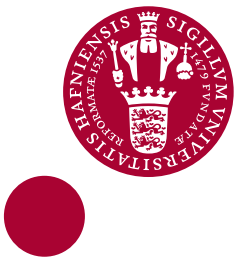

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The effects of yeasts involved in fermentation of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*

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Abstract

The effects of *Pichia anomala*, *P. 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 yeasts 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 to 4.7, which was found to have no effect on spore germination of *A. ochraceus*.

Pichia 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. *Hanseniaspora uvarum* did not affect production of OTA by *A. ochraceus* on CA medium.

Key words: 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, P. 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 (EC No 472 / 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 *A. ochraceus*, *A. carbonarius* and *A. 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 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 *P. roqueforti* has been found to be stimulated by *Debaryomyces hansenii*; while *Candidum geotricum* and Yar*rowia 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; Vinas 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 *P. 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 Bactopeptone (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 Bactopeptone (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 ($Na_2HPO_4.12H_2O$) (Merck) and 1 g bactopeptone (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 ^{o}C for 48 hours. After propagation cells were harvested by centrifugation at 3000 x 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 ^{o}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 yeasts 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 ^oC. Spores of *A. ochraceus* were also inoculated in yeasts cells free supernatant, which was obtained by propagation of each yeast in 25 ml of MYGP broth at 25 ^oC for 24 hours. Where after yeasts cultures were centrifuged at 3000 x 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 ^oC, germination of the fungal spores was inspected by microscopy where five regions of each sample with about 10 to 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. *Aspergillus 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 ^oC, 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* was 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). Same findings on the effects of yeasts on growth of *A. ochraceus* B677 were obtained (results not shown).

Pichia anomala was reported to inhibit a number of fungi like *Botrytis cinerea* (Masih et al., 2000), *P.* roqueforti, *A. 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 yeasts 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 2 D, E, 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 2 G). Spores of *A. ochraceus* started to swell after 48 h, but germ tubes were not observed (Fig 2 H). After 72 h, some spores germinated with very short germ tubes (Fig 2 I) compared to the control (Fig 2 A, B, 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 yeasts 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 yeasts free supernatant 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 cause reduction of spore germination in yeasts free supernatant. Spadaro and Gullino

(2004) reported that the mechanisms behind the antagonist activity of yeasts against fungi responsible for fruit diseases can be 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 *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. 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 virto* (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-phenlethyl acetate, ethyl propionate and isoamyl alcohol (unpublished results). The effect of ethyl acetate on growth of *P. roqueforti* was studied by Fedlund 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 *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*

Of the most important aspects during coffee processing is to prevent production of OTA. *Aspergillus ochraceus* B722 was found to produce OTA when grown on yeast free MEA and CA plates (Fig 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 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-culture together on MEA or on wheat (Petersson et al., 1998). Reduction of OTA might be as a result of its degradation or adsorption by yeasts. It has been reported that *S. cerevisiae* and *S. 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 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 *A. flavus* in vitro (Coallier-Ascah and Idziak, 1985). Mellon and Moreau (2004) found that a class of polyamine conjugates inhibited aflatoxin B_1 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 on of OTA in both MEA and CA media. *Pichia 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. kluyeri* 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.

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Legends to figures

Figure 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 (fugal colony diameter on free yeast plates). Bars represent standard deviations.

Fig 2. Germination of *A. ochraceus* B722 spores incubated at 30 ^{o}C when inoculated in MYGP broth without yeast after 24 h (A), 48 h (B), 72 h (C); when co-cultured with cells of *P. 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 (H), 48 h(I), 72 h (J).

Figure 3. Production of OTA by *A. ochraceus* B722 co-cultured with strains of *P. 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. *A. 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).

Figure 4. Production of OTA by *A. ochraceus* B722 co-cultured with strains of *P. kluyveri* and *H. uvarum*. Lane numbers from 3-8 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 10-15 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 with out yeasts (lanes 1, 2) and on MEA (lanes 16, 17) were used as controls. OTA standard 10 µg / l, (lane 9).

