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EFFECT OF ALTITUDE ON MICROBIAL SUCCESSION DURING TRADITIONAL ENSET (ENSETE VENTRICOSUM) FERMENTATION

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Abstract: Effect of altitude on microbial succession during traditional enset fermentation was determined at enset culture sites, 2908 (high altitude) and 2252 (mid altitude) meters above sea level. Counts of aerobic mesophilic bacteria and lactic acid bacteria showed differences between the study sites. The warmer temperature of the fermenting mass at the mid altitude site contributed to the rapid proliferation of microorganisms and rapid fermentation process and hence shorter fermentation time (112 days) than at the high altitude site (142 days). The microorganisms isolated from traditional enset fermentation process, at both sites were similar. Bacillus spp. and Lactic acid bacteria dominated the fermentative microflora at the initial and later stages of the fermentation, respectively. The coliforms and other members of enterobacteriaceae contributed to initial lowering of the pH of the fermenting mass. At about day 15, the homofermentative lactobacilli took over the process and dominated the flora till the end of fermentation. Following the proliferation of the lactobacilli, the pH decreased and titratable acidity increased. Count of yeasts remained low throughout the fermentation process. The coliforms and enterobacteriaceae were undetectable following domination of the microflora by LAB. The decline in moisture content of the fermenting mass was relatively faster at the mid altitude than at the high altitude site. Some Gram-negative rods and other Gram-positive cocci were occasionally encountered at lower levels. Almost all isolates were amylolytic than proteolytic and lipolytic due to high carbohydrate content of the substrate. This study showed there could be differences in microbial succession from place to place depending on various factors.

Keywords: Ensete ventricosum, fermentation, LAB, altitude

INTRODUCTION

Enset (Ensete ventricosum) food products are used as staple or co-staple food for about 20% of the Ethiopian population (Brandt et al., 1997). Over 80% of enset produced in the country is grown in the south and south-western parts of the country (Belehu, 1993). Enset plant has been traditionally fermented for generations in an earthen pit into carbohydrate rich food product, kotcho. This age-old traditional method has been used for

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generations without any scientific modification. Traditional fermentation techniques, limited to a particular country, are less likely to advance in technologies because of less research effort on indigenous foods.

Studies done so far on traditional enset fermentation and its food products mainly focused on evaluation of effect of length of fermentation on carbohydrate and calcium content (Bezuneh, 1984), microbial dynamics and accompanying changes in the fermenting mass (Gashe, 1987a), microbial spoilage and safety issues of enset food products (Girma and Gashe, 1985; Nigatu and Gashe, 1993; Ashenafi and Abebe, 1996) and biochemical changes during fermentation (Bisrat et al., 1980; Urga et al., 1997).

Traditional enset fermentation process differs from one locality to another (Gebremariam, 1993). It is not known, though, whether fermenting microbes differ from one locality to another and whether environmental factors in respective localities have any influence on microbial activities. Understanding these effects can help to scientifically define and improve the product. Improving, standardizing and modernizing of traditional enset fermentation process will help to minimize time and energy needed, enhance quality and quantity of the food product and also minimize wastage and related public health problems. The aim of this study was, therefore, to investigate the effect of altitude on the succession of different microorganisms and other parameters over fermentation period.

MATERIALS AND METHODS

Preliminary Survey

A preliminary survey was done in enset growing areas of West Shewa Zone of Oromia Regional State, Ethiopia during September 2007. Two different sites were selected to represent enset fermentation at high altitude (2908 masl) and mid altitude (2252 masl). Actual experimental pits were prepared on enset farms of a selected household at both sites. As processing of the enset plant for fermentation is traditionally the responsibility of the woman (Belay et al, 2008), knowledgeable women were employed for this purpose. Steps in enset fermentation at both sites are shown in Fig. 1.

Sample collection

At both study sites, about 500g of samples of fermenting mass were collected at day 0, 5, and later at 15 days interval until the final stage of fermentation process. Samples were collected randomly from different parts of the fermenting mass aseptically using sampling tongs. Portions from a pit were pooled into a sterile polyethylene bag. The samples were taken to the laboratory and analysis was done within two hours of collection.

