Molecular identification and proteinase activity of yeasts isolated from fermented milk

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Introduction

Fermented milk is an important addition to the local diet and it provides essential building blocks for good health. Yeasts are some of the commonly found microorganisms in both raw and processed dairy products. The acid environment created by Lactic acid bacteria (LAB), creates a selective environment for a broad diversity of yeast growth [1, 2, 3, 4]. The fact that some yeasts may be associated with opportunistic infections and adverse conditions in humans and hence food safety threat, there is a growing interest in the biodiversity and ecology of yeast associated with different foods [5]. The realization that yeasts are able to interact with themselves and other microbial species in various ecosystems with outcome possibly affecting their roles they have in foods, increases curiosity to identify yeasts associated with different foods [6].

Moreira and others [7] reported the richness of yeasts in yoghurt from Brazil, up to 2723 CFU g⁻¹, with the most abundant yeasts being Debaryomyces hansenii, Debaryomyces castellii, Candida parapsilosis Saccharomyces cerevisiae, Mrakia frigida, Hansenula spp., and Candida maltosa. Similar studies from Turkey reported Candida sp., Kluyveromyces sp., Saccharomyces sp., Trichosporon sp., Geotrichum sp., Pichia sp., Yarrowia sp., and Rhodotorula sp. [3] with more than 10⁷ CFU of yeast counts being exhibited in retail marketed yoghurt [8]. Up to log 6 CFU/g of yeasts count was reported in yoghurt samples from retail outlets in Karachi, Pakistan [9]. The diversity and richness of yeasts in yoghurt have further been reported in Egypt (10³ to 10⁵ CFU/ml) [10] and in Pakistan (10⁷ CFU/ml) [9].

Yoghurt from Pakistan was reported to be rich in Candida sp., Cryptococcus sp., Debaryomyces sp. and Pichia sp. [10].

Mtindi is a spontaneously fermented bovine milk beverage consumed in Tanzania. Like many other African countries such as Burkina Faso and Kenya, more or less similar methods of preparation of mtindi are practiced across Tanzania with slightly variation in fermentation time due to local indigenous microflora and also the climatic conditions from one region to the other [4, 11, 12]. In rural Tanzania, raw milk is left to spontaneously ferment in gourds at...
ambient temperature varying from 25°C to 34°C from 24 to 36 hours, where the cream is skimmed away and the remaining is the protein rich fermented milk which is called mtindi. In urban settings, mtindi is made by addition of a portion of remnant of previous fermentation on to pre-heated milk (back-slopping) and left for 12 hours, the cream and the protein rich part are all mixed up together to form mtindi as well.

Like many other spontaneously fermented foods, the nature of mtindi is different from one region to another and the acidic environment found in fermented milk provides favourable environment for growth of yeasts [13, 14]. The presence of yeast in fermented milk indicates their ability to proliferate and positively interact with LAB and hence playing both beneficial and detrimental role in fermented milk safety and quality [5,15,16,17]. Some yeasts like Saccharomyces sp., which have been isolated from Kouniss ([18] have shown to contribute to the development of desirable flavour in fermented milk products like cheese, koumiss and kefir [19,12]. The improved flavour in traditional fermented milk is attributed by the presence of yeasts and thus, formation of volatile compounds like acetalddehydes [2].

Extracellular proteases can affect the quality of milk products in various ways, but largely by producing bitter peptides. Large numbers of somatic cells in raw milk affect the shelf life of pasteurized milk. Increased somatic cell numbers are reported to have a positive correlation with the concentration of plasmin, a heat stable protease and of lipoprotein lipase in freshly produced milk [20]. The activity of these proteolytic enzymes can supplement those of bacterial hydrolases hence fasten spoilage. The initial numbers of cells, their generation times, their ability to form volatile compounds like acetalddehydes [2].

Yeasts like Candida lipolytica are important in rendering longer shelf life of certain foods due to its ability of producing extracellular protease with antimicrobial activities [12, 21, 23]. There are studies reporting Candida species to be effective in enhancing longevity and activity of LAB and are now being used with some dairy starter cultures [31].

