from Samson et al., 2010).

1.3.1.2 Physiology, ecology and occurrence in food

P. expansum is considered to be a psychrophile. In particular, minimum temperatures for growth have been reported as -6°C (Pitt and Hocking, 2009), -3°C (Panasenko, 1967) and -2°C (Mislivec and Tuite, 1970). The maximum growth temperature is near 35°C (Panasenko, 1967). Baert et al. (2007b) provided excellent data regarding the optimum growth temperature varying between 24 to 27°C, which is in accordance with optimal growth temperature of 25-26°C earlier reported by Panasenko (1967) and 24°C (pH 5.1 and a_w 0.99) as predicted recently by Tannous et al. (2015). The minimum a_w value permitting germination are reported between 0.82-0.83 (Hocking and Pitt, 1979a; Mislivec and Tuite, 1970) and 0.85 (Judet-Correia et al., 2011). Growth of *Penicillium* species can occur over a wide pH range of 3-8 and 2.5-7 as reported in Pitt and Hocking (2009) and Tannous et al. (2015), respectively. The species has very low requirements for oxygen given that its growth was virtually unaffected by oxygen levels as low as 2.1%. When a reduction in growth rate did occur, it was rather correlated with higher temperatures (Golding, 1945). Growth of certain species including *P. expansum*, seemed to be stimulated by carbon dioxide (CO_2) concentrations up to 15% in air, but growth rates declined at higher CO_2 levels (Pitt and Hocking, 2009).

Filamentous fungi are capable of synthesizing and accumulating primary and secondary metabolites. While primary metabolites like enzymes, DNA, polysaccharides, etc. are directly needed for vegetative growth, the role and factors controlling the production of secondary metabolites like mycotoxins is not always as clear and can only be explained in specific cases rather than explaining the phenomenon as a whole (Reverberi et al., 2010).

At first, *P. expansum* was reported to be capable of producing no fewer than 50 different secondary metabolites. Later reports, suggested the spectrum of different metabolites to be more limited: patulin, citrinin, chaetoglobosins A, B, C and others, communesins A and B, roquefortines C and D, expansolides A and B (Andersen et al., 2004; Frisvad and Samson, 2004). The presence of *P. expansum* on a particular substrate does not necessarily mean that these metabolites are produced. Both the primary and secondary metabolism is influenced to a large extent by a variety of environmental factors, and by a network of interactions between the different factors (Calvo et al., 2002; McCallum et al., 2002; Northolt et al., 1978).

P. expansum mostly occurs in temperate climate regions where it is commonly distributed in the soil and sometimes in the indoor air (Domsch et al., 1980). In addition, the species has been found on household waste, lumber and wall paper (Frisvad and Samson, 2004). *P. expansum* has been established as the principle cause of spoilage of pomaceous fruits (Raper and Thom, 1949) (Figure 1.7).

Figure 1.7 Blue mould rot on apple caused by *P. expansum* (obtained during the course of this thesis).

Isolates are therefore predominantly originating from apples and pears, but have also been isolated from a wide variety of other fruits including tomatoes, strawberries, avocados, mangoes, grapes, cherries, peaches, plums, papaya, gooseberries, rowanberries, black currants, apricots and prunes (Andersen et al., 2004; Frisvad and Samson, 2004; Pitt and Hocking, 2009).

The species has been repeatedly found on nuts including pecans, pistachios, peanuts, walnuts, acorns and pine cones (Aziz et al., 2006; Frisvad and Samson, 2004; Pitt and Hocking, 2009). Its presence on meat and meat products is widespread as well (Lacumin et al., 2009; Pitt and Hocking, 2009). Although the occurrence on cereals and grains is rare, especially in comparison with other *Penicillium* species, isolations have been reported from maize, wheat, rice, barley, corn and a variety of retail cereal products (Andersen et al., 2004; Aziz et al., 2006; Pitt and Hocking, 2009). *P. expansum* isolates from vegetables such as onions, carrots, cabbages, Brussels sprouts, beetroots and licorice roots are uncommon but have been reported as well (Andersen et al., 2004; Lugauskas et al., 2005).

Other records from which *P. expansum* isolates originate include olives, cheese, margarine, beans, dried beans, rapeseed, dried fish, frozen fruit pastries, fruit yoghurt, jellied fruit desserts, apple sauce and apple juice, and some Southeast Asian commodities like for example soybeans (Hayaloglu and Kirbag, 2007; Hocking, 1994; Pitt et al., 1994; Pitt and Hocking, 2009).

1.3.2 Patulin

1.3.2.1 History

Following the discovery of penicillin by Alexander Flemming in 1928, great interest has arisen concerning the production of fungal substances that inhibit bacterial growth and development. Consequently, in the 1940's, the compound patulin was isolated as an antibiotic. This antibiotic was simultaneously detected and named by various research groups as a metabolite of different fungal species such as *Aspergillus clavatus* (clavacin) (Moake et al., 2005), *Penicillium claviforme* (*Penicillium vulpinum*) (claviformin) (Chain et al., 1942; Wilkins and Harris, 1942), *Penicillium patulum* (*Penicillium urticae*, *Penicillium griseofulvum*) (patulin) (Birkinshaw et al., 1943) and *A. clavatus* (clavatin) (Moake et al., 2005). Anslow et al. (1943) found that patulin, previously isolated as a metabolic product from *P. patulum*, was also produced by various strains of *P. expansum* isolated from rotting fruit. Although research first suggested it to be useful in the treatment of nasal congestion and a common cold, not long after, it was proven to be not only toxic to fungi and bacteria but also to animals and higher plants (Moake et al., 2005). Mycotoxins are mostly consumed through contaminated food and feed. Consequently, the presence of patulin in food and feed implies substantial risks to animal and human health due to its toxicological properties.

1.3.2.2 Chemical properties, toxicology and legislation

Patulin or 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one is an α,β-unsaturated, heterocyclic γ-lactone (Figure 1.8). It is a relatively small molecule represented by the empirical formula $C_7H_6O_4$ and a molecular weight of 154.12 Da. Patulin is a neutral substance, which is soluble in water and most organic solvents including ethyl acetate, ethanol, acetone and diethyl ether (Ciegler et al., 1976). It is stable in acidic conditions (pH 3.3-6.3) and relatively stable to thermal degradation in the pH range of 3.5 to 5.5, with a lower pH leading to greater stability (Lovett and Peeler, 1973). *P. expansum* is capable of producing organic acids during sugar metabolism, by which the stability of patulin is improved (McCallum et al., 2002). Patulin is unstable in an alkaline solution due to hydrolysis of the lactone ring (Dombrink-Kurtzman and Blackburn, 2005).

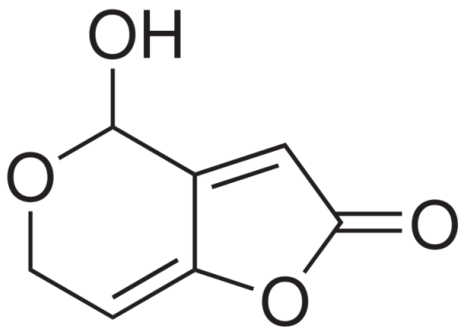


Figure 1.8 Structural formula of patulin.

The genotoxic and cytotoxic properties are believed to be related to the high reactivity of patulin with cellular nucleophiles (Glaser and Stopper, 2012). In fact, patulin has a strong affinity for sulfhydryl-containing amino acids (e.g. cysteine), glutathione or proteins (Fliege and Metzler, 1999). This affinity explains its potential to inhibit many enzymes (Arafat et al., 1985). Patulin adducts formed with cysteine are less toxic than the unmodified compound in acute toxicity, teratogenicity, and mutagenicity studies. However, it is unlikely that the toxicity is systemic since patulin is degraded quickly after penetrating the gastric wall. This degradation is caused partly by its reaction with glutathione and probably also proteins. However, the significant depletion of glutathione in gastric tissue can lead to local toxic effects (Rychlik et al., 2004; Rychlik, 2015).

More recently, the mechanisms of patulin-induced genotoxicity were further elucidated by Glaser et al. (2012). As a mechanistic hypothesis, patulin was suspected to cause structural DNA damage by cross-linking, yielding nucleoplasmic bridges and as a later consequence, micronucleus formation.

Patulin has been reported to be acutely toxic (Escoula et al., 1988; Hayes et al., 1979; WHO, 1995), genotoxic (Alves et al., 2000; Liu et al., 2003), cytotoxic (Liu et al., 2007; Riley and Showker, 1991), teratogenic (Ciegler et al., 1976) and immunosuppressive (Escoula et al., 1988; Puel et al., 2010). Few studies have been performed on the long-term toxicity effect of patulin in experimental animals. Based on those, there is inadequate evidence for carcinogenicity, hence, the International Agency for Research on Cancer (IARC) classified patulin as category 3, i.e. "not classifiable as to its carcinogenicity to humans" (IARC, 1986). Due to its toxicological properties, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 µg per kg bodyweight per day. This PMTDI is based on a no-observed effect level (NOEL) of 43 µg per kg bodyweight per day and a safety factor of one hundred (WHO, 1995). Based on this TDI, the European Commission (EC) (2006a) established maximum limits for patulin of 50 µg kg^{-1} in apple juices, 25 µg kg^{-1} in apple purees and 10 µg kg^{-1} for apple products intended for infants and young children.

1.3.2.3 Patulin producing fungi

Early studies reported patulin to be produced by more than 60 fungal species belonging to over 30 genera. Several more recent studies, however, allowed a revision of these numbers of patulin producing genera and species (Puel et al., 2010). Patulin producers have been isolated from the genera *Penicillium*, *Aspergillus*, *Byssochlamys* and *Paecilomyces*. An overview by Frisvad et al. (2004) described 14 patulin producing species among the *Penicillium* genus: *Penicillium carneum*, *Penicillium clavigerum*, *Penicillium concentricum*, *Penicillium coprobium*, *Penicillium dipodomyicola*, *P. expansum*, *Penicillium formosanum*, *Penicillium glandicola*, *Penicillium gladioli*, *P. griseofulvum*, *Penicillium marinum*, *Penicillium paneum*, *Pencillium slerotigenum* and *P. vulpinum*. Houbraken et al. (2010) and Vansteelandt et al. (2012) further reported the isolation of patulin from the species *Penicillium psychrosexualis* and *Penicillium antarcticum* cultures, respectively. The genus *Aspergillus* is probably limited to only three patulin producers belonging to the Clavati group: *A. clavatus*, *Aspergillus giganteus* and *Aspergillus longivesica* (Varga et al., 2007).

A comparative study of all *Byssochlamys* and *Paecilomyces* species showed that only *Byssochlamys nivea* and *Paecilomyces saturates* are capable of producing patulin (Samson et al., 2009).

1.3.2.4 Occurrence in food

The presence of patulin producing fungi on food products does not necessarily guarantee that patulin will be produced. As mentioned before, fungal growth and mycotoxin production is influenced to a large extent by environmental and intrinsic factors typical for the substrate. However, defining ranges and optimal conditions for patulin production is not as easy and straightforward as one might think. The production is not only influenced by each individual factor but also by interactions between factors, which makes it a very complex issue. In the case of patulin production by *P. expansum*, the fungus is capable of production over a broad temperature range of 0 to 30°C, with optimal reported at 16°C (CYA medium), 17°C (apples) and 25°C (pears) (Paster et al., 1995; Tannous et al., 2015). While *P. expansum* is capable of growing over a broad pH range and at rather low a_w, patulin production by *P. expansum* is more restricted with a minimum a_w value reported around 0.95 at 25°C (bread analogue). The bread analogue consisted of bread dough (flour, margarine, salt, baking powder, yeast extract), which was sterilised and aseptically cut into pieces and placed onto Petri dishes (Patterson and Damoglou, 1986). A recent study of Tannous et al. (2015) described the detection of traces of patulin at a_w 0.90 and 0.95 (CYA medium) with pH and temperature values fixed to 5.2 and 25°C, respectively.

A scientific Cooperation (SCOOP) study conducted by the Directorate – General Health and Consumer Protection of the European Union (EU) on the assessment of the dietary intake of patulin by the population of EU member states showed that apple juice and apple nectar are the main sources of patulin intake in most countries, particularly for young children (SCOOP, 2002).

An overview of reports regarding worldwide contamination of apple juice by patulin is given in Table 1.4. Although, apples and their respective products are of highest concern for patulin contamination, compiling research has shown patulin to occur naturally in a variety of other food sources as well. An overview of reports regarding patulin contaminated fresh and processed foods is presented in Table 1.5.

Table 1.4 Reports regarding worldwide contamination of apple juice by patulin (- = not specified).

Country	Amount of samples positive/total	Patulin levels (range) in positive samples (µg/l)	Reference
Australia	-/-	5 - 646	Reddy et al., 2010
Austria	-/-	-	Moake et al., 2005
Belgium	22/177	6 - 123	Baert et al., 2006
	-/-	-	Gillard et al., 2009
	35/43	3 - 39	Tangni et al., 2003
Brazil	1/30	17	de Sylos and Rodriguez-Amaya, 1999
	4/100	3 - 7	Iha and Sabino, 2008
	-/16	15 - 46	Welke et al., 2009
Canada	-/-	-	Moake et al., 2005
China	2/25	34 - 39	Zhou et al., 2012
Czech Republic	5/6	4 - 28	Vaclavikova et al., 2015
Finland	21/84	30 - 16400	Lindroth and Niskanen, 1978
France	27/27	<610	Barkai-Golan, 2008
Germany	12/12	6 - 26	Rychlik and Schieberle, 1999
Greece	29/29	1 - 12	Moukas et al., 2008
Holland	1/36	>25	Boonzaaijer et al., 2005
India	10/40	24 - 1839	Saxena et al., 2008
Iran	47/65	15 - 285	Cheraghali et al., 2005
	64/72	15 - 151	Forouzan and Madadlou, 2014
	54/58	11 - 122	Karimi et al., 2008
	17/64	11 - 191	Rahimi and Jeiran, 2015
Italy	-/-	0 - 1150	Beretta et al., 2000
	28/57	1 - 69	Piemontese et al., 2005
	6/15	1 - 56	Ritieni, 2003
	25/53	<48	Spadaro et al., 2007
	12/22	1 - 22	Versari et al., 2007
Japan	15/76	1 - 46	Ito et al., 2004
	9/30	1 - 15	Kataoka et al., 2009
	9/188	6 - 15	Watanabe and Shimizu, 2005
Malaysia	1/13	27	Lee et al., 2014
Portugal	28/68	<42	Barreira et al., 2010
Romania	41/50	<102	Oroian et al., 2014
Saudi Arabia	-/120	57 - 104	Gashlan, 2009
South Africa	4/17	5 - 45	Llegott and Shephard, 2001
	5/22	10 - 45	Moake et al., 2005
South Korea	3/24	3 - 9	Cho et al., 2010
Spain	30/71	<25	Cano-Sancho et al., 2009
	5/17	2 - 51	Gonzàlez-Osnaya et al., 2007
	2/28	3 - 6	Marín et al., 2011
	4/12	2 - 25	Marsol-Vall et al., 2014
	66/100	1 - 119	Murillo-Arbizu et al., 2009
	21/47	<37	Piqué et al., 2013
	82/100	<170	Prieta et al., 1994
Sweden	-/-	-	Moake et al., 2005
	5/39	<50	Thuvander et al., 2001
Taiwan	12/105	15 - 40	Lai et al., 2000

Tunisia	11/30	0 - 167	Zaied et al., 2013
	12/42	4 - 122	Zouaoui et al., 2015
Turkey	27/45	19 - 733	Yurdun et al., 2001
	215/215	7 - 376	Gökmen and Acar, 1998
	-/482	<376	Gökmen and Acar, 2000
USA	23/40	10 - 350	Brackett and Marth, 1979
	-/-	<2700	Harris et al., 2009
	8/13	44 - 309	Moake et al., 2005

Table 1.5 Reports regarding patulin occurrence in fresh and processed food products (- = not specified).

Fresh or processed food products	Amount of samples positive/total	Patulin levels (range) in positive samples (µg/l)	References
Apple	-/-	-	Beretta et al., 2000
	32/35	1010 - 120400	Celli et al., 2009
	-/-	150 - 267	de Sylos and Rodriguez-Amaya, 1999
	28/61	154 - 136154	Harwig et al., 1973
	100/100	150000 - 1000000	Hasan, 2000
	10/21	1 - 44572	Piemontese et al., 2005
	1/3	415	Vaclavikova et al., 2015
	-/-	-	Walker, 1969
Apple cider	92/493	-	Harris et al., 2009
	2/8	5 - 10	Leggott and Shephard, 2001
	-/-	-	Moake et al., 2005
	2/8	1 - 4	Piemontese et al., 2005
	3/7	3 - 6	Tangni et al., 2003
	2/2	12 - 48	Vaclavikova et al., 2015
	-/-	244 - 3993	Wheeler et al., 1987
	9/100	<45000	Wilson and Nuovo, 1973
Apple jam	6/26	17 - 39	Funes and Resnik, 2009
	1/1	-	Lindroth and Niskanen, 1978
	5/15	5 - 554	Zouaoui et al., 2015
Apple juice			see Table 1
Apple leather	35/35	7 - 2559	Montaseri et al., 2014
Apple puree	4/8	22 - 221	Funes and Resnik, 2009
	6/18	8 - 28	Gonzàlez-Osnaya et al., 2007
	6/46	6 - 50	Piqué et al., 2013
	3/6	16 - 74	Ritieni, 2003
	7/35	2 - 77	Zouaoui et al., 2015
Apricot juice (concentrate)	2/2	12 - 15	Moukas et al., 2008
	7/27	2 - 32	Spadaro et al., 2008
Baby food	5/76	3 - 6	Barreira et al., 2010
	-/-	-	Beretta et al., 2000
	79/120	3 - 9	Bonerba et al., 2010
	42/124	<10	Cano-Sancho et al., 2009
	6/16	5 - 20	Leggott and Shephard, 2001
	3/3	4 - 7	Moukas et al., 2008
	10/40	1 - 13	Piemontese et al., 2005
	2/10	13 - 18	Ritieni, 2003
	-/-	-	Sarubbi et al., 2016
	2/24	2 - 5	Vaclavikova et al., 2015
	7/25	0 - 165	Zaied et al., 2013
Beer with 35% apple concentrate	1/3	10	Marsol-Vall et al., 2014
Blackcurrant jam	-/-	-	Moake et al., 2005
Blackcurrant juice	1/1	<1	Rychlik and Schieberle, 1999
Blueberry jam	-/-	-	Moake et al., 2005
Bread	-/-	-	Moake et al., 2005
Cheddar cheese	-/-	-	Moake et al., 2005

Cherry juice	-/-	-	Moake et al., 2005
	1/1	<1	Rychlik and Schieberle, 1999
Corn	-/-	-	Moake et al., 2005
Dairy products (apple-based)	3/6	4 - 15	Gonzàlez-Osnaya et al., 2007
Dried figs	-/-	5 - 152	Karaca and Nas, 2006
Fruit juices	2/6	5	Leggott and Shephard, 2001
	12/12	3 - 11	Moukas et al., 2008
	1/1	1	Rychlik and Schieberle, 1999
	22/82	2 - 55	Spadaro et al., 2007
	9/29	2 - 25	Spadaro et al., 2008
	12/30	0 - 125	Zaied et al., 2013
	17/34	10 - 56	Zouaoui et al., 2015
Fruit leather (apple- or pear-based)	14/36	4 - 58	Maragos et al., 2015
Fruit salad	1/1	14	Vaclavikova et al., 2015
Grape juice	21/55	-	Altmayer et al., 1982
	4/24	5 - 16	Cho et al., 2010
	-/-	-	Moake et al., 2005
	3/20	5 - 17	Rahimi and Jeiran, 2015
	2/2	5	Rychlik and Schieberle, 1999
Hawthorn beverages	6/43	20 - 207	Li et al., 2007
Hawthorn juice	1/13	12	Zhou et al., 2012
Lychee juice	1/6	13	Lee et al., 2014
Orange juice	2/24	10 - 31	Cho et al., 2010
	3/3	3 - 11	Moukas et al., 2008
	1/1	<1	Rychlik and Schieberle, 1999
Passion fruit juice	-/-	-	Moake et al., 2005
Peach	-/-	92 - 174	de Sylos and Rodriguez-Amaya, 1999
Peach juice (concentrate)	4/15	9 - 21	Marín et al., 2011
	3/30	5 - 35	Rahimi and Jeiran, 2015
	2/30	2 - 5	Spadaro et al., 2008
Pear	-/-	134 - 245	de Sylos and Rodriguez-Amaya, 1999
	1/3	42	Vaclavikova et al., 2015
Pear jam	1/6	25	Funes and Resnik, 2009
	7/16	17 - 325	Zouaoui et al., 2015
Pear juice (concentrate)	5/10	67 - 74	Marín et al., 2011
	-/-	-	Moake et al., 2005
	5/15	1 - 61	Piemontese et al., 2005
	2/15	5 - 31	Rahimi and Jeiran, 2015
	25/39	2 - 33	Spadaro et al., 2008
	2/3	12 - 39	Vaclavikova et al., 2015
	20/42	5 - 231	Zouaoui et al., 2015
Pineapple juice (concentrate)	1/6	33	Lee et al., 2014
	-/-	-	Moake et al., 2005
	1/1	8	Moukas et al., 2008
Pomegranate juice	1/12	8	Rahimi and Jeiran, 2015
Red fruits (soft)	4/50	-	Van de Perre et al., 2014
Strawberry jam	-/-	-	Moake et al., 2005
Sweet bell pepper	5/44	-	Van de Perre et al., 2014
Tomato	17/158	-	Van de Perre et al., 2014

1.3.2.5 Biosynthesis pathway: precursors and enzymology

Patulin is like most fungal secondary metabolites and several major mycotoxins a polyketide metabolite. Fungal polyketides are synthesized by type I polyketide synthases (PKSs), which are multidomain proteins that are related to eukaryotic fatty-acid synthases and contain similar domain structures. For a PKS, short-chain carboxylic acids (usually acetyl coenzyme A (acetyl CoA) and malonyl CoA) are condensed to form carbon chains of varying lengths. In the fungal PKSs, the ketoacyl CoA synthase, acyl transferase and acyl carrier domains are essential for polyketide synthesis (Keller et al., 2005). Historically, 6-methylsalicylic acid synthase (6MSAS), involved in the first step of the patulin biosynthesis pathway, was the first PKS to be studied and characterized *in vitro*. The biosynthetic pathway of patulin additionally involves a number of different post-polyketide synthesis steps. In fact, the patulin biosynthesis pathway consists of about ten steps as suggested by several biochemical studies and by the identification of several mutants that were blocked at various steps in the pathway (Puel et al., 2010) (Figure 1.9 A).

Subsequent to the original isolation of patulin from the growth filtrates of *P. griseofulvum* (Birkinshaw et al., 1943), the first pathway intermediate 6-methylsalicylic acid (6MSA) was isolated from these filtrates. The enzyme 6MSAS is responsible for the formation of 6MSA by the condensation of one acetyl-CoA and three malonyl-CoA units (Tanenbaum and Bassett, 1959). In addition to patulin and 6MSA, investigation succeeded in isolating various other metabolites including gentisyl alcohol, gentisaldehyde, gentisic acid (Birkinshaw et al., 1943), *m*-hydroxybenzyl alcohol (Rebstock, 1964), *m*-cresol, *m*-hydroxybenzaldehyde, *m*-hydroxybenzoic acid and toluquinol (Puel et al., 2010) (Figure 1.9 A).

Identification of these *P. griseofulvum* metabolites provided the basis for determining the sequence of reactions in the conversion of 6MSA into patulin. A first step of this conversion involves the 6MSA decarboxylase-mediated decarboxylation of 6MSA into *m*-cresol (Light, 1969; Puel et al., 2010). Based on studies using radioactively labeled intermediates, methyl hydroxylation of *m*-cresol to *m*-hydroxybenzyl alcohol was suggested to represent an important reaction in the pathway leading to patulin formation. On the other hand, ring hydroxylation of *m*-cresol to toluquinol appeared to be a side reaction functional at higher concentrations of *m*-cresol.

Both hydroxylation reactions seemed to be catalyzed by typical mixed-function oxidases involving an NADPH-dependent reductase and a pigment-like cytochrome P450 (Murphy et al., 1974).

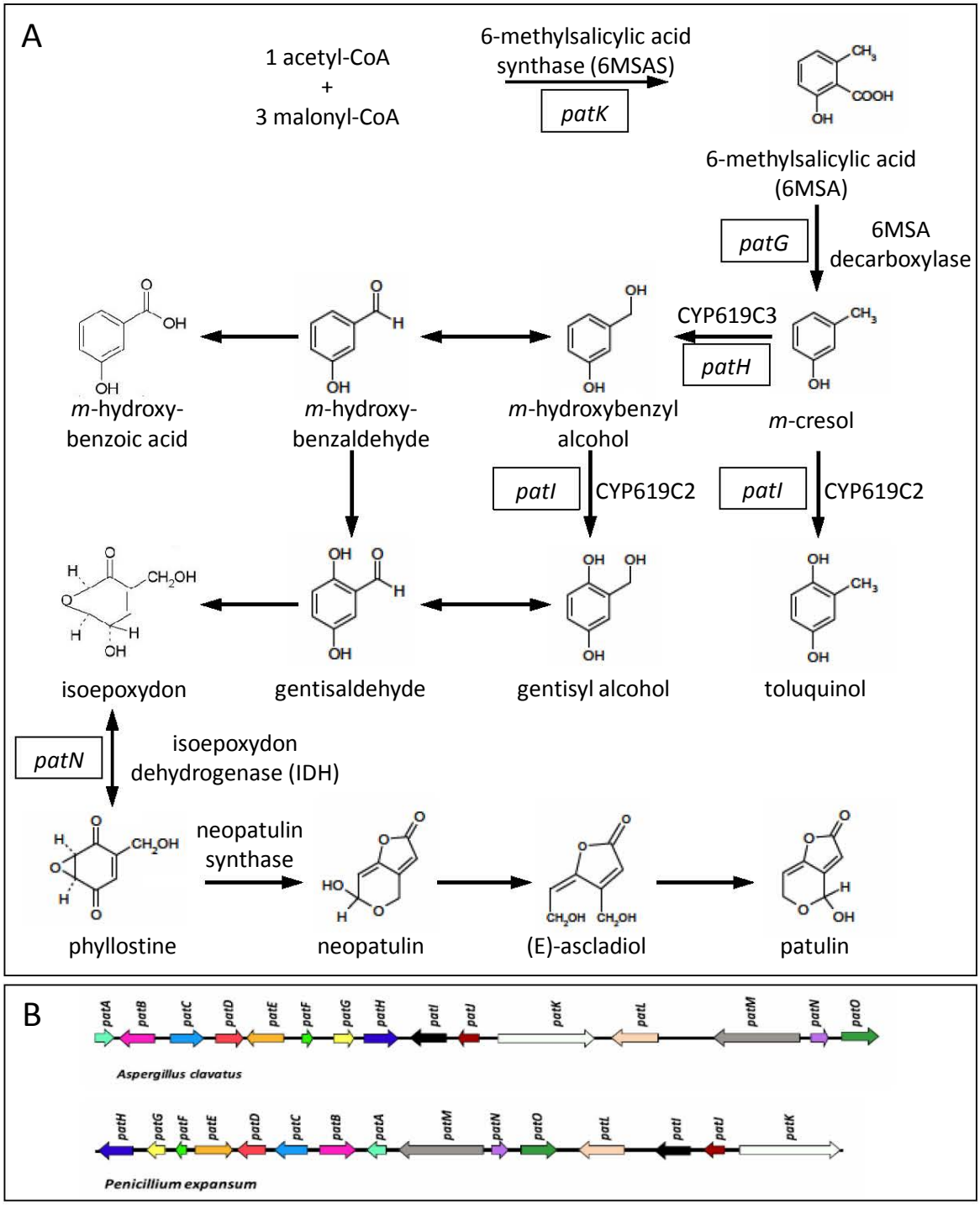


Figure 1.9 Schematic representation of the patulin biosynthesis pathway. (A) Metabolites, enzymology and genes encoding enzymes. (B) Patulin gene cluster and comparison of the positional organisation of the genes in *A. clavatus* and *P. expansum* (adapted from Artigot et al. (2009) and Tannous et al. (2014)).

The study by (Murphy and Lynen, 1975) was able to characterize the ring hydroxylation of *m*-hydroxybenzyl alcohol to gentisyl alcohol. As with both *m*-cresol hydroxylases, *m*-hydroxybenzyl alcohol hydroxylase was suggested to be catalyzed by a mixed-function oxidase reaction of the cytochrome P450, requiring oxygen and NADPH for activity. More recently, the involvement of cytochrome P450 monooxygenases in the hydroxylation of *m*-cresol and *m*-hydroxybenzyl alcohol was confirmed by Artigot et al. (2009). The first cytochrome that catalyzed the hydroxylation of *m*-cresol to yield *m*-hydroxybenzyl alcohol was termed CYP619C3. The second, termed CYP619C2, catalyzed the hydroxylation of *m*-hydroxybenzyl alcohol and *m*-cresol to gentisyl alcohol and toluquinol, respectively. Forrester and Gaucher (1972) described the partial purification of an alcohol dehydrogenase specific for the interconversion of *m*-hydroxybenzyl alcohol and *m*-hydroxybenzaldehyde. Scott et al. (1973) reported that *m*-hydroxybenzaldehyde was an efficient precursor of patulin. However, neither Scott and Beadling (1974), nor Murphy and Lynen (1975) were able to demonstrate direct conversion of the aldehyde to patulin. According to Murphy and Lynen (1975), the failure to detect an *m*-hydroxybenzaldehyde 2-hydroxylase activity in preparations of *P. griseofulvum* under their laboratory conditions, implies that patulin biosynthesis must proceed from *m*-hydroxybenzyl alcohol through gentisyl alcohol, rather than *m*-hydroxybenzaldehyde to gentisaldehyde and patulin. The possibility exists that the enzyme involved is extremely unstable and, hitherto undetectable. Although its conversion to *m*-hydroxybenzyl alcohol and *m*-hydroxybenzoic acid are demonstrable, the importance as a patulin precursor remains uncertain. Scott and Beadling (1974) isolated and partially purified two inseparable dehydrogenase activities catalyzing the reversible conversions of *m*-hydroxybenzyl alcohol and gentisyl alcohol to their corresponding aldehydes. They were also able to show that the rate of gentisaldehyde production from gentisyl alcohol in crude fungal extracts was of the same order of magnitude as the detectable gentisaldehyde to patulin conversion rate, suggesting a dioxygenase mechanisms for patulin synthesis from gentisaldehyde.

Until 1978, the post-gentisaldehyde portion of the pathway was believed to consist of a single, dioxygenase-mediated ring cleavage. Since then, extensive studies of patulin-minus mutants have shown that patulin is synthesized via isoepoxydon, phyllostine, neo-patulin (isopatulin) and (E)-ascladiol (Sekiguchi et al., 1983; Sekiguchi and Gaucher, 1979a; Sekiguchi and Gaucher, 1979b) (Figure 1.9 A).

The authors suggested isoepoxydon to be an obligatory precursor of phyllostine. Both structures suggest they are metabolically interconverted by a simple alcohol dehydrogenase-mediated redox reaction. The enzymes involved in the interconversion of isoepoxydon and phyllostine, and the conversion of phyllostine to neopatulin, have been purified and termed isoepoxydon dehydrogenase (IDH) and neopatulin synthase, respectively (Puel et al., 2010). Following neopatulin, literature data reported the production of the E and Z isomers of ascladiol by *P. griseofulvum*. Sekiguchi et al. (1983) suggested that neopatulin was first reduced to (E)-ascladiol by NADPH and then either oxidized to patulin or isomerized to a side product, (Z)-ascladiol.

1.3.2.6 Biosynthesis pathway: gene cluster

Fungal genes encoding enzymes involved in the production of secondary metabolites are usually contained in clusters on chromosomes (Keller et al., 2005; Keller and Hohn, 1997). A large number of gene clusters related to secondary metabolite production have been discovered and particularly those responsible for the biosynthesis of several mycotoxins, e.g. aflatoxins, fumonisins, trichothecenes, ergot alkaloids and zearalenone (Puel et al., 2010). In contrast, the gene cluster related to patulin biosynthesis has been elucidated only recently by Artigot et al. (2009) and Tannous et al. (2014) (Figure 1.9 B).

The 6-methylsalicylic acid synthase gene (*6msas*) and isoepoxydon dehydrogenase gene (*idh*) were the first isolated and sequenced, both from *P. griseofulvum* (Beck et al., 1990; Gaucher and Fedeshko, 2000). Next, White et al. (2006) reported the cloning of the full-length *idh* gene and a partial fragment of the *6msas* gene from *P. expansum*. In addition, the authors cloned a partial fragment of a putative ATP binding cassette (ABC) transporter gene (*peab1*). Dombrink-Kurtzman MA (2007) compared the partial gene fragment and corresponding amino acid sequences of *idh* between *P. griseofulvum* and *P. expansum* strains. They found 12 amino acid differences between the coding regions of both species. The differences correlated with the amount of patulin previously described for *in vitro* production, with strains of *P. griseofulvum* producing the greatest amounts of patulin. The absence or substitution of certain key amino acids at positions involved in protein folding or in binding a cofactor may lead to lower enzyme activity.

As the genes involved in patulin biosynthesis are most likely arranged in a cluster, the same authors performed a Gene Walking approach to identify genes upstream and downstream of the *idh* gene of *P. griseofulvum* (Dombrink-Kurtzman, 2008). They were the first to report the presence of an isoamyl alcohol oxidase gene (*iao*). However, at this point, further research was necessary to determine its relevance for the patulin biosynthesis pathway.

Artigot et al. (2009) identified two cytochrome P450 genes in the genome sequence of the patulin-producing *A. clavatus*. Both genes are located in a 40 kb cluster consisting of approximately 15 genes: one putative transcription factor gene (*patL*); three transporter genes, namely one ABC transporter gene (*patM*), one Major Facilitator Superfamily (MFS) gene (*patC*) and one acetate transporter gene (*patA*); one gene encoding a putative dioxygenase (*patJ*); one gene with unknown function (*patF*); and nine biosynthesis genes, i.e. the *6msas* gene (*patK*), the genes encoding CYP619C2 and CYP619C3 (*patI* and *patH*), the *idh* gene (*patN*), a putative *iao* gene (*patO*), a gene encoding a putative decarboxylase displaying an amido hydrolase conserved domain (*patG*), and two genes encoding, respectively, a putative Zn-dependent alcohol dehydrogenase and a glucose-methanol choline oxidoreductase (*patD* and *patE*) (Figure 1.9 B). Continuous research regarding the patulin gene cluster of *A. clavatus* resulted in the identification of *patG* as the gene encoding the 6MSA decarboxylase (Snini et al., 2014). Recently, the same research institute was able to elucidate the patulin biosynthetic gene cluster from *P. expansum*. The study consisted of the identification and positional organization of above described genes (Tannous et al., 2014) (Figure 1.9 B). Li et al. (2015) and Ballester et al. (2015) independently identified the gene cluster involved in patulin biosynthesis of *P. expansum*. The first authors also revealed that *patL* encodes a Cys_6-zinc finger regulatory transcription factor. Characterisation of *patL*, encoding a Cys_6-zinc finger regulatory factor, has been reported around the same time by Snini et al. (2016).

Chapter 2

Detection and identification of xerophilic fungi in Belgian chocolate confectionery factories

The content of this chapter is based on:

De Clercq, N., Van Coillie, E., Van Pamel, E., De Meulenaer, B., Devlieghere, F. and Vlaemynck, G. 2015. Detection and identification of xerophilic fungi in Belgian chocolate confectionery factories. Food Microbiology 46: 322-328

CHAPTER 2 DETECTION AND IDENTIFICATION OF XEROPHILIC FUNGI IN BELGIAN CHOCOLATE CONFECTIONERY FACTORIES

ABSTRACT

Chocolate confectionery fillings are generally regarded as microbiologically stable. The stability of these fillings is largely due to the general practice of adding either alcohol or preservatives. Consumer demands are now stimulating producers to move away from adding alcohol or other preservatives to their confectionery fillings and instead to search for innovative formulations. Such changes in composition can influence the shelf life of the product and may lead to spoilage by xerophilic fungi. The aim of this chapter was to test whether the production environments of Belgian chocolate confectionery factories and common ingredients of chocolate confectioneries could be potential sources of contamination with xerophilic fungal species. In the factory environment, the general and strictly xerophilic fungal spore load was determined using an RCS Air Sampler device in combination with DG18 and MY50G medium, respectively. Four basic ingredients of chocolate confectionery fillings were also examined for fungal spore levels using a direct plating technique. Detected fungi were identified to species level by a combination of morphological characterisation and sequence analysis. Results indicated a general fungal spore load in the range of 50-250 colony forming units per cubic meter of air (CFU/m³ air) and a more strict xerophilic spore load below 50 CFU/m³ air. These results indicate rather low levels of fungal spores present in the factory environment. The most prevalent fungi in the factory environment were identified as *Penicillium* spp., particularly *P. brevicompactum*. Examination of the basic ingredients of confectionery fillings revealed nuts to be the most likely potential source of direct contamination. In nuts, the most prevalent fungal species identified were *Eurotium*, particularly *E. repens*.

