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The Inhibitory Potential of Dominating Yeasts against Moulds in Maize Fermentation

M. Halm [a] and A. Olsen [b]

[a] Food Research Institute,
Council for Scientific and Industrial Research,
P.O. Box m 20,
Accra, Ghana.

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

Abstract

The inhibitory effects of the dominating yeasts from different stages of *kenkey* production i.e. *Candida krusei* (35 isolates) and *Saccharomyces cerevisiae* (29 isolates) against 3 moulds *Penicillium citrinum, Aspergillus flavus* and *Aspergillus parasiticus* were investigated. Distinct growth inhibitory effects of the two yeasts was demonstrated against all isolates of the 3 mould species obtained from maize as well as mycotoxin producing strains (15) of the same mould species. The *Candida* isolates were found to have a greater inhibitory potential than the *Saccharomyces* isolates. The *Penicillium* spp. were more sensitive to the yeasts than the *Aspergillus spp.* Preliminary investigations to determine the mechanism of inhibition indicated that mould growth was not affected by either yeast supernatant or cell free extracts suggesting that the inhibitions were not due to compounds produced by the yeasts. It was concluded that the inhibitory potential of the yeasts was due to competition for nutrients between the yeasts and the moulds.

Keywords: Inhibitory potential; yeasts; moulds; maize fermentation

Introduction

Fermented maize products contribute to a large proportion of the daily food intake in Ghana and other West African countries. The dough is produced by spontaneous fermentation leading to a decrease of pH, from 5.9 at the beginning of maize steeping to 3.7, after 24-48 hours of dough fermentation (Halm *et al.*, 1993).

Concomitant with the drop in pH, lactic acid , the major acid produced in the highest concentration in the dough, rises from an initial level of 0.2% (w/w) at the beginning of fermentation to 1.2% (w/w) by 24 h of fermentation and thereafter to about 1.4% (w/w) at the

advanced stage of fermentation (unpublished data). Other acids found in the dough include acetic and propionic acids in concentrations of 0.15 - 0.30% (w/w) and 0. 002 - 0.02% (w/w) respectively.

Within 24 hours of fermentation, moulds and catalase positive Gram positive and Gram negative bacteria are reduced from 10^5 - 10^6 cfu/g to below 10^2 cfu/g. During the same period a microbial succession takes place leading to a dominance of lactic acid bacteria and yeasts. The lactic acid bacteria consist mainly of obligative heterofermentative lactic acid bacteria namely, *Lactobacillus fermentum* and *Lactobacillus reuteri* (Halm *et al.*, 1993; Hounhouigan *et al.*, 1993; Nyako and Danso, 1992). Previous investigations by Olsen *et al.*, 1995, showed that each processing stage of maize fermentation has its own micro environment with strong antimicrobial activity. They screened 241 isolates of lactic acid bacteria representative of the various processing stages, and demonstrated a widespread occurrence of antimicrobial compounds effective against closely related organisms as well as catalase-positive, Gram-positive and Gram-negative bacteria. These antimicrobial effects explain the apparent elimination of both these groups of bacteria during the early stages of fermentation. Further widespread microbial interactions were demonstrated amongst lactic acid bacteria within the various processing stages explaining the microbial succession taking place amongst lactic acid bacteria during maize fermentation. The succession amongst yeasts involves the change from a mixed population , including a variety of species, to a flora consisting of 60 - 90 % *Candida krusei* and 5 - 20% *Saccharomyces cerevisiae* (Jespersen *et al.*, 1994).

The mould flora forming part of the surface micro flora of maize kernels comprises mainly the genera *Penicillium, Aspergillus,* and *Fusarium* (Akinrele, 1970, Fields *et al.* 1981, Halm *et al.*, 1993, Jespersen *et al.*, 1994), of which *Penicillium citrinum, Aspergillus flavus, Aspergillus parasiticus, Aspergillus wentii, and Fusarium subglutinans* seem to be the dominating species (Jespersen *et al.*, 1994).