Microbiological analysis

After aseptically mixing the samples thoroughly, a 25g sample was aseptically blended in 225 ml of sterile water using a laboratory blender (Wagtech international Ltd, UK). Further dilutions were made using 0.1 % peptone water.

Microbial count

From appropriate dilutions, volumes of 0.1 ml aliquots were spread-plated in duplicates on pre-dried surfaces of Nutrient Agar (NA) plates to count aerobic mesophilic bacteria. Colonies were counted after incubation at 30 to 32 ^{o}C for about 48 hrs.

To count staphylococci, 0.1 ml aliquots from appropriate dilutions were spread-

plated in duplicate on pre-dried surfaces of Mannitol Salt Agar. The plates were incubated at 30 to 32°C for 36 h after which yellow colonies were counted as staphylococci.

Coliforms were counted by spread-plating 0.1 ml of appropriate dilutions on pre-dried surfaces of Violet Red Bile (VRB) agar plates and incubating at 30 to 32°C for 24 hrs. Purplish red colonies surrounded by reddish zone of precipitated bile were counted as coliforms.

To count enterobacteriacea, 0.1 ml of appropriate dilutions was spread-plated in duplicate on pre-dried surfaces of Violet Red Bile agar plates supplemented with 1% glucose. The plates were incubated at 30 to 32°C for 24 hrs after which pink to red colonies with or without haloes of bile precipitation were enumerated as members of Enterobacteriaceae.

Lactic acid bacteria (LAB) were counted by spread-plating 0.1 ml of appropriate dilutions in duplicate on pre-dried surfaces of MRS (de-Mann Rogosa and Sharp) agar plates. The plates were incubated under anaerobic condition, using anaerobic candle jar at 30 to 32°C for 48 hrs. All colonies were counted as lactic acid bacteria.

Yeasts and molds were counted by spread-plating 0.1 ml of appropriate dilutions in duplicate on pre-dried surfaces of Chloramphenicol Bromophenol Blue (CBB) agar plates (yeast extract, 5.0g; glucose, 20.0g; chloramphenicol, 0.1g; bromophenol blue, 0.01g; agar, 15g; distilled water, 1000ml; pH, 6.0 to 6.4. Plates were incubated at 25 to 28°C for three to five days. Smooth (non-hairy) colonies without extension at periphery (margin) were considered as yeasts whereas hairy colonies with extension at periphery were considered as molds.

To count aerobic bacterial spores, homogenized samples were heated at 80°C for 10 minutes in a water bath to kill vegetative cells. A volume of 0.1ml of appropriate dilutions was spread plated in duplicate on pre-dried surfaces of Nutrient Agar (NA) plates. The plates were incubated at 30 to 32°C for 24 to 48 hrs after which colonies were counted as aerobic bacterial spores. All dehydrated media used in this study were from Oxoid.

Microbial flora analysis

After colony counting of aerobic mesophilic bacteria, 10 to 15 colonies were randomly picked from countable NA plates and separately inoculated into tubes containing about 5 ml nutrient broth (Oxoid). The isolates were further purified by repeated plating and were differentiated to genus level using the following characteristics.

Cell shape, grouping and presence or absence of endospores were determined microscopically. Test on lipopolysaccharide (KOH test), which corresponded to the Gram reaction, was done according to Gregersen (1978). The catalase test was conducted by flooding colonies with a 3% solution of hydrogen peroxide (H_2O_2). Formation of bubbles indicated catalase production. The utilization of glucose was assessed by O/F test (Hugh and Leifson, 1953). Cytochrome oxidase test was conducted following the method outlined by Bisen and Verma (1994).

Starch hydrolysis was detected by streak plating a bacterial suspension on starch agar and incubating it at 32°C for 48 hrs. The plate was flooded with dilute Lugol's iodine solution for 30 seconds. Clear zones around colonies indicated amylase production.

The ability of the isolates to degrade the protein casein was tested by growing the

isolates on proteinase agar plates. The medium was spot inoculated with a loopful of bacterial suspension and incubated at 32°C for 7 days. Clear zones around colonies indicated proteolytic activity.