The diversity and richness of yeasts with different activities like probiotics [5] flavour and aroma of foods like egg odour in cheese [22]; and bio-control like the inhibition of growth of spoilage bacteria [23] calls for more studies on yeasts from milk from different settings and preparations around the globe. This is because, each country prefers indigenous fermented milk products according to their own palatal satisfaction which the aroma may be contributed by yeast strain present in fermented milk. Therefore, the main objective of this study was to isolate yeasts from selected samples of fermented milk, genetically identify them and examine their ability to hydrolyze casein.

Materials and methods

Sample collection, preparation and pH determination

A total of 25 yeasts were isolated from mtindi and yoghurt samples from local markets in four regions of Tanzania between the months of April to June in 2009 - North-west Tanzania (Mwanza), Eastern Zone (Dar es Salaam and Morogoro) and Northern zone (Kilimanjaro) were studied. The samples included 4 samples from Mwanza rural, coded as TFMMw1, 2, 3 and 4 and were prepared by natural fermentation of raw milk at room temperature for 24 to 36 hours in earthenware which is not sanitized after previous fermentation. Three samples from Morogoro peri-urban with codes TFMM1, 2 and 3 (a portion of mtindi from previous fermentation which was used as starter culture [back-slopping] in pre-boiled and cooled milk); 1 sample from Kilimanjaro (TFMK) and 7 samples from Dar es Salaam with codes TFMD 1 – 7 in which a portion of yoghurt was added in pre-boiled and cooled milk in to a well sanitized and covered plastic containers for 12 hours.

Samples were collected in sterile 250 mL glass containers and immediately placed in ice box and transported to University of Dar es Salaam, Molecular Biology and Biotechnology laboratory and preserved on refrigerator at 4°C to 7°C for analysis within 6 hours of sampling. The pH of the samples was determined by direct insertion of the electrode into the sterilized jar containing 25mL of fermented milk at 30°C using a pH metre (pH 209 Hanna Instruments, USA).

Enumeration and Isolation of yeasts

One gram of each yoghurt and mtindi sample was diluted in 9mL sterile 0.1% Ringer’s solution (0.9% NaCl, 0.042% KCl, 0.48% CaCl₂·6H₂O, 0.02% NaHCO₃, and 1% Glucose) followed by decimal dilution up to 10⁻⁶ and 1mL was used for pour plating in duplicate on malt extract agar, MEA (HIMEDIA, India) at 30°C for 24 hours. About 0.1mL of 10⁻¹, 10⁻² and 10⁻⁶ dilutions each was spread on Malt extract agar supplemented with Chloromphenicol (100mg/L) to inhibit bacterial growth then incubated at 30°C for 24 to 48 hours. The average sum of colonies formed was used to determine the total yeasts populations.

Yeast isolates per plate were selected based on their microscopic appearance of the cells grown, morphological differences, colour, margin and elevation of the colonies [3] further sub-cultured overnight at 30°C on Malt extract broth supplemented with Chloromphenicol (100mg/L). Overnight broth cultures were then streaked on malt extract agar plates supplemented with Chloromphenicol (100mg/L) for purification. The strains were then cultured on malt extract slants at 30°C for 24 to 48 hours and kept at 4°C.

DNA isolation

A modified technique for yeast DNA extraction by [24] was applied. Briefly, an aliquot of 1 mL overnight Malt extract broth cultures was centrifuged at 10,000 rpm for 1min. A pellet was collected and suspended in 500 µL lysis buffer (50 mMmLL⁻¹ Tris; 250 mMmLL⁻¹ NaCl; 50 mMmLL⁻¹ EDTA; 0.3% w/v SDS; pH 8). Glass beads equivalent to a volume of 200 µL 425-600 µm was added to remove/crush cell membranes (Sartorius Mikro dismembrator U) for 1.5 min, and then the tubes were heated in water bath for 20 min at 99°C. The suspensions were then centrifuged for 30 min at 4°C. Supernatant was collected and diluted 1: 750; 5 µL was used for polymerase chain reaction (PCR).

PCR assay

The amplification of the 26S rDNA D1/D2 region was done by NLI (5’ GCATATCAATAAGCGGAGGAAAAG
Mean yeast counts and pH isolated from yoghurt (I), Kinondoni (K), Temeke (T) to 3 ng DNA. The mixture was subjected to 30 PCR cycles; 1 minute of denaturation at 94°C, annealing at 60°C for 1 minute; extension at 72°C for 2 minutes and then final extension at 72°C for 7 minutes.

