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THE SIGNIFICANCE OF YEASTS AND MOULDS OCCURRING IN MAIZE DOUGH FERMENTATION FOR 'KENKEY' PRODUCTION

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1993

ABSTRACT

Yeasts and moulds associated with the fermentation of maize dough during the processing of the West African traditional food 'kenkey' were investigated. A mixed flora comprising Candida, Saccharomyces, Trichosporum, Klyveromyces and Debaryomyces species were isolated from raw maize, during steeping and early phases of fermentation. After 24-48 h of fermentation, Candida krusei and Saccharomyces cerevisiae dominated reaching counts exceeding 10^6 cfu/g. This succession of yeast populations and the significant multiplication of C. krusei and Sacch. cerevisiae were observed in all cases for both the fermentations and the production sites investigated.

Penicillium, Aspergillus and Fusarium species, including potential mycotoxin producers were isolated from raw maize. Initial high counts of 10^5 cfu/g for moulds were reduced to less than 10^2 cfu/g within 24 h of fermentation. High levels of aflatoxins were observed in raw maize and they were not affected during the fermentations.

Keywords: Maize dough, fermentation, yeasts, moulds, aflatoxins.

INTRODUCTION

Fermented maize forms the basis of a variety of foods in Ghana and other West African countries, contributing to a large proportion of the daily food intake. Despite the importance of fermented foods, they are still produced by traditional spontaneous and largely uncontrolled fermentations. The most popular way of consuming fermented maize dough will be in the form of 'kenkey' where a mixture of cooked and non-cooked dough is moulded into balls, wrapped in corn husk and cooked.

The traditional production method for fermented maize doughs in Ghana has been described in a previous publication (Halm et al., 1993) concluding that a homogeneous group of obligatively heterofermentative lactobacilli related with Lactobacillus fermentum and Lactobacillus reuteri play a dominating role. Yeasts were observed in levels 10^5-10^6 cfu/g as reported from other investigations (Arinkele, 1970; Hamad et al., 1992; Hounhouigan et al., 1992; Nyako and Danso, 1992). The yeast species associated with maize dough fermentations appear to be mainly Candida tropicalis, Candida kefyr , Candida mycoderma and Saccharomyces cerevisiae. However, systematic yeast studies have not been carried out, and whether the yeast take part in the fermentation process is not clear.

The occurrence of moulds has been dealt with by Arinkele (1970), who investigated the surface microflora of maize kernels for production of African starch cake and demonstrated the mould genera Penicillium, Aspergillus, Fusarium and Cephalosporium, however, they disappeared during the maize steeping. Fields et al. (1981) found moulds present in unfermented maize dough but they were not detectable from the doughs after two days of fermentation. It thus appears that the content of viable moulds is reduced significantly.

The fate of mycotoxins, preformed in the raw material, during fermentation is not known. High aflatoxin contents have been found in several foods in tropical Africa (Ibeh et al., 1991).

It has been the objective of the present work to perform quantitative and qualitative studies on yeasts and moulds occurring on maize, during maize steeping and maize dough fermentations. Further it has been the objective to elucidate the occurrence of aflatoxin producing moulds and the contents of aflatoxins during the complete process from steeping to the final product 'kenkey'. To obtain relevant information, main emphasis was given to larger commercial production sites with capacities of several tons per week. The sites were investigated by sampling on several occasions at months intervals.

MATERIALS AND METHODS

Samples

Samples were collected from two larger commercial production sites and four markets in Accra, Ghana, on several occasions over a period of 12 months.

The samples, 500-1000 g, comprised whole maize kernels, steeping water at 0 and 24 h, fresh dough, dough fermented for 24, 48 and 72 h, as well as the final product 'kenkey'. Surface layers were removed before sampling. Analyses were performed within two hours of sampling.

For examination of the mould flora of maize kernels, 150 kernels per batch were incubated individually on the substrates described below. Miscoloured kernels were selected for the examinations.

