



**The diversity of yeasts in wet processing of coffee, their role in
mucilage degradation and interaction with ochratoxin A (OTA)
producing *Aspergillus ochraceus***

Ph.D. Thesis

Wafa Masoud

**Department of Food Science, Food Microbiology
The Royal Veterinary and Agricultural University
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Preface

This Thesis reports the results obtained as a part of the project “An integrated approach to prevent ochratoxin A contamination in post-harvest processing of coffee in East Africa”. The project (contract number ICA4-CT-2001-10060) was financially supported by the INCO programme sponsored by the European Commission 5th framework programme. This financial support is highly acknowledged. The work was carried out at the Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

I am grateful to my supervisor Professor Mogens Jakobsen for the giving me the opportunity to carry out this work, his excellent scientific guidance and support throughout the study. Many thanks to my colleagues at Food Microbiology for their help and creating an enjoyable work atmosphere. Furthermore, I would like to thank Associate Professor Lene Jespersen for collaboration in paper I and IV and Associate Professor Lief Poll for collaboration in paper II. Thanks to Lene B. Cesar and Christa H. Kaltoft for participation in paper I and III, respectively.

I would like to thank my family and friends for their support and encouragement. Finally, I am indebted to my beloved husband Mohammed for his belief in me and being there.

Summary

It was the main aim of this thesis to identify the yeast species predominant along the coffee processing chain from picking of cherries to fermentation and drying and investigate their role in coffee fermentation. For this purpose, coffee samples were collected from two coffee processing sites in Tanzania. Yeasts were identified by the classical conventional methods and by the culture independent technique Denaturing Gradient Gel Electrophoresis (DGGE). Furthermore, the ability of the yeasts to degrade pectin was investigated and the antagonist activity of the predominant yeasts during coffee fermentation against ochratoxin A (OTA) producing *Aspergillus ochraceus* were studied.

The yeasts in coffee samples were determined by classical methods of cultivation and isolation. It was observed that the yeasts counts were in the range of 4.0×10^4 to 5.0×10^7 cfu / g along the coffee processing chain with an increase during fermentation. Further, the isolated yeasts were identified by genotyping using ITS-PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene. In addition, DGGE of PCR-amplified 26S rRNA gene was performed to detect yeasts directly from coffee samples without cultivation. Three predominant yeasts were detected by the classical methods of isolation and cultivation and by the DGGE technique. They include *Pichia kluyveri*, *Pichia anomala* and *Hanseniaspora uvarum*. The predominant yeast species during fermentation and drying was *P. kluyveri*. *Pichia anomala* was observed in high numbers at the beginning of fermentation and during drying of coffee beans. *Hanseniaspora uvarum* dominated during fermentation but decreased in numbers during drying. *Kluyveromyces marxianus*, *Candida pseudointermedia*, *Issatchenkia orientalis*, *Pichia ohmeri* and *Torulaspora delbrueckii*, which were found in low numbers of 10^3 cfu / g or below during the different stages of processing were not detected by the DGGE method. On the other hand, *Saccharomyces cerevisiae* and *Candida xestobii* were only observed in the DGGE profile for a sample of drying beans i.e. they were not detected by cultivation. With regard to the predominant yeasts in coffee samples, a good agreement was observed between classical methods of isolation and cultivation and the DGGE profiles of the same coffee samples.

All strains of *P. anomala*, *P. kluyveri* and *H. uvarum* were found to have pectinolytic activity with *P. anomala* S16 and *P. kluyveri* S13Y4 showing the strongest activity. Enzymatic assays showed that the three yeast species were observed to secrete polygalacturonase (PG) but not pectin esterase (PE) or pectin lyase (PL). *Pichia anomala* S16 and *P. kluyveri* S13Y4 were found to produce higher amounts of PG when grown in coffee broth (CB) than in the yeast polygalacturonic acid (YPA) substrate.

The maximum amounts of PG produced by *P. anomala* S16 and *P. kluyveri* S13Y4 were obtained at pH 6.0 and an incubation temperature of 30 °C, conditions close to coffee fermentation. For PG secreted by *P. anomala* S16, the optimum pH and temperature for the enzymatic activity were 5.5 and 40 °C. For PG produced by *P. kluyveri* S13Y4 the strongest activity was observed at pH 5.0 and 50 °C.

To solve the problem of OTA contamination in coffee, the antifungal activity of *P. anomala*, *P. kluyveri* and *H. uvarum* against *A. ochraceus* was investigated. The three yeasts were observed to inhibit growth of *A. ochraceus* when co-cultured on malt yeast extract (MEA) and coffee agar (CA) with a significant higher inhibition on MEA than on CA. In addition, *P. anomala* and *P. kluyveri* were found to have a stronger effect on growth of *A. ochraceus* than *H. uvarum*. The three yeasts prevented spore germination of *A. ochraceus* in yeast glucose peptone (MYGP) broth. In yeast-free supernatant of MYGP broth after incubation periods of 24 and 48 h, spores of *A. ochraceus* did not germinate; after 72 h very short germ tubes were observed but further development of the germ tubes was inhibited. Furthermore, *P. anomala*, *P. kluyveri* and *H. uvarum* were able to prevent production of OTA by *A. ochraceus* when co-cultured on MEA. On CA medium, *P. anomala* and *P. kluyveri* prevented *A. ochraceus* from producing OTA. However, OTA formation on CA was not affected by the presence of *H. uvarum*.

For the understanding of the antifungal properties observed, the effects of volatile compounds produced by *P. anomala*, *P. kluyveri* and *H. uvarum* on growth of *A. ochraceus* and production of ochratoxin A (OTA) were studied. The main volatile compounds produced by the three yeasts were analyzed by gas chromatography-mass spectrometry (GC-MS). Exposure of *A. ochraceus* to the gaseous phase of malt yeast glucose peptone (MYGP) plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* inhibited fungal growth with the two *Pichia* spp.

showing the strongest effect. The main esters and alcohols produced by the three yeasts were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate and isoamyl alcohol. The individual esters and alcohols were found to affect fungal growth. The most effective compound in inhibiting fungal growth was 2-phenyl ethyl acetate. Exposure of *A. ochraceus* to MYGP plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* prevented production of OTA. On CA plates, only the headspace of *P. anomala* and *P. kluyveri* prevented OTA production. A comparison of the effects of different headspace concentrations of the individual volatiles on OTA formation by *A. ochraceus* showed that 2-phenyl ethyl acetate was also the most effective compound in reduction of OTA.

In conclusion, *P. anomala* and *P. kluyveri* were the predominant yeasts during coffee fermentation and drying and they are for the first time shown to have a role in degradation of mucilage surrounding the coffee beans. Furthermore, the antifungal effect of *P. anomala* and *P. kluyveri* against growth and OTA formation by *A. ochraceus* demonstrate the possibility of using the two yeasts as starter cultures in coffee fermentation. However, further *in vivo* studies i.e. use of yeasts as starter cultures should be carried out to confirm the inhibition of fungal growth and OTA formation. *In vivo* starter cultures should also be conducted to disclose any undesired effect on the quality of coffee beans in terms of colour and aroma.

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Appendix I:

Masoud, W., Cesar, L.B, Jespersen, L. and Jakobsen, M. (2004). Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis (DGGE). *Yeast* 21: 549-556.

Appendix II:

Masoud, W., Poll, L. and Jakobsen, M. (2005). Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and Ochratoxin A (OTA) production by *Aspergillus ochraceus*. *Yeast* 22: 1133-1142.

Appendix III:

Masoud, W. and Kaltoft, C.H. (2006). The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and Ochratoxin A (OTA) production by *Aspergillus ochraceus*. *International Journal of Food Microbiology* 106: 229-234

Appendix IV:

Masoud, W. and Jespersen, L. (2006). Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa. *International Journal of Food Microbiology* 210: 291-296.

1. Introduction

According to an ancient story, coffee was first known 12 centuries ago by an Arabian goat-herd named Kaldi, who realized that his goats acting unusually frisky after eating cherries from a bush. Kaldi then tried eating the cherries himself and found that they gave him a renewed energy. The news of this energy fruits then spread throughout the region and the stimulating effect was exploited by monks monasteries to stay a wake during long hours of praying. In the 13th Century coffee was discovered as a delicious beverage in the Arabic region. The name coffee is derived from the Arabic term kahwa, which later gave rise to the English word coffee. Coffee was introduced to Europe by the late 1500 and spread all over the world becoming the most popular and enjoyable beverage in the world until present.

Green coffee beans are obtained after a natural fermentation, which is accompanied by other processing steps. It is generally accepted that the main aim of coffee fermentation is to degrade the pectinolytic mucilage surrounding coffee beans. The microbiota of coffee fermentation is composed mainly of bacteria, yeasts and fungi; however, their role in fermentation is poorly understood.

Since 1974, varying levels of ochratoxin A (OTA) were reported in green coffee beans imported to Europe from coffee producing countries in Africa, Asia, and Central and South America. In tropical areas, OTA in coffee is mainly produced by the toxigenic strains of *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus niger*. It is not exactly known when OTA is produced along the coffee processing chain. Furthermore, there are few studies on the complex microbiota of coffee processing. No studies, which deal with the interactions between OTA producing fungi and other microorganisms predominant during coffee fermentation have been carried out.

Of the promising tools to prevent fungal growth and OTA formation in coffee might be the use of other microorganisms with antifungal activity as biocontrol agents. Yeasts are among

the microorganisms, which have been reported to control several plant pathogenic fungi. The use of yeasts as biocontrol agents against OTA producing fungi might help to prevent or reduce OTA contamination in coffee.

The main objectives of this thesis were:

- To identify yeasts predominant during different stages of wet processing of coffee using the classical techniques of isolation of yeasts and cultivation as well as identification of yeasts directly from coffee samples using the culture independent method denaturing gradient gel electrophoresis (DGGE).
- To investigate the role of the predominant yeasts in degradation of mucilage surrounding coffee beans.
- To study the ability of the predominant yeasts during coffee processing to inhibit growth and OTA production by *A. ochraceus*.

This thesis is based on the following appendices referred to in the text by their Roman numerical:

I. Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis (DGGE).

Masoud, W., Cesar, L.B., Jespersen, L. and Jakobsen, M. Yeast 2004, 21: 549-556.

II. Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and Ochratoxin A (OTA) production by *Aspergillus ochraceus*.

Masoud, W., Poll, L. and Jakobsen, M. Yeast 2005, 22: 1133-1142.

III. The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and Ochratoxin A (OTA) production by *Aspergillus ochraceus*.

Masoud, W. and Kaltoft, C.H. International Journal of Food Microbiology 2006, 106: 229-234.

IV. Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa.

Masoud, W. and Jespersen, L. International Journal of Food Microbiology 2006. 210: 291-296.

The present thesis contains seven chapters (2-8) that point out the information obtained from the existing literature on coffee and topics having relations to the results achieved. The results are discussed when appropriate through out the thesis. In addition, two chapters (9-10) deal with conclusions and future studies and activities are also included.

2. The coffee plant

The coffee plant belongs to the genus *Coffea* of the family *Rubiaceae*. This family consists of many genera including *Gardenia*, *Ixora*, *Cinchona*, and *Rubia*, from which the name *Rubiaceae* was derived (Wintgens, 2004). The family *Rubiaceae* has about 500 genera and over 600 species, which are mostly trees and shrubs but rarely herbs (Wrigley, 1988). The coffee tree was first botanically described by Antoine de Jussieu in 1714 who gave it the name *Jasminum arabicum laurifolia* (Wrigley, 1988). In 1737, Linnaeus classified it as a separate genus *Coffea* with only one known species *Coffea arabica* (Charrier & Berthaud, 1985; Wrigley, 1988). Many species of *Coffea* were then identified; now they include about 80 species (Anzueto et al., 2005). From a commercial point of view, the most important species are *Coffea arabica* and *Coffea canephora* var. *robusta* known as arabica and robusta, respectively. *Coffea arabica* represents about 70% of the worldwide production and mainly grown in Latin America and East Africa. *Coffea canephora* var. *robusta* accounts for one third of worldwide production; it is mainly grown in Asia, South America and Africa (Fowler et al., 1998). *Coffea liberica* and *Coffea excelsa* which are cultivated less extensively in West Africa and Asia are also present and account for only 1-2 % of world production (Wintgens, 2004).

The coffee plant grows by lengthening of vertical main stem and successive growth of horizontal branches arising in pairs opposite to each other (Wrigley, 1988). The leaves of coffee tree are borne in opposite pairs on the sides of the branches; they are elliptical in shape, shiny, waxy and green in colour (Wrigley, 1988). Depending on the species and environmental conditions, a one year old coffee plant develops about 6 to 10 levels of its branches and after two years the coffee tree can reach a height of 1.5 to 2 meters and start flowering (Wintgens, 2004). After about three years the coffee tree is full mature and starts to produce cherries, which grown in clusters attached to the limb by a short stem (Wintgens, 2004).

2.1. *Coffea arabica*

Coffea arabica is originated from Ethiopia in high areas of 1300 to 2000 meters altitudes (Wintgens, 2004). It is the first and the only species that has been known for the longest time and the most widespread through out the world. *Coffea arabica* can be grown in a variety of soils of different geographical origin but it prefers acid soils of 5.5 to 6.0 pH (Varnam & Sutherland, 1994). It requires a temperature of 15 to 24 °C and an average rainfall of 1500 to 2000 mm (Varnam & Sutherland, 1994). A rainfall over a 9 month wet season and 3 month dry season is ideal. However, in Equatorial Kenya, Northern Tanzania and Colombia, there are two wet and two dry seasons resulting in double cropping (Varnam & Sutherland, 1994). Cherries of arabica are ovoid or sub-globular, red when ripe, 10-15 mm wide and 16-18 mm long (Fig 1, Coste, 1992).



Figure 1. Arabica coffee cherries grown in Tanzania

2.2. *Coffea canephora* var. *robusta*

Coffea canephora var. *robusta* takes second place in the world after *C. arabica*; it accounts for one third of the coffee world production (Coste, 1992). It grows at altitudes of less than 600 meters, mainly in Asia, South America and Africa (Fowler et al., 1998). Robusta grows in hot and very humid climate, which is unfavourable for arabica (Coste, 1992). Cherries of robusta are ovoid or sub-globular, red when ripe and are 8-16 mm long (Coste, 1992).

2.3. Coffee cherry

The coffee fruit is a drupe, which referred to as a cherry or a berry; botanically, the term cherry is the correct name (Coste, 1992). Depending on the coffee species, the mature cherries show a variety of colours from green through red and purple to black; some species have a clear yellow colour (Wrigley, 1988).

The cherry normally contains two coffee beans that vary in size, shape and density depending on growing conditions and genotype. Occasionally, the coffee cherry contains one round bean called a pea berry, or three slightly triangular beans (Arunga, 1982; Illy & Viani, 1995). Figure 2 shows a longitudinal section of a coffee cherry. The cherry usually contains two beans (endosperm) surrounded by a thin integument generally known as the silver skin (spermoderm). The coffee beans and the silver skin are sheltered by a hard, horny endocarp, called the parchment to which a pulpy mucilaginous mesocarp is adhered firmly, enclosing each bean. The inner layer of the mesocarp is referred to as the mucilage varying in thickness from 0.5 to 2.0 millimetres whereas the outer layer is called the pulp, which is covered by the coloured fruit skin (exocarp) (Arunga, 1982; Illy & Viani, 1995).

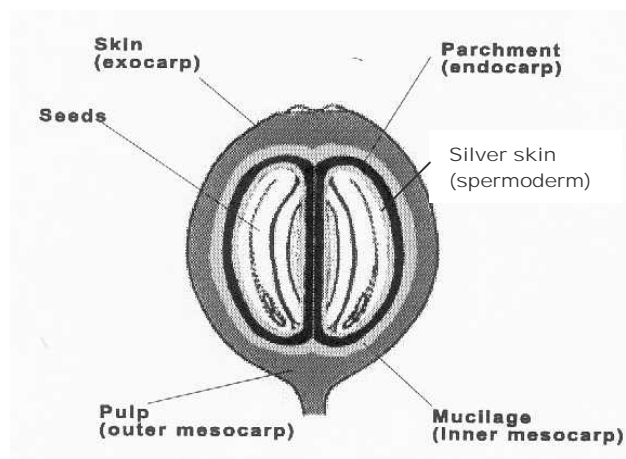


Figure 2. Longitudinal section of a coffee cherry (modified from Avallone et al., 2002)

The ripe coffee cherry of arabica consists roughly of 39 % pulp, 17 % mucilage, 7 % parchment and skin, and 37 % beans (Wrigley, 1988). Ripe coffee cherries have moisture content of 65 to 70 % (Wrigley, 1988). The mesocarp (pulp and mucilage) has a water content of 70-85 %; it consists mainly of protopectin, (a group of pectic substances insoluble in water,

which are hydrolysed to pectin or pectinic acid) associated with sugars, cellulose, lipids and proteins (Arunga, 1982). During coffee processing, the pulp is removed and coffee cherries are left for natural fermentation where it is assumed that degradation of the pectinolytic mucilage takes place by microbial enzymes.

The size and shape of coffee bean differs depending on the variety, environmental conditions and cropping practices (Coste, 1992). A typical arabica coffee bean is oval, flat and deeply grooved (Arunga, 1982; Illy & Viani, 1995). On the average, they are 10 mm long, 6 to 7 mm wide and 3 to 4 mm thick (Coste, 1992). The beans of rubosta are ovoid and generally very small in size (Coste, 1992). The coffee bean has 10.5 % to 11.5 % moisture content when dry (Viani & Clarke, 2001) and it contains polysaccharides, lipids, glucose, fructose, sucrose, polyphenols and caffeine (Illy, 2002). Carbohydrates constitutes about half of the green coffee bean and they are considered as key precursors for generation of flavour during roasting (Bradbury, 2001). Arabinogalactan has been reported as the main polysaccharide in green coffee beans, which beside sucrose and proteins was considered as an important precursor for development of flavour volatiles compounds during roasting of coffee (De Maria et al., 1994).

3. Coffee processing

Processing of coffee cherries involves removal of the different layers; pulp, mucilage, parchment, and silver skin surrounding the coffee beans. Coffee processing consists of several steps, which will end in production of the green coffee beans that will be roasted and milled before consumption. Depending on the coffee species and climatic conditions, coffee cherries are processed either by the dry (natural) method or the wet (washing) method. The dry process is mainly used for robusta coffee because the cherry has a thin pulp that allows direct drying. Furthermore, using the dry process requires limited rainfall and enough sunshine to ensure the long drying necessary to dry the whole cherry, while rainfall throughout the year only allows wet processing (Wrigley, 1988; Illy & Viani, 1995). The wet process is mainly used for arabica coffee because the pulp is thick with high water content, which will delay drying of the entire cherry. However, more than 80 % of arabica coffee in Brazil, Yemen and Ethiopia are processed by the dry method (Brando, 2004). Wet processed coffee is considered to be of higher quality than the dry processed coffee (Varnam & Sutherland, 1994; Illy & Viani, 1995). Wet processed coffee also known as washed coffee is characterized by a bright and clean taste with a fine body (mouth feel) and an acidic aroma (Illy & Viani, 1995).

3.1. Dry processing

Dry processing of coffee involves three basic steps, which include picking and sorting of cherries, drying of the whole cherries and dehusking (Fig 3). After picking, unripe, overripe or damaged cherries are removed. Mature coffee cherries then spread out on patios or racks in shallow layers of approximately 10 cm, which are exposed to the sunlight. Frequent mixing over a period of 10 to 30 days allows the cherries to dry evenly down to levels ranging from 9% to 14% moisture content (Fowler et al., 1998). Drying is also done using machines *i.e.* hot air drying, which reduces the drying time to 3 days (Varnam & Sutherland, 1994). After drying, the dried mesocarp forms the husk, which is then removed mechanically in a process called dehusking or dehulling (Varnam & Sutherland, 1994).

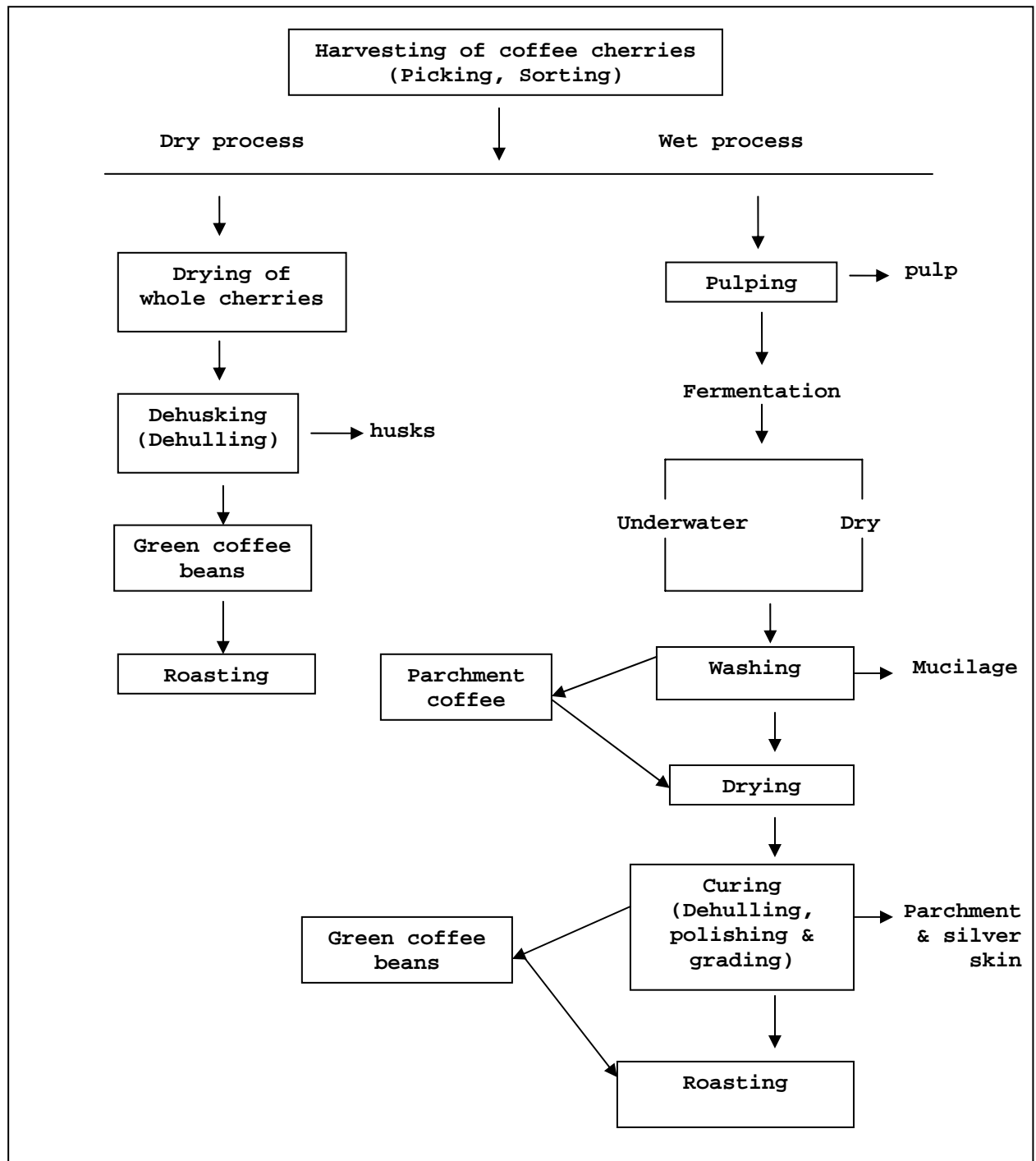


Figure 3. General flow chart of dry and wet processing of coffee from harvesting of cherries to production of roasted coffee beans

3.2. Wet processing

In the wet processing, the skin and pulp are removed from coffee cherries by machines called pulpers. Then, the fresh pulped beans are left for natural fermentation either soaked in water or under dry conditions (Fig 3). Coffee fermentation takes about 24-48 hours and results in degradation of the pectin rich mucilage (Fowler et al., 1998). Where after, the fermented beans known as parchment coffee are washed to remove any trace of the mucilage. Drying follows washing and parchment coffee is mainly sun dried on patios or tables for eight to ten days to reach a moisture content of about 12 % (Varnam & Sutherland, 1994). Afterwards, the parchment coffee are subjected to curing, which involves removal of parchment and silver skin to obtain the green coffee beans.

3.2.1. Harvesting

Harvesting is an essential stage because the degree of ripeness of the cherries determines the final coffee quality. Arabica coffee cherries should be harvested when the cherries have a full red colour. If the coffee cherries are not picked when ripe, they turn dark red to violet and start to ferment on the trees and might be invaded by insects or fungi (Wrigley, 1988; Illy & Viani, 1995). Harvesting is performed by handpicking, stripping or by harvesting machine. The two later methods can easily result in harvesting of ripe, unripe and overripe cherries. Unripe coffee cherries are hard and difficult to pulp without damaging the beans while overripe coffee cherries easily cause development of stinker beans. Stinker beans are associated with a fruity or rotten smell caused by over fermentation with microbial production of dimethylsulphide and short chain aliphatic esters and acids (Illy & Viani, 1995). Stinker beans present at even very low levels are able to contaminate large batches of coffee beans (Bade-Wegner *et al.*, 1997). Coffee cherries do not mature at once but ripen over an extended season, and thus it is necessary to handpick the cherries individually to guarantee a good quality coffee (Wrigley, 1988). Finally, cherries fallen to the ground should be avoided because of increased risk of microbial attack (Pitt *et al.*, 2001). After picking, cherries are subjected to sorting where leaves, sticks, unripe and overripe cherries are removed. Sorting is done manually but often in combination with a gravity method where coffee cherries are fed into a large hopper from where they pass into a concrete tank of water; overripe cherries will float and therefore are easy to sort out, while ripe and unripe cherries will sink in water (Wrigley, 1988).

3.2.2. Pulping

Pulping is the process which involves mechanical removal of the skin and the soft pulpy part of coffee cherries. Pulping should be done as soon as possible after harvesting to avoid the onset of fermentation and development of off-flavours (Varnam & Sutherland, 1994). There are four different types of pulping machines; the drum or cylinder, the disc, the screen and the Raoeng pulpers. All types of pulpers squeeze the coffee cherries to release the beans (Brando, 2004). The drum pulper is generally forced by hand and used by small holders handling small volumes of cherries, whereas the disc, screen and Raoeng pulpers are used by middle-size and large coffee farms having sufficiently power supply and where large volumes of coffee cherries are processed. In connection with pulping by disc-pulper the fully pulped beans are separated from damaged beans, un-pulped beans and pulp. Screen pulper allows separation of un-ripe cherries and the pulping of the ripe ones (Brando, 2004). Unlike other types of pulpers, Raoeng pulper removes both pulp and mucilage in a single operation that consumes large amount of water and power (Brando, 2004). Despite the type of pulper used, water is added during pulping to ease the process and reduce damage of the coffee beans (Wrigley, 1988). It is important that the pulping machine is effective because large amounts of pulp present during fermentation may cause off-flavours (Wrigley, 1988).

3.2.3. Fermentation

The main aim of coffee fermentation is to degrade the slimy mucilage adhering firmly to beans. As mentioned above, the mucilage is assumed to be degraded by pectinolytic enzymes produced by natural occurring microorganisms (Illy & Viani, 1995). The beans are fermented either under dry conditions (dry fermentation), soaked in water (under-water fermentation), or by a two-stage fermentation with a dry fermentation during the initial stage followed by under-water fermentation (Coste, 1992). When the mucilage is exposed to air, it rapidly turns brown, which can diffuse inside and change the colour of the beans (Wrigley, 1988). During under-water fermentation the undesirable brown colour is leached out to the water, and result in a good quality coffee especially in terms of appearance of the beans.

In small-scale growing sites, coffee fermentation takes place in wooden drums or boxes. While in large processing site, pulped coffee are fermented in concrete tanks. Metal containers should not be used as metals are corroded by fermented beans giving them a metallic fla-

avour (Wrigley, 1988). The duration of fermentation process is a very important factor regarding the quality of the final product. According to Bee et al. (2005), fermentation takes from 12 to 36 hours and fermentation longer than 36 hours increases the risk of development of stinker beans. However, fermentation time is very variable depending upon ambient and water temperature, pH, aeration, humidity, and on ripeness of the coffee cherries (Coste, 1992). Fermentation can take from 12 hours in warm areas to 96 hours in cold areas (Wrigley, 1988). At high altitudes where the temperatures are lower, fermentation takes 36 to 48 h or longer; while fermentation takes 12 to 24 in hot low-lying areas (Coste, 1992). Completion of fermentation is decided when the parchment loses the slimy feel of the mucilage, which is tested by hand feeling (Wrigley, 1988). In case of doubt, it is better to slightly over ferment, because under-fermented coffee beans will have a remaining of the mucilage after washing, which will provide adequate substrate for growth of undesirable microorganisms (Wrigley, 1988; Illy & Viani, 1995).

3.2.4. Washing

The objective of washing is to remove all traces of mucilage and breakdown products from the fermentation ending up with parchment coffee (Wrigley, 1988). The washing step is important and should be done immediately after fermentation to avoid the remaining mucilage from further fermentation, which will result in flavour defects and beans discolouration (Thompson et al., 1997). Washing is generally done in large tanks or by slowly moving the coffee beans into open-topped channels (Coste, 1992). When the water leaving the channels or washtub is as clear as it entered, washing is considered to be completed, (Coste, 1992).

3.2.5. Drying

After washing, coffee beans are dried to reduce their moisture content from 50-60 % to about 12 % (Wrigley, 1988; Coste, 1992; Fowler et al., 1998). Drying should start as soon as possible to prevent a second fermentation, which will have serious adverse effects on coffee quality (Wrigley, 1988; Coste, 1992; Fowler et al., 1998; Bee et al., 2005). The coffee beans are either sun dried on patios or tables or artificially dried in mechanical dryers. In sun drying, the coffee beans are spread out in thin layers and mixed frequently to accelerate the drying and prevents development of mouldy beans and further fermentation. Mouldy beans develop a greyish colour and a musty flavour (Bee et al., 2005). Drying time is variable and depends on the climate, the temperature, the sun intensity, the relative humidity, the ventilation and the nature of the material on which the coffee is placed on (Coste, 1992). In areas with low rela-

tive humidity and very intense sun shine, drying might take only six to eight days, while ten to fifteen days may be required in less-favoured regions (Coste, 1992). Drying of coffee beans on tables will accelerate the process compared with drying on patios because the air can pass on all sides of the beans, which will avoid second fermentation and development of mouldy beans (Bee et al, 2005). In case of rain and also at night, coffee beans should be covered to avoid rewetting. Rewetting extracts water soluble materials from beans, which then will oxidize when exposed to air forming brown colour (Wrigley, 1988).