Analysis of PCR products and sequencing

PCR products were analyzed electrophoretically with 1.5% agarose gel containing 0.5 µg/mL ethidium bromide and visualized with transilluminator. Gene Ruler DNA ladder mix (100-1000bp) (Invitrogen) was run as a molecular-size marker in one of the lanes for size comparison. PCR products (600bp – 650bp) were sequenced at a commercial facility in South Africa (INQABA Biotechnology Ltd). Identity and similarity of the sequences were tested using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence data were then assembled and submitted at the NCBI GenBank for acquisition of accession numbers.

Screening of proteolytic activity

The ability of yeasts to produce extracellular proteolytic enzymes was examined by means of agar plates containing milk-agar [25]. About 5 µL of an overnight yeast suspension (2.0 x 10^3 cells) was added into bore holes previously made in milk agar plates, pH 5. The inoculated plates were incubated at 30°C for 5 days. The plates were observed for an increasing opacity around the holes caused by growing fungi. Clearing of opacity by hydrolysis of precipitated casein was affected by protease of the fungi. The millimetric zone measurements were evaluated as the level for proteolytic enzymes as: no proteolytic activity (0); low proteolytic activity (d<10mm); medium proteolytic activity (10mm < d<20mm) and high proteolytic activity (d > 20mm).

Statistical analysis

The significance of the yeasts load in mtindi and yoghurt samples was evaluated by comparing means using one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test. Results were expressed as means ± SEM and differences were considered significant when p < 0.05. Statistics were performed with Graphpad Instat version 3 for Windows (Graphpad Software, San Diego CA, USA).

Results

Yeast total counts, pH and diversity in yoghurt and mtindi samples

Based on [26], thirteen and sixteen yeast cultures were isolated from yoghurt and mtindi, respectively. The total yeast counts for mtindi varied from 10^3 + 0.92 to 10^5 + 0.34 CFU/mL with pH values ranging from 4.16 + 0.08 to 4.8 + 0.14 (Fig. 1) while yeast counts in yoghurt samples were generally higher than Log 3.9 but slightly lower than log 5 CFU/mL while the pH values ranged from 4.20 + 0.10 to 4.40 + 0.13 (Fig.2).

Identification of yeast isolates

Sequences data obtained from the three isolates TFMMw1B, TFMMw2B and TFMMw4B showed a 99% sequence identity to Candida pararugosa. The BLAST results for DNA sequence data for isolate TFMMw1A showed 99% sequence similarity to that of Candida tropicalis (GenBank accession number DQ099738 - a Chinese botanical sample). TFMM3, TFMD6, and TFMD7 showed 99% homology similarity with that of Pichia guilliermondii FJ468466 in GenBank database (Chinese marine yeast) and Meyerozyma guilliermondii HM988686, native Italian wine yeast. The Blast results for sequence data for isolates A4 and B3 showed 99% homology to Debaryomyces hansenii JF781387 isolated from material and environment in process of Chinese liquor fermentation and D. hansenii HM988696 isolated from Italian wine production materials. Isolates TFMMw2A exhibited 99% homology to that of Pichia kudriavzevii EU394711 isolated from Chinese lignoncelloses hydrolysate while TFMMw3B showed 87% homology to Issatchenkia sp. JF781389 isolated from materials of Chinese liquor fermentation. Isolate TFMMw3A had 99% homology to Clavispora lusitaniae FJ627984 isolated from Egyptian traditional fermented dairy products. The results for blast search for sequences of isolate B2 showed 99% sequence identity to Yarrowia lipolytica GQ121611 isolated from Tibetan natural fermented milk. The isolates A2, A3, TFMK, TFMB and TFMD3 and TFMD4 showed 99% sequence homology to Galactomyces geotrichum. Isolate A1 showed 99% sequence similarity to that of Geotrichum sp. However, the BLAST sequence data for isolate TFMD2 showed 97% sequence identity to that of G. geotrichum.