2.1 INTRODUCTION

Chocolate, cocoa and confectioneries represent 13.2% of the Belgian food industry's global turnover and 14.6% of Belgium's food exports. Currently, other European countries are the main importers (75%) of Belgian chocolate confectionery products. The international fame and distribution of these products demands a high level of quality and a sufficiently long shelf life. Chocolate confectionery fillings are intermediate moisture food (IMF) products with water activity values (a_w) of 0.70 to 0.90. These fillings are generally regarded as microbiologically stable, often because alcohol or preservatives are added to reduce the risk of microbial spoilage. But consumer demands for clean label products are on the rise, and addition of alcohol in confections is not allowed in some countries. Confectionery producers are therefore stimulated to reduce or eliminate the use of preservatives and search for more innovative formulations (Vermeulen et al., 2015). Changes in composition can potentially influence the stability, and consequently the shelf life, of these chocolate confectionery products.

Intermediate moisture foods are susceptible to spoilage by xerophilic fungi and osmophilic yeasts (Beuchat and Hocking, 1990; Kinderlerer, 1997; Pitt and Hocking, 1997). Spoilage of sweet IMF products is mainly caused by *Eurotium* species and xerophilic *Aspergillus* and *Penicillium* species (Abellana et al., 2000). All *Eurotium* species are sexual forms of *Aspergillus*. The most important factors determining the growth of these organisms on food products are water activity, pH and temperature (Fustier et al., 1998; Guynot et al., 2002; Huang et al., 2010). Industrial processing of cocoa beans results in edible chocolate with low water activity values that does not allow for microbial growth (Copetti et al., 2011; ICMSF, 2005; Kinderlerer, 1997; Schwan and Wheals, 2004). Yet xerophilic fungi have been isolated from chocolate products. Such contamination was suggested to be the result of post-process contamination due to increased water availability at the interface of the chocolate and its packaging (Beckett, 2000; ICMSF, 2005; Kinderlerer, 1997).

Little is known about the mycoflora of chocolate confectionery factories. Bakeries and chocolate confectionery factories are both "dry food processing environments", thus data for bakeries may have some relevance for the little-studied situation of chocolate confectionery factories. A difference between bakeries and chocolate confectionery is the load of dust and particles. This load is usually much higher in bakeries and consequently leads to a higher fungal load in the environment.

Studies concerning mould spoilage of bakery products suggest two possible sources of contamination: 1) exposure to airborne fungal spores that may originate directly from the air or that originate from dry ingredients and spread throughout the processing environment; or 2) direct introduction of fungal spores via ingredients such as sugars and nuts (Abellana et al., 1999; Fustier et al., 1998; Legan, 1993; Ooraikul et al., 1987).

Final chocolate confectionery fillings are made by mixing basic ingredients in a kitchen processor. Depending on the final product, liquid chocolate may be poured in premade forms, after which a cooling step is applied. The fillings may be added onto the cooled chocolate base or fillings may be directly added onto the production belt, after which toppings or coatings are added. The final products are immediately brought to the packing area, where the products are packed manually.

The aim of this chapter was to determine the presence and prevalence of fungal spoilage organisms in both the production environment of Belgian chocolate confectionery factories as well as in some of the ingredients used in chocolate confectionery fillings. Environmental and ingredient samples were examined for their load of general and xerophilic fungal spores. The xerophilic fungi detected were subsequently isolated and identified to species level. This information provides insight into some of the potential sources and species of fungi that may negatively affect the quality and shelf life of chocolate confectionery products.

2.2 MATERIAL AND METHODS

2.2.1 Media

Three media were used in this study: Malt Extract Agar (MEA) (Oxoid Ltd, Basingstoke, Hampshire, UK), Dichloran 18% Glycerol agar (DG18) (Oxoid Ltd) and Malt extract Yeast extract 50% Glucose agar (MY50G). DG18 and MY50G are selective media with relatively low water activity (a_w ± 0.95 and ± 0.85, respectively). These were chosen for sampling and subsequent detection and enumeration. DG18 medium is recommended for the detection and enumeration of filamentous fungi in dry indoor environments such as chocolate confectionery factories, and in products with a water activity less than or equal to 0.95.

DG18 has been proven suitable for detecting a broad range of food and indoor fungi, ranging from moderately xerophilic to xerotolerant species (ISO 21527-2, 2008; Samson et al., 2010). MY50G is a selective medium specifically for xerophilic fungi (Samson et al., 2010). MEA is a general-purpose medium used for the detection and enumeration of fungi. For isolation, morphological characterization and DNA extraction, we used both MEA and DG18 (see Methods below). MEA and DG18 were prepared according to the manufacturer's instructions (Oxoid Ltd). DG18 was prepared without the addition of chloramphenicol. MY50G consisted of 3 g malt extract (Oxoid Ltd), 3 g yeast extract (Oxoid Ltd), 5 g neutralized bacteriological pepton (Oxoid Ltd), 15 g bacteriological agar nr. 1 (Oxoid Ltd), 500 g dextrose (Oxoid Ltd), and 474 g demineralized water for a total volume of 1000 g medium. MY50G was sterilized by autoclaving at 115°C for 10 minutes. Water activity values of the final media were checked using an Aqualab Series 3 with an accuracy of ± 0.003 a_w units (Decagon Devices, Pullman, WA, USA).

2.2.2 Sampling

Chocolate confectionery factories were sampled during August 2012, October 2012 and April 2013 in Belgium. In August 2012, production air and ingredients of seven chocolate confectionery factories were sampled. During October 2012 and April 2013, three of these seven factories were sampled for a second and third time to evaluate the effect of the season. Air samples were obtained using a Reuter Centrifugal Air Sampler (RCS) (HYCON® Biotest AG, Dreieich, Germany) operating at a flow rate of 40 L/min. Within each factory, three sites of the manufacturing area were sampled, i.e. the kitchen (i.e. the processing station containing an industrial kitchen processor for mixing up the ingredients in order to prepare fillings), the production line and the packing area. Air was sampled during 8 min using the RCS Air Sampler, which was loaded with DG18 and MY50G agar strips. At each sampling moment, various ingredients of chocolate confectionery fillings (nuts, fruit filling, ganache (a mixture of cream and chocolate) and fondant sugar) were also collected.

2.2.3 Enumeration of fungi

After transfer to the laboratory, air sample agar strips (DG18, MY50G) were incubated at 25°C for 2 weeks. Ingredients were directly plated by distributing 1 g of each ingredient onto three DG18 plates as well as three MY50G plates.

Nuts were first aseptically transferred to a Stomacher® bag and ground into small pieces. As needed for ease of plating, ganache was transferred to a Stomacher bag and melted in a water bath at 40°C prior to plating. The standard incubation regime is 5-7 days at 25°C. The petri dishes were incubated at 25°C for two weeks. The agar strips and petri dishes were examined after five days as well as two weeks of incubation to ensure sufficient grow-out time. Results were expressed in colony forming units per cubic meter of air (CFU/m³) and colony forming units per gram of sample (CFU/g), respectively. Almost no fungi were detected after 5 days on MY50G. Therefore, the results of enumeration on MY50G were expressed after 2 weeks of incubation instead of 5 days incubation for DG18.

2.2.4 Morphological identification

After incubation of the samples collected during the second sampling in October 2012, fungal spores and/or mycelium of macroscopically different colonies were isolated for each sample and purified by streaking onto MEA and DG18. These plates were incubated at 25°C for seven days. Purity of the isolates was confirmed visually and pure fungal cultures were subsequently added to the laboratory culture collection of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium). Sporulating mycelia of isolated fungal colonies were scraped off the surfaces and three-point inoculated onto MEA and DG18. After incubation for seven days at 25°C, macroscopic characteristics (colony diameter and color) as well as microscopic characteristics were determined for classification to genus level using the identification key published in Samson et al. (2010).

2.2.5 Sequence analysis

Genomic DNA extraction and subsequent visualization was carried out as described by Van Pamel et al. (2012). Molecular identification of the isolates was performed using the internal transcribed spacer (ITS) region and/or partial *β-tubulin* gene sequence, depending on the identified genus (Samson et al., 2010). PCR amplification was conducted using the forward primer ITS1 and reverse primer ITS4 for the ITS region (White et al., 1990) and the forward primer Bt2a and reverse primer Bt2b for the *β-tubulin* gene (Glass and Donaldson, 1995). The PCR assay and PCR conditions were performed as described by Van Pamel et al. (2012), with some minor modifications as follows: the PCR mixture contained 3 µL of template DNA and 1x buffer II (Applied Biosystems).

The primer used annealed for 30 s at 50°C. A 100 bp DNA ladder (Invitrogen Ltd) was used as control for DNA size. A negative control using water and a positive control using *P. expansum* (CBS 325.48^{T}) genomic DNA were included in all PCR runs. The PCR products were visualized after staining a 1.5% (w/v) Seakem LE agarose gel (Lonza, Rockland, ME, USA) with ethidium bromide (2 µg/mL). DNA fragment purification and DNA sequencing were performed at Macrogen Europe (Amsterdam, the Netherlands). Identification was conducted by comparing the DNA sequences against those available in the EMBL database conducting a BLAST search. A similarity percentage of at least 98% with a sequence of a strain of a reputable culture collection (CBS, ATCC and others), was taken into account for identification of the fungal isolates to species level.

2.2.6 Statistical analysis

Statistical analysis was done using the Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA), except for the non-parametric analysis, which was done using SPSS® version 22 (IBM, New York, USA). CFU data were transformed prior to statistical analysis to y = log (CFU/m³). A variety of statistics were calculated to characterize the data distribution of the variables. Data were obtained during the summer of August 2012 from seven chocolate confectionery factories (A-G). Within each factory, the air of three processing stations (kitchen, production line and packing area) was sampled using agar strips. The data obtained from these sampling events did not follow a normal distribution. Therefore, a non-parametric Kruskal-Wallis test was performed with CFU as dependent variable and with either factory or manufacturing site as independent variable. For the CFU data obtained from MY50G agar strips (dependent variable), a one-way analysis of variance (ANOVA) was carried out with either chocolate confectionery factory or manufacturing site as independent variable. Post-hoc comparison was conducted using a Bonferroni test. The CFU data of three out of seven chocolate confectionery factories (B, C and F) at three time points using DG18 and MY50G media follow a normal distribution. To test the effect of time and location (independent variables) on CFU using DG18 and MY50G (independent variables), mixed regression models were performed. Factory was included as a random effect to correct for repeated measurements (three time points) within one factory. Post-hoc comparison was done using a Bonferroni test. *P* values ≤0.05 were considered significant.

2.3 RESULTS

2.3.1 General and xerophilic fungal spore load in the production air of chocolate confectionery factories

During the summer of August 2012, three processing stations (kitchen, production line and packing area) of seven chocolate confectionery factories (A-G) were sampled (Figure 2.1) using an air sampler (RCS) fitted with DG18 (Figure 2.1 I) and MY50G (Figure 2.1 II) agar strips (7 factories × 3 sites × 2 media). After five days of incubation at 25°C on DG18, a general fungal spore load in the range of 50-250 CFU/m³ air was detected in 63% of all air samples. The remaining 37% had a fungal spore load below 50 CFU/m³ air. After two weeks of incubation at 25°C on MY50G, 80% of all air samples were characterized by a xerophilic fungal spore load below 50 CFU/m³ air. No fungal spore load above 250 CFU/m³ air was detected either on DG18 or on MY50G. On DG18, chocolate confectionery factory D showed a fungal spore load below 50 CFU/m³ air, unlike the majority of DG18 samples obtained from other factories. The fungal spore load measured in chocolate confectionery factory D is significantly lower (p = 0.04) from factories A, B and C. On MY50G, a significant difference (p = 0.05) was found in between factory D and factory B. In general, when comparing the different manufacturing sites, no significant differences (p > 0.05) were observed between the kitchen, production line and packing area.

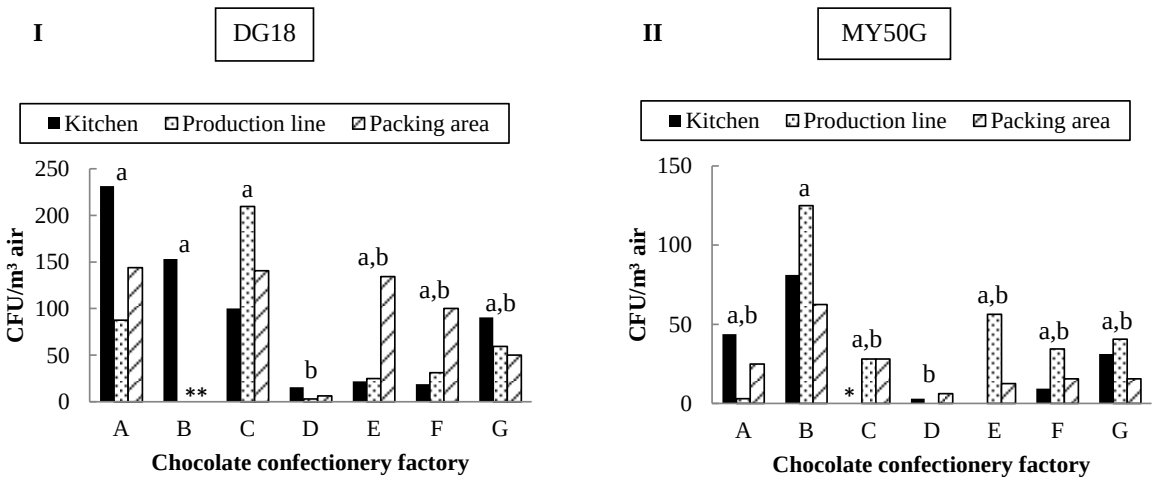


Figure 2.1 Fungal spore load expressed in CFU/m³ on DG18 (I) and MY50G (II) at seven chocolate confectionery factories (A-G). * No data available due to overgrow by fast-growing mould genera such as *Mucor, Rhizopus*, etc. Bars with different letters (a-b) are significantly different at *P* values ≤0.05.

2.3.2 General and xerophilic fungal spore load in the factory air at different time points

The fungal spore load in the air of three out of the seven chocolate confectionery factories (B, C and F) was further investigated at three time points (3 factories × 3 sites × 3 time points × 2 media) (Figure 2.2). After five days of incubation at 25°C on DG18 (Figure 2.2 I), 76% of all air samples was characterized by a general fungal spore load in the range of 50-250 CFU/m³ air. The other 24% was distributed below 50 CFU/m³ air and above 250 CFU/m³ air, with an upper range of 940 CFU/m³ air. After two weeks of incubation on MY50G at 25°C, enumeration of the air samples revealed that 78% of all samples had a xerophilic fungal spore load below 50 CFU/m³ air (Figure 2.2 II). For the majority of all DG18 air samples and all of the MY50G air samples, fungal spore loads detected were within a relatively small range of 50-250 CFU/m³ air and below 50 CFU/m³ air, respectively. No significant differences between time points ($p > 0.05$) were detected. In addition, no significant differences among the processing stations of a factory were found. The results of the sampling in April 2013 at the production line of factory B on both DG18 and MY50G showed the highest fungal loads, namely 940 CFU/m³ air and 910 CFU/m³ air, respectively.

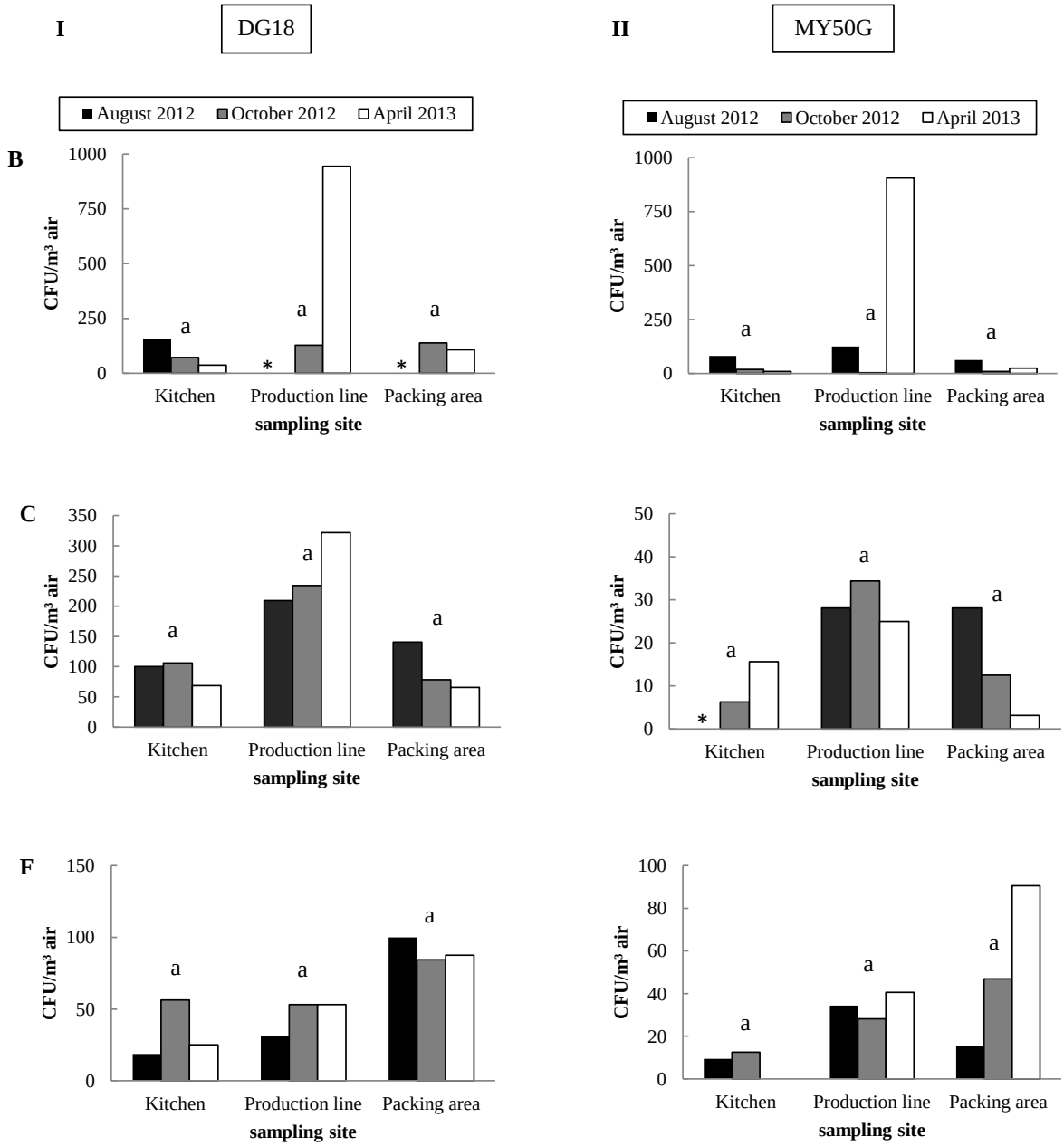


Figure 2.2 Fungal spore load expressed in $\log_{10}$ CFU/m³ on DG18 (left panel) and MY50G (right panel). Samples were taken at three manufacturing sites; the kitchen, production line and packing area of chocolate confectionery factory B (upper panel), factory C (middle panel) and factory F (lower panel). * No data available due to overgrow by fast-growing mould genera such as *Mucor*, *Rhizopus*, etc. Bars with different letters (a-b) are significantly different at *P* values ≤0.05.

2.3.3 Fungal spore levels of ingredients

Of all four ingredients examined, the majority of samples of ganache, fruit filling and fondant sugar showed no or low (<10 CFU/g) fungal counts. Of a total of 12 samples of ganache collected and plated on DG18 and MY50G, no fungal spore contamination was observed in 8 and 9 out of 12 samples, respectively. Also, no viable fungal spores were detected on DG18 in 11 out of 12 samples of fruit filling; no viable fungal spores could be detected in 10 out of 12 samples plated on MY50G. The results of the fondant sugar showed no fungal spore contamination for 8 (DG18) and 9 (MY50G) out of 13 samples investigated. Samples of the abovementioned ingredients (37 samples in total) plated on DG18 resulted in one sample of ganache with a general fungal load in the range of 10-100 CFU/g and one sample of fondant sugar contaminated with more than 100 fungal CFU/g. For the sample of fondant sugar examined on MY50G, a xerophilic fungal spore load of more than 100 CFU/g was detected. The majority of samples of nuts (hazelnuts, walnuts, etc.) contained some fungal contamination (Table 2.1). On DG18, 15 out of 17 samples of nuts were contaminated, five of which had viable fungal spores with levels above 10 CFU/g. Even on MY50G, more than half of the samples of nuts collected showed fungal spore loads. Six of these had fungal counts above 10 CFU/g. The highest levels of fungal contamination were found in walnuts.

Table 2.1 Distribution of fungal spore levels in CFU/g, determined on DG18 and MY50G, of different types of nuts.

Ingredient	Medium	No. of samples[a]	No. of samples[b]			
			<1 CFU/g	[1-10] CFU/g	[10-100] CFU/g	>100 CFU/g
Hazelnuts	DG18	7	1	5	1	0
	MY50G		5	1	1	0
Walnuts	DG18	4	0	0	1	3
	MY50G		1	0	1	2
Others (almond, pistachio, brésilienne)	DG18	6	1	4	0	0
	MY50G		2	2	1	1
Total	DG18	17	2	9	2	3
	MY50G		8	3	3	3

[a] Number of samples of each type of nut (ingredient) collected and analyzed.
[b] Number of samples of each type of nut with a fungal spore load within one of the above described ranges.

2.3.4 Species identification

After enumeration of the CFU detected in the samples obtained during the second sampling round in the three factories, a total of 116 fungi were isolated from both DG18 and MY50G media (Table 2.2). For both air and ingredients, 73% were isolated from DG18 medium, while 27% were isolated from MY50G. Of a total of 83 fungal isolates originating from the air of three chocolate confectionery factories, 20 were isolated from the kitchen area, 31 came from the production line and 32 came from the packing area. Of the four types of ingredients analyzed, nuts were found to contain the highest number of isolates. The 116 fungal isolates, of which 85 isolates were identified to species level, were classified into 14 genera and 35 species (data not shown). The most common genera were *Penicillium*, *Eurotium* and *Aspergillus*, comprising 38%, 27% and 14% of the total number of isolates, respectively. Most (72%) of the total number of isolates originated from the air, while 28% came from the ingredients. This distribution was also found in the isolates belonging to the genera *Penicillium* and *Aspergillus*. Exactly 75% and 25% originated from the air and from the ingredients, respectively, for both *Penicillium* and *Aspergillus*. *Eurotium* isolates showed a less pronounced distribution: 61% of these isolates were derived from the air and the remaining 39% from ingredients.

Table 2.2 Total number of fungal isolates originating from the air of the factory environments and ingredients of three out of seven chocolate confectionery factories (B, C and F), during October 2012.

Source		Total no. of samples[a]	Total no. of isolates	Medium[b]	
				DG18	MY50G
Air	Kitchen	3	20	13	7
	Production line	3	31	25	6
	Packing area	3	32	23	9
	Total	9	83	61	22
Ingredients	Nuts	6	29	20	9
	Fruit filling	2	0	0	0
	Ganache	3	3	3	0
	Fondant sugar	3	1	1	0
	Total	14	33	24	9
Total			116	85	31

[a] Total number of samples taken from the air at three manufacturing sites, and from different types of ingredients both at three chocolate factories (B, C and F).
[b] Number of isolates detected on DG18 and MY50G medium.

Table 2.3 lists the identification results of all fungal isolates originating from the air and ingredients of chocolate confectionery factories B, C and F. *P. brevicompactum* and *E. repens* were the most commonly identified species. *Penicillium crustosum*, *Penicillium chrysogenum* and *E. herbariorum* were also isolated repeatedly. Three out of four *W. sebi* isolates originated from ingredients. *Cladosporium* sp. could only be detected on DG18 and was seven out of eight times isolated from the air. Common species such as *Aspergillus sydowii, E. herbariorum, E. repens, P. brevicompactum*, *P. chrysogenum*, *Penicillium corylophilum* and *P. crustosum* were often detected in more than one manufacturing site of the same factory. A total of 14 species could be identified from different types of nuts. All except for *Penicillium scabrosum* were detected on DG18. Besides *P. scabrosum*, *P. brevicompactum*, three *Eurotium* species and *W. sebi* could also be detected on MY50G.

Table 2.3 Species identification results of all fungal isolates originating from the air and ingredients of chocolate confectionery factories B, C and F. Isolates listed as sp. could not be identified to species level with a similarity percentage of at least 98% if possible with a sequence of a type strain, or a strain of a reputable culture collection (CBS, ATCC, and others).

Species	Chocolate confectionery factory	Manufacturing site/ ingredient	Medium	Identity %	Reference strain	Accession number
Alternaria arborescens	B	Production line	DG18	100	DHMJ20	JN986772
Alternaria sp.	B	Kitchen	DG18			
Apiospora montagnei	B	Production line	DG18	100	MUCL 1684	AB220322
Aspergillus niger	C	Packing area	DG18	99.1	IHEM 17902	EF422213
A. niger	F	Packing area	DG18	99.8	CBS 113.33	HQ632731
Aspergillus penicillioides	C	Production line	MY50G	98.9	NRRL 4548	EF651928
Aspergillus sp.	B	Kitchen	MY50G			
Aspergillus sp.	B	Nuts (walnuts)	DG18			
Aspergillus sp.	C	Packing area	MY50G			
Aspergillus sp.	F	Packing area	MY50G			
Aspergillus sp.	F	Production line	MY50G			
Aspergillus sp.	F	Production line	DG18			
Aspergillus sp.	C	Nuts (almonds)	DG18			
Aspergillus sydowii	B	Packing area	DG18	100	NRRL 4768	JN853936
A. sydowii	B	Production line	DG18	100	CBS 593.65	JN853935
A. sydowii	F	Kitchen	DG18	100	NRRL 5585	EF428373
Aspergillus tubingensis	C	Nuts (almonds)	DG18	100	ATCC 10550	HQ632759
A. tubingensis	F	Nuts (walnuts)	DG18	100	IHEM 22370	AB574056
Aspergillus westerdijkiae	B	Kitchen	DG18	100	CBS 588.68	FN185743
Cladosporium cladosporioides	C	Packing area	DG18	100	isolaat 258	FJ490620
C. cladosporioides	F	Packing area	DG18	100	T3B1c. 10P	JQ780660
Cladosporium sp.	B	Kitchen	DG18			

Cladosporium sp.	B	Production line	DG18			
Cladosporium sp.	C	Production line	DG18			
Cladosporium sp.	F	Nuts (almonds)	DG18			
Cladosporium sp.	B	Packing area	DG18			
Cladosporium sp.	C	Production line	DG18			
Cladosporium sphaerospermum	F	Production line	DG18	99.8	CBS 109.14	DQ780350
Epicoccum nigrum	B	Production line	DG18	100	CBS 115825	FJ427109
Ep. nigrum	C	Production line	DG18	100	CBS 161.73	GU563404
Eurotium amstelodami	B	Packing area	DG18	100	NRRL 35696	EF651901
E. amstelodami	B	Production line	DG18	100	NRRL 4716	EF651899
E. amstelodami	C	Production line	MY50G	99.7	CCF 4069	FR775356
Eurotium appendiculatum = *Aspergillus appendiculatus**	B	Production line	DG18	100	CBS 101746	HE801334
Eurotium chevalieri = *Aspergillus chevalieri**	C	Nuts (almonds)	DG18	100	KACC 46341	JN696382
Eurotium herbariorum = *Aspergillus glaucus**	B	Nuts (walnuts)	MY50G	98.6	NRRL 114	EF651885
E. herbariorum = *A. glaucus**	C	Kitchen	MY50G	99.3	NRRL 71	EF651885
E. herbariorum = *A. glaucus**	C	Packing area	MY50G	98.4	NRRL 71	EF651888
E. herbariorum = *A. glaucus**	F	Production line	DG18	100	NRRL 117	EF651886
E. herbariorum = *A. glaucus**	F	Nuts (hazelnuts)	DG18	100	KACC 46352	JN696391
Eurotium repens = *Aspergillus pseudoglaucus**	B	Nuts (walnuts)	MY50G	100	NRRL 13	FR775359
E. repens = *A. pseudoglaucus**	B	Packing area	MY50G	100	CCF 3283	FR775359
E. repens = *A. pseudoglaucus**	B	Production line	MY50G	100	KACC 46361	JN696398
E. repens = *A. pseudoglaucus**	C	Nuts (almonds)	DG18	100	CCF 1454	EF651915
E. repens = *A. pseudoglaucus**	C	Packing area	DG18	99.6	NRRL 13	JN696398
E. repens = *A. pseudoglaucus**	F	Kitchen	MY50G	100	NRRL 13	EF651915
E. repens = *A. pseudoglaucus**	F	Nuts (almonds)	MY50G	100	NRRL 13	EF651915
E. repens = *A. pseudoglaucus**	F	Nuts (hazelnuts)	DG18	99.7	KACC 46363	JN696396
E. repens = *A. pseudoglaucus**	F	Nuts (hazelnuts)	MY50G	100	CCF 1454	FR775360
E. repens = *A. pseudoglaucus**	F	Packing area	DG18	98.8	KACC 46363	EF651915
Eurotium rubrum = *Aspergillus ruber**	F	Nuts (hazelnuts)	DG18	100	KACC 46366	JN696401
E. rubrum = *A. ruber**	F	Nuts (walnuts)	MY50G	100	NRRL 52	EF651920
Eurotium sp. = *Aspergillus* sp.*	B	Packing area	DG18			

Eurotium sp. = *Aspergillus* sp.*	C	Packing area	DG18			
Eurotium sp. = *Aspergillus* sp.*	F	Nuts (walnuts)	MY50G			
Eurotium sp. = *Aspergillus* sp.*	C	Nuts (almonds)	DG18			
Eurotium sp. = *Aspergillus* sp.*	C	Packing area	DG18			
Eurotium sp. = *Aspergillus* sp.*	C	Packing area	DG18			
Eurotium sp. = *Aspergillus* sp.*	C	Packing area	MY50G			
Eurotium sp. = *Aspergillus* sp.*	C	Production line	DG18			
Eurotium sp. = *Aspergillus* sp.*	F	Production line	DG18			
Hypocrea sp.	F	Packing area	DG18			
Paecilomyces variotii	F	Nuts (almonds)	DG18	100	CBS 121581	EU037080
Penicillium bialowiezense	F	Nuts (walnuts)	DG18	99.3	CBS 110104	AY674440
P. bialowiezense	F	Packing area	DG18	99.7	CBS 112.882	AY674441
Penicillium brevicompactum	B	Kitchen	DG18	98.1	CBS 110067	EU587345
P. brevicompactum	B	Nuts (walnuts)	MY50G	100	SCCM 10-B8	EU587359
P. brevicompactum	B	Packing area	DG18	100	CBS 48084	DQ645795
P. brevicompactum	B	Production line	DG18	99.3	CBS 25729	AY674434
P. brevicompactum	C	Ganache	DG18	99.4	DAOM 215331	AY674436
P. brevicompactum	C	Production line	DG18	100	ATCC 10111	AY674438
P. brevicompactum	C	Production line	MY50G	100	CBS 48084	AY674438
P. brevicompactum	F	Kitchen	DG18	97.9	NRRL 28139	EU587352
P. brevicompactum	F	Nuts (hazelnuts)	DG18	99.0	CBS 110067	AY674438
P. brevicompactum	F	Packing area	DG18	98.1	CBS 110067	AY674434
Penicillium chrysogenum	B	Kitchen	MY50G	98.5	CBS 478.84	AY495981
P. chrysogenum	B	Packing area	DG18	100	CBS 302.67	GQ498300
P. chrysogenum	B	Packing area	MY50G	100	NRRL_A_6200	EU597708
P. chrysogenum	B	Production line	DG18	98.4	CBS 306.48	JX996926
P. chrysogenum	F	Packing area	MY50G	100	L4	AY495988
Penicillium commune	B	Production line	DG18	100	CBS 311.48	AY213672
Penicillium corylophilum	B	Kitchen	DG18	100	CBS 330.79	GU944519
P. corylophilum	F	Packing area	DG18	99.1	CBS 330.79	GU944519
P. corylophilum	F	Production line	MY50G	99.5	CBS 330.79	GU944519
Penicillium crustosum	B	Kitchen	DG18	100	CBS 47184	FJ930934
P. crustosum	C	Kitchen	DG18	99.6	CBS 101025	FJ930935
P. crustosum	F	Kitchen	DG18	100	CBS 101025	AY674351
P. crustosum	F	Kitchen	MY50G	99.6	CBS 101025	AY674351
P. crustosum	F	Nuts (hazelnuts)	DG18	100	CBS 101025	FJ930935
P. crustosum	F	Nuts (walnuts)	DG18	99.5	CBS 101025	FJ930934
P. crustosum	F	Packing area	DG18	99.7	CBS 101025	AY674351
P. crustosum	F	Production line	DG18	100	CBS 47184	AY674351
Penicillium expansum	B	Nuts (walnuts)	DG18	98.7	CBS 325.48	JQ965099

Penicillium glabrum	F	Packing area	MY50G	100	CBS 125543	GU981619
Penicillium multicolor	B	Kitchen	MY50G	100	CBS 501.73	JN799645
Penicillium polonicum	F	Nuts (walnuts)	DG18	99.8	CBS 101479	AY674306
Penicillium roseopurpureum	B	Packing area	MY50G	100	CBS 28139	JN606839
Penicillium scabrosum	F	Nuts (walnuts)	MY50G	100	DAOM 214786	DQ285610
Penicillium solitum	B	Packing area	DG18	99.5	CBS 14786	AY674355
P. solitum	B	Production line	DG18	100	KACC 45929	AY674355
P. solitum	F	Packing area	DG18	99.7	CBS 14786	JF521535
Penicillium sp.	B	Kitchen	DG18			
Penicillium sp.	C	Kitchen	MY50G			
Penicillium sp.	C	Production line	DG18			
Penicillium sp.	F	Ganache	DG18			
Penicillium sp.	F	Kitchen	DG18			
Penicillium spinulosum	B	Nuts (walnuts)	DG18	100	CBS 271.35	GQ367505
Phaeosphaeria sp.	F	Ganache	DG18			
Talaromyces ruber	F	Production line	DG18	100	CBS 195.88	JX965350
Thysanophora penicillioides	C	Production line	DG18	98.2	WCN 1152	AB175272
Trichoderma atroviride	B	Kitchen	DG18	100	UASWS 0364	HM236004
Trichoderma sp.	B	Packing area	DG18			
Wallemia sebi[+]	F	Fondant sugar	DG18			
W. sebi[+]	F	Nuts (almonds)	DG18			
W. sebi[+]	F	Nuts (almonds)	MY50G			
W. sebi[+]	F	Production line	DG18			

* Newly proposed species names (Hubka et al., 2013; Samson et al., 2014). [+] Identification based on morphological characteristics.

2.4 DISCUSSION

In this chapter, the fungal spore load and fungal species present in the production air and ingredients of Belgian chocolate confectionery factories was examined. The aim was to determine whether fungal spoilage organisms, which have a potentially important role in the shelf life and quality of chocolate confectionery products, were present in the factory air and the ingredients used in confectionery fillings. The majority of the processing stations of the chocolate confectionery factories showed a general fungal spore load in the range of 50-250 CFU/m³ air, with 250 CFU/m³ as the upper limit of spore load detected. No quantitative standards for air quality in dry food processing environments are currently available. As part of a preventive health policy, the Government of Flanders (Belgium) issued a decision in 2004 regarding health risks posed by indoor pollution of building offices, houses, etc. This decision includes a guideline to maintain fungal spore loads below 200 CFU/m³ air (Flanders' FOOD, 2010).

Rao et al. (1996) reviewed and compared some existing quantitative standards and guidelines for indoor airborne fungi. Those quantitative standards/guidelines ranged from <100 CFU/m³ to >1,000 CFU/m³ as the upper limit for non-contaminated indoor environments. Currently, no studies are available regarding the fungal contamination of chocolate confectionery factories. A number of studies have been conducted on air quality in bakeries, however. The average fungal spore load in the air of six British bakeries was found to be in the range of 85 to 2,850 CFU/m³. Fungal spore concentrations in German bakeries ranged from 85 to 5,000 CFU/m³ in the storage rooms, although these concentrations were considerably higher in some production areas (approximately 90,000 CFU/m³). Canadian bakeries showed a total of yeast and fungal spore counts from 50 to 2,000 CFU/m³ air (Legan, 1993; Ooraikul et al., 1987). Bakeries and chocolate confectionery factories have important differences, however. Bakeries use dry ingredients with low water activity values, but the final products do not necessarily have low water activity. Microbial spoilage of baked goods is therefore a major problem. Fungal counts of bakeries found in literature are very high compared to those of the chocolate confectionery factories observed in this study. This can largely be explained by the use of dry ingredients such as flour. It is well known that flour and other dry ingredients contain substantial amounts of spores (Legan, 1993). The lightweight flour dust spreads widely throughout the bakery and leads to post-process contamination of bakery products.