The ideal dry beans should have a greyish blue colour and the beans should be hard but not brittle (Wrigley, 1988). After drying the parchment coffee should have a water activity below 0.80 and the green beans should have a water activity between 0.50 and 0.60 (Viani & Clarke, 2001). If the beans become over dried to a water activity below 0.50 the beans develop a yellow-green colour (Wrigley, 1988).

3.2.6. Curing

Curing of coffee beans involves dehulling, polishing and grading. During dehulling, the parchment and silver skin are removed mechanically from the beans often followed by polishing to remove remaining silver skin. After dehulling and polishing, the green beans are graded according to their size and density and stored until sale (Wrigley, 1988; Illy & Viani, 1995).

3.2.7. Roasting

Roasting is a pyrolytic (heat-driven) process that greatly increases the chemical complexity of coffee (Illy, 2002). It is an important step in the development of the flavour associated with coffee. The aroma of green coffee contains about 250 various volatile compounds, which increase to more than 800 in the roasted coffee (Illy, 2002). The roasting temperature ranges from 185 to 240 °C with an optimum of 210 to 230 °C (Coste, 1992). Roasting above 240 °C will cause charring of beans and disappearance of aroma (Wrigley, 1988). The roasting time depends on the required type of roasting and the temperature used, and may vary from 45 seconds to 25 minutes, although 1.5 minutes to about 6 minutes are typical range for modern commercial roasting (Parliament, 2000). The colour intensity of the roasted coffee beans correlates with the final roasting temperature. The temperatures range from about 193°C for a light roast, through about 205°C for a medium roast, and to about 208°C for a dark roast (Parliament, 2000). At the beginning of roasting and at 100 °C, the residual water inside beans will be converted into steam, which will promote complicated chemical reactions among sugars, proteins lipids and minerals (Illy, 2002). At higher temperatures i.e. 185-240 °C, sugars

will combine with amino acids, peptides and proteins by a chemical reaction known as Maillard's reaction (Parliament, 2000). Simultaneously, the aroma compounds developed and the final beans will be expanded, cracked with shiny exudates collected on their surface (Coste, 1992). At the end of roasting and to ensure that the chemical reactions are stopped, cold air or water is sprayed in a fine mist throughout the beans (Wrigley, 1988; Illy & Viani, 1995).

4. Microbiology of coffee fermentation

4.1. Microenvironment of coffee fermentation

The environmental conditions mainly the nature of the substrate, moisture content, water activity (a_w) and pH play an important role in growth of bacteria, yeasts, and filamentous fungi. Due to the high moisture content and the presence of a wide variety of nutrients in pulp and mucilage, coffee cherries seem to be a suitable medium for growth of microorganisms. The mucilage is mainly composed of 30 % pectin, 18 % polysaccharides, 8 % cellulose, 14 % protein and 30 % monosaccharides (Avallone et al., 2000). In another study, Avallone et al. (2001) demonstrated that 60 % of glucose, fructose and sucrose present in mucilage are consumed by natural microbiota present during coffee fermentation. It has also been reported that the initial concentration of glucose in the aqueous phase of fermenting coffee beans was in the range of 2-6 g / L, which decreased markedly with increasing time of fermentation (Jackels & Jackels, 2005).

Along the coffee processing chain, the moisture content in coffee and thus water activity is reduced. The initial moisture content in fresh coffee cherries is about 60 to 70 %, which corresponds to about 0.99 a_w (Viani & Clarke, 2001). The pulp has a moisture content of about 70 % while the moisture content of mucilage is about 86 % (Bressani & Braham, 1980). The moisture content of parchment coffee ranges from 14 to 18 % and from 10.5 to 11.5 % moisture in green coffee beans corresponding to a_w of 0.70-0.80 and 0.50-0.60, respectively (Viani & Clarke, 2001).

In general, most Gram-positive bacteria are able to grow at a lower water activity than Gram-negative bacteria. The minimum water activity for growth of Gram-positive bacteria is in a range of 0.90 to 0.92. However, *Staphylococcus aureus* has been found to grow at a water activity of 0.86 (Sperber, 1983). For most Gram-negative bacteria the minimum water activity for growth is in a range of 0.95 to 0.99 (Sperber, 1983). Due to the decreasing of water activity during the different stages of coffee processing, bacteria might be present in high numbers at beginning of coffee fermentation but will decrease later with decreasing water activity.

Yeasts are more tolerant to a reduced water activity than bacteria. The minimum water activity for growth of most yeasts is about 0.88 to 0.90; on the other hand, some yeasts species like *Zygosaccharomyces rouxii* can grow in high sugar content at a water activity as low as 0.67 (Tokuoka & Ishitani, 1991). The minimum water activity for growth of most filamentous fungi is reported to be about 0.80 (Northolt *et al.*, 1979; Pitt & Hocking, 1997). The low water activity for growth of filamentous fungi seem to help in providing a suitable environment for fungal growth and OTA contamination in coffee.

During fermentation, the pH value decreases due to microbial metabolism, in particular organic acids formation. The initial pH in fresh pulped cherries is reported to be between 5.5 and 6.0, which is then reduced during fermentation to a final pH of 3.5 after 25 hours of fermentation (Avallone *et al.*, 2001). A final pH value as high as 4.3 after 36 hours of fermentation has also been reported (Van Pee & Castelein, 1972). Recently, a pH of 5.5 to 5.7 during the first 15 hours of coffee fermentation has been recorded, which decreased to 4.7 and 4.4 after 18 and 20 hours, respectively (Jackels & Jackels, 2005). The differences of the final pH value might be due to variation in predominant microflora. The organic acids responsible for the reduction in pH are mainly lactic and acetic acids, which were reported to be produced by the heterofermentative LAB such as *Leuconostoc mesenteroids* and *Lactobacillus brevis* found in coffee fermentation (Avallone *et al.*, 2001). Ethanol has also been detected during coffee fermentation with a rapid increase in the last 5 hours of coffee fermentation. Ethanol is produced mainly by yeasts such as *Saccharomyces cerevisiae*, *Kloeckera* spp., and *Candida* spp. (Fleet, 1997) and by heterofermentative lactic acid bacteria such as *Leuconostoc* spp. and some *Lactobacillus* spp. (Montville, 1997).

Most bacteria are found to have an optimal pH for their growth of 6.0 to 8.0 (Lebert *et al.*, 1998; Leuschner *et al.*, 1998). This supports the assumption that bacteria predominate at the beginning of coffee fermentation but decrease at later stages due to the decreasing pH. *Leuconostoc mesenteroids*, which dominate at first stages of coffee fermentation was found to decreased sharply after 15 h of fermentation (Avallone *et al.*, 2001). On the other hand, yeasts were predominant from the beginning to the end of coffee fermentation (Avallone *et al.*, 2001). In Tanzanian coffee samples collected at different stages of coffee processing, it was found that yeasts were predominant in the whole process (Appendix I). Several yeast species are found to have an optimal growth pH close to 6.0 but are observed to grow at a pH of 2.5

(Betts *et al.*, 1999). It has been reported that changes of pH in a range of 4.7-6.0 (Sørensen & Jakobsen, 1997), 4.0-6.0 (Van den Tempel & Jakobsen, 2000) or 5.5-7.5 (Masoud & Jakobsen, 2005) have no significant effect on growth of *Debaryomyces hansenii*. Toxigenic filamentous fungi such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. can grow in a pH range of 2.0 to 11.0 (Wheeler *et al.*, 1991). Due to the wide range of pH for growth of filamentous fungi, coffee appear to be at a high risk of fungal contamination.

4.2. Microorganisms involved in coffee fermentation

Concerning the predominant microbiota in coffee and their role in the coffee fermentation, few studies have been carried out. In a preliminary study from Brazil, Silva *et al.* (2000) showed that Gram-negative and Gram-positive bacteria, yeast and filamentous fungi were present in high numbers during the different stages of processing and the predominant microorganisms were found to vary considerably between farms and at different stages of maturation. Bacteria represented the majority group constituting up to 80 % of the microorganisms present at the cherry surface (Frank *et al.*, 1965; Silva *et al.*, 2000) followed by yeasts and filamentous fungi (Silva *et al.*, 2000). This is in contrast to the findings of Panneerselvam *et al.* (2001), who reported the predominance of yeasts on surface of coffee cherries and during fermentation.

4.2.1. Bacteria

Bacteria isolated at different stages of coffee processing represent a wide range of genera and species. A preliminary study on dry processing of arabica coffee in Brazil showed that the majority of bacteria present on coffee cherries and fermentation were Gram-positive bacteria, which include the genera *Bacillus*, *Cellulomonas*, *Arthrobacter* and *Lactobacillus* and Gram-negative bacteria of the genera *Aeromonas*, *Pseudomonas*, *Enterobacter* and *Serratia* (Silva *et al.*, 2000). Gram-negative bacilli mainly *Erwinia* and *Klebsiella* followed by *Aeromonas*, *Pseudomonas* and *Chrysomonas* were also isolated during coffee fermentation in Mexico (Avallone *et al.*, 2001). An increase in the numbers of lactic acid bacteria, which belong mainly to *Lactobacillus brevis* and *Leuconostoc mesenteroides*, was reported later in coffee fermentation (Avallone *et al.*, 2001). In other studies, *Streptococcus*, *Pseudomonas*, *Flavobacterium*, *Proteus* (Agate & Bath, 1966), *Erwinia* and *Escherichia* (Frank *et al.*, 1965; Van Pee & Castelein, 1972), were isolated from coffee cherries and during fermentation. It has

been found that in Tanzanian coffee samples that LAB, mainly *Lactobacillus plantarum*, *Leuconostoc fallax* and *Leuconostoc citreum* were predominant during fermentation (INCO: ICA4-CT-2001-10061, second annual report, 2003). From the same coffee samples, Gram negative bacteria belonging to the Genera *Enterobacter*, *Serratia*, *Chryseomonas*, *Pseudomonas* and *Aeromonas* were isolated (INCO: ICA4-CT-2001-10061, second annual report, 2003). In coffee samples collected from Ethiopia, *Leuconostoc citreum*, *Leuconostoc mesenteroids* and *Leuconostoc pseudomesentroids* were the predominant LAB found during fermentation (INCO: ICA4-CT-2001-10061, fourth annual report, 2005). *Enterobacteriaceae cloacae*, *Klebsiella oxytoca* and *Hafnia alevi* were the main Gram negative bacteria isolated from Ethiopian coffee samples (INCO: ICA4-CT-2001-10061, fourth annual report, 2005).

4.2.2. Yeasts

Studies of yeasts populations during coffee processing revealed the presence of yeasts on surface of coffee cherries and at the different steps of the fermentation process. The main yeasts species isolated from coffee fermentation are *Kluyveromyces marxianus* (Agate & Bhat, 1966; Van Pee & Castelein, 1971), *Saccharomyces cerevisiae* (Agate & Bhat, 1966; Van Pee & Castelein, 1971; Silva *et al.*, 2000) and *Schizosaccharomyces* spp. (Agate & Bhat, 1966; Silva *et al.*, 2000). In a study of robusta coffee in Congo, Van Pee and Castelein (1971) identified 11 yeast species and found *Candida* spp. to be dominant and most commonly *C. guilliermondii* var. *membranefaciens*. In addition, *Pichia* spp., *Arxula* spp. (Silva *et al.*, 2000), *Kloeckera* spp. and *Cryptococcus* spp. (Avallone *et al.*, 2001) have been isolated from arabica coffee. In the present study, it was found that *Pichia anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum* were the main yeasts found in Tanzanian coffee samples at the different steps of wet processing (Appendix I).

In order to obtain a more complete picture of the yeasts community during wet processing of arabica coffee in Tanzania, both culture-dependent and culture-independent techniques were applied (Appendix I). Yeasts were isolated from coffee samples and identified by genotyping and sequence analysis of the D1/D2 region of the 26S rRNA gene. Furthermore, denaturing gradient gel electrophoresis (DGGE) was used to identify yeasts directly from coffee samples without cultivation. The traditional determination of colony forming units (cfu) showed that yeasts counts were in a range of 4.0×10^4 to 5.0×10^7 cfu / g with an increase during ferment-

tation (Table 1) indicating that they seem to have a role. *Pichia kluyveri* was the predominant yeast during fermentation and drying. *Hanseniaspora uvarum* was also predominant during fermentation but decreased during drying. *Pichia anomala* was found in high numbers in coffee cherries, at the beginning of fermentation and during drying. *Kluyveromyces marxianus*, *Candida pseudointermedia*, *Issatchenkia orientalis*, *Pichia ohmeri* and *Torulaspora delbrueckii* were encountered in low numbers and in few coffee samples (Table 1). The DGGE profile of pulp and coffee beans during fermentation also showed the predominance of *P. kluyveri* and *H. uvarum* (Fig 4). The other yeasts found in low numbers by traditional isolation and cultivation were not detected by the DGGE technique. For example, *Pichia anomala* was not present in the DGGE profiles of the pulp and beans during the first day of fermentation even though this yeast was isolated from same samples by traditional culture dependent techniques. It has been demonstrated that DGGE can only detect numerically dominant yeast species (Ampe et al., 1999; Cocolin et al., 2000; Boon et al., 2002). However, *Saccharomyces cerevisiae* and *Candida xestobii* were identified directly from drying coffee beans by the DGGE technique but they were not isolated by the traditional cultivation method (Appendix I). This might be explained by the fact that yeasts in question might entered a viable non cultural state or due to the presence of the yeast DNA as a result of cell lysis (Cocolin et al., 2000).

Table 1. The number of colony forming units (cfu / g) of *Pichia kluyveri* (*P. kluyveri*), *Candida pseudointermedia* (*C. pseudointermedia*), *Pichia anomala* (*P. anomala*), *Kluyveromyces marxianus* (*K. marxianus*), *Issatchenkia orientalis* (*I. orientalis*), *Torulaspora delbrueckii* (*T. delbrueckii*) and *Hanseniaspora uvarum* (*H. uvarum*) isolated from coffee samples collected from two processing sites in Tanzania (Appendix I).

Coffee processing stage	Number of colony forming units (cfu / g) of yeasts species ¹						
	<i>P. kluyveri</i>	<i>C. pseudointermedia</i>	<i>P. anomala</i>	<i>K. marxianus</i>	<i>I. orientalis</i>	<i>T. delbrueckii</i>	<i>H. uvarum</i>
Cherries ²	4.5 x 10 ⁵	— ⁴	3.0 x 10 ⁴	— ⁴	— ⁴	— ⁴	7.5 x 10 ⁵
Pulp ³	5.2 x 10 ⁵	— ⁴	— ⁴	— ⁴	— ⁴	— ⁴	6.5 x 10 ⁵
Fresh beans ²	6.0 x 10 ⁵	— ⁴	1.5 x 10 ⁴	— ⁴	— ⁴	— ⁴	6.0 x 10 ⁵
Beans during 1 st day of fermentation ³	2.0 x 10 ⁶	— ⁴	1.0 x 10 ³	1.3 x 10 ³	— ⁴	— ⁴	1.6 x 10 ⁶
Beans during 2 nd day of fermentation ³	3.4 x 10 ⁷	— ⁴	— ⁴	— ⁴	— ⁴	— ⁴	1.4 x 10 ⁷
Beans after fermentation and washing ³	2.1 x 10 ⁶	— ⁴	— ⁴	— ⁴	— ⁴	1.4 x 10 ³	1.7 x 10 ⁶
Beans during 3 rd day of drying ³	2.6 x 10 ⁵	1.3 x 10 ³	6.5 x 10 ⁵	— ⁴	— ⁴	— ⁴	2.6 x 10 ⁵
Beans during 7 th day of drying ³	6.4 x 10 ⁴	— ⁴	9.6 x 10 ⁴	— ⁴	— ⁴	— ⁴	— ⁴
Beans > 7 days of drying ³	1.6 x 10 ⁴	— ⁴	2.0 x 10 ⁴	— ⁴	4.0 x 10 ³	— ⁴	— ⁴

¹ identified by sequencing of D1/D2 domain of the 26S rRNA gene

² samples collected from processing site e.

³ samples collected from processing site f.

⁴ < 10³ cfu/g

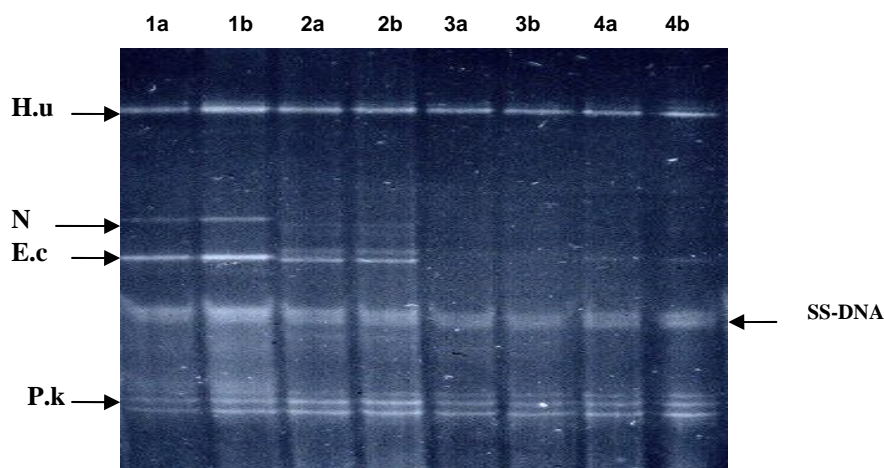


Figure 4. DGGE profiles of PCR-products of the 26S rRNA gene fragments obtained from selected coffee samples. The lane number refers to the coffee sample: lane 1, beans during the 1st day of fermentation; lane 2, pulp; lane 3, beans during the 2nd day of fermentation; lane 4, beans after fermentation and washing. The lane letter corresponds to the PCR conditions: a, NL-1A and NL-4 primers were used; b, NL-1A and LS2 primers were used. The bands common to all samples (SS-DNA) are single stranded DNA artefacts. Abbreviations: H.u., *Hanseniaspora uvarum*; E.c., *Eremothecum coryli* (a yeast plant parasite); P.k., *Pichia kluyveri*; N, unsequenced (Appendix I)

Pichia anomala is ascomycetous heterothallic yeast, which reproduces asexually by budding and sexually by forming hat-shaped ascospores (Kurtzman, 1998). *Candida pelliculosa* is the asexual state of *P. anomala* (Kurtzman, 1998). *Pichia anomala* has been detected in foods. It has been reported as spoilage yeast in yoghurt (Kosse et al, 1997) cheese (Westall & Filtenborg, 1998), the animal feed maize silage (Kitamoto et al., 1999) and coffee (Silva et al., 2000; Appendix I). This yeast species has also been reported as non-Saccharomyces wine yeast (Mingorance-Cazorola et al., 2003; Rojas et al., 2003). *Pichia anomala* has been reported as a biocontrol yeast against a number of filamentous fungi such as *Botrytis cinerea* responsible for grey mould disease in grape-vine (Masih et al., 2000), *Rhizoctonia solani*, *Fusarium equiseti*, *Botrytis fabae*, *Phytophthora infestans* (Walker et al., 1995), *Penicillium roqueforti*, *Aspergillus candidus* (Björnberg and Schnürer, 1993), *Penicillium verrucosum* (Petersson et al., 1998) and *A. ochraceus* (Appendix II & III).

Pichia kluyveri is mainly heterothallic yeast but few strains appear homothallic. This yeast reproduces asexually by budding and sexually by producing two to four hat-shaped spores in each ascus (Kurtzman, 1998). The asexual state of *P. kluyveri* is *Candida eremophila*. *Pichia kluyveri* has been found in vegetables, milk and dairy products (Tudor & Board, 1993), fruits

(Spenser *et al.*, 1992; Abranches *et al.*, 2000), cocoa (Jespersen *et al.*, 2005; Nielsen *et al.*, 2005) and coffee (Appendix I). This yeast species has been reported to produce killer toxins against other yeasts (Radler *et al.*, 1985; Zorg *et al.*, 1988; Stamer *et al.*, 1992; Abranches *et al.*, 1997, 2000). To our knowledge, *P. kluyveri* was not reported before to have antagonist activity against filamentous fungi. In this thesis, it was found strains of *P. kluyveri* were able to prevent growth and OTA formation by *A. ochraceus* isolated from coffee (Appendix II & III).

Hanseniaspora uvarum is ascomycetous yeast that reproduces asexually by bipolar budding and sexually by producing 1 to 2 warty spherical ascospores with equatorial or subequatorial ledge per ascus (Smith, 1998). The asexual state of *H. uvarum* is *Kloeckera apiculata*. *Hanseniaspora uvarum* has been isolated from various fermented food and beverages such as wine (Constanti *et al.*, 1997), yoghurt (Kosse *et al.*, 1997), grapes, banana, cocoa, cucumber brine, tanning fluid, water, cider (Barnett *et al.*, 1990) and coffee (Avallone *et al.*, 2001; Appendix I). *Hanseniaspora uvarum* has also been reported to secrete killer toxins, which are lethal to other yeasts (Radler *et al.*, 1985; Schmitt & Neuhausen, 1994; Abranches *et al.*, 1997). In the present study, it was observed that the antifungal activity of *H. uvarum* against growth and OTA formation by *A. ochraceus* was comparatively weak compared to the antifungal activities of *P. anomala* and *P. kluyveri* (Appendix II, III).

4.2.3. Fungi

Filamentous fungi have also been isolated from surfaces of coffee cherries and from green coffee beans. On surface of arabica and robusta coffee cherries, *Cladosporium*, *Fusarium*, and *Penicillium* were the predominant fungi (Alves & Castro, 1998; Silva *et al.*, 2000; Panneerselvam *et al.*, 2001). In several studies, the most frequent fungus isolated from coffee beans was *Aspergillus niger* (Nakajima *et al.*, 1997; Joosten *et al.*, 2001; Ngabirano *et al.*, 2001; Pitt *et al.*, 2001). *Aspergillus carbonarius* and *A. ochraceus* were also isolated from green coffee beans (Nakajima *et al.*, 1997; Téren *et al.*, 1997; Joosten *et al.*, 2001; Ngabirano *et al.*, 2001; Pitt *et al.*, 2001; Varga *et al.*, 2001; INCO: ICA4-CT-2001-10061, second annual report, 2003). Species like *Aspergillus flavus*, *A. tamarii*, *A. glaucus*, *A. wentii*, *A. versicolor*, *A. candidus*, *A. fumigatus*, *A. oryzae*, and *A. paraciticus* have been isolated less frequently

from coffee (Levi *et al.*, 1974; Mislivec *et al.*, 1983; Nakajima *et al.*, 1997; Téryen *et al.*, 1997; Joosten *et al.*, 2001; Varga *et al.*, 2001).

All of the isolated filamentous fungi are related to plants surfaces (Pitt & Hocking, 1997). A number of *Aspergillus* (Varga *et al.*, 1996; Abarca *et al.*, 1994) and *Penicillium* (Varga *et al.*, 2001) species were reported to produce ochratoxin A (OTA), mainly *Aspergillus ochraceus* (Mantle & Chow, 2000; Taniwaki *et al.*, 2001; INCO: ICA4-CT-2001-10061, second annual report, 2003), *Aspergillus carbonarius* (Joosten *et al.*, 2001; Bucheli *et al.*, 2001), *Aspergillus niger* (Abarca *et al.*, 1994; Heenan *et al.*, 1998), and *Penicillium verrucosum* (Pitt, 1987) as shall be discussed in details in Chapter 5..

5. Pectin degradation during coffee fermentation

5.1. Pectin in coffee

As mentioned above, the mucilage surrounding coffee beans is mainly composed of pectic substances associated with sugars, cellulose, proteins and lipids. During coffee fermentation the mucilage is degraded, which will result in production of green coffee beans. However, the group of microorganisms responsible for pectin degradation in coffee is not known.

In general, pectic substances are mixtures of polysaccharides that present mainly in the cell wall of higher plants. They are found in the middle lamellae between primary cell walls and account for about one third of the macromolecules present in the primary cell wall (Smith & Harris, 1999). Pectic substances are responsible for the control of the cell wall porosity and they are the major adhesive materials between cells (Willats et al., 2001). Chemically, pectic substances are complex colloidal acid polysaccharides with a backbone of galacturonic acid residues linked by α -(1-4) linkages (Be Miller, 1986). The carboxyl groups of galacturonic acid are partially esterified by methyl groups and totally or partially neutralized by sodium, potassium or ammonium ions (Kashyap et al., 2001). Galacturonic acid occurs in two major structures, which form the backbone of three polysaccharide domains; they include homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Albersheim et al., 1996). HGA is a linear homopolymer of (1-4)- α -linked-D-galacturonic acid known as the smooth region (Be Miller, 1986). RG-I is an acidic pectic domain consisting of about 100 repeats of the disaccharide (1-2)- α -L-rhamnose-(1-4)- α -D-galacturonic acid known as the hairy region (Albersheim et al., 1996). RG-II is a branched pectic domain containing an HGA backbone, which has around nine galacturonic acid residues that are (1-4)- α -linked and is substituted by heteropolymeric side chains containing 11 different sugars (O'Neill et al., 1996).

Based on the type of the backbone chain modifications, pectic substances are classified into four main types; they include protopectin, pectic acids, pectinic acid and pectin (Be Miller, 1986). Protopectin is a parent pectic substances from which pectin or pectic acid are produced upon restricted hydrolysis (Kashyab et al., 2001). Pectic acids are galacturonans that contain

negligible amounts of methoxyl groups, while pectinic acids are galacturonans that contain various amounts of methoxyl groups (Kashyab et al., 2001). Pectin is a mixture of widely differing compositions containing pectinic acid as the major component (Kashyap, 2001). The percent of methoxyl groups present in pectin is referred to as the degree of esertification (DE). Pectin with DE of more than 50 % is called high-methoxyl pectins, while low-methoxyl pectin has a DE of less than 50 % (Be Miller, 1986).

Garcia et al. (1991) found that pectin present in the pulp and mucilage of three varieties of arabica coffee from Guatemala have low DE ranging from about 19 to 31 %. Contrary, Aval-lone et al. (2000) reported an average of 62 % DE in pectin of the mucilage of Mexican arabica coffee. Avallone et al. (2000) demonstrated that the low DE obtained by Garcia et al (1991) might be as a result of partial demethoxylation occurred due to the strong acidic medium used for precipitation of pectin from coffee samples. However, low DE were found in coffee pectin obtained by precipitation using both acidic medium or 95 % ethanol (Garcia et al., 1991).

5.2. Pectinolytic enzymes

Pectinolytic enzymes or pectinases are heterogeneous group of enzymes that hydrolyze the pectic substances; they have been found in higher plants and microorganisms. They play important roles in plants by helping in cell wall extension and softening of some plant tissue during maturation and storage (Jayani et al., 2005). Pectinolytic enzymes are divided into propectinases, pectin esterases (PEs) and depolymerases (Sakai, 1992). Propectinases degrade the insoluble propectin into highly polymerized soluble pectin. Pectin esterases catalyze the de-esterification of pectin by the removal of methoxy esters. The third group of pectinases, the depolymerases catalyze the hydrolytic cleavage of the α -(1-4) glycosidic bonds in the D-galacturonic acid chain of the pectic substances (Sakai, 1992). Depending on the preference of the enzyme for substrate, the mechanism of cleavage and the splitting of the glycosidic bonds, depolymerases can be subdivided into four categories (Jayani et al., 2005). The first two are polygalacturonases (PGs) and polymethylgalacturonases (PMGs), which catalyze hydrolysis of α -(1-4) glycosidic linkages in pectic acid (polygalacturonic acid) and pectin, respectively. The other two groups of depolymerases are pectin lyases (PLs) and pectin methyl lyase (PMLs), which breakdown pectate and pectin by β elimination, respectively (Jayani et al., 2005). Furthermore, depolymerases are termed as endo or exo depending on the pattern of

their action. The enzyme that act by random cleavage of the α -(1-4) glycosidic bonds is called endo, while the term exo is given to the enzyme, which act in sequential cleavage of α -(1-4) glycosidic bonds (Kashyap et al., 2001).

5.2.1. Pectinolytic enzymes in yeasts

Secretion of pectinolytic enzymes has been reported for several yeast species. The main pectinolytic enzyme produced by yeasts is endo-PG i.e. it randomly cleaves the α -(1-4) glycosidic bonds by hydrolysis (Blanco et al., 1999). This enzyme has been found in *Rhodotorula* spp. (Vaughn et al., 1969), *Cryptococcus albidus* (Federici, 1985), *K. marxianus* (Barnby et al., 1990; Schwan & Rose, 1994; Schwan et al., 1997), *S. cerevisiae* (Blanco et al., 1994) and several species of *Candida* (Call et al., 1985; Sanchez et al., 1984; Stratilova et al., 1998). Recently, we found that PG was the only pectinolytic enzyme secreted by *P. anomala*, *P. kluyveri* and *H. uvarum* isolated from coffee fermentation (Appendix IV). Only in two cases, other pectinolytic enzymes have been found in yeasts; the first one was in *Rhodotorula* spp. involved in the softening of olives, which was reported to secrete pectin methyl esterase (Vaughn et al., 1969). In addition, Gainvors et al. (1994) found that a strain of *S. cerevisiae* isolated from champagne wine was able to secrete PL and PE. However, in both studies the main enzyme produced by the two yeasts was PG (Vaughn et al., 1969; Gainvors et al., 1994).

It has been reported that pectin, polgalacturonic acid and galacturonic acid are not required to induce PG production by most yeast species (Blanco et al., 1999). On the other hand, galacturonic acid was found to induce PG production by a strain of *Cryptococcus albidus* (Federici, 1985) and a strain of *S. cerevisiae* (Blanco et al., 1994). In another study, it has also been reported that polygalacturonic acid was required for secretion of PG by two strains of *S. cerevisiae* (Mckay, 1990). In the present study, it was found that *P. anomala*, *P. kluyveri* required polygalacturonic acid to produce PG (Appendix IV). Furthermore, the two yeast species showed scarce growth and no PG activity when grown in a laboratory medium without addition of glucose (Appendix IV), which indicates that the investigated yeasts were unable to utilize polygalacturonic acid as a sole carbon source. This agrees with the findings of Sanchez et al. (1984), who reported that the pectinolytic yeasts isolated from cocoa were unable to grow or to produce PG in a pectin medium not supplemented with glucose. However, high

glucose concentrations were reported to suppress PG production by *S. cerevisiae* (Blanco et al., 1994 & 1997), *C. albidus* (Federici, 1985) and *K. marxianus* (Schwan and Rose, 1994).