Phylogenetic reconstruction based on D1/D2 26S rDNA gene sequences grouped these isolates into 8 groups (Fig. 3). Group one is of G. geotrichum comprising of isolates TFMD1, TFMF3, TFMKA, TFMB and TFMD4 showed 99% sequence homology to Galactomyces geotrichum. Isolate TFMD6 and TFMD7 form group fifth of P. guilliermondii (M. guilliermondii). C. pararugosa is the sixth group made by isolates TFMMw1B and TFMMw4B. P. kudriavzevii (I. orientalis) group made by isolates TFMMw2A and TFMD5 while TFMMw3A formed C. lusitaniae group are the seventh and eighth groups respectively.

Figure 1: Mean yeast counts and pH isolated from Traditional fermented milk (TFM) mtindi bought from Dar es Salaam (DSM; Ilala (I), Kinondoni (K), Temeke (T) municipalities), Kilimanjaro and Morogoro (K&M)
Proteolytic activity
Protease activity was revealed as a clearance zone around the hole filled 5ul of 18 hours yeast culture on Milk agar medium (Table 1). Of 25 yeast isolates tested 21 (84%) were protease positive. Of the yeast isolates examined in this study, the highest proteolytic extracellular enzymes were revealed by the strains belonging to among the Candida isolates tested (Fig.5). C. pararugosa (TFMMw2B) produced the strongest protease activity (37±5.487) as well as C. tropicalis (TFMMw1A) with (27±1.202).

Table 1: Some yeasts found with strong proteolytic activity

<table>
<thead>
<tr>
<th>Name</th>
<th>Reference No.</th>
<th>Inhibitory zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. guilliermondii</td>
<td>TFMM3</td>
<td>17.667±0.333</td>
</tr>
<tr>
<td>P. kudriavzevii</td>
<td>TFMMw2A</td>
<td>19.667±1.856</td>
</tr>
<tr>
<td>Cl. lusitaniae</td>
<td>TFMMw3A</td>
<td>10.667±0.333</td>
</tr>
<tr>
<td>G. geotrichum</td>
<td>TFMD2</td>
<td>29.333±2.333</td>
</tr>
</tbody>
</table>

Figure 2: Mean yeast counts isolated from major Tanzanian yoghurt brands; A, B and C bought from Dar es Salaam City.

Figure 3: The evolutionary history inferred by using the Maximum Likelihood method. The analysis involved 25 nucleotide sequences. Evolutionary analyses were conducted in MEGA5. *Isolate TFMD2 diverging from the rest of the G. geotrichum group.
Discussion
The mean yeast counts in mtindi ranged from $10^3$ to $10^5$ CFU/ml with a pH ranging from 4.16 to 4.82 and selective environment for yeast proliferation which compliments [30] findings. Mtindi samples from Mwanza (TFMMw) showed the highest yeasts count of $10^5$ CFU/ml unlike that from Dar es Salaam (DSMK, DSMI, and DSMT), Kilimanjaro (KLM) and Morogoro (Mo) which exhibited yeast counts below $10^4$ CFU/ml (Fig.2). This may be contributed by the fact that raw milk is used for preparation of mtindi in Mwanza. The mean yeast counts in Tanzania's mtindi were lower (p = 0.0001) than that reported by [27] in Traditional Tunisian Leben and [28] in Sudanese fermented milk.

Yeast counts in yoghurts were lower (p = 0.0052) than that reported by [8] and [9] but were similar to the counts observed in yoghurt samples of Tunisian industrial Leben [27], which were reported to be in excess of $10^4$ CFU/g, while, values between $10^3$ to $10^5$ CFU/ml were reported in dairy products from Egypt [29].

The presence of C. tropicalis in mtindi from Mwanza was an interesting observation. The source of this pathogenic yeast may have been from the handler’s hands at the time of preparations or during milking. C. tropicalis has previously been found in Zimbabwe traditionally fermented milk [30] also in Turkish-style homemade dairy products [13] in Tunisia traditional fermented milk, Leben [27] and in raw milk and cheese [33, 36].