To determine the lipolytic activity of the bacterial isolates, a loopful of the broth culture was surface streaked on Tributyrin agar plates and incubated at 32°C for 7 days. Clear zones around colonies indicated lipolytic activity.

Isolates from MRS agar plates which were Gram-positive, non-sporing, catalase negative rods or cocci were considered as LAB and were grouped as homofermentative and heterofermentative by their ability to produce gas in 5% glucose in MRS broth after incubation at 30-32°C for 4-5 days.

Temperature determination

Temperature of the fermenting mass was measured within the pit just before taking the sample. Reading was taken after dipping a glass thermometer cleaned with 70% alcohol into the fermenting mass for about five minutes. This was done at three points at equilateral distances from the center.

Physico-chemical determination

The pH of the samples was measured from 1/10 dilution of sample using a digital pH meter. Titratable acidity (TA) was measured according to Antony and Chandra (1997). The result was expressed as g lactic acid/100g. To determine moisture content, a known quantity of the sample was allowed to dry to a constant weight.

Statistical analysis

Data analysis was done using SAS version 8.2 (SAS institute). GLM procedure was followed for data analysis. Means were compared at 5% significance level, using Duncan Multiple Range Test. All charts and trend lines were plotted by Microsoft Excel 2003.

RESULTS AND DISCUSSION

General steps like decortication and pulverization of the pseudostem, wrapping of fermentable mass with enset leaves, digging of the pit for fermentation around the homestead and allowing fermentation of the mass in the pit are basically similar in all enset traditions. Reports hitherto indicated the similarity of these processing steps in the different enset cultures found in low, mid and high altitude parts of Ethiopia (Bezuneh, 1984; Gashe, 1987a; Tedla and Abebe, 1994) Certain details of the traditional enset processing for kotcho fermentation were, however, different from what was observed in this study. Even within this study, notable differences in processing were observed between the two altitude sites.

At the high altitude site in this study, nine mature enset plants were decorticated (Figure 1) and further processed and divided into about seven different portions. The fermentation pit was prepared and the floor and walls of the pit were longitudinally lined with layers of fresh enset leaves (Figure 2.). The leaves of an enset plant may be five meters long and nearly one meter wide. Each portion was put in to a separate pit and the portions of the lining leaves that extended out from the pit floor were used to tightly cover the upper surface of the mass. Layers of more fresh leaves were put on the surface. Heavy logs and stones were put as weight over the tightly wrapped and sealed mass possibly to ensure the creation of airtight conditions in the pit (Ashenafi, 2006). Lining of pit with enset leaves was done

Figure 1 Women decorticating the pseudostem (left) and smashing the lower piece of the pseudostem.

Figure 2 Lining the inside of the pit with enset leaves and putting the mass into the pit

Figure 3 Mixing the mass with Gamma by pounding with feet, and wrapping the mass in the pit.

to collect and prevent the juicy part from leaking into the ground (Gashe, 1987a). The mass was put in the fermentation pit on the same day of decortication. After one month of fermentation, the fermenting mass was taken out of the pit, mixed thoroughly with a starter (gamma) (Figure 3), put back into the pit following the steps

of pit lining and wrapping of the mass as described above. The starter (gamma) consisted of pulverized, previously fermented enset parts and other herbs. In other localities, an already fermented kotcho from a previous fermentation is used as a starter (Ashenafi, 2006). The starter may contain fermentative microorganisms and speed up the fermentation process (Urga, et al., 1993). Fermentation continued for about 2 to 5