Other parameters, which influence PG production by yeasts, are the pH of growth medium, incubation time and incubation temperature. Differences in the optimum pH for PG production by different yeast species have been reported. An optimum pH of 5.0 for growth medium was recorded for PG secretion by *K. lactis* (Murad & Foda, 1992). For *Kluyveromyces wickerhammi* the optimum pH for PG secretion was in a range of 3.8-4.5 (Moyo et al., 2003). In a range of 3.0 to 8.0, the optimum pH of growth medium for production of PG by *P. anomala* S16 and *P. kluyveri* S13Y4 and a strong pectinolytic strain of *K. marxianus* CCT 3172 isolated from cocoa (Schwan & Rose, 1994) was 6.0 (Fig 5; Appendix IV). The optimum pH of growth medium for production of PG by *P. anomala* S16 and *P. kluyveri* S13Y4 appears to be close to the initial pH during coffee fermentation i.e. 5.5-6.0.

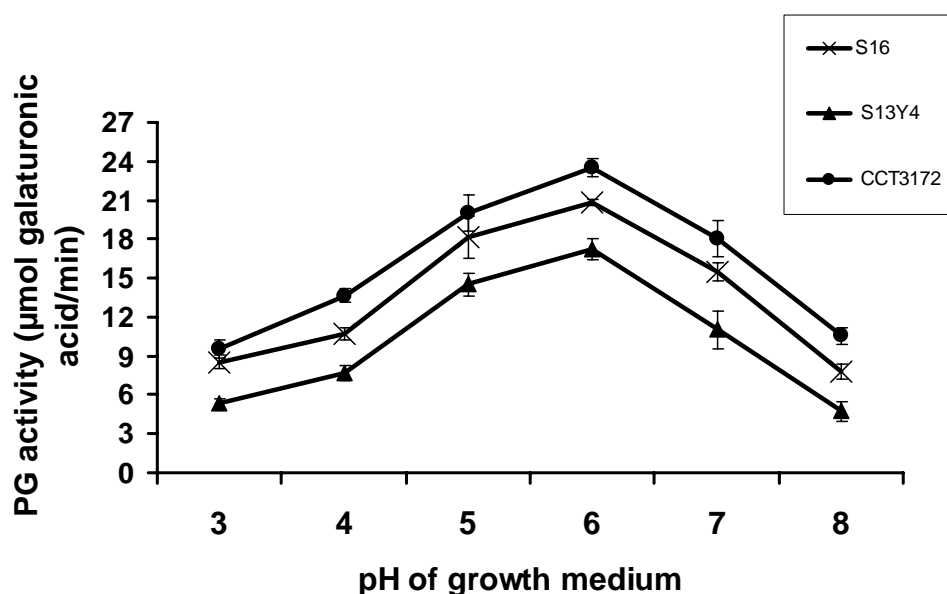


Figure 5. Production of polygalacturonase (PG) by *Pichia anomala* S16, *Pichia kluyveri* S13Y4 and a reference strain *Kluyveromyces marxianus* CCT 3172 when grown in yeast polygalacturonic medium (YPA) adjusted to different pH values (Appendix IV)

Incubation temperature has also an influence on secretion of PG by yeasts. This was investigated by Moyo et al. (2003), who found that the optimum temperature for production of polygalacturonase by *Kluyveromyces wickerhammi* was in a range of 28.5-35.5 °C. In the present work, it was observed that increasing incubation temperature from 15 to 40 °C for *P.*

anomala S16, *P. kluyveri* S13Y4 and *K. marxianus* CCT 3172 showed a maximum secretion of PG at 30 °C followed by a sharper decrease at higher temperature (Fig 6; Appendix IV).

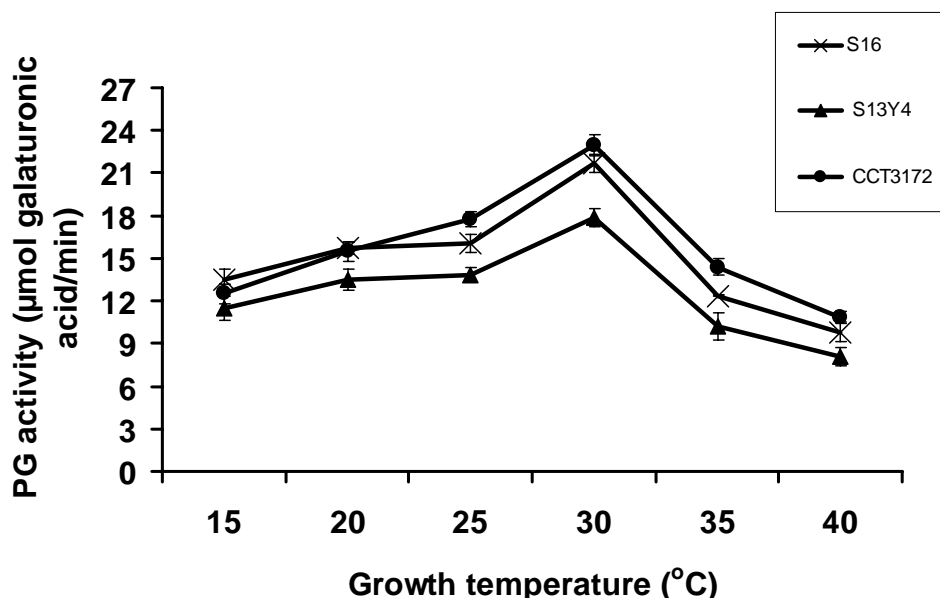


Figure 6. Production of polygalacturonase (PG) by *Pichia anomala* S16, *Pichia kluyveri* S13Y4 and a reference strain *Kluyveromyces marxianus* CCT 3172 when grown in yeast polygalacturonic medium (YPA) and incubated at different temperatures (Appendix IV)

Table 2. The amounts of Polygalacturonase (PG) produced by *Pichia anomala* S16 and *Pichia kluyveri* S13Y4 at different times of incubation.

Yeast	PG activity (μmol galaturonic acid /min)		
	24 h	48 h	72 h
<i>P. anomala</i> S16	19.2 ± 0.40 [†]	23.1 ± 0.50	4.7 ± 0.60
<i>P. kluyveri</i> S13Y4	13.1 ± 0.60	16.6 ± 0.80	3.3 ± 0.30

[†] Standard deviation for two duplicate trials

Incubation time is another parameter, which was reported to affect production of PG by yeasts. It was found that after 3 days of incubation, the amounts of PG produced by *C. albidus* (Federici, 1985), *S. cerevisiae* (Blanco et al., 1994) and *K. lactis* (Murad & Foda, 1992) decreased with longer periods of incubation. Schwan et al. (1997) reported that *K. marxianus* CCT3172 isolated from cocoa started to secrete PG in the exponential phase with maximum amount after 24 h of incubation. The maximum amounts of PG produced by *P. anomala* S16 and *P. kluyveri* S13Y4 were observed after 48 h of incubation time and markedly decreased at

72 h of incubation (Table 2). It appears that production of the maximum amounts of PG by both strains of *P. anomala* and *P. kluyveri* occurs during the time of coffee fermentation i.e. 24 to 48 h.

The stability of the PGs produced by different yeasts species under different pH and temperature has been examined in several studies (Table 3). The pH and the temperature optima of PGs activities seem to vary between yeast species as well as strains within the same species. However, the optimum pH of PGs activity for the different yeasts appear to be within the acidic region i.e. 3.5-6.0 and their optimum temperature is between 40 to 55 °C with the exception of three strains of *Saccharomyces chevalieri*, *Candida albidus* and *Saccharomyces cerevisiae* var. *chevalieri* showing optimum activity at 25, 37 and 30 °C, respectively (Table 3).

Table 3. The optimum pH and temperature for PGs activity produced by different yeast species

Yeast	Optimum pH	Optimum temperature (°C)	Reference
<i>Rhodotorula</i> spp.	6.0	50	(Vaughn et al., 1969)
<i>Trulopsis candida</i>	5.0	60	(Sanchez et al., 1984)
<i>Candida norvegensis</i>	5.0	50	(Sanchez et al., 1984)
<i>Kluyveromyces fragilis</i>	5.0	50-60	(Sanchez et al., 1984)
<i>Saccharomyces chevalieri</i>	5.0	25	(Sanchez et al., 1984)
<i>Candida albidus</i>	3.75	37	(Federici, 1985)
<i>Saccharomyces cerevisiae</i> IMI-8b	4.5	45	(Blanco et al., 1997)
<i>Saccharomyces cerevisiae</i> 1389	5.0	45	(Blanco et al., 1997)
<i>Saccharomyces cerevisiae</i> var. <i>chevalieri</i>	4.5	30	(Schwan et al., 1997)
<i>Kluyveromyces marxianus</i>	5.0	40	(Schwan et al., 1997)
<i>Kluyveromyces marxianus</i>	3.0-5.0	55	(Serrat et al., 2002)
<i>Saccharomyces cerevisiae</i>	3.5-5.5	50	(Fernández-González et al., 2004)
<i>Pichia anomala</i> S16	5.5	40	(Appendix IV)
<i>Pichia kluyveri</i> S13Y4	5.0	50	(Appendix IV)

In the present study, the PG produced by *P. anomala* S16 was found to increase by increasing pH above 3.0 with an optimum activity at pH 5.5. For PG produced by *P. kluyveri* S13Y4,

the maximum activity was observed at pH 5.0. For the two yeasts, PG activity decreased rapidly at pH above 6.0 and being inactive at pH 8.0 (Appendix IV). The pH range for the increasing activity of PG produced *P. anomala* S16 and *P. kluyveri* S13Y4 is within the pH range reported for coffee fermentation which indicates that the yeasts in the present study originating from coffee fermentation might have a role in degradation of pectinolytic mucilage surrounding coffee beans. Contrary, pectin lyase produced by *Klebsiella pneumoniae* and *Erwinia hericola* isolated from coffee has an optimum activity at pH 8.5 (Avallone et al., 2002) and pectin lyase secreted by *Paenibacillus amylolyticus* has an optimum activity at 7.9 (Sakiyama et al., 2001), which is far from the acidic coffee fermentation conditions.

5.3. Mucilage degradation

It is generally assumed that the main aim of coffee fermentation is to degrade the pectin rich mucilage adhering to the coffee beans. Due to that reason, pectinolytic microorganisms seem to be of particular importance in the fermentation of coffee. In studies that have been carried out to date, disagreements exist concerning the dominant group of microorganisms present during fermentation and their role in the degradation of mucilage. Early studies of arabica coffee fermentation in Mexico and Colombia (Pederson & Breed, 1946), demonstrated that several species of bacteria, filamentous fungi and yeasts were associated with the fermentation. It was shown that during fermentation, acid-producing bacteria became dominant, which lead to the conclusion that the fermentation was a typical lactic acid fermentation by strains of *Leuconoctoc mesenterioides*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and strains related to *Enterococcus faecalis* (Pederson & Breed, 1946). On the other hand, Vaughn *et al.* (1958) reported the dominance of the pectinolytic Gram-negative bacteria belonging to the genera *Enterobacter* and *Escherichia* in dry and semi-dry processed Brazilian coffee. From wet processed coffee in Hawaii, Frank et al. (1965) found that the pectinolytic *Enterobacter dissolvens* predominated during fermentation with very low incidence of lactic acid bacteria and yeasts. However, Agate and Bhat (1966) isolated pectinolytic yeasts from fermented cherries of robusta harvested in India. The isolated yeasts were identified as *K. marxianus*, *S. bayanus*, *S. cerevisiae* var. *ellipsoideus*, and *Schizosaccharomyces* species. *Streptococcus* species and Gram-negative bacteria belonging to the genera *Pseudomonas*, *Flavobacterium*, and *Proteus* were also isolated from the same coffee samples. Agate and Bhat (1966) suggested that in the absence of other pectinolytic microorganisms and in the presence of large numbers of pectinolytic yeasts, the yeasts play an important role in the degradation of pulp and mucilage.

lage during coffee fermentation. Van Pee and Castelein (1971) isolated several *Candida* species and *K. marxianus* from robusta coffee samples; but no pectinolytic activity was detected in the isolated yeasts. Contrary, *K. marxianus* which was isolated from wastewater of coffee wet processing was found to be a strong producer of PG (Serrat et al., 2002). In another study, Van Pee and Castelein (1972) reported that bacteria isolated from robusta coffee harvested in Congo belong to the family *Enterobacteriaceae*; *Enterobacter dissolvens* was dominant and found to have a pectinolytic activity; but further investigation of the specific enzyme produced by *E. dissolvens* showed that this bacterium was only able to form pectinase capable to break down de-esterified pectic substances, such as pectic acid and not the esterified pectin. A subsequent study by Castelein and Pilnik (1976), who investigated the properties of pectin lyase produced by *E. dissolvens*, showed that the isolated enzyme of *E. dissolvens* only act on partially or fully de-esterified pectic substances and that the optimum pH of the enzyme activity was 8.5.

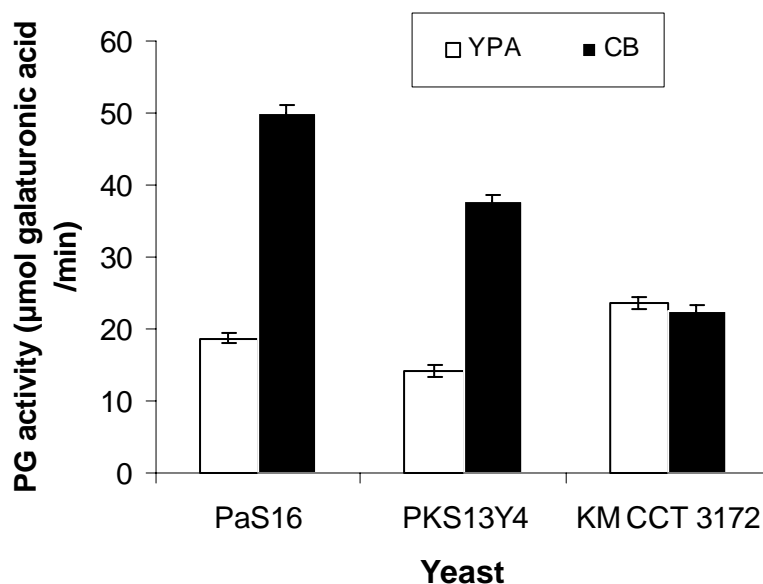


Figure 7. Production of polygalacturonase (PG) by *Pichia anomala* S16, *Pichia kluyveri* S13Y4 and a reference strain *Kluyveromyces marxianus* CCT 3172 grown in yeast polygalacturonic medium (YPA) and in coffee broth (CB)

In this study, *P. anomala* S16 and *P. kluyveri* S13Y4 were found to significantly produce higher amounts of PG when grown in coffee broth (CB) compared to yeast polygalacturonic

acid broth (YPA) (Fig 7). On the other hand, the amount of PG secreted by the reference strain *K. marxianus* CCT 3172 isolated from cocoa (Schwan & Rose, 1994) was slightly higher when this yeast was grown in YPA broth compared to CB. It appears that coffee is a good substrate for *P. anomala* S16 and *P. kluyveri* S13Y4 to produce high amounts of PG. In addition, the PG secreted by *P. anomala* S16 and *P. kluyveri* S13Y4 has optimum activity within the acidic region during coffee fermentation. The two yeast species seem to have a role in degradation of coffee mucilage.

6. Ochratoxin A (OTA) in coffee

Ochratoxin A (OTA) is a secondary fungal metabolite of toxigenic species of *Aspergillus* and *Penicillium*. OTA was first identified by Van der Merwe et al. (1965), who found that *Aspergillus ochraceus* found in tropical regions is responsible for production of this toxin. In temperate regions, OTA was reported to be secreted by *Penicillium viridicatum* (Ciegler et al., 1973), which was then shown to be *Penicillium verrucosum* but not *P. viridicatum* (Pitt, 1987).

Ochratoxin A is a colourless, crystalline compound that is soluble in polar organic solvents and dilute sodium bicarbonate solution and slightly soluble in water (Scott, 1994). The chemical structure of OTA is shown in Fig 8. OTA contains a 7-carboxy-5-chloro-8-hydroxy-3, 4-dihydro-3R-methylisocoumarin ring linked through the 7-carboxy group to the amino acid L- β -phenylalanine by an amide bond, which is very stable with regard to temperature (Petzinger & Ziegler, 2000).

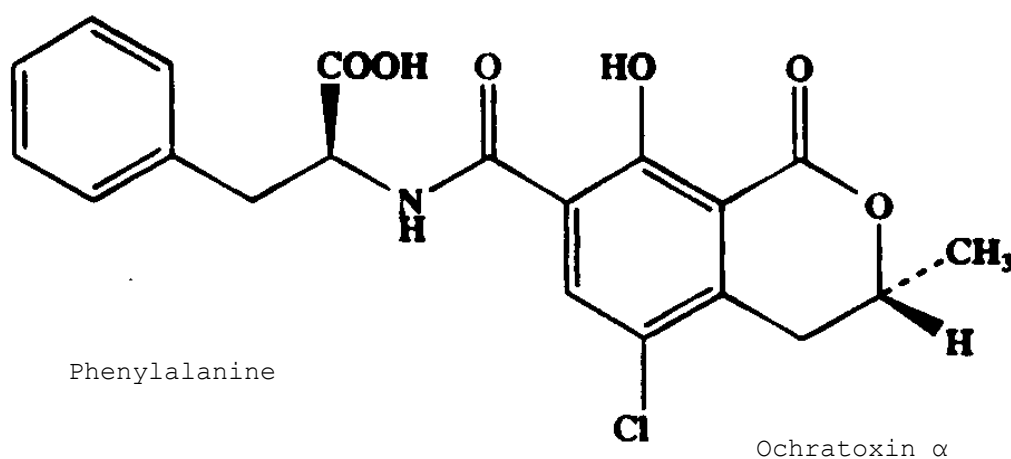


Figure 8. Chemical structure of ochratoxin A (OTA)

Ochratoxin A has been shown to be nephrotoxic, hepatotoxic, genotoxic, immunotoxic, teratogenic, and possibly carcinogenic (Petzinger and Ziegler, 2000). It has been implicated in the irreversible and fatal kidney disease referred to as Balkan Endemic Nephropathy (Krogh *et al.*, 1977). Furthermore, it is the main cause of renal disease accompanied with a high risk factor for urinary tract tumours (Höhler, 1998; Petzinger and Ziegler, 2000). The mode of action of OTA is poorly understood. It has been suggested that the mechanism of toxicity of OTA might be explained by inhibition of mitochondrial respiration associated with ATP depletion, enhanced lipid peroxidation or inhibition of tRNA synthetase accompanied by a reduced protein synthesis (Röschenthaler *et al.*, 1984; Marquardt & Frohlich, 1992).

Ochratoxin A is found in a variety of plant and animal products. It has been detected in cereals, cocoa, coffee, beer, wine (Petzinger & Ziegler, 2000) as well as in pork and poultry meat (Jørgensen, 1998). Due to consumption of OTA contaminated food, OTA has been detected in human blood serum (Radic *et al.*, 1997) and in human breast milk (Miraglia *et al.*, 1995).

6.1. Occurrence of Ochratoxin A in coffee

Among food and beverages, coffee has been considered to be one of the significant sources of OTA in human diet (JECFA, 2001; Anonymous, 2005). Recently, the European Commission established maximum levels of OTA in coffee, wine and grape juice (Anonymous, 2005). The maximum levels of OTA were set to be 5.0 µg/kg for roasted coffee beans and ground roasted coffee, 10.0 µg/kg for soluble coffee and 2.0 µg/kg for wine, grape must based drinks, grape juice and grape juice ingredients in other drinks (Anonymous, 2005).

Several studies on the occurrence of OTA and OTA producing fungi in green coffee beans have been carried out (Levi *et al.*, 1974; Levi, 1980; Mislivic *et al.*, 1983; Micco *et al.*, 1989; Studer-Rohr *et al.*, 1995; Nakajima *et al.*, 1997; Romani *et al.*, 2000; Joosten *et al.*, 2001; Ottender & Majerus, 2001; Urbano *et al.*, 2001; Pittet and Royer, 2002; Pardo *et al.*, 2004; Suárez-Quiroz *et al.*, 2004; Palacios-Cabrera *et al.*, 2004; Nehad *et al.*, 2005). Micco *et al.* (1989) studied 29 samples of green coffee and found that 58 % of examined samples to be contaminated with OTA at values of 0.2 µg/kg to 15 µg/kg. In addition, Studer-Rohr *et al.* (1995) examined 25 samples of green coffee beans and found 52 % contaminated with OTA levels of 1.2 µg/kg to 56.0 µg/kg. Furthermore, Nakajima *et al.* (1997) examined 47 samples of commercial green beans imported from Central and South America, Africa and Asia col-

lected in Japan (1988-1993); they detected OTA in 30 % of samples with values of 0.1 to 17.4 µg/kg. The OTA content in coffee products from the German market (1995-1999) was evaluated by Ottender and Majerus (2001), who examined 82 samples of green beans of unknown origin; they found that 27 % were contaminated with OTA with levels ranged from less than 0.23 to 24.5 µg/kg. The contamination rate and the levels of OTA appear to differ considerably with the rate of contaminated samples varying from less than 1 % (Levi, 1980) to 65 % (Romani *et al.*, 2000) and OTA levels ranging from 0.1 µg/kg (Nakajima *et al.*, 1997) to 360 µg/kg (Levi *et al.*, 1974). Furthermore, green coffee beans from African origin were found to have significantly higher OTA values than beans from America and Asia (Pardo *et al.*, 2004). The prevalence of OTA contamination was suggested to depend on the processing method with higher risk of OTA contamination in coffee beans obtained by the dry process compared to those obtained from the wet process (Ngabirano *et al.*, 2001). The bean type of coffee i.e. arabica or robusta were reported to have no influence on the OTA content (Micco *et al.*, 1989; Nakajima *et al.*, 1997).

It has been demonstrated that OTA in green coffee beans can be reduced by roasting. The effect of roasting on both artificial and natural OTA contaminated coffee samples was found to reduce OTA contamination in percentages of 48 % to 87 % and 90 % to 100 %, respectively (Micco *et al.*, 1989). Furthermore, Blanc *et al.* (1998) examined coffee samples naturally contaminated with low levels of OTA (4.0-22.1 µg/kg) and found significant reduction of 84 % after roasting. It was suggested that OTA degradation during roasting could be attributed to both thermal destruction and silver skin removal. Same findings were reported by van der Stegen *et al.* (2001), who showed that a 69 % reduction of the OTA content during roasting of naturally contaminated green coffee beans. Urbano *et al.* (2001) investigated artificially contaminated green coffee beans at different temperatures (200 °C, 210 °C, 220 °C) and time (10 min, 15 min). It was found that OTA reduction depended on both roasting temperature and time with the most efficient roasting at 220 °C for 15 minutes with 94 % OTA reduction. On the other hand, Tsubouchi *et al.* (1987) found that only 0 % to 12 % of OTA reduction was obtained after roasting of artificial contaminated coffee beans at 200 °C. Studer-Rohr *et al.* (1995) reported that roasting at 250 °C for 150 seconds of naturally contaminated green beans or beans inoculated with *A. ochraceus* resulted in a reduction of less than 30 % in the OTA levels. A percentage of about 30 % reduction in OTA content was reported as a result of roasting at 180 °C for 10 min of *A. ochraceus* inoculated green coffee beans (Nehad *et al.*,

2005). The differences between the findings obtained from studies carried out might be due to the different methods used in contaminating coffee beans with OTA. The way of contamination varied over natural contamination, artificial inoculation with *A. ochraceus* or direct spiking with OTA. Levi *et al.* (1974) and Micco *et al.* (1989) spiked pure OTA directly to the coffee beans, and it is possible that OTA is removed with the loss of silver skin during roasting. On the other hand, Nehad *et al.* (2005) inoculated green beans with OTA producing strain of *A. ochraceus* and suggested from the low percentages of OTA reduction that the produced OTA may be more strongly heat-resistant due to mode of OTA binding to the beans than spiked OTA. This is in contrast to other studies using natural OTA contamination (Micco *et al.*, 1989; Blanc *et al.*, 1998; van der Stegen *et al.*, 2001; Urbano *et al.* 2001), where high percentages of OTA reduction during roasting were observed. Inhomogeneity and roasting conditions may also be reasons for the discrepancy between findings.

Subsequent operations on coffee such as soluble (instant) coffee manufacturing and brewing were also reported to reduce OTA content in coffee (Blanc *et al.*, 1998; Perez de Obanos *et al.*, 2005). In a study by Blanc *et al.* (1998), a reduction of 20 % in OTA after soluble coffee manufacturing was observed. Recently, different percentages of OTA reduction were reported depending on the brewing machine used with OTA reduction of about 50, 32 and 15 % when using espresso coffee maker, moka brewing and auto-drip, respectively (Perez de Obanos *et al.*, 2005). On the other hand, Van der Stegen *et al.* (1997) were unable to detect any OTA reduction after coffee brewing. In a recent study, Suarez-Quiroz *et al.* (2005) observed that the degree of roasting i.e. light to medium to dark is proportional to OTA loss in coffee; however, after coffee brewing OTA content detected was more than that found in the roasted coffee when using filter or plunger coffee brewing, which was not observed in espresso coffee due to the short time spent in contact with water. It was suggested that OTA bound to coffee components might be masked by reactions occurred during roasting; which lead to the conclusion that it is better to have green coffee beans free or with low content of OTA than accounting on reduction of OTA during roasting or brewing (Suarez-Quiroz *et al.*, 2005).

6.2. Ochratoxin A producing fungi in coffee

Coffee is grown and processed in tropical and subtropical climates, which favour growth of some species of *Aspergillus* that are known to produce OTA. OTA producing strains of *A. ochraceus*, *A. carbonarius* and *A. niger* have been isolated from coffee beans. A survey on

OTA producing fungi from 944 samples collected in 1979 to 1981 from 31 different coffee producing countries in Africa, Asia, and Central and South America showed *A. ochraceus* and *A. niger* were found to be dominant (Mislivec *et al.*, 1983). In addition, Joosten *et al.* (2001) found *A. carbonarius* in 50 % samples of Thai coffee whereas *A. ochraceus* was only isolated from one sample. A rare occurrence of *A. ochraceus* was also reported by Nakajima *et al.* (1997). On the contrary, several studies reported that *A. ochraceus* to be more common than *A. carbonarius* in green coffee beans (Téren *et al.*, 1997; Ngabirano *et al.*, 2001; Pitt *et al.*, 2001; Varga *et al.*, 2001). From 86 unidentified fungal isolates obtained from Tanzanian green coffee beans, three isolates of *A. ochraceus* were found to produce OTA (INCO: ICA4-CT-2001-10061, second annual report, 2003). Pitt *et al.* (2001) examined 407 samples of Brazilian coffee taken at different stages of processing and found 872 isolates, which belong to *A. niger* (63 %), *A. ochraceus* (31 %), and *A. carbonarius* (6 %). Although *A. niger* is frequently isolated as the predominant species of green coffee beans and known to produce OTA, the production was found to be minimal compared with *A. ochraceus* and *A. carbonarius* (Téren *et al.*, 1996; Nakajima *et al.*, 1997; Heenan *et al.*, 1998; Pitt *et al.*, 2001; Taniwaki *et al.*, 2003). It seems that *A. niger* is probably a relatively unimportant source of OTA in coffee.

Fungal growth and OTA production are affected by water activity of the coffee cherries and beans as well as climatic and geographical conditions in coffee growing areas. *Aspergillus ochraceus* grows at temperatures ranging from 8°C to 37 °C (Norholt *et al.*, 1979) with optimum between 24 °C and 37 °C (Sweeney & Dobson, 1998). *Aspergillus ochraceus* grows well at pH values of 3 and 10, with optimum of 3.5 and 4.0 at temperatures of 25°C and 30°C, respectively (Wheeler *et al.*, 1991). While the optimum water activity for growth of *A. ochraceus* is between 0.95 and 0.99 (Sweeney & Dobson, 1998), *A. ochraceus* is able to grow at a water activity as low as 0.771 due to its xerotolerant nature (Pitt & Christian, 1968). *Aspergillus carbonarius* grows at 8 °C to 41 °C with optimum between 35 °C and 37 °C (Viani, 2002) and at a water activity of or above 0.90 (Bucheli *et al.*, 2001). Taniwaki *et al.* (2001) evaluated the influence of water activity on OTA production by *A. ochraceus* and *A. carbonarius* in green coffee beans and found that both species were able to produce OTA above a water activity of 0.80 at 25 °C. On the other hand, neither Bucheli *et al.* (2001) nor Joosten *et al.* (2001) detected OTA production by *A. carbonarius* at a water activity below 0.94 at 25 °C. In general, OTA production was favoured by high water activity, with an optimum at 0.99 for both *Aspergillus* species (Norholt *et al.*, 1979; Bucheli *et al.*, 2001; Taniwaki *et al.*, 2001).

Comparing the conditions for growth and OTA production of *A. ochraceus* and *A. carbonarius*, *A. ochraceus* is able to grow at lower water activities than *A. carbonarius*, whereas both produce OTA under same conditions.

It is not exactly known when fungal growth and production of OTA take place along the coffee processing chain. Coffee beans can be infected by OTA producing fungi at the different stages of coffee production, which will lead to OTA contamination. It has been demonstrated that accumulation of OTA in coffee seems to occur before storage of green coffee beans and it appears to be associated with post-harvest conditions of coffee processing (Bucheli *et al.*, 1998, 2000). It was found that ripe cherries were not contaminated with OTA at harvest, whereas damaged and overripe cherries were commonly contaminated with OTA (Bucheli *et al.*, 2000). Panneerselvam *et al.* (2001) reported that the genera *Aspergillus* and *Penicillium* were able to easily penetrate into overripe cherries compared to ripe cherries. Teixeira *et al.* (2001) found that overripe cherries sampled from the ground were more infected with *A. ochraceus* and *A. carbonarius* than ripe cherries picked from trees. Same findings were obtained by Taniwaki *et al.* (2003), who reported that coffee cherries taken from ground and coffee beans during drying and storage were higher in OTA contamination compared to coffee cherries picked directly from trees. It has been demonstrated that storage of contaminated green coffee beans with *A. ochraceus* at high humidity showed high levels of OTA at changing temperatures compared to a constant temperature (Palacios-Cabrera *et al.*, 2004).