Fig 1

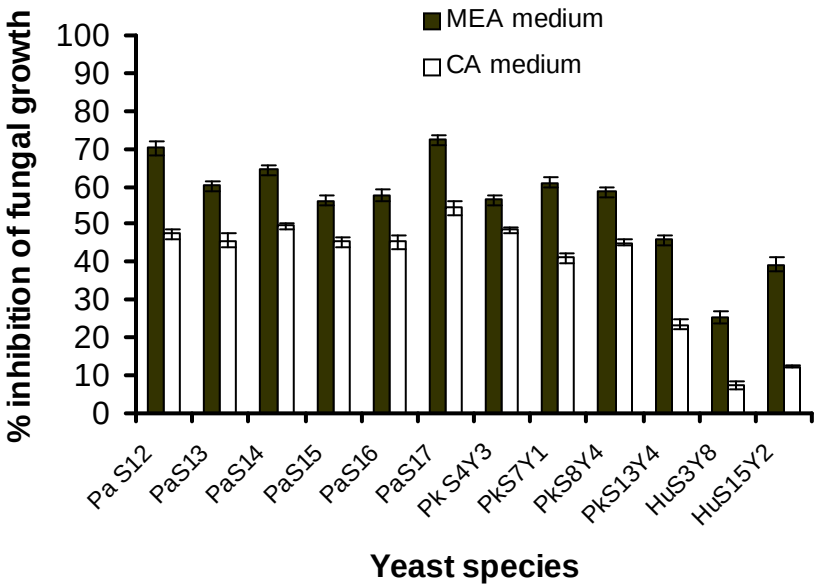
MEA medium
CA medium
% inhibition of fungal growth
100
90
80
70
60
50
40
30
20
10
0
Pa S12
PaS13
PaS14
PaS15
PaS16
PaS17
Pk S4Y3
PkS7Y1
PkS8Y4
PkS13Y4
HuS3Y8
HuS15Y2
Yeast species

