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Chemical changes during spontaneous and lactic acid bacteria starter culture fermentation of *bushera*

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Abstract

Bushera is a fermented sorghum beverage which is widely consumed in Uganda. It is not clear whether the process of fermentation affects some nutrient attributes. Thus a study was conducted to monitor changes in dry matter, total soluble solids (TSS), protein content, and composition of sorghum *(Sorghum bicolor)* storage proteins (kafirins) during spontaneous and lactic acid bacteria starter culture fermentation of *bushera* for up to 96 h at 30°C. Results show that dry matter and TSS decreased by about 41% and 58%, respectively, during spontaneous fermentation of *bushera*. Starter cultures had very little effect on the dry matter and TSS of *bushera*. Fermentation had no marked effect on protein content of *bushera*. The SDS-PAGE banding pattern showed the presence of various proteins of different molecular weights, in addition to α_1-, α_2-, β-, and γ-kafirins. The predominant kafirin protein was α-kafirin. SDS-PAGE did not show any protein degradation during fermentation.

Key words: Kaffirins, protein content, SDS-PAGE, soluble solids

Introduction

Sorghum *(Sorghum bicolor)* is a staple food for millions of people who live in the semi-arid tropical regions of Africa, Asia and Latin America (Chandrashekar and Kirleis, 1988; Rom *et al.*, 1992; Watterson *et al.*, 1993; Oria *et al.*, 1995a; Charlotte *et al.*, 1998). For consumers of sorghum-based diets, the grain represents a high percentage of protein and energy intake (Oria *et al.*, 1995a). Sorghum proteins are grouped as albumins (water-soluble protein), globulins (salt-soluble proteins), prolamins (alcohol-soluble protein) and glutelins (alkali-soluble proteins). The prolamin fraction of sorghum, kafirins is further divided into α-, β-, and γ-kafirins based on differences in solubility, molecular weight and structure (Shull *et al.*, 1991: Watterson *et al.*, 1993; El-Nour *et al.*, 1998).

A major problem associated with sorghum as a food is the poor nutritional quality of its proteins (Sastry *et al.*, 1986). The factors contributing to low-quality of protein are low solubility in aqueous media, insolubilisation of proteins by tannins present in the grain pericarp and testa and deficiencies in essential amino acids especially lysine. Additionally, sorghum proteins are unique among the plant food proteins in that they become markedly less digestible after cooking (Oria *et al.*, 1995b; Charlotte *et al.*, 1998). Studies using human subjects have shown that protein from tannin free sorghum porridge and Indian bread is poorly digested in comparison to other cereal proteins (MacLean *et al.*, 1981; Oria *et al.*, 1995b).

Some processing methods such as fermentation and extrusion have been shown to increase digestibility (Chavan and Kadam, 1989). A study on sorghum germination revealed that proteins are degraded during the process (Mazhar and Chandrashekar, 1993). However, such studies have not been extended to the effects of fermentation on the sorghum protein in traditional fermented sorghum

products such as *bushera*. *Bushera* is one of the traditional fermented sorghum beverage widely consumed in Uganda. This study was aimed at investigating the effect of spontaneous and lactic acid bacteria (LAB) starter culture fermentation on sorghum protein content and composition, total soluble solids (TSS), dry matter (DM) and sugars of *bushera*, a traditional sorghum based spontaneously fermented beverage.

Materials and methods

Sorghum flour (*Sorghum bicolor* (L) Moench) made from germinated sorghum grains was purchased from local markets in Kabale district in western region of Uganda. The flour was stored at -40°C, to prevent insect infestation, until airfreighted to the Department of Food Science, Agricultural University of Norway, and then stored at 3-4°C until *bushera* was produced.

Preparation of starter cultures

Lactic acid bacteria starter cultures were isolated from traditionally fermented *bushera*, characterised and identified using biochemical test and API 50 CH strips and API CHL medium according to manufacturer's instructions (API system, Bio-Merieux, France). Detailed procedure for isolation and characterisation of LAB are described by Muyanja *et al.* (2002). Five pure LAB starter strains were selected and used for fermentation of *bushera* under controlled conditions. The strains used were *Lactobacillus* (Lb) *fermentum* MINF99, *Weissella* (W) *confusa* MINF8, *Lb plantarum* MINF277, *Lb brevis* MINF226 and *Lb paracasei* subsp *paracasei* MINF98. Each strain was grown in 250 ml of MRS broth, incubated for 18 hours at 30°C and centrifuged at 6000 rpm (5440 x g) for 10 minutes at 4°C (Sorvall 5RB, du pont Instruments, Delaware, USA). The cell pellets were resuspended in 25 ml of Ringers solution containing 10% glycerol and stored at -80°C until required for use.