High quality processed green coffee beans free from fungal and OTA contamination and good storage conditions appear to be the key factors for lowering the risk of OTA contamination. Application of Good Manufacturing Practice (GMP) and implementation of Hazard Analysis Critical Control Point (HACCP) system along the coffee processing chain i.e. from picking of cherries through drying of coffee beans and storage might help to reduce or prevent OTA contamination in coffee beans. Furthermore, biological control (biocontrol) could provide additional protection against OTA contamination in green coffee beans. The use of microorganisms predominating during coffee fermentation as starter cultures i.e. LAB and yeasts, which are capable of inhibiting fungal growth and OTA formation seems to be a promising tool to reduce or prevent OTA contamination in coffee. In the present thesis, it was found that *P. anomala*, *P. kluyveri* and *H. uvarum* isolated from coffee were able to inhibit growth and OTA formation by *A. ochraceus* (Appendix II, III). Preliminary study of the antifungal activ-

ity of LAB isolated from Tanzanian coffee, showed that *Lactobacillus* spp. and *Lactobacillus plantarum* strongly inhibited growth of *A. ochraceus* (INCO: ICA4-CT-2001-10061, second annual report, 2003).

Another way to eliminate OTA from contaminated green coffee beans is the use of microorganisms that are capable of degrading or adsorbing OTA. It has been reported that some filamentous fungi and bacteria are able to degrade OTA. Varga *et al.* (2000) examined several species of *Aspergillus* for their ability to degrade OTA added to a laboratory substrate; it was found that only isolates of the toxigenic *Aspergillus fumigatus* and only one nontoxigenic *A. niger* strain were found to effectively eliminate OTA. In addition, Stander *et al.* (2000) found that lipase from *A. niger* was able to degrade OTA to the non toxic compound ochratoxin α (OT α). In a recent study, it was reported that OTA added to liquid medium was degraded by some strains of *Rhizopus stolonifer*, *R. microsporus*, *R. homothallicus* and *R. oryzae* (Varga *et al.*, 2005). Addition of *Lactobacillus* and *Streptococcus* and *Bifidobacterium* was observed to decrease OTA content in contaminated milk (Skrinjar *et al.*, 1996). To our knowledge, yeasts were not reported to degrade OTA. It has been found that *S. cerevisiae* and *S. bayanus* were able to significantly reduce the amount of OTA in synthetic and natural grape juice by adsorption of the toxin to yeast cells but not by degradation as both heat and acid treated cells enhanced removal of OTA (Bejaoui *et al.*, 2004). Same findings were obtained by Cecchini *et al.* (2006) on the effect of *S. cerevisiae* and other yeast species in red and white wine, which were also explained by OTA adsorption to yeast cells but not by degradation. Eliminating OTA from green coffee beans by addition of a particular microorganism that is capable of degrading or adsorbing OTA appears to be a good tool. However, the addition of microorganisms to green coffee beans after fermentation and drying might cause off flavour and colour defects of coffee beans.

7. The antifungal activity of yeasts in coffee processing

7.1. Yeasts as biocontrol agents during coffee processing

As mentioned earlier, varying levels of ochratoxin A (OTA) were found in green coffee beans from coffee producing countries in Africa, Asia, and Central and South America. At present, the information about production of OTA in coffee and how the problem should be addressed is inadequate. The major reasons appear to be lack of knowledge of the complex microbiology in the coffee environment and the lack of effective quality systems in the coffee producing countries. Development of simple biocontrol method, which is based on a deep understanding of the underlying microbiological and biochemical processes during coffee fermentation can help in minimizing mould growth and OTA production. Yeasts are among microorganisms, which were found to control many pathogens in fruits and vegetables. Several yeast species were reported to have antagonist activity towards several plant pathogenic fungi. It has been found that pathogens of sweet cherries *Alternaria alternata*, *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer* were inhibited by *Trichosporon pullulans*, *Cryptococcus laurentii*, *Rhodotorula glutinis* and *Pichia membranefaciens* (Qin et al., 2004). In addition, *Debaryomyces hansenii* was found to control growth of *Penicillium digitatum* on grape fruit (Droby et al., 1989). *Candida sake* was also found to be very effective against *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus nigricans* (Viñas et al., 1998). *Pichia anomala* was reported to be antagonistic against several fungal species such as *Botrytis cinerea* responsible for grey mold disease in grape-vine (Masih et al., 2000), wood decay basidiomycetes, plant pathogenic fungi (Walker et al., 1995). Furthermore, *P. anomala* was found to inhibit a number of filamentous fungi in high moisture wheat with the strongest effect on growth of *P. roqueforti* (Petersson & Schnürer, 1995). Growth and production of OTA by *P. verrucosum* were also found to be reduced markedly by *P. anomala* (Petersson et al., 1998). In this study, we found that the yeasts predominating during coffee fermentation and drying i.e. *P. anomala*, *P. kluyveri* and *H. uvarum* inhibited growth and OTA production by two strains of *A. ochraceus* when grown together on malt extract agar (MEA) and on coffee agar (CA) (Appendix III). However, differences were found between yeasts species and between strains of the same species in inhibition of fungal growth with strains of *P. anomala* and *P. kluyveri*

showing stronger effect than *H. uvarum* (Fig 9). To prevent fungal growth and OTA formation, strains of *P. anomala* and *P. kluyveri* might be used as biocontrols during coffee processing. However, the effects of the two yeasts on the quality of green coffee beans should be studied.

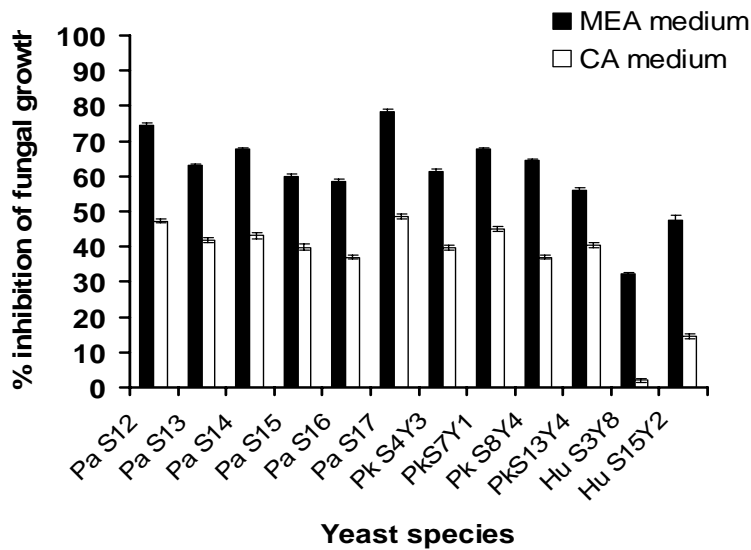


Figure 9. Growth inhibition of *Aspergillus ochraceus* B722 on MEA and CA plates inoculated with six strains of *Pichia anomala* (S12, S13, S14, S15, S16, S17), four strains of *Pichia kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4) and two strains of *Hanseniaspora uvarum* (S3Y8, S15Y2). Inhibition is expressed as the percentage of reduction of the fungal colony diameter compared to the control (fungal colony diameter on free yeast plates). Bars represent standard deviations. (Appendix III).

7.2. Mechanisms of yeast antifungal activity

Due to difficulties encountered when studying complex interactions between host, pathogen, antagonist and other microorganisms present, the mechanism of the antagonist activity of yeasts against filamentous fungi is not fully explored. A good understanding of the mode of action of the antagonist activity will help to clarify the mechanism behind it. In most studies carried out, the antagonist activity of yeasts was explained by a combination of two mechanisms or more. Various mechanisms behind the antagonist activity of yeasts against filamentous fungi have been suggested. Among others, they include competition for nutrients, production of killer toxins, production of volatile metabolites, parasitism and resistant induction in the host tissue.

7.2.1. Competition for nutrients

The mode of action of the antagonist activity of several yeasts against filamentous fungi has been suggested to be explained by competition for nutrients. Droby et al. (1989) suggested that the mechanism of the antagonist activity of *D. hansenii* against *P. digitatum* in grapefruit might be due to competition for nutrients because the antagonist activity was overcome by the addition of exogenous nutrients to grapefruit. It has also been demonstrated that the antagonist activity of *Sporobolomyces roseus* and *Cryptococcus laurentii* against the grey mould *Botrytis cinerea* was due to competition for sugars (Filonow, 1998). Furthermore, competition for nutrients and production of toxic metabolites were suggested to be the main reasons for growth inhibition of postharvest pathogenic fungi of apple fruit by *Metschnikowia pulcherrima* (Spadaro et al., 2002). In the present study, it was found that *P. anomala* inhibited spore germination of *A. ochraceus* when grown together in malt yeast glucose peptone (MYGP) broth (Appendix III, Fig 10). In MYGP yeasts free supernatant, spores of *A. ochraceus* were able to germinate only after 72 h of inoculation but with very short germ tubes (Fig 10). Changes of the pH of growth medium by yeast cells were found to have no effect on germination of fungal spores (Appendix III). Same findings were obtained for *P. kluyveri* and *H. uvarum* (Appendix III). It seems that depletion of the amounts of glucose in MYGP and/or production of toxic metabolites by the investigated yeasts might result in reduction of spore germination of *A. ochraceus*.

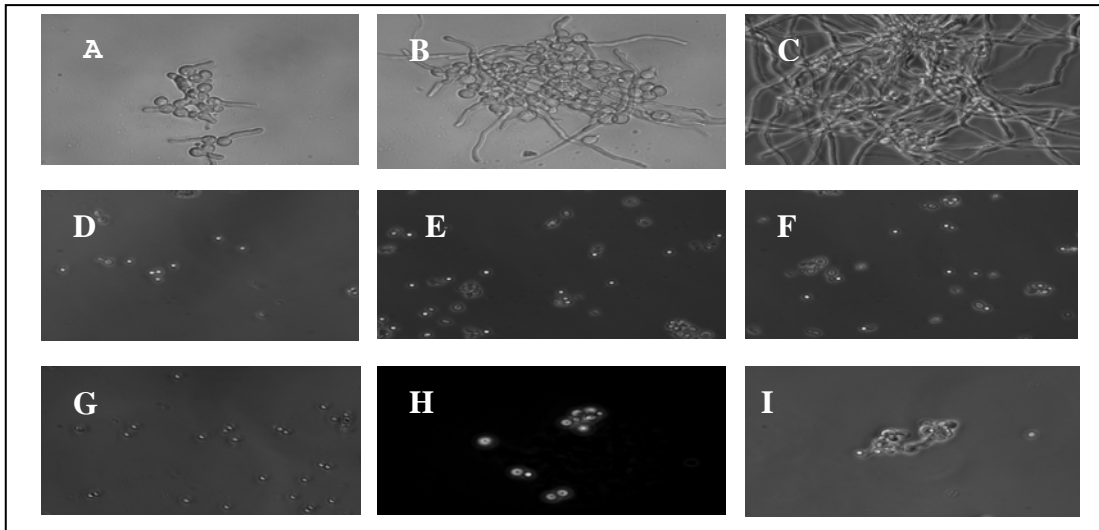


Figure 10. Germination of *Aspergillus ochraceus* B722 spores incubated at 30 °C when inoculated in MYGP broth without yeast after 24 h (A), 48 h (B), 72 h (C); when co-cultured with cells of *Pichia anomala* S12 in MYGP broth after 24 h (D), 48 h (E), 72 h (F); when inoculated in MYGP yeast free supernatant after 24 h (G), 48 h (H), 72 h (I). (Appendix III).

7.2.2. Production of killer toxins

Killer toxins can be defined as low molecular mass protein or glycoprotein produced by certain yeasts, which kill sensitive cells of other yeasts and fungi without direct cell-cell contact. Production of killer toxins is a widespread phenomenon among several yeasts such as the genera *Saccharomyces*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Hanseniaspora* (Philliskrik & Young, 1975; Stumm et al., 1977; Radler et al, 1985). Most studies carried out reported the effect of toxins produced by killer yeasts against other yeasts. The effect of yeasts killer toxins on filamentous fungi has been reported as well in few cases. Walker et al. (1995) found that strains of *S. cerevisiae* and *P. anomala* produced killer toxins, which were effective against wood decay basidiomycetes and plant pathogenic fungi. Furthermore, the killer strain of *Pichia membranifaciens* was reported to inhibit growth of *Botrytis cinerea* responsible for spoilage of onion, potato, strawberry, grapes and wine (Santos et al., 2004). The killer toxin produced by a strain *P. anomala* was suggested to contribute to the antifungal activity against *Penicillium roqueforti* (Fredlund et al., 2002).

Beside *P. Anomala*, strains of *P. kluyveri* and *H. uvarum* are known as producers of killer toxins (Radler et al, 1985; Zorg et al., 1988; Walker et al., 1995; Abranches et al., 1997; Zagroc et al., 2001). In this thesis, the antagonist activity of *P. anomala*, *P. kluyveri* and *H.*

uvarum against *A. ochraceus* (Appendix III) might also be contributed by killer toxins produced by the three yeasts. However, a study on the ability of the investigated strains of *P. anomala*, *P. kluyveri* and *H. uvarum* to produce killer toxins is needed.

7.2.3. Production of volatile metabolites

Volatile compounds produced by yeasts include alcohols, esters, fatty acids, aldehydes, ketones and sulfur containing compounds (Dufour et al., 2003). Yeasts volatiles are important flavour determinants in wine, beer, other alcoholic beverages and fermented food. Volatile metabolites produced by yeasts seem also to contribute in their antifungal activity. Taczman-Brückner et al. (2005) demonstrated the inhibitory effect of volatile compounds produced by *K. lactis* against growth of *Penicillium expansum*. It has been reported that among the volatile compounds produced by *Muscodor albus*, esters were reported to be the most effective class of inhibitory compounds against plant and human pathogenic fungi and bacteria (Strobel et al., 2001). Fredlund et al. (2004) reported the inhibitory effect of ethyl acetate, which is a major ester produced by *P. anomala*, on growth of *Penicillium roqueforti*. In brewers yeasts, volatile compounds are produced by biochemical transformation originating from sugar or nitrogen metabolism (Fig 11). Esters in yeasts are synthesized either from alcohols and acetyl coenzyme A catalyzed by the enzyme alcohol acetyltransferase or from alcohols and acetic acid by the reversed reaction of the esterase enzyme (Yoshioka & Hashimoto, 1981). The use of one or both of the two pathways for esters synthesis depends on the yeast species as well as the type of ester produced. It has been reported that synthesis of ethyl acetate in *S. cerevisiae* takes place by both pathways, while isoamyl acetate was only produced by the alcohol acetyltransferase pathway (Yoshioka & Hashimoto, 1981; Inoue et al., 1997; Fukuda et al., 1998). On the other hand, *P. anomala* was reported to mainly use the reverse reaction of esterase for production of ethyl acetate and isoamyl acetate (Yoshioka & Hashimoto, 1981; Rojas et al., 2002)

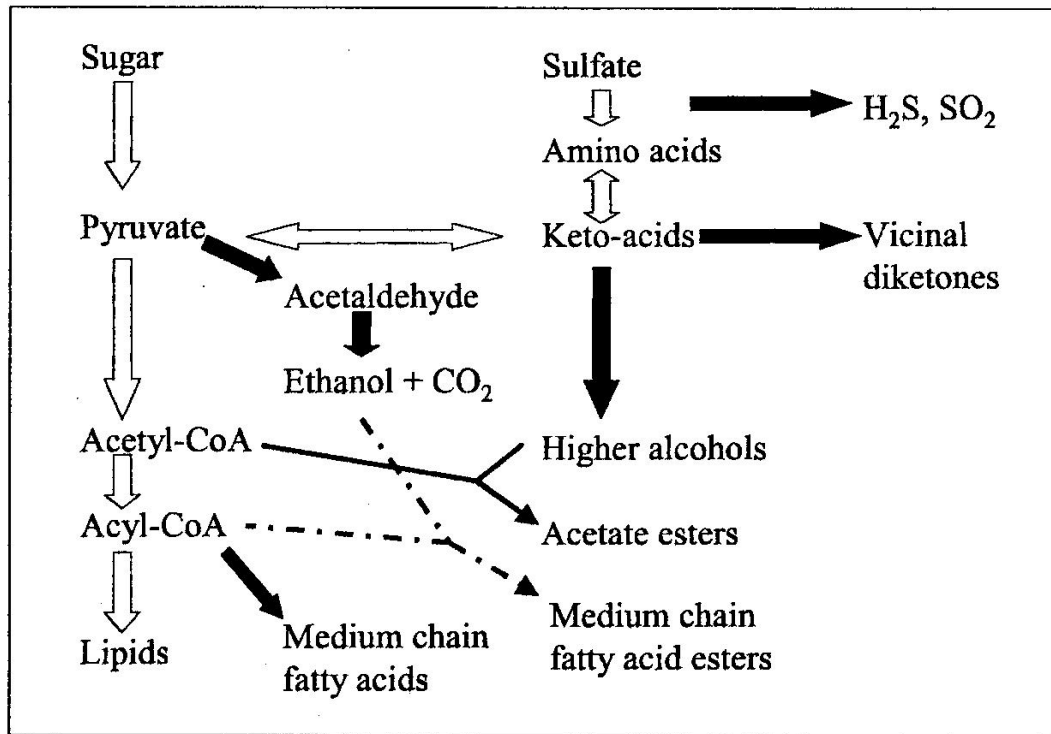


Figure 11. Formation of alcohols, esters, fatty acids, aldehydes, ketones and sulfur containing compounds in brewers yeasts (Dufour et al., 2003).

In this study, the main volatiles produced by *P. anomala*, *P. kluyveri* and *H. uvarum* were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate and isoamyl alcohol (Table 4). Furthermore, the volatiles produced by the three yeasts were found to have inhibitory effects against growth and OTA production by *A. ochraceus* (Appendix II). In our investigation, small differences were found in the profiles of volatiles produced by *P. anomala*, *P. kluyveri* and *H. uvarum* (Table 4). The effect of the individual esters and alcohols produced by the three yeasts on growth of *A. ochraceus* showed that 2-phenyl ethyl acetate was the most effective compound against fungal growth and OTA formation (Appendix II). On the other hand, ethyl acetate reduced fungal growth only at high concentrations while OTA formation was not affected at all concentrations tested (Appendix II). It seems that the volatile compounds, mainly 2-phenyl ethyl acetate produced by the three yeasts contribute largely to their antifungal activity.

Table 4. The profiles of volatile compounds identified for four strains of *Pichia anomala* (P.a), two strains of *Pichia kluyveri* (P.k), one strain of *Hanseniaspora uvarum* (H.u) in headspace above MYGP plates after 48 h of incubation at 25°C (Appendix II).

Compound	Concentrations of volatiles in the headspace above yeasts cultures (µg / l) ¹						
	P.a S12	P.a S13	P.a S16	P.a S17	P.k S8Y4	P.k S7Y1	H.u S15Y2
Ethyl acetate	65.0	42.0	31.0	74.0	69.0	54.0	49.0
Ethyl propionate	10.0	14.0	12.0	6.0	2.0	5.0	3.0
Propyl acetate	8.0	10.0	3.0	14.0	-	-	2.0
Isobutyl acetate	50.0	37.0	23.0	62.0	34.0	53.0	21.0
Propyl propionate	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	-
Butyl acetate	2.0	≤ 1	≤ 1	5.0	≤ 1	≤ 1	≤ 1
Isobutyl alcohol	3.0	4.0	≤ 1	5.0	≤ 1	≤ 1	≤ 1
Isopentyl acetate	3.0	4.0	≤ 1	5.0	4.0	3.0	-
Butanoic acid octyl ester	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Isoamyl alcohol	7.0	4.0	6.0	10.0	7.0	5.0	≤ 1
Acetoin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
2-phenyl ethyl acetate	23.0	19.0	17.0	27.0	16.0	17.0	5.0
Phenyl ethyl alcohol	3.0	≤ 1	≤ 1	4.0	≤ 1	≤ 1	≤ 1
Isobutyl propanoate	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1

¹ average values for three trials for each volatile compound determined for each yeast. Standard deviations for all trials were in a range of 0.5 to 2.0

Growth substrate seems to affect the inhibitory effect of the volatile compounds against *A. ochraceus* (Appendix II). The effect of exposure of yeasts to *A. ochraceus* was significantly stronger when yeasts were grown on MYGP plates compared to coffee agar (Appendix II). This is probably due to the higher concentration of glucose in MYGP substrate i.e. 1 % w/v compared to CA. In green coffee beans, the concentrations of glucose and fructose was reported to be around 0.03% and 0.04% dry weight, respectively (Rogers et al., 1999). Druvefors et al. (2005) found that addition of glucose to wheat increased production of ethanol and ethyl acetate by *P. anomala* as well as its antagonist activity against *P. roqueforti*. *Saccharomyces cerevisiae* was also found to produce higher amounts of esters and alcohols when glucose or fructose was used as a sole carbon source compared to maltose with even higher concentrations of esters and alcohols by increasing glucose concentration (Younis & Stewart, 1998).

7.2.4. Parasitism

The antagonist yeasts and pathogenic fungi can interact by direct parasitism. The yeast species *Pichia guilliermondii* showed a strong adhesion to the mycelium of *Botrytis cinera* (Wisniewski et al., 1991). Furthermore, El-Ghaouth et al. (1998) reported that co-cultivation of *Candida saitoana* and *Botrytis cinera* in wounded apple tissue resulted in yeast attached to fungal hyphal walls causing cytological damage. Degradation of the fungal cell wall by production of lytic enzymes such as Exo- β -1,3-glucanase, chitinase and protease has also been reported in *P. anomala* (Jijakli & Lepoivre, 1998) and *Candida oleophila* (Bar-Shimon et al., 2004). In the present study, inoculation of *P. anomala*, *P. kluyveri* or *H. uvarum* with *A. ochraceus* in MYGP broth completely inhibited fungal spore germination (Appendix III, Fig 10). This might be due to cytological damage of fungal cells caused by adhesion of the yeasts cells to fungal cell walls or by production of cell wall degrading enzymes by the three yeasts. However, this needs further studies.

7.2.5. Resistance induction in the host tissue

The induction of pathogen resistance in the host tissue has also been considered as one of mechanisms behind the antifungal activity of yeasts. Droby et al. (2002) demonstrated that treatment of grapefruit by *C. oleophila* induced host resistance against *P. digitatum*. In addition to limiting fungal colonization, *C. saitoana* was reported to induce formation of structural responses in apple tissue (El-Ghaouth et al., 1998). In another study, El-Ghaouth et al. (2003) found that *C. saitoana* induced accumulation of β -1,3-glucanases, chitinase and preoxidases in apple which will result in resistance against pathogenic fungi. In this study, the antagonist activity of *P. anomala*, *P. kluyveri* or *H. uvarum* against *A. ochraceus* was only investigated in laboratory and coffee substrates but not in coffee beans during fermentation. Further studies on the antagonist activity of the three yeasts species in fermented coffee beans might help to clarify if resistance in the host cells i.e. coffee bean is one of the mechanisms behind their antifungal activity.

8. Effect of yeasts on Ochratoxin A (OTA) formation

Beside their antagonist activity against growth of OTA producing fungi yeasts were also reported to inhibit or reduce OTA formation by toxigenic filamentous fungi. In this study, it was found that *Pichia anomala*, *P. kluyveri* and *H. uvarum* isolated from coffee were able to inhibit production of OTA by *A. ochraceus* (Appendix II, III). The same findings were obtained by Petersson et al. (1998), who investigated the inhibitory effect of *P. anomala* and *S. cerevisiae* on growth and OTA formation by *P. verrucosum* *in vitro* as well as in wheat. It was found that the two yeasts reduced mould growth and OTA formation with a stronger effect on OTA production than on mould growth. Furthermore, it has been demonstrated that production of OTA by *Aspergillus alutaceus* var. *alutaceus* was significantly reduced when grown in un sterile barely compared to barely sterilized by irradiation suggesting an inhibitory effect by the competitive undefined indigenous microbiota (Chelack et al., 1991).

In this thesis, we investigated the effect of co-culturing of *A. ochraceus* with *P. anomala*, *P. kluyveri* or *H. uvarum* on OTA formation in MEA and CA media (Appendix III). It was found that OTA was not detected on MEA plates co-cultured with *A. ochraceus* and *P. anomala*, *P. kluyveri* or *H. uvarum* (Appendix III, Fig 12). This might be explained by the depletion of nutrients from growth substrates, which are important for mycotoxins production (Luchese & Harrigan, 1993). On CA plates inoculated with *H. uvarum* and *A. ochraceus*, OTA was detected which agrees with our findings on the weak effect of this yeast on growth of *A. ochraceus* in the same substrate (Appendix III).

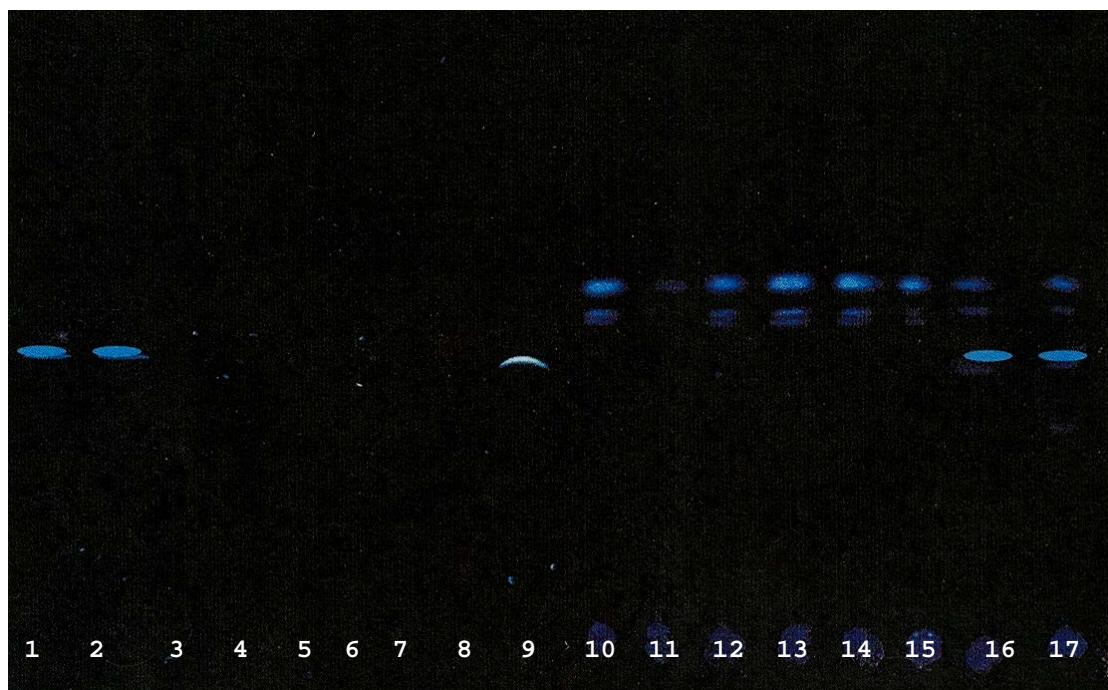


Figure 12. Production of OTA by *Aspergillus ochraceus* B722 co-cultured with strains of *Pichia anomala*. Lane numbers from 3-8 refer to *A. ochraceus* co-cultured on CA with: lane 3, *P. anomala* S12; lane 4, *P. anomala* S13; lane 5, *P. anomala* S14; lane 6, *P. anomala* S15; lane 7, *P. anomala* S16; lane 8, *P. anomala* S17. Lane numbers from 10-15 refer to *A. ochraceus* co-cultured on MEA with: lane 10, *P. anomala* S12; lane 11, *P. anomala* S13; lane 12, *P. anomala* S14; lane 13, *P. anomala* S15; lane 14, *P. anomala* S16; lane 15, *P. anomala* S17. *Aspergillus ochraceus* grown on CA with out yeasts (lanes 1, 2) and on MEA (lanes 16, 17) were used as controls. OTA standard 10 µg / l (lane 9)

Furthermore, the effect of exposure of *A. ochraceus* to the three yeasts and the effect of individual volatile compounds produced by yeasts on OTA formation by *A. ochraceus* were investigated (Appendix II). Beside their effect on fungal growth, the volatile compounds produced by *P. anomala*, *P. kluyveri* and *H. uvarum* inoculated on MYGP plates were found to reduce or inhibit OTA formation by *A. ochraceus* (Appendix II). Growth of *H. uvarum* on CA plates and exposure to *A. ochraceus* had no effect on OTA formation. This can be explained again by the weak antagonist activity of this yeast especially on CA substrate, which has low content of carbohydrates compared to the laboratory substrate (Appendix II).

Table 5. Ochratoxin A (OTA) production by *Aspergillus ochraceus* B722 on MEA when exposed to the individual volatile compounds at the stated headspace concentrations (Appendix II).

Compound	Detection of OTA produced by <i>A. ochraceus</i> B722 when exposed to different concentrations of volatiles in the headspace above plates					
	4 µg / l	8 µg / l	12 µg / l	24 µg / l	48 µg / l	96 µg / l
Ethyl Acetate	+ ¹	+	+	+	+	+
Ethyl Propionate	+	+	+	+	+	+
Isobutyl Acetate	+	+	+	+	+	- ²
Butyl Acetate	+	+	+	+	+	+
Propyl Acetate	+	+	+	+	+	+
Propyl Propionate	+	+	+	+	+	+
Isopentyl Acetate	+	+	+	+	+	+
Isobutyl Propionate	+	+	+	+	+	+
Isoamyl Alcohol	+	+	+	+	+	-
2-Phenyl Ethyl Acetate	+	+	+	-	-	-
Isobutyl Alcohol	+	+	+	+	-	-
Phenyl Ethyl Alcohol	+	+	+	+	-	-
Butanoic Acid Octyl Ester	+	+	+	+	+	+

¹ OTA ≥ 6 µg / l² OTA < 6 µg / l

Differences among the individual esters and alcohols in their ability to inhibit or reduce OTA production were observed with 2-phenyl ethyl acetate being the most effective compound (Appendix II, Table 5). Contrary, exposure of *A. ochraceus* to the headspace of various concentrations of ethyl acetate did not inhibit OTA formation. It was also observed that OTA was not detected when the fungal biomass was reduced to more than 50 % indicating that reduction of the biomass might to be the main reason for absence of OTA (Appendix II).