Additionally, several studies have been published on the fungal spore load of houses, apartments or office buildings with and without fungal problems (Burge et al., 2000; Codina et al., 2008; Herbarth et al., 2003; Hunter et al., 1988; Lehtonen and Reponen, 1993; Verhoeff et al., 1990). Most of these studies used different air sampling devices and media for the enumeration of viable fungal propagules. Verhoeff et al. (1990) compared several air sample devices in combination with various collection media. Air sampling devices can differ in flow rate and filter particle size. Because fungi can vary distinctly in structure and spore size, certain species will be able to penetrate the mesh while others will be retained. This suggests that care should be taken when comparing CFU data and indicates the need for standardized protocols.

One chocolate confectionery factory (factory D) clearly showed lower concentrations of fungal spores compared to the other factories studied. A possible hypothesis is the application of different manufacturing and hygienic practices (Codex alimentarius, 2013). Using the RCS Air Sampler, Singh et al. (1986) studied the effectiveness of UV rays and microbial filters for air disinfection.

They found that air filters were most effective, with a reduction in fungal load from 2,100 to 0 CFU/m^3 in 60 min. The type of filters installed in the ventilation system may vary from simple dust filters to High Efficiency Particulate Air (HEPA) filters (Codex alimentarius, 2013). The type of filter used may affect the fungal load of a factory as well. In addition to good ventilation systems, fungal spore concentrations may be reduced by proper disinfection systems and guidelines for factory workers entering the work environment, controlled entry of material and ingredients, and stringent dry cleaning practices.

No clear differences in fungal spore load could be observed among the processing stations of the chocolate confectionery factories. In addition, the most commonly identified species in the air were mostly found in more than one manufacturing site of the same factory. Possible explanations for this is the proximity of the kitchen, production line and packing area to each other; the lack of separation between the areas; and easy access between areas. All of these factors promote distribution of dust and fungal spores from one area to another.

The enumeration data of sampling during August, October and April showed no significant differences. These data suggest that the fungal spore loads are not subjected to large variations. In contrast, several other studies have described a seasonal variation in the total indoor fungal spore concentrations, showing higher concentrations in summer months (Herbarth et al., 2003; Koch et al., 2000). Nevertheless, more data are needed before conclusions can be drawn regarding seasonal variation of indoor fungal concentrations. In addition to seasonal variation, spore concentrations are also reported to be subjected to possible local increases in air movement. Some studies described the increase in spore concentrations associated with increasing air velocity (Pasanen et al., 1991), construction work and vacuum cleaning (Hunter et al., 1988). Spicher et al. (1967) found that bakery activities such as cleaning and production operations clearly affected the spore load.

The more strictly xerophilic spore load (MY50G counts) present in the air of the chocolate factories was found mostly to be below 50 CFU/m^3 air. This xerophilic load was partly found on DG18, which detects a broad range of species. Some extremely xerophilic species will only grow on MY50G due to its lower water activity. This results in less competition with other fungal species and implies that detection of species diversity can depend on the media used.

Previous air sampling of a confectionery factory described *Penicillium*, *Aspergillus* and *Cladosporium* as the main genera isolated from the air of a confectionery factory (Singh et al., 1986). This was confirmed in the air sampling during our study, which showed the highest prevalence of *Penicillium*, *Eurotium* and *Aspergillus* species as well as the occurrence of *Cladosporium.* The most prevalent species isolated from the production environment was *P. brevicompactum*. This species has not yet been described as being a potential spoiler of chocolate products, but *P. brevicompactum* has been isolated from bakery products with intermediate water activity (Membré and Kubaczka, 2000). *P. brevicompactum* is a common food and indoor fungal species that can tolerate rather low water activities, i.e. 0.75-0.79 (Samson et al., 2010; Scott et al., 2008). The water activity of chocolate itself is too low for spoilage by this species, but chocolate confectionery fillings, with their water activity in the range of 0.70 to 0.90, may be susceptible to spoilage from *P. brevicompactum*.

Of all the ingredients of chocolate confectionery fillings investigated in our study, nuts were shown to be most frequently and most highly contaminated. The highest levels of fungal contamination were found on walnuts. One explanation for this could be the lack of heat treatment of walnuts, in comparison to other types of nut preparations such as *brésilienne*. Nearly the same number of nut samples showed fungal levels in the range of 10-100 CFU/g and above 100 CFU/g on DG18 as well as on MY50G, suggesting contamination of nuts is mainly caused by more strictly xerophilic species. Most of the isolates belong to the genus *Eurotium*: the species detected were *E. repens*, *E. herbariorum* and *E. rubrum*. All of these *Eurotium* species can tolerate very low water activities (in the range of 0.70 to 0.74) (Pitt and Hocking, 1997; Samson et al., 2010), which explains how they could be detected on both media. In literature, pistachio nut samples were reported with fungal counts of 10^3-10^4 CFU/g and 10^5-10^6 CFU/g for harvest and storage, respectively (Heperkan et al., 1994). In retail cashew nuts from Lagos, Nigeria, total fungal counts in the range of 10^2-10^4 CFU/g on malt extract agar with 40% sucrose (MA40) were detected, with the most predominant isolates belonging to the genus *Aspergillus* (Adebajo and Diyaolu, 2003). After plating Brazilian nut kernels on tap water agar (TWA), Czapek yeast extract agar (CYA), malt agar with 20% sucrose (M20) and malt agar with 40% sucrose (M40), *Aspergillus flavus* was found to be the most dominant species, followed by *A. niger* (Freire et al., 2000). No *Eurotium* species were detected. Total fungal counts with a wide range between 10^3-10^4 CFU/g were found for six types of nuts from Saudi Arabia on glucose-Czapek and glycerol agar media.

A. flavus, A. niger and *P. chrysogenum* were the most prevalent fungal species on both media. In contrast, *Eurotium* species were detected in high frequency on all six types of nuts when plated on glycerol agar media, while no *Eurotium* species were detected on glucose-Czapek medium (Abdel-Gawad and Zohri, 1993). These results suggest that *A. flavus* occurs commonly on nuts, although this species was not found in the present study.

2.5 CONCLUSIONS

Microbiological stability of chocolate confectionery products could be compromised in the search for alternative (non-alcoholic or "clean label") formulations. Xerophilic fungi are potential spoilage organisms of these chocolate confectionery fillings. These organisms may originate either in the factory environment or in the ingredients used. Results of this study in seven Belgian companies demonstrate rather low levels of fungal spores present in the confectionery factory environment. Among the ingredients investigated, nuts seem to represent an important source of contamination. *Penicillium*, particularly *P. brevicompactum*, was most dominantly present in the factory environment. Nuts were shown to be mostly contaminated by *Eurotium*, specifically with the species *E. repens*. Further research is needed to examine whether these frequently occurring fungal species are of concern for the quality and shelf life of chocolate confectionery products.

Chapter 2 presents a survey on the presence and prevalence of xerophilic fungi in the production environment of Belgian chocolate confectionery factories as well as in common ingredients of chocolate confectionery fillings. Nuts seemed to represent a possibly important source of xerophilic fungal contamination of confectionery fillings. Therefore, chapter 3 focused profoundly on the nuts and investigated some preventive measures to reduce initial fungal loads on walnuts.

Chapter 3

Potential preventive measures against fungal contamination of chocolate confectionery fillings introduced via nuts

The content of this chapter is based on:

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CHAPTER 3 POTENTIAL PREVENTIVE MEASURES AGAINST FUNGAL CONTAMINATION OF CHOCOLATE CONFECTIONERY FILLINGS INTRODUCED VIA NUTS

ABSTRACT

Nuts are a common ingredient of confectionery fillings and a potential source of xerophilic fungal contamination. The present chapter investigated some preventive measures against fungal contaminants of chocolate confectionery fillings that are introduced by nuts. Microbiological analysis of the fungal load on a variety of nuts and their corresponding nut-based fillings indicated that walnuts, and the fillings in which they were used, were highly contaminated (>2.5 $\log_{10}$ CFU/g). A challenge test with three xerophilic species (*Penicillium brevicompactum*, *Eurotium repens* and *Wallemia sebi*) was conducted on almond-based marzipan (a_w 0.84 and pH 5.76) without preservatives, with 0.15% potassium sorbate and with 1% ethanol. The results showed that common xerophiles *E. repens* and *W. sebi* are capable of growing on marzipan without preservatives. *E. repens* showed some resistance to 0.15% potassium sorbate, hence, spoilage could only be inhibited for at least 25 days if 1% ethanol was added. The effect of dry and humid heating with various heating and drying cycle times was tested on walnuts. Humid heating completely eliminated the initial fungal load, while dry heating did not induce any measurable change. According to sensorial analysis, humid heated and unprocessed walnuts tasted significantly different, with a slight indication of preference for the heated walnuts. Moreover, hexanal analysis of walnuts demonstrated humid treatment to increase the oxidative stability of walnuts, delaying the onset of rancidity during storage.

3.1 INTRODUCTION

A strategy to prevent early spoilage of sweet intermediate moisture food (IMF) is by controlling the growth of spoilage organisms in the final product. Predictive growth/no growth (G/NG) models are an important tool to predict the long-term microbial stability of innovative food products, e.g. confectionery fillings with reduced sugar or fat content or without preservatives. *W. sebi* and *E. herbariorum* are two important xerophilic moulds known to cause spoilage of sugar-rich products (Deschuyffeleer et al., 2015; Samson et al., 2004a). G/NG models for both species were developed including parameters a_w (0.75 – 0.90), pH (5.0 – 6.2) and ethanol concentration (0 and 5% (w/w) in water phase) (Deschuyffeleer et al., 2015). Growth could only be inhibited for a prolonged time (>3 months) if an ethanol concentration of 5% was present. Based on these predictive models, it seems difficult to guarantee the microbial stability of sweet IMF products without the addition of alcohol or preservatives.

In addition to predictive modeling, the spoilage problem can also be addressed by controlling the initial sources of contamination. In CHAPTER 2, a survey study of both the production environment of Belgian chocolate confectionery factories as well as some common ingredients used in chocolate confectionery fillings was conducted. It was noted that among the ingredients investigated, nuts were by far most contaminated and may negatively affect the quality and shelf life of the final products. A total of 35 distinct mould species were identified, of which *P. brevicompactum* and *E. repens* were most frequently isolated from the factory environment and ingredients.

Continuing the results obtained in CHAPTER 2, the present chapter now focuses on the importance of nuts as potential source of xerophilic fungal contamination in confectionery fillings. First, the fungal load of a variety of nuts (raw material) and their corresponding nut-based fillings (final product) was examined. Subsequently, a challenge test was conducted to investigate the potential growth of some prevalently occurring mould species on a nut-based confectionery filling with and without preservatives. Finally, dry and humid heat-treatment as possible preventive measures against initial fungal loads was evaluated on the basis of their effect on the microbiological load, sensorial quality, and oxidative stability of walnuts.

3.2 MATERIAL AND METHODS

3.2.1 Collection and fungal enumeration of nuts and their corresponding nut-based confectionery fillings

Twenty nut samples, consisting of almonds (8), hazelnuts (7), walnuts (3), coconut (1), and pecans (1), were collected from four Belgian chocolate confectionery factories. At the same time, their corresponding nut-based chocolate confectionery products were collected as well.

Additionally, hazelnut samples were collected in triplicate from one Belgian chocolate confectionery factory at different steps of processing: before roasting, after 60 min of roasting at 185°C, and after grinding into oil.

Nut samples were aseptically transferred to a Stomacher® bag and ground into small pieces. Chocolate confectionery products were aseptically opened and the nut-based fillings were transferred to a Stomacher® bag and homogenized. Nuts and nut-based confectionery fillings were directly plated by distributing 1 g onto three plates with Dichloran 18% Glycerol agar medium (DG18, Oxoid Ltd) as well as three plates with Malt extract Yeast extract 50% Glucose agar medium (MY50G). These selective media were prepared as described in § 2.2.1, and have an a_w around 0.95 and 0.85, respectively. Incubation and subsequent enumeration of fungi was conducted as described in § 2.2.3.

3.2.2 Challenge test marzipan

3.2.2.1 Marzipan recipes

A preservative-free marzipan was prepared by adding water (9.1%, w/w) to a bowl of icing sugar (50.9%, w/w), and the dissolved sugar was homogeneously mixed with almond powder (40%, w/w) by means of a food processor (Kenwood Cooking Chef KM084, Havant, Hampshire, UK). Both products were purchased in a horeca specialist store. In addition to the reference marzipan, two other variant were prepared, one with 0.15% potassium sorbate (Solina-group, Bréal-Sous-Monfort, France) and the other with 1% alcohol (GDC, Jumet, Belgium). The a_w of the three marzipans was analyzed using an Aqualab Series 3 device (Decagon Devices, Pullman, WA, USA) and the acidity was evaluated with a SevenCompact pH meter (Mettler Toledo, Greifensee, Switzerland).

3.2.2.2 Preparation of inoculum and design of a challenge test

P. brevicompactum FC207, *E. repens* FC182 and *W. sebi* FC255 isolates were selected from an in-house laboratory culture collection at the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium). All three strains originated from nuts that were purchased as raw material by chocolate confectionery factories.

Malt Extract Agar (MEA; Oxoid Ltd, Basingstoke, Hampshire, UK) was prepared according to the manufacturer's instructions and poured into Petri dishes. MEA was inoculated with each strain by streaking the stock culture onto the plate with an inoculation needle. Inoculated MEA plates were incubated at 25°C for seven days, after which they were evaluated for purity. Pure cultures were transferred to new MEA plates and incubation was repeated. Finally, plates with well-grown fungal cultures were directly used as inoculum for the marzipan samples.

For each marzipan recipe a challenge test was designed by filling 80 sterile 200 ml polypropylene containers with about 40 g marzipan. Twenty containers were used as controls (without inoculum) and the remainders were divided into three equally sized batches, which were inoculated with one of the strains by applying the inoculum with a sterile toothpick. All inoculated and control samples were incubated at 25°C during 25 days. Samples were visually inspected for mould growth at various moments in time.

3.2.3 Heat-treatment of walnuts

Shelled walnuts were purchased in closed containers in a horeca specialist store. Two types of heating (dry/humid) and various heating and drying cycle times were compared by means of a multifunctional chamber (DRKBGO-SE-I-1A, Gernal, Komen, Belgium) that has the purpose of processing food products. The parameters for dry and humid heating were based on laboratory trials (temperature and cycle times) by means of this particular instrument allowing the two types of heating to be compared within the same chamber under the same temperature. Cycle times were chosen based on preliminary sensorial testing. Walnuts were spread in the chamber onto a shelf with a sieve-like structure. Dry heating was performed at 75 – 80°C and 50% relative humidity (RH), while humid heating was achieved at 75 – 80°C through the injection of steam. Humid treatment was directly followed by a drying cycle at 60°C.

The effects of heating and drying were evaluated by varying the duration of each cycle. All treatments were repeated three times. Walnuts with and without dry or humid heat-treatment were microbiologically analyzed on DG18 as described in § 3.2.1. The a_w was measured using an Aqualab Series 3 device (Decagon Devices).

Sensory analysis was performed on walnut samples without treatment and samples heated for 3 min under humid conditions, followed by a drying cycle of 15 min. These treatment conditions were selected on the basis of the results of the microbiological analysis and a_w values. Untreated and treated walnuts were compared by means of a triangle test (ISO 4120:2004(E), 2004) of 40 assessors. The analysis was organized in a sensory lab that complies with ISO 8589:2007. The walnuts were cut into small pieces, homogenized, and about 15 g of the homogenate was transferred to separate cups for blind tasting. Score sheets contained one primary question for which assessors indicated which sample was different from the other two. A comment section was included to indicate if the assessor was guessing and if he had a preference for a specific sample.

The oxidative stability of the treated and untreated walnuts was evaluated by measuring the amount of hexanal, one of the major secondary oxidation products of polyunsaturated fatty acids as linoleic acid. A homogenous batch of untreated and treated walnuts (humid heating for 3 min and drying during 15 min) were divided over 200 ml polypropylene containers, and stored in the dark for 9 weeks at 20°C and 55% RH. At different moments in time, both walnut batches were analyzed for their hexanal concentration. To this end, whole walnuts were ground in a kitchen processor and 5 g of the homogenized sample was carefully weighed in a 50 ml Falcon tube. Exactly 15 ml triacetin (Sigma-aldrich, St. Louis, MO, USA) was added as extraction solvent, together with 5 µg hexanal-d_{12} as internal standard. The centrifuge tube was capped, thoroughly shaken for 1 h on a horizontal shaker, and centrifuged at 4000 *g* for 10 min. The supernatant beneath the pellet was collected with a syringe fitted with needle, and exactly 10 ml was transferred to a 20 ml headspace vial. Each vial was capped and stored at -20°C o/n.

3.2.4 Analysis of hexanal with gas chromatography/mass spectrometry (GC/MS)

Hexanal in the solvent extracts was analyzed with automated SPME using a MPS2 multi-purpose auto sampler (Gerstel GmbH, Mülheim an der Ruhr, Germany), followed by GC/MS (7890A/5975C, Agilent Technologies inc., Santa Clara, CA, USA). The sample was equilibrated for 20 min at 60°C and extraction was carried out with a DVD/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) during 10 min. Desorption and reconditioning of the fiber was achieved directly in the flow of the GC injector for 15 min at 270°C. Helium was used as a carrier gas with a column flow of 1 ml/min. A DB5-MS capillary column (30 m x 0.25 mm, film thickness: 1 µm), fitted with a retention gap (5 m x 0.25 mm), allowed for separation of hexanal and hexanal-d_{12} during a temperature programmed run. The oven program was as follows: 35°C (hold 1 min), 3.5°C/min to 100°C, 30°C/min to 210°C. The mass spectrometer was used in selected ion monitoring mode for detection of m/z 82, 72, and 67 (hexanal) and m/z 92, 80 and 74 (hexanal-d_{12}). The temperatures of the transfer line, source and quadrupole mass analyzer were set at 250, 230 and 150°C, respectively.

Single ion chromatograms reconstructed at m/z 82 (hexanal) and 92 (hexanal-d_{12}) were used for quantification by the internal standard method, and standard solutions in triacetin were used for response calibration. The method gave a linear response for hexanal concentrations between 0.17 and 15 µg/ml (R^2 = 0.998) and the precision was less than 5%. Based upon results for fortified samples, the extraction efficiency of hexanal was found to be quantitative (data not shown). Comparison of results with standard addition experiments showed the method to be accurate, with little or no matrix effect (<4%, data not shown). The method detection limit for hexanal was estimated from 9 replicate blanks fortified at 10 ng/ml (3 x σ) and was found to be 4 ng/ml, corresponding to a concentration of 12 ng/g in walnut.

3.2.5 Statistical analysis

Statistical analysis was performed using the Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA), except for the triangle test (difference test only), which was conducted by means of FIZZ Calculations (version 2.50, Biosystèmes, Couternon, France). Spearman's rank correlation (r_s) was used to test the association between the fungal load of nuts and the fungal load of their corresponding nut-based confectionery fillings. Data obtained during the hexanal analysis of treated and untreated walnuts were analyzed and a linear regression analysis was performed using hexanal content as dependent variable and time and treatment as independent variables. Post-hoc comparison was conducted using a Scheffé test. *P* values ≤0.05 were considered statistically significant.

3.3 RESULTS

3.3.1 Fungal load of nuts and nut-based confectionery fillings

An initial survey of 14 Belgian chocolate confectionery factories identified hazelnuts, almonds and walnuts as the varieties most commonly used in fillings and as topping of chocolate confectionery products. Table 3.1 presents the fungal (spore) levels that were detected on these commonly used nuts and their corresponding nut-based confectionery fillings. Among the 20 samples of different nut types from four factories, walnuts (B6, C4 and C5) exhibited the highest fungal load (>2.5 $\log_{10}$ CFU/g) on both DG18 and MY50G media. The corresponding fillings of the final products also contained relatively high fungal loads on DG18, with values exceeding 1.4 $\log_{10}$ CFU/g. The more selective MY50G medium detected stricter xerophilic fungi with values ranging from less than 0 to more than 2.5 $\log_{10}$ CFU/g. Five of six fillings obtained from chocolate confectionery factory A showed a fungal (spore) load ≥1.0 $\log_{10}$ CFU/g, while fungal levels for the nuts used in these fillings were ≤0.6 $\log_{10}$ CFU/g. No statistically significant correlation ($p > 0.05$) was found between the fungal load of nuts and the fungal load of their corresponding chocolate confectionery fillings.

Table 3.1 Fungal spore levels (log_{10} CFU/g) observed on DG18 and MY50G media for a variety of nuts and corresponding nut-based confectionery fillings. Countable range = 0-2.5 log_{10} CFU/g.

Confectionery factory	Sample code	Sample of nut and corresponding confectionery filling	Log_{10} CFU/g	
			DG18	MY50G
A	A1	Coconut	0.0	<0.0
		Buttercream with small coconut slides	<0.0	<0.0
	A2	Almonds	0.6	0.5
		Marzipan	1.2	0.8
	A3	Hazelnuts	<0.0	<0.0
		Praliné containing small pieces of hazelnut	1.2	0.6
	A4	Hazelnuts	0.3	<0.0
		Praliné containing a complete hazelnut	1.1	0.5
	A5	Hazelnuts	<0.0	<0.0
		Praliné containing small pieces of hazelnut	1.0	<0.0
	A6	Hazelnuts	0.3	<0.0
		Praliné containing a complete hazelnut	1.1	<0.0
B	B1	Pecans	0.6	<0.0
		Filling containing a complete pecan	<0.0	<0.0
	B2	Almonds	0.0	<0.0
		Praliné with small almond slides	0.0	<0.0
	B3	Almonds	<0.0	<0.0
		Truffel with almond slides on top	0.3	<0.0
	B4	Hazelnuts	1.0	0.8
		Praliné containing small pieces of hazelnut	0.3	0.0
	B5	Hazelnuts	1.1	0.5
		Paste based on hazelnuts	0.8	0.5
	B6	Walnuts	>2.5	>2.5
		Filling containing small pieces of walnut	>2.5	>2.5
C	C1	Almonds	0.3	<0.0
		Praliné with complete almond on top	1.9	1.7
	C2	Almonds	<0.0	<0.0
		Praliné containing small almond slides	1.2	1.1
	C3	Hazelnuts	1.0	1.5
		Praliné containing a complete hazelnut	0.0	0.3
	C4	Walnuts	>2.5	>2.5
		Praliné containing a complete walnut	1.4	<0.0
	C5	Walnuts	>2.5	>2.5
		Praliné with a complete walnut on top	>2.5	1.7
D	D1	Almonds	0.8	0.0
		Marzipan (33% almonds)	0.7	0.0
	D2	Almonds	0.3	<0.0
		Marzipan (20% almonds)	<0.0	<0.0
	D3	Almonds	0.3	<0.0
		Marzipan (40% almonds)	<0.0	<0.0

The effect of nut processing on the fungal spore load of hazelnuts is detailed in Table 3.2. A relatively high fungal (spore) load of 2.1 ± 0.2 and 1.8 ± 0.1 log_{10} CFU/g was observed for raw (unprocessed) hazelnuts using DG18 and MY50G media, respectively, while no fungal load was detected after roasting (Table 3.2). Depending on the purpose, roasted hazelnuts are further ground into a hazelnut oil. Fungal enumeration of hazelnut oil samples resulted in very low amount of spores (0.1 ± 0.2 log_{10} CFU/g) on DG18 and no spores on MY50G.

Table 3.2 Fungal spore levels (log_{10} CFU/g) observed on DG18 and MY50G media for unprocessed hazelnuts, hazelnuts after roasting, and after roasting and grinding. Countable range = 0-2.5 log_{10} CFU/g.

Processing	Medium	Log_{10} CFU/g[a]
Unprocessed	DG18	2.1 ± 0.2
	MY50G	1.8 ± 0.1
Roasted	DG18	<0.0
	MY50G	<0.0
Roasted and grinded	DG18	0.1 ± 0.2
	MY50G	<0.0

[a] Average ± standard deviation of triplicate analysis

3.3.2 Challenge test marzipan

The reference (preservative-free) marzipan had a_w and pH values of 0.84 and 5.76, respectively. The addition of preservatives resulted in similar a_w and pH values, i.e. 0.83 and 5.96 for marzipan with potassium sorbate, and 0.84 and 5.77 for marzipan with alcohol, respectively. For each marzipan, the fraction of samples showing fungal growth during 25 days of incubation at 25°C is presented in Figure 3.1. The fungal species *P. brevicompactum* was not capable of growing in any of the marzipans during the first 25 days after inoculation. Both, *E. repens* and *W. sebi* were capable of growing on the preservative-free marzipan. Initiation of growth by *E. repens* and *W. sebi* was visually observed after six and seven days, respectively. Ninety percent of the inoculated reference samples presented growth by *E. repens* and *W. sebi* after 14 and 15 days, respectively.

The addition of 0.15% potassium sorbate had a complete inhibitory effect on the growth of *W. sebi* but only partially inhibited growth of *E. repens* (25% of the samples showed visible growth) over a 25 day period. The addition of 1% alcohol completely inhibited growth of all three fungal species.

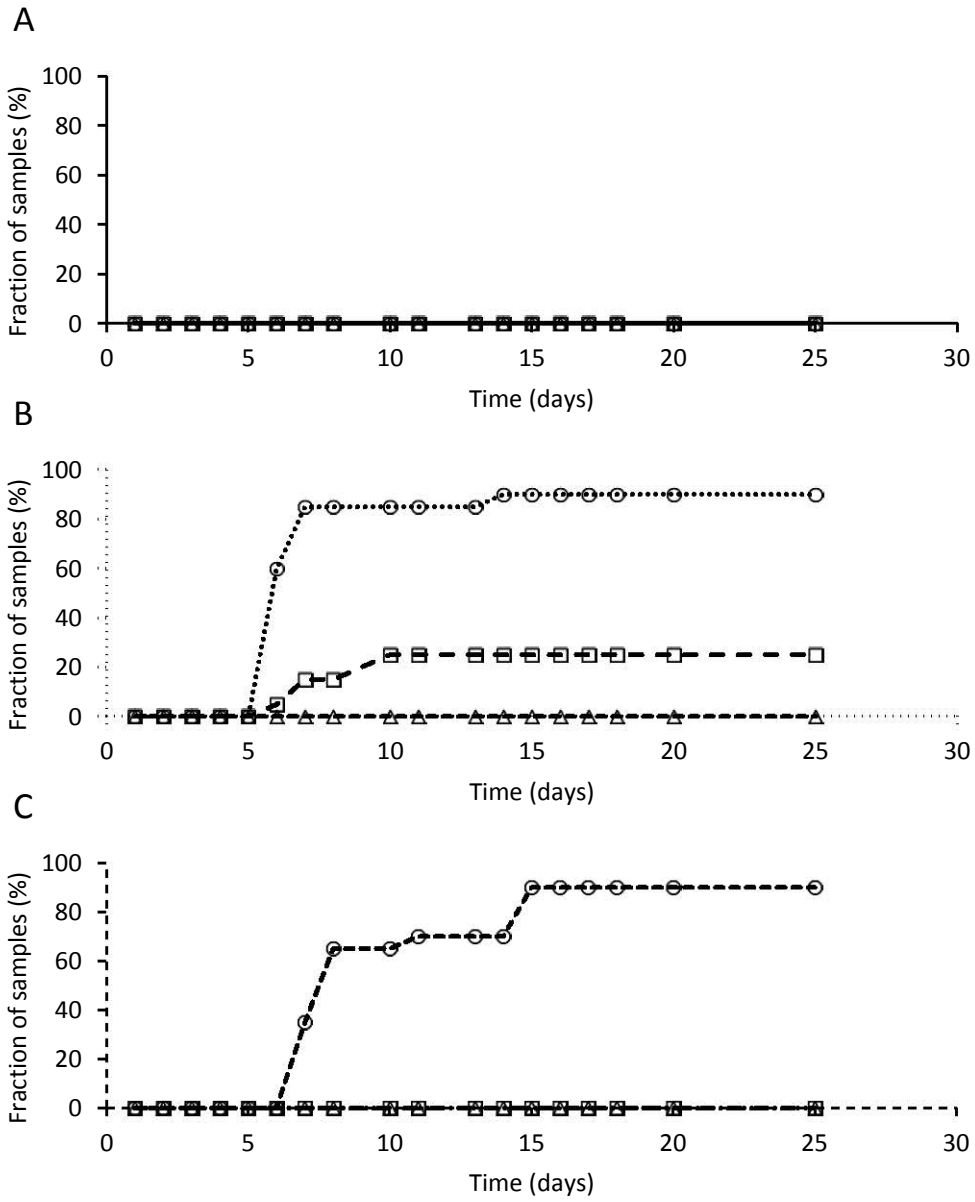

Figure 3.1 Fraction of marzipan samples (%) showing growth of (A) *P. brevicompactum*, (B) *E. repens*, and (C) *W. sebi* over time. Legend: preservative free marzipan, marzipan + 0.15% potassium sorbate, marzipan + 1% alcohol.

3.3.3 Effect of heat-treatment on the fungal load, sensorial quality and hexanal content of walnuts

Table 3.3 presents the a_w values and the results of microbiological analysis of walnuts with and without heat-treatment. Raw (untreated) walnuts had an a_w value of 0.60 and a fungal load above 2.5 $\log_{10}$ CFU/g. Dry heating at 75 – 80°C during 5 and 30 min reduced the a_w to values of 0.40 ± 0.00 and 0.25 ± 0.03, respectively, while the fungal load remained unaffected (>2.5 $\log_{10}$ CFU/g). A humid heating for 3 and 5 min at 75 – 80°C, followed by a drying process for 10, 15 and 20 min at 60°C, all resulted in a complete reduction of the initial fungal load. The a_w values ranged from 0.65 to 0.73, depending on the time of heating and drying.

Table 3.3 Water activity (a_w) and fungal spore levels on DG18 ($\log_{10}$ CFU/g) for untreated and heat-treated walnuts. Countable range = 0-2.5 $\log_{10}$ CFU/g.

Treatment	Heating cycle (min)[a]	Drying cycle (min)[a]	a_w[b]	Log_{10} CFU/g[b]
Untreated	0	0	0.60	>2.5
Dry heating	5	0	0.40 ± 0.00	>2.5
Dry heating	30	0	0.25 ± 0.03	>2.5
Humid heating	3	10	0.71 ± 0.00	<0.0
Humid heating	3	15	0.68 ± 0.01	0.1 ± 0.2
Humid heating	3	20	0.65 ± 0.01	<0.0
Humid heating	5	10	0.73 ± 0.01	<0.0
Humid heating	5	15	0.70 ± 0.00	<0.0
Humid heating	5	20	0.67 ± 0.02	<0.0

[a] Heating at 75-80°C and drying at 60°C
[b] Average ± standard deviation for triplicate analysis

The triangle test for evaluating sensorial differences between untreated and humid treated walnuts was performed by 40 assessors of which 19 gave a correct answer. This result statistically gives a 95.6% certainty that the humid heated walnuts taste differently. Of those assessors that gave a correct answer, 42% and 32% had a preference for the treated and untreated walnuts, respectively. The remaining 26% indicated to have no specific preference.

The hexanal content of untreated and treated walnuts was determined after storage in the dark at 20°C and 55% RH (Figure 3.2). Initially (week 0), untreated and treated walnut samples contained 4.76 ± 0.12 and 6.46 ± 2.73 µg/g hexanal, respectively. After 9 weeks of storage, untreated and treated samples contained 28.9 ± 0.3 and 10 ± 4 µg/g hexanal, respectively. The untreated walnuts showed a clear increase in hexanal concentration over time. For the treated walnuts, concentrations varied between 5 ± 2 µg/g and 16 ± 5 µg/g. After 4 weeks of storage, hexanal concentrations in humid treated walnuts were significantly lower ($p < 0.05$) compared to untreated walnuts.

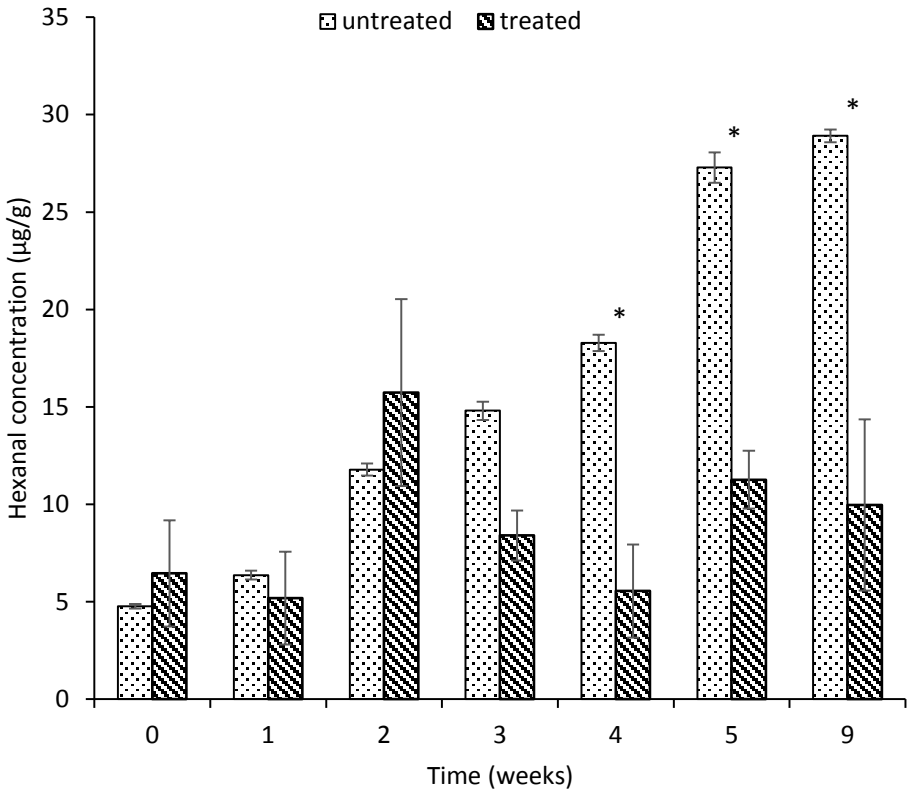

Figure 3.2 Hexanal concentration (µg/g) in untreated and humid treated walnuts stored in the dark at 20°C and 55% RH. Bars and error flags represent average and standard deviation of triplicate analysis. Significant differences (*P* ≤0.05) between treated and untreated samples are indicated with an asterisk.

3.4 DISCUSSION

Microbiological analysis of the most commonly used types of nuts and nut-based confectionery fillings clearly indicated that the nuts and fillings were contaminated with moulds, especially walnuts (>2.5 $\log_{10}$ CFU/g) and the fillings in which they are used. Sejiny et al. (1989) found fungal densities in the range of 5 – 5.5 $\log_{10}$ CFU/g (Sabourad dextrose agar) on walnuts, almonds and hazelnuts collected from markets (Saudi Arabia). Walnuts contained the highest average of mould contamination in comparison with the other types of nuts. Another study on the microbiology of six types of nuts from Saudi Arabia (almond, cashew nut, chestnut, hazelnut, pistachio nut and walnut) detected total fungal counts between 3.3 and 3.9 $\log_{10}$ CFU/g on glucose-Czapek and glycerol agar media (Abdel-Gawad and Zohri, 1993). Tournas et al. (2015) analyzed the fungal contamination on almond, pecan and walnut samples purchased from local supermarkets in the Washington D.C. area. Again, the highest yeast and mould counts (DG18) were found on walnuts (2.65 – 5.34 $\log_{10}$ CFU/g), followed by almonds (<2 – 4 $\log_{10}$ CFU/g) and pecans (<2 – 2 $\log_{10}$ CFU/g). So, based on our results and other studies, walnuts seem among the highest contaminated types of nuts. However, compared to literature, our study detected relatively low levels of fungal contamination on the other types of nuts. The lack of processing of walnuts in comparison to other types of nuts (such as hazelnuts) could be a plausible explanation for this result. Hazelnuts are often roasted to improve their sensorial quality, hence, roasted hazelnuts are purchased as raw material for chocolate confectionery products and were analyzed as such. Indeed, the microbiological analysis of hazelnuts at different steps in the processing chain demonstrated that the roasting process of hazelnuts completely eliminated the fungal contamination that was initially present.

No significant correlation was found between the levels of fungal contamination on the nuts and those on the corresponding fillings. In fact, our results indicated that nut-based fillings could present small amounts of fungal contamination, irrespective of whether the nuts showed any contamination or not. In CHAPTER 2, we found rather low levels of fungal spores in the factory environment and except for nuts, very few or no fungal spore levels on the other three investigated and commonly used ingredients (sugar, fruit fillings and ganache). All the same, small contaminations on fillings of confectionery products may be introduced through the air or other ingredients.

It was also found that the species *P. brevicompactum* occured most prevalently in the production environment of Belgian chocolate confectionery factories. Samson et al. (2010) describes it as a common food and indoor fungal species, with minimum a_w for germination and growth of 0.78 at 25°C (Hocking and Pitt, 1979b), categorizing it as one of the most xerophilic Penicillia. Moreover, *P. brevicompactum* has been previously isolated from dried foods, such as nuts, and intermediate moisture bakery products (Membré et al., 2001; Pitt and Hocking, 2009). A study by Membré et al. (2001) reported growth on preservative-free cakes (a_w 0.9 and pH 5) stored at 20°C. However, *P. brevicompactum* seems to be very restrictive to certain intrinsic and extrinsic factors, since the conditions of our challenge test did not support its growth, while both *E. repens* and *W. sebi* were able to spoil the preservative-free marzipan (a_w 0.84 and pH 5.76) stored at 25°C. *E. repens* even showed resistance to 0.15% potassium sorbate, but none was resistant to 1% alcohol. As such, the latter had a stronger preservative effect than potassium sorbate.