The kind and number of microorganisms, growing with the mould and the volatile metabolites from these organisms, affect mould growth and sporulation (Barr, 1976; More-Landecker and Stotzky, 1972, 1973, 1974). Reports can be found on the inhibitory and stimulatory effects of associative growth of bacteria and moulds on mycotoxin production (Barr, 19769; Coallier-Ascha and Idziak, 1985; Cuero *et al.*, 1987; Nout, 1989 and Wechbach and Marth, 1977).

In addition, yeasts produce a variety of compounds including organic acids, esters, alcohols and aldehydes (Janssens *et al.*, 1992) of which some, e.g. ethylacetate are known to reduce mould growth and spore germination (Saksena and Tripathi, 1987) and killer toxins, which can inhibit both yeasts (Rosini, 1983) and moulds (Polonelli *et al.* (1990).

The objective of the present study was to determine the inhibitory effects in a laboratory medium of strains of *Sacch. cerevisiae* and *C. krusei* isolated from maize dough against strains of *P. citrinum, A. flavus* and *A. parasiticus* isolated from maize and known mycotoxin producing strains of the same species obtained from a culture collection.

Materials and methods

Microorganisms

The yeasts and moulds investigated were isolated from samples collected from two large commercial *kenkey* production sites in Accra on 5 occasions over one year period. The samples comprised whole maize kernels, steeping water, fresh dough, 24 and 48 h fermented doughs. Isolation of yeasts was carried out for all samples, whereas moulds were isolated from maize kernels only. All were identified as previously described (Halm *et al.*, 1993; Jespersen *et al.*, 1994). The yeasts included 35 strains of *C. krusei* and 29 strains of *Sacch. cerevisiae.* The moulds comprised 6 isolates of *P. citrinum*, 4 isolates of *A. flavus,* and 2 isolates of *A. parasiticus* used for the first series of experiments. In addition, 5 isolates each

of *P. citrinum, A. flavus,* and *A. parasiticus* obtained from the culture collection of the Department of Biotechnology at the Technical University of Denmark (DTU) were used for the second series of experiments. The yeasts were maintained on Malt Agar slants (MA, Difco 0024-01-1) at 5°C and the moulds on Czapek Yeast Agar (CYA) consisting of 5 g Yeast extract DIFCO 0127-17-4; 35 g Czapek Dox Broth DIFCO 0338 -17 4; 15 g DIFCO Bacto Agar; and 1 ml spore metal solution made up of 1 g $ZnSO_4$,$7H_2O$ MERCK 8883; 0.5 g $CuSO_4 5H_2O$ MERCK 2787; 100 ml distilled water.

Growth inhibition studies of yeast cultures and culture supernatants against moulds

The inhibitory effects of yeast cultures and yeast supernatants against moulds were investigated using the well assay as described by Schillinger and Lucke (1989). Broth cultures of yeasts were prepared by growing the yeasts in Yeast morphology (YM, DIFCO 0711 - 17 - 1) broth at 30°C for 24 h. Yeast culture supernatants were prepared by centrifuging 24 h old yeast cultures in YM broth at 5,000 g, for 10 minutes and sterilized by filtration through a 0.45 µm disposable filter (Satorius Minisart NM6 165 55K, Sartorius GmbH, Gottingen, Germany).

Mould spore suspensions were prepared from CYA incubated at 30°C for 5 days by wetting the plates with 5 ml diluent (Bacteriological Peptone OXOID L 32, 0.1%; NaCl MERCK 6404, 0.9%; pH 7.2 added 1% Tween 80 MERCK Schuchardt 822187). One ml were transferred from the plates to 9 ml diluent with 1% Tween 80 and mixed. Spore counts were recorded using Thomas counting chamber and the suspension was adjusted to 10^7 cfu /ml with diluent. For the well assay, 15 ml of MA were poured into petri dishes and allowed to set and dry. Six circular wells, 8 mm in diameter, were made per dish. One hundred µl of an overnight culture of yeast or culture supernatant were inoculated into each well and after drying the plates at room temperature for 6 h, they were overlaid with 8 ml of CYA seeded with 100 µl spore suspension containing 10^7 spores/ml. The assay was incubated at 30°C for 48 h and inhibitory reactions recorded visually.