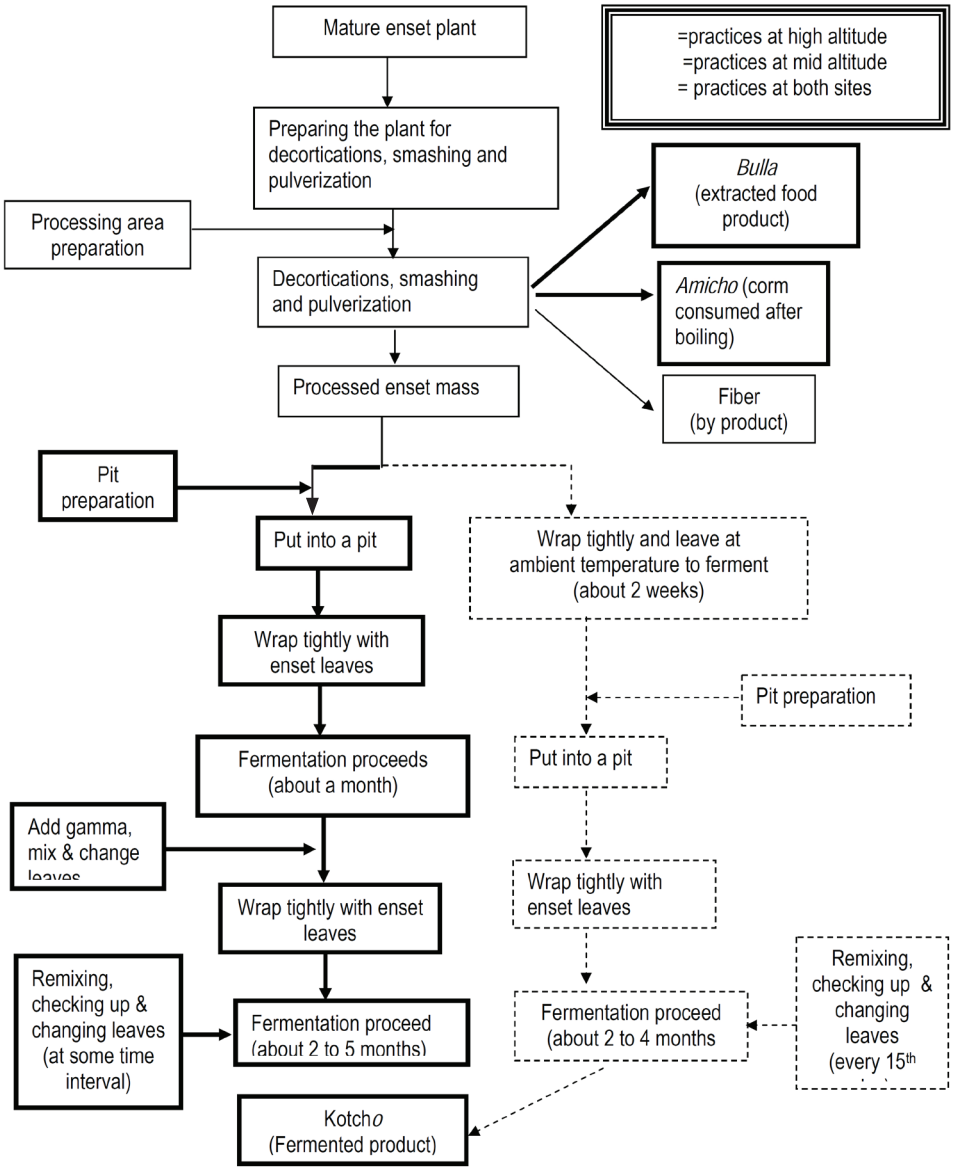

Figure 4 Flow chart of traditional enset fermentation process at high and mid altitude sites

months. During this time regular checking was done to detect incipient spoilage of the fermenting mass. Checking was usually done by making a small narrow opening in the fermenting mass and detecting for undesirable odor. Blackening and slime formation were also considered undesirable and were detected on the surface of the packed fermenting mass visually. Remixing and wrapping with new leaves was undertaken only when undesirable odor was detected. When this happened, the whole mass was removed from the pit and re-mixed thoroughly. The pit was again lined with fresh leaves and the remixed mass was put back into the pit and tightly sealed with fresh leaves.

At the mid-altitude site, however, about six smaller mature plants were processed producing about 300 kg of fermentable mass. The mass was tightly wrapped with fresh enset leaves and left at ambient temperature for two weeks. This was then remixed, put in one deep and wide pit lined with fresh enset leaves and sealed as described for the high altitude site. Fermentation proceeded for about 2 to 4 months. During this period, at 15-day intervals, the whole mass was taken out of the pit, remixed at the mouth of the pit, and put back into the same pit lined with fresh leaves and wrapped with fresh enset leaves. The number of enset trees used for fermentation and the duration of fermentation varies among various enset cultures (Gashe, 1987a; Ashenafi, 2006). The similarities and differences in enset processing at the two study sites are shown in Figure 4.