Preparation of bushera

The *bushera* samples were prepared in 320 ml screw capped glass jars by mixing the prepared sorghum flour (30 g) with 250 ml of distilled water, and then steamed at 98°C for 30 minutes. The steamed samples were cooled to 30°C before inoculation. The *bushera* samples to be fermented spontaneously and by LAB starter cultures were treated in a similar manner except that sorghum malt (75 g) was used to initiate spontaneous fermentation. Samples for LAB starter culture fermentation were inoculated at about 7 log cfu ml^{-1}. The mixtures were incubated at 30°C and samples taken after 0, 4, 8, 12, 24, 48, 72 and 96 h. Each sampling interval was allocated a separate fermentation jar. All samples were analysed for total soluble solids and dry matter. Samples for crude protein determination and gel electrophoresis were freeze-dried (Heto Drywinner, 85, Model DW 6-85, Copenhagen, Denmark). The experiment was repeated using two independent times.

Dry matter determination

Dry matter was determined according to AOAC method (AOAC, 1995). Samples (5 g) of spontaneously and starter culture fermented *bushera* were weighed (Mettler AE, Delta Range, Switerzland) in pre-weighed aluminium dishes and dried overnight in a hot air oven at 100°C. Thereafter, samples were cooled in a desiccator for 1 h. The loss in weight was used to calculate the dry matter content. Dry matter was determined at zero time and after fermentation for 96 h. Determinations were carried out in duplicate.

Determination of total soluble solids (TSS)

Total soluble solids (°Brix) of fermenting or fermented *bushera* were determined at 20°C using an Abbe refractometer (Model IT, Atago, Japan) according to the method of Joslyn (1970).

Determination of sugars

Maltose, glucose and fructose were determined during spontaneous fermentation by high performance liquid chromatography according to Narvhus *et al.* (1998). The sugar detection was done by a Refractive Index detector (Series 2000, Perkin Elmer, Norwalk, USA). Standard sugar solutions (Sigma, St Louis, MO, USA) were used for calibration.

Determination of crude protein

Crude protein of freeze-dried fermented *bushera* was determined by the micro-Kjeldahl method (AOAC, 1995). The sorghum protein conversion factor of 5.65 was used as reported by Mossé (1990). Samples from each fermentation interval were analysed in duplicate.

Protein extraction

Proteins were extracted from freeze dried fermented *bushera* samples according to the method described by Wallace *et al.* (1990) as modified by Oria *et al.* (1995a). Freeze dried fermented *bushera* (200 mg) was weighed into a 15 ml plastic screw cap test tube and extracted with 6 ml 0.0125 M sodium borate (Merck, Dermstadt, Germany) buffer, pH 10, containing 1% (w/v) Sodium dodecyl sulphate (SDS) (Koch-Light Laboratories, Colnbrok-Bucks, England) and 2% (v/v) 2-mercaptoethanol (2-ME) for 16 h on an orbital shaker at 25°C and 280 revolutions/minute (Gallenkamp, UK). The suspension was centrifuged at 9000 rpm (8160 x g) for 10 minutes at 4°C (Beckman J2-MC, Beckman Instruments, California, USA). The supernatants were frozen at -80°C overnight and then freeze dried (Heto Drywinner, 85, Model DW 6-85, Copenhagen, Denmark).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a horizontal Pharmacia Phast (Pharmacia, Sweden) electrophoresis system. The running gels used were Phast Gel Homogenous (Pharmacia) with 20% polyacrylamide. Freeze-dried protein extracts (0.05 g) were diluted in 5 ml sample buffer 10% (w/v) Tris-HCl, pH 8.8, containing 1% (w/v) SDS, 2% (v/v) 2-ME and 0.05% (w/v) Bromophenol blue. The samples were boiled for 3 min and immediately cooled with ice. One microlitre of protein solution was loaded into each well. Proteins were separated and stained according to the protocol as described in Phastsystem Owners Instruction manual (1987). The proteins were fixed using a solution of 25% (v/v) glutaraldehyde, 15% (v/v) iso-propanol, 30% (v/v) ethanol and 0.03% (w/v) sodium acetate at 30°C. The gels were then washed in 10% ethanol (v/v) and 5% (v/v) acetic acid and then stained using 0.4% (w/v) silver nitrate. Gels were then developed using a solution containing 25% (w/v) sodium carbonate, 16% (w/v) sodium thiosulphate and 37% (w/v) Tris-HCl, pH 8.8). Gels were preserved in a solution with 10% (v/v) acetic acid and 10% (v/v) glycerol.