From the results obtained on the effect of co-culturing of *A. ochraceus* with *P. anomala*, *P. kluyveri* or *H. uvarum* (Appendix III) as well as the effect of fungal exposure to the volatile compounds produced by the three yeasts (Appendix II), it seems that at least two mechanisms are involved in inhibition of fungal growth and OTA formation; they include an effect of a competition for nutrients and production of volatile metabolites by yeasts with antifungal effect.

The use of yeasts, which have antifungal activity as starter cultures during coffee processing appears to be a promising tool for preventing fungal growth and OTA contamination. There are several properties in yeasts, which make them ideal biocontrol agents against fungal growth and OTA formation. Yeasts have simple nutritional requirements, they can grow in cheap substrates and they rapidly utilize the available nutrients, which make them good competitive agents against toxigenic fungi (Janisiewicz & Korsten, 2002). Furthermore, yeasts are resistant to many pesticides (Wilson & Wisniewski, 1994). Regarding human health, the antagonist microorganism should be safe as well. Yeasts are considered to be safe as they do not produce mycotoxins as filamentous fungi do or antibiotics as many of the antagonistic bacteria do. To obtain a successful biocontrol, the environmental conditions that favour growth of the antagonist should be similar to those favoured by the undesired microorganism (Janisiewicz & Korsten, 2002). During coffee fermentation, pH drops from about 5.5 or 6.0 to 3.5, which in general will not affect growth of yeasts. During drying the water activity decreases to about 0.50-0.60 and yeast growth eventually will be inhibited. At the same time growth of filamentous fungi will be inhibited. Furthermore, the antagonists used should not have a negative role on the quality of the food product like off flavour. In the present thesis, *P. anomala*, *P. kluyveri* and *H. uvarum* dominating the different stages of coffee processing i.e. indigenous microbiota were found to inhibit growth and OTA production by *A. ochraceus* (Appendix II, III). Furthermore, two strains of *P. anomala* and *P. kluyveri* were found to possess strong pectinolytic activity by producing high amounts of polygalacturonase especially in coffee substrate, which indicates that they have a role in mucilage degradation during coffee fermentation. However, before using the two yeasts species as starter cultures in coffee fermentation, their capability to degrade mucilage surrounding coffee beans and their effect on quality of the green coffee beans should be investigated.

Coffee is processed by small, medium or large processing sites in the coffee producing countries. Application of the biocontrol microorganisms in coffee processing might be difficult for

small or even medium processing sites if the expenses are high. As mentioned earlier yeasts are easy to propagate and they need cheap substrates, which makes it feasible to propagate them by small, medium and large processing sites. In Africa, it is practice to use solar dried yeast as starter culture for fermentation of local beers (Glover et al., 2005). The technology may be transferable to coffee fermentation.

9. Conclusions

The main conclusions drawn from this thesis can be summarised as follows:

- In samples of Tanzanian arabica coffee collected at different wet processing steps from picking of cherries to drying of green beans, yeasts counts were in a range of 4.0×10^4 to 4.8×10^7 cfu/g with the highest numbers during fermentation. *Pichia kluyveri*, *P. anomala* and *H. uvarum* were the predominant yeasts. *Pichia kluyveri* was found to dominate during the whole process including drying. *Pichia anomala* was isolated from cherries, pulp, fresh beans and beans during first day of fermentation and during the drying stage. *Hanseniaspora uvarum* was found to be predominant yeast on coffee cherries and during fermentation but it decreased in numbers during drying.
- For the first time, the culture independent technique DGGE was used for identification of yeasts directly from coffee samples at different stages of coffee processing. Regarding the predominant yeasts in coffee samples, a good agreement was found between the profiles obtained by the culture independent technique DGGE and the findings obtained by the classical culturing, isolation and further identification of yeasts indicating that DGGE is an efficient tool for studying microbial diversity during natural fermentation.
- Strains of *P. anomala*, *P. kluyveri* and *H. uvarum* were found to have pectinolytic activity with *P. anomala* S16 and *P. kluyveri* S13Y4 showing the strongest activity. Only polygalacturonase (PG) was produced by the three yeasts; neither pectin esterase (PE) nor pectin lyase (PL) was secreted by the investigated yeasts. In coffee broth (CB), *P. anomala* S16 and *P. kluyveri* S13Y4 secreted significantly higher amounts of PG than in yeast polygalacturonic acid (YPA) broth. This indicates that coffee is a good substrate for the investigated yeasts to produce PG. In addition, the highest amounts of PG produced by *P. anomala* S16 and *P. kluyveri* S13Y4 were obtained at

growth medium with pH 6.0 and incubation temperature of 30 °C, which are similar to conditions during coffee fermentation.

- *Pichia anomala*, *P. kluyveri* and *H. uvarum* were observed to inhibit growth of *A. ochraceus* when co-cultured on MEA and on CA media. The effect of *H. uvarum* on growth of *A. ochraceus* was significantly lower than that caused by *P. anomala* and *P. kluyveri*. Spore germination of *A. ochraceus* was fully inhibited when co-cultured with the three yeasts. Co-culturing of *A. ochraceus* with *P. anomala*, *P. kluyveri* and *H. uvarum* on MEA plates also inhibited OTA formation. On CA plates, only *P. anomala*, *P. kluyveri* inhibited OTA formation by *A. ochraceus*.
- Exposure of *A. ochraceus* to the gaseous phase of MYGP or CA plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* inhibited fungal growth with the two *Pichia* spp. showing the strongest effect. The main esters and alcohols produced by *P. anomala*, *P. kluyveri* and *H. uvarum* were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate and isoamyl alcohol. These compounds were observed to reduce growth of *A. ochraceus* with 2-phenyl ethyl acetate being the most effective compound. In addition, exposure of *A. ochraceus* to MYGP plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* prevented formation of OTA. On CA medium, only exposure of *A. ochraceus* to the headspace of *P. anomala* and *P. kluyveri* prevented OTA formation. The effect of the individual volatiles produced by the three yeasts on OTA formation by *A. ochraceus* showed that 2-phenyl ethyl acetate was the most effective in preventing OTA formation. When fungal growth was reduced to more than 50% caused by the individual volatiles, OTA was under the detection limit i.e. 6 µg/l indicating that reduction of the fungal biomass might be the main reason for absence of OTA.

The results obtained suggest that *P. anomala* and *P. kluyveri* could be useful starter cultures in coffee fermentation to increase the rate of mucilage degradation and to prevent OTA formation. The use of the two yeasts as starter cultures in coffee fermentation as well as application of Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Point (HACCP) during coffee processing are seen as primary tools to produce good quality coffee beans free from OTA.

10. Future studies and activities

In the present thesis, two strains of *P. anomala* and *P. kluyveri* were found to produce high amounts of polygalacturonase (PG) especially in green coffee substrate with optimum pH and temperature close to those occurring in coffee fermentation, which indicates that they have a role in mucilage degradation. Other microorganisms might also participate in mucilage degradation by secretion of PG, pectin esterase (PE) and pectin lyase (PL), which needs further investigations.

For the purpose of preventing fungal growth and OTA contamination in coffee, the present thesis demonstrates the possible use of *P. anomala* and *P. kluyveri* as biocontrol agents against OTA producing fungi in coffee. Further *in vivo* studies on the effects of *P. anomala* and *P. kluyveri* on fungal growth and OTA formation, their role in mucilage degradation and effect on coffee quality i.e. colour, appearance and aroma are needed.

In this work, volatile compounds produced by the three yeasts contribute to the antifungal activity against growth and OTA formation by *A. ochraceus* (Appendix II). In addition, competition for nutrient was suggested to be one of the mechanisms behind the antifungal activity of *P. anomala*, *P. kluyveri* and *H. uvarum* (Appendix III). Involvement of other mechanisms behind the antifungal activity of the investigated yeasts needs further studies.

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Appendix I

Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis (DGGE)

Wafa Masoud, Lene Bjørg Cesar, Lene Jespersen and Mogens Jakobsen

Department of Food Science, Food Microbiology
The Royal Veterinary and Agricultural University
Denmark

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Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis

Wafa Masoud*, Lene Bjørg Cesar, Lene Jespersen and Mogens Jakobsen

Department of Dairy and Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

*Correspondence to:
Wafa Masoud, Department of
Dairy and Food Science, Food
Microbiology, The Royal
Veterinary and Agricultural
University, Rolighedsvej 30,
DK-1958 Frederiksberg C,
Denmark.
E-mail: wm@kvl.dk

Abstract

Samples of *Coffea arabica* were collected during the different stages of the fermentation from two production sites in Tanzania. The yeasts community was identified by genotyping using ITS-PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene. For confirmation, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 26S rRNA gene was performed to detect yeast directly from coffee samples without cultivation. Yeast counts were in the range 4.0×10^4 – 5.0×10^7 CFU/g with an increase during fermentation. Three yeasts species were dominant. The predominant yeast found during fermentation and drying was *Pichia kluyveri*. *Pichia anomala* was found in high numbers during drying of coffee beans. *Hanseniaspora uvarum* was the predominant yeast during fermentation but decreased during drying. *Kluyveromyces marxianus*, *Candida pseudointermedia*, *Issatchenkia orientalis*, *Pichia ohmeri* and *Torulaspora delbrueckii* occurred in concentrations of 10^3 CFU/g or below in coffee samples. *Saccharomyces cerevisiae* and *Candida xestobii* were not isolated by cultivation, but by the DGGE technique. A good agreement was found between the sequence analysis of the D1/D2 domain of the 26S rRNA gene and sequencing of the DGGE bands. Sequences of yeast isolates determined in this study have been deposited in the GenBank database under Accession Nos AY305664–AY305669 and AY305672–AY305683. Sequences of DGGE bands have also been deposited in the GenBank database under Accession Nos AY314789–AY314802. Copyright © 2004 John Wiley & Sons, Ltd.

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Introduction

The main objective of coffee processing is removal of the pulp, mucilage, parchment and silver skin surrounding the coffee beans, which leaves the so-called 'green' coffee beans (Fowler *et al.*, 1998). Coffee can be processed by two different methods, referred to as the 'wet' or the 'dry' method. The wet method is used for *arabica* coffee and involves removal of the outer skin and part of the mucilage by machines. The remaining mucilage is then removed by fermentation in water for 24–48 h, followed by drying (Fowler *et al.*, 1998). The dry

method is mainly used for *robusta* coffee, which has a thin pulp that allows direct drying (Fowler *et al.*, 1998).

During processing of *Coffea arabica*, Gram-negative and Gram-positive bacteria, yeast and filamentous fungi are present in high numbers during the different stages (Silva *et al.*, 2000). Limited information is available in the yeast community of coffee fermentation. *Kluyveromyces marxianus* (Agate and Bhat, 1966; Van Pee and Castelein, 1971), *Saccharomyces cerevisiae* (Agate and Bhat, 1966; Van Pee and Castelein, 1971; Silva *et al.*, 2000) and *Schizosaccharomyces* spp. (Agate and

Bhat, 1966; Silva *et al.*, 2000) have been isolated. In addition, *Pichia* spp. and *Arxula* spp. (Silva *et al.*, 2000), *Kloeckera* spp. and *Cryptococcus* spp. (Avallone *et al.*, 2001) have been isolated from *arabica* coffee.

It is assumed that degradation of the pectin-rich mucilage adhering to coffee beans is the major role of yeasts during coffee fermentation. In a study of fermented *robusta* coffee cherries harvested in India, Agate and Bhat (1966) have isolated yeasts identified as *K. marxianus*, *S. bayanus*, *S. cerevisiae* and *Schizosaccharomyces* spp., which were found to have strong pectinolytic activity. In another study, it has been reported that yeasts isolated were non-pectinolytic (Avallone *et al.*, 2001). In a recent study, Avallone *et al.* (2002) reported that the mucilage degradation seems to be due to acidification rather than to microbial pectinolytic enzymes.

Cultivation and isolation of yeasts as applied in previous studies can be combined with culture-independent methods to give a complete picture of the microbial diversity. Culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), have been developed to differentiate rRNA genes directly purified from complex microbial communities (Muyzer *et al.*, 1993). Recently, DGGE has been shown to be a good tool for monitoring microbial dynamics, such as in fermentation of Mexican fermented maize dough (Ampe *et al.*, 1999), wine (Cocolin *et al.*, 2000) and sausages

(Cocolin *et al.*, 2001). It was demonstrated that the DGGE of PCR-amplified 26S rRNA genes provided a qualitative assessment of the yeast diversity in wine fermentation (Cocolin *et al.*, 2000). So far, culture-independent techniques seem not to have been used for studies on coffee fermentation.

The objective of the present study was to investigate the yeast community in samples from different coffee fermentation sites in Tanzania. Conventional microbiological cultivation methods were combined with DGGE of PCR-amplified 26S rRNA gene.

Materials and methods

Coffee samples

Samples of *Coffea arabica* were collected during different stages of the wet processing method from two processing sites in Arusha, Tanzania (Table 1). Samples were placed aseptically in sterile plastic bags and transferred in ice boxes to a nearby laboratory in Arusha for immediate analysis by cultivation and isolation of yeasts. For extraction of DNA for the DGGE analysis, coffee samples were frozen at -20°C until analysed.

Conventional microbiological analysis and yeasts isolation

Ten g of each sample were added to 90 ml diluent saline peptone (SPO) [0.1% bactopectone (Difco,

Table 1. The number of colony forming units (CFU/g) of yeast species isolated from coffee samples collected from two processing sites in Tanzania

Coffee processing stage	Number of colony forming units (CFU/g) of yeast species ¹						
	<i>P. kluyveri</i>	<i>C. pseudointermedia</i>	<i>P. anomala</i>	<i>K. marxianus</i>	<i>I. orientalis</i>	<i>T. delbrueckii</i>	<i>H. uvarum</i>
Cherries ²	4.5×10^5	— ⁴	3.0×10^4	— ⁴	— ⁴	— ⁴	7.5×10^5
Pulp ³	5.2×10^5	— ⁴	— ⁴	— ⁴	— ⁴	— ⁴	6.5×10^5
Fresh beans ²	6.0×10^5	— ⁴	1.5×10^4	— ⁴	— ⁴	— ⁴	6.0×10^5
Beans during 1st day of fermentation ³	2.0×10^6	— ⁴	1.0×10^3	1.3×10^3	— ⁴	— ⁴	1.6×10^6
Beans during 2nd day of fermentation ³	3.4×10^7	— ⁴	— ⁴	— ⁴	— ⁴	— ⁴	1.4×10^7
Beans after fermentation and washing ³	2.1×10^6	— ⁴	— ⁴	— ⁴	— ⁴	1.4×10^3	1.7×10^6
Beans during 3rd day of drying ³	2.6×10^5	1.3×10^3	6.5×10^5	— ⁴	— ⁴	— ⁴	2.6×10^5
Beans during 7th day of drying ³	6.4×10^4	— ⁴	9.6×10^4	— ⁴	— ⁴	— ⁴	— ⁴
Beans > 7 days of drying ³	1.6×10^4	— ⁴	2.0×10^4	— ⁴	4.0×10^3	— ⁴	— ⁴

¹ Identified by sequencing of D1/D2 domain of the 26S rRNA gene (see Table 2).

² Samples collected from processing site e.

³ Samples collected from processing site f.

⁴ $<10^3$ cfu/g.

Detroit, MI, USA), 0.85% (w/v) NaCl (Merck, Darmstadt, Germany), 0.03% Na₂H₂PO₄ · 2H₂O (Merck), adjusted with 1 M NaOH (Merck) and 1 M HCl (Merck) to pH 5.6]. After mixing in a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 30 s, 10-fold dilutions were prepared and spread onto malt yeast glucose peptone (MYGP) agar [3 g yeast extract (Difco), 3 g malt extract (Difco), 5 g bactopectone (Difco), 10 g glucose (Merck), 100 mg chloramphenicol (Oxoid) and 20 g Agar (Difco)] per litre of distilled water, adjusted with 1 M NaOH and 1 M HCl to pH 5.6] and incubated at 25 °C for 5 days. The number of colony forming units (CFU) was recorded. From a suitable dilution of each sample, 10 representative colonies were picked and recultivated in MYGP broth at 25 °C for 2 days, and further purified by streaking onto MYGP agar. A total of 110 yeast isolates were obtained.

Typing of yeast isolates by ITS PCR

From a colony of a pure culture on MYGP agar incubated at 25 °C for 5 days, a loop-full was transferred to 200 µl Tris–EDTA (TE) buffer [10 mM Tris–HCl (Sigma), 1 mM EDTA (Sigma) (Merck)], boiled for 15 min and centrifuged at 1400 × *g* for 2 min. The supernatant was used as a template for the PCR reaction. ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers were used for amplification of the ITS1–5.8S rDNA–ITS2 region (White *et al.*, 1990). DNA amplification was performed in a 100 µl volume containing 10 µl template, 1 µM of each primer, 200 µM of each nucleotide [dATP, dCTP, dGTP, dTTP (Promega, Madison, WI)], 2.5 U *Taq* polymerase (Amersham Pharmacia Biotech), 10 µl 10× PCR buffer (Amersham Pharmacia Biotech) and adjusted to 100 µl by addition of MilliQ water. The reactions were performed in an automatic thermal cycler (GeneAmp® PCR System 9700, Perkin Elmer, Norwalk, CT) with initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s; final extension at 72 °C for 7 min. The PCR products were analysed by electrophoresis in 2% (w/v) Nusieve agarose (FMC BioProducts, Rockland, ME, USA) gel in 1× TBE [89 mM Tris-base (Sigma), 89 mM boric acid (Sigma), 2 mM EDTA (Sigma)] at 80 V for 2 h. GenRuler DNA ladder mix (Fermentas, Vilnius, Lithuania) was used as a

marker. The amplified fragments were visualized by ethidium bromide staining and UV transillumination. The sizes of the amplified ITS1–5.8S rDNA–ITS2 regions were determined with Fragment Manager (Amersham Pharmacia Biotech).

Sequence analysis of the D1/D2 domain of the 26S ribosomal RNA gene for selected yeast isolates

For amplification of the D1/D2 domain, the external primers NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman and Robnett, 1998) were used. The reactions were performed in an automatic thermal cycler as described above. The amplified products were purified using the QIAGEN PCR purification kit (QIAGEN, Dorking, UK). Sequencing of the purified PCR products was performed using the CEQ 2000 Dye Terminator Cycle Sequencing kit (Beckman Coulter, Fullerton, USA) and the primers NL-1 and NL-4, NL-2A (5′-CTT GTT CGC TAT CGG TCT C-3′) and NL-3A (5′-GAG ACC GAT AGC GAA CAA G-3′) (Kurtzman and Robnett, 1998) were used for cycle sequencing following the instructions of the manufacturer. Sequences were aligned to the 26S rRNA gene sequences obtained from the National Center for Biotechnology Information (NCBI) Genbank database, using the BLAST algorithm.

DNA extraction from coffee samples

Portions (10 g) of each sample resuspended in 90 ml SPO were homogenized with a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 2 min; 30 ml each suspension were centrifuged at 1000 × *g* for 15 min; 500 µl lysis buffer [2% Triton X-100, 1% SDS (Sigma), 100 mM NaCl (Merck), 10 mM Tris–HCl (Sigma), 1 mM EDTA (Sigma)], 500 µl phenol:chloroform:isoamylalcohol (50:48:2) and 0.30 g glass beads were added to each pellet and the mixtures were shaken in a bead mill (MM 2000, Retsch, Germany) for 20 min. The aqueous phase was obtained by centrifugation at 12 000 × *g* for 10 min. The DNA was precipitated with 2.5 vols 96% ethanol, centrifuged at 16 000 × *g* for 10 min; after which the pellets were washed with 70% ethanol, dried and resuspended in MilliQ water containing 2 IU RNase (Sigma).

DNA extraction from pure cultures

From a 72 h culture in MYGP broth, 1.5 ml was centrifuged at $16000 \times g$ for 10 min. The pellet were resuspended in 200 μ l lysis buffer, 200 μ l phenol:chloroform:isoamylalcohol (50:48:2) and 0.30 g glass beads; the mixture was homogenized in a bead mill (MM 2000, Retch) for 20 min. The DNA was obtained as described previously.

PCR–DGGE analysis

For amplification of the D1/D2 domain of the 26S rRNA gene, an indirect approach with two types of nested PCR (a and b) was performed. Type (a) included a first PCR round using primers NL-1A (5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3') and NL-4 (indicated above) (O'Donnell, 1993). Type (b) included a first PCR round, using primers NL-1A and LS2 (5'-ATTT CCC AAA CAA CTC GAC TC-3') according to Cocolin *et al.* (2000). Templates obtained from either (a) or (b) were used in the second PCR round; primers NL1-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3') (the GC clamp sequence is underlined) and LS2 were used to amplify the 253 bp fragment with the GC clamp.

The PCR products were then analysed by DGGE using a DCode system apparatus (Bio-Rad, Hercules, CA, USA). Polyacrylamide gels (8% w/v acrylamide–bisacrylamide) were prepared with a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad) using solutions containing 40% and 60%

denaturant (100% denaturant corresponds to 7 M urea and 40% v/v formamide). Gels were run at 60 °C for 16 h at 70 V. The amplified fragments were visualized by SYBR-GOLD (Molecular Probes, Eugene, OR) staining and UV transillumination.

Sequence analysis of DGGE bands

Selected bands were excised from the DGGE gels with a sterile scalpel and the DNA was eluted in 50 μ l distilled water overnight at 4 °C. DNA was reamplified using the same reaction mixture described above for the second PCR round. To confirm their electrophoretic mobility relative to the fragment from which they were excised, the PCR products were analysed by DGGE, as described previously. PCR products that migrated as the original bands were sequenced using CEQ 200 Dye Terminator Cycle Sequencing kit (Beckman Coulter) following the manufacturer's instructions.

Results

Yeast species determined by cultivation, ITS–PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene

Yeasts counts were in a range $4.0 \times 10^4 - 5 \times 10^7$ CFU/g with an increase during fermentation (Table 1). According to fragment sizes obtained by amplification of the ITS1–5.8S rDNA–ITS2 region, a total of 110 yeast isolates were divided into eight groups, with three being dominant (Table 2). The square root of the number of yeast

Table 2. Groups of yeast isolates obtained from coffee samples according to PCR amplification of the ITS1–5.8S rDNA–ITS2 region and further identified by sequencing D1/D2 domain of the 26S rRNA gene

ITS-Group	Number of isolates	Band size (bp)	26S rRNA gene sequence	Homology ¹ (%)	GenBank Accession No.
G1 ²	37	439	<i>Pichia kluyveri</i>	99.4	AY305664
G2 ³	3	399	<i>Candida pseudointermedia</i>	99.1	AY305666
G3 ²	39	604	<i>Pichia anomala</i>	99.5	AY305668
G4 ³	3	679	<i>Kluyveromyces marxianus</i>	99.4	AY305673
G5 ³	1	520	<i>Issatchenkia orientalis</i>	99.4	AY305674
G6 ³	1	831	<i>Torulaspora delbrueckii</i>	100	AY305680
G7 ²	25	746	<i>Hanseniaspora uvarum</i>	99.3	AY305678
G8 ³	1	410	<i>Pichia ohmeri</i>	98.9	AY305683

¹ Percentage of identical nucleotides of the closest relative found in the GenBank database.

² The square root of the number of yeasts isolates were sequenced.

³ One isolate was sequenced.

isolates in each group were identified by sequencing the D1/D2 domain of the 26S rRNA gene, and the three dominant groups, G1, G3 and G7, were identified as *Pichia kluyveri*, *Pichia anomala* and *Hanseniaspora uvarum*, respectively. For each yeast species, the number of CFU was calculated for the various samples (Table 1). *Pichia kluyveri* was isolated from cherries, pulp and beans during fermentation and drying; it accounted for $1.6 \times 10^4 - 3.4 \times 10^7$ CFU/g. *Pichia anomala* was found in coffee cherries, pulp, fresh beans and beans during the first day of fermentation in the range $1.0 \times 10^3 - 3.0 \times 10^4$ CFU/g. Later in fermentation, *P. anomala* was not detected, but during drying it was found to be predominant, accounting for $2.0 \times 10^4 - 6.5 \times 10^5$ CFU/g in drying coffee samples. *Hanseniaspora uvarum*, the third dominating yeast, was isolated from cherries and during fermentation and during the third day of drying; it accounted for $2.6 \times 10^5 - 1.5 \times 10^7$ CFU/g, but decreased to less than 10^3 CFU/g later in the drying process (Table 1). Other yeast species, which included *Kluyveromyces marxianus*, *Candida pseudointermedia*, *Issatchenkia orientalis*, *Pichia ohmeri* and *Torulaspora delbrueckii*, were found at low levels and accounted for about 10^3 CFU/g or less in the coffee samples examined.

Yeast species detected by PCR–DGGE profiles

As seen from Figure 1, no differences in the intensity of the bands were observed for the two types of nested PCR used, indicating that both primer sets used gave PCR products that allowed differentiation by DGGE. Sequence analysis of excised DGGE bands showed that *H. uvarum* was observed in all samples examined by DGGE. *Pichia kluyveri* was also identified in all samples and it was present as three bands that migrated very close to each other. Sequencing of the three bands showed that all the three bands were belonging to *P. kluyveri* with a homology of 98.5% for the first band, 99.0% for the second band and 99.4% for the third band. The sequences of the three bands were aligned together and it was found that the first band was different in two base pairs from the second band and in one base pair from the third band. *Pichia anomala* was not detected in any of the samples examined. It should be noted that dried samples were not included. *Eremothecium coryli* was

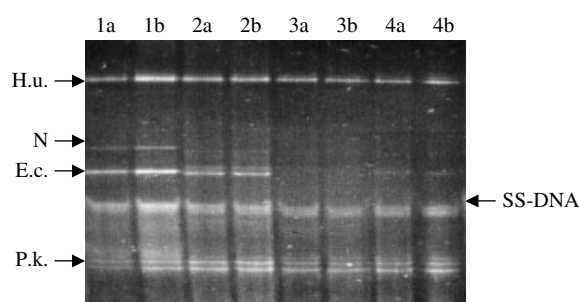


Figure 1. DGGE profiles of PCR-products of the 26S rRNA gene fragments obtained from selected coffee samples. The lane number refers to the coffee sample: lane 1, beans during the first day of fermentation; lane 2, pulp; lane 3, beans during the second day of fermentation; lane 4, beans after fermentation and washing. The lane letter corresponds to the PCR conditions: a, NL-1A and NL-4 primers were used; b, NL-1A and LS2 primers were used. The bands common to all samples (SS-DNA) are single-stranded DNA artefacts. H.u., *Hanseniaspora uvarum*; E.c., *Eremothecium coryli* (a yeast plant parasite); P.k., *Pichia kluyveri*; N, unsequenced

detected in the pulp and beans during the first day of fermentation. We did not succeed in sequencing a band (Figure 1, N) found in the yeast profiles of pulp and beans during the first day of fermentation.

For comparison, the DGGE profiles for one sample of coffee beans during the fourth day of drying and five yeasts cultivated and isolated from the same sample are shown in Figure 2. Six bands were obtained from the coffee sample. Sequence analysis of the bands from the DGGE gel revealed that *P. anomala* and *P. burtonii* were detected in samples by DGGE and by cultivation. The other yeast species identified by DGGE were *Candida xestobii* and *Saccharomyces cerevisiae*. One band was identified as *Mitchella repens*, which is a plant that belongs to the family Rubiaceae (Greth Van Wijk, 1962). However, the homology of this band to *M. repens* was only 97.2%.

Discussion

In the present study, increasing yeasts counts in the range $4.0 \times 10^4 - 4.8 \times 10^7$ CFU/g were observed during coffee fermentation, which indicates that yeasts might play a role. An increase of yeasts counts during fermentation of *Coffea arabica* in Brazil has also been reported by Silva *et al.* (2000). In the present work, *Pichia kluyveri* was a predominant yeast during the whole process,

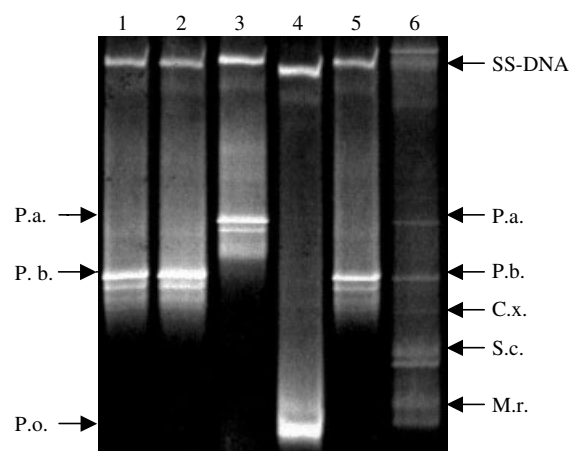


Figure 2. DGGE profiles of PCR products of the 26S rRNA gene fragments obtained from coffee beans, isolated during the fourth day of drying. Lanes 1–5 correspond to the bands obtained from pure cultures isolated from the same coffee sample: lanes 1, 2, 5, P.b. (*Pichia burtonii*); lane 3, P.a. (*Pichia anomala*); lane 4, P.o. (*Pichia ohmeri*). Lane 6 refers to the bands obtained from DNA extracted from the coffee sample: P.a., *Pichia anomala*; P.b., *Pichia burtonii*; C.x., *Candida xestobii*; S.c., *Saccharomyces cerevisiae*; M.r., *Mitchella repens* (a plant belonging to the botanical genus *Mitchella* of the family Rubiaceae, to which coffee also belongs); P.o., *Pichia ohmeri*. The bands common to all samples (SS-DNA) are single-stranded DNA artefacts

including drying. This yeast species has not previously been isolated from coffee; however, it was found in cocoa (Kurtzman, 1998) and in fruits (Spenser *et al.*, 1992; Abranches *et al.*, 2000). *Hanseniaspora uvarum* was found to be the predominant yeast on coffee cherries and during fermentation but it decreased in numbers during drying. *Kloeckera apiculata*, which is the imperfect form of *H. uvarum*, has also been isolated from coffee fermentation (Avallone *et al.*, 2001) and from fruits (Arias *et al.*, 2002; Heras-Vazquez *et al.*, 2003). *Pichia anomala* was isolated from cherries, pulp, fresh beans and beans during the first day of fermentation but it disappeared later in the fermentation. However, *P. anomala* dominated during the drying stage; it constituted $2.0 \times 10^4 - 6.5 \times 10^5$ CFU/g in drying beans. *Pichia* spp., including *P. anomala*, were reported to constitute 39.0% of 107 yeasts isolated from *Coffea arabica* in Brazil (Silva *et al.*, 2000).