Various studies already compared the effect of different concentrations of typical weak-acid preservatives (calcium propionate, potassium sorbate and sodium benzoate) in combination with common levels of pH and a_w of bakery products on growth of *Aspergillus*, *Eurotium* and *Penicillium* isolates. Of those, potassium sorbate was found to be the most effective in preventing fungal spoilage of this type of products, regardless of a_w. However, at pH 5.5, fungal growth was observed even after the addition of 0.3% potassium sorbate. The authors suggested that the latter could be valuable in bakery products of slightly acidic pH (near 4.5) (Guynot et al., 2005; Marín et al., 2003). These studies support our findings regarding the observed growth of *E. repens* on marzipan containing 0.15% potassium sorbate (pH 5.96). It is generally known that the effectiveness of weak-acid preservatives depends on the pH of the product. Sorbate only acts as a preservative at a pH below 6.0-7.5 (Suhr and Nielsen, 2004). *Aspergillus* and *Penicillium*, although commonly found in bakery products, did not represent an important risk if the industrial process is well controlled and the a_w is below 0.80. *Eurotium* species, on the other hand, did show a major spoiling potential (Guynot et al., 2002; Marín et al., 2002). Another study examined the resistance of *E. amstelodami, E. chevalieri, E. herbariorum, E. rubrum* and *W. sebi* against some weak-acid preserving agents (Vytrasová et al., 2002).

The authors found the tested *Eurotium* species to be more resistant to potassium sorbate (concentrations between 0.5 and 1%) than *W. sebi*. These studies confirm our findings that *E. repens* was more resistant to 0.15% potassium sorbate compared to *W. sebi*. The resistance of *Eurotium* species could be associated with the presence of thick-walled ascospores. A drawback of using a challenge test is that the obtained shelf life is only applicable for the intrinsic and extrinsic parameters of the studied product. On the other hand, the challenge test tells us that commonly occurring species such as *E. repens* and *W. sebi* are capable of spoiling preservative-free confectionery fillings (such as the tested marzipan) when they are present on nuts or in the factory environment.

Since the roasting of hazelnuts was highly effective for decreasing the fungal contamination, it was tested if different heat-treatments (with various heating and drying cycle times) could similarly decrease the spore levels of highly contaminated walnuts. Regardless of the duration of heating and drying cycles, humid heating completely eliminated the initial fungal contamination. This in contrast to dry heating, which did not induce any measurable changes in fungal spore levels. Dry heating for more than 30 min was not considered since the sensorial properties of these walnuts were unacceptable.

Within the context of potential application in the food industry, our study further examined the effect of humid heating on sensorial quality and oxidative stability of walnuts, the latter being an important indicator of quality and shelf life. Humid treated and untreated walnuts tasted significantly different, with a slight indication of preference for the heated walnuts. Moreover, humid treatment seemed to increase the oxidative stability of walnuts during storage. Walnuts have a lipid content of about 60 – 70%. Walnut oil contains high concentrations of polyunsaterated fatty acids including linoleic acid, oleic acid and linolenic acid, which makes them more susceptible to oxidation. Maillard reaction between amino acids and reducing sugars is one of the most well known reactions in heat-treated food products. These reactions have already been linked to the increased oxidative stability of different roasted seed oils (Cai et al., 2013; Chandrasekara and Shahidi, 2011; Wijesundera et al., 2008). Apart from amino acids and proteins, amino group containing PLs, such as phosphatidylethanolamine (PE), are also known to take part in the Maillard type browning reaction (Lederer and Baumann, 2000).

Various studies regarding the PL composition of walnut oil identified PE, phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidic acid (PA) (Angelova-Romova et al., 2013; Pasini et al., 2013). Zamora et al. (2011) showed that Maillard type reaction products between PE and reducing sugars are lipophilic and have free radical scavenging activity. More particular, studies regarding the roasting of mustard seed oil identified PL and its Maillard type reaction products to increase the oxidative stability of roasted mustard seed oil, and demonstrated potent antioxidant activity of the identified reaction products of PE (Shrestha et al., 2013; Shrestha and De Meulenaer, 2014). Considering these facts, we hypothesise that the increased oxidative stability of walnuts after humid heating may be explained by the formation of Maillard type reaction products of PE. The lipid fraction of walnuts also contains natural antioxidants such as tocopherol, which preserves oil quality by retarding the production of off-flavour and rancidity during storage (Abdallah et al., 2015). Vaidya and Eun (2013) investigated the effect of roasting on the oxidative stability of walnut oil. Initially, roasting seemed to increase the oil peroxide value, but during storage the rate of oxidation was significantly lower in roasted than in unroasted walnut oil. Our results of decreased hexanal formation in the heat-treated walnuts support these observations.

3.5 CONCLUSIONS

Nuts as common ingredient of confectionery fillings are a potential source of xerophilic fungal contamination. This chapter investigated some preventive measures against fungal contamination of confectionery fillings introduced by nuts. Results indicated walnuts, and related fillings to be highly contaminated. A challenge test on preservative-free marzipan, a typical nut-based confectionery filling, demonstrated that growth of commonly occurring *E. repens* and *W. sebi* could only be inhibited for over a month with the addition of 1% ethanol. The effect of different heat-treatments was tested on walnuts. Humid heating completely eliminated the initial fungal contamination. The walnuts tasted significantly different, with a slight indication of preference for the heated walnuts. Based on the hexanal content in walnuts, humid heating was found to increase the oxidative stability, delaying the onset of rancidity during storage.

Chapter 4

Optimization and validation of a method without alkaline clean-up for patulin analysis on Apple Puree Agar Medium (APAM) and apple products

The content of this chapter is based on:

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CHAPTER 4 OPTIMIZATION AND VALIDATION OF A METHOD WITHOUT ALKALINE CLEAN-UP FOR PATULIN ANALYSIS ON APPLE PUREE AGAR MEDIUM (APAM) AND APPLE PRODUCTS

ABSTRACT

A sensitive High Performance Liquid Chromatography-UV (HPLC-UV) method, based on the AOAC Official method 2000.02, was developed and validated for the high-throughput analysis of patulin in *in vitro* experiments on Apple Puree Agar Medium (APAM). The importance of repeating the ethyl acetate extraction step at liquid-liquid extraction (LLE) was examined, as well as the extent of patulin degradation during the sodium carbonate clean-up. In addition to this alkaline clean-up, the efficiency of using an Oasis HLB or C_{18} cartridge as solid-phase extraction (SPE) clean-up was compared. This resulted in a two-step ethyl acetate LLE, followed by an Oasis HLB SPE clean-up, without alkaline clean-up conditions. The method was fully validated for APAM, cloudy apple juice and apple puree. Average patulin recoveries at levels of 100, 500 and 1000 µg kg^{-1} of APAM varied between 95% and 113% over three independent days, with an interday precision (RSD_R) of 5 to 10%. Recovery experiments carried out with the spiked apple juice (at 50 µg kg^{-1}) and apple puree (10 µg kg^{-1}) showed average recovery rates laying between 80-101% (RSD_R = 12%) and 77-100% (RSD_R = 9%), respectively. This method offered a detection limit of 3-4 µg kg^{-1} and a quantification limit of 5-8 µg kg^{-1} for APAM, apple juice and puree.

4.1 INTRODUCTION

The major human dietary exposure to patulin is through the consumption of apple-based products, e.g. apple juice made from affected fruit. Patulin has been reported to be acutely toxic (Broom et al., 1944), genotoxic (Alves et al., 2000), cytotoxic (Riley and Showker, 1991), teratogenic (Ciegler et al., 1976) and immunosuppressive (Escoula et al., 1988). As a consequence, the European Commission (EC) (2006a) established maximum limits for patulin of 50 µg kg^{-1} in apple juices, 25 µg kg^{-1} in apple purees and 10 µg kg^{-1} for apple products intended for infants and young children. These maximum limits have to be enforced and therefore, analytical methods are required.

Throughout the years, several analytical methods have been developed for patulin determination in food products. A collaborative study led to the first AOAC Official Method 974.18 for patulin determination in apple juice using thin-layer chromatography (TLC) and silica gel plates for detection. Traditionally, TLC was widely used as it had the advantage to quantitatively analyse large amounts of samples with a low operating cost. Later on, High Performance Liquid Chromatography (HPLC) became the standard for mycotoxin detection in the food industry. It is less time consuming, achieved a higher sensitivity and gave an improved resolution for patulin (Shephard and Leggott, 2000). LC coupled to UV detection is particularly well suited to determine patulin, since the toxin is relatively polar and exhibits a specific absorption wavelength at 276 nm (Gökmen and Acar, 1996; Shephard et al., 2013). Besides UV-based detection, patulin detection by LC coupled to diode array detection (DAD) (Katerere et al., 2008; Zhou et al., 2012), and especially mass spectrometry (MS) (Beltran et al., 2014; Zhang et al., 2014) have been well described. Although HPLC-UV detection is a fast and reliable method and so, the method of choice for routine determination of patulin, a number of gas chromatography (GC) methods have been developed over time (Kharandi et al., 2013; Xiao and Fu, 2012). GC is specific for volatile compounds or compounds that can be made volatile, while LC is characterised by a wider application field because it relies on the solubility of compounds in the mobile phase. GC methods for patulin analysis generally involved the formation of trimethylsilyl ether derivatives with detection by electron-capture or MS (Shephard and Leggott, 2000).

A collaborative study performed by MacDonald et al. (2000) resulted in the AOAC Official Method 2000.02 for the determination of patulin at >25 µg kg^{-1} in clear and cloudy apple juice and apple puree. This method includes a liquid-liquid extraction (LLE), followed by a sodium carbonate clean-up and HPLC-UV analysis. However, the clean-up procedures using sodium carbonate have been described to degrade patulin since the compound is unstable in alkaline conditions. Although this is an often-reported phenomenon, almost no data are available demonstrating the extent of this inactivation. Moreover, all these methods have been validated for food/feed-based matrices. However, *in vitro* experiments studying the underlying molecular genetics of patulin biosynthesis often make use of the laboratory reference/simulation medium "Apple Puree Agar Medium (APAM)". To carry out this type of research, effective and simple analytical methods are needed as well.

The aim of this chapter was to optimize the official method described by MacDonald et al. (2000) for patulin analysis of *P. expansum* isolates grown on APAM. During optimization, the phenomenon of patulin degradation during sodium carbonate clean-up was tested. In order to eliminate impurities not removed during LLE, an alternative SPE clean-up step was searched for and incorporated. Finally, the optimized method was validated for laboratory APAM, and successfully applied for commercial cloudy apple juice and apple puree.

4.2 MATERIAL AND METHODS

4.2.1 Reagents and chemicals

Pure patulin standard (≥98%), 5-hydroxymethyl furfural (HMF, ≥99%), acetic acid (99,99%), sodium carbonate ($NaHCO_3$) and anhydrous sodium sulfate (Na_2SO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate (AcOEt, Pesti-S), hexane (Pesti-S) and acetonitrile (ACN, LC-grade) were supplied by Biosolve BV (Valkenswaard, The Netherlands). Oasis HLB 6 ml LP extraction cartridges (500 mg sorbent) were obtained from Waters (Milford, MA, USA) and Octadecyl (C_{18}) 6 ml extraction cartridges (1000 mg sorbent) were purchased from JT Baker (Center Valley, PA, USA). HPLC-grade water was generated by a Milli-Q grade purification system (Millipore, Darmstadt, Germany) and adjusted to pH 4.0 with acetic acid.

4.2.2 Standard solutions

Stock solutions of patulin and HMF (1 mg ml^{-1}) were prepared in ACN and stored at -20°C. Working solutions were prepared freshly by evaporating the appropriate volume of the stock solution under a stream of nitrogen (N_2) at room temperature, after which they were dissolved in HPLC-grade water pH 4.0.

4.2.3 APAM preparation

APAM was prepared as described by Baert et al. (2007a). Jonagold was used as apple cultivar.

4.2.4 Optimization of the AcOEt LLE and $NaHCO_3/Na_2SO_4$ clean-up step

The method for patulin analysis described by MacDonald et al. (2000) was used as a starting point for the extraction of patulin from APAM. First, the importance of repeating the AcOEt extraction step was examined. Second, the phenomenon of patulin degradation in alkaline conditions was studied by measuring the patulin concentrations of the method with and without a $NaHCO_3/Na_2SO_4$ clean-up step. The procedure for optimization is illustrated in Figure 4.1 and described below. Prior to extraction, all glassware was rinsed with HPLC-grade water pH 4.0 and AcOEt to remove possible alkaline residues present as a result of the washing of the glassware.

P. expansum isolate FC094, from the laboratory culture collection of the Institute For Agricultural and Fisheries Research (ILVO, Melle, Belgium), was grown on Malt Extract Agar (MEA) (Oxoid Ltd, Basingstoke, New Hampshire, UK) during seven days at 25°C. Sporulating mycelium was scraped off the surface and one-point inoculated on three APAM plates ($N = 3$). The Petri dishes were incubated at 25°C and samples were taken when colony diameters reached 0.5 cm. For each APAM plate, exactly half of the mould colony (10 g sample of fungus and medium mix) was transferred to a 50 mL Falcon tube, 20 mL of AcOEt was added and the Falcon tube was put on a horizontal shaker for 30 min. After centrifugation at 1,500 *g* for 5 min, the supernatant was transferred to a new 50 mL tube (protocol 1 and 2). For the other half of the same mould colony, the same extraction was performed and repeated a second time, after which both organic phases were combined in one 50 mL tube (protocol 3 and 4).

All extracts obtained were split into two parts and treated differently. One of the two parts of each extract was subjected to a $NaHCO_3/Na_2SO_4$ clean-up step (protocol 2 and 4), while the other part of each extract was left untreated (protocol 1 and 3). During this clean-up, samples were first extracted with 4 mL of 1.5% $NaHCO_3$ solution by shaking carefully for 10 s. The organic phase was dried by immediately transferring it into a round-bottemed flask through a funnel with filter paper containing 15 g anhydrous Na_2SO_4. The remaining aqueous phase was extracted immediately with 10 mL ethyl acetate by shaking for 10 s. The organic phase was also dried with anhydrous Na_2SO_4 and both organic phases were combined. The untreated as well as treated ($NaHCO_3/Na_2SO_4$ clean-up) extracts were now evaporated under vacuum at 35°C until a volume of about 3 mL. The obtained volume was passed to a glass tube and further evaporated to dryness under a gentle stream of N_2 at 35°C. The residue was redissolved in 1 mL HPLC-grade water pH 4.0 and vortexed for 1 min. Finally, the extracts were filtered into microvials using 0.45 µm Millex-HV syringe Driven Filter Units (Millipore). Prior to injection, samples were 1:10 diluted with HPLC-grade water pH 4.0. Quantification was performed by means of an internal calibration curve of increasing concentrations (0-10,000 ng mL^{-1}) of a pure patulin standard.

4.2.5 Alternative SPE clean-up

Based on the results obtained from the optimization of LLE extraction and $NaHCO_3/Na_2SO_4$ clean-up, it was decided to perform a twofold AcOEt extraction and exclude the clean-up step based on the use of alkaline $NaHCO_3$. An alternative clean-up step using an SPE-cartridge was tested. The efficiency of an Oasis HLB cartridge and a C_{18} cartridge was compared. Extraction solvents used were based on a procedure described by Valle-Algarra et al. (2009).

In order to compare the clean-up efficiency of two different SPE-cartridges (Oasis HLB and C_{18}), blank APAM (10 g) was spiked with 20 µL of patulin standard (250 ng μL^{-1}) at two different time points during the extraction procedure. Namely, for each of the cartridges, two blank APAM extracts were spiked after LLE but before SPE clean-up (a), and two were spiked after SPE clean-up but before evaporating to dryness (b). The spiked extracts of both situations were left to equilibrate for 30 min. The extraction efficiency (%) was calculated by means of the observed concentrations of a) relative to the observed concentrations of b). The complete procedure was the following.

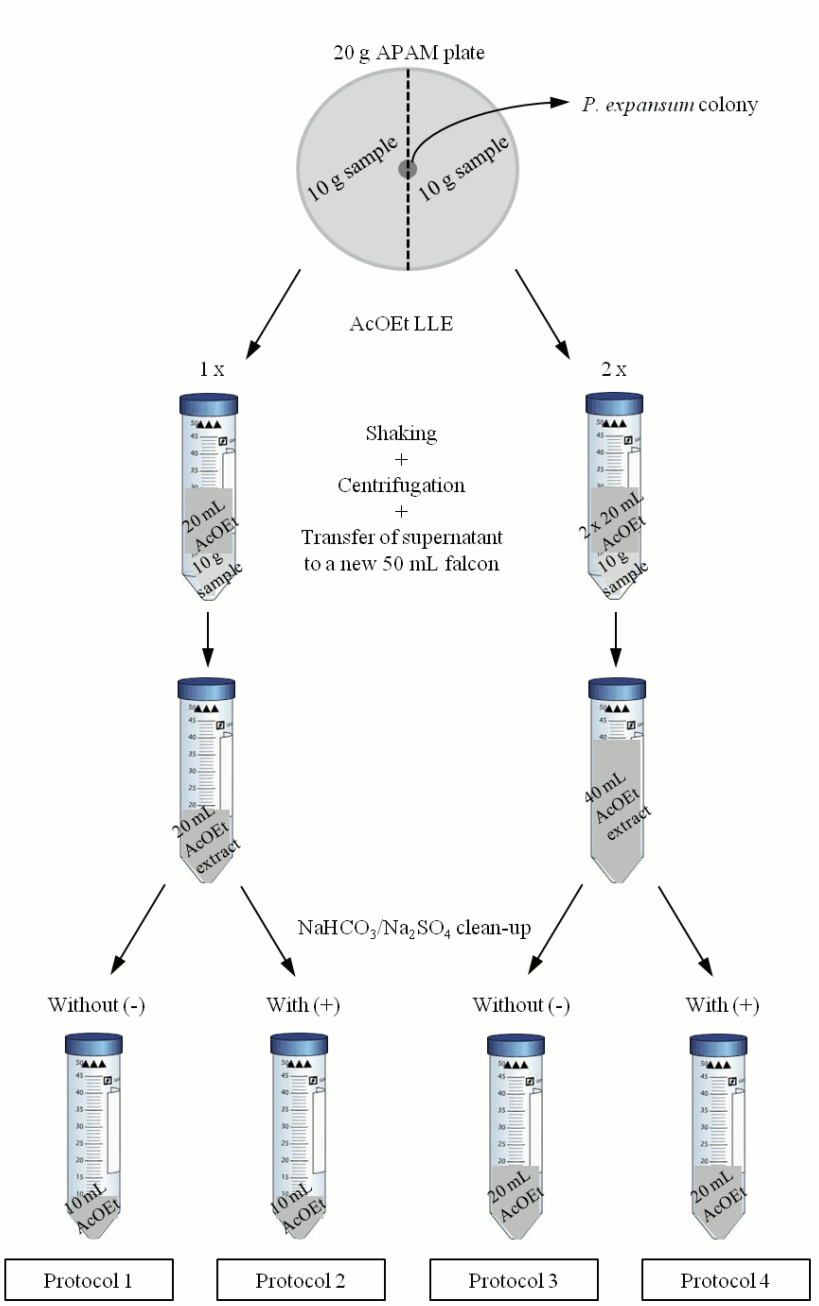


Figure 4.1 Schematic illustration of the evaluation of the AcOEt LLE and $NaHCO_3/Na_2SO_4$ clean-up step.

Blank APAM (10 g) was twofold extracted by means of AcOEt and subsequently evaporated, first under vacuum to a volume of about 3 ml, and finally to dryness under a gentle stream of N_2 at 35°C. Concerning the volume used on the SPE cartridges, 2 mL was chosen based on a study of Gökmen et al. (2005) in which the effect of sample volume on the recovery of patulin from cartridges was determined. Therefore, the residue was redissolved in 2 mL of HPLC-grade water pH 4.0 and vortexed for 1 min. The Oasis HLB and C_{18} cartridges were equilibrated by subsequently passing 5 mL AcOEt-hexane (80/20) and 5 mL HPLC-grade water pH 4.0 over the columns. The redissolved extracts were passed onto each of the cartridges. Each column was washed with 5 mL hexane. Subsequent elution in glass tubes containing 30 µL of acetic acid, was performed by adding 4 mL AcOEt-hexane (80/20). Eluates were evaporated to dryness under a gentle stream of N_2 at 35°C. Afterwards, the residues were redissolved in 1 mL HPLC-grade water pH 4.0 and vortexed for 1 min. The samples were filtered (0.45 µm Millex-HV syringe Driven Filter Units, Millipore) into microvials prior to injection in the HPLC system.

4.2.6 HPLC-UV apparatus and conditions

Liquid chromatography was performed using an HPLC PE series 200 equipped with a Detector UV/VIS series 200 set at 276 nm (Perkin Elmer, Waltham, MA, USA). Chromatographic separation was carried out with an XBridge C_{18} column (150 × 2.1 mm, 5 µm; Waters) preceded by a C_{18} Alltima guard column (7.5 × 2.1 mm, 5 µm; Grace/Alltech, Columbia, MD, USA). Column and sample temperature were maintained at 20°C and 4°C, respectively. The injection volume was set at 6 µL and the sample flow rate at 0.1 mL min^{-1}. Solvent A was an ACN/acetic acid (0.1%) (5/95, v/v) solution and solvent B was ACN. Gradient elution was initiated at 100% A. After 15 min at 100%, in a time period of 5 min, the flow rate and solvent changed gradually to 0.3 mL min^{-1} and 100% B. This condition was maintained for 10 min after which solvent B changed gradually back to 100% A (5 min) and was held for 20 min at 100% A. Subsequently, the flow rate decreased to 0.1 mL min^{-1} in 2 min.

4.2.7 Method validation

For the final method optimized in this chapter and illustrated in Figure 4.2, following validation parameters were evaluated: specificity, linearity, recovery (R_A), repeatability (RSD_r), reproducibility (RSD_R), limit of detection (LOD) and quantification (LOQ). Specificity was tested by analyzing peak separation of patulin and HMF at a level of 1 ng μl^{-1} pure standard containing both compounds. The compound HMF, created when sugar is heated such as during pasteurization, is the main interference of patulin as it shares the same UV chromophore at 276 nm. Linearity was assessed in terms of R^2 and least squares regression. This was performed by duplicate injections of 10 concentrations of a pure patulin standard in the range of 50 ng ml^{-1} to 2,000 ng ml^{-1}. R_A percentages for APAM were measured at three concentration levels (100-500-1,000 µg kg^{-1}). Increasing appropriate concentration ranges were spiked in the matrix to obtain a calibration curve. For each concentration level considered, the observed concentrations were calculated based on peak areas and by using the calibration curve. Finally, the recovery was expressed as a percentage of the measured concentration relative to the concentration actually spiked. These data were used to determine the intraday precision or repeatability by calculating the relative standard deviation. Reproducibility or interday precision was measured by repeating the experiments on three different days. The limit of detection and the limit of quantification were calculated as three and six times the standard error of the intercept divided by the slope of the calibration curve, respectively. The LOD value was checked in practice by evaluating chromatographically if a signal-to-noise of 3 was obtained.

After validating our method for APAM, it was validated for cloudy apple juice and apple puree. The apple products were commercially purchased in the local supermarket. R_A was measured for cloudy apple juice and apple puree at the maximum regulatory limit of 50 µg kg^{-1} and 10 µg kg^{-1}, respectively. RSD_r, RSD_R, LOD and LOQ were determined as described above. The expanded measurement uncertainty U (coverage factor 2, 95% confidence level) of the method for patulin analysis on both apple product matrices, was determined according to the protocol described by FASFC (2008). The decision limit CCα (α = 5%) was calculated, according to the Commission Decision 2002/657/EC (2002). Finally, in order to test the accuracy of the method, a cloudy apple juice sample was purchased at FAPAS (T1652QC, Fera Science Ltd, Sand Hutton, York, UK), and analyzed in our laboratory (N = 6). An assigned value of 38.6 µg kg^{-1}

and a concentration range for $|z| \leq 2$ of 21.6-55.6 µg kg^{-1} was delivered as a fit-for-purpose quality control measure.

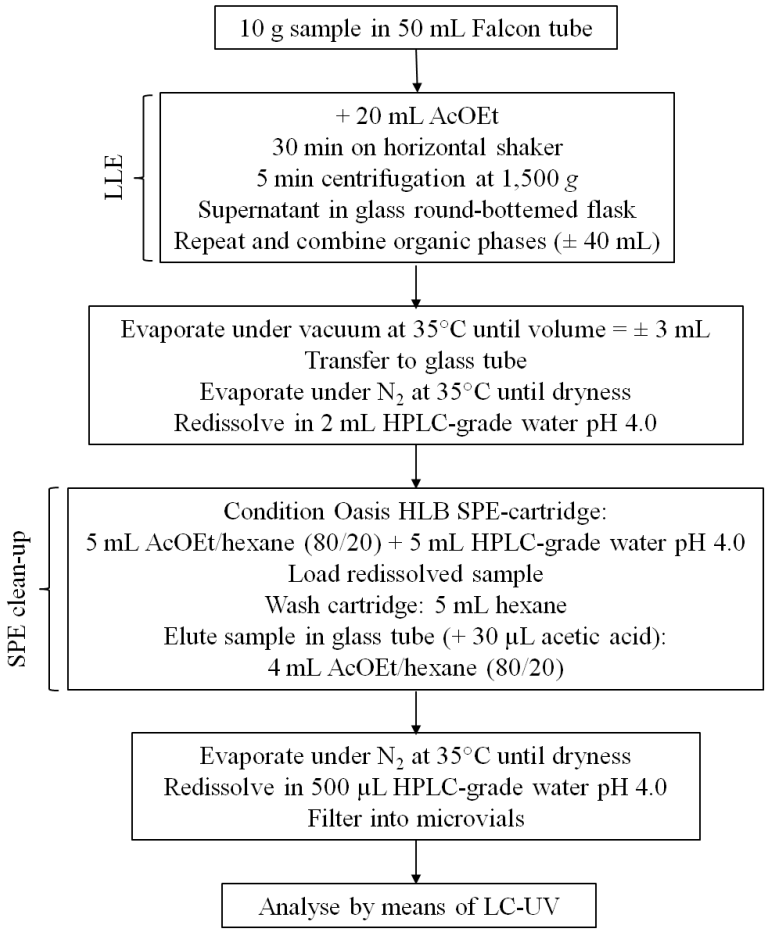


Figure 4.2 Final method for patulin analysis on APAM and apple products.

4.2.8 Statistical analysis

Statistical analysis, of the data obtained at optimization of the AcOEt LLE and $NaHCO_3/Na_2SO_4$ clean-up step, was done using SPSS® version 22 (IBM, New York, USA). A non-parametric Kruskal-Wallis test was performed with patulin concentration as dependent variable and protocol as independent variable. Post-hoc comparison was conducted using a Bonferroni test. *P* values ≤0.05 were considered statistically significant. The recoveries obtained during method validation were checked with the Grubbs test for outliers. This was performed with R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

4.3 RESULTS AND DISCUSSION

The AOAC official method 2000.02 for the determination of patulin in apple juice and apple puree (MacDonald et al., 2000) includes a liquid-liquid extraction (LLE), followed by a sodium carbonate clean-up and HPLC-UV analysis. As APAM has become the medium of choice in most research studying the molecular genetics of patulin biosynthesis, this method was first optimized, validated and later implemented for high-throughput experiments of *P. expansum* isolates grown on APAM. A LLE consisting of several AcOEt extraction steps and the overall use of glassware can be time-consuming. Therefore, the necessity of a twofold extraction was examined and the use of disposables, e.g. falcons was incorporated. As the clean-up procedure using sodium carbonate has been described to degrade patulin, this possible effect was also tested and the use of an alternative SPE clean-up was investigated.

4.3.1 Evidence of patulin decreasing in alkaline conditions

On the one hand, the importance of a twofold extraction by means of AcOEt was examined. On the other hand, the phenomenon of patulin degradation under alkaline conditions was investigated by measuring the patulin concentration of the method with and without a $NaHCO_3/Na_2SO_4$ clean-up step. The results are shown in Table 4.1.

Table 4.1 Average concentration (µg kg^{-1}) and relative standard deviation (RSD) (%) of patulin produced by *P. expansum* on APAM. Comparison of four different extraction protocols (*N* = 3).

Protocol	Amount of AcOEt liquid-liquid extractions	With (+) or without (-) $NaHCO_3/Na_2SO_4$ clean-up	Average patulin concentration ± SD (µg kg^{-1})	Relative standard deviation (RSD) (%)
1	1	-	6285 ± 1635	26
2	1	+	2047 ± 500	24
3	2	-	9234 ± 203	2
4	2	+	5922 ± 512	9

Applying only one AcOEt extraction step with and without $NaHCO_3/Na_2SO_4$ clean-up (protocol 2 and 1) resulted in an average patulin concentration of 2,047 ± 500 µg kg^{-1} (RSD = 24%) and 6,285 ± 1,635 µg kg^{-1} (RSD = 26%) for *P. expansum* grown on APAM, respectively.

Average patulin concentrations of 5,922 ± 512 µg kg^{-1} (RSD = 9%) and 9,234 ± 203 µg kg^{-1} (RSD = 2%) were measured when performing two AcOEt extraction steps with and without a subsequent $NaHCO_3/Na_2SO_4$ clean-up (protocol 4 and 3), respectively. Both protocol 3 and 4, containing a twofold AcOEt extraction step resulted in higher patulin concentrations and lower relative standard deviations than protocol 1 and 2, respectively. An alkaline clean-up step clearly resulted in a decrease of patulin recovery. All together, protocol 3 consisting of two AcOEt extractions without subsequent alkaline clean-up led to the highest patulin concentrations. Moreover, a significant difference (p = 0.014) was found between protocol 3 and the other protocols. Katerere et al. (2007) who conducted an evaluation of four methods for patulin analysis assumed that a greater variability in the results of certain methods was due to the use of the alkaline clean-up. Gökmen et al. (2005) used an alkaline solution in an attempt to remove impurities from SPE cartridges. According to the authors, this resulted in a drastic decrease of patulin recovery, which made purification of patulin by washing the cartridge with alkaline solution questionable. Arranz et al. (2005) noted that washing with alkaline solutions should be performed as quickly as possible to avoid any loss. The results obtained in our study confirm the studies describing the instability of patulin in alkaline conditions. Moreover, our results demonstrate the extent by which the patulin concentration is diminished by comparing the same method with and without alkaline clean-up. Average patulin concentrations obtained with protocol 2 were 67% lower than those obtained with protocol 1. Protocol 4 resulted in 36% lower average patulin concentrations than protocol 3. Altogether, these findings strongly suggest that methods for patulin analysis should be performed without applying any alkaline solution for clean-up, as this negatively influences the patulin content.

Nevertheless, a clean-up step is necessary to prolong the shelf life of the XBridge C_{18} column. Therefore, an alternative clean-up without involving alkaline conditions was searched for by comparing the efficiency of an Oasis HLB cartridge and a C_{18} cartridge. The patulin SPE clean-up efficiency of both cartridges is expressed as a percentage of the average patulin concentration of the extracts spiked before clean-up compared to those spiked after clean-up. The Oasis HLB clean-up cartridge showed an efficiency of 73% compared to only 58% obtained with the C_{18} cartridge (data not shown). Given the higher efficiency obtained with an Oasis HLB cartridge, the latter one was chosen to use for SPE clean-up in our method for patulin analysis.

The last years, various methods for patulin analysis have included the use of SPE cartridges for clean-up. A study of Boonzaaijer et al. (2005) described a method starting an LLE and first clean-up step using a C_{18} column, followed by a second clean-up step by means of a Romer #224 column. This method however showed an LOD and LOQ of 25 µg kg^{-1}, not sensitive enough for the maximum regulatory limit of patulin in baby food set at 10 µg kg^{-1}. Zhou et al. (2012) compared clean-up results of a home-made PVPP-F column with those of a MycoSep®228 AflaPat column. Both gave good clean-up performances. The PVPP-F column itself is cheaper, but home-made columns are more time-consuming for high-throughput and routine analysis and could be less standardized. Desmarchelier et al. (2011) published a QuEChERS procedure involving an initial liquid extraction followed by an alkaline partitioning and a final clean-up based on dispersive SPE (dSPE). According to Marsol-Vall et al. (2014), the above mentioned method resulted in interfering compounds during the chemical analysis. An extra clean-up step was added to overcome these interferences, making the latter more cumbersome. In our study, the method without performing a clean-up step already showed a clear baseline separation of patulin from any interfering compounds. However, the use of an Oasis HLB SPE cartridge for sample clean-up, prior to injection on the HPLC, resulted in a prolonged shelf life of the XBridge C_{18} column. All together, the optimization of extraction and clean-up steps in our study resulted in a final method for patulin analysis consisting of a simple two-step AcOEt LLE and a subsequent Oasis HLB SPE clean-up. This method was subsequently validated for APAM, cloudy apple juice and apple puree.

4.3.2 Analytical method characteristics

The method developed fulfils the criteria of specificity. In Figure 4.3, chromatograms of patulin spiked samples of apple puree, cloudy apple juice and APAM are presented at concentration levels of 10, 50 and 100 µg kg^{-1}, respectively. Alongside the spiked samples, the figure also exhibits the blank samples of each matrix. A clear baseline separation is shown between patulin and its possible interfering compounds (HMF and other matrix compounds). As for the linearity, R^2 of the calibration curve of a pure patulin standard showed to be very adequate (R^2 >0.99). Moreover, the regression analysis indicates that it is highly likely that there is a strong relationship between the patulin concentration (*x* value) and instrument response (*y* value) ($p = 2.2\ 10^{-12}$).

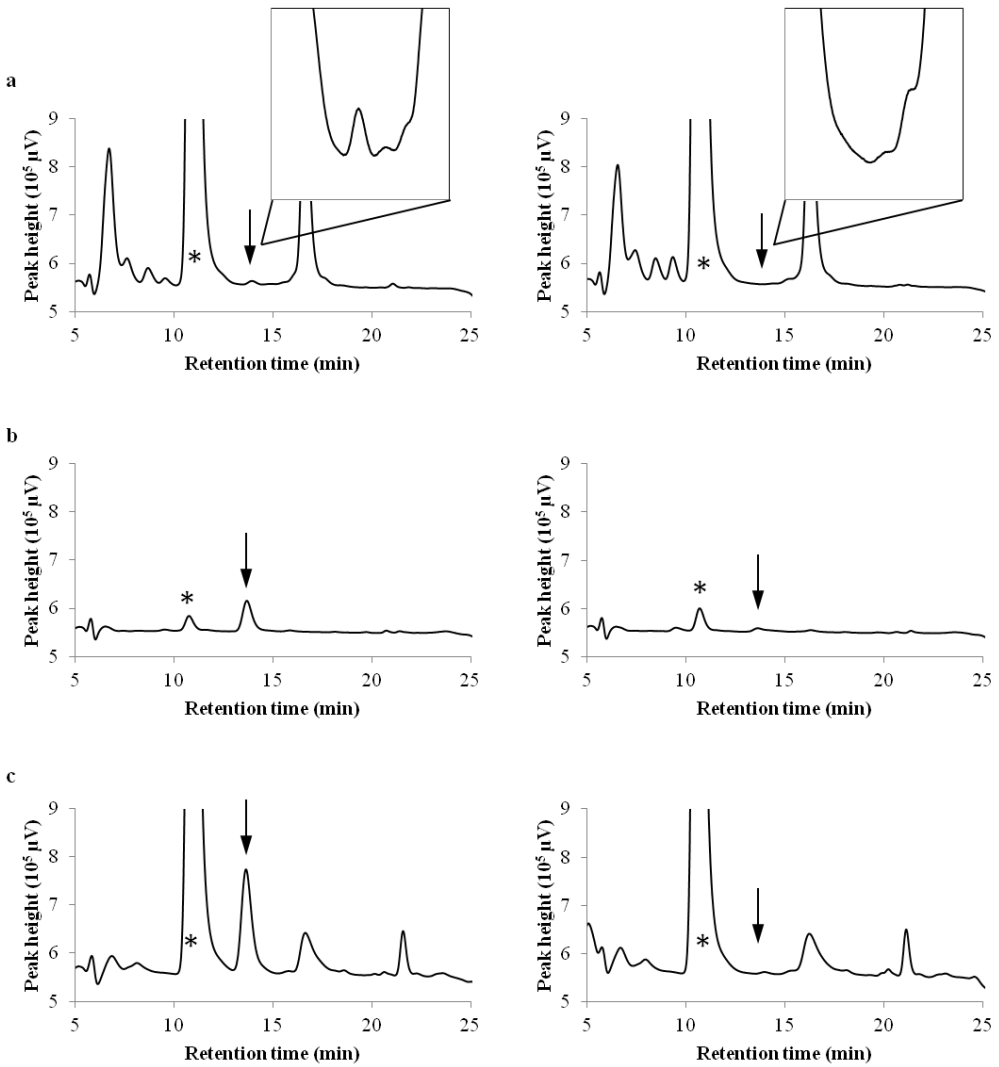


Figure 4.3 Chromatograms of spiked samples (left panel) and blank samples (right panel) of a) apple puree (10 µg kg^{-1}), b) cloudy apple juice (50 µg kg^{-1}), and c) APAM (100 µg kg^{-1}). The arrow refers to the retention time of patulin. The asterisk refers to the retention time of HMF.