Microbiological analyses

From all samples 10 g were homogenized in 90 ml sterile diluent (0.1% peptone, 0.8% NaCl, pH 7.2) by use of a stomacher (LabBlender, Model 4001, Seward Medical, London, England) for 30 seconds. Maize kernels were milled by use of a hand mill under aseptic conditions before homogenization.

From appropriate tenfold dilutions, spread plate countings were carried out using Malt Agar (MA, Merck 5398 Darmstadt, Germany) added per 100 ml 100 mg chloramphenicol (Chloramphenicol Selective Supplement, Oxoid, Hampshire, England) and 50 mg chlortetracycline (Sigma c-4881, St Louis, MO. USA), with incubation at 25°C for at least 7 days. The medium was used for counting both yeasts and moulds. Dichloran Glycerol agar (DG-18, Oxid CM 729) added 50 mg chloramphenicol (Sigma c-0378) and 50 mg chlortetracycline (Sigma c-4881) with incubation at 25°C for at least 7 days, was included as an extra medium for mould counts.

Individual maize kernels were examined for mould infections by incubation of 5 kernels on each of 10 petri dishes (90 mm) of each of the following 3 media (Lillie and Jakobsen, 1992): i) DG 18, ii) Potato Dextrose Agar (PDA, Difco 0013-01-42, Difco Detroit Michigan, USA) added per 1000 ml: 50 mg chloramphenicol (Sigma c-0378), 50 mg chlortetracycline (Sigma c-4881), 10 mg $ZnSo_4$, 7 H_2O (Merck 8883), 5 mg $CuSo_4$, 5 H_2O (Merck 2790) and 0.5 g $MgSO_4$ (Merck 5886), pH = 6.5, and iii) (PDAID) consisting of PDA added per 1000 ml: 2 mg dichloran (Fluka AG 360103 Fluka, Buchs, Switzerland) and 6 mg iprodione (Rovral 50 wp, Rhone-Poulenc Agro-chemie, France). Incubation was carried out at 25°C for at least 7 days and PDAID was incubated at 12 h alternating UV-light (360 nm) and darkness.

Characterization and identification of the dominating yeasts and moulds.

To determine the composition of the yeast population of a particular sample all colonies from a sector (>15% of the plate area and at least 20 colonies) were subcultured in Malt extract Broth (MB, Merck 5397) for 48 h before streaking onto MA until pure cultures were obtained.

The yeast isolates were grouped according to their colony appearance and cell morphology. Representative isolates of the groups were identified to the level of species according to Kreger-Van Rij (1984) by observation of pellicle and sediment formation in MB, spore formation, growth at 37°C, growth in 50% (w/w) glucose, nitrate assimilation, fermentation of carbohydrates (glucose, galactose, saccharose, maltose, lactose and raffinose) and carbon compound assimilation (API 20 C AUX, Bio Merieux SA, Marcy-L'Etoile, France).

For isolates of Sacch. cerevisiae chromosomal patterns were performed by pulse field gel electrophoresis of spheroplasts as described by Donhauser et al. (1990) with minor modifications. The pulse field electrophoresis (pulsaphor control unit 2015, power supply 2301, both from Pharmacia-LKB, Upsala Sweden) was performed at 10°C and the following conditions: 100-120 mA, 165 V; pulse: 90 sec for 14 h, 105 sec for 12 h and 120 sec for 14 h. Yeast DNA-markers (Pharmacia-LKB) were used as yeast chromosome size DNA-standards.

Moulds were identified to the level of genus directly from the MA and DG18 plates according to their colony appearance and micromorphology. Selected isolates were subcultured and identified to the level of species according to Pitt (1979) and Onions et al. (1981).