The use of starter in the high altitude area corresponded with low frequency of spoilage during fermentation. Although microbiological analysis was not made on the starter 'gamma', its incorporation in the fermenting mass could result in higher initial population of fermenting microorganisms compensated for possible delayed microbial growth at low temperatures at the high altitude site. At the mid altitude site remixing of the fermenting mass was done at regular intervals indicating the frequent appearance of incipient spoilage during the fermentation process.

Initial pH of the pulverized and decorticated mass was 6.11 and 6.05 at high and mid altitude, respectively (Figure 5). The pH of fermenting mass was significantly lower at the mid-altitude than at high altitude site ($p<0.05$) between days 15 and 82. An initial sharp decline in pH corresponded to a similar increase in count of all groups of microorganisms. The aerobic mesophilic count reached levels as high as over log 9 cfu/g in both sites (Figure 6).

As all LAB were homofermentative, more acid would be produced per mole of fermentable sugar and the rate of pH fall would be faster. The lowest pH achieved during the fermentation was 4.30 at the high altitude site at day 97 and this pH was maintained until the end of fermentation at day 142. At the mid-altitude site, similar pH value was reached only towards the end of fermentation. Inclusion of a starter (gamma) at the high altitude site could help in faster rate of pH fall and a product with low pH value for longer fermentation duration. Although the final pH value of the fermented mass in both sites was similar, the longer duration of fermentation at the high altitude site could result in the production of more acid thereby retarding the growth of undesirable microorganisms. The dominance of LAB during the active stages of enset fermentation is in agreement with the report of Gashe (1987a). However, according to this author, heterofermentative cocci were important in initiating enset fermentation.