Table 4.2 gives the results of the average recoveries obtained for APAM, cloudy apple juice and apple puree, as well as the repeatability, reproducibility, and limits of detection and quantification. The average patulin recoveries for the three concentration levels (100-500-1,000 µg kg^{-1}) of APAM varied between 95% and 113% over three independent days, with an interday precision (RSD_R) of 5 to 10%. The intraday precision (RSD_r) shown for each day was calculated as the average relative standard deviation of the four samples analyzed on that particular day.

RSD_r for patulin analysis on APAM medium varied between 5% and 13%. Cloudy apple juice at a maximum regulatory limit of 50 µg kg^{-1} gave recovery percentages in the range of 80 to 101% with an RSD_r varying from 8 to 17% and an RSD_R of 12%. The recoveries at a maximum regulatory limit of 10 µg kg^{-1} for apple puree varied between 77% and 100% and exhibited an RSD_r between 2% and 14% and an RSD_R of 9%. The results for apple juice and apple puree comply with the performance criteria required by the Commission Regulation (EC) No 401/2006 for patulin in food products (2006b). Limits of detection of all three matrices show similar values of 3 or 4 µg kg^{-1}. The average expanded measurement uncertainty U, for the method for patulin analysis, was 46% for both cloudy apple juice and apple puree. This percentage lies in the expected order for concentrations <100 µg kg^{-1}, according to the report (the relationship between analytical results, measurement uncertainty, recovery factors and the provisions of EU Food and Feed legislation) supporting Commission Regulation (EC) 401/2006 (2006b). The decision limit CCα, at the permitted limits for apple juice and apple puree, was 55.45 µg kg^{-1} and 10.89 µg kg^{-1}, respectively. Patulin analysis of the FAPAS QC material resulted in an average patulin concentration of 36.4 ± 9.9 µg kg^{-1}. This result confirms the accuracy of the method.

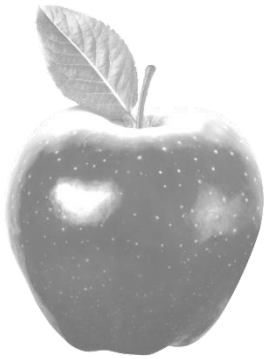
Table 4.2 Validation characteristics: recovery (R_A), repeatability (RSD_r), reproducibility (RSD_R), limit of detection (LOD), and limit of quantification (LOQ) of patulin on APAM, cloudy apple juice, and apple puree (N = 4).

Matrix	Concentration (µg kg^{-1})	Day 1		Day 2		Day 3		RSD_R (%)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
		R_A (%)	RSD_r (%)	R_A (%)	RSD_r (%)	R_A (%)	RSD_r (%)			
APAM	100	113	6	105	10	107	6	8	3	5
	500	95	5	96	5	102	6	5		
	1000	102	10	107	6	103	13	10		
Cloudy apple juice	50	85	17	80	8	101	11	12	4	8
Apple puree	10	100	14	77	11	86	2	9	4	5

4.4 CONCLUSIONS

The AOAC official method 2000.02 for patulin analysis in commercial apple-products was optimized and validated for laboratory APAM, the reference medium in most research studying the molecular genetics of patulin biosynthesis. The optimized method was subsequently validated for commercial cloudy apple juice and apple puree at maximum regulatory limits. During optimization, the protocol consisting of two AcOEt extractions without subsequent $NaHCO_3/Na_2SO_4$ clean-up gave the best results. These results strongly suggest that methods for patulin analysis should be performed without applying any alkaline solution, as this negatively influences the patulin content. Nevertheless, an alternative clean-up step seemed necessary to prolong the shelf life of the HPLC column. Therefore, the new method consists of a simple two-step AcOEt LLE, followed by an Oasis HLB SPE clean-up and HPLC-UV analysis. This sensitive HPLC-UV method for patulin analysis is very useful as monitoring system or in our case for further research on the underlying molecular mechanisms of patulin biosynthesis, with the aim of minimizing the patulin problem in food.

An HPLC-UV method for the high-throughput analysis of patulin in in vitro *experiments on laboratory APAM, was optimized and validated in chapter 4. This method is now implemented to characterise the patulin production capacity of a collection of* Penicillium expansum *isolates from Belgian apples and some reference strains under classical controlled conditions and under representative conditions of long-term apple storage.*



Chapter 5

Patulin production by *Penicillium expansum* isolates from apples during different steps of long-term storage

The content of this chapter is based on:

De Clercq, N., Vlaemynck, G., Van Pamel, E., Colman, D., Heyndrickx, M., Van Hove, F., De Meulenaer, B., Devlieghere, F. and Van Coillie, E. 2016. Patulin production by *Penicillium expansum* isolates from apples during different steps of long-term storage. World Mycotoxin Journal 9: 379-388.

CHAPTER 5 PATULIN PRODUCTION BY *PENICILLIUM EXPANSUM* ISOLATES FROM APPLES DURING DIFFERENT STEPS OF LONG-TERM STORAGE

ABSTRACT

Penicillium expansum is the principal cause of blue mould rot and associated production of patulin, a weak mycotoxin, in apples worldwide. *P. expansum* growth and patulin production is observed during improper or long-term storage of apples. We have investigated the extent to which each successive step during long-term storage contributes to patulin production in various *P. expansum* isolates. Fungal isolates collected on apples from several Belgian orchards/industries were identified to species level. Random amplification of polymorphic DNA (RAPD) analysis and *β-tubulin* gene sequencing identified *P. expansum* and *Penicillium solitum* as the most prevalent *Penicillium* species associated with Belgian apples. All 27 *P. expansum* isolates and eight reference strains were characterized for their patulin production capacity on apple puree agar medium (APAM) for five days under classical constant temperature and atmosphere conditions. Under these conditions, a large range of patulin production levels was observed. Based on this phenotypic diversity, five *P. expansum* isolates and one reference strain were selected for *in vitro* investigation of patulin production under conditions representative of each step of long-term apple storage. Patulin accumulation seemed highly strain dependent and no significant differences between the storage steps were observed. The results also indicated that a high spore inoculum may lead to a strong patulin accumulation even at cold temperatures (1°C) combined with controlled atmosphere (CA) (3% O_2, 1% CO_2), suggesting that future control strategies may benefit from considering the duration of storage under CA conditions as well as duration of deck storage.

5.1 INTRODUCTION

Blue mould is one of the most severe post-harvest diseases of apples and pears worldwide (Rosenberger, 1990). Although the disease may be caused by various *Penicillium* species, *P. expansum* is considered to be the principal cause of blue mould rot (Dombrink-Kurtzman and Blackburn, 2005; Moss, 2008). Low quality, over-mature or long-stored fruit is more susceptible to infection by this fungus. Blue mould rot has an important economic impact on the fresh fruit industry (Rosenberger, 1990) as well as the fruit-processing industry, as *P. expansum* is a major producer of several toxic metabolites, e.g. patulin and chaetoglobosins (Andersen et al., 2004). Those metabolites may eventually end up in final products such as fruit juices. Although patulin is not a very potent mycotoxin, the European Commission (EC) has regulated patulin levels in apple-derived products (Commission Regulation (EC), 2006a). The main legal focus is on apples and apple products, especially apple juice, which represents the largest source of patulin intake by humans (Moake et al., 2005). Patulin remains stable in the acidic conditions of apple juice, even after pasteurisation (Damoglou and Campbell, 1986; Wheeler et al., 1987). Control strategies are therefore necessary to minimize the risk of *P. expansum* growth and subsequent patulin contamination of the final product.

Apple storage at low temperature combined with controlled atmosphere (CA) (i.e. reduced O_2 and elevated CO_2) is commonly applied to extend the shelf life of apples during long-term storage (Morales et al., 2010). In practice, long-term storage (≥3 months) involves several steps under varying conditions (Figure 5.1). First, apples are stored for one day at ambient temperature (deck storage), and then a short period (one to six days) at 1°C before CA is achieved. CA storage, typically maintained for several months, is at 0.5 to 3.5°C, an O_2 level between 1 and 3% and a CO_2 level between 0 and 3%, depending on the cultivar. When the stored apple lot is prepared for processing, the composition of the atmosphere is set equal to that of the air. This atmospheric change typically lasts only 1-7 days. After delivery at the apple juice producer, apples are kept for one day maximum at ambient temperature (deck storage) before processing (Baert et al., 2012).

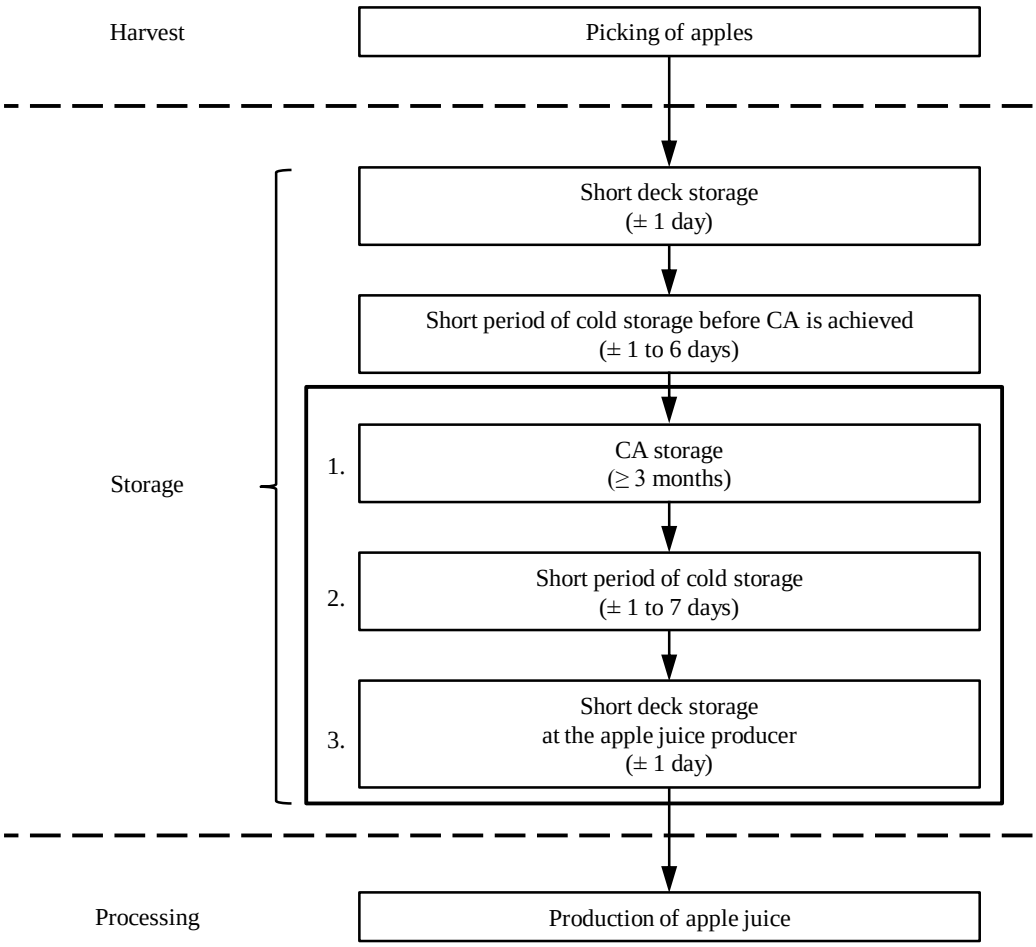


Figure 5.1 Flow diagram of the long-term storage (≥3 months) of apples, based on data obtained by Baert et al. (2012). The box indicates the three storage steps simulated during the last experiment of this study. The numbers (1, 2 and 3) in the box represent the different storage steps.

Several authors have investigated the effect of temperature and/or atmosphere on *P. expansum* growth and patulin production on apples (Baert et al., 2007a; Baert et al., 2007b; Morales et al., 2007a; Morales et al., 2007b; Morales et al., 2007c; Salomão et al., 2009; Sydenham et al., 1997; Welke et al., 2011). Baert et al. (2012) developed a quantitative risk assessment model (QRAM) to evaluate different strategies for reducing patulin contamination of apple products. One of their conclusions was that the duration of deck storage, between the delivery at the apple juice producer and the processing of apples, should become a critical control point (CCP) in HACCP systems.

Besides extrinsic parameters like temperature and atmospheric composition, fungal growth and mycotoxin production are also influenced by intrinsic parameters such as water activity, pH, microbial interactions and implicit factors such as the physiological state of the mould and its genetic information. Several published studies highlight the effect of some of these factors on the growth and mycotoxin production of fungal species. Some reports showed high variability between the strains tested (Baert et al., 2007a; Garcia et al., 2011; Menniti et al., 2010; Santos et al., 2002), while others found few differences among isolates of the same species (Bellí et al., 2004; Pardo et al., 2005).

In the current chapter, we have investigated the influence of strain variability by including a large collection of fungal isolates. Fungal isolates from apples of different Belgian orchards and industries were gathered and identified to species level to get better insight into the diversity of *Penicillium* species present. All of the *P. expansum* isolates and reference strains were characterised for their patulin production under classical controlled conditions. Based on this phenotypic diversity, six *P. expansum* isolates with a low, medium or high level of patulin production capacity were selected for *in vitro* investigation of patulin production under conditions representative of each step of long-term apple storage.

5.2 MATERIAL AND METHODS

5.2.1 *P. expansum* reference strains

P. expansum strains MUM 00.01, MUM 99.19, MUM 99.20, MUM 99.22, MUM 99.23 and MUM 99.24 originated from Portuguese grapes; the strains were obtained from the Centro de Engenharia Biológica da Universidade do Minho in Portugal (Braga). Strain MUCL 20453 and MUCL 29189 come from the agro-industrial fungi-yeast collection of the Mycothèque de l'Université catholique de Louvain, member of the Belgian Coordinated Collections of Micro-organisms (BCCM/MUCL, Louvain-la-Neuve, Belgium). *P. expansum* MUCL 20453 originated from Belgian apples and MUCL 29189 from Californian grapes.

5.2.2 Sampling of apples and fungal isolation

Sound apples (cv. "Jonagold"), collected from organic and conventional orchards in Belgium, were sampled at 3 time points (December 2008, February 2009 and April 2009) during long-term storage. At each sampling moment, 15 apples were taken from the lots originating from four organic and five conventional farms. Ten of those apples were placed in a 500 ml jar and then immersed in sterile demineralised water with 0.05% Tween® 80 (Merck KGaA, Darmstadt, Germany). The filled jar was then placed on a stirrer at 115 rpm during 15 min. 100 µl of the rinse water were plated onto Malt Extract Agar (MEA) (Oxoid Ltd, Basingstoke, Hampshire, UK) with oxytetracycline (0.5%) (Oxoid Ltd). The remaining five apples were directly imprinted onto MEA. 10 and 100 ml of the same rinse water were also filtered through a Plain White Sterile filter (0.22 µm, 47 mm, Millipore, Darmstadt, Germany). The filters were subsequently placed on MEA plates and incubated. In addition, mouldy apples from different varieties (Jonagold, Belle de Boskoop and Elstar) were gathered from a Belgian fruit-processing factory. Visible fungal mycelium was transferred directly from the apples onto MEA. Petri dishes were incubated at 25°C for seven days. After incubation, isolates from each sample were further purified by streaking them onto fresh MEA plates followed by incubation at 25°C for seven days. Sporulating mycelia of the isolated fungal colonies were scraped off the surface and three-point inoculated onto MEA, Czapek Yeast Autolysate agar (CYA) (BD Difco™, NJ, USA), Creatine Agar (CREA) and Yeast Extract Xucrose agar (YES). MEA and CYA were prepared according to the manufacturer's instructions. CREA and YES were prepared as described by Samson et al. (2004b). After incubation for seven days at 25°C, macroscopic characteristics (colony diameter and colour) as well as microscopic characteristics were determined for classification to genus level using the identification key published in Frisvad and Samson (2004). Pure fungal cultures were added to the laboratory culture collection of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium).

5.2.3 Random amplification of polymorphic DNA analysis of *Penicillium* spp.

Genomic DNA extraction and subsequent visualisation was carried out as described by Van Pamel et al. (2012). RAPD-PCR, performed using random primer Ari1, was done according to Geisen et al. (2001), with the exception that 45 cycles were used instead of 42 for PCR amplification. PCR was carried out in a 30 µL-reaction mixture containing 15 U Red GoldStar buffer (Eurogentec, Seraing, Belgium), 2.5 mM $MgCl_2$, 0.4 mM dNTP, 90 pmol primer, 0.6 U Red GoldStar DNA polymerase (Eurogentec) and 1 µL of template DNA. Separation and visualisation of the PCR fragments was performed by subsequent gel electrophoresis on a 1.5% Seakem LE agarose gel (Lonza, Rockland, ME, USA) and staining with ethidium bromide (2 µg/ml). Normalisation and analysis of similarity in band patterns was conducted using the Bionumerics 3.5 software package (Applied Maths, St-Martens-Latem, Belgium). The relationship between all *Penicillium* spp. isolates and *P. expansum* reference strains was scored by the Pearson correlation coefficient. Clustering was based on the unweighted pair group method with arithmetic average (UPGMA). A similarity percentage of at least 90% between isolates was taken into account for discriminating into groups. At least one representative of each group was chosen for further identification to species level.

5.2.4 *β-tubulin* gene sequencing

For each of the representative isolates, the partial *β-tubulin* gene sequence was determined. PCR amplification was performed using the forward primer Bt2a and reverse primer Bt2b (Glass and Donaldson, 1995). The PCR mix was prepared as described by Van Pamel et al. (2012). Initial denaturation at 95°C for 1 min was followed by 30 cycles of denaturation at 95°C for 15 s, primer annealing at 56°C for 15 s and extension at 72°C for 30 s, with a final extension at 72°C for 8 min. The PCR products were visualised after staining of a 1.5% Seakem LE agarose gel with ethidium bromide (2 µg/ml). PCR products were purified using the High Pure DNA Purification kit (Roche Applied Science, Mannheim, Germany). The purified PCR fragments were sequenced using the forward primer Bt2a and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and ABI prism 3130xl Genetic Analyzer System (Applied Biosystems).

After analysing the obtained DNA sequences with the Sequencing Analysis 5.2 software (Applied Biosystems), the sequences were compared against those available in the EMBL database using the FASTA function (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). A similarity percentage of at least 97% with a sequence of a CBS strain was taken into account for identification of the fungal isolates to species level.

5.2.5 Detection of *P. expansum* by PCR

Isolates were analysed by PCR, based on a specific sequence of the polygalacturonase (*peg*) gene, using forward primer PEF and reverse primer PEG as described by Marek et al. (2003). PCR mix, conditions and product visualisation were performed as described above with the exception that the primer annealing temperature used was 60°C instead of 56°C.

5.2.6 Analysis of patulin production capacity of *P. expansum*

All *P. expansum* apple isolates obtained in this study and reference strains from apple or grapes were grown on MEA for seven days at 25°C. Sporulating mycelium was picked off and one-point inoculated onto Apple Puree Agar Medium (APAM) plates using a sterile toothpick (N = 3). APAM of cv. "Jonagold" (pH 3.6) was prepared according to Baert et al. (2007a). In house experiments of fungal spore counting showed that the inoculum size using this toothpick method was 5×10^5-1×10^6 spores. The toothpick technique was preferred over inoculation with a liquid volume to avoid liquid interference. After incubating the inoculated APAM plates for five days at 25°C, patulin analysis was performed as described by Baert et al. (2007a).

5.2.7 Simulation of long-term apple storage steps and corresponding patulin analysis

The three steps of long-term storage of apples were simulated *in vitro* using APAM plates. Five *P. expansum* isolates and one reference strain MUM 99.19 were selected based on their diversity in patulin production capacity under classical controlled conditions (two strains with a high patulin production capacity, two average producers and two low patulin producers). Selected strains were first grown on MEA during seven days at 25°C. Sporulating mycelium was picked off and one-point inoculated onto APAM plates using a sterile toothpick (N = 3).

To determine the effect of CA storage (storage step 1) on patulin production, inoculated Petri dishes were placed separately in polypropylene trays (oxygen transmission rate of 192 cm^3/m^2 24h) and a gas mixture of 3% O_2 – 1% CO_2 – 96% N_2 (Air Liquide, Paris, France) was introduced. A tray sealer (TS400, VC999 Packaging Systems, Herisau, Switzerland) was used to seal the trays with a PET/CPP NPAF foil with an oxygen transmission rate of 190 cm^3/m^2 24h at 25°C and 50% R.H. Trays were stored under these controlled atmospheric conditions at 1°C for two months. The gas composition in the trays was checked using a headspace gas analyzer (PBI-Dansensor A/S, Denmark). Trays were repacked every 10 days to limit the variation (<1.5%) in the O_2 level. After two months, APAM plates of each of the selected isolates and reference strain were analysed for their patulin content (N = 3). Patulin was quantified using the HPLC-UV method described in CHAPTER 4. All remaining trays were opened to allow the atmosphere composition to equalise to that of the surrounding air. After three days under these normal atmospheric conditions at 1°C (storage steps 1+2), APAM plates of each of the isolates and reference strain were analysed as above for patulin content (N = 3). The last remaining trays were subsequently transferred for one day to 20°C (storage steps 1+2+3). Patulin concentration was measured for each of the isolates and the reference strain (N = 3). As a reference condition, the selected isolates and reference strain were grown at 20°C for three days and their patulin production capacity was analysed (N = *3*).

5.2.8 Statistical analysis

Statistical analysis was performed using the Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). The data obtained on the patulin production of all *P. expansum* isolates and reference strains, incubated for five days at 25°C on APAM, followed a normal distribution. Therefore, a one-way analysis of variance (ANOVA) was conducted with patulin concentration as dependent variable and origin of the isolate/reference strain as independent variable. A post-hoc comparison was conducted using a Bonferroni test. The data obtained during the *in vitro* storage experiment did not follow a normal distribution. For this data set, a non-parametric Friedman two-way ANOVA was carried out with patulin concentration as dependent variable and *P. expansum* isolate and storage step as independent variables. Post-hoc comparison was conducted using a Scheffé test. P values ≤ 0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 Identification of *Penicillium* spp. isolated from Belgian apples

Fungal isolation on apples resulted in a collection of 103 isolates of which 56 isolates (54%) were morphologically identified as *Penicillium* species. Of those, 31 out of 64 isolates and reference strains were selected for gene sequencing (Figure 5.2). Gene sequencing resulted in the species identification of 24 out of 26 isolates (two unidentified isolates) (Table 5.1). Subsequently, RAPD combined with gene sequencing resulted in the species identification of 54 *Penicillium* isolates. Of those, 27 and 12 isolates were *P. expansum* and *Penicillium solitum*, respectively. The other identified species were as follows: four *Penicillium bialowiezense* isolates, three *Penicillium polonicum*, two *P. carneum*, one *P. corylophilum*, one *Pencillium venetum*, one *Penicillium citreonigrum*, one *P. brevicompactum*, one *P. chrysogenum* and one *Penicillium spinulosum*. Of the 17 fungal isolates originating from mouldy apples collected from a Belgian fruit-processing factory, 14 were identified as *P. expansum*. Two of the three remaining isolates belonged to the species *P. solitum;* the third isolate was identified as *P. polonicum*. To confirm the identity of the *P. expansum* isolates, all *Penicillium* strains were subjected to a species-specific *peg*-PCR (Marek et al., 2003). All isolates identified as *P. expansum* showed the expected 404 bp DNA fragment, while all the others were negative (Figure 5.2).

Table 5.1 Species identification of the *Penicillium* field isolates using the partial *β-tubulin* gene.

Penicillium isolate	Identity %	Reference strain[1] (Accession number)	Accession number	Species
FC111	100	CBS 14686 (AY674356)	KT588910	*Penicillium solitum*
FC097	100	CBS 14686 (AY674356)	KT588909	*P. solitum*
FC122	99.8	CBS 14686 (AY674356)	KT588912	*P. solitum*
FC112	99.8	CBS 14686 (AY674355)	KT588911	*P. solitum*
FC083	99.8	CBS 14686 (AY674356)	KT588908	*P. solitum*
FC126	99.0	CBS 40592^{T} (AY674334)	KT588914	*Penicillium venetum*
FC088	98.8	DTO 034-E9^{T} (KM088694)	KT588898	*Penicillium citreonigrum*
FC103				*Penicillium* sp.
FC119				*Penicillium* sp.
FC110	97.2	CBS 25729^{T} (KF499573)	KT588894	*Penicillium brevicompactum*
FC114	99.8	CBS 306.48^{T} (AY495981)	KT588897	*Penicillium chrysogenum*
FC093	99.5	CBS 325.48^{T} (JQ965099)	KT588900	*Penicillium expansum*
FC094	99.8	CBS 325.48^{T} (JQ965099)	KT588901	*P. expansum*
FC124	99.8	CBS 325.48^{T} (JQ965099)	KT588903	*P. expansum*
FC136	99.8	CBS 325.48^{T} (JQ965099)	KT588905	*P. expansum*
FC134	99.3	CBS 325.48^{T} (JQ965099)	KT588904	*P. expansum*
FC116	99.0	CBS 325.48^{T} (AY674400)	KT588902	*P. expansum*
FC090	98.3	CBS n 271.35 (GQ367505)	KT588913	*Penicillium spinulosum*
FC084	99.8	CBS 112297^{T} (AY674386)	KT588895	*Penicillium carneum*
FC107	99.6	CBS 112297^{T} (AY674386)	KT588896	*P. carneum*
FC102	98.8	CBS 312.48^{T} (JX141042)	KT588899	*Penicillium corylophilum*
FC100	97.8	CBS 22728^{T} (AY674439)	KT588893	*Penicillium bialowiezense*
FC092	97.9	CBS 22728^{T} (AY674439)	KT588891	*P. bialowiezense*
FC098	98.0	CBS 22728^{T} (AY674439)	KT588892	*P. bialowiezense*
FC106	99.5	CBS 101479 (AY674306)	KT588907	*Penicillium polonicum*
FC095	99.8	CBS 101479 (AY674306)	KT588906	*P. polonicum*

[1] The isolates were identified to species level with a similarity percentage of at least 97% with a sequence of a CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands) strain, and a GenBank accession number was obtained for each identified isolate.

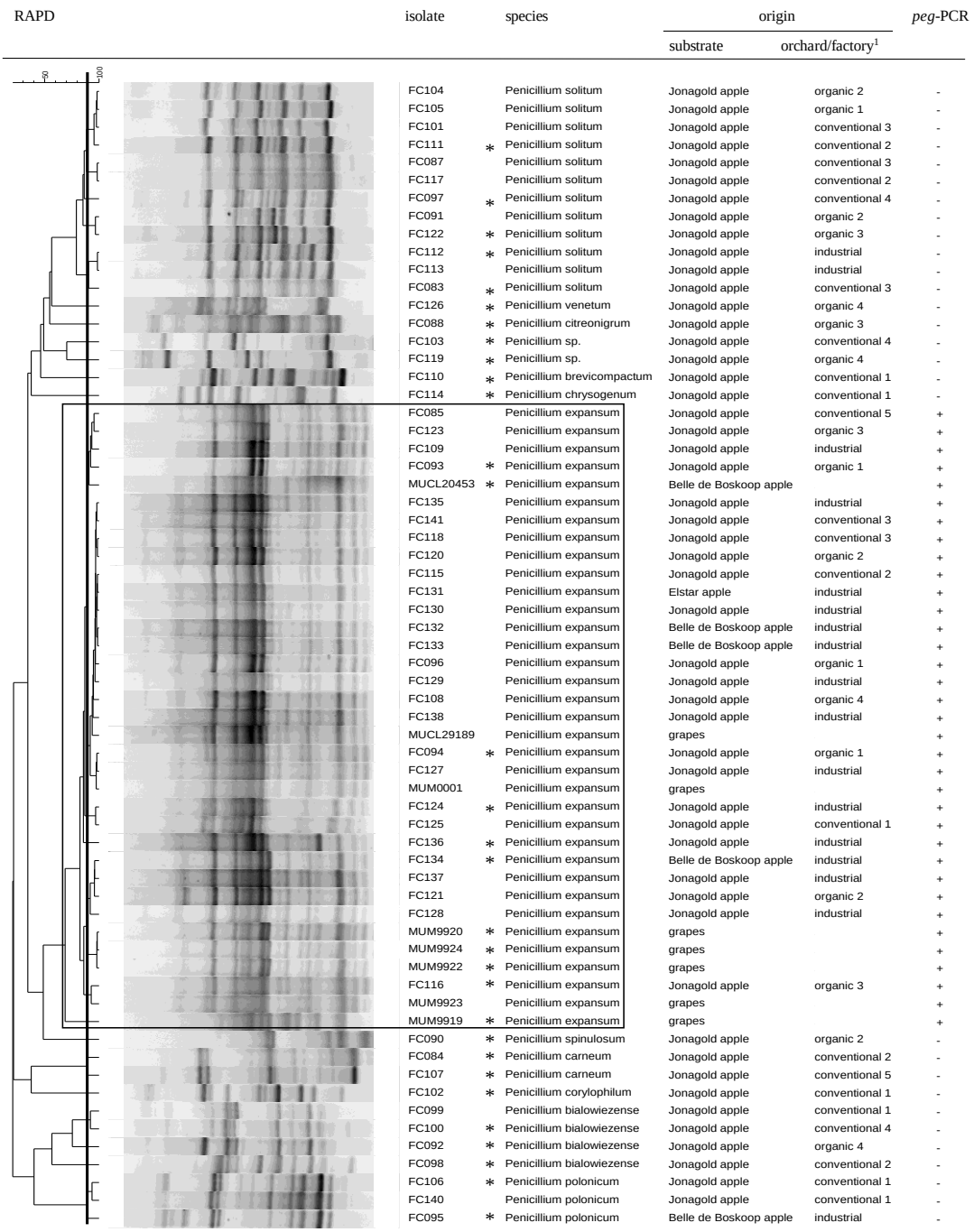


Figure 5.2 Dendrogram generated with the Unweighted Pair Group Method with Arithmetic Means (UPGMA) and Pearson coefficient (optimization 3%) for the RAPD fingerprints generated with random primer Ari1 for the *Penicillium* spp. field isolates and *P. expansum* reference strains of different origin (substrate, orchard/industry). A delineation level of 90% (indicated by the vertical line) was considered for discrimination between groups. At least one representative (indicated by *) of each group was chosen for species identification based on *β-tubulin* gene sequencing. The box indicates the group of *P. expansum* field isolates and reference strains. The last column represents the results of the *peg*-PCR. [1] Sound apples were sampled from storage facilities of conventional and organic orchards, and mouldy apples from an apple juice factory (industrial). The five conventional and four organic orchard farms from which isolates were sampled are identified by a number.

5.3.2 Patulin production capacity of *P. expansum* strains

A wide diversity in patulin production capacity was observed between all tested *P. expansum* strains (Figure 5.3). Average patulin concentrations varied from 15 ± 2 mg/kg to 747 ± 98 mg/kg, with the reference strain MUCL 29189 showing the lowest average patulin concentration and isolate FC116 showing the highest, respectively. The group of *P. expansum* contains isolates originating from sound "Jonagold" apples of conventional or organic orchards, and isolates originating from mouldy apples from a fruit-processing factory (cv. "Jonagold", "Belle de Boskoop" and "Elstar"). One reference strain originated from Belle de Boskoop apples and seven reference strains were isolated from grapes. The group of *P. expansum* isolates from Jonagold apples of conventional and organic origin showed average patulin concentrations significantly higher ($p < 0.001$) than those of the *P. expansum* reference strains from apple and grapes. Medium patulin amounts (no significant differences) were found with the group of *P. expansum* isolates of mouldy apples of different varieties.

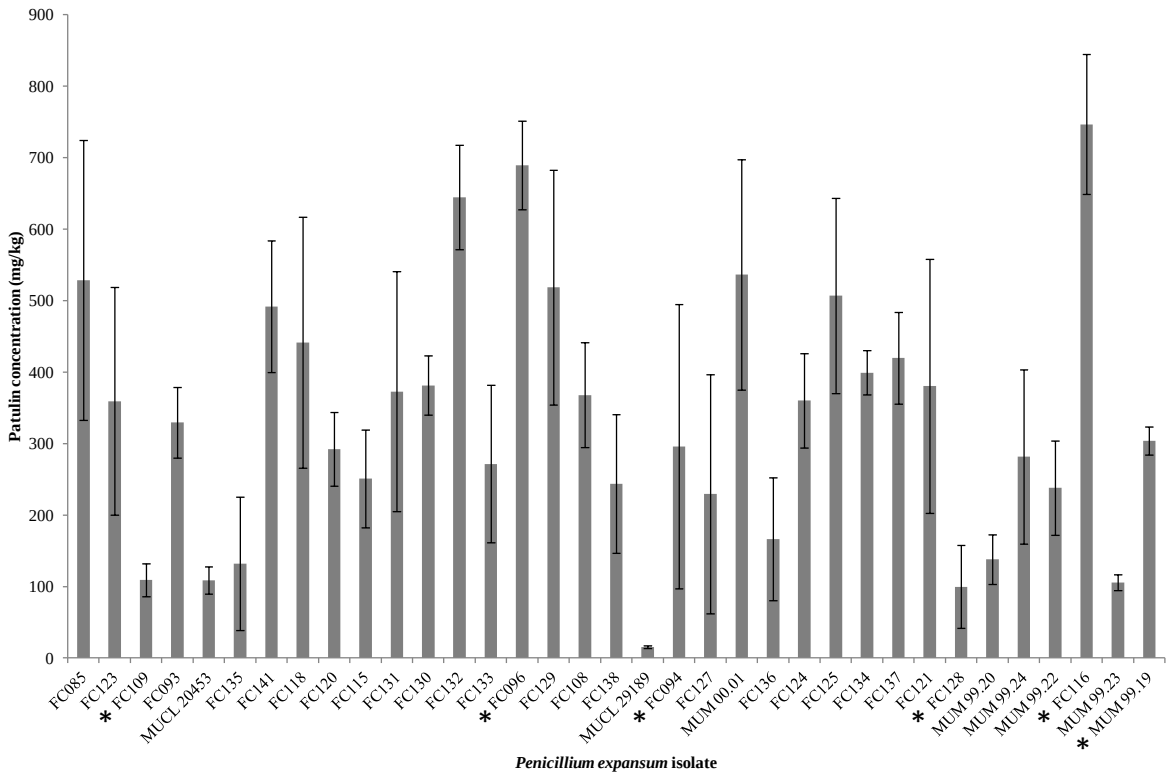


Figure 5.3 The patulin production capacity expressed as the average patulin concentration (mg/kg) on Apple Puree Agar Medium (APAM) of *P. expansum* strains after five days incubation at 25°C. *N* = 3 (except for FC129 and FC132; *N* = 2). * Isolates and reference strain selected for the *in vitro* long-term storage experiment on APAM.

5.3.3 Comparison of patulin production during different steps of long-term storage

Under the reference condition, average patulin concentrations varied between 12 ± 0 mg/kg and 33 ± 7 mg/kg APAM, with isolate FC128 producing the lowest and FC116 producing the highest amounts of patulin, respectively. Figure 5.4 shows the patulin content measured for each of the *P. expansum* strains after each storage step. Although no statistically significant differences were found in patulin concentrations ($p > 0.05$) measured between the three different steps of storage of each strain *in vitro*, two trends in patulin accumulation during long-term storage could be observed. The isolates FC109, FC094 and FC096, the highest producers under the first cold CA storage step, tended to have a peak in patulin production. These isolates showed a slight increase in patulin from the first to the second step of storage, followed by a decrease from the second to the third storage step. On the other hand, the isolates FC128, FC116 and reference strain MUM 99.19 tended to accumulate patulin slowly over time. For the tested *P. expansum* isolates and reference strain, a significant difference ($p < 0.001$) in their patulin production capacities during long-term storage was observed. Under the long-term conditions, the highest average patulin concentrations were produced by strains FC109 and FC096, while the strains FC116 and FC128 produced the lowest patulin amounts. Isolate FC094 and reference strain MUM 99.19 were average producers compared to the others ($p < 0.05$). During the three steps of long-term storage, strain FC128 (lowest patulin production capacity) formed patulin at concentrations of 3 ± 5 mg/kg, 3 ± 1 mg/kg and 8 ± 5 mg/kg APAM at each of the three storage steps, respectively. Strain FC096, one of the highest patulin producing *P. expansum* strains, was characterised by average patulin concentrations of 249 ± 144 mg/kg, 317 ± 116 mg/kg and 276 ± 143 mg/kg APAM during the successive steps of storage, respectively.