Gels were scanned to determine molecular weights using a computerised densitometer (Colour Image Scanner Model JX-330, Sharp Twain/Win Version 22x soft ware, Sharp, Corporation, Japan) and Labscan, version, 201 (Pharmacia, Sweden) The scanned gel images were analysed for band quantification using Image Master 1D Elite, version 201 (Pharmacia) soft-ware.

Molecular weights were determined from a standard curve obtained by plotting log molecular weight against relative mobility. A low molecular weight protein reference standard (LMW, 14-97 kDa)

containing phosphorylase b (97 kDa), bovine serum albumin (66 kDA), ovalbumin (45 kDA) carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) from Pharmacia Biotech (Sweden) was used.

Results

Dry matter

The changes in dry matter during fermentation are shown in Table 1. The dry matter of *bushera* fermented by the starter cultures slightly decreased from an average of 10.3% at zero time to between 9.9 and 10.1% after 96 h of fermentation depending on the starter. In contrast, the dry matter of spontaneously fermented *bushera* significantly decreased from 13.5 to about 7.94% during the fermentation period. Initially, spontaneously fermented *bushera* had higher dry matter than starter fermented *bushera* due to the sorghum flour added to initiate the spontaneous fermentation.

Total soluble solids (TSS)

The TSS of *bushera* with or without added LAB starter cultures remained unchanged about 7.2 °Brix during the first 24 h of fermentation (Table 2). A slight decrease in TSS (6.4 - 6.8 °Brix) was observed in *bushera* inoculated with starters after 24 h of fermentation. Spontaneously fermented *bushera* showed a significant decrease in TSS after 24 h. The TSS of spontaneously fermented *bushera* was reduced from 7.2 to 3.0 °Brix after 96 h of fermentation.

Table 1. Changes in dry matter (%) in bushera during fermentation.

Time (h)	Starter cultures					
	Spontaneous fermentation	*Lb. *paracasei* MINF98	Lb. *plantarum* MINF227	Lb. *brevis* MINF8	*W. confusa* MINF8	Lb. *fermentum* MINF99
0	13.5±0.2	10.3±0.07	10.1±0.0	10.3±0.01	10.4±0.00	10.1±0.03
96	7.94±0.01	9.94±0.02	10±0.1	10.1±0.02	9.98±0.01	9.97±0.00

Values are means of two experiments. *: *Lactobacillus* (Lb.) *paracasei* subsp. *paracasei*.

Table 2. Changes in total solube solids (°Brix) in bushera during fermentation.

Time (h)	Starter cultures					
	Spontaneous fermentation	*Lb. *paracasei* MINF98	Lb. *plantarum* MINF227	Lb. *brevis* MINF226	*W. confusa* MINF8	Lb. *fermentum* MINF99
0	7.2±0.0	7.2±0.0	7.2±0.1	7.1±.0.1	7.2±0.0	7.2±0.0
4	7.1±0.1	7.0±0.0	7.0±0.0	7.0±.0.0	7.0±0.0	7.0±0.0
8	7.0±0.0	7.0±0.1	7.0±0.0	7.0±.0.0	7.0±0.0	7.0±0.0
12	7.0±0.0	7.0±0.1	7.0±0.0	7.1±.0.1	7.0±0.0	7.0±0.0
24	7.0±0.0	7.0±0.1	7.0±0.0	7.0±.0.0	7.1±0.1	7.0±0.0
48	5.0±0.0	6.5±0.0	6.9±0.1	6.5±.0.0	6.7±0.1	6.5±0.1
72	3.5±0.0	6.5±0.1	6.8±0.0	6.8±.0.4	6.7±0.1	6.4±0.0
96	3.0±0.0	6.5±0.1	6.8±0.0	6.7±.0.2	6.9±0.1	6.4±0.0

Results given as averages of duplicate determination ±S.D.