Inhibitory effects of cell free extracts

For preparation of cell free extract, One litre of a 24 h yeast culture in YM broth was centrifuged at 5000 rpm for 10 min at 4°C. The pellet was washed twice with phosphate buffer pH 7 (39.2 ml 0.1 M $NaH_2PO_4.H_20$ + 60.8 ml 0.1 M $Na_2HPO_4.H_2O$); I ml phosphate buffer per each gram of cells and ten times amount of 0.5mm glass beads per each gram of cells were added to the pellet. The suspension was then mixed for 1 min at high speed and placed on ice for 1 min. This procedure was repeated until complete disruption of the cells as controlled by microscopy. The suspension was hereafter centrifuged 3 times at 5000 rpm for 15 min, the clear supernatant collected each time and finally filtered through 0.2 µm Sartorius ministart filter to remove any intact cells.

The inhibitory potential of yeast cell free extracts against moulds was investigated using the spot test. Two hundred µl of a 10^7 spores/ml mould spore suspension were added to 15 ml CYA, mixed well and poured into a petri dish and kept at room temperature to solidify. 30 l of the yeasts cell free extracts were spotted on the plates and incubated at 30°C for 48 h. Inhibitory effect was observed as a clear zone where the extract was placed.

Results

Distinct growth inhibitory effects of the yeasts *Candida krusei* and *Saccharomyces cerevisiae* against *Penicillium citrinum, Aspergillus flavus* and *Aspergillus parasiticus* strains were observed (Tables 1 to 4).

Table 1 and 2 show The inhibitory effects of yeast strains against mould species isolated from maize. The inhibitory potential against all three mould species was particularly pronounced for the *C. krusei* strains as compared to the *Sacch. cerevisiae* strains. Strong inhibition was observed as no growth of mycelia or sporulation of the mould around the well where the yeast grew. Weak inhibition was recorded as sparse growth of mycelia with less sporulation around

Table 1:

The inhibitory effect of *Candida krusei* (18 strains) isolated from maize fermentation on growth of *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus* from maize. (The Table shows percentage inhibitory yeast strains)

	Growth Inhibition		No Inhibition
	Strong[a]	Weak[b]	
Penicillium citrinum Aj-2	22.2	61.1	11.1
Penicillium citrinum Aj-4	83.3	5.5	11.1
Penicillium citrinum Aj-8	88.9	0.0	11.1
Penicillium citrinum Aj-10	66.7	5.5	27.7
Penicillium citrinum Maj-2	27.7	61.1	11.1
Penicillium citrinum Maj-15	27.7	66.7	5.5
Aspergillus flavus Aj5-1	77.8	22.2	0.0
Aspergillus flavus Maj-1	61.1	27.1	11.1
Aspergillus flavus Mam-2	66.7	22.2	11.1
Aspergillus flavus Mam-7	61.1	5.5	33.3
Aspergillus parasiticus Mam-13	72.2	16.7	0.0
Aspergillus parasiticus Maj-14	88.8	11.1	0.0

[a] Strong inhibition- no growth of mycelia or sporulation around the well where the yeast is growing

[b] Weak inhibition- sparse growth of mycelia with less sporulation around the well where the yeast is growing

Table 2 :

The inhibitory effect of *Saccharomyces cerevisiae* (12 strains) isolated from maize fermentation on growth of *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus* from maize. (The Table shows percentage inhibitory yeast strains)

	Growth Inhibition		No Inhibition
	Strong[a]	Weak[b]	
Penicillium citrinum Aj-2	25.0	16.6	58.3
Penicillium citrinum Aj-4	33.3	33.3	33.3
Penicillium citrinum Aj-8	0	50.0	50.0
Penicillium citrinum Aj-10	50.0	8.3	41.7
Penicillium citrinum Maj-2	41.8	0.0	58.3
Penicillium citrinum Maj-15	50.0	25.0	25.0
Aspergillus flavus Aj5-1	33.3	8.3	58.3
Aspergillus flavus Maj-1	33.3	0.0	66.7
Aspergillus flavus Mam-2	50.0	0.0	50.0
Aspergillus flavus Mam-7	25.0	41.7	33.3
Aspergillus parasiticus Mam-13	33.0	50.0	16.6
Aspergillus parasiticus Maj-14	25.0	25.0	50.0