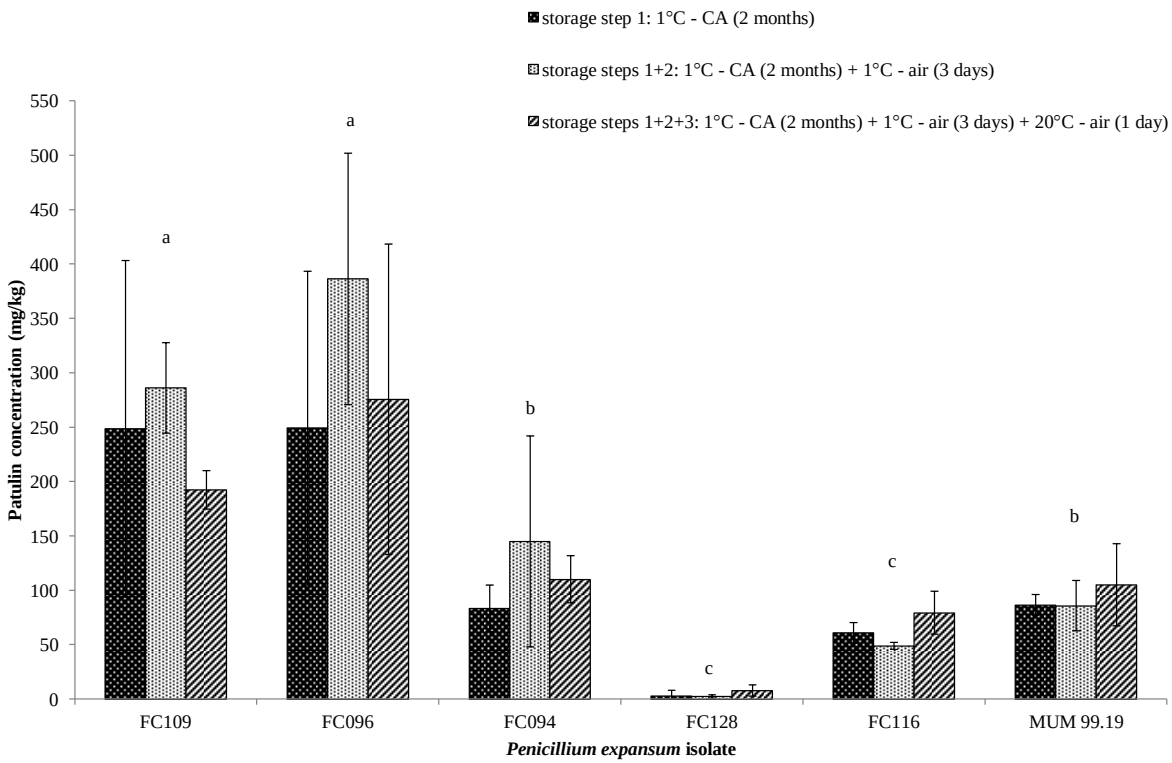


Figure 5.4 The patulin production capacity expressed as the average patulin concentration (mg/kg) on Apple Puree Agar Medium (APAM) of *P. expansum* during the different steps of long-term storage. Significant differences ($P \leq 0.05$) in patulin production during storage between strains are indicated with different letters.

5.4 DISCUSSION

The primary and secondary metabolism of fungi are influenced by a variety of intrinsic, extrinsic and implicit factors (McCallum et al., 2002; Northolt et al., 1978). *P. expansum* growth and patulin production occur mainly during long-term storage of apples. To reduce these risks, different conditions of temperature and atmosphere composition are applied successively during long-term storage. In this chapter, the successive steps of this storage period were investigated to determine whether and how these steps contribute to possible differences in patulin concentrations.

The possible presence and potential effect of strain variability on patulin production capacity was tested on a large set of *Penicillium* isolates originating from Belgian apples. RAPD was used because it may discriminate between species, genotypes within a species or even between strains (Geisen et al., 2001).

In the study conducted in this chapter, RAPD was mainly used to reduce the number of gene sequencing reactions for species identification. This revealed that *P. expansum* formed one group, clearly separated from the other *Penicillium* species. Of all *Penicillium* sp. isolated from Belgian apples, *P. expansum* and *P. solitum* were the most encountered species in our collection. Research on fungal diversity in apple orchards and storage facilities from Uruguay, France and the USA (states of Oregon and Washington) found the same commonly-occurring species (Amiri and Bompeix, 2005; Pianzzola et al., 2004; Sanderson and Spotts, 1995). These findings indicate that *P. solitum* and *P. expansum* frequently appear together in apple orchards and storage facilities in different parts of the world. Pitt et al. (1991) showed that *P. solitum* and *P. expansum* were both capable of growing over the tested temperature range of 5 to 30°C on MEA. The optimum temperature for growth of *P. expansum* was 25°C, and for *P. solitum* between 20 and 25°C (no growth at 37°C). However, growth of *P. solitum* was slower at all temperatures. Other data regarding *in vitro* growth of *P. solitum* and *P. expansum* on CYA and MEA during 7 days at 25°C also indicated that the growth rate of *P. solitum* was slower. More specific, *P. solitum* reached colony diameters of 22-28 mm on CYA and 20-28 mm on MEA, while *P. expansum* reached colony diameters of 30-40 mm on CYA and 20-40 mm on MEA (Pitt and Hocking, 2009). Pitt et al. (1991) analysed pathogenicity of both species on apple and pear fruits and found that lesion diameters were significantly smaller than those of *P. expansum*. The authors concluded that although *P. solitum* is ubiquitous in fruit packinghouses, it is much less aggressive compared to *P. expansum*. A more recent study found *P. solitum* in marine sediments from Antarctica (Gonçalvez et al., 2013). The authors find similarly that *P. solitum* was capable of growing over the tested temperature ranges of 5 to 30°C. Based on Frisvad and Samson (2004), *P. solitum* does not produce mycotoxins, and no recent data in contraction to this report could be found. Although the fungus seems to be a weaker pathogen than *P. expansum*, it has been demonstrated that *P. solitum* could enhance the establishment of *P. expansum* (Sanderson and Spotts, 1995). Fruits were treated with a *P. expansum* inoculum in challenge inoculations of 0, 1, 7 or 28 days following initial treatment with either *P. solitum* or *P. commune*, in order to determine the competitive ability of *P. expansum* relative to these species. After incubation, *P. expansum* became established in wounds (0, 1 or 7 days) after initial treatments with *P. solitum* but not after treatment with *P. commune*. Incidence of infection was even greater after initial treatment with *P. solitum* than in water. The authors suggested that *P. solitum* may act as a predisposing agent that allows the entry of *P. expansum*, a far more destructive decay, into the wound. Initial infection by *P. solitum*, served to allow

entry of *P. expansum* into wounds otherwise protected by a wound-healing or other host-resistance response. Further research is needed to elucidate the nature of these responses. Finally, *P. solitum* has been reported to directly cause decay of pomaceous fruits and showed resistance to fungicides that are used to control growth of *P. expansum* (Pitt and Hocking, 2009).

When comparing the results of identification with the origin of the fungal isolates, most of the *Penicillium* isolates originating from mouldy apples were identified as *P. expansum*. This confirms previous research that indicates *P. expansum* as the principal cause of blue mould rot.

In contrast to the rather small differences found in the genetic profiles of the *P. expansum* group, a wide diversity in patulin production was observed between all *P. expansum* isolates and reference strains grown on APAM under classical culture conditions. Moreover, many of our *P. expansum* field isolates from apples of conventional and organic origin produced significantly more patulin on APAM than the reference strains originating from apple and grapes. This lower patulin production observed in all reference strains may be explained by fungal strain instability. Especially older, frequently used laboratory collection strains are known for degeneration in secondary metabolite production (Kale and Bennett, 1992).

Based on the phenotypic diversity observed under classical culture conditions, two strains each with high, average and low patulin production capacities were selected to investigate how each step of long-term storage may influence their patulin production. Our study showed no significant difference between the storage steps but demonstrated that patulin accumulation is mostly strain dependent. Various studies have been conducted on the effect of temperature and/or atmosphere on patulin production. Salomão et al. (2009) reported that apples incubated at 20.5°C yielded significantly higher patulin amounts than those incubated at 11°C. Welke et al. (2011) stated that the time apples are left at deck storage (25°C) after one month of cold storage (4°C) might be critical for patulin increase. This was also confirmed by Morales et al. (2007a) who observed a significant increase at the second day at 20°C after a period of storage at 1°C. A clear patulin accumulation over time was shown when apples were deck stored at the apple juice producer (Sydenham et al., 1997). Based on the QRAM developed by Baert et al. (2012), the authors concluded that the duration of deck storage is critical. In contrast, we did not find the *in vitro* simulated deck storage to be the most critical point. This may be partly explained by the inoculum size.

In our study, an inoculum density of 5×10^5-1×10^6 spores was achieved. Above mentioned studies used inoculum densities around 2-3×10^4 spores (Morales et al., 2007a; Welke et al., 2011). Baert et al. (2008) and Morales et al. (2008b) reported that a higher inoculum led to a shorter lag phase, i.e. the period between the spore reaching an apple wound and germination, of the mould. Therefore, the high inoculum size emulates a worst case scenario. This may explain the high patulin concentrations measured during the first two months of storage at 1°C under controlled atmosphere. These results imply that high spore concentrations in storage facilities may lead to a strong apple decay and patulin accumulation earlier in the process of long-term storage. This finding emphasises the importance of hygiene in the storage facilities and suggests that future control strategies may benefit from considering the duration of storage under CA conditions as well as duration of deck storage.

Our study regarding the storage effect of apples on patulin production by *P. expansum* strains was performed *in vitro* on apple simulation medium. Use of a simulation medium has the advantage that it eliminates the high natural variability present in entire apples. Baert et al. (2007a) found APAM to be a good model system to evaluate patulin production by *P. expansum* on apples, although a higher patulin production at 1°C was observed on APAM compared to apples.

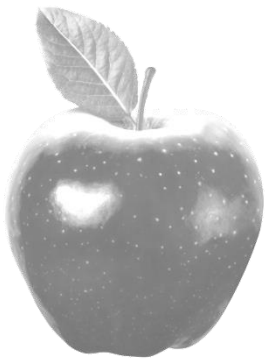
The patulin accumulation under classical and long-term storage conditions is strain dependent. However, it is worthwhile to note that the strain variability in patulin production observed at 25°C under classical conditions was different from the variability that was found during long-term storage conditions starting at 1°C. For example, isolate FC116 showed the highest patulin production at 25°C (5 days) and 20°C (3 days) but one of the lowest during long-term storage (1°C), while the opposite pattern was seen for isolate FC109. Garcia et al. (2011) obtained similar results and suggested that a strain specific adaptation to stress conditions may be a plausible explanation. On the other hand, a similar variability in the patulin production among strains assayed at different temperatures was described by Reddy et al. (2010). These inconsistencies demonstrate that a great number of isolates needs to be included when investigating the influence of specific conditions on patulin production.

Although no significant differences were found in patulin concentrations measured between the different steps of storage, two trends in patulin accumulation could be observed. The highest patulin producing isolates tend to have a peak production in patulin over time, while the others showed a slower and more continual increase in patulin over time. Both types of patulin production patterns over time have been previously described by Garcia et al. (2011). An earlier study by Damoglou et al. (1985) also described a period of patulin accumulation followed by a decrease. They suggested that patulin may be metabolised by intracellular or extracellular enzymes released by the fungus itself under a certain stress condition such as the exhaustion of carbohydrates. Our results indicate that time may exert a different influence on patulin production in long-term storage depending on the *P. expansum* strain.

5.5 CONCLUSIONS

P. expansum and *P. solitum* were the most prevalent *Penicillium* species associated with Belgian apples. The collection of *P. expansum* isolates showed a high phenotypic diversity based on their patulin production on APAM. The *in vitro* study on the effect of each successive step of long-term storage on the patulin production of different *P. expansum* strains revealed no significant differences in patulin between the different storage steps. The results suggested that a high spore inoculum may lead to a stronger patulin accumulation already during the first steps of long-term storage under controlled atmosphere at 1°C. This finding emphasises the importance of hygiene in the storage facilities and suggests that future control strategies may benefit from considering the duration of storage under CA conditions as well as duration of deck storage. Finally, strain variability is an important factor influencing patulin accumulation during long-term storage of apples.

In the previous chapter, patulin production capacity of a large collection of Penicillium expansum *isolates from Belgian apples was studied under different conditions of temperature and atmosphere. The current chapter will now focus on the effect of temperature and atmosphere on both the expression of the isoepoxydon dehydrogenase gene (*idh*), involved in patulin biosynthesis, as well as on the patulin production itself. To measure* idh *gene expression, a sensitive RT-qPCR technique was developed.*



Chapter 6

Isoepoxydon dehydrogenase (*idh*) gene expression in relation to patulin production by *Penicillium expansum* under different temperature and atmosphere

The content of this chapter is based on:

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CHAPTER 6 ISOEPOXYDON DEHYDROGENASE (*IDH*) GENE EXPRESSION IN RELATION TO PATULIN PRODUCTION BY *PENICILLIUM EXPANSUM* UNDER DIFFERENT TEMPERATURE AND ATMOSPHERE

ABSTRACT

Penicillium expansum growth and patulin production mainly occur at post-harvest stage during the long-term storage of apples. Low temperature in combination with reduced oxygen concentrations is commonly applied as a control strategy to extend their shelf life and supply the market throughout the year. Our *in vitro* study investigated the effect of temperature and atmosphere on *idh* gene expression in relation to patulin production of *P. expansum*. The *idh* gene encodes the isoepoxydon dehydrogenase enzyme, a key enzyme in the patulin biosynthesis pathway. First, a reverse transcription real-time PCR (RT-qPCR) method was optimized to measure accurately the *P. expansum idh* mRNA levels relative to the mRNA levels of three reference genes (*18S*, *β-tubulin*, *calmodulin*), taking into account important parameters such as PCR inhibition and multiple reference gene stability. Subsequently, two *P. expansum* field isolates and one reference strain were grown on Apple Puree Agar Medium (APAM) under three conditions of temperature and atmosphere: 20°C – air, 4°C – air and 4°C – controlled atmosphere (CA; 3% O_2). When *P. expansum* strains reached a 0.5 and 2.0 cm colony diameter, *idh* expression and patulin concentrations were determined by means of the developed RT-qPCR and an HPLC-UV method, respectively. The *in vitro* study showed a clear reduction in patulin production and down-regulation of the *idh* gene expression when *P. expansum* was grown under 4°C – CA. The results suggest that stress (low temperature and oxygen level) caused a delay of the fungal metabolism rather than a complete inhibition of toxin biosynthesis. A good correlation was found between *idh* gene expression and patulin production, corroborating that temperature and atmosphere affected patulin production by acting at the transcriptional level of the *idh* gene. Finally, a reliable RT-qPCR can be considered as an alternative tool to investigate the effect of control strategies on the toxin formation in food.

6.1 INTRODUCTION

P. expansum growth and patulin production mainly occur at post-harvest stage during the long-term storage of apples. Cold temperature is often applied in combination with other strategies, e.g. fungicide treatments and controlled atmosphere (CA; 1 to 3% O_2 and <1 to 3% CO_2), in order to improve the effect and extend the shelf life of apples during long-term storage (Morales et al., 2010). Various studies have been conducted on the effect of temperature and/or atmosphere on *P. expansum* growth and patulin production in apples (Baert et al., 2007a; Baert et al., 2007b; Salomão et al., 2009; Sydenham et al., 1997). Thus strategies for reducing patulin contamination and risk assessment models were developed (Baert et al., 2012). However, beside the direct effect on the *P. expansum* growth and patulin production, no information is available regarding the effect of temperature and atmosphere on the expression of genes involved in the patulin biosynthesis. The patulin biosynthesis pathway consists of about ten enzymatic steps (Puel et al., 2010). Isoepoxydon dehydrogenase, encoded by the *idh* gene, is a precursor in the late steps of the patulin biosynthesis pathway. Real-time reverse transcription polymerase chain reaction (RT-qPCR) is the method of choice for sensitive, specific and reproducible quantification of mRNA amounts transcribed by a gene (Bustin, 2000). However, the transcriptome is context-dependent, i.e. mRNA amounts vary with physiology, pathology or development, making the information contained within the transcriptome intrinsically flexible and variable. Therefore, transcriptome analysis requires successful application of RT-qPCR, considering each stage of the experimental protocol, starting with the laboratory setup and proceeding through sample acquisition, template preparation, RT, and finally the PCR step. Every one of these stages needs to be properly validated before the biological significance of the data can be reported (Bustin and Nolan, 2004; Vandesompele et al., 2002).In this chapter, the effect of temperature and atmosphere was examined on the *idh* gene expression and patulin production of *P. expansum*. The first objective was to develop a sensitive and reliable molecular technique to quantify the expression of the *idh* gene involved in the patulin biosynthesis of *P. expansum*. By means of the newly developed RT-qPCR, the *idh* mRNA levels were determined under different conditions of temperature and atmosphere. In addition, these *idh* gene expression levels were compared with patulin production, which was quantified by means of the HPLC-UV method developed in CHAPTER 4.

6.2 MATERIAL AND METHODS

6.2.1 *P. expansum* strains

P. expansum strain MUM 99.19, from Portuguese grapes, was obtained from the Centro de Engenharia Biológica da Universidade do Minho in Portugal (Braga). Based on the patulin production capacity of *P. expansum* field isolates on Apple Puree Agar Medium (APAM) under classical controlled conditions (CHAPTER 5), a medium (isolate FC094) and high (isolate FC116) patulin producing field isolate, obtained from Belgian Jonagold apples of organic orchards, were randomly selected from our laboratory culture collection at the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium).

6.2.2 Optimization of an RT-qPCR method to measure the *idh* gene expression of *P. expansum*

6.2.2.1 RNA extraction and DNase treatment

A pure culture of *P. expansum* was inoculated, by means of streaking, onto Malt Extract Agar (MEA) (Oxoid Ltd, Basingstoke, Hampshire, UK). MEA was prepared according to the manufacturer's instructions. Inoculated MEA plates were incubated at 25°C for seven days. After incubation, sporulating mycelium was one-point inoculated onto APAM of Jonagold cultivar using a sterile toothpick (§ 5.2.6). APAM was prepared as described by Baert et al. (2007a). After two days incubation at 25°C, 100 mg of fungal spores/mycelium was scraped off the surface and transferred to a 2 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany). The sample material was immediately frozen under liquid nitrogen and stored at -80°C until further analysis. When all samples had been collected, cells were disrupted under liquid nitrogen by grinding the mycelium with a pestle. The disrupted cell mixture was homogenized and RNA was extracted by means of the Invitrap® Spin Plant RNA mini kit (Stratec Molecular, Berlin, Germany) according to the manufacturer instructions. Total RNA was eluted in a final volume of 60 µl nuclease-free H_2O (Qiagen, Germantown, MD, USA).The TURBO DNA-*free*™ kit (Ambion®, Carlsbad, CA, USA) was used to remove any residual DNA. A 10x TURBO DNase buffer and 2 U TURBO DNase were added to a 200 µl centrifuge tube containing 15 µl of total RNA.

After gentle inversion, the mixture was incubated for 30 min at 37°C. The RNA mixture and 2 µl of DNase inactivation reagent were well resuspended by smooth pipetting, and incubated for 5 min at room temperature with occasional mixing. Centrifugation was conducted for 2 min at 10,000 *g*. The supernatant was transferred to a 1.5 ml microcentrifuge tube and stored at -20°C. Further analysis was conducted within seven days.

RNA samples were visually checked after staining a 1.5% (w/v) Seakem LE agarose gel (Lonza, Rockland, ME, USA) with ethidium bromide (2 mg/mL). The Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure the RNA concentration and nucleic acid purity based on the A_{260}/A_{280} absorbance ratio. A pure RNA sample should exhibit an absorbance ratio ~ 2.0.

6.2.2.2 cDNA synthesis

Prior to cDNA synthesis by means of reverse transcription (RT), a heat shock treatment was applied to the mixture of RNA and primers for sequence denaturation. In this way, the absence of secondary structures of the RNA and/or primers is assured, guaranteeing a more efficient primer annealing and cDNA synthesis. Hence, a total of 2 µl RNA and 2.5 µM random hexamers (Applied biosystems, Foster City, CA, USA) was subjected to a heat shock of 2 min at 95°C followed by 2 min cooling on ice. After the heat shock, 5 mM $MgCl_2$, 10x PCR buffer II (Applied Biosystems), 1 mM dNTP's, 40 U RNase inhibitor (Applied Biosystems) and 50 U Multiscribe reverse transcriptase (Applied Biosystems) were added to the reaction mixture obtaining a final volume of 40 µl. RT was carried out with a GeneAmp PCR System 2400 (Applied Biosystems) with the following incubation conditions: 10 min at 22°C, 15 min at 42°C, 5 min at 99°C and 5 min at 5°C. To check for residual genomic DNA (gDNA), each RNA sample was also subjected to a cDNA synthesis reaction without addition of RT-enzyme (noRT) (Werbrouck et al., 2007).

6.2.2.3 Quantitative real-time PCR

Optimization of the quantitative real-time PCR (qPCR) is essential for an accurate quantification (Werbrouck et al., 2007). *Idh* gene expression levels were quantified relative to the expression levels of three reference genes: *18S, β-tubulin* and *calmodulin*.

Primer selection

Based on the alignment of DNA sequences of different *P. expansum* strains obtained from the EMBL database, primers were designed with the aid of Primer Express® software v2.0 (Applied Biosystems) (Table 6.1). If a single-nucleotide polymorphism (SNP) was present in the selected primer-binding site, the nucleotide in the corresponding primer was replaced by an inosine. For each gene, three primer sets were designed and tested, taking into account crucial parameters such as the quantification cycle (C_q) that should be as low as possible, the slope of the standard curve ideally near to -3.3 and primer dimer formation should be absent (qBase+, 2008).

Table 6.1 Primer nucleotide sequence, annealing position and length of the DNA product amplified by each of the developed primer sets for the gene of interest *idh* and for the reference genes *18S*, *β-tubulin* and *calmodulin*.

Gene	Primer set (forward/reverse) (F/R)	F primer sequence (5' to 3')	R primer sequence (5' to 3')	5' F/R primer annealing position* (bp)	Product length (bp)
Idh[a]	RT-PCR1_F/EKRT1R	TCACCAATACiGAGTATGATGACTTC	GCGATAATCACGTCAATTCGTC	176/332	157
	EK-RT-2F/EK-RT-2R	GCAGTTTCGCGATCGATGT	GTAGGGAGTAGCCGCCTTGA	433/491	59
	EK-RT-3F/EK-RT-3R	CAAAGATCAACCCGGAATGG	TCCCAAACGCTTAAGAGGAATC	706/767	62
18S[b]	Do_18S_1_F/Do_18S_1_R	GGTCTCGTAATTGGAATGAGAACA	CGCTATTGGAGCTGGAATTACC	497/597	101
	Do_18S_2_F/Do_18S_2_R	CAGGTCCAGACAAAATAAGGATTGA	AGCAGACAAATCACTCCACCAA	1210/1310	101
	Do_18S_3_F/Do_18S_3_R	TCTGTGATGCCCTTAGATGTTCTG	GGGTTTAACAAGATTACCCAiACCT	1428/1529	102
β-tubulin[c]	BTub-1F/BTub-1R	GCCAGCGGTGACAAGGTACGT	TACCGGGCTCCAAATCGA	311/365	55
	BTub-2F/BTub-2R	GGTCCCTTCGGCAAGCTT	TGTTACCAGCACCGGACTGA	386/449	64
	BTub-3F/BTub-3R	GCAGATGTTCGACCCCAAGA	TAGCGGCCGTTACGGAAGT	426/482	57
Calmodulin[d]	Do_Cmd_1_F/Do_Cmd_1_R	CAGAACCCCTCCGAGTCTGA	TCAGTGGGTTATGGTCAAGTACCA	276/376	101
	Do_Cmd_2_F/Do_Cmd_2_R	CAACAACGGCACCATTGACT	AGCCATCATGGTGAGGAATTCT	329/429	101
	Do_Cmd_3_F/Do_Cmd_3_R	CTCACCATGATGGCTCGTAAGA	GCGGAAATGAAACCGTTGTT	415/515	101

[a] Primer sequences were designed based on the partial *idh* sequence of *P. expansum* strain NRRL 32289 (GenBank accession number DQ343639).
[b] Primer sequences were designed based on the partial *18S* sequence of *P. expansum* strain 898 (GenBank accession number DQ912698).
[c] Primer sequences were designed based on the partial *β-tubulin* sequence of *P. expansum* strains MUCL 29129 and NRRL 976 (GenBank accession numbers AY674400 and FJ457079).
[d] Primer sequences were designed based on the partial *calmodulin* sequence of *P. expansum* strain CBS 325.48 (GenBank accession number DQ911134).

qPCR assay

The 25 µl reaction-mixtures contained 2x SYBR Green PCR master mix buffer (Applied Biosystems), 600 nM of each of the appropriate gene-specific primers and 5 µl of template cDNA. Reaction mixtures were added to a white Lightcycler®480 96-well plate (Roche Diagnostics, Mannheim, Germany) and sealed using adhesive Lightcycler®480 sealing foil (Roche Diagnostics). For each sample, a quantification of the mRNA levels of the gene of interest and the reference genes towards their corresponding standard curves was performed using a Lightcycler®480 real-time PCR system (Roche Diagnostics). Thermal cycling conditions were as follows: 5 min incubation at 95°C to activate the polymerase enzyme, followed by 35 cycles of denaturation at 95°C for 10 s, and a one-step annealing and elongation during 30 s at 60°C. C_q was defined as the PCR cycle at which the sample fluorescence, generated within a reaction, exceeds a chosen threshold based on the background fluorescence.

This threshold was determined for each gene separately. The obtained C_q-values of the samples were quantified by means of a standard curve based on a dilution series of control plasmids. Control plasmids consisted of a pIDTSmart Amp vector with the specific partial gene sequence inserted. The partial sequences are based on the reference sequences and selected primers represented in Table 6.1. These constructs were ordered at Integrated DNA Technologies Inc. (Coralville, IA, USA). The four plasmids (for the *idh* gene and the three reference genes) were tenfold serial diluted, in the range of 2×10^6 to 2×10^2 DNA copies/µl, and analyzed in triplicate. Samples were tested for residual gDNA by comparing the C_q-values of RT cDNA and noRT cDNA reactions. When a ΔC_q >7 was obtained, gDNA contamination was considered negligible (Werbrouck et al., 2006). A negative control (without cDNA) was included in all PCR runs.

6.2.2.4 qPCR quality control

High RNA yields and pure RNA samples do not guarantee a cDNA synthesis and/or qPCR free of inhibition. To detect the possible presence of inhibition, RNA dilution series were tested to check linearity of standard curve. One *P. expansum* strain (isolate FC094) was middle-point inoculated onto two APAM Petri dishes (sample A and B) and incubated at 25°C until a growth stage of 0.5 cm colony diameter was achieved. RNA was extracted and subsequently DNase treated as described above.

RNA concentration and A_{260}/A_{280} absorbance ratio were determined for both samples. Sample A was twofold serial diluted in water. Each dilution was converted to cDNA and tested by means of qPCR for all four genes. Inhibition was analysed by comparing the obtained C_q-values. A twofold dilution, in the absence of inhibition and considering a 100% efficient PCR reaction, should be characterized by an increase in C_q-value (ΔC_q) ≈ 1. Based on the results obtained for the dilution series of sample A, sample B was twofold and tenfold serial diluted and tested by means of a qPCR for two of the four genes (*18S* and *calmodulin*).

6.2.2.5 Data analysis

After quantifying the expression levels of all four genes by means of their corresponding standard curves, relative expression of the gene of interest (*idh*) was determined by calculating the ratio of *idh* expression relative to the expression of the three reference genes using the qBase$^+$ software program (Biogazelle, Zwijnaarde, Belgium). First, the stability of each reference gene was evaluated by calculating two crucial parameters using the qBase$^+$ software program: the stability M-value (M) and the coefficient of variation (CV). If M <1 and CV <0.5, the gene was considered as stably expressed (Hellemans et al., 2007). Next, a normalization factor was calculated for each stable reference gene. qBase$^+$ is based on a mathematical model for normalization by geometric averaging of multiple stable reference genes, taking into account gene-specific amplification efficiencies. Calculations and algorithms are detailed in Hellemans et al. (2007).

6.2.3 *In vitro* assay

Pure cultures of two *P. expansum* isolates and one reference strain (CHAPTER 5) were grown on MEA during seven days at 25°C. Sporulating mycelium was scraped off and one-point inoculated onto APAM plates using a sterile toothpick (N = 3) (§ 5.2.6). To determine the effect of different atmospheric conditions, inoculated APAM plates were packed in polypropylene trays (307 x 240 x 50 mm; oxygen transmission rate of 192 cm^3/m^2 24h). For each strain, six trays with three APAM plates within each tray, were incubated under conditions of: a) 4°C – CA, b) 4°C – air and c) 20°C – air. To simulate CA, trays were introduced with a gas mixture of 3% O_2 – 1% CO_2 – 96% N_2 (Air Liquide, Paris, France). A PET/CPP NPAF foil with an oxygen transmission rate of 190 cm^3/m^2 24h at 25°C and 50% R.H. was used to seal the trays by means of a tray sealer (TS400, VC999 Packaging Systems, Herisau, Switzerland).

Trays with inoculated APAM plates under CA were stored at 4°C. The gas composition in the trays was checked using a headspace gas analyzer (PBI-Dansensor A/S, Ringsted, Denmark). Trays that were incubated for a longer time period (2.0 cm fungal colony diameters) were repacked after 10 days. As such, a rather stable O_2 level (3 ± 1% variation) was maintained. Trays stored at an atmospheric condition of air were sealed without the introduction of any gas mixture and stored at 4 or 20°C. Three trays, of each strain at each temperature/atmosphere condition, were collected when fungal colonies reached a 0.5 and a 2.0 cm colony diameter. For each tested condition, all strains reached the fixed diameters with a difference of maximum 24 h. More specific, a 0.5 cm colony diameter was reached after two days and 7-8 days incubation at 20°C – air and 4°C – air/CA, respectively. A 2.0 cm colony diameter was reached after five days and 20-21 days incubation at 20°C – air and 4°C – air/CA, respectively. In case of a 2.0 cm colony diameter, one half of the mould colony was used as sample material for RNA extraction and subsequent gene expression analysis by means of RT-qPCR. The other half of the APAM and mould colony was used for patulin analysis according to the HPLC-UV method described in CHAPTER 4. The method is characterized by a detection limit (LOD) of 3 µg/kg and a quantification limit (LOQ) of 5 µg/kg for patulin analysis on APAM. In the case of a 0.5 cm colony diameter, one half of the mould colony did not provide enough mycelium to guarantee successful RNA extraction. Therefore, mycelium of two extra colonies, grown on the other APAM plates in the same tray, were used for RNA extraction. Half of one APAM and mould colony was used for patulin analysis.

6.2.4 Statistical analysis

Statistical analysis was performed using the Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). Data obtained during the *in vitro* assay did not follow a normal distribution. Therefore, a non-parametric Friedman two-way analysis of variance (ANOVA) was carried out, with *idh* expression and patulin concentration as dependent variables and *P. expansum* strain and temperature/atmosphere condition (20°C – air, 4°C – air and 4°C – CA) as independent variables. Post-hoc comparison was conducted using a Scheffé test. Spearman's rank correlation (r_s) was used to test the association between the *idh* expression levels and patulin concentrations. *P* values ≤0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 Optimization and data analysis of the RT-qPCR assay

Three primer sets were designed for the gene of interest and each of the reference genes (Table 6.1). Based on the absence of primer dimers, lowest C_q-values, and a standard curve slope near -3.3, the primer sets EK-RT-2F/ EK-RT-2R (*idh*), Do_18S_3_F/ Do_18S_3_R (*18S*), BTub-2F/BTub-2R (*β-tubulin*) and Do_Cmd_3_F/ Do_Cmd_3_R (*calmodulin*) were selected. The possible presence of sample inhibition was tested by means of RNA dilution series. At a higher dilution (>1/8), inhibition was never observed. However, this was often the case at a dilution ≤1/8. Therefore, all DNase treated RNA extracts of the *in vitro* assay were $1/10^{th}$ diluted before converting to cDNA. Based on Nanodrop® ND-1000 spectrophotometer measurements, all RNA samples exhibited A_{260}/A_{280} absorbance ratios ~ 2.0, confirming their nucleic acid purity (data not shown). The cDNA was used as a template for qPCR, and gDNA contamination was tested by comparing the C_q-values of the RT and noRT cDNA samples. No amplification was detected in the noRT reactions, implying that all cDNA samples were free of gDNA. The characteristics of the qPCR standard curves, based on a dilution series (10^7-10^3 DNA copies) of control plasmids containing the specific partial sequence of the genes *idh*, *18S*, *β-tubulin* and *calmodulin*, are presented in Table 6.2. Calculated by means of the slope of the standard curves varying from -3.4 to -3.6, PCR efficiencies in the range of 91-99% were obtained. The Y-intercept of the standard curve, the C_q-value at which one DNA copy is detected, was reached around 38-41.8 C_q-values.

Table 6.2 Characteristics of the standard curves based on a dilution series (10^7-10^3 DNA copies) of control plasmids containing the specific partial sequence of the gene of interest *idh* and the reference genes *18S*, *β-tubulin* and *calmodulin*.

Gene	Primer set (forward/reverse) (F/R)	Slope standard curve	qPCR efficiency (%)	Y-intercept (C_q-value)
Idh	EK-RT-2F/EK-RT-2R	-3.5	95	38.8
18S	Do_18S_3_F/Do_18S_3_R	-3.4	99	40.3
β-tubulin	BTub-2F/BTub-2R	-3.5	95	38.0
Calmodulin	Do_Cmd_3_F/Do_Cmd_3_R	-3.6	91	41.8

The melting curves showed only one melting point, indicating that no primer dimer formation took place. The qPCR results of the amplified partial *idh* sequence obtained during the *in vitro* assay are shown in Figure 6.1. All unknown samples fall inside the range of the standard curve. No amplification was observed for the negative controls and the noRT cDNA samples.

Finally, the obtained qPCR data were analyzed by means of the qBase^{+} software program. The reference gene stability values M (CV) were: 0.69 (CV = 0.24) for *18S*, 0.78 (CV = 0.33) for *β-tubulin* and 0.63 (CV = 0.23) for *calmodulin*. Based on these M- and CV-values, all three reference genes were considered suitable internal control genes, and subsequently used for normalization.

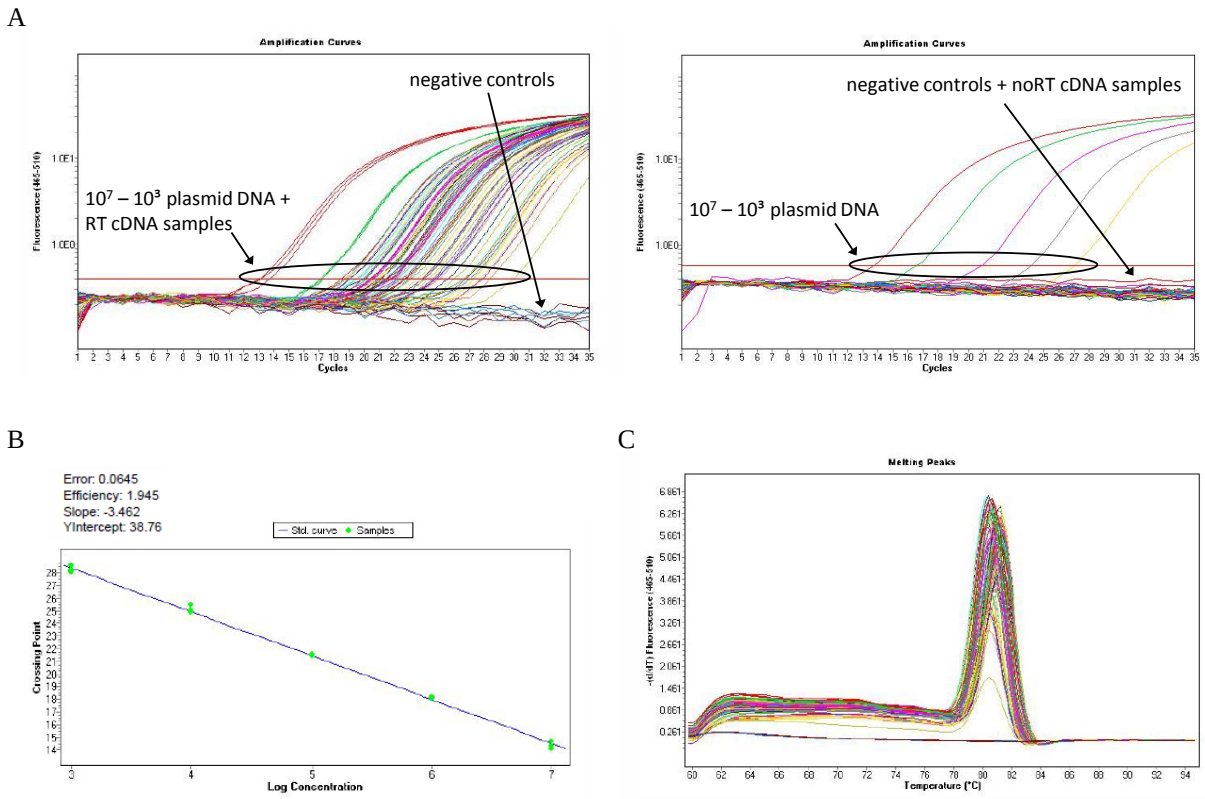

Figure 6.1 *In vitro* assay qPCR results of the partial *idh* gene fragment amplified by means of primer set EK-RT-2F/EK-RT-2R. A) Amplification curves of the dilution series of plasmid DNA in triplicate, all unknown RT cDNA samples and seven negative controls (left), and amplification curves of the dilution series of plasmid DNA, all noRT cDNA samples and three negative controls (right). B) Standard curve based on tenfold serial dilutions of plasmid DNA in the range of 10^7-10^3 DNA caopies, in triplicate. C_q-values were obtained for each dilution and plotted against known DNA copy amounts. C) The melting curves representing the change in fluorescence observed when the amplicon, bound with SYBR Green dye, dissociates as the temperature of the reaction increases. The melting point (T_m) of the amplicon, the temperature at which a peak fluorescence is followed by a sudden decrease due to the dissociation and subsequent release of the dye, occurs at ± 81°C.