Protein content

The protein content expressed as percent of dry matter (DM), of spontaneously fermented *bushera* and of *bushera* with added starters is shown in Table 3. The protein %DM of *bushera* with LAB starters was between 9.4 and 9.6 % at zero time and varied between 9.0 and 10.2 % after 96 h of fermentation. The protein %DM of spontaneously fermented *bushera* showed about the same development as observed for *bushera* with added starters up to 48 h. After 48 h, however, an increase up to 16.5% protein in DM was observed (Table 3).

Changes in sugar content

The changes in sugar content during spontaneous fermentation are shown in Figure 1. Maltose content increased during the first 48 h from 12181 to 50233 mg kg^{-1}, and then decreased rapidly during the following 48 h to 2826 mg kg^{-1}. The glucose levels of spontaneously fermented *bushera* increased markedly from 12 to 48 h (from 6136 to 29349 mg kg^{-1}), but then decreased to undetectable level after 96 h. Fructose levels decreased from 1700 to 500 mg kg^{-1} during the fermentation period.

Table 3. Changes protein content (%) in bushera during fermentation.

Time (h)	Starter cultures					
	Spontaneous fermentation	*Lb. *paracasei* MINF98	Lb. *plantarum* MINF227	Lb. *brevis* MINF226	*W. confusa* MINF8	Lb. *fermentum* MINF99
0	10.0±0.4	9.4±0.1	9.5±0.2	9.4±.0.1	9.60±0.1	9.6±0.1
4	10.5±0.1	9.5±0.1	9.4±0.2	9.6±.0.5	10.8±0.1	10±0.1
8	10.8±0.1	9.5±0.2	9.5±0.1	9.5±.0.0	10.3±0.4	9.9±0.1
12	9.6±0.2	9.6±0.3	9.7±0.1	9.8±.0.0	10.4±0.6	9.6±0.3
24	9.9±0.1	9.8±0.1	9.5±0.2	9.5±.0.1	10.1±0.2	9.8±0.1
48	9.4±0.1	9.5±0.1	9.9±0.2	9.7±.0.1	10.1±0.0	9.5±0.1
72	12.6±0.0	9.2±0.1	10±0.2	9.3±.0.1	9.8±0.0	9.2±0.1
96	16.5±0.1	9.3±0.1	10±0.2	9.2±.0.1	10.2±0.0	9.0±0.1

Results given as averages of duplicate determination ±S.D.

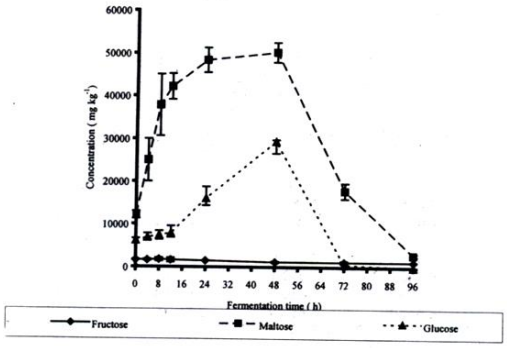

Figure 1. Changes in sugar content in bushera made from germinated sorghum flour during spontaneous fermentation. Results given as averages and standard deviation indicated by bars.

Relationship between protein content and dry matter loss

Figure 2 shows the relation between protein content and dry matter loss before and after spontaneous fermentation and fermentation with starter cultures. The loss of dry matter in form of maltose reduction was found to be high in spontaneously fermented *bushera* and negligible in *bushera* fermented with starters. For *bushera* fermented using starters, the protein content (0.97g 100 g^{-1} *bushera*) expressed on wet weight basis remained unchanged after 96 h fermentation. Protein content in spontaneously fermented *bushera* before fermentation, expressed on the wet weight basis was 13.5 g 100 g^{-1} of *bushera*. The protein content remained constant throughout the fermentation period when expressed on both wet and dry weight basis. The results indicated that the loss of dry matter does not have any effect on the actual protein content in the fermenting mixtures.

SDS-PAGE

The SDS-PAGE electrophoretograms of the *bushera* samples showed no change in band patterns during the fermentation period (Fig. 3a and b). Seven bands were detected with molecular weight 93, 63, 42, 28, 25, 23, 19, and 15 kDa. Bands of lower molecular weight between 13 and 11 kDa were also detected. Kafirins have been classified into α (M_r 25 and 23 kDa), β (20, 18 and 16 kDa), and γ-kafirins (28 kDa) on the basis of solubility, molecular weights and structure (Shull *et al.*, 1991).