Coliforms and other members of enterobacteriaceae grew faster and reached

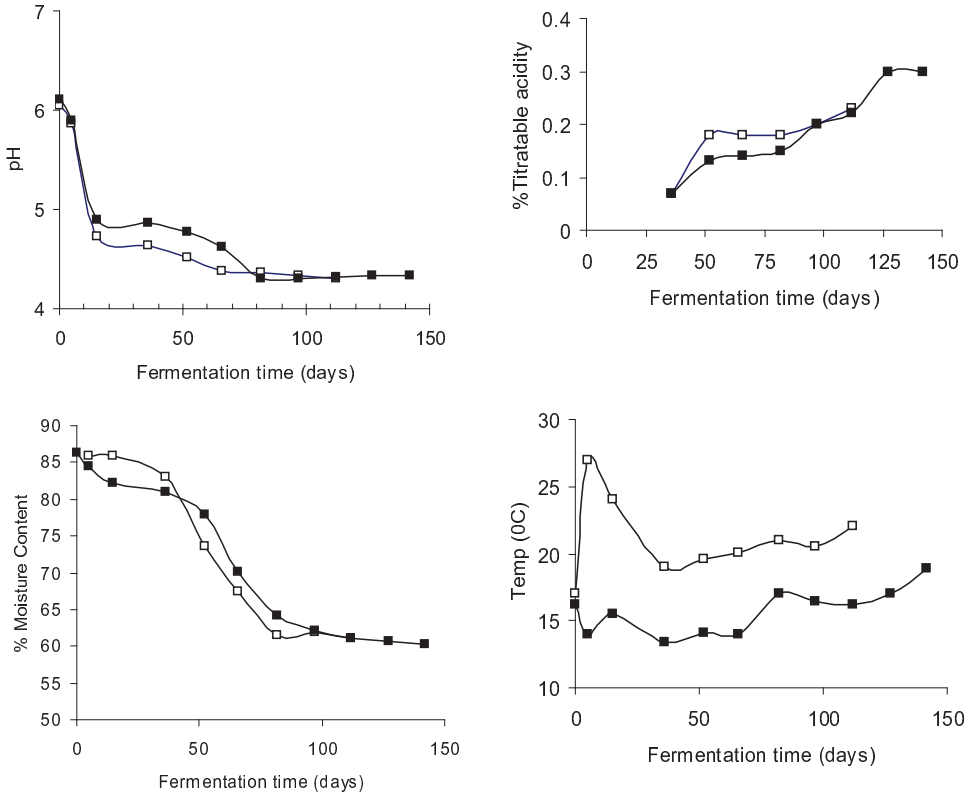

Figure 5 Changes in pH, titratable acidity, temperature and moisture content in fermenting enset at high (closed square) and mid (open square) altitudes.

significantly higher counts ($p<0.05$) than those at the high altitude site until day 36 (Figure 6), possibly favored by the warmer temperature and available fermentable sugars produced by the amylolytic microorganisms. Coliforms were called "pioneer species" of the sauerkraut fermentation as they produce acids and lower pH of the fermenting sauerkraut, setting appropriate condition for the lactic acid bacteria to colonize the system (Scott and Sullivan, 2008). The temperature at the high altitude site was significantly lower than that at the mid-altitude site ($p<0.05$) throughout the fermentation (Figure 5). However, these groups of microorganisms were later not detected following the proliferation of LAB. LAB have been known to produce antimicrobial substances during fermentation of different foods (Steinkraus, 1992; Jay et al, 2005; Tadesse et al., 2005) and play important role in the preservation and production of wholesome foods. The increase in LAB counts and the corresponding fall in pH thereafter, resulted in low levels of these groups of bacteria and they could not be encountered with the limit of detection used in this study. Despite the unhygienic handling of the fermenting mass and the possible introduction of food borne pathogens, the fermentation appeared to reduce possible food borne pathogens. The fermented mass is consumed after steam baking and it will guarantee complete elimination of any contaminating food borne pathogens.

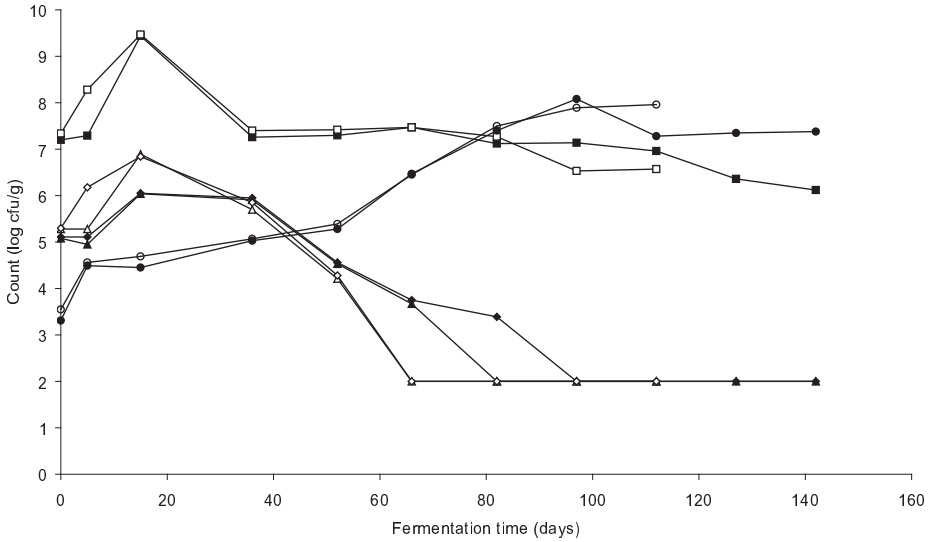

Figure 6 Changes in count of aerobic mesophilic bacteria (square), coliforms (triangle), other enterobacteriaceae (diamond) and LAB (circle) during enset fermentation at high (closed symbols) and mid (open symbols) altitudes.