6.3.2 *Idh* gene expression and patulin production of *Penicillium expansum* grown under different conditions of temperature and atmosphere

The effect of temperature and atmosphere on the *idh* gene expression and patulin production of three *P. expansum* strains, was investigated *in vitro* on APAM. Figure 6.2 showed the results of their average *idh* expression levels (A.U.) and patulin concentrations (mg/kg) measured at two growth stages (0.5 cm and 2.0 cm colony diameter, Figure 6.2 A and B, respectively) (*N* = 3).

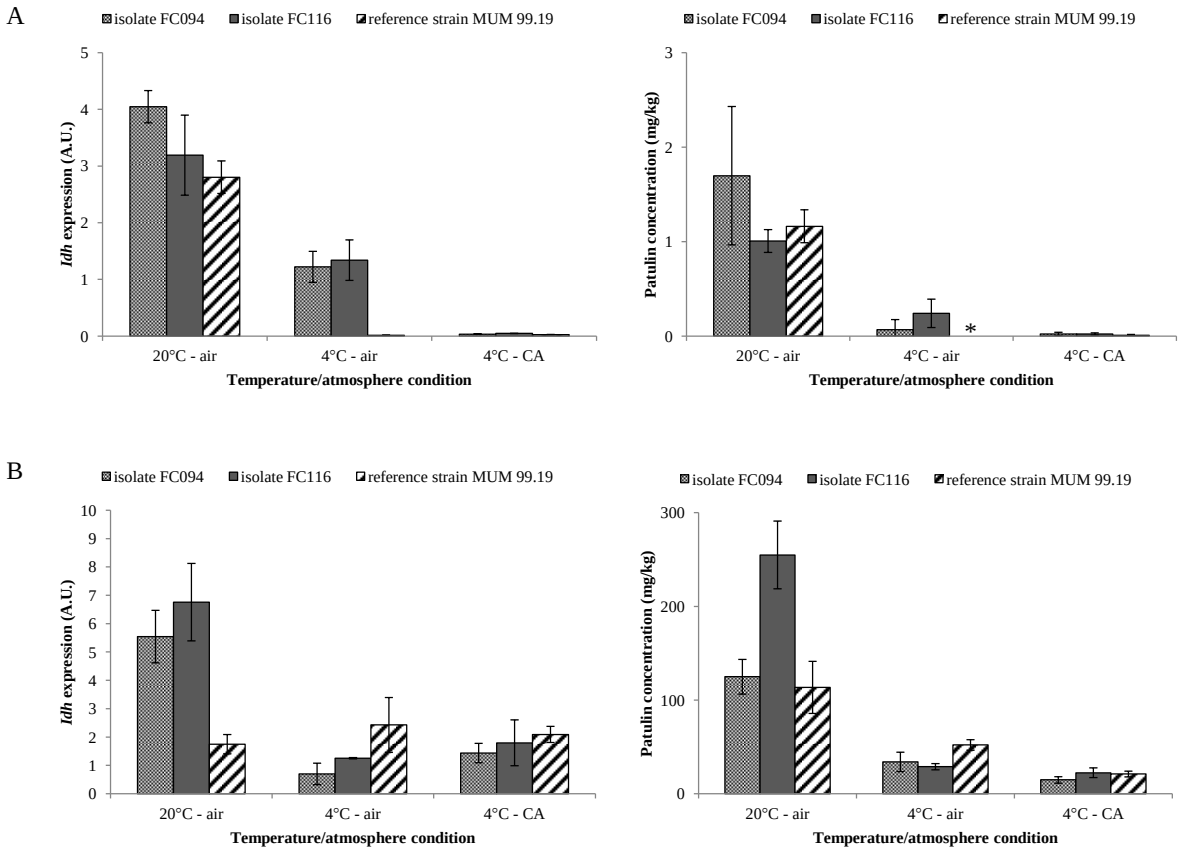

Figure 6.2 Average *idh* gene expression levels (A.U.) (left panel) and patulin concentrations (mg/kg) (right panel) of two *P. expansum* field isolates and one reference strain, grown under different temperature/atmosphere conditions (20°C-air, 4°C-air and 4°C-CA), until a growth stage of 0.5 cm (A) and 2.0 cm (B) colony diameter. *N* = 3 (except at 4°C-CA: 0.5 cm colony (FC094) and 2.0 cm colony (FC094 and MUM 99.19), *N* = 2). * Patulin concentrations below the detection limit of 3 µg/kg.

APAM plates of 0.5 cm *P. expansum* colonies (Figure 6.2 A) contained less than 50 µg/kg patulin after incubation under cold temperature combined with CA (4°C – CA). Under 4°C and 20°C – air, the detected patulin concentrations were in the range of <3 (LOD)-500 µg/kg and 800-2,500 µg/kg APAM, respectively. Colonies grown at 4°C – CA, producing less than 50 µg/kg patulin on APAM, showed very low (≤0.05 A.U.) *idh* mRNA transcript levels. All three *P. expansum* strains grown at 4°C – air expressed *idh* in a low range of 0.02-1.34 A.U., while at 20°C – air higher expression profiles in between 2.80 and 4.05 A.U. were observed. Temperature and O_2 concentration seemed to have a statistically significant effect ($p < 0.001$) on both the *idh* gene expression and the patulin production of 0.5 cm *P. expansum* colonies. In particular, the *P. expansum* strains grown at 20°C – air showed significantly higher *idh* expression levels and produced significantly more patulin than those grown at cold temperature (4°C – air) and cold temperature combined with CA (4°C – CA). No statistically significant differences ($p > 0.05$) in *idh* expression and patulin production were observed between the different *P. expansum* strains within each temperature/atmosphere condition.

The patulin concentrations of 2.0 cm large *P. expansum* colonies grown at 4°C – CA and 4°C – air, accumulated to ranges of 10,000-30,000 µg/kg and 20,000-60,000 µg/kg APAM, respectively (Figure 6.2 B). Incubation of inoculated APAM plates at 20°C – air led to an increase of patulin to a range of 80,000-300,000 µg/kg. Again, low *idh* mRNA transcript levels in ranges of 1.44-2.09 A.U. and 0.70-2.43 A.U. were measured at 4°C – CA and 4°C – air, respectively. At 20°C – air, higher average *idh* expression profiles in between 1.75 and 6.76 A.U. were observed. As also observed for 0.5 cm *P. expansum* colonies, the temperature and O_2 concentration continued to have a statistically significant effect ($p < 0.05$) on the *idh* gene expression and patulin production of the 2.0 cm large colonies. On average, significantly different concentrations of patulin were produced under all three temperature/atmosphere conditions. No statistical differences in *idh* gene expression profiles were observed. In particular, the 2.0 cm large colonies of *P. expansum* reference strain MUM 99.19 showed similar *idh* mRNA amounts under all three temperature/atmosphere conditions (1.80-2.55 A.U.). Those amounts were more or less equal to those found when the strain reached 0.5 cm growth at 20°C – air (2.97 A.U.), but much higher than the 0.5 cm colony incubated at 4°C – air (0.01 A.U.) and 4°C – CA (0.02 A.U.).

The graphs in Figure 6.3 represent the correlation between all measured *idh* expression levels and patulin production data of 0.5 cm and 2.0 cm large *P. expansum* colonies. The two parameters were positively correlated ($p < 0.001$) at both growth stages. Based on the Spearman correlation coefficient, a high correlation ($r_s = 0.89$) was observed between the *idh* expression and patulin production at the start of *P. expansum* growth (0.5 cm colony diameter).

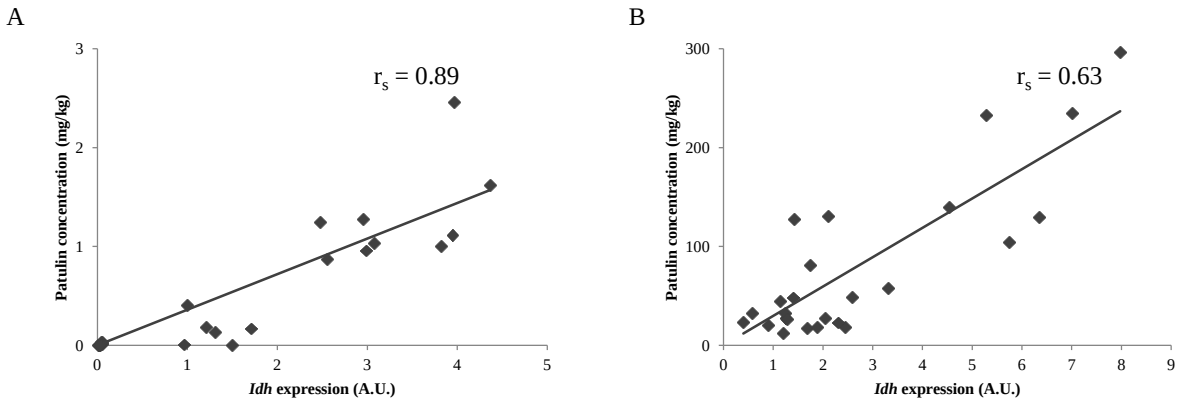

Figure 6.3 Correlation between *idh* gene expression levels (A.U.) and patulin concentrations (mg/kg) of *P. expansum* strains, grown under different temperature/atmosphere conditions, until a growth stage of 0.5 cm (A) and 2.0 cm (B) colony diameter ($N = 3$). r_s = Spearman's rank correlation coefficient.

6.4 DISCUSSION

Real-time reverse transcription PCR is a molecular technique that makes it possible to quantify fungi in environmental samples and to study host-pathogen interactions and changes in gene expression in response to certain treatments (Schena et al., 2004). However, as its application has become the method of choice for quantitatively assessing steady-state mRNA levels, it has become clear that the reliability to detect transcriptional differences between samples is affected by several factors. Therefore, the gene expression assay in the *in vitro* study was developed with great care and important variables such as gDNA contamination, primer dimer formation, PCR inhibition, sensitivity and efficiency, as well as multiple reference gene stability were taken into account.

After selecting gene-specific primer sets with high sensitive and efficient PCR reactions in the absence of primer dimer formation, the presence of a certain inhibition during the reverse transcription and/or real-time PCR step was noted. PCR inhibitors may originate from the sample matrix or may be introduced during processing or nucleic acid extraction. Their presence is a major drawback of the PCR as it may decrease sensitivity or give false-negative results. A general method to remove PCR inhibitors is the dilution of the sample or extracted nucleic acid (Schrader et al., 2012). In our study, a 1/10 dilution of the RNA was sufficient to minimize the PCR inhibition effect and was applied to all DNase treated RNA samples before conversion to cDNA.

Most studies regarding the expression kinetics of fungal genes involved in mycotoxin biosynthesis were quantified relative to only one reference gene (Doohan et al., 1999; Jiao et al., 2008; Mayer et al., 2003; Sanzani et al., 2009; Sweeney et al., 2000). According to the studies of Thellin et al. (1999) and Vandesompele et al. (2002), at least two or three stable reference genes should be used as internal controls to calculate a normalization factor, as the conventional use of a single gene leads to relatively large errors. Therefore, in our study, the three genes *18S*, *β-tubulin* and *calmodulin* were chosen as internal controls. The qBase^{+} software measures the gene stability and pairwise variation of the selected reference genes, based on the principle that the expression ratio of two ideal internal control genes is identical in all samples. Subsequently, the program calculates the normalization factor by means of the geometric mean of the multiple stable reference genes (Vandesompele et al., 2002). This normalization strategy was applied as it has been proposed as a prerequisite for accurate RT-qPCR expression profiling.

Overall, the results of our *in vitro* study demonstrate that the temperature and atmosphere affect the patulin production of *P. expansum* on APAM, by acting at the transcriptional level of the *idh* gene. A good correlation was found between *idh* expression and patulin production of *P. expansum* strains under the different temperature/atmosphere conditions. Especially at small contamination levels (low *P. expansum* colony diameters), the developed RT-qPCR may be an interesting alternative approach to detect whether certain patulin amounts are present or not.

A lower patulin production and a down-regulation of the *idh* gene was observed when inoculated APAM plates were stored under a combination of cold temperature and controlled atmosphere. These observations confirm the results of the study of Baert et al. (2007a) who proved that a combination of different stress conditions (e.g. low temperature and low oxygen) will result in a reduced formation of patulin. It has to be noted that 4°C – CA resulted in a very low expression of the *idh* gene at the start of colony growth (0.5 cm), while this additional reducing effect of controlled atmosphere at the *idh* mRNA expression level disappeared once a larger growth stage (2.0 cm) was achieved. These results suggest that this combination of low temperature and CA causes a delay of the fungal metabolism rather than a complete inhibition of the toxin biosynthesis. In our study, the highest *idh* mRNA levels and patulin amounts were observed at 20°C – air. Other authors reported independently that the duration of apples stored at ambient temperature (20°C – air) is critical for both lesion diameter and patulin accumulation (Baert et al., 2012; Morales et al., 2007a; Sydenham et al., 1997; Welke et al., 2011). Those studies investigated the direct effect of storage on the *P. expansum* growth and patulin production but no information was available regarding the underlying molecular genetics. Another *in vitro* study by Sanzani et al. (2009) provided evidence that the combined application of the two phenolic compounds quercetin and umbelliferone, reduced patulin accumulation by acting on the transcript level of five genes, among which *idh*, involved in the patulin biosynthesis.

Interestingly, in our study, the 2.0 cm large colonies of reference strain MUM 99.19 showed a similar expression of the *idh* gene under all three temperature/atmosphere conditions. The extent of expression was comparable to the one found when the strain reached 0.5 cm under 20°C – air, but much higher than the 0.5 cm growth obtained under stress conditions (4°C; CA). This differential expression might be due to the fact that the host of origin of the reference strain was grapes instead of apples. Sanzani et al. (2013) investigated the influence of the origin on *P. expansum* pathogenicity/virulence and found that the *P. expansum* strains produced more patulin when grown on the host from which they originated. However, this was not found in our study as the patulin amounts of all three strains were more or less similar within each temperature/atmosphere condition or the difference was rather strain dependent than origin dependent.

In fact, our results suggest that the effect of certain stress (cold temperature and/or reduced oxygen) on the *idh* expression and patulin production is more pronounced in the reference strain in comparison with the field isolates at the early stage of growth (0.5 cm). However, once the strain reaches a later growth stage (2.0 cm), this difference in effect was not observed.

The use of RT-qPCR to monitor gene expression related to mycotoxin biosynthesis has been described for other fungal species as well. Sweeney et al. (2000) monitored the mRNA levels of two genes involved in the aflatoxin biosynthesis pathway of *Aspergillus parasiticus* grown in aflatoxin permissive and non-permissive media. Transcription of both genes was observed in cultures grown in Yeast Extract Sucrose (YES) but was absent in Yeast Extract Peptone (YEP) which did not support aflatoxin production. Mayer et al. (2003) quantified the *nor-1* aflatoxin biosynthetic gene transcription in parallel with the aflatoxin B_1 production of *Aspergillus flavus* in wheat samples. Two days after the first *nor-1* mRNA amounts were measured by means of RT-qPCR, aflatoxin B_1 could be detected by means of HPLC-UV. Doohan et al. (1999) developed an RT-PCR to quantify *Tri5* gene expression of *Fusarium culmorum* under *in vitro* conditions, and found that the *Tri5* expression was directly correlated with an increase in deoxynivalenol production over time. The levels of *Tri5* expression, and, therefore, the levels of trichothecene production appeared to be host plant tissue dependent and were significantly influenced after a fungicide treatment. Jiao et al. (2008) investigated the effect of different carbon sources on the trichothecene production and on the trichothecene induction mechanisms of *Fusarium graminearum*, a disease agent of cereal crops. The authors suggested that *F. graminearum* recognizes the sucrose molecules, activates *Tri* gene expression, and induces thrichothecene production.

Our study and the studies mentioned above all describe a certain relationship between the expression of genes involved in the mycotoxin biosynthesis and the toxin formation itself in response to parameters influencing the metabolism of the fungal species. An accurate and specific RT-qPCR method to measure the expression of genes involved in mycotoxin biosynthesis is an alternative tool to investigate the effect of potential new control strategies on the toxin formation in food.

6.5 CONCLUSIONS

Until now, studies regarding the effect of temperature and atmosphere on patulin contamination of apples were focused directly on the *P. expansum* growth and patulin production itself. This chapter investigated the effect of temperature and atmosphere on the *idh* gene expression in relation to the patulin production of *P. expansum* strains. Although RT-qPCR has become the method of choice for quantitatively assessing the mRNA levels transcribed by genes, transcription analysis poses some challenges that still need to be considered. Therefore, the gene expression assay was developed with great care and important variables, such as reverse transcription and/or real-time PCR inhibition and multiple reference gene stability, were taken into account. A good correlation, especially at low colony diameter, was found between the *idh* expression and patulin production data. Our results indicate that temperature and atmosphere affect the patulin production by acting at the transcriptional level of the *idh* gene. Moreover, a clear reduction in patulin production and a down-regulation of the *idh* gene expression were observed under stress (combination of low temperature and controlled atmosphere). These findings suggest that this stress condition causes a delay of the fungal metabolism rather than a complete inhibition of the toxin biosynthesis. Finally, a reliable RT-qPCR can be considered as an alternative tool to investigate the effect of control strategies on the toxin formation in food.

Chapter 7

General discussion and perspectives

CHAPTER 7 GENERAL DISCUSSION AND PERSPECTIVES

Different foods have different fungal problems that need to be addressed

In the food industry, the intentional (purposeful) application of moulds for the manufacture of desirable food products presumably predates written historical records. Unfortunately, there are two sides to every coin and it is no different for the role of moulds in foods. Mould growth is in many cases associated with spoilage of food and feed commodities and in certain cases with mycotoxin contamination. Mould and mycotoxin contamination pose a major obstacle on the way towards more sustainable food products and increased international trade. Fungal growth negatively affects the microbiological stability and consequently shelf life of the product. Moreover, it may lead to important economic and environmental losses due to the disposal of contaminated food and feed. In addition, mycotoxins significantly impact human and animal health through loss of human and animal life, increased disability-adjusted life years (DALYs) and veterinary costs, and reduced live stock production. For these reasons, the presence of fungi in food products and feedstuffs is undesirable.

A comprehensive view on typical fungal problems that occur in foods and beverages is not easily defined. Each product however has its own mycobiota (fungal flora) that may cause these problems. Shortly, one might say "certain products have certain fungi". *P. expansum* for example does not typically grow on citrus fruit, it grows on pomaceous fruit, especially apples. One way of describing these problems is by categorising the associated mycobiota as follows: (1) mycotoxigenic moulds, e.g. *Fusarium* species producing fusarium-toxins in corn, (2) xerophilic moulds, e.g. *E. herbariorum* on cake, (3) heat-resistant moulds, e.g. *Byssochlamys* species in non-transparent sportdrink bottles, and (4) preservative-resistant moulds, e.g. *Trichoderma* species on margarine. In this PhD, the first case of chocolate confectionery (CHAPTER 2 and CHAPTER 3) and the second case of apples (CHAPTER 4, CHAPTER 5 and CHAPTER 6) studied the problems posed by the category of xerophilic moulds and mycotoxigenic moulds, respectively.

During the course of this PhD, several food safety alerts concerning our cases popped up. In 2012 for example, the Minnesota Department of Agriculture (MDA) and Pepin Heights Orchards advised consumers to discard apple cider after routine surveillance confirmed the product was affected with patulin levels exceeding the FDA regulatory limits (Food Poison Journal, 2012). In 2013, biological apple juice and biological cranberry juice had to be withdrawn from American supermarkets for the same reason (VMT Voedselveiligheid, 2013). And only last year, government controls found considerable exceedance (65 and 147 µg/kg) of the limits for patulin in apple juices originating from Belgium and The Netherlands (VMT Voedselveiligheid, 2015b). Next to recalls of all sorts of confectionery and nuts infested with high concentrations of non-pathogenic moulds, border rejections of nuts contaminated with aflatoxins that exceed regulatory limits are an almost daily issue. In Europe alone, 143,000 tons of chocolate confectionery products are discarded each year because of physical-chemical and microbiological spoilage, summing up to an annual cost of 1.2 billion euro for the chocolate industry (Svanberg, 2012).

It is therefore unquestionable that mould spoilage of chocolate confectionery products and patulin contamination of apples have important economic implications and still pose problems that need to be addressed. Within the general scope of this thesis, focus is put on optimizing and developing methodologies to detect, identify and characterise the associated mycobiota of chocolate confectionery and apples. As such, a polyphasic or multidisciplinary research approach was applied which formed the basis to acquire more knowledge that will contribute in generating solutions.

Culture-based methods, a solid foundation for identifying fungi in foods

For starters, culture-based methods were applied to study the associated mycobiota of chocolate confectionery and apples. Culture-based methods are very useful for qualitative (e.g. identification) as well as quantitative (e.g. enumeration) measurements, when adhering to the context-dependent restrictions. In fact, studying the presence of fungi in food and feed commodities still largely relies on the traditional subsequent detection, isolation, purification, and macro-/microscopic identification on laboratory media.

Unfortunately, a single all-purpose medium does not exist since food and feed matrices have very different characteristics (a_w, pH, nutrient status, etc.). Consequently, the first and utmost important issue is the selection of suitable media representative for the food of interest and their associated mycobiota. In the first case of this thesis, MEA, DG18 and MY50G medium were used for the detection, enumeration, isolation and/or morphological identification of xerophilic fungi from (1) air of the production environments of Belgian chocolate confectionery factories, (2) common ingredients of chocolate confectionery fillings (CHAPTER 2), and (3) nuts and their corresponding nut-based confectionery fillings (CHAPTER 3). In the second case, MEA, CYA, CREA and YES medium were used for the detection, isolation and/or morphological identification of fungal isolates, e.g. *Penicillium* spp., on Belgian apples (CHAPTER 5). Choices of culture media were carefully thought through and based on solid literature data and recommendations by Samson et al. (2010) and Pitt and Hocking (2009). However, sampling size needs to be feasible which puts a restriction on the amount of different culture media that can be included. Hence possibly certain species will not be isolated/detected or will be outcompeted by other faster-growing ones. For example, literature has reported isolation of the extreme xerophilic species *X. bisporus* from chocolate, fruit cakes and cookies, and suggests it to be probably much more widespread than current data point out (CBS, 2007; Pitt and Hocking, 2009). The species is very restrictive in growth, which could explain why it was not identified during our survey in the chocolate confectionery industry. So, it has to be noted that selection of cultivation media and incubation conditions (temperature and time) will have had its influence on the fungal biodiversity reported in this doctoral thesis.

Besides the drawback posed by laboratory media, conventional plating is also limited in the amounts of CFU countable, and it is relatively slow since it may take two to three weeks for certain fungi, especially xerophiles (e.g. *X. bisporus*), to grow. Morphological identification to species level requires a great deal of mycological expertise. Consequently, morphological plate identification to genus level followed by species identification through gene sequencing, based on a fit for purpose locus, is a good objective approach for obtaining reliable data regarding the fungal mycobiota of foods. Nowadays, molecular identification by means of outsourcing the gene sequencing part is quite cost-effective and worth considering if sample size is feasible.

In CHAPTER 5, RAPD was used to cluster the isolates to reduce the number of gene sequencing reactions for species identification of *Penicillium* spp. isolates from Belgian apples. In the future, when taking into account cost-effectiveness, a fungal collection as such could be immediately outsourced for gene sequencing.

Important to be mentioned is the fact that with the culture-based approach, only fungal cells that are able to grow on the selected medium are detected, this in contrast to emerging culture-independent methods, e.g. targeted metagenomics. It is based on high-throughput sequencing (HTS) and bioinformatics, which allows full characterisation of the total microbiota (i.e. thousands of microorganisms), and it highlights the presence of not well-known or yet undescribed organisms, in one single sample. In particular, it enables a profoundly detailed, semi-quantitative analysis of the diversity of microbial communities in large sample sets. On the downside, targeted metagenomics is only semi-quantitative and mostly does not allow identification to species level. Moreover, these methods involve a large series of steps from sampling, via laboratory handling to bioinformatics, each going hand in hand with some challenges and pitfalls (e.g. repeated sampling of single individuals, advanced data analysis, etc.) that need to be considered as well (Lindahl et al., 2013).

At present, HTS methods have been considerably used as a research tool in studying diversity of bacterial communities (based upon 16S rRNA) in a wide range of environmental samples of water, soil, human gut, food, etc. (Ercolini, 2013; Golebiewski et al., 2014; Maccaferri et al., 2011; Yergeau et al., 2012). However, the last years, these methods are taking over in fungal biodiversity studies as well. In particular, it has been used as primary tool to assess fungal communities (based upon 18S rRNA and ITS) inhabiting soil and associated with plants, since these are greatly subjected to temporal variations in response to local weather events or cyclic in relation to seasons and host plants phenology (Anderson et al., 2003; Hunt et al., 2004).

In contrast to culture-based techniques, targeted metagenomics detects living as well as dead microorganisms. In our case of studying xerophilic spoilage mould diversity, it is possibly more relevant to focus only on the living organisms, as these are the ones that will be able to grow out and spoil the particular substrate. On the other hand, investigation on health implications by fungal organisms in indoor environments of hospital setting, public buildings, houses, etc., may be favoured by focusing on both living and dead organisms as the latter ones can also give allergic reactions.

In conclusion, the metagenomics approach could be interesting for a more in-depth study on the mycobiota of chocolate confectionery. These data could be compared with our results obtained by means of conventional plating. As such, the relevance and suitability for potential application, for example routine air surveillance system, in this food sector could be evaluated.

A case of xerophilic moulds as spoilers of chocolate confectionery: sources of contamination and possible solutions

Legislation/guidelines

Legislation in Belgium implemented the regulation (EC) No 178/2002 of the European Union through the Royal Decision of 14th November 2003 (KB-14/11/2003) regarding self-checking (autocontrol), compulsory notification and traceability in the food chain (Belgisch Staatsblad, 2003). KB-14/11/2003 states that it is mandatory for all business operators with activities in the food chain to introduce, implement and sustain a self-checking system to ensure the safety of its products. This self-checking system should include good hygiene practices (GHPs) and good manufacturing practices (GMPs), which constitute the foundation of Hazard Analysis – Critical Control Point (HACCP). Good hygiene practices comprise the conditions and measures necessary to ensure the safety and suitability of food at all stages of the food chain (FAO/WHO, 2007).

On behalf of the biscuit, chocolate, pralines and confectionery industry as well as the industry of breakfast cereals, The Belgian association Choprabisco developed an "Autocontrol guide" to help the companies implement KB-14/11/2003 on autocontrol, obliged notification and traceability. Their most important objective is to create a summary document containing all legal requirements on the one side and to share a valuable tool on the other. In addition, the "Code of Hygienic Practice for Low-Moisture Foods", addresses GMPs and GHPs that will help control microbial hazards associated with all stages of the manufacturing of low-moisture foods including dried fruits and vegetables, cereals, dry protein products (e.g. dried dairy products), confections (e.g. chocolate), snacks (e.g. chips), nuts, nut pastes (e.g. peanut butter), seeds for consumption, as well as spices and dried aromatic plants (e.g. teas) (Codex alimentarius, 2013).

All together, these documents provide a good base of information for each chocolate confectionery company to develop, implement and sustain its own solid and functional self-checking system. As mentioned earlier, a self-checking system is in the first place mandatory to ensure food safety, i.e. effective regulatory limits for certain pathogens and contaminants. However, it goes without saying that good GMP and GHP substantially impact food quality as well.

Air quality, an indicator for fungal contamination problems

Based on "prevention rather than cure", CHAPTER 2 and CHAPTER 3 of this doctoral thesis focused on exploring initial potential sources of fungal contamination (e.g. factory air and ingredients) that may impede quality and shelf life of the final products. CHAPTER 2 demonstrated that rather low levels of fungal spores were isolated from the air of chocolate confectionery factories. More specific, a general fungal spore load in the range of 50-250 CFU/m^3 air was detected using DG18 medium in combination with an RCS Air Sampler operating for 8 min at a flow rate of 40 L/min.

So far, no real (quantitative) standards exist to estimate fungal contamination in relation to hygienic or health implications in food plants, agricultural barns, health care facilities, building offices, houses etc. CHAPTER 2 reports the first data regarding fungal loads present in the air of the production environment of chocolate confectionery companies. Worthwhile noticing is that one chocolate confectionery factory clearly showed lower concentrations of fungal spores compared to the other factories studied. The latter could be explained by the application of different manufacturing and hygienic practices.

Therefore, a first recommendation would be to develop and validate GHP and GMP guidelines that are used similarly throughout the whole sector. National workshops on personal hygiene and technical trainings, specific to each sector in the food industry, could be very helpful for plant workers to raise awareness. Once these are established, our method could be implemented as a diagnostic tool to regularly monitor xerophilic mould levels in the factory air of the chocolate confectionery industry. Finally, it would be interesting to adjust autocontrol guide documents with earlier described validated GMP guidelines, GHP trainings and an air sampling method as routine air quality monitoring system.

This could help the industry to rapidly detect indoor air quality problems caused by fungi and to react accordingly by eliminating conditions that promote amplification of these potentially hazardous organisms e.g. dampness, humid spots, ventilation/air movement, and manipulation of potentially contaminated ingredients in the production environment.

Remediation of fungal hazards may involve thoroughly cleaning affected areas (e.g. floor and wall), decontaminating the Heating, Ventilation and Air Conditioning (HVAC) systems, removing contaminated materials (e.g. ingredients), repairing or replacing damaged materials or structures, and modifying the environmental conditions in the affected area (e.g. air filters) (Federal-Provincial Advisory Committee on Environmental and Occupational Health, 1995).

Thermal humid treatment of walnuts, a possible solution

Our research concerning the fungal contamination of commonly used ingredients of chocolate confectionery fillings pointed out that nuts, and especially walnuts, were highly contaminated and seemed to represent a potential contamination source (CHAPTER 2 and CHAPTER 3). For that reason, decontamination of the initial fungal spore load on walnuts was investigated in CHAPTER 3.

Microbiological decontamination of food can be accomplished by means of a variety of methods including thermal decontamination methods (e.g. steam and hot air) chemical decontamination methods (e.g. chlorine dioxide) and non-chemical and non-thermal decontamination methods (e.g. high hydrostatic pressure and irradiation) (Demirci and Ngadi, 2012). However, food regulations in the European Union are strict. Foods or ingredients of foods may or may not be treated with certain methods, others need to be labelled as such in order to allow consumers to make an informed choice or others may fall within the novel food legislation No 2015/2283 (Commission Regulation (EC), 2015). Historically, thermal treatments have been the most common means of food decontamination, either through sterilisation or pasteurisation. Such treatments do not pose significant health risks and, as a result, are appealing to consumers. However, they affect important properties of the food product such as sensorial quality, texture, oxidative stability, etc. Moreover, heat-resistance of microorganisms increases in food products of low a_w or with a high lipid content, e.g. walnuts (Goepfert and Biggie, 1968; Laroche et al., 2005).

Against that background, CHAPTER 3 evaluated dry and humid heating (with varying heating and drying cycle times) as fungal decontamination strategy on the base of their effect on microbiological load, sensorial quality, and oxidative stability of walnuts. Taking these factors into account, a humid thermal treatment of 3 min at 75-80°C, followed by a drying cycle for 15 min at 60°C is proposed as a possible solution to decontaminate walnuts.

However, nuts are not only dealing with spoilage problems due to mould contamination, they are also facing problems that concern food safety (e.g. mycotoxins and pathogenic bacteria). During the last decade for example, different types of nuts, e.g. macadamia nuts, walnuts and hazelnuts have been reported to contain worrying levels of *Salmonella* contamination. At the moment, both FDA and EFSA are conducting risk analysis on nuts. Anticipating the outcome, hazelnut farmers in Oregon (USA) already took action by implementing a steam pasteurization process achieving a 5 $\log_{10}$ CFU reduction of *Salmonella* and other pathogenic bacteria (VMT Voedselveiligheid, 2015a). A study on the effect of hot water blanching on *Salmonella* spp. on almonds for example, observed that a minimum time/temperature of 3.09 min at 82.2°C, 2.49 min at 85°C and 2.00 min at 87.8°C was necessary to obtain a 5-log reduction in *Salmonella* (GMA, 2010). These findings together with our results obtained in CHAPTER 3 suggest one single humid thermal decontamination strategy could be beneficial for both minimizing/eliminating initial fungal contamination involved in spoilage and pathogenic bacterial contamination of importance for its health implications.

All together, the quality and safety of nuts is of great concern for the different steps in the food and feed supply chain (e.g. harvest, storage and processing industry), as well as for animal and consumer health. Today even more than ever with the emerging rise of the so called "superfoods" and the shift and awareness of our society towards a 'healthier' life style. In conclusion, good manufacturing and hygienic practices from farm to fork is an important issue that needs to be further dealt with in the near future. A mandatory decontamination of all types of nuts is a prevention strategy worthwhile considering. CHAPTER 3 of this doctoral thesis presents a method for the fungal decontamination of walnuts. After validation, this method could be used as a control strategy and should therefore be implemented in the food/feed supply chain at the stage of final storage prior to entering the market or processing industries. Last but not least, this humid thermal treatment also led to an increased oxidative stability of walnuts. This may be translated in a possible delayed onset of rancidity during storage, which is an additional advantage in terms of product shelf life.

A case of **P. expansum** *as patulin producer on apples*

Why do fungi produce mycotoxins?

Mycotoxin biosynthesis is influenced by a variety of extrinsic and intrinsic factors, e.g. temperature, a_w, pH, and nutrient status, and by a network of interactions between these factors. For example, the effect of pH on toxin biosynthesis is also influenced by the composition of the growth media (Calvo et al., 2002). In addition, fungi react to light in various ways. Although the precise relationship still needs to be established, it has been demonstrated that light initiates considerable adaptations in metabolic pathways (e.g. fatty acid metabolism) and in the regulation of production of secondary metabolites (Tisch and Schmoll, 2010). Moreover, plant volatile organic compounds, such as methyl salicylate and oxylipins, trigger sporulation (Hountondji et al., 2006) and modulate mycotoxin biosynthesis in pathogenic fungi (Gao and Kolomiets, 2009). Consequently, it is evident that we can, as yet, explain only specific cases and specific interactions rather than the complexity and diversity of the phenomenon as a whole (Reverberi et al., 2010).

Several factors (e.g. temperature, pH and fruit variety) have been suggested to affect patulin accumulation by *P. expansum*. Analysis of patulin accumulation in different apple cultivars demonstrated that patulin accumulation was highly cultivar-dependent (Snini et al., 2016). Storage conditions (i.e. temperature and O_2 level), carbon, nitrogen and pH have also been evaluated as possible inducers of patulin accumulation. In fact, studies indicated that a low pH (around 3.5-5.5), or acidic content, might significantly induce patulin accumulation. For example, differences in apple acidity affect patulin accumulation, with higher patulin accumulation in more acidic apples (Marín et al., 2006; Zong et al., 2015). Zong et al. (2015) also found that glucose-containing sugars and complex nitrogen sources were favourable conditions for patulin production. Baert et al. (2007) found patulin production to be stimulated when temperature decreased from 20 to 4°C, while a further decrease of the temperature to 1°C caused a reduction in patulin production. The temperature at which the stimulation changed into suppression was strain dependent. Similar results were observed for the O_2 level. A reduction in O_2 level from 20 to 3% stimulated or suppressed patulin production depending on the strain, while a clear decrease in patulin production was observed when O_2 level was further reduced from 3 to 1%. Their results showed that the induction of limited stress to the fungus, such as lowering the temperature or the O_2 level, stimulates patulin production.

However, a combination of different stress conditions (e.g. low temperature and O_2 level) results in a reduced formation of the toxin. Altogether, these studies indicate that patulin accumulation in apples can be affected by environmental, host, and fungal factors including: (1) fungal isolates of *P. expansum*, (2) fruit cultivar and pH, and (3) storage conditions (Barad et al., 2016).

But this still does not answer the question why fungi synthesize mycotoxins? Their role is not always clear and still remains to be elucidated. Mycotoxins may provide ecological advantages. The most commonly held idea is that mycotoxin-producing fungi are better protected against other organisms sharing the same trophic niche (Fox and Howlett, 2008). An example is the inhibitory effect mycotoxins exert on the quorum-sensing system of bacteria. Patulin has been found to be a potent quorum sensing inhibitor (Rasmussen et al., 2005). Another study on ochratoxin A in relation to plant growth reported a clear phytotoxic effect, by inducing an evident oxidative burst in the leaves with an increase of ROS and concomitant down-regulation of antioxidant defense enzymes (Peng et al., 2010; Ponts, 2015). Conclusively, it can be stated that mycotoxins can act as pathogenicity factors or as virulence factors (Hof, 2007).