Aflatoxin determinations

The clean-up procedure (method of extraction) used was based on that of Pons (1979) involving extraction with methanol, addition of zinc acetate to precipitate colour pigments, further extraction with dichloromethane and clean-up by column chromatography using cellulose and silica gel. The final residual was dissolved in 0.1 - 1.0 ml of methanol: acetonitrile: water (10:30:60 v/v) and used for HPLC analysis.

The HPLC instrument system used included a Model 501 Solvent Delivery System (Waters Associates, Milford, MA, USA), a Rheodyne Model 7125 injector with a 20µl fixed volume loop, Waters Temperature Control System, Model 470 Scanning Flourescence detector and a Model 746 Data Module (Waters Associates).

Identification and quantification of aflatoxins were by reversed phase liquid chromatography with postcolumn iodine derivatisation and fluorescence detection as described by Shepherd and Gilbert (1984). Separation of aflatoxins was carried out on a Spherisorb S5 ODS-1 column (250mm x 4.6mm) maintained at 35°C. Derivatisation tubing (5m x 0.3mm) was held at 75°C. Excitation and emission wavelengths were 360 and 440 nm, respectively.

RESULTS

Yeasts

The number of yeasts in the maize kernels varied considerably as seen in Table I. The examples presented refer to samples taken from the two commercial production sites at the time of harvest. The maize with the low yeast content (site A) was from the previous harvest (12 month old) and the maize with the high content (site B) was freshly harvested maize. The average yeast concentration for 6 samples of maize taken over the 9 months period with 3 months intervals was 1.9×10^5 cfu/g with a standard deviation of 1.79 (results not shown).

The differences observed in the yeast contents on the maize kernels influenced yeast numbers during steeping and fermentation and it also seemed to influence the composition of the yeast populations (Table I). A moderate increase of yeast concentration during steeping and fermentation was observed at all occasions and the maximum yeast concentrations were in the order of 10^5 - 10^6 cfu/g observed after 24 - 48 h fermentation followed by a reduction. The result from analysis of the steeping water at start of steeping reflected the result obtained for the maize. The water used for steeping did not contain (<10/ml) yeasts (results not included).

The yeast populations on the maize kernels included a variety of Candida, Saccharomyces, Trichosporon, Kluyveromyces and Debaryomyces spp. (data not shown). However, during steeping and maize dough fermentation a selection was observed with Candida krusei and Saccharomyces cerevisiae becoming the dominating yeasts (Table I). Although the increase in yeast number was limited as mentioned above a significant multiplication took place for C. krusei and Sacch. cerevisiae. This succession of yeasts was observed for all fermentations examined. The pH of the fresh dough was around 5.7 falling rapidly below pH 4.0 (Table I) which is likely to be one of the factors controlling the microbial succession.

The isolates of C. krusei were characterized by being flat, off-white to greyish colonies with irregular margin fringed with pseudomycelium on malt agar, some with rapidly spreading colonies, formation of a creeping pellicle when grown in broth, fermentation of glucose, inability to ferment galactose, saccharose, maltose, lactose and raffinose, growth at 37°C, growth in 50% (w/w) glucose and inability of nitrate assimilation.

The isolates of Sacch. cereviseae were characterized as cream-coloured colonies which were semi-glossy, smooth, slightly raised with entire margin on malt agar, absence of pellicle when grown in broth, multilateral budding, formation of ascospores, fermentation of glucose, galactose, saccharose, maltose, maltotriose and raffinose, inability of lactose fermentation, growth at 37°C, growth in 50% (w/w) glucose and inability of nitrate assimilation.

Within the two dominating species C.krusei and Sacch. cerevisiae significant strain variations were observed. For C. krusei the variation also included colony morphology with one type showing rapidly spreading colonies sometime covering the whole surface of the agar plate as mentioned above. Biochemically strain variation were most pronounced for Sacch. cerevisiae as illustrated in Table II, showing the assimilation characteristics of 26 and 22 strains of C. krusei and Sacch. cerevisiae respectively. The C. krusei isolates had quite similar assimilation profiles characterized by assimilation of very few of the compounds included. For Sacch. cerevisiae more heterogeneous assimilation patterns were observed, with five various assimilation patterns (data not shown).