With regard to the predominant yeast species (*P. kluyveri* and *H. uvarum*) identified in coffee samples, a good agreement was found between

sequence analysis of the D1/D2 region of the 26S rRNA gene for yeast obtained by cultivation and sequencing of the DGGE bands obtained from the same samples (Figure 1). In the present study, *P. kluyveri* was found as three bands that migrated very close to each other. The sequences of the three bands belonging to *P. kluyveri* were found to have differences of one to two base pairs between the three bands, indicating that the three bands might represent three different strains of *P. kluyveri*. According to Nübel *et al.* (1996) and Rosado *et al.* (1998), the presence of such triple bands for the same strain indicates that the DNA molecules have slightly different migration behaviour, which might be due to incomplete extension of the same template caused by the GC clamp. *Pichia anomala*, which was isolated from the pulp and during the first day of fermentation, was not detected by DGGE analysis of the same samples. This could be due to the low numbers of this yeast species encountered in the samples investigated. It has been demonstrated that the DGGE patterns are related to the numerically dominant species (Ampe *et al.*, 1999; Boon *et al.*, 2002). In addition, Cocolin *et al.* (2000) found that during wine fermentation, the PCR–DGGE was unable to detect yeast species when their number was $<10^3$ cells/ml. *Kluyveromyces marxianus*, *T. delbrueckii* (Table 1) and *P. ohmeri* (Figure 2) were isolated from some coffee samples but they were not detected from the DGGE profiles of the same samples. This might be explained by the fact that these yeast species were encountered occasionally in some samples and only in low numbers. On the other hand, *S. cerevisiae* and *C. xestobii* were identified in the DGGE profile for a sample of drying beans, but they were not detected by cultivation. This was also the case for *Eremothecum coryli*, which was detected from the DGGE profiles of two coffee samples examined. According to Cocolin *et al.* (2000), reasons could be that the yeast cells have entered a viable non-cultural (VBNC) state, or the presence of the DNA of the yeast in question as a result of cell lysis. *Eremothecum coryli* has been reported to be a yeast plant parasite in tropical and subtropical areas (Hoog *et al.*, 1998). It has not been previously isolated from coffee fermentation. The sequence of band 5, lane 6, in the DGGE profile of drying beans (Figure 2) was found to have a homology of 97.2% with five different nucleotides from the

sequence of *Mitchella repens*, which indicates that the sequence of this band does not belong to *M. repens*, which is a plant that belongs to the botanical genus *Mitchella* of the family Rubiaceae (Greth Van Wijk, 1962), to which the genus *Coffea* belongs. The sequence of this band might belong to *Coffea arabica*. Unfortunately, no sequence for the D1/D2 domain of the 26S rRNA gene for *Coffea* spp. was found in the GenBank database.

In the present work, *P. kluyveri*, *P. anomala* and *H. uvarum* were found to dominate during different stages of coffee processing. These yeast species are reported to be fermentative and have been found in soil, fruits and trees (Kurtzman, 1998; Smith, 1998). Furthermore, *P. kluyveri* and *P. anomala* are able to grow on pectin as a sole carbon source (unpublished results). Therefore, it is assumed that they may play a role in coffee fermentation among other yeast species identified, which were encountered occasionally in few samples and in low numbers. However, further studies are needed to investigate the technological properties of the predominant yeast species and their interactions with other microorganisms, such as lactic and acetic acid bacteria and Gram-negative bacteria present during fermentation. With regard to predominant yeast species in coffee samples, a good agreement was found between the profiles obtained by the DGGE and the findings obtained by traditional isolation and further identification of yeasts. DGGE seems to be an efficient tool for studying microbial diversity during natural fermentation. In addition, DGGE is a fast technique compared to time-consuming cultivation and isolation methods, especially when investigating large numbers of samples.

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Appendix II

Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*

Wafa Masoud ^{1*}, Leif Poll ² and Mogens Jakobsen ¹

¹ Department of Food Science, Food Microbiology

² Department of Food Science, Food Technology

The Royal Veterinary and Agricultural University

Denmark

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Research Article

Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*

Wafa Masoud^{1*}, Leif Poll² and Mogens Jakobsen¹

¹Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

²Department of Food Science, Food Technology, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

*Correspondence to:

Wafa Masoud, Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark.
E-mail: wm@kvl.dk

Abstract

The effects of volatile compounds produced during coffee processing by *Pichia anomala*, *P. kluyveri* and *Hanseniaspora uvarum* on growth of *Aspergillus ochraceus* and production of ochratoxin A (OTA) were studied. On malt extract agar (MEA) and on coffee agar (CA), exposure of *A. ochraceus* to the gaseous phase of malt yeast glucose peptone (MYGP) plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* inhibited fungal growth, with the two *Pichia* spp. showing the strongest effect. The main esters and alcohols produced by the three yeasts were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate and isoamyl alcohol. The individual esters and alcohols were found to affect fungal growth. The most effective compound in inhibiting fungal growth was 2-phenyl ethyl acetate; which at 48 µg/l headspace completely inhibited growth of *A. ochraceus*. Exposure of *A. ochraceus* to the gaseous phase of MYGP plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* prevented production of OTA. On CA medium, only the headspace of *P. anomala* and *P. kluyveri* prevented OTA production. Furthermore, when *A. ochraceus* was exposed to the headspace of the individual volatile compounds, 2-phenyl ethyl acetate was the most effective in preventing OTA production. Prevention of OTA seems to be due to reduction of fungal biomass. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: coffee; *Pichia anomala*; *Pichia kluyveri*; *Hanseniaspora uvarum*; *Aspergillus ochraceus*; OTA; volatile compounds

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Introduction

The coffee plant belongs to the genus *Coffea* of the family Rubiaceae. *Coffea arabica* and *Coffea canephora* var. *robusta* are the two species that have commercial importance. Coffee cherries are processed by dry or wet method to separate the beans from the pulp. The dry method is mainly used for Robusta coffee, which has a thin pulp that allows direct drying (Fowler *et al.*, 1998). In wet processing of coffee, which is mainly used for Arabica coffee, the ripe coffee cherries are pulped

followed by fermentation and drying (Fowler *et al.*, 1998).

The microbiota during coffee processing is composed of Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi (Silva *et al.*, 2000). In coffee samples collected at different steps of the wet processing of Arabica coffee in Tanzania three predominant yeast species were isolated in a previous study (Masoud *et al.*, 2004). They included *Pichia anomala*, which was found in high numbers at the beginning of fermentation and during the drying process, *P. kluyveri*

which predominated during the whole process and *Hanseniaspora uvarum* which was detected in high numbers during fermentation but decreased markedly in the drying process (Masoud *et al.*, 2004).

Ochratoxin A (OTA) is a secondary metabolite of some toxigenic species of *Aspergillus* and *Penicillium*, which has been shown to be genotoxic, nephrotoxic, teratogenic, immunotoxic and possibly carcinogenic (Petzinger and Ziegler, 2000). Levi *et al.* (1974) were the first to report occurrence of OTA in coffee beans. Since then, several studies have detected both OTA-producing fungi and OTA in green coffee beans (Levi, 1980; Mislivec *et al.*, 1983; Micco *et al.*, 1989; Studer-Rohr *et al.*, 1995; Nakajima *et al.*, 1997; Romani *et al.*, 2000; Ottender and Majerus, 2001; Pittet and Royer, 2002). The main filamentous fungi that have been found in coffee with the potential to produce OTA were *A. ochraceus*, *A. carbonarius* and *A. niger* (Nakajima *et al.*, 1997; Joosten *et al.*, 2001; Ngabirano *et al.*, 2001; Pitt *et al.*, 2001). The origin of OTA in coffee is not known. Bucheli *et al.* (1998) studied the accumulation of OTA during storage of green coffee beans and found no growth of OTA-producing fungi and no consistent production of OTA at different storage conditions. It appeared that accumulation of OTA in coffee beans occurred before storage and it was suggested to be linked mainly to post-harvest conditions of coffee processing (Bucheli *et al.*, 1998, 2000). However, for Brazilian coffee, it was found that the highest levels of contamination with OTA and OTA-producing fungi occurred in coffee beans during drying and storage (Taniwaki *et al.*, 2003). Recently, Palacios-Cabrera *et al.* (2004) reported that storage of contaminated green coffee beans with *A. ochraceus* at high humidity resulted in high levels of OTA at changing temperatures within the range 14–15 °C compared to a constant temperature of 25 °C.

It has been reported that some yeasts can inhibit growth of filamentous fungi. *Pichia anomala* was shown to be antagonistic to several fungi including *Botrytis cinerea* responsible for grey mould disease in grape-vine (Masih *et al.*, 2000), plant pathogenic fungi such as *Rhizoctonia solani*, *Fusarium equiseti*, *Botrytis fabae* and *Phytophthora infestans* (Walker *et al.*, 1995), *Penicillium roqueforti* and *A. candidus* (Björnberg and Schnürer, 1993) and *Penicillium verrucosum* (Petersson *et al.*, 1998). It

has been found that *P. anomala* and *P. kluyveri* were able to inhibit growth and OTA production by *A. ochraceus* when grown together on malt extract agar (MEA) or on coffee agar (CA) (unpublished results).

For biological control purposes in solid state fermentations, the antimicrobial effects of volatiles produced by some microorganisms against other microorganisms could be of a particular interest. It has been reported that growth of a number of plant pathogenic fungi can be inhibited by volatile compounds produced by *Muscodor albus* (Strobel *et al.*, 2001; Mercier and Jiménez, 2004) and by *Gliocladium* spp. (Stinson *et al.*, 2003). *Trichoderma* spp. were also found to produce volatile compounds, which were effective against wood decay fungi (Wheatley *et al.*, 1997). The effect of ethyl acetate, which is a major volatile compound produced by *P. anomala*, on growth of *Penicillium roqueforti* was investigated by Fredlund *et al.* (2004), who found that fungal growth was inhibited when plates inoculated with *Penicillium roqueforti* were exposed to gaseous phase containing ethyl acetate.

The aim of the present study was to determine the spectrum of volatiles produced by *P. anomala*, *P. kluyveri* and *H. uvarum* during coffee processing and the effects of the individual compounds on growth and OTA production by *A. ochraceus*.

Materials and methods

Cultures

Yeasts used in this study were obtained from coffee samples collected from the Arusha region, Tanzania (Masoud *et al.*, 2004). They included six strains of *P. anomala* (S12, S13, S14, S15, S16 and S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4 and S13Y4) and two strains of *H. uvarum* (S3Y8 and S15Y2). A non-fermentative laboratory strain of *Debaryomyces hansenii* CBS798 (CBS, Baarn and Delft, The Netherlands) was included as a control. Two strains of *A. ochraceus* (B677, B722) with the potential to produce OTA were studied. They were also isolated from coffee samples collected from Arusha region, Tanzania (Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition and Food, Karlsruhe, Germany).

Culture media

Malt yeast glucose peptone agar (MYGP) was prepared by dissolving 3 g malt extract (Difco, Detroit, MI, USA), 3 g yeast extract (Difco), 5 g bacto-peptone (Difco), 10 g D(+)-glucose monohydrate (Merck, Darmstadt, Germany) and 20 g agar (Difco) in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck). For malt extract agar (MEA), 20 g malt extract (Difco), 10 g D(+)-glucose monohydrate (Merck), 5 g bacto-peptone (Difco) and 20 g agar (Difco) were dissolved in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck). Diluent saline peptone (SPO) was prepared by dissolving 8.5 g NaCl (Merck), 0.3 g disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Merck) and 1 g bacto-peptone (Difco) in 1 l distilled water. SPO was adjusted to pH 5.6 by the addition of 1 M HCl and 1 M NaOH. Coffee agar (CA) was prepared by adding 20 g grounded green coffee beans (Levi Farm, Arusha, Tanzania) and 20 g agar (Difco) to 1 l distilled water. The diluent and all substrates were autoclaved at 121 °C for 15 min.

Effect of exposure of *A. ochraceus* to volatiles produced by *P. anomala*, *P. kluyveri* and *H. uvarum* on fungal growth and OTA production

The yeasts were propagated in 25 ml MYGP broth at 25 °C for 48 h. After propagation, the cells were harvested by centrifugation at $3000 \times g$ for 10 min, and resuspended in SPO. Cell concentrations were estimated by microscopy using a counting chamber (Neubauer) and the suspensions were diluted to final concentrations of 10^6 cells/ml; after which 100 µl each yeast suspension were spread on the MYGP plates.

Malt extract agar plates inoculated with *A. ochraceus* after an incubation period of 7 days at 30 °C were used to harvest fungal spores. Spores were suspended in SPO and the concentration was estimated by microscopy as just described and the suspensions were diluted in SPO to 10^6 spores/ml; 100 µl spore suspensions were spread onto MEA plates. The plates inoculated with yeasts and fungal spores were left to dry for 4 h at 25 °C. The lids were removed and the MYGP plate inoculated with yeast was inverted facing down the MEA plate inoculated with *A. ochraceus* and the two plates were sealed with tape and three layers of parafilm

and incubated at 30 °C for 7 days. A plate inoculated with *A. ochraceus* sealed with yeast free agar plates was included as well. Six trials were done for each yeast; three plates to estimate fungal growth and three plates to detect OTA as described below. The same experiment was carried out on CA medium in which both of the yeasts and fungi were grown on CA.

Growth of *A. ochraceus* was estimated by measuring the dry weight of the fungal biomass. The agar with fungal growth was cut and placed in 200 ml distilled water, which was then heated in a microwave oven until the residual agar was melted. The fungal biomass was recovered on a pre-weight filter paper washed with distilled water and dried at 80 °C for 24 h, whereafter, the dry weight of the fungal biomass was determined (Fredlund *et al.*, 2004).

Identification and quantification of volatile compounds produced by yeasts

The volatiles produced by four strains of *P. anomala* (S12, S13, S16 and S17), two strains of *P. kluyveri* (S7Y1 and S8Y4), one strain of *H. uvarum* (S15Y2) and a laboratory strain of *D. hansenii* (CBS 798) were identified. MYGP plates were inoculated with 100 µl of 10^6 cells/ml of each yeast, respectively, and left to dry for 4 h at 25 °C. Then the plates were sealed with tape and three layers of parafilm and incubated at 25 °C for 48 h. A yeast-free MYGP plate was used as a control. To analyse volatiles in the headspace above the plates inoculated with the yeasts, a hole was made in the top plate and capped with a rubber septum to allow sampling from the headspace with a syringe. The volatiles were collected in a trap of 250 mg Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Netherlands) using a gas pump (Handy check-8000, PBI Dansensor, Denmark) at a flow rate of 100 ml/min for 3 min. Desorption of volatiles was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, Buckinghamshire, UK). Desorption temperature of the first trap to the second cold trap (30 mg Tenax GR, 5 °C) was 250 °C for 15 min with a helium flow of 60 ml/min. Desorption temperature of the cold trap was 300 °C for 4 min with a helium flow of 31 ml/min and an outlet split ratio of 1 : 30. Separation was performed by a gas chromatography–mass

spectrometry (HP G1800 A GCD system, Palo Alto, CA) under the following conditions: column, DB Wax form, J&W Scientific, CA (30 m \times 0.25 μ m film thickness); carrier gas, helium; flow rate, 1 ml/min (constant); column pressure (constant), 48 kPa; oven programme, 45 °C for 10 min, then rising at 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV) and the m/z (mass/charge) ratio range was 10–425. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard, Palo Alto, CA).

Quantification of volatiles was based on comparisons of retention times and integrated peak areas of 14 reference compounds (see Table 1). A 10 μ l aliquot of each reference compound was dissolved in 10 ml heptane. A quantity of 2 μ l of the reference compound in heptane solution was injected into the GC–MS with the same flow, time and temperature conditions as just described. The peak area obtained for each reference compound was used to calculate the concentration of the compound in the headspace. The experiment was done in triplicate.

Effect of individual volatile compounds on growth and OTA production by *A. ochraceus*

The effect of the 14 individual volatile compounds identified in the headspace of yeasts cultures (Table 1) on growth of *A. ochraceus* and OTA production were investigated at concentrations of 4, 8, 12, 24, 48 and 96 μ g/l headspace. To control the concentration of volatiles in the headspace, 2 μ l each volatile compound purchased as a pure chemical, were added to 20 ml distilled water and placed in a Petri dish, which was sealed with tape and three layers of parafilm and left for 2 h at 25 °C, whereafter the concentrations of volatiles were determined by headspace analysis as described above. To obtain 4, 8, 12, 24, 48 and 96 μ g/l headspace over plates, the amounts of each volatile compound in distilled water that corresponded to the above concentrations in the headspace were calculated from the peak area obtained for each reference compound, as described above, assuming a linear relation between the concentrations in distilled water and in the headspace.

Aspergillus ochraceus was inoculated at a rate of 100 μ l of 10^6 spores/ml on MEA plates, which were left to dry. Then each plate was placed facing down another plate containing distilled

Table 1. The profiles of volatile compounds identified for four strains of *P. anomala* (P.a), two strains of *P. kluyveri* (P.k), one strain of *H. uvarum* (H.u) and one strain of *D. hansenii* (D.h) in headspace above malt yeast glucose peptone (MYGP) plates after 48 h of incubation at 25 °C

Compound	Concentrations of volatiles in the headspace above yeast cultures (μ g/l) ¹							
	P.a S12	P.a S13	P.a S16	P.a S17	P.k S8Y4	P.k S7Y1	H.u S15Y2	D.h CBS 798
Ethyl acetate	65.0	42.0	31.0	74.0	69.0	54.0	49.0	— ²
Ethyl propionate	10.0	14.0	12.0	6.0	2.0	5.0	3.0	—
Propyl acetate	8.0	10.0	3.0	14.0	—	—	2.0	—
Isobutyl acetate	50.0	37.0	23.0	62.0	34.0	53.0	21.0	—
Propyl propionate	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	—	—
Butyl acetate	2.0	≤ 1.0	≤ 1.0	5.0	≤ 1.0	≤ 1.0	≤ 1.0	—
Isobutyl alcohol	3.0	4.0	≤ 1.0	5.0	≤ 1.0	≤ 1.0	≤ 1.0	6.0
Isopentyl acetate	3.0	4.0	≤ 1.0	5.0	4.0	3.0	—	—
Butanoic acid octyl ester	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	—
Isoamyl alcohol	7.0	4.0	6.0	10.0	7.0	5.0	≤ 1.0	3.0
Acetoin	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	—
2-phenyl ethyl acetate	23.0	19.0	17.0	27.0	16.0	17.0	5.0	—
Phenyl ethyl alcohol	3.0	≤ 1.0	≤ 1.0	4.0	≤ 1.0	≤ 1.0	≤ 1.0	—
Isobutyl propanoate	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	—

¹ Average values for three trials for each volatile compound determined for each yeast. Standard deviations for all trials were in the range 0.5–2.0.

² Not detected.

water with the concentration estimated for each volatile compound, respectively. The two plates were sealed as just described and incubated at 30 °C for 7 days. Six trials were done for each volatile to estimate fungal growth and OTA formation in triplicates. The dry weight of the fungal biomass was determined as described above and OTA was detected as described below.

Determination of OTA in MEA and CA by thin layer chromatography (TLC)

The ability of *A. ochraceus* to produce OTA on MEA and CA plates when exposed to yeasts or volatiles was investigated by thin layer chromatography (TLC) as described by Samson *et al.* (2002). Agar plugs were aseptically removed from mould colonies on MEA and CA plates inoculated with *A. ochraceus* and one drop of chloroform:methanol mixture (1:2) was added to each plug. The plug was placed onto a TLC plate silica gel 60 (Merck Art 5721) with mycelium side towards the gel. 10 µg/l OTA in toluene:acetic acid (99:1) was added to TLC plates as a standard. Then the TLC plates were developed in toluene:acetone:methanol (5:3:2) and left to dry in a fume hood for 10 min. The TLC plates were examined visually under UV light at 366 nm wave length. The OTA detection limit of this analysis was determined to be 6 µg/l.

Results

Effect of exposure of *A. ochraceus* to volatiles produced by *P. anomala*, *P. kluyveri* and *H. uvarum* on fungal growth

As seen from Figure 1, exposure of *A. ochraceus* B722 grown on MEA to the headspace of MYGP plates inoculated with yeasts inhibited fungal growth. Growth of *A. ochraceus* B722 on CA was also inhibited when exposed to CA plates inoculated with strains of the three yeast species; however, the inhibition was less pronounced. On both MYGP and CA media, strains of *P. anomala* and *P. kluyveri* showed stronger inhibition than *H. uvarum*. When yeasts were grown on MYGP, the strongest inhibition of fungal growth was observed for *P. anomala* S12 and S17. The inhibition amounted to 70% compared to the control. On CA, *P. anomala* S17 was observed to inhibit

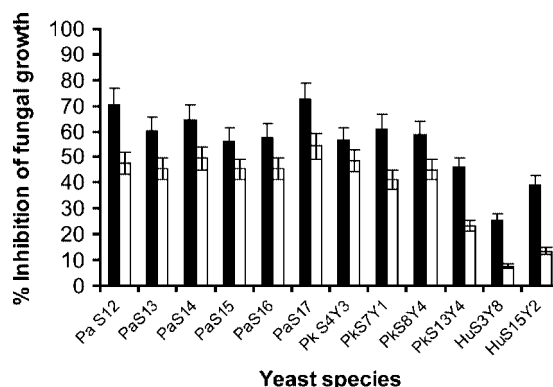


Figure 1. Growth inhibition of *A. ochraceus* B722 exposed to volatiles produced by six strains of *P. anomala* (Pa S12, Pa S13, Pa S14, Pa S15, Pa S16 and Pa S17), four strains of *P. kluyveri* (Pk S4Y3, Pk S7Y1, Pk S8Y4 and Pk S13Y4) and two strains of *H. uvarum* (Hu S3Y8 and Hu S15Y2). *A. ochraceus* grown on MEA was exposed to yeasts grown on MYGP (black bars). *A. ochraceus* grown on CA was exposed to yeasts grown on CA (white bars). Inhibition is expressed as the percentage reduction of the fungal biomass compared to the control (mould grown on plates exposed to yeast-free medium). Indicators above bars represent standard deviations

growth of *A. ochraceus* B722 by about 55%. The remaining strains of *P. anomala* and three strains of *P. kluyveri*, S4Y3, S7Y1 and S8Y4, grown on CA, inhibited fungal growth by 40–50%. For the two strains of *H. uvarum* grown on MYGP, the inhibition of fungal growth was about 25% and 40%. When grown on CA medium, the two strains of *H. uvarum* S3Y8 and S15Y2 showed weak inhibition, with percentages of growth inhibition of ca. 10%. For all trials, similar observations were made for *A. ochraceus* B677 (results not shown).

Identification and quantification of volatile compounds produced by yeasts

The major volatile compounds produced by the yeasts on MYGP agar are shown in Table 1. The profiles of volatile compounds produced by *P. anomala*, *P. kluyveri* and *H. uvarum* were similar. However, the concentrations of volatiles such as isoamyl alcohol, ethyl propionate, isobutyl acetate, isopentyl acetate and 2-phenyl ethyl acetate in the headspace above cultures of *H. uvarum* on MYGP plates were lower than those above cultures of *P. anomala* and *P. kluyveri*. The major volatile produced by the three yeasts was ethyl acetate, followed by isobutyl acetate, 2-phenylethyl acetate,

ethyl propionate and isoamyl alcohol. No ester was found in the headspace above MYGP agar inoculated with *D. hansenii*. For this yeast, only isobutyl alcohol and isoamyl alcohol were detected in the headspace.

Effect of the individual volatile compounds on growth of *A. ochraceus*

The effect of exposure of *A. ochraceus* B722 inoculated on MEA plates to the headspace of different concentrations of esters and alcohols produced by *P. anomala*, *P. kluyveri* and *H. uvarum* are shown in Table 2. Differences between volatiles in their ability to affect fungal growth were evident. Ethyl acetate, the main ester produced by the three yeasts (Table 1), started to inhibit growth of *A. ochraceus* at a concentration of 48 µg/l headspace with a 15% reduction of the dry weight of fungal mycelia compared with the control. However, 2-phenyl ethyl acetate started to inhibit fungal growth at 4 µg/l headspace and the percentage of inhibition increased strongly with increasing headspace concentration. At 48 µg/l headspace, 2-phenyl ethyl acetate was able to completely inhibit fungal growth under the conditions of the trial. Other volatiles, such as ethyl propionate,

isobutyl acetate, isoamyl alcohol, isobutyl alcohol and phenyl ethyl alcohol, were found to reduce growth of *A. ochraceus* at initial concentrations of 12 and 24 µg/l headspace and the reduction increased with increasing volatile concentrations (Table 2).

Effect of exposure of *A. ochraceus* to the headspace of agar plates inoculated with yeasts on OTA formation

The effect of exposure of MEA plates inoculated with *A. ochraceus* B722 to the headspace of MYGP plates inoculated with strains of *P. anomala*, *P. kluyveri*, *H. uvarum* and *D. hansenii* on the production of OTA is shown in Figure 2. All strains of *P. anomala*, *P. kluyveri* and *H. uvarum* were found to prevent production of OTA by *A. ochraceus* at a detection limit of 6 µg/l. Exposure of *A. ochraceus* to the headspace of plates inoculated with *D. hansenii* did not prevent production of OTA. When both yeasts and *A. ochraceus* B722 were grown on CA, the headspace of *P. anomala* and *P. kluyveri* prevented OTA production; while the headspace of *H. uvarum* and *D. hansenii* did not prevent OTA production (Table 3). Although exposure of *A. ochraceus* B722 to the headspace of MYGP and CA agar inoculated with the strains

Table 2. The effect of individual volatile compounds on growth of *A. ochraceus* B722 at the headspace concentrations stated

Compound	Dry weight of fungal biomass at different concentrations of volatiles in the headspace above plates ¹						
	0	4 µg/l	8 µg/l	12 µg/l	24 µg/l	48 µg/l	96 µg/l
Ethyl acetate	61 ²	62 (–) ³	61 (–)	62 (–)	61 (–)	52 (15%)	33 (46%)
Ethyl propionate	59	61 (–)	60 (–)	58 (–)	52 (11%)	50 (15%)	38 (36%)
Isobutyl acetate	62	61 (2%)	62 (–)	57 (8%)	54 (13%)	30 (52%)	17 (73%)
Butyl acetate	61	62 (–)	60 (2%)	60 (2%)	59 (3%)	54 (11%)	35 (43%)
Propyl acetate	58	60 (–)	59 (–)	58 (–)	58 (–)	60 (–)	32 (45%)
Propyl propionate	62	60 (3%)	59 (5%)	62 (–)	61 (2%)	55 (11%)	39 (37%)
Isopentyl acetate	59	59 (–)	58 (–)	60 (–)	60 (–)	51 (14%)	34 (42%)
Isobutyl propionate	60	58 (3%)	60 (–)	59 (2%)	58 (3%)	49 (18%)	29 (52%)
Isoamyl alcohol	62	60 (3%)	58 (6%)	56 (10%)	52 (16%)	41 (34%)	17 (73%)
2-Phenyl ethyl acetate	61	54 (12%)	39 (36%)	32 (48%)	16 (74%)	0 (100%)	0 (100%)
Isobutyl alcohol	59	61 (–)	60 (–)	53 (10%)	48 (19%)	25 (58%)	16 (73%)
Phenyl ethyl alcohol	61	59 (3%)	61 (–)	54 (12%)	39 (36%)	17 (72%)	0 (100%)
Butanoic acid octyl ester	61	63 (–)	59 (3%)	58 (5%)	60 (2%)	54 (11%)	43 (30%)

¹ Average values of dry weight for three trials for each concentration of each volatile tested. Standard deviations for all trials were in the range 1–2.5.

² Dry weight of fungal biomass not exposed to volatiles.

³ The values between brackets are the percentages of reduction of the dry weight of the fungal biomass compared to the dry weight of fungal biomass not exposed to volatiles. (–), no reduction in the dry weight of fungal biomass.

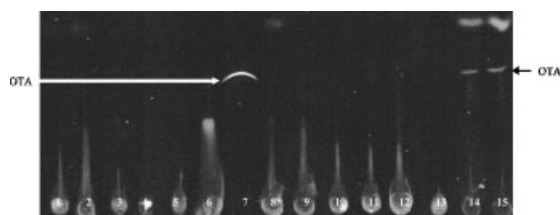


Figure 2. Production of OTA by *A. ochraceus* B722 grown on MEA plates exposed to the headspace of yeasts grown on MYGP plates. The lane numbers refer to *A. ochraceus* exposed to plates inoculated with: lane 1, *P. anomala* S12; lane 2, *P. anomala* S13; lane 3, *P. anomala* S14; lane 4, *P. anomala* S15; lane 5, *P. anomala* S16; lane 6, *P. anomala* S17; lane 7, OTA standard (10 µg/l); lane 8, *P. kluyveri* S4Y3 P; lane 9, *P. kluyveri* S7Y1; lane 10, *P. kluyveri* S8Y4; lane 11, *P. kluyveri* S13Y4; lane 12, *H. uvarum* S3Y8; lane 13, *H. uvarum* S15Y2; lane 14, *D. hansenii*; lane 15, MYGP without yeast (control)

Table 3. Ochratoxin A production (≥ 6 µg/l) by *A. ochraceus* B722 and B677 on malt extract agar (MEA) and coffee agar (CA) when exposed to agar plates inoculated with *P. anomala*, *P. kluyveri*, *H. uvarum* and *D. hansenii* grown on malt yeast glucose peptone (MYGP) and CA media

Yeast	<i>A. ochraceus</i> B722		<i>A. ochraceus</i> B677	
	MYGP ¹	CA ²	MYGP ¹	CA ²
Control ³	+	+	+	+
<i>P. anomala</i> S12	— ⁵	—	—	—
<i>P. anomala</i> S13	—	—	—	—
<i>P. anomala</i> S14	—	—	—	—
<i>P. anomala</i> S15	—	—	—	—
<i>P. anomala</i> S16	—	—	—	—
<i>P. anomala</i> S17	—	—	—	—
<i>P. kluyveri</i> S4 Y8	—	—	—	—
<i>P. kluyveri</i> S7 Y1	—	—	—	—
<i>P. kluyveri</i> S8 Y4	—	—	—	—
<i>P. kluyveri</i> S13 Y4	—	—	—	—
<i>H. uvarum</i> S3Y8	—	+	—	+
<i>H. uvarum</i> S15Y2	—	+	—	+
<i>D. hansenii</i> CBS 798	+	+	+	+

¹ *A. ochraceus* grown on MEA exposed to MYGP plate inoculated with yeasts.