[a] Strong inhibition- no growth of mycelia or sporulation around the well where the yeast is growing

[b] Weak inhibition- sparse growth of mycelia with less sporulation around the well where the yeast is growing

Table 3:
The inhibitory effect of *Candida krusei* (17 strains) isolated from maize fermentation on growth of *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus* from a culture collection (The Table shows percentage inhibitory yeast strains)

	Growth Inhibition		No Inhibition
	Strong[a]	Weak[b]	
Penicillium citrinum 16147	100	0.0	0.0
Penicillium citrinum 16258	100	0.0	0.0
Penicillium citrinum 16146	100	0.0	0.0
Penicillium citrinum 16209	100	0.0	0.0
Penicillium citrinum 16212	94.1	0.0	5.9
Aspergillus flavus 15915	94.1	5.9	0.0
Aspergillus flavus 16343	47.1	52.9	0.0
Aspergillus flavus 16338	52.9	47.1	0.0
Aspergillus flavus 16350	100	0.0	0.0
Aspergillus flavus 16341	52.9	41.2	5.9
Aspergillus parasiticus 15628	70.6	29.4	0.0
Aspergillus parasiticus 15660	100	0.0	0.0
Aspergillus parasiticus 15444	100	0.0	0.0
Aspergillus parasiticus 11783	100	0.0	0.0
Aspergillus parasiticus 15002	94.1	0.0	5.9

[a] Strong inhibition- no growth of mycelia or sporulation around the well where the yeast is growing.

[b] Weak inhibition- sparse growth of mycelia with less sporulation around the well where the yeast is growing.

Table 4:
The inhibitory effect of *Saccharomyces cerevisiae* (17 strains) isolated from maize fermentation on growth of *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus* from a culture collection (The Table shows percentage inhibitory yeast strains)

	Growth Inhibition		No Inhibition
	Strong[a]	Weak[b]	
Penicillium citrinum 16147	0.0	100	0.0
Penicillium citrinum 16258	100	0.0	0.0
Penicillium citrinum 16146	100	0.0	0.0
Penicillium citrinum 16209	100	0.0	0.0
Penicillium citrinum 16212	70.6	29.4	0.0
Aspergillus flavus 15915	0.0	100	0.0
Aspergillus flavus 16343	0.0	100	0.0
Aspergillus flavus 16338	0.0	100	0.0
Aspergillus flavus 16350	0.0	29.4	70.6
Aspergillus flavus 16341	11.8	88.2	0.0
Aspergillus parasiticus 15628	0.0	100	0.0
Aspergillus parasiticus 15660	23.5	76.5	0.0
Aspergillus parasiticus 15444	5.9	94.1	0.0
Aspergillus parasiticus 11783	0.0	100	0.0
Aspergillus parasiticus 15002	0.0	94.1	5.9

[a] Strong inhibition- no growth of mycelia or sporulation around the well where the yeast is growing.

[b] Weak inhibition- sparse growth of mycelia with less sporulation around the well where the yeast is growing.

the well. The results of the effects of yeast strains against mycotoxin producing mould species, are presented in Tables 3 and 4. Clear inhibitory activity was demonstrated against the 3 mycotoxin producing mould species with *Candida* isolates being the most inhibitory. The *P. citrinum* and *A. parasiticus* strains were more inhibited than the *A.flavus* strains (Table 3).

The *P. citrinum* strains were inhibited by all the *Saccharomyces* strains (Table 4) except *P. citrinum* 16147 which was only weakly inhibited. On the other hand, all the *A. flavus* and *A. parasiticus* strains were only weakly inhibited by the *Saccharomyces* strains with *A. flavus* 16350 being the least inhibited.