To investigate the differences between strains within the same species, furthermore chromosomal pattern analysis was carried out for Sacch. cerevisieae isolates as shown in Fig. 1. All strains had a large number of bands with size distribution patterns typical for Sacch. cerevisiae as known from previous examinations of isolates of wine and brewing yeasts (unpublished results). However, length polymorphism is evident and it was seen for strains from different production sites as well as for strains from the same batch of fermentation.

Among the carbohydrates present in the maize dough (unpublished data) all C. krusei isolates fermented glucose whereas all Sacch. cerevisiae fermented glucose, maltose and maltotriose.

Moulds and aflatoxins.

The average initial numbers of moulds on milled maize kernels were in the range 10^5 cfu/g (Table III). The number of moulds remained the same during the steeping period. Immediately after mixing, mould numbers of 10^5 cfu/g were observed in the fresh dough, however, during the first 24 h of fermentation the numbers

were reduced significantly and after 24 h less than 10^2 cfu/g were observed. The strong reduction of mould numbers was observed for all samples investigated, as reported earlier (Halm et al., 1993). High levels of aflatoxins were detected during fermentations (Table IV). However, the results seem to reveal that fermentation does not affect aflatoxin levels. Table IV also indicates that the level of contamination is rather uniform within the same batch of fermenting dough. The presence of aflatoxins in the end product 'kenkey' was evidenced by the fact that out of sixteen 'kenkey' samples from four Accra markets fifteen were positive for aflatoxins with a total aflatoxin range of 6.15 to 196.10 μgkg^{-1} with a mean value of 50.88 μgkg^{-1}.

The dominating mould genera on the maize kernels were Penicillium, Aspergillus and Fusarium. For Fusarium spp. the highest numbers occurred in the freshly harvested maize (data not shown).

The composition of the mould population of the maize kernels remained the same during steeping and in the fresh dough (data not shown). Investigations of miscoloured whole kernels incubated on the surface of solid substrates revealed the same three genera to be dominating. Detailed investigations showed the species with highest frequency to be Penicillium citrinum, Aspergillus flavus, Aspergillus parasiticus, Aspergillus wentii and Fusarium subglutinans. In some cases miscoloured kernels were overgrown by species belonging to the A. flavus group. The presence of aflatoxin producers on the maize kernels was verified by aflatoxin determinations showing high levels in the kernels (unpublished results).

Table I

Numbers of yeasts (cfu/g), pH and composition of yeast populations on maize kernels, in steeping water and during maize dough fermentation at two production sites, A and B.

SAMPLE	PRODUCTION SITE A		PRODUCTION SITE B	
Maize kernels (milled)	$<10^3$		3.9×10^5	Mixed population dominated by Candida spp.
Steeping water (24 h)	5.0×10^3 (pH 4.1)	Mixed population of Candida and Saccharomyces spp.	1.3×10^6 (pH 4.1)	Mixed population of Candida and Saccharomyces spp.
Fermentation (24 h)	3.2×10^5 (pH 3.7)	C. krusei (78%) Sacch. cerevisiae (6%) others (16%)	1.5×10^6 (pH 4.1)	Sacch. cerevisiae (65%) C. krusei (5%) others (19%)
Fermentation (48 h)	8.6×10^4 (pH 3.5)	C. krusei (89%) Sacch. cerevisiae (4%) others (7%)	9.3×10^4 (pH 3.7)	Sacch. cerevisiae (95%) C. krusei (5%)
Fermentation (72 h)	8.0×10^3 (pH 3.8)	C. krusei (71%) Sacch. cerevisiae (14%) others (15%)	3.9×10^4 (pH 3.7)	C. krusei (62%) Sacch. cerevisiae (18%) others (20%)

Table II

Assimilation of carbon compounds by strains of C. krusei and Sacch. cerevisiae.