² *A. ochraceus* grown on CA exposed to CA plates inoculated with yeasts.

³ *A. ochraceus* exposed to MYGP and CA plates without yeasts.

⁴ OTA ≥ 6 µg/l.

⁵ OTA < 6 µg/l.

of *P. anomala* and *P. kluyveri* did not inhibit fungal growth completely (Figure 1), production of OTA by *A. ochraceus* B722 under the same experimental conditions was under the detection limit of 6 µg/l. Similar observations were made for *A. ochraceus* strain B677, as shown in Table 3.

Effect of exposure of *A. ochraceus* to individual volatile compounds on OTA formation

The exposure of *A. ochraceus* B722 to different concentrations of the individual volatile compounds similar to those produced by the three yeasts on OTA formation showed that the most effective compound which affected OTA formation was 2-phenyl ethyl acetate; OTA was not detected at a concentration of 24 µg/l headspace or higher (results not shown). Phenyl ethyl alcohol and isobutyl alcohol affected OTA production at 48 and 96 µg/l headspace concentrations (results not shown). Only at 96 µg/l headspace of isobutyl acetate and isoamyl alcohol, OTA was not detected. The remaining volatiles, ethyl acetate, ethyl propionate, butyl acetate, propyl acetate, propyl propionate, isopentyl acetate, isobutyl propionate and butanoic acid octyl ester were found to have no effect on OTA formation by *A. ochraceus* in the range of concentrations investigated (results not shown).

Discussion

In the present work, exposure of *A. ochraceus* to the headspace of MYGP or CA plates inoculated with strains of *P. anomala*, *P. kluyveri* and *H. uvarum*, the predominant yeasts involved in coffee processing, was found to inhibit mould growth. Differences were observed in the degrees of growth inhibition among yeast species as well as between strains of the same species. Two strains of *P. anomala* S12 and S17 showed the strongest effect on growth of *A. ochraceus*, followed by the remaining strains of *P. anomala* and strains of *P. kluyveri*. Growth inhibition of *A. ochraceus* caused by the headspace of strains of *H. uvarum* was comparatively weak, especially on CA medium. Furthermore, exposure of *A. ochraceus* to the headspace of *P. anomala*, *P. kluyveri* and *H. uvarum* grown on MYGP plates prevented production of OTA. On the other hand, exposure of *A. ochraceus* to MYGP plates inoculated with *D. hansenii* did not affect OTA production. When yeasts were grown on CA medium, only exposure of *A. ochraceus* to the headspace of *P. anomala* and *P. kluyveri* prevented OTA production. Similar findings were obtained on the effects of co-culturing of *P. anomala*, *P. kluyveri* and *H.*

uvarum with *A. ochraceus* on MEA and CA substrates, as mentioned above. Although exposure of *A. ochraceus* to the headspace of CA plates inoculated with *P. anomala* and *P. kluyveri* showed less inhibition of mould growth than when the two yeasts were grown on MYGP plates, exposure of the fungus to the headspace of CA plates with the two yeasts prevented OTA formation. This could be due to reduction of fungal biomass where OTA was under the detection limit, i.e. $<6 \mu\text{g/l}$. It might also be because volatiles produced by the investigated yeasts can inhibit biosynthesis of OTA by *A. ochraceus* under conditions where fungal growth takes place.

The effect of exposure of *A. ochraceus* grown on MEA to the headspace of MYGP plates inoculated with *P. anomala*, *P. kluyveri* and *H. uvarum* was found to be stronger in reduction of fungal biomass compared to exposure of *A. ochraceus* grown on CA to yeasts grown on CA medium. It was also found that *P. anomala*, *P. kluyveri* and *H. uvarum* have a stronger inhibitory effect on growth and OTA production by *A. ochraceus* on MEA than on CA (unpublished results). The reason could be that the three yeasts were found to show weaker growth on CA than on MYGP. De Maria *et al.* (1994) found that in Arabica fermented green coffee beans the main carbohydrates were polysaccharides, sucrose and trace amounts of galactose, arabinose, mannose, glucose and xylose. It was also reported that in Arabica coffee beans the concentrations of glucose and fructose by the end of maturation and at the time of picking of the cherries, i.e. before fermentation, were about 0.03% and 0.04% dry weight, respectively (Rogers *et al.*, 1999). The three yeasts were found to produce acetate esters, which are produced from alcohols and acetyl co-enzyme A by the alcohol acetyltransferase (Yoshioka and Hashimoto, 1981). It has been reported that production of esters and alcohols by *Saccharomyces cerevisiae* increases when glucose or fructose are used as a sole carbon source compared to maltose, and increasing the concentrations of glucose increased the amounts of esters and alcohols produced (Younis and Stewart, 1998). This might explain the lower antagonist activity of the yeasts when grown on CA, with its low content of carbohydrates. Furthermore, it has been reported that the number and quantities of volatiles, which included alcohols, esters and ketones, produced by the antagonist fungus *Muscodor albus* grown on

low nutrient medium, were also found to be less than those produced on a more enriched medium with a high level of carbon source (Ezra and Strobel, 2003). It has also been found that exposure of plant pathogenic fungi to the antagonist fungi *M. albus* (Ezra and Strobel, 2003) and *Trichoderma* spp. (Wheatley *et al.*, 1997) grown on a medium with a low sugar content was less effective in inhibiting fungal growth than when the two antagonists were grown on a more sugar-enriched medium.

The major esters produced by *P. anomala*, *P. kluyveri* and *H. uvarum* were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate and ethyl propionate. On the other hand, only isobutyl alcohol and isoamyl alcohol at 3 and $6 \mu\text{g/l}$ were detected in the headspace of plates inoculated with a laboratory strain of *D. hansenii*. Exposure of *A. ochraceus* to this yeast species did not affect fungal growth (unpublished results) or OTA production. With the exception of ethyl acetate, the estimated quantities of esters and alcohols in the headspace above yeast cultures showed that *H. uvarum* produced the lowest amounts compared to *P. anomala* and *P. kluyveri*, which might explain the weak antagonist activity of this yeast species.

The main esters and alcohols produced by the three yeasts were individually tested against growth of *A. ochraceus* and OTA formation. Various inhibition levels were observed among the tested compounds. When *A. ochraceus* was exposed to $48 \mu\text{g/l}$ headspace of ethyl acetate, the fungal biomass reduction was only 15%. At $96 \mu\text{g/l}$ headspace, which is higher than that estimated in the headspace above yeast cultures, the biomass reduction increased to 46%. Furthermore, OTA formation was not affected when the fungus was exposed to the various headspace concentrations of ethyl acetate. This means that ethyl acetate is not the major ester responsible for inhibition of *A. ochraceus*. On the contrary, 2-phenyl ethyl acetate reduced the biomass of *A. ochraceus* at an initial headspace concentration of $4 \mu\text{g/l}$ and the percentage of biomass reduction increased with increasing headspace concentration. The growth of *A. ochraceus* was inhibited completely at $48 \mu\text{g/l}$ headspace of 2-phenyl ethyl acetate. Furthermore, OTA was not detected when *A. ochraceus* was exposed to a concentration of $24 \mu\text{g/l}$ headspace of 2-phenyl ethyl acetate. It seems that 2-phenyl ethyl acetate plays a major role in the antagonist

activity of the tested yeasts against *A. ochraceus*. It has been reported that 2-phenyl ethyl acetate and phenyl ethyl alcohol were among the bioactive compounds produced by the antagonist fungi *M. albus* (Strobel *et al.*, 2001) and *Gliocladium* spp. (Stinson *et al.*, 2003).

It was observed that when the percentage of reduction of the fungal biomass caused by the individual volatiles was more than 50% (Table 3), OTA was not detected on TLC plates (results not shown). It seems that reduction of the fungal biomass is the main reason for the absence of OTA.

For the purpose of preventing production of OTA in coffee, the present work demonstrated the possibility of using *P. anomala* and *P. kluyveri* in biological control of OTA-producing fungi during coffee fermentation. From this work and a recent study (unpublished results), it appears that two mechanisms are involved, i.e. an effect of volatiles and a competition for nutrients. Further studies are needed on the effects of *P. anomala* and *P. kluyveri* on other OTA-producing fungi present in coffee. In addition, studies should be conducted of interactions between the two yeasts and OTA-producing fungi *in vivo*, i.e. during coffee processing.

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Appendix III

The effects of yeasts involved in fermentation of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*

Wafa Masoud and Christa Høj Kaltoft

Department of Food Science, Food Microbiology
The Royal Veterinary and Agricultural University
Denmark.

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Short Communication

The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*

Wafa Masoud*, Christa Høj Kaltoft

Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

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Abstract

The effects of *Pichia anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum* predominant during coffee processing on growth of *Aspergillus ochraceus* and production of ochratoxin A (OTA) on malt extract agar (MEA) and on coffee agar (CA) were studied. The three yeasts were able to inhibit growth of *A. ochraceus* when co-cultured in MEA and CA. Growth inhibition was significantly higher on MEA than on CA. Furthermore, *P. anomala* and *P. kluyveri* were found to have a stronger effect on growth of *A. ochraceus* than *H. uvarum*. The three yeasts were able to prevent spore germination of *A. ochraceus* in yeast glucose peptone (MYGP) broth. In yeast-free supernatant of MYGP broth after an incubation period of 72 h, spores of *A. ochraceus* were able to germinate with very short germ tubes, but further development of the germ tubes was inhibited. The three yeasts decreased the pH of MYGP broth from 5.6 to a range of 4.4–4.7, which was found to have no effect on spore germination of *A. ochraceus*.

P. anomala, *P. kluyveri* and *H. uvarum* were able to prevent production of OTA by *A. ochraceus* when co-cultured on MEA. On CA medium, *P. anomala* and *P. kluyveri* prevented *A. ochraceus* from producing OTA. *H. uvarum* did not affect production of OTA by *A. ochraceus* on CA medium.

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Keywords: Coffee; *Pichia anomala*; *Pichia kluyveri*; *Hanseniaspora uvarum*; *Aspergillus ochraceus*; OTA

1. Introduction

Wet processing of coffee is mainly used for *arabica* coffee, where the ripe coffee cherries are pulped followed by fermentation and drying (Fowler et al., 1998). The main goal of fermentation is to degrade the slimy mucilage adhering firmly to coffee beans by pectolytic enzymes produced by natural occurring microbiota (Illy and Viani, 1995). At all steps of coffee processing, Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi are present at high levels (Silva et al., 2000). In a previous study (Masoud et al., 2004) on the yeasts community of *arabica* coffee in East Africa, we found that the total yeasts counts were in a range of 4.0×10^4 to 5×10^7 cfu/g with an increase during fermentation. *Pichia anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum* were the

three predominant yeasts during the different stages of processing (Masoud et al., 2004).

Ochratoxin A (OTA) is a secondary metabolite of toxigenic species of *Aspergillus* and *Penicillium*, which has been detected in foods such as cereal products, wine, beer, coffee, spices and grape juice (European Commission [EC] No. 472 of 12/03/2002). In a literature survey, Varga et al. (2001) reported that in temperate regions, OTA is mainly produced by *Penicillium* species, whereas in tropical and subtropical areas, OTA is produced by *Aspergillus* species. Several studies have reported the occurrence of both OTA-producing fungi and OTA in green coffee beans (Levi et al., 1974; Levi, 1980; Mislivic et al., 1983; Micco et al., 1989; Studer-Rohr et al., 1995; Nakajima et al., 1997; Romani et al., 2000; Ottender and Majerus, 2001; Pittet and Royer, 2002). Taniwaki et al. (2003) isolated *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus niger* from Brazilian coffee cherries and beans and found that 3%, 75% and 77% of *A. niger*, *A. ochraceus* and *A. carbonarius* isolates produced OTA, respectively. A survey on

* Corresponding author. Tel.: +45 35 28 32 87; fax: +45 35 28 32 14.

E-mail address: wm@kv1.dk (W. Masoud).

stored green coffee beans from various origins has shown that coffee samples from African origin have significantly higher levels of OTA than those from America and Asia (Pardo et al., 2004). Little is known about the origin of OTA in coffee and when exactly it is produced along the coffee processing chain.

Growth of yeasts and moulds together on same substrate can lead to positive or negative interaction. During ripening of the blue mould cheese Danablu, growth of *Penicillium roqueforti* has been found to be stimulated by *Debaryomyces hansenii*; while *Candidum geotricum* and *Yarrowia lipolytica* were found to inhibit growth of *P. roqueforti* under same conditions (Van den Tempel and Nielsen, 2000; Van den Tempel and Jakobsen, 2000). Furthermore, *D. hansenii*, *Candida sake* and *P. anomala* were reported to control growth of some plant pathogenic fungi (Droby et al., 1989; Viñas et al., 1998; Walker et al., 1995; Masih et al., 2000). Biological control of OTA-producing fungi during the different stages of coffee processing might help to reduce the accumulation of OTA in green coffee beans. Petersson et al. (1998) found that *P. anomala* significantly reduced growth and production of OTA by *Penicillium verrucosum* in malt extract agar as well as in wheat.

The aim of the present study was to investigate the effects of *P. anomala*, *P. kluyveri* and *H. uvarum* predominant during coffee processing on growth and OTA production by *A. ochraceus* in laboratory substrates including coffee-based agar medium.

2. Materials and methods

2.1. Cultures

Yeasts used in this study were obtained from coffee samples collected from Arusha region, Tanzania (Masoud et al., 2004). They included six strains of *P. anomala* (S12, S13, S14, S15, S16, S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4) and two strains of *H. uvarum* (S3Y8, S15Y2). In addition, two strains of *A. ochraceus* (B677, B722) with the potential to produce OTA were studied. They were also isolated from coffee samples collected from Arusha region, Tanzania (Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition and Food, Karlsruhe, Germany).

2.2. Culture media

Malt yeast glucose peptone medium (MYGP) was prepared by dissolving 3 g malt extract (Difco, Detroit, MI, USA), 3 g yeast extract (Difco), 5 g bacto-peptone (Difco) and 10 g D(+) glucose monohydrate (Merck, Darmstadt, Germany) in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck). For malt extract agar (MEA), 20 g of malt extract (Difco), 10 g D(+) glucose monohydrate (Merck), 5 g bacto-peptone (Difco) and 20 g of agar (Difco) were dissolved in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck). Diluent saline peptone (SPO) was prepared by dissolving 8.5 g NaCl (Merck), 0.3 g disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Merck) and 1 g bacto-peptone (Difco) in 1 l distilled water. SPO was adjusted

to pH 5.6 by the addition of 1 M HCl and 1 M NaOH. Coffee agar (CA) was prepared by adding 20 g of grounded green coffee beans (Levi Farm, Arusha, Tanzania) and 20 g of agar (Difco) to 1 l distilled water.

2.3. Effect of yeasts predominant in coffee on growth of *A. ochraceus*

Strains of *P. anomala*, *P. kluyveri* and *H. uvarum* were propagated in 25 ml of MYGP broth at 25 °C for 48 h. After propagation, cells were harvested by centrifugation at $3000 \times g$ for 10 min and resuspended in SPO. Cell concentrations were estimated by microscopy using a counting chamber (Neubauer) and the suspensions were diluted to final concentrations of 10^4 and 10^6 cells/ml. Suspensions of yeasts were mixed with 20 ml of melted MEA and poured in Petri dishes, which were left for 2 h to solidify. Spores of *A. ochraceus* were harvested from MEA plates and suspended in SPO. Spore concentration was estimated by microscopy as described above and the suspension was diluted in SPO to 10^6 spores/ml. After solidification of the MEA plates inoculated with yeasts, spots of 10 µl of *A. ochraceus* spore suspension (10^6 spore/ml) were placed on three sites of each plate. Spots of *A. ochraceus* spore suspension were also placed on three sites of yeast-free MEA plate, which was used as a control. The plates were incubated at 30 °C for 7 days where after, growth of fungi was determined by measuring the fungal colony diameter. The experiment was done in triplicates. The same experiment was done on CA medium.

2.4. Effects of yeasts cells and yeast-free supernatant on germination of *A. ochraceus* spores

In this assay, the effects of the six strains of *P. anomala*, the four strains of *P. kluyveri* and the two strains of *H. uvarum* on spore germination of *A. ochraceus* B722 were investigated. Spores of *A. ochraceus* B722 (10^6 spores/ml) were inoculated together with 10^6 cells/ml of each yeast in 10 ml MYGP broth (pH 5.6) and incubated at 30 °C. Spores of *A. ochraceus* were also inoculated in yeast cell-free supernatant, which was obtained by propagation of each yeast in 25 ml of MYGP broth at 25 °C for 24 h. Whereafter, yeasts cultures were centrifuged at $3000 \times g$ for 10 min and the supernatant was filtered through a 0.22-µm nitro-cellulose filter (Osmonics, Minnetonka, MN, USA). The pH of supernatant was determined. Spores of *A. ochraceus* inoculated in MYGP broth (pH 5.6) was used as a control. Furthermore, spores of *A. ochraceus* were inoculated in MYGP broth adjusted to pH 4.4, 4.5, 4.6 and 4.7. After 24, 48 and 72 h of incubation at 30 °C, germination of the fungal spores was inspected by microscopy where five regions of each sample with about 10–20 spores in each region were inspected. The experiment was carried out in triplicates.

2.5. Effects of yeasts on production of OTA by *A. ochraceus*

The ability of the two strains of *A. ochraceus* B722 and B677 to produce OTA when co-cultured with the six strains of

P. anomala, the four strains of *P. kluyveri* and the two strains of *H. uvarum* on MEA and CA plates was investigated. Yeasts were co-cultured with *A. ochraceus* on MEA and CA plates as described above. *A. ochraceus* was also inoculated in yeast-free MEA and CA plates, which were used as controls. After an incubation period of 7 days at 30 °C, production of OTA was estimated by thin-layer chromatography (TLC) (Samson et al., 2002). Agar plugs were aseptically removed from mould colonies on MEA and CA plates and one drop of chloroform/methanol mixture (1:2) was added to each plug. The plug was placed onto a TLC plate silica gel 60 (Merck Art 5721) with mycelium side towards the gel. OTA of 10 µg/l in toluene/acetic acid (99:1) was used as a standard. Then the TLC plates were developed in toluene/acetone/methanol (5:3:2) and left to dry in a fume hood for 10 min. The TLC plates were examined visually under UV light at 366 nm wave length.

3. Results and discussion

3.1. Effect of yeasts predominant in coffee on growth of *A. ochraceus*

The effect of six strains of *P. anomala*, four strains of *P. kluyveri* and two strains of *H. uvarum* at 10^4 cells/ml on growth of *A. ochraceus* B722 on MEA and CA media is shown in Fig. 1. The three yeasts were found to inhibit growth of *A. ochraceus* when grown together. On both MEA and CA media, strains of *P. anomala* and *P. kluyveri* were found to have stronger effect on growth of *A. ochraceus* compared to *H. uvarum*. On CA medium, the levels of growth inhibition of *A. ochraceus* by the two strains of *H. uvarum* were extremely low. The two strains of *P. anomala* S12 and S17 were found to have the highest percentages of inhibition against *A. ochraceus*. The percentage of fungal growth inhibition caused by *P. kluyveri* S13Y4 was lower than those caused by *P. anomala*

and the other three strains of *P. kluyveri*. Small differences in the degree of inhibition among the remaining strains of *P. anomala* and *P. kluyveri* were observed. Increasing concentration of yeasts to 10^6 cells/ml increased growth inhibition of *A. ochraceus* (results not shown). The same findings on the effects of yeasts on growth of *A. ochraceus* B777 were obtained (results not shown).

P. anomala was reported to inhibit a number of fungi like *Botrytis cinerea* (Masih et al., 2000), *P. roqueforti*, *Aspergillus candidus* (Petersson and Schnürer, 1995) and *P. verrucosum* (Petersson et al., 1998). *P. kluyveri* and *H. uvarum* were found to produce killer toxins against other yeasts (Zorg et al., 1988; Abranches et al., 1997). However, the antagonist activities of those two yeasts against filamentous fungi have not been investigated. A good understanding of the mode of action of the antagonist activity will help to clarify the mechanism behind it. In the present study, the degree of inhibition was found to be dependent on the yeast species and the substrate used. On both MEA and CA media, strains of *P. anomala* and *P. kluyveri* were found to have stronger effect on growth of *A. ochraceus* compared to *H. uvarum*. On CA medium, the levels of growth inhibition of *A. ochraceus* by the two strains of *H. uvarum* was significantly lower. For all yeasts, inhibition of fungal growth was significantly higher on MEA compared to that on CA medium. The three yeasts showed less growth on CA compared to MEA medium (results not shown), which might explain the lower inhibition of fungal growth by the three yeasts on CA. The CA medium may contain less specific nutrients essential for growth of yeasts. On the other hand, *A. ochraceus* showed very good and equal growth in both yeasts free plates of CA and MEA (results not shown).

3.2. Effects of yeasts cells and yeast-free supernatant on germination of *A. ochraceus* spores

Germination of *A. ochraceus* B722 spores when co-cultured with *P. anomala* S12 in MYGP broth and when inoculated in the cell-free supernatant of *P. anomala* S12 is shown in Fig. 2. Co-culture of *P. anomala* S12 with *A. ochraceus* B722 totally inhibited fungal spore germination after 24, 48 and 72 h of incubation (Fig. 2D–F). Similar results were obtained for the effect of the other strains of *P. anomala*, *P. kluyveri* and *H. uvarum* on spore germination of *A. ochraceus* B722 (results not shown). In yeast-free supernatant, spores of *A. ochraceus* did not germinate after 24 h (Fig. 2G). Spores of *A. ochraceus* started to swell after 48 h, but germ tubes were not observed (Fig. 2H). After 72 h, some spores germinated with very short germ tubes (Fig. 2I) compared to the control (Fig. 2A–C). The same observations were obtained for the other five strains of *P. anomala*, the four strains of *P. kluyveri* and the two strains of *H. uvarum* (results not shown). The pH of the yeast-free supernatant was determined; it was found that the pH decreased from 5.6 to a range of 4.4–4.7 by the three yeasts species. Germination of *A. ochraceus* spores in MYGP broth at pH values 4.4–4.7 was not affected (results not shown). It seems that inhibition of spore germination in yeast-free supernatant

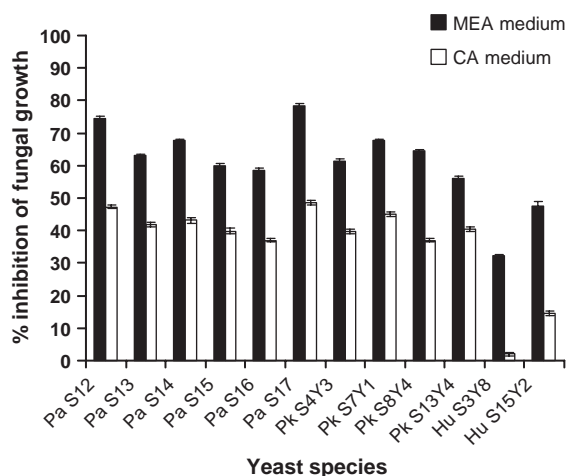


Fig. 1. Growth inhibition of *A. ochraceus* B722 on MEA and CA plates inoculated with six strains of *P. anomala* (S12, S13, S14, S15, S16, S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4) and two strains of *H. uvarum* (S3Y8, S15Y2). Inhibition is expressed as the percentage of reduction of the fungal colony diameter compared to the control (fungal colony diameter on free yeast plates). Bars represent standard deviations.

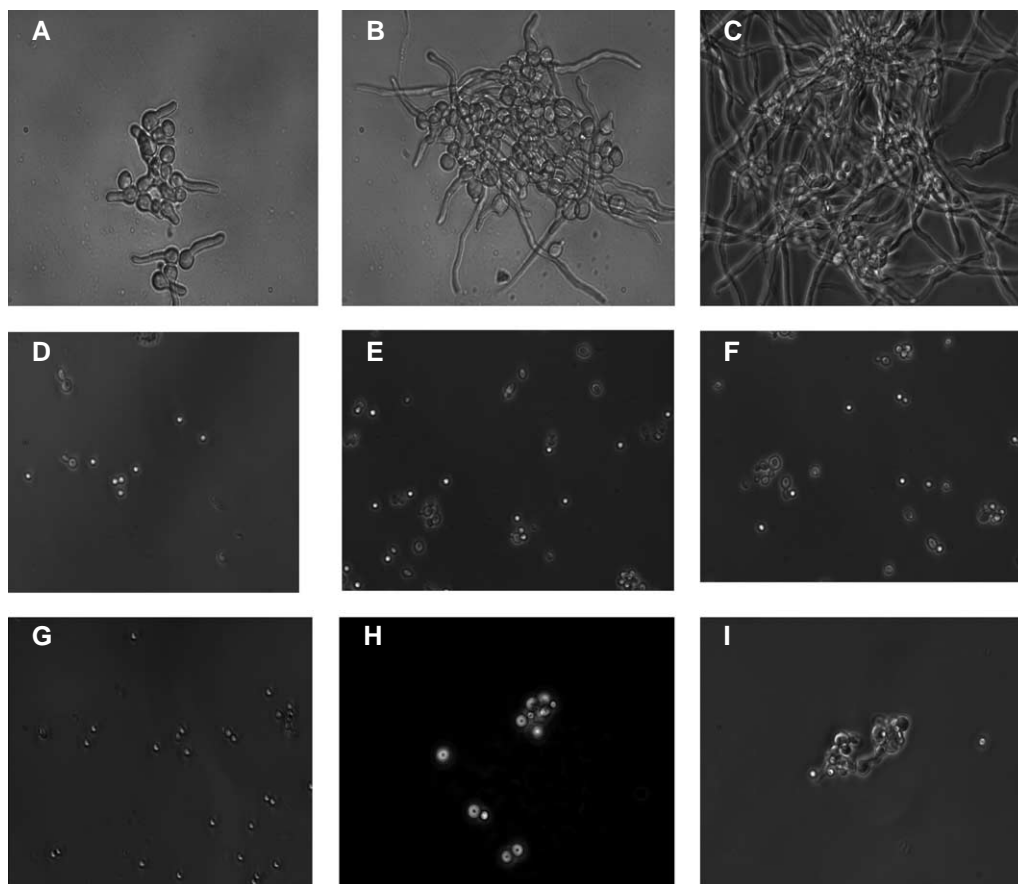


Fig. 2. Germination of *A. ochraceus* B722 spores incubated at 30 °C when inoculated in MYGP broth without yeast after 24 h (A), 48 h (B) and 72 h (C); when co-cultured with cells of *P. anomala* S12 in MYGP broth after 24 h (D), 48 h (E) and 72 h (F); when inoculated in MYGP yeast-free supernatant after 24 h (G), 48 h (H), 48 h (I) and 72 h (J).

was not due to changes in the pH of medium caused by the yeasts.

Depletion of the amounts of glucose in MYGP broth by the investigated yeasts might result in reduction of spore germination of *A. ochraceus*. The three yeasts might also produce extra-cellular metabolites toxic to *A. ochraceus* which caused the reduction of spore germination in yeast-free supernatant. Spadaro and Gullino (2004) reported that the mechanisms behind the antagonist activity of yeasts against fungi responsible for fruit diseases can be a competition for nutrients and space, adhesion of the antagonist cells to the mycelium of the fungi or by inducing resistance in the host tissue. Droby et al. (1989) suggested that the mechanism of the antagonist activity of *D. hansenii* against *Penicillium digitatum* in grapefruit might be due to the competition for nutrients because the antagonist activity was overcome by the addition of exogenous nutrients to grapefruit. The yeast *Metschnikowia pulcherrima* was found to inhibit growth of postharvest pathogenic fungi of apple fruit; it was suggested that the antagonist activity seems to be due to a combination of competition for nutrients and production of toxic metabolites in vitro (Spadaro et al., 2002). Strains of *P. anomala*, *P. kluyveri* and *H. uvarum* used in this study have been found to be strong producers of some volatile compounds, mainly ethyl acetate, acetate, 2-phenylethyl acetate, ethyl propionate

and isoamyl alcohol (unpublished results). The effect of ethyl acetate on growth of *P. roqueforti* was studied by Fredlund et al. (2004), who found that only high concentrations of ethyl acetate reduced fungal growth. It has been reported that growth of a number of plant pathogenic fungi can be inhibited by volatile compounds produced by the endophytic fungi *Muscodor albus* (Strobel et al., 2001; Mercier and Jiménez, 2004) and by the *Gliocladium* spp. (Stinson et al., 2003). Other non-volatile metabolites toxic to *A. ochraceus* might also be produced by the three investigated yeasts.