Fig 2

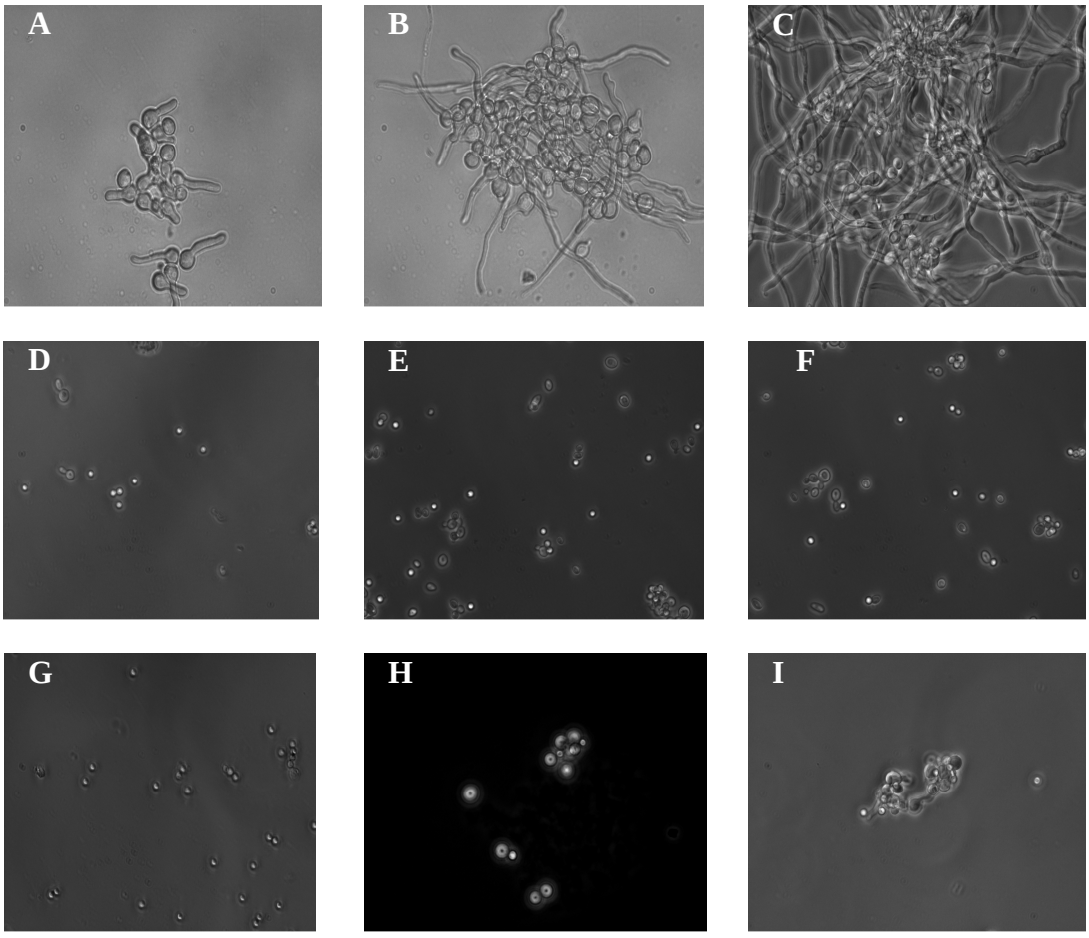

Fig 3

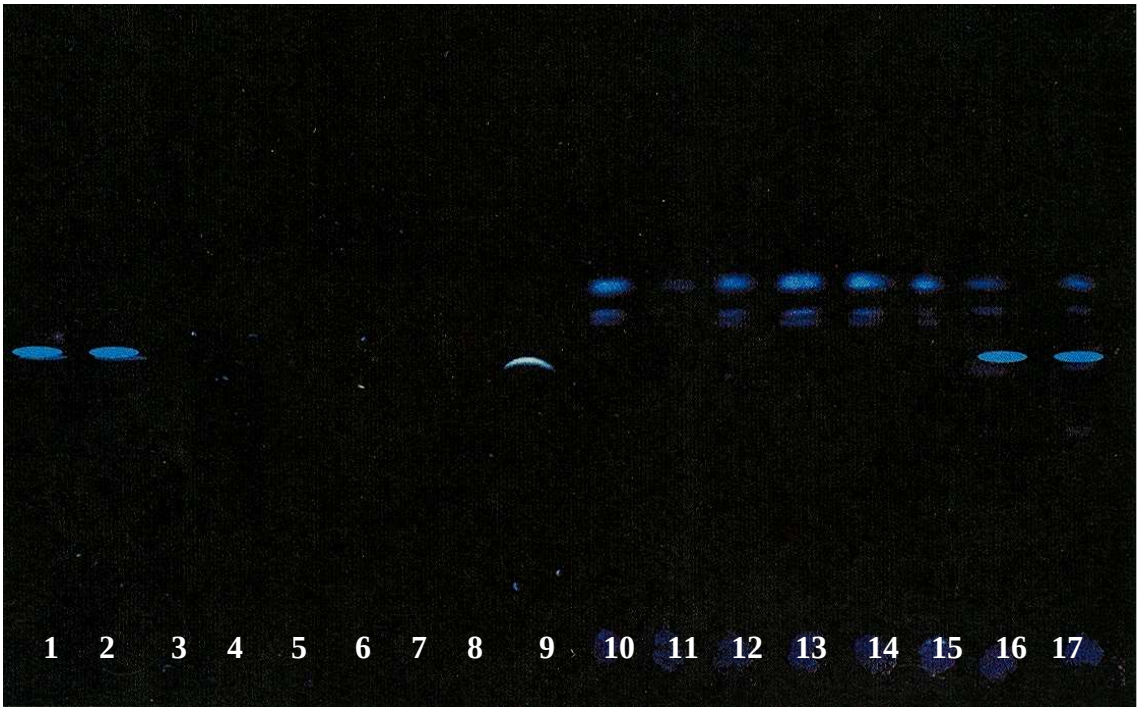

Fig 4