As the enset plant is rich in carbohydrate but poor in protein and lipid content (Urga et al. 1997; Bezuneh, 1984), most of the bacterial isolates were found to be amylolytic than proteolytic and lipolytic. According to Jay et al (2005), products that contain polysaccharides but no significant levels of simple sugars are normally stable to the activities of yeasts and lactic acid bacteria due to the lack of amylase in most of these organisms. Bacillus species are reported to break down starch and make fermentable sugars available during cassava fermentation (Oyewole, 1992) and the fermentation of a wild legume (Harper and Collins, 1992). The faster growth of LAB and the corresponding increase in titratable acidity (Figure 5) after day 36 could thus be due to the availability of fermentable sugars produced by activities of Bacillus spp.

A major difference in the two sites was seen in composition of the amylolytic flora (Figure 7). Of the isolates picked from countable PC plates at different fermentation times from each fermentation site, 74% and 86% showed amylolytc activity at the mid and high altitude sites, respectively. At the high altitude site, Bacillus isolates were the major amylolytic groups (80%) at the initiation of fermentation. However, the amylolytic activity was overtaken by lactobacilli between days 36 and 97. Amylolytic lactobacilli are known to develop during the fermentation of plant materials (Fitzsimons et al, 1994). At the mid altitude site, amylolytic groups had lower frequencies at the initiation of fermentation and Gram +ve cocci, consisting of micrococci, staphylococci and streptococci were more important initiators of amylolysis and constituted about 50% of the amylolytic flora.

As the count of the different groups of microorganisms decreased towards the completion of fermentation, Bacillus spp., in the form of their spores, appeared at a higher frequency. Otherwise the active

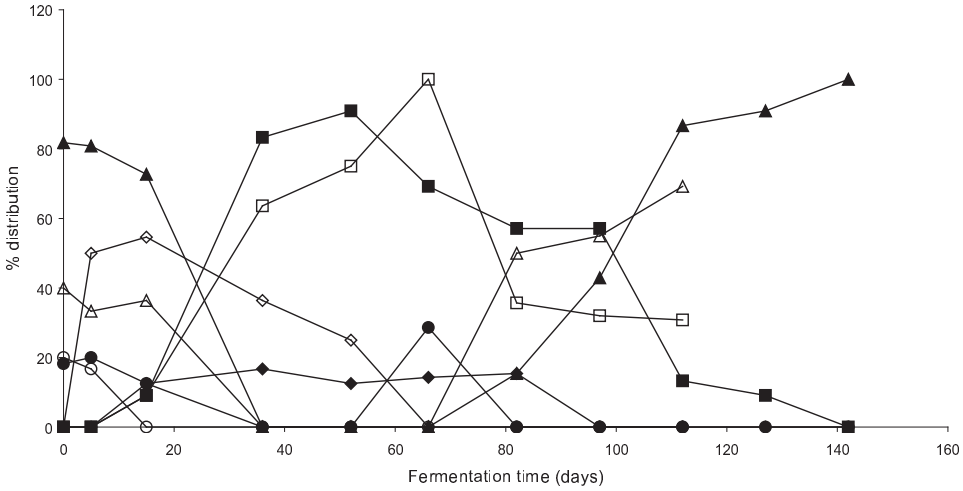

Figure 7 Chages in frequency distribution of amylolytic Bacillus (triangle), lactobacilli (square), Gram positive cocci (circle) and Gram negative rods (diamond) in fermenting enset at high (closed symbols) and mid (open symbols) altitudes.