Different inhibitory potentials were observed for the same species from steeping, 24 h and 48 h fermentation. Generally, the yeasts from 24 h and 48 h fermentation were more inhibitory than from the steeping stage. 49 % strong inhibitions were recorded for all the combinations tested between the steeped maize and the moulds. For the yeasts from 24 h and 48 h dough fermentation, the values were 61 and 58 % respectively (results not shown).

Preliminary investigations were carried out using cell free culture supernatants to determine whether the inhibition was caused by competitive growth of the yeasts or by secondary metabolites produced by the yeasts. Also inhibitory effects of yeast cell free extracts were examined in order to determine whether intracellular compounds were involved.

Ten isolates each of *C. krusei* and *Sacch. cerevisiae* which showed strong inhibitory effects against the moulds were selected and tested against three most sensitive mould strains ie. *P. citrinum* 16209, *A. flavus* 15915 and *A. parasiticus* 11783 using the well assay. No moulds were inhibited by the culture supernatants (results not shown). Growth and sporulation covering the whole plates indicated that the moulds were unaffected by the culture supernatants. The same 20 yeasts and 3 moulds investigated in the tests with cell free culture supernatants were used in testing the effects of cell free extracts. Again no inhibitions were observed (results not shown). The moulds grew normally where the intracellular extract was spotted as well as on the rest of the plate.

Discussion and conclusions

The results of the present investigations demonstrated that *Candida krusei* and *Saccharomyces cerevisiae*, the dominating yeasts in *kenkey* production, inhibit growth of mycotoxin producing moulds *Penicillium citrinum, Aspergillus flavus* and *Aspergillus parasiticus.*

Generally, *Candida krusei* strains were more inhibitory than the *Saccharomyces cerevisiae* strains and this is likely to be associated with their rapid growth and spreading pellicle. The *Candida* isolates grew rapidly and spread out of the wells whereas the *Saccharomyces* isolates grew inside the wells and hence showed smaller zones of inhibition.

That the isolates of the same yeast species showed differences in their inhibitory potential towards a particular mould support the finding by Jespersen *et al.* (1994) that significant strain variation exist amongst the dominating yeasts in *kenkey* production process.

In the first series of experiments, using strains of moulds isolated from maize, *A. parasiticus* was the most sensitive to all the *Candida krusei* strains tested whereas *P. citrinum* and the *A. flavus* strains were equally sensitive. The three moulds were however equally sensitive to inhibition by *Saccharomyces* strains. In the second series of trials with known mycotoxin producing moulds, *A. flavus* was clearly the least inhibited by the two yeast types *Candida* and *Saccharomyces.*

P. citrinum and *A. parasiticus* were found to be inhibited to the same degree by the *Candida* isolates and *P. citrinum* strains were more sensitive than *A. parasiticus* to the *Saccharomyces* isolates. The differences observed in the sensitivity of the mould strains to the yeasts in the two sets of trials, indicate that mould sensitivity is strain specific.

In the investigation to determine the mechanism of inhibition, the tests using yeast supernatant and cell-free extract could not verify that a secondary metabolite or an intracellular compound of the yeast caused the inhibitions observed. That the inhibition zone was only observed where the yeast colony was growing, seem to suggest that the inhibitions were due to competition for nutrients. In addition, that the *Candida* isolates with larger biomass, rapid growth and spreading abilities , showed greater inhibitory potential support the theory about competition for nutrients. This finding support the statement by Janissiewicz (1988) that the rapid growth of yeasts may restrict the availability of nutrients and sites for colonisation essential for germination of mould spores. Competitive effects of yeasts and moulds have also been described by Bjornberg and Schnurer (1993) in co-culture experiments with *Pichia anomala* (Hansen) Kurtzman, *Aspergillus candidus* and *Penicillium roqueforti*. Reports can also be found on the use of yeasts in the biological control of postharvest mould diseases of fruits and vegetables (Wilson and Chalutz, 1989; Droby *et al.*, 1989; 1991; McLaughlin *et al.* 1990).

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