Different degrees of yeast diversity were observed between yoghurt and mtindi samples. Mtindi from Mwanza contained diverse yeast flora consisting of five species whereas yoghurt brand C and traditionally inoculated fermented milk from Kilimanjaro and Morogoro contained a single but different yeast species. P. guilliermondii, G. geotrichum, Y. lipolytica, D. Hansenii were commonly identified across different products. While P. guilliermondii was only detected in mtindi samples, Y. lipolytica and D. Hansenii were detected in yoghurts only. Interestingly, G. geotrichum was the only species that was detected in both mtindi and yoghurt. However, C. tropicalis, C. pararugosa, Cl. lusitaniae (C. lusitaniae) and I. Orientalis were only detected in mtindi from Mwanza. Fermented milk from Mwanza is usually made by spontaneous fermentation of raw milk for about two days with the removal of cream and it has shown in this study to contain the most diverse yeast flora than the rest. The technic or utensils used in removing the cream may be associated with the introduction of more yeast in to mtindi in Mwanza. Mtindi from Kilimanjaro had single yeast species G. geotrichum while both G. geotrichum and P. guilliermondii were isolated from mtindi from Dar es Salaam.

In this study, the most frequently yeast isolated from both yoghurt and mtindi was G. geotrichum (Anamoph: Geotrichum candidum). The proteases of G. geotrichum has been associated with development of distinctive protein breakdown of milk proteins which may result in an increased protein bioavailability and have a positive impact on health and nutrition.

Figure 4: Neighbor-Joining phylogenetic tree of the polymorphic sequences of the D1/D2 domains of the 26S rDNA of Galactomyces geotrichum.
flavours and other qualities of traditionally made cheese and also reduction of bitterness in industrially made cheese [32]. Another enzyme, lipases from *G. geotrichum* were also reported to contribute to its good qualities. Of all *G. geotrichum* tested, isolate TFMD2 produced the strongest protease activity (29±2.333; *p*=0.0119). The observed variation in proteolytic activity among *G. geotrichum* in this study might be explained by two circumstances first, the possibility of slight genetic variation in isolate TFMD2 from the rest of *G. geotrichum* of this study, Maximum likelihood tree based on 26S rDNA sequences (Fig.4) displayed isolate TFMD2 and *Geotrichum* sp. CICC1745 (Accession number, DQ912852) from China which is a novel *Geotrichum* species. Secondly, could be due to the three basic morphological variations among *G. geotrichum* strains. Yeast like colonies with abundant arthrospores are associated with low proteolytic activity while those with white colonies and filamentous fungi look-aliike and hyphae have high proteolytic activity, and others fall in between [32, 34]. Another fascinating observation was the detection of *G. geotrichum* in yoghurt and mtindi made by addition of a portion of inoculums of previously fermentation in to pre-boiled milk only and not in naturally fermented mtindi (TFMMw1, TFMMw2, TFMMw3, TFMMw4 and TFMM3). Marian and others [33] reported that *G. geotrichum* appeared to be one of the most dominating microbial ecology of biofilms. Their presence in yoghurt may be due to their ability to form biofilm on the surface on the factory while in mtindi it may be due to the use of the same holding containers with biofilm dominated by *G. geotrichum*. We may also speculate the possible association between protease activity and biofilm formation.

**Conclusions**

Even though the microbiology of dairy products is generally subjected to debates of Lactic acid bacteria, an extensive literature is now describing the importance of the role of yeasts in flavour and texture development of a
number of dairy products. Since fermented dairy products have got favourable growth conditions for yeasts, it is not surprising to find yeasts proliferating in these products. Indeed, the presence of yeasts and their proteases among other metabolites in dairy products is of major importance as they may either have beneficial or harmful outcome with contribution to flavour development and rippening. This study demonstrates that diverse yeast species existed in the examined Tanzanian traditional (mtindi) and industrially fermented milk (yoghurt) in which mtindi from Mwanza contains the most diverse yeast flora. To our knowledge this reports for the first time genetically characterized yeast isolated from Tanzanian fermented milk and their proteolytic characteristics with either potential biotechnological benefits or harmful effects. In the present study, we amplified and sequenced the domains (D1 and D2) regions of the 26S rRNA gene for the identification of yeasts from industrially fermented milk, yoghurt and traditional Tanzanian fermented milk, mtindi obtained from different farms and localities of Tanzania. Findings showed a diverse degree of yeast population and protease activity in examined products. Further research concerning characterization of the protease from yeasts is necessary to explore the mechanism of their actions in relation to the biochemical nature of the fermentation processes. This may further explore the identity of extracellular protease for novel agents in bio-control of food spoilage and the identity of yeast with the ability to produce them. 

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