Substrate	C. krusei (%)[1]	Sacch. cerevisiae (%)[1]
Glucose	100	100
Glycerol	92	9
2-Keto-D-gluconate	0	5
L-Arabinose	0	0
D-Xylose	0	0
Adonitol	0	5
Xylitol	0	0
Galactose	0	100
Inositol	0	0
Sorbitol	0	5
α-Methyl-D-glucoside	0	0
N-Acetyl-D-glucosamine	92	5
Cellobiose	0	0
Lactose	0	0
Maltose	0	100
Saccharose	0	100
Trehalose	0	50
Melizitose	0	5
Raffinose	0	100

[1]Percentage of assimilation positive strains for 26 isolates of C. krusei and 22 isolates of Sacch. cerevisiae.

Table III

Numbers of moulds (cfu/g) occuring on maize kernels, in steeping water and during maize dough fermentation[1].

	$\bar{x}$	sd
Maize kernels	1.9×10^5	(0.75)
Steeping water (0 h)	7.0×10^4	(5.69)
Steeping water (24 h)	9.8×10^4	(8.40)
Fresh dough	1.2×10^5	(1.22)
Fermented dough (24 h)	$<10^2$	(-)
Fermented dough (48 h)	$<10^2$	(-)
Fermented dough (72 h)	$<10^2$	(-)

1. The figures given are mean ($\bar{x}$) and standard deviations (sd) for 10 samples from each of the two production sites (A and B) examined, sampled on 10 occasions over a 12 months period.

Table IV

Aflatoxin contents (μgkg^{-1}) of maize dough during fermentation.

	B_1	B_2	G_1	G_2	Total Aflatoxin
Fresh dough	19.58	2.93	72.75	4.94	100.20
Fermented dough I[1]	63.91	5.59	106.46	7.78	183.74
(24h) II	30.18	4.16	86.08	3.87	124.29
Fermented dough I	54.09	5.39	91.11	6.76	157.35
(48h) II	49.33	4.66	87.48	5.77	147.24
Fermented dough I	34.12	3.50	77.27	4.49	119.38
(72h) II	42.95	5.19	84.13	6.00	138.27

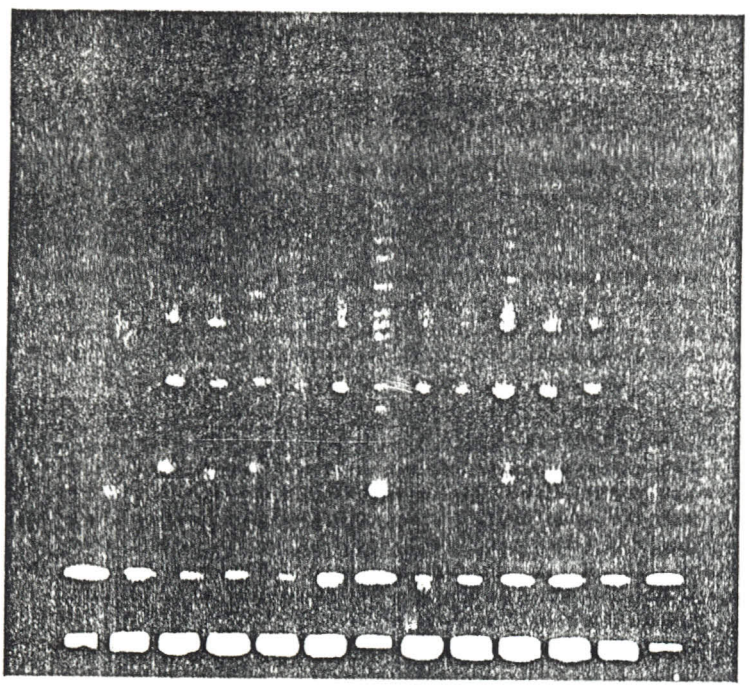
[1] I and II refer to two samples from different locations within the same batch (Production site A).