3.3. Effects of yeasts on production of OTA by *A. ochraceus*

The most important aspect during coffee processing is the prevention of the production of OTA. *A. ochraceus* B722 was found to produce OTA when grown on yeast-free MEA and CA plates (Figs. 3 and 4). When the six strains of *P. anomala* were co-cultured with *A. ochraceus* B722, OTA was not detected on both MEA and CA plates (Fig. 3). *P. kluyveri* also prevented OTA production by *A. ochraceus* on both MEA and CA media (Fig. 4). However, the two strains of *H. uvarum* did not prevent OTA production on CA medium; it was only prevented on MEA (Fig. 4). The same observations were made for *A. ochraceus* B677 (results not shown). Although the yeasts did not inhibit growth of *A. ochraceus* completely (Fig. 1), they



Fig. 3. Production of OTA by *A. ochraceus* B722 co-cultured with strains of *P. anomala*. Lane numbers from 3 to 8 refer to *A. ochraceus* co-cultured on CA with lane 3, *P. anomala* S12; lane 4, *P. anomala* S13; lane 5, *P. anomala* S14; lane 6, *P. anomala* S15; lane 7, *P. anomala* S16; lane 8, *P. anomala* S17. Lane numbers from 10 to 15 refer to *A. ochraceus* co-cultured on MEA with lane 10, *P. anomala* S12; lane 11, *P. anomala* S13; lane 12, *P. anomala* S14; lane 13, *P. anomala* S15; lane 14, *P. anomala* S16; lane 15, *P. anomala* S17. *A. ochraceus* grown on CA without yeasts (lanes 1, 2) and on MEA (lanes 16, 17) were used as controls. OTA standard 10 µg/l (lane 9).

were able to prevent production of OTA. It has been found that *P. anomala* reduced both growth of *A. verrucosum* and OTA production when co-cultured together on MEA or on wheat (Pettersson et al., 1998). Reduction of OTA might be as a result of its degradation or adsorption by yeasts. It has been reported that *Saccharomyces cerevisiae* and *Saccharomyces bayanus* adsorbed about 45% of OTA present in synthetic grape juice medium (Bejaoui et al., 2004). Production of extra-cellular compounds by the three yeasts might also inhibit the production of OTA by *A. ochraceus*. *Streptococcus lactis* was reported to produce a heat-stable low molecular weight compound that inhibits production of aflatoxin by *Aspergillus flavus* in vitro (Coallier-Ascah and Idziak, 1985). Mellon and Moreau (2004) found that a class of polyamine conjugates inhibited aflatoxin B₁ biosynthesis in *A. flavus* but they did not reduce growth of that fungus.

In the present study, the two strains of *A. ochraceus* showed very good growth and production of OTA in both MEA and CA media. *P. anomala*, *P. kluyveri* and *H. uvarum* were found to reduce growth of *A. ochraceus* and prevent biosynthesis of OTA on MEA medium. On CA medium, *P. anomala* and *P. kluyveri* were able to reduce growth of *A. ochraceus* and prevent production of OTA. For the purpose of preventing production of OTA in coffee, the present work indicated the possibility of using *P. anomala* and *P. kluyveri* in biological control of OTA-producing fungi during coffee fermentation. Further studies on the effects of *P. anomala* and *P. kluyveri* on other OTA-producing fungi present in coffee are needed. The mechanisms behind the antagonist activity of those yeasts need to be clarified. In addition, studies of interactions between those two yeasts and OTA-producing fungi in vivo, i.e., during coffee processing, have to be conducted.

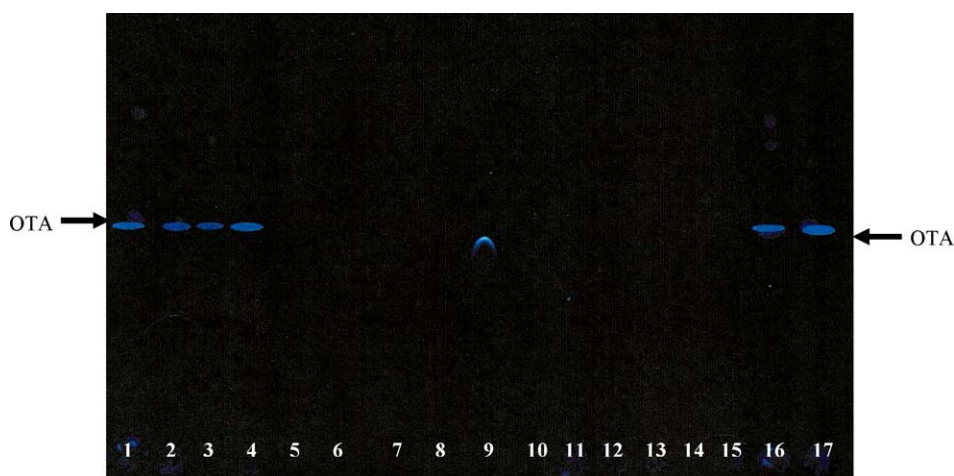


Fig. 4. Production of OTA by *A. ochraceus* B722 co-cultured with strains of *P. kluyveri* and *H. uvarum*. Lane numbers from 3 to 8 refer to *A. ochraceus* co-cultured on CA with lane 3, *H. uvarum* S3Y8; lane 4, *H. uvarum* S15Y2; lane 5, *P. kluyveri* S4Y3; lane 6, *P. kluyveri* S7Y1; lane 7, *P. kluyveri* S8Y4; lane 8, *P. kluyveri* S13Y4. Lane numbers from 10 to 15 refer to *A. ochraceus* co-cultured on MEA with lane 10, *H. uvarum* S3Y8; lane 11, *H. uvarum* S15Y2; lane 12, *P. kluyveri* S4Y; lane 13, *P. kluyveri* S7Y1; lane 14, *P. kluyveri* S8Y4; lane 15, *P. kluyveri* S13Y4. *A. ochraceus* grown on CA without yeasts (lanes 1, 2) and on MEA (lanes 16, 17) were used as controls. OTA standard 10 µg/l, (lane 9).

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Appendix IV

Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa

Wafa Masoud and Lene Jespersen

Department of Food Science, Food Microbiology
The Royal Veterinary and Agricultural University
Denmark.

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Short communication

Pectin degrading enzymes in yeasts involved in fermentation of
Coffea arabica in East Africa

Wafa Masoud *, Lene Jespersen

Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

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Abstract

The ability of six strains of *Pichia anomala*, four strains of *Pichia kluyveri* and two strains of *Hanseniaspora uvarum* predominant during coffee processing to produce polygalacturonase (PG), pectin esterase (PE) and pectin lyase (PL) in yeast polygalacturonic acid medium (YPA) and in coffee broth (CB) was studied. For comparison, a reference strain of *Kluyveromyces marxianus* CCT 3172 isolated from cocoa and reported to produce high amount of PG was included.

Initial screening of PG activity using YPA medium showed that *K. marxianus* CCT 3172, *P. anomala* S16 and *P. kluyveri* S13Y4 had the strongest activity. Enzymatic assays showed that the four yeast species secreted PG, but none of the yeasts investigated was found to produce PE or PL. *P. anomala* S16 and *P. kluyveri* S13Y4 were found to produce higher amounts of PG when grown in CB than in YPA. When *K. marxianus* CCT 3172, *P. anomala* S16 and *P. kluyveri* S13Y4 were grown in YPA broth adjusted to pH of 3.0–8.0 and incubated at temperatures of 15–40 °C, the three yeast species secreted the highest amount of PG at pH 6.0 and at 30 °C. For PG secreted by *K. marxianus* CCT 3172 and *P. anomala* S16, the optimum pH and temperature for the enzymatic activity were 5.5 and 40 °C, respectively. On the other hand, PG produced by *P. kluyveri* S13Y4 showed the highest activity at pH 5.0 and 50 °C.

Significant differences in the extracellular activity of PG were found between the yeasts species as well as between strains within same species. High amounts of PG were produced by two strains of *P. anomala* and *P. kluyveri*. It is therefore likely that strains of those two species may be involved in the degradation of pectin during coffee fermentation.

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Keywords: Coffee; *Pichia anomala*; *Pichia kluyveri*; *Hanseniaspora uvarum*; Polygalacturonase

1. Introduction

Coffee is mainly grown in tropical and subtropical regions but is consumed worldwide. A large number of species of the genus *Coffea* have been identified. The commercial coffee beans belong to the two species *Coffea arabica* and *Coffea canephora* var. *robusta*. To separate beans from pulp coffee is processed by dry or wet method. The dry method is mainly used for *robusta* coffee, which has a thin pulp that allows direct drying (Fowler et al., 1998). In general, cherries of *C. arabica* worldwide are processed by the wet method. However, more than 80% of arabica coffee in Brazil, Yemen and Ethiopia are processed by

the dry method (Brando, 2004). In wet processing of coffee, the ripe coffee cherries are pulped followed by fermentation and drying (Fowler et al., 1998).

It is a main aim of coffee fermentation to remove the pectinous mucilage adhering to coffee beans. According to studies that have been carried out, disagreements about which microorganisms are responsible for pectin degradation exist. Vaughn et al. (1958) examined dry and semi-dry processed Brazilian coffee and found mainly Gram-negative bacteria with pectinolytic activity belonging to the genera *Aerobacter* and *Escherichia*. In addition, pectinolytic species of *Bacillus* and a variety of pectinolytic filamentous fungi were isolated. From wet processed coffee in Hawaii, species of *Erwinia*, *Escherichia* and most commonly *Erwinia dissolvens*, were isolated to which degradation of mucilage was related (Frank et al., 1965). On the other hand, *Kluyveromyces marxianus*, *Saccharomyces*

* Corresponding author. Tel.: +45 35 28 32 87; fax: +45 35 28 32 14.
E-mail address: wm@kvl.dk (W. Masoud).

bayanus, *Saccharomyces cerevisiae* var. *ellipsoideus*, and *Schizosaccharomyces* species, which were found to have pectinolytic activity, were isolated from fermented robusta coffee in India (Agate and Bhat, 1966).

Pectin is a complex heteropolysaccharide composed of D-galacturonic acid residues joined by α -1,4-linkages, which form homogalacturonan chains (Be Miller, 1986). Enzymes that act on pectin molecules include the pectinesterases (PE) and the depolymerases. PE are able to de-esterify pectin by hydrolysis of the methyl ester group, while depolymerases split the main chain (Be Miller, 1986). The depolymerases are divided into polygalacturonases (PG), which cleave the glycosidic bonds by hydrolysis, and lyases (PL), which break the glycosidic bonds by β -elimination at esterified D-galacturonic acid units. Pectinolytic enzymes from yeasts are mainly *endo*-PG; they have been reported in *Rhodotorula* spp. (Vaughn et al., 1969), *Cryptococcus albidus* (Federici, 1985), *K. marxianus* (Lim et al., 1980; Barnby et al., 1990; Schwan and Rose, 1994; Schwan et al., 1997), *S. cerevisiae* (Blanco et al., 1994), and several species of *Candida* (Call et al., 1985; Sanchez et al., 1984; Stratilova et al., 1998).

The aim of the present work was to study the ability of *P. anomala*, *P. kluyveri* and *H. uvarum* predominant during wet processing of arabica coffee in Tanzania (Masoud et al., 2004) to produce pectinolytic enzymes in a laboratory substrate as well as in coffee broth. Furthermore, the effects of pH and incubation temperature on the production of polygalacturonase and its activity were also investigated.

2. Materials and methods

2.1. Cultures

Yeasts used in this study were obtained from arabica coffee samples collected from the Arusha region, Tanzania (Masoud et al., 2004). They included six strains of *P. anomala* (S12, S13, S14, S15, S16, S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4) and two strains of *H. uvarum* (S3Y8, S15Y2). One strain of *K. marxianus* CCT 3172, which was isolated from cocoa and has been reported to have a strong pectinolytic activity (Schwan and Rose, 1994) was also included.

2.2. Screening for polygalacturonase activity

The yeasts were propagated in 25 ml of malt yeast glucose peptone (MYGP) broth [3.0 g yeast extract (Difco, Detroit, MI, USA), 3.0 g malt extract (Difco), 5.0 g bactopectone (Difco), 10.0 g glucose (Merck, Darmstadt, Germany) per 1 l distilled water] at 25 °C for 48 h. After propagation cells were harvested by centrifugation at 3000 \times g for 10 min, and resuspended in diluent saline peptone (SPO) [0.1% (w/v) bactopectone (Difco), 0.85% (w/v) NaCl (Merck), 0.03% (w/v) Na₂H₂PO₄, 2H₂O (Merck), adjusted with 1.0 M NaOH (Merck) and 1.0 M HCl (Merck) to pH 5.6]. Cell concentrations were estimated by microscopy using a counting chamber (Neubauer) and the suspensions were diluted to final concentrations of 10⁶ cells/ml. Screening for pectinolytic activity was modified from a procedure described by Zink and Chatterjee (1985). Three spots of 10 μ l of each yeast suspension

were placed on plates of polygalacturonic acid specific medium [7.0 g yeast nitrogen base (YNB) (Difco), 5.0 g glucose (Merck), 5.0 g polygalacturonic acid (Sigma), 20 g agar (Difco) per 1 l distilled water]. The plates were incubated for 48 h at 25 °C and then flooded with 6.0 M HCl (Merck), where clear halo around yeast colonies indicated pectinolytic activity. The experiment was done in triplicates.

2.3. Polygalacturonase enzyme assay in yeast polygalacturonic acid broth (YPA) and in coffee broth (CB)

Yeasts were grown in 50 ml of yeast polygalacturonic acid medium (YPA) [7.0 g YNB (Difco), 5.0 g glucose (Merck), 5.0 g polygalacturonic acid (Sigma) per 1 l distilled water adjusted to pH 5.5 with 1.0 M NaOH (Merck)]. Yeasts were also propagated in 50 ml of coffee broth (CB) [20 g grounded green coffee beans (Levi Farm, Arusha, Tanzania), 5.0 g glucose per 1 l distilled water adjusted to pH 5.5 with 1.0 M NaOH (Merck)]. Yeast cultures were incubated on a rotary shaker at 30 °C for 48 h. Then yeast cells were centrifuged at 7000 \times g for 20 min at 4 °C. The cell free supernatant was used for PG and the other enzymatic assays described below. For determination of PG activity, a reaction mixture composed of 0.5 ml of supernatant, 0.5 ml of 0.5% (w/v) polygalacturonic acid in 0.05 M sodium acetate buffer (pH 5.5) and 9.0 ml of 0.05 M sodium acetate buffer (pH 5.5) was prepared and incubated in a water bath at 45 °C for 1 h. PG activity was determined by estimation of the amounts of reducing sugar groups as described by Miller (1959). 1.0 ml of the reaction mixture was added to 1.0 ml of 3,5-Dinitrosalicylic acid (DSN) reagent [1% (w/v) DSN (Merck), 0.2% (w/v) phenol (Merck), 0.05% (w/v) sodium sulfite (Merck) and 1% (w/v) sodium hydroxide (Merck)] and boiled for 15 min then cooled under tap water. To stabilized colour, 1.0 ml solution of 40% (w/v) potassium sodium tartarate (Merck) was added subsequent to colour development and before cooling. After cooling the optical density of resulting coloured mixture was measured by spectrophotometry at 575 nm. Galacturonic acid (Sigma) was used as a standard. One unit of PG activity was defined as the amount of the enzyme which catalysed the formation of 1.0 μ mol of galacturonic acid per min at 45 °C. The enzyme activity was expressed as units per milligram dry weight per ml (U/mg DW/ml) of the supernatant. The experiment was done in duplicates for each yeast strain.

PG was also investigated when yeasts were grown in YPA without addition of glucose or in YNB with glucose but without addition of polygalacturonic acid.

2.3.1. The effects of pH and incubation temperature on production of polygalacturonase by yeasts

Yeasts were propagated in YPA medium at pH values of 3.0–8.0 and incubated at temperatures of 15–40 °C for 48 h. Production of PG by yeasts was determined as described above.

2.3.2. The effects of pH and temperature on polygalacturonase activity

PG activity was determined at different pH values by incubation of the reaction mixture described above with different phosphate buffers pH 3.0–8.0. Furthermore, the effect of

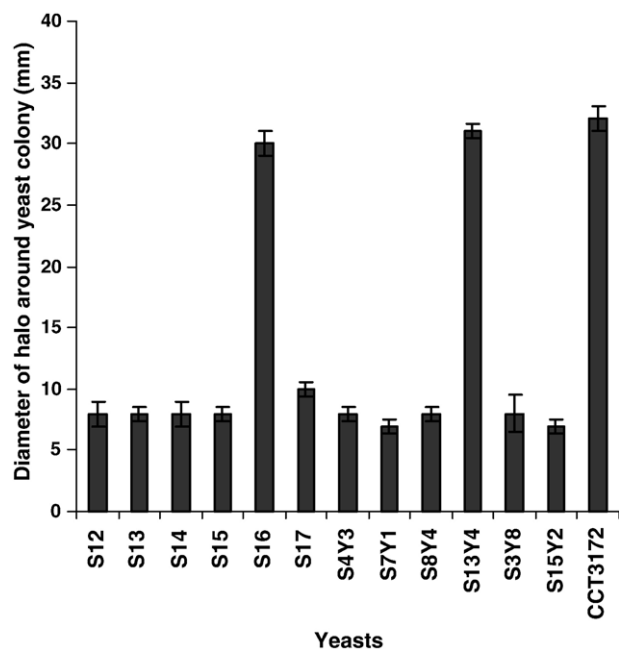


Fig. 1. Screening for pectinolytic activity of six strains of *Pichia anomala* (S12, S13, S14, S15, S16, S17), four strains of *Pichia kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4), two strains of *Hanseniaspora uvarum* (S3Y8, S15Y2) and the reference strain *Kluyveromyces marxianus* CCT 3172 grown on yeast polygalacturonic acid medium (YPA). Pectinolytic activity was determined by the diameter of the clearing zone around yeasts' colonies. Bars represent standard deviations.

incubation temperature of the reaction mixture on PG activity was investigated at 10–70 °C.

2.4. Assay for pectin esterase activity

A solution of 1% (w/v) of pectin (47% esterified) (Sigma) in 0.1 M sodium chloride (Merck) was adjusted to pH 7.5 with 0.5 M NaOH (Merck). Quantities of 0.5–5.0 ml of yeasts free supernatant prepared as described above were added to 20 ml of the pectin solution and the pH was maintained 7.5 for 30 min by addition of 0.02 M NaOH (Merck). The enzyme activity is proportional to the volume of NaOH added (Barnby et al., 1990). The experiment was done in duplicates for each yeast strain.

2.5. Assay for pectin lyase activity

One half ml of the yeast free supernatant prepared as described above was added to 0.25% (w/v) pectin in 0.1 M Tris–HCl buffer, pH 7.5. The activity was determined spectrophotometrically by monitoring the increase in absorbance at 240 nm (Barnby et al., 1990). The experiment was done in duplicates for each yeast strain.

3. Results and discussion

Besides *K. marxianus* CCT 3172 being isolated from cocoa and reported to have strong PG activity (Schwan and Rose, 1994), *P. anomala* S16 and *P. kluyveri* S13Y4 were found to

exhibit the strongest extracellular PG activity on agar plates of the polygalacturonic acid medium with diameters of clearing zones around colonies of 29–32 mm (Fig. 1). The two strains of *H. uvarum* and the remaining strains of *P. anomala* and *P. kluyveri* showed significantly weaker PG activity with diameters of clearing zones around colonies of 7.0–11 mm.

Secretion of pectinolytic enzymes by yeasts was also investigated in YPA and CB media. In YPA broth, all yeasts were found to secrete PG (Table 1) but no pectin lyase or pectin esterase was found to be produced by the yeasts examined. It has been reported that the most common enzyme found to be secreted by pectinolytic yeasts is PG (Federici, 1985; Barnby et al., 1990; McKay, 1990; Blanco et al., 1994; Schwan et al., 1997). However, in few cases, other pectinolytic enzymes were detected in yeasts such as pectin esterase and pectin lyase, secreted by a strain of *S. cerevisiae* isolated from wine (Gainvors et al., 1994) and pectin esterase secreted by *Rhodotorula* spp. associated with softening of olives (Vaughn et al., 1969).

In the present study, the highest amounts of PG were found to be secreted by *K. marxianus* CCT 3172, *P. anomala* S16 and *P. kluyveri* S13Y4, while other yeast strains produced scarce amounts of the enzyme, which agrees with screening of PG activity, determined by the plate method for hydrolysis of polygalacturonic acid (Fig. 1). Furthermore, the PG was found to be secreted in higher amounts when *P. anomala* S16 and *P. kluyveri* S13Y4 were grown in CB than when grown in PYA broth (Table 1). It appears that CB is a good substrate for production of PG by the two strains of *P. anomala* and *P. kluyveri*. When yeast strains were grown in YPA without addition of glucose, they showed scarce growth and no PG activity was detected in yeasts free supernatant (results not shown), which indicates that the investigated yeasts were unable to utilize polygalacturonic acid as a sole carbon source. This is in accordance with the findings of Sanchez et al. (1984), who found that the pectinolytic yeasts

Table 1

Production of polygalacturonase (PG) by six strains of *Pichia anomala* (S12, S13, S14, S15, S16, S17), *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4), two strains of *Hanseniaspora uvarum* (S3Y8, S15Y2) and *Kluyveromyces marxianus* CCT 3172 grown in yeast polygalacturonic acid medium (YPA) and in coffee broth (CB)

Yeasts	PG activity (μmol galaturonic acid/min)	
	YPA±SD ^a	CB±SD
S12	5.5±0.3	5.2±0.4
S13	5.3±0.3	4.7±0.5
S14	4.5±0.6	5.0±0.3
S15	5.1±0.3	5.5±0.6
S16	20.0±0.7	46.0±0.4
S17	5.7±0.7	5.3±0.6
S4Y3	7.2±0.4	5.1±0.7
S7Y1	5.3±0.7	5.8±0.3
S8Y4	6.1±0.3	5.2±0.5
S13Y4	17.0±0.7	36.5±0.5
S3Y8	3.4±0.8	3.5±0.7
S15Y2	2.7±0.4	3.2±0.8
CCT3172 ^b	24.5±0.3	16.6±0.6

^a Standard deviation for two trials.

^b Reference strain *Kluyveromyces marxianus* isolated from cocoa (Schwan and Rose, 1994).

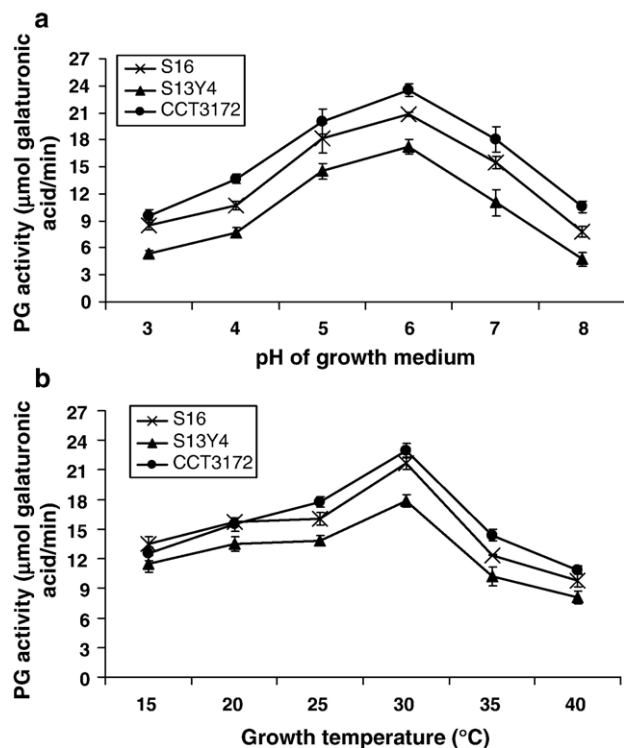


Fig. 2. Polygalacturonase (PG) production by *P. anomala* S16, *P. kluyveri* S13Y4 and reference strain *K. marxianus* CCT 3172 when grown in yeast polygalacturonic medium (YPA) adjusted to different pH (a) and incubated at different temperatures (b). Bars represent standard deviations.

isolated from cocoa were unable to grow or to produce PG in a pectin medium not supplemented with glucose. In addition, when yeasts were grown in YNB, which only contained glucose but no polygalacturonic acid, PG activity was not detected in the yeasts free supernatant (results not shown). Same observation for two strains of *S. cerevisiae*, NCYC 365 and NCYC 73 was reported by McKay (1990), who suggested that this might be due to the absence of enzyme induction during growth on glucose without polygalacturonic acid. In addition to polysaccharides and sucrose, it has been reported that in beans of arabica coffee the concentrations of glucose and fructose at the end of maturation and at the time of picking of cherries i.e. before fermentation were about 0.03 and 0.04% dry weight, respectively (Rogers et al., 1999). The presence of monosaccharides in coffee seems to be a prerequisite for secretion of polygalacturonase during fermentation by the pectinolytic yeasts.

The effect of growing of *P. anomala* S16, *P. kluyveri* S13Y4 and *K. marxianus* CCT 3172 in YPA broth adjusted to different pH values in the range of 3.0 to 8.0 on secretion of polygalacturonase is shown in Fig. 2a. It was found that the yeasts investigated showed an optimum pH of 6.0 for enzyme secretion. The effect of incubation temperature for the yeasts to secrete polygalacturonase is shown in Fig. 2b. Increasing temperature from 15 to 40 °C for the three yeasts showed a maximum at 30 °C followed by a sharper decrease in enzyme secretion. From another study, it has been reported that the optimum pH and temperature for production of polygalacturonase by *Kluyveromyces wickerhamii* were in the range of 3.8–4.5 and 28.5–

35.5 °C, respectively (Moyo et al., 2003). The difference in the optimum pH for PG production from our investigations might be due to the different yeast species studied.

The stability of the PG produced by the three yeasts was investigated in different buffers at pH values from 3.0 to 8.0 as shown in Fig. 3a. Optimum pH for the enzyme activity produced by the reference strains *K. marxianus* CCT 3172 and *P. anomala* S16 was 5.5; while PG produced by *P. kluyveri* S13Y4 was found to have a maximum activity at pH 5.0. Furthermore, the enzyme activity was examined at different temperatures in the range of 10–70 °C. For *K. marxianus* CCT 3172 and *P. anomala* S16, the maximum PG activity was observed at 40 °C (Fig. 3b). The optimal temperature for activity of PG produced by *P. kluyveri* S13Y4 was 50 °C. For the three yeasts, PG activity decreased rapidly above 50 °C and the enzyme was inactive at 70 °C. Vaughn et al. (1969) reported that the optimum pH and temperature for PG produced by *Rhodotorula* spp. were 6.0 and 50 °C, respectively. The PGs produced by *Trulopsis candida*, *Candida norvegensis*, *Kluyveromyces fragilis* and *Saccharomyces chevalieri* were found to have the same optimum pH of 5.0, however different optimum temperatures for the PGs produced by the four yeasts were obtained (Sanchez et al., 1984). In another study, two optimum pH values of 4.5 and 5.0 were reported for the PG activity produced by two strains of *S. cerevisiae*; on the other hand, an optimum temperature of 45 °C was reported for both strains (Blanco et al., 1997). It appears that the optimum pH and temperatures for PGs produced by yeasts might vary between yeast species and also between strains within the same species. From this study and previous studies, it can also be observed that

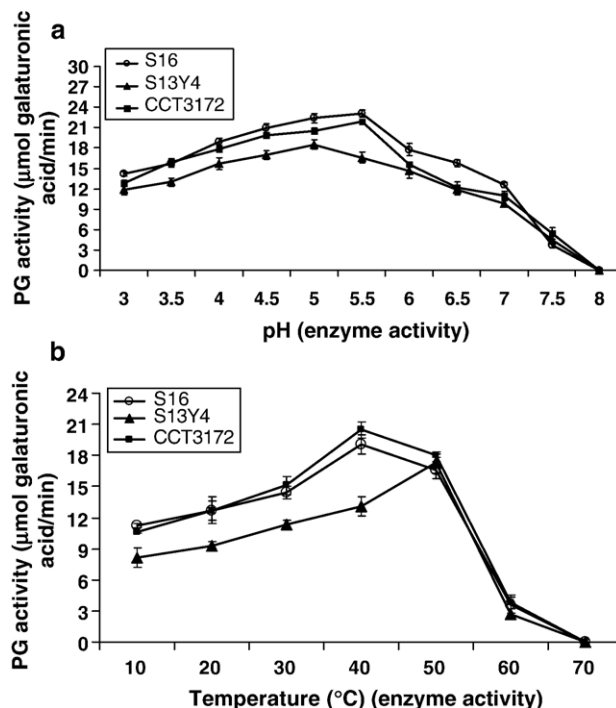


Fig. 3. Polygalacturonase (PG) activity of *P. anomala* S16, *P. kluyveri* S13Y4 and reference strain *K. marxianus* CCT 3172 when the enzyme reaction mixtures were incubated in phosphate buffers of pH 3.0 to 8.0 (a) or when the enzyme reaction mixtures were incubated at temperatures of 10 to 70 °C (b). Bars represent standard deviations.

the optimum pH of PGs produced by yeasts is within the acidic region. During coffee fermentation, the initial pH in fresh pulped cherries is reported to be between 5.5 and 6.0 (Wootton, 1963), which after 20 to 25 h of fermentation is reduced to 3.5 (Avallone et al., 2001). A final pH of 4.3 after 36 h of coffee fermentation has also been recorded (Van Pee and Castelein, 1972). The pH values reported during fermentation are within the range of pH at which the PGs secreted by the investigated yeasts are active, which indicates that the yeasts in the present study originating from coffee fermentation could have a role in mucilage degradation. On the contrary, *Klebsiella pneumoniae* and *Erwinia hericola*, which have been isolated from coffee fermentation, were found to secrete pectin lyase with optimum pH of 8.5, which is far from the acidic coffee fermentation conditions i.e. pH 5.3–3.5 (Avallone et al., 2002). Sakiyama et al. (2001) also found a pectin lyase with an optimum pH of 7.9 to be secreted by *Paenibacillus amylolyticus*; a bacterium isolated from arabica coffee cherries.

From the present study, it can be seen that only PG was produced by the investigated yeasts. Significant differences in the amounts of PG secreted were found between the yeast species as well as between strains of the same species. The extracellular PG produced by *P. anomala* S16 and *P. kluyveri* S13Y4 has an optimum activity at pH of 5.5 and 5.0 respectively, which is within the range of pH conditions that occur during coffee fermentation. In addition to their ability to secrete PG, *P. anomala* S16 and *P. kluyveri* S13Y4 were among the yeasts, which have been found to inhibit growth and ochratoxin A (OTA) production by *Aspergillus ochraceus* (Masoud and Kaltoft, 2005; Masoud et al., 2005). Therefore, the strong pectinolytic strains of *P. anomala* and *P. kluyveri* appear to have potential to be used as starter cultures for mucilage degradation and biological control against OTA producing fungi during coffee fermentation. Further studies of the PG produced by these yeasts are needed and the ability of the investigated yeasts to degrade mucilage *in vivo* i.e. during coffee processing shall be conducted, which will be accompanied by evaluation of the quality of coffee beans i.e. appearance, colour and aroma.

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