Supported by the fact that the importance of mycotoxins in plant pathogenesis has been described previously for other diseases, some studies have focused on the role of patulin in the pathogenicity of *P. expansum*. Sanzani et al. (2012) disrupted the *patK* gene, encoding the biosynthetic enzyme 6MSAS, involved in the first step of patulin biosynthesis. The authors found that disease incidence and severity were lower in Golden Delicious apples inoculated with the mutant strain than in apples inoculated with the wild-type strain. Barad et al. (2014) that generated *idh*-RNAi mutants exhibiting a down-regulation of *idh*, observed similarly a reduction in disease incidence and severity. However, mutants in both studies still produced some patulin, making it more difficult to draw definitive conclusions. In contrast to previous studies, Ballester et al. (2015) and Li et al. (2015) demonstrated, by using *patK* and *patL* knockout mutants, that patulin is not required to infect Golden Delicious and Fuji apples, respectively. However, a very recent publication by Snini et al. (2016) found a different effect. The authors made a deletion of *patL*, encoding the specific regulatory transcription factor of patulin biosynthesis, and studied pathogenicity by inoculating the mutant and wild-type strain on several apple cultivars, followed by a 14 days incubation period.

During the first 4 days, the mutant, which did not produce patulin in the apple, induced disease in a manner similar to the wild-type strain. Then, a clear decrease in the rate of rot progression was observed for the mutant compared with the wild-type strain. These results clearly demonstrated that patulin acts as an aggressiveness factor (disease severity) that contributes to the colonisation of most apple cultivars. The results regarding the cultivar-dependent aggressiveness suggest that the composition of apples (sugars, organic acids, phenolic compounds) and oxidant ability of the host in general may greatly influence the development of the disease. The authors suggested that the differential results obtained by Ballester et al. (2015) and Li et al. (2015) are due to the short incubation period and the apple cultivars used.

It goes without saying that continuous research in this area is necessary as a full understanding of the role and underlying mechanisms of toxigenesis is still incomplete. A profound knowledge on the mechanisms underlying the biosynthesis of mycotoxins will contribute to defining/identifying new strategies to tackle mycotoxin contamination problems. In order to study these mechanisms, reliable techniques are necessary.

A chemical and molecular approach to characterise patulin production by *P. expansum* in a fast and sensitive way

Besides culture-based methods, an analytical and molecular method were developed to further elucidate characteristics of patulin producing *P. expansum* on Apple Puree Agar Medium (APAM).

APAM has become the reference medium in most *in vitro* experiments studying the molecular genetics of patulin biosynthesis. CHAPTER 4 of this doctoral thesis reports the first HPLC-UV method, optimised and validated for the high-throughput analysis of patulin in *in vitro* experiments on APAM. The optimized method was subsequently validated for commercial cloudy apple juice and apple puree. As a result, the analytical method documented in this thesis is a useful tool in future research on the underlying mechanisms of patulin biosynthesis on APAM, and as routine surveillance method for the apple industry. It has to be noted that APAM is a simulation medium for apples, the latter being prone to a high natural variability. For future research, it is recommended that studies on apple simulation medium, which may lead to the development of new control strategies for reducing patulin contamination, are validated on apples as well.

Andersen et al. (2004) demonstrated that *P. expansum*, when contaminating fruits and fruit products, can produce toxic metabolites other than patulin. For example, a sample of windfall apples contained chaetoglobosins A and C, communesin B, roquefortine C, and patulin. Such samples, should they ever reach the consumers, could have unforeseen toxicities, due to possible additive or synergistic effects. These results emphasise to focus the attention of legislators and people in the food industry, as well as researchers, on the potential problem of co-occurring *P. expansum* metabolites in fruit products. Within this context, our method is limited as it is developed to target one specific metabolite. In this case, a targeted metabolomics approach, such as multi-mycotoxin LC-MS, could be considered. Targeted metabolomics is the unbiased global study of a large range of metabolites in a biological sample under a given set of conditions. As a final version of the 'omics cascade', it represents the final step in an organism's phenotype, and thus can address the quantitative expression of each metabolite in a sample (Johnson and Gonzalez, 2012). A great advantage of this technique is obviously the high-throughput capability of spectroscopic and structural information that permits characterising a wide range of metabolites simultaneously, with high analytical precision. On the other hand, analysing multiple mycotoxins in one single run poses some challenges as well. Van Pamel et al. (2011) developed a multi-mycotoxin LC MS/MS method to determine mycotoxins produced by pure fungal isolates grown on YES agar. The authors specifically pointed out to the possible pitfalls for accurately identifying patulin in between other metabolites.

Real-time reverse transcription PCR (RT-qPCR) is a molecular technique that enables detection and quantification of small amounts of nucleic acids in a variety of samples from numerous sources in the field of life sciences, agriculture, and medicine. It has become the method of choice for sensitive, specific and reproducible quantification of mRNA amounts transcribed by a gene. Some pitfalls concerning qPCR assays that need to be taken into account include template quality, poor choice of primers, PCR inhibition, efficiency and sensitivity, normalization and subjectivity in data analysis and reporting (Bustin and Nolan, 2004; Schrader et al., 2012). In 2009, the Minimum Information for Publication of Quantitative real-time PCR Experiments (MIQE) guidelines were published to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency (Bustin et al., 2009). MIQE is a set of guidelines (for authors, reviewers and editors) that describe the minimum information necessary for evaluating and reproducing qPCR experiments.

Based on these potential pitfalls and proposed guidelines, a robust and reliable RT-qPCR method was developed for evaluating expression levels of the *idh* gene, a key gene for patulin biosynthesis in *P. expansum* (CHAPTER 6). In particular, it is the first report of an RT-qPCR method regarding the expression of genes involved in mycotoxin biosynthesis that relatively quantified on the base of multiple stably expressed reference genes. Although the presence of patulin in by-products of apples is under strict regulatory control, patulin continues to be detected in these products so there is further need for new or adapted control strategies. Therefore, the relevance of our work lies on the possibility to investigate the effect of abiotic parameters on patulin production by *P. expansum*. Moreover, the carefully detailed method can be proposed and incorporated by other research facilities as an interesting tool for testing future control strategies that may lead to effectively minimizing patulin formation in food.

One of the major drawbacks of this method is that the expression of only one gene involved in patulin biosynthesis by *P. expansum* is evaluated. This issue could be handled with a transcriptomics approach e.g. RNA sequencing. With the aim of developing control strategies for mycotoxigenic moulds or other pathogenic microorganisms in the food supply chain, there is a need to determine the physiological state of these organisms when present on the particular foods under certain conditions (Bergholz et al., 2014). Over the past 10 years, studies mostly assessed transcriptomics of bacteria (e.g. *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*) under conditions simulating those that a pathogen may experience in food (Goudeau et al., 2013; Kocharunchitt et al., 2012; Liu and Ream, 2008). In the case of mycotoxigenic moulds, a transcriptomics approach could be interesting to study genes involved in signaling and mycotoxin biosynthesis pathways and as such to further investigate the still largely elusive role of secondary metabolism and infection. A drawback of RNA sequencing is the fact that the entire genome of the organisms has to be known. For now, databases with entire genome sequences of organisms are already available but many still remain to be elucidated as well. However, in the case of *P. expansum*, the entire genome sequence of three *P. expansum* strains has been reported recently by Ballester et al. (2015). This provides a great source of information which may lead to a better understanding of how these regulatory pathways directly or indirectly control fungal development and secondary biosynthesis, hence this could help in finding control strategies to reduce or eliminate contamination.

Strain variability, an important factor influencing patulin contamination

CHAPTER 5 of this doctoral thesis investigated *in vitro* the patulin production capacity of a large collection of *P. expansum* isolates and reference strains in function of apple storage conditions (temperature and atmosphere). Interestingly, within the same test conditions, it was noted that variability between strains had a considerable impact on patulin accumulation. Besides our findings, a previous study by McCallum et al. (2002) already reported differences in growth rates between *P. expansum* isolates.

In general, fungal development and mycotoxin synthesis are subject to multiple intrinsic and extrinsic parameters. Moreover, it is worth mentioning that both are differentially influenced by a very complex network of parameter interactions. The last decade, predictive microbiology emerged as a practical tool in the food industry to predict the behaviour of microorganisms under certain conditions, through the development of mathematical models. At first, these tools were largely employed for bacterial growth control, however later on they found their application in modeling fungal growth as well. In the case of *P. expansum*, studies have been conducted to characterise on the one side growth rate as a function of storage temperature, a_w and oxygen levels (Baert et al., 2007b; Judet-Correia et al., 2011; Marín et al., 2006) and independently on the other patulin production as a function of temperature, pH, and fruit varieties (Morales et al., 2008a; Salomão et al., 2009). Recently, Tannous et al. (2015) studied *in vitro* the individual effects of temperature, pH and a_w on both the growth and patulin production by *P. expansum*, and subsequently developed a mathematical model which enables accurate prediction of optimal and marginal conditions for *P. expansum* growth. Mostly, the development of these predictive models is based on data obtained by one single *P. expansum* strain. However, to accurately forecast its behaviour and predict its potential risks on the food sector and consumer health, it is recommended to include various strains as our findings clearly indicated that strain variability seems to be a very important factor influencing patulin accumulation by *P. expansum* (CHAPTER 5). As such, the relevance of CHAPTER 5 additionally lies in the potential application of this diverse collection of patulin producing *P. expansum* strains in validating these predictive models.

As mentioned earlier, mycotoxins may have a role as pathogenicity or virulence factors. Clarifying how certain fungal strains manage to survive, grow and reproduce without mycotoxin synthesis and why their metabolism produces lower oxidative bursts without compromising their growth is essential if control over mycotoxins has to be achieved. In this regard, future research concerning the role and underlying mechanisms of patulin biosynthesis may benefit from using a diverse collection of patulin producing *P. expansum* strains.

To conclude ...

The problem of mould and mycotoxin contamination of food and feed commodities is a very complex issue subject to a complex network of extrinsic, intrinsic and implicit factors. On top of that, one needs to consider the variability and diversity of the moulds themselves. So it is imperative to approach these problems in a multidisciplinary way. Within this framework, one has to take into account that the outcome of *in vitro* experiments cannot just be extrapolated to the *in vivo* situation. This emphasises the importance of having reliable and rapid methods, which is not always a straightforward matter, available to us. Compared to bacterial infections and intoxications, mould and mycotoxin contamination still seems a somewhat neglected area. Despite the fact that for years now, they are of high economic burden and may pose severe public health risks. Together with the tangible shift in climate change, these problems will not simply go away, on the contrary, challenges to prevent them will only increase. Altogether, this highlights the relevance of research on the stimuli and molecular regulatory mechanisms involved in mould and mycotoxin contamination, and underlies the importance to continue this type of work in the future.

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Summary

SUMMARY

The ubiquitous nature of fungi leads to losses worldwide because of deterioration of food and feed, as well as infections in plants, humans and animals. Their presence in food and feed commodities is undesirable as this often causes spoilage (e.g. off-flavour, discolouration, reduced nutritional value), and in certain cases mycotoxin contamination and related public health concerns. Despite efforts to control or avoid contamination by moulds and their mycotoxins, they remain to occur frequently in food commodities widespread. Chocolate confectionery and apples are two important Belgian food products, each characterised by their own fungal problems and related economic implications. The aspects are highlighted in CHAPTER 1 of this doctoral thesis.

The correct identification and physiology of those fungi of relevance for specific food products, and the mechanisms involved in regulating fungal growth and mycotoxin biosynthesis, are very complex and specific, and still remain to be elucidated. Therefore, prevention and control strategies can only be successful when the identity and characteristics of the associated mycobiota are well known. Hence, the general scope of this PhD thesis were the detection, identification and characterisation of the mycobiota associated with chocolate confectionery and apples. Within this scope, focus was put on optimizing and developing methodologies through the application of a multidisciplinary research approach, i.e. a combination of conventional, molecular and/or chemotaxonomical analysis.

Chocolate confectionery fillings are generally regarded as microbiologically stable, mainly due to a low water activity (<0.60) inhibiting microbial growth. Stability of fillings with a a_w 0.60-0.85 is maintained through the general practice of adding either preservatives or alcohol, or by maintaining a relatively short shelf life. Increasing consumer demands for high-quality products containing less sugar, fat and preservatives direct the industry towards the production of innovative formulations. In addition, exportation of these products is of great economic importance to the Belgian food industry and requires sufficiently long shelf life. Changes in the composition of chocolate confectionery fillings can impede the stability of these products and may lead to spoilage by xerophilic fungi.

In this respect, the aim of CHAPTER 2 was to test whether the production environment of Belgian chocolate confectionery factories and common ingredients of chocolate confectioneries could be potential sources of contamination with xerophilic fungal species. In the factory environment, the general and strictly xerophilic fungal spore load was determined using an RCS Air Sampler device in combination with DG18 and MY50G medium, respectively. In addition, four basic ingredients (ganache, fruit fillings, nuts and fondant sugar) of chocolate confectionery fillings were examined for fungal spore levels using a direct plating technique. Results demonstrated a general fungal spore load in the range of 50-250 CFU/m³ air and a strictly xerophilic spore load below 50 CFU/m³ air. These results indicate that rather low levels of fungal spores were present in the factory environment. Analysis of the basic ingredients of confectionery fillings revealed nuts to be the most likely source of direct contamination. Besides detection and enumeration, fungi were also identified to the species level by a combination of macro-/microscopic characterisation, and gene sequence analysis based upon their *β-tubulin* and/or ITS region. The most prevalently isolated fungi in the factory environment were identified as *Penicillium* spp., particularly *P. brevicompactum*. In nuts, the most commonly isolated species identified were *Eurotium*, particularly *E. repens*.

Based on the survey study conducted in CHAPTER 2, CHAPTER 3 focused profoundly on nuts as the potential initial source of xerophilic contamination of chocolate confectionery fillings. In addition, some preventive measures for reducing the initial fungal contamination on walnuts were investigated based on their effect on microbiological load, sensorial quality, and oxidative stability. Microbiological analysis of the fungal load on a variety of nuts and their corresponding nut-based fillings indicated that walnuts, and the fillings in which they were used, were highly contaminated (>2.5 $\log_{10}$ CFU/g). In addition, a challenge test on almond-based marzipan (a_w 0.84 and pH 5.76) showed that the previously isolated common xerophiles *E. repens* and *W. sebi* are capable of growing in such commodity. *E. repens* even showed some resistance to 0.15% potassium sorbate, whereas spoilage could only be inhibited for over a month if 1% ethanol ((w/w) in water phase) was added. Within the framework of finding a possible decontamination strategy, the effect of dry and humid thermal treatment of walnuts with various heating and drying cycle times was tested. Humid heating completely eliminated the initial fungal load, while dry heating did not induce any detectable change.

According to sensorial analysis, humid heated and unprocessed walnuts tasted significantly different, with a slight indication of preference for the heated walnuts. Moreover, hexanal analysis of walnuts demonstrated humid treatment to increase the oxidative stability of walnuts, hence, delaying the onset of rancidity during storage.

Apples are a seasonal product and so, need an enlarged shelf life to allow furnishing the market all along the year. *Penicillium expansum* is a commonly occurring fungal species on apples in temperate regions, easily disseminated by different vectors in the orchard and by field equipment at harvest. After harvest, apples are transported to storage rooms of packinghouses. Long-term storage of apples at low temperature combined with controlled atmosphere (i.e. reduced O_2 and elevated CO_2) is a commonly applied strategy to extend their shelf life. However, *P. expansum* has a psychrophilic nature, allowing the fungus to invade the apple during post-harvest storage. Moreover it is capable of producing the mycotoxin patulin, which may end up in apples as well as by-products such as apple juice. Therefore, the second part of this thesis, focused further on the effect of temperature and atmosphere on patulin biosynthesis of various *P. expansum* strains.

In vitro experiments studying the underlying molecular genetics of patulin biosynthesis often make use of the laboratory reference/simulation medium "Apple puree agar medium (APAM)". Within this context, CHAPTER 4 started with the development and validation of a sensitive High Performance Liquid Chromatography-UV (HPLC-UV) method, based on the AOAC Official method 2000.02, for the high-throughput analysis of patulin in *in vitro* experiments for *P. expansum* grown on APAM. The importance of repeating the ethyl acetate extraction step at liquid-liquid extraction (LLE) was examined, as well as the extent of patulin degradation during the sodium carbonate clean-up. In addition to this alkaline clean-up, the efficiency of using an Oasis HLB or C_{18} cartridge as solid-phase extraction (SPE) clean-up was compared. This resulted in a two-step ethyl acetate LLE, followed by an Oasis HLB SPE clean-up, without alkaline clean-up conditions. After optimization, the method was fully validated for APAM, cloudy apple juice and apple puree. Average patulin recoveries at levels of 100, 500 and 1000 µg kg^{-1} of APAM varied between 95% and 113% over three independent analysis days, with an interday precision (RSD_R) of 5 to 10%. Recovery experiments carried out with the spiked apple juice (at 50 µg kg^{-1}) and apple puree (10 µg kg^{-1}) showed average recovery rates between 80-101% (RSD_R = 12%) and 77-100% (RSD_R = 9%), respectively.

This method was characterised by a detection limit of 3-4 µg kg^{-1} and a quantification limit of 5-8 µg kg^{-1} for APAM, apple juice and puree. In conclusion, this method is a useful monitoring system in the apple industry or for further research on the regulation of mechanisms involved in patulin biosynthesis on APAM.

The analytical method for patulin analysis on APAM developed (CHAPTER 4), was implemented to investigate the extent to which each successive step during long-term storage contributes to patulin production by *P. expansum* (CHAPTER 5). Therefore, Belgian apples from several orchards/industries were collected and the fungi isolated were identified to the species level. Random amplification of polymorphic DNA (RAPD) analysis and *β-tubulin* gene sequencing identified *P. expansum* and *P. solitum* as the most prevalent *Penicillium* species associated with Belgian apples. All 27 *P. expansum* isolates and eight reference strains were characterised for their patulin production capacity on APAM after five days growth under classical constant temperature and atmospheric conditions. Under these conditions, a large range of patulin production levels was observed between the different isolates. Based on this phenotypic diversity, five *P. expansum* isolates and one reference strain were selected for *in vitro* investigation of patulin production under representative conditions in each step of long-term apple storage. The results indicated that a high spore inoculum leads to a strong patulin accumulation already during the first step of long-term storage under cold temperature (1°C) combined with CA (3% O_2, 1% CO_2). Finally, patulin accumulation was highly strain dependent and for each separate strain were no significant differences between the storage steps observed.

Whereas CHAPTER 5 focused on the patulin production capacity of a large collection of *P. expansum* strains, CHAPTER 6 investigated the effect of temperature and atmosphere on the *idh* gene expression in relation to the patulin production of *P. expansum*. The *idh* gene encodes the isoepoxydon dehydrogenase enzyme, a key enzyme in the patulin biosynthesis pathway. First, a reverse transcription real-time PCR (RT-qPCR) method was optimised to measure accurately the *P. expansum idh* mRNA levels relative to the mRNA levels of three reference genes (*18S*, *β-tubulin*, *calmodulin*), taking into account important parameters such as PCR inhibition and multiple reference gene stability. Subsequently, two *P. expansum* field isolates and one reference strain were grown on APAM under three conditions of temperature and atmosphere: 20°C – air, 4°C – air and 4°C – CA.

When *P. expansum* strains reached a 0.5 cm and 2.0 cm colony diameter, the *idh* expression and patulin concentrations were determined by means of the developed RT-qPCR and HPLC-UV method, respectively. This *in vitro* study showed a clear reduction in patulin production and down-regulation of *idh* expression when *P. expansum* was grown under 4°C – CA. The results suggest that stress (low temperature and oxygen level) caused a delay of the fungal metabolism rather than a complete inhibition of the toxin biosynthesis. A good correlation was found between the *idh* expression and patulin production, corroborating that temperature and atmosphere affecte patulin production by acting at the transcriptional level of the *idh* gene. Finally, our RT-qPCR developed can be considered as a reliable alternative tool to investigate for example the effect of control strategies on the toxin formation in food.

In general, this PhD thesis made use of a multidisciplinary research approach to identify/characterise the mycobiota associated with two food commodities, namely chocolate confectionery and apples. A rapid, sensitive and reliable chemical (HPLC-UV) and molecular (RT-qPCR) method were developed and proposed as useful research tools in future studies aiming to develop new control strategies for patulin contamination in apples. Furthermore, these methods along with others (e.g. conventional plating and gene sequencing) were applied to gather knowledge on the factors involved in mould and mycotoxin contamination. For example, *P. expansum* exhibited a large strain variability, which seemed an important and not to be neglected aspect affecting patulin accumulation. Within the case of chocolate confectionery, nuts, and especially walnuts, were highlighted as an important source of fungal contamination. Humid thermal treatment offers an interesting solution to reduce this initial contamination on walnuts, and hence, extending shelf life of the confectionery products.

Samenvatting

SAMENVATTING

Het wijdverspreide karakter van schimmels heeft een immens negatieve impact wereldwijd; dit in de zin van zowel kwalitatieve achteruitgang van voedsel en voeder, als infecties van plant, dier en mens. Bijgevolg is hun aanwezigheid in voedsel en voeder ongewenst aangezien ongewenste schimmelgroei vaak aanleiding geeft tot bederf (bvb. afwijkende smaak, verkleuring, verminderde nutritionele waarde), en in sommige gevallen zelfs tot mycotoxine contaminatie en de daarmee gepaard gaande volksgezondheidsimplicaties. Ondanks de grote inspanningen om contaminatie ten gevolge van schimmels en mycotoxines te beheersen/vermijden, blijven beide alomtegenwoordig en worden ze regelmatig aangetroffen doorheen de voedselketen. Zo ook in zoetwaren en appels, welke van economisch belang zijn voor de Belgische voedingsindustrie. Tot op heden hebben beide matrixen eigen schimmel-gerelateerde problemen en daarmee gepaarde gaande economische gevolgen die oplossingen vereisen. In HOOFDSTUK 1 worden deze aspecten belicht.

De juiste identificatie en fysiologische eigenschappen van voedsel-gerelateerde schimmels, en de mechanismen bepalend voor groei en mycotoxine synthese, zijn zeer complex en specifiek, en tot op heden nog onduidelijk. Beheersmaatregelen zijn enkel succesvol wanneer de identiteit en karakteristieken van de geassocieerde mycobiota gekend zijn. Dit doctoraatsonderzoek focust zich op de detectie, identificatie en karakterisering van de geassocieerde mycobiota van zoetwaren en appels. Binnen dit kader werd gefocust op de optimalisatie en ontwikkeling van methodieken via een multidisciplinaire onderzoeksbenadering, d.w.z. een combinatie van conventionele, moleculaire en/of chemotaxonomische analyses.

Zoetwarenvullingen zijn doorgaans microbiologisch stabiel. Deze stabiliteit is grotendeels te wijten aan een voldoende lage water activiteit, de toevoeging van bewaarmiddelen of alcohol, of door het aanhouden van een relatief korte houdbaarheid. Toenemende vraag van de consument naar zeer kwalitatieve producten met minder suiker, vet en bewaarmiddelen zorgt voor een continu veranderende productontwikkeling (zonder alcohol of “clean-label”). Daarnaast is export een heel belangrijk economisch facet van de voedingsindustrie. Producten dienen daarom een voldoende lange houdbaarheid te kunnen garanderen.

Verandering van de receptuur kan ten koste gaan van de stabiliteit en bijgevolg tot bederf leiden ten gevolge van xerofiele schimmels.

Derhalve werd in de eerste plaats een studie uitgevoerd naar de aanwezigheid en prevalentie van xerofiele schimmels in de productieomgeving van Belgische zoetwarenbedrijven en in de grondstoffen van zoetwarenvullingen. De algemene en strikt xerofiele schimmeldruk in de lucht van de productieomgeving werd bepaald aan de hand van een RCS lucht staalname toestel gevuld met respectievelijk DG18 en MY50G medium. De aanwezige schimmeldruk in vier types grondstoffen (ganache, fruitvullingen, noten en suiker) werd onderzocht door middel van een directe uitplatingsmethode. Er werden een algemene en strikt xerofiele schimmeldruk van respectievelijk 50-250 KVE/m^3 lucht en <50 KVE/m^3 lucht teruggevonden. Deze resultaten wijzen op een relatief lage schimmeldruk in de productieomgeving van de onderzochte zoetwarenbedrijven. Uit het onderzoek rond de aanwezige schimmeldruk in grondstoffen bleken de noten een mogelijke bron van contaminatie te kunnen zijn. De gedetecteerde schimmels werden verder geïsoleerd en geïdentificeerd tot op soortniveau door middel van een combinatie van macro-/microscopische karakterisering en moleculaire sequenering op basis van het *β-tubulin* gen en/of ITS region. *Penicillium* spp., en meer specifiek de soort *P. brevicompactum*, werden overwegend geïsoleerd uit de lucht van de productieomgeving. Het genus *Eurotium*, en meer specifiek de soort *E. repens*, werd het meest frequent geïsoleerd uit de noten.

Op basis van de survey uitgevoerd in HOOFDSTUK 2, werd in HOOFDSTUK 3 de focus gelegd op noten als potentieel belangrijke initiële bron van schimmelcontaminatie van zoetwarenvullingen. Bijkomend werden een aantal beheersmaatregelen uitgetest teneinde deze initiële contaminatie mogelijk te reduceren. Deze maatregelen werden onderzocht op basis van hun effect op de microbiologische schimmeldruk, sensorische kwaliteit en oxidatieve stabiliteit. Uit microbiologische analyse van de schimmeldruk op noten en de noot-gebaseerde zoetwarenvullingen bleek dat walnoten, alsook de vullingen op basis van walnoten, het sterkst gecontamineerd waren (>2.5 $\log_{10}$ KVE/g). Een challengetest op marsepein (a_w 0,84 en pH 5,76) toonde aan dat de voorheen geïsoleerde xerofiele schimmelsoorten, *E. repens* en *W. sebi*, in staat zijn uit te groeien. Daarenboven vertoonde *E. repens* enige resistentie tegen 0.15% kalium sorbaat. Enkel toevoeging van 1% ethanol kon een groei-inhibitie van minstens een maand waarborgen.

In het kader van een mogelijke verwijdering van contaminatie, werd het effect van droge en vochtige verhittingen met variërende tijdsintervallen uitgetest op walnoten. Vochtige verhitting zorgde voor een volledige reductie van de initiële schimmeldruk, daar waar een droge verhitting geen direct detecteerbare verandering teweeg bracht. Op basis van een sensorische triangle test bleek dat er wel degelijk een verschil in smaak waarneembaar is tussen de vochtig verhitte en onbehandelde walnoten, met een lichte voorkeur voor de vochtig verhitte walnoten. Daarenboven toonde analyse van de hexanalconcentratie in vochtig verhitte en onbehandelde walnoten aan dat een vochtige verhitting resulteerde in hogere oxidatieve stabiliteit en daaruit volgend een uitstel van ranzig worden tijdens bewaring.

Appels zijn een seizoensproduct dat op de markt beschikbaar dient te zijn gedurende het ganse jaar door. De schimmelsoort *Penicillium expansum* is alomtegenwoordig op appels in gematigde gebieden. Deze schimmel wordt zeer gemakkelijk verspreid door allerhande vectoren aanwezig in de boomgaard, en door gereedschap gebruikt tijdens het oogsten. Na de oogst worden deze appels getransporteerd naar bewaarruimtes. Lage temperatuur in combinatie met gecontroleerde atmosfeer (d.w.z. verlaagd zuurstofgehalte en verhoogd stikstofgehalte) is een zeer algemeen toegepaste strategie om appels gedurende lange termijn te bewaren en de markt te kunnen bevoorraden gedurende het ganse jaar. *P. expansum* is een psychrofiele schimmel, wat toelaat appels te infecteren na de oogst gedurende deze lange-termijn bewaring. Daarnaast is de schimmel ook in staat om het mycotoxine patuline te produceren. Patuline kan bijgevolg naast appels ook verder in de keten terechtkomen in afgeleide producten van appels zoals bvb. appelsap. Het tweede luik van dit doctoraatsonderzoek legde de focus op de effecten van temperatuur en atmosfeer op de vorming van patuline door verscheidene *P. expansum* stammen.

In vitro onderzoek naar de mechanismen van patuline biosynthese maakt vaak gebruik van een simulatiemedium van appels, met name “appel puree agar medium (APAM)”. Binnen deze context beschrijft hoofdstuk 4 de optimalisatie en validatie van een gevoelige hoge performantie vloeistofchromatografie-UV (HPLC-UV) methode, gebaseerd op de AOAC officiële method 2000.02, voor de high-throughput analyse van patuline in *in vitro* experimenten op APAM. Tijdens de optimalisatie werd in eerste instantie het belang onderzocht van een tweevoudige ethylacetaat extractie tijdens vloeistof-vloeistof extractie. Daarnaast werd aandacht geschonken aan de mate van patulinedegradatie tijdens de traditionele staalopzuivering op basis van natriumcarbonaat.

Naast deze alkalische opzuivering werd de efficiëntie uitgetest van een vaste-fase extractie op basis van een Oasis HLB en C_{18} opzuiveringskolom. Dit resulteerde in een tweevoudige ethylacetaat vloeistof-vloeistof extractie, gevolgd door een staalopzuivering met behulp van een Oasis HLB kolom in een niet-alkalische milieu. Deze geoptimaliseerde methode werd vervolgens gevalideerd voor APAM, appelsap en appelmoes. De gemiddelde terugvindingspercentages van patuline aan concentraties van 100, 500 en 1000 µg kg^{-1} APAM waren over drie onafhankelijke dagen tussen 95 en 113%, met een interdag precisie (RSD_R) van 5 tot 10%. Experimenten uitgevoerd ter bepaling van de terugvinding van patuline in appelsap (aan 50 µg kg^{-1}) en appelmoes (10 µg kg^{-1}) vertoonden een gemiddelde terugvinding respectievelijk tussen 80-101% (RSD_R = 12%) en 77-100% (RSD_R = 9%). Een detectielimiet van 3-4 µg kg^{-1} en een kwantificatielimiet van 5-8 µg kg^{-1} werden vastgesteld voor APAM, appelsap en appelmoes. Tot besluit, deze methode is een nuttig hulpmiddel als controlesysteem in de appelindustrie of voor verder onderzoek omtrent de regulatie van mechanismen die betrokken zijn bij de vorming van patuline in APAM.

De eerder ontwikkelde analytische methode ter bepaling van patuline in APAM (HOOFDSTUK 4), werd geïmplementeerd in HOOFDSTUK 5 ter vaststelling van de mate waarin elke opeenvolgende stap tijdens lange-termijn bewaring bijdraagt tot de productie van patuline door *P. expansum*. In dit kader, werd in eerste instantie een staalname uitgevoerd van Belgische appels afkomstig van verscheidene boomgaarden/industrieën. Aanwezige schimmels werden gedetecteerd, geïsoleerd en geïdentificeerd tot op soortniveau. Op basis van Random amplification of polymorphic DNA (RAPD) analyse en *β-tubuline* gensequenering bleken *P. expansum* en *P. solitum* de meest frequent geïsoleerde schimmelsoorten geassocieerd met Belgische appels. Karakterisering van de patulineproductie van 27 *P. expansum* isolaten en acht referentiestammen op APAM onder klassieke constante temperatuur en atmosferische condities duidde op een zeer hoge fenotypische diversiteit. Op basis van deze diversiteit werden vijf isolaten en één referentiestam geselecteerd voor een *in vitro* onderzoek rond de patulineproductie onder representatieve omstandigheden van lange-termijn bewaring van appels. Dit onderzoek toonde aan dat een hoog schimmelsporeninoculum reeds aanleiding geeft tot een hoge patulineaccumulatie tijdens de eerste stap van lange-termijn bewaring onder lage temperatuur en gecontroleerde atmosfeer. Tot slot bleek dat er een zeer hoge variabiliteit is in patulineaccumulatie tussen verschillende stammen. Significante verschillen werden niet waargenomen tussen de verschillende stappen van lange-termijn bewaring.

HOOFDSTUK 5 legde de focus op de verscheidenheid aan patulinevorming tussen een grote verzameling aan stammen, terwijl in HOOFDSTUK 6 werd gefocust op de *idh* genexpressie in relatie tot de patulineproductie door *P. expansum*. *Idh* codeert voor het isoepoxydondehydrogenase, een essentieel enzyme betrokken in de biosyntheseweg van patuline. In eerste instantie werd een reverse transcription real-time PCR (RT-qPCR) methode ontwikkeld ter bepaling van de hoeveelheid *idh* mRNA in relatie tot de hoeveelheid mRNA van drie referentiegenen (*18S*, *β-tubuline*, *calmoduline*). Tijdens de ontwikkeling van deze methode werd rekening gehouden met belangrijke parameters zoals PCR-inhibitie en stabiliteit van meerdere referentiegenen. Twee *P. expansum* isolaten en één referentiestam werden geïnoculeerd op APAM en geïncubeerd onder verschillende omstandigheden van temperatuur en atmosfeer: (1) 20°C – lucht, (2) 4°C – lucht, en (3) 4°C – gecontroleerde atmosfeer. *Idh* expressie en patulinevorming werden bepaald wanneer een koloniediameter van 0,5 cm en 2 cm werden bereikt. Genexpressie en patulineproductie werden gekwantificeerd met behulp van de respectievelijk ontwikkelde RT-qPCR en HPLC-UV methodes. Onder 4°C – gecontroleerde atmosfeer werd een sterk verminderde patulineproductie en *idh* expressie geobserveerd. Dit suggereert dat dergelijke stres stimuli (lage temperatuur en laag zuurstofgehalte) geen aanleiding geven tot een volledige inhibitie van de patulinevorming, maar eerder tot een vertraagd metabolisme. Daar *idh* expressie en patulineproductie sterk gecorreleerd zijn, blijkt dat temperatuur en atmosfeer patulineproductie beïnvloeden op het niveau van transcriptie van het *idh* gen. Tot slot kan gesteld worden dat een betrouwbare RT-qPCR werd ontwikkeld, welke kan gebruikt worden als hulpmiddel voor bijvoorbeeld verder onderzoek naar mogelijke strategieën ter beheersing van de patulinevorming in voedsel.

Samengevat, dit doctoraatsonderzoek maakte gebruik van een multidisciplinaire onderzoeksbenadering ter identificatie/karakterisering van de geassocieerde mycobiota van zoetwaren en appels. Een snelle, gevoelige en accurate chemische (HPLC-UV) en moleculaire (RT-qPCR) methode werden ontwikkeld en vooropgesteld als bruikbare hulpmiddelen in verder onderzoek gericht op de totstandkoming van succesvolle maatregelen ter beheersing van de patulinecontaminatie van appels. Deze methoden in combinatie met andere methoden werden ook toegepast om inzicht te verschaffen in de parameters die betrokken zijn bij schimmelgroei en mycotoxinevorming.

Bijvoorbeeld, er dient gewezen te worden op het feit dat de waargenomen hoge fenotypische variabiliteit tussen *P. expansum* stammen een zeer belangrijk, niet te negeren facet is van invloed op de patulinecontaminatie in appels. In het geval van zoetwaren, bleken noten, en voornamelijk walnoten, een belangrijke initiële bron van schimmelcontaminatie te zijn. Vochtige verhitting werd hier voorgesteld als beheersmaatregel teneinde de initiële schimmeldruk van walnoten te minimaliseren en bijgevolg de impact op de houdbaarheid van zoetwaren te verlengen.

Curriculum vitae

CURRICULUM VITAE

Personalia

First name:	Nikki
Last name:	De Clercq
Address:	Molenstraat 17, 9750 Huise
Telephone:	+32 489916239
E-mail:	nikki_dc@hotmail.com
Place of birth:	Waregem
Date of birth:	November 20, 1985
Nationality:	Belgian

Education

1997-2003	Sciences-mathematics
	Bernarduscollege, Oudenaarde, Belgium
2003-2009	Biology
	Faculty of Sciences, Ghent University, Ghent, Belgium

Professional record

2009-2011	Scientific research (Santiago Grisolia Fellowship 09/41)
	Faculty of Sciences, Department of Biochemistry and Molecular Biology, University of Valencia, Valencia, Spain

2011-2015 PhD research (projects: FOD Patuline RF6198 and IWT TETRA sweetshelf 110193)

Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium – Technology and Food Science Unit, ILVO, Melle, Belgium

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Workshop Clear and engaging writing. Miriam Levenson, ILVO. November 21, 2013, Merelbeke, Belgium.

QX200™ Droplet Digital PCR™ System Training. Eddy Van Collenburg, Bio-Rad Laboratories. September 8, 2014, Merelbeke, Belgium.

Project Management course. Tom Jacobs, Ghent University. January 21 and 28 – February 4, 2016, Ghent, Belgium.

Students

Identificatie van schimmels in zoetwaren. 2013. Siebert Denys, KaHo Sint-Lieven, Ghent, Belgium.

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