Fig. 1

Chromosome profiles for 10 isolates of Sacch. cerevisiae determined by pulse field gel electrophoresis (DNA - standards in outer and middle lane).

Lanes 1, 2, 3, 9 and 10 refer to 5 isolates from the same batch fermented at production Site A.

Lanes 4, 5, 6, 7 and 8 refer to 5 isolates from different batches fermented at production Site B.


M 1 2 3 4 5 M 6 7 8 9 10 M

DISCUSSION AND CONCLUSION

An increase in yeast numbers during maize dough fermentations has been demonstrated. Further a succession of yeast species took place with C. krusei and Sacch. cerevisiae becoming the dominating species. The number of yeast present and the significant multiplications of the dominant species are likely to influence the organoleptic and structural quality of the doughs. Yeasts are known to produce a wide range of aromatic compounds, such as organic acids, esters, alcohols, aldehydes, lactones and terpenes as reviewed by Janssens et al. (1992), and to be involved in leaving. Another possible effect of the yeasts would be growth stimulation of lactic acid bacteria as well as stimulation of their lactic acid production, as shown for Saccharomyces florentinus and Lactobacillus hilgardii, both isolated from sugary kefir grains (Leroi and Pidoux, 1993). The results obtained suggest that strains of C. krusei and Sacch. cerevisiae be used as starter cultures in combination with lactic acid bacteria.

By inoculation studies, for 'fufu', a traditional African fermented food, C. krusei has been shown to have significant influence on the typical odour of 'fufu' and reaching level of 10^7 - 10^8 cfu/g at the end of fermentation (Oyewole, 1990). Hamad et al. (1992) found that fermented sorghum doughs with high numbers of C. krusei (10^6 cfu/g) had a more pleasant smell than dough with less yeasts, and Nyako and Danso (1992) found that inoculation of maize dough with 10^6 cfu/g of Sacch. cerevisiae in pure culture or in combination with various Candida spp. increased the organoleptic scores of the doughs significantly.

From the detailed examinations performed on isolates of both C. krusei and Sacch. cerevisiae, several strains of the two species seem to be present during fermentation. Similar findings have been reported by Owuama and Saunders (1990) finding physiological variants of Sacch. cerevisiae to be involved in fermentation of palm wine. The chromosomal analyses carried out in the present investigation gave additional information on strain variations. Promising results have been obtained for identification of wine yeast strains of Sacch. cerevisiae by use of chromosomal analyses (Degré et al., 1989; Vezinhet et al., 1992). According to results obtained for pure cultures of brewing yeast, chromosome profiles are characteristic and stable for a particular culture, which means that within the same brewing yeast all isolates have identical profiles (unpublished results). To achieve a consistent product quality as well as uniform and controllable fermentations starter cultures should be well defined. Only one strain should be present characterized by a number of properties including a chromosomal profile. The latter could be used for selection and control of starter cultures for maize dough fermentations.

The decrease in viable mould counts during the early stage of fermentation was distinct and in agreement with other investigations performed on fermented maize dough (Arinkele, 1970; Fields et al., 1981; Hounhouigan et al., 1992; Halm et al., 1993). Since lactic acid bacteria are present in high numbers in the dough (about 10^9 cfu/g) according to previous work (Halm et al., 1993) they may be responsible. Bacteria have been shown to suppress the growth of moulds (El-Gendy and Marth, 1980), but their ability to inactivate moulds including potential toxin producers like A. flavus and P. citrinum seems not to be reported.

The apparent widespread occurrence of aflatoxins in fermented maize doughs is a health risk which is being further investigated. However, the predominance of potential aflatoxins producing moulds in mouldy and miscoloured kernel emphasize the need for selection of high quality maize.

Acknowledgement

The present work has been financed by DANIDA (Danish International Development Assistance) and the Government of Ghana.

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