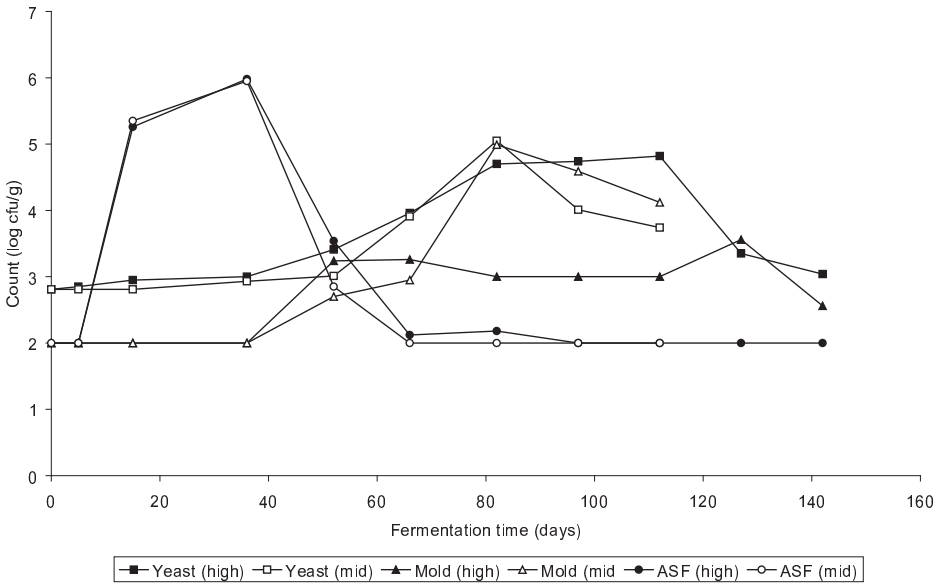

Figure 8 Changes in count of yeasts (square), molds (triangle) and aerobic spore formers (circle) during enset fermentation at high (closed symbols) and mid (open symbols) altitudes.

vegetative forms are usually inhibited at the pH values observed towards the completion of fermentation. Gram +ve cocci and Gram –ve rods, consisting of Acinetobacter, Pseudomonas and members of enterobacteriaceae, might have contributed to amylolytic actions at lower levels (Figure 7). However, amylolysis may not be desirable in the fermented product. Amylolytic Gram-negative rods such as Acinetobacter and non-lactic Gram-positive cocci such as micrococci and staphylococci were isolated from spoiled kotcho and bulla (Ashenafi and Abebe, 1996).

Enset plant processed for fermentation had high initial moisture content of about 86% in both sites. The moisture content of the fermenting mass showed gradual uniform decrease and reached values around 60% at both sites. At both study sites, moisture content of the fermenting mass showed significant difference ($p<0.05$) over the fermentation time. The fermenting mass loses a significant amount of moisture during the course of fermentation and according to Bizuneh (1984), only 25-33% of the fresh weight yield was retrieved as fermented product. The low moisture content did not, however, markedly affect the high counts of LAB and aerobic mesophilic bacteria.

Yeasts and molds had initial counts of <3 log cfu/g at both sites (Figure 8). At the mid altitude site, they reached counts around log 5 cfu/g at around day 82 and the number declined thereafter. At the high altitude site, yeasts were more important than molds. Although the yeast count was not high enough to play any significant metabolic role, they may produce some flavor components upon autolysis. Some report also indicated the fermentative activities of yeast along with lactic acid bacteria during similar fermentation processes (Urga et al., 1997; Amoa-Awua, et al., 1997; Amoa-Awua et al., 1996; Oyewole, 1992). Gashe (1987a) also isolated four yeast genera; Trichosporon, Torulopsis, Rhodotorulla and Candida from fermenting enset mass. The proliferation of yeasts requires an abundant continuous supply of oxygen. The low number of yeasts in fermenting enset could be due to unavailability of sufficient oxygen in the tightly packed and sealed fermenting mass. Surviving mold spores may later result in post-processing food loss and health risk by mycotoxin producing species. Development of dark discoloration on loosely wrapped market kocho and bulla, products of enset fermentation, was caused by molds (Ashenafi and Abebe (1996). According to Gashe (1987b), kotcho became easily contaminated with microorganisms when removed from fermenting pits and the major spoilage fungi belonged to Penicillium, Trichoderma and Chaetomium species.

This study showed notable differences from previous reports based on different fermentation conditions. It indicated that enset fermentation varied from place to place depending on various factors. Thus, the microbial succession and the accompanying chemical changes have to be analyzed at different geographical sites to optimize processing techniques and produce microbial starter cultures for large scale kotcho fermentation.

BIOGRAPHY

Mogessie Ashenafi obtained a BSc and an MSc in Biology/Microbiology at Addis Ababa University, Ethiopia, and a PhD in Food Microbiology from the Technical University of Munich, Germany. He is a Professor of Food Microbiology at the Institute of Pathobiology, Addis Ababa University. He taught graduate courses like

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