Discussion

The protein content of *bushera* products did not change during fermentation when expressed on a wet weight basis (Fig. 2). However, if expressed as a percent of dry matter an increase was observed for the protein concentration (Table 3). The protein content on dry weight basis, in spontaneously fermented *bushera* after 96 h, was higher than at start of fermentation due to starch degradation to maltose and glucose followed by utilisation. This increase is apparent but the absolute amount of protein in *bushera* was unchanged and identical to 13.5g protein (Fig. 2). The apparent increase in protein became obvious when the population of yeast was at its highest (7 log cfu ml^{-1}) during spontaneous fermentation (Muyanja, 2001). This indicates that the major factor influencing the dry matter loss was the presence of yeasts since these were not present in *bushera* produced with starters.

Using the starters, the decrease in dry matter was minimal and the actual protein content remained the same. Fields *et al.* (1981) observed that dry matter losses increased from about 6% to 16.5% for maize solids: water ratio of 1:1 and 1:8, respectively after 4-day fermentation of maize meal. Wang

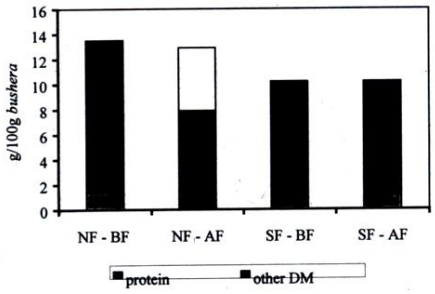

Figure 2. Changes in dry matter components during bushera fermentation. NF: Natural/spontaneous fermentation, SF: starter fermentation, BF: before fermentation and AF: after fermentation.

and Fields (1978) reported about 50% and 14% dry matter loss within 3 days during maize (1:10, solids: water) fermentation when *Saccharomyces cerevisiae* and *Candida tropicalis*, respectively were used as starters. These findings indicated that loss in dry matter is influenced by the solids:water ratio, the nature of microorganism involved and the duration of fermentation.

Spontaneously fermented *bushera* contained active amylases from the germinated flour, and this was responsible for the increase in fermentable sugars observed during the first half of fermentation. These sugars were then fermented by yeasts in the spontaneously fermented *bushera*, probably an alcoholic fermentation, producing carbon dioxide, water and ethanol. Some volatile components would be lost from the fermenting *bushera* and most probably during the drying of the samples for dry matter analysis. In *bushera* produced by starter cultures, not only was the availability of fermentable sugars much lower (Muyanja, 2001) but also the acidic fermentation became self-limiting due to the low pH. This probably accounts for the greater loss of dry matter in spontaneously fermented *bushera* as opposed to *bushera* fermented by starter cultures. The loss of dry matter can be deducted from Figure 1, where there was an increase in the fermentable sugars, which later decreased. It can be seen that the decrease in maltose and glucose (Fig. 1) corresponds approximately to the loss of DM in spontaneously fermented *bushera*.

Numerous authors have reported increase in protein content during fermentation Yousif and El-Tinay (2000) reported increase in protein during the first period of maize dough fermentation. Shayo *et al.* (2000) indicated that the protein content of *orubisil/amarwa* increased from 2.0 to 2.7% during 120 h fermentation. Azoulay (1978) reported 15 - 30% increase in protein as a result of maize fermentation with *Candida tropicalis*. Ikemefuna and Atti (1994) reported protein increase in pearl millet *(Eleuseine coracana)* with the fermentation period. El-Tinay *et al.* (1979) indicated that there was a slight increase in protein content as a result of *kisra* fermentation. Other authors have suggested that the increase in protein content is due to synthesis of proteins by microorganisms (Abdelmoneim

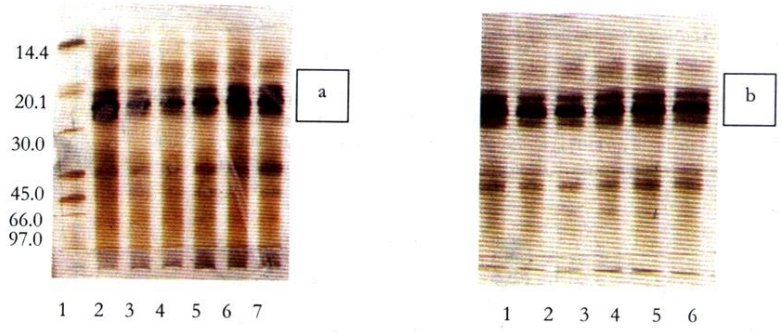

Figure 3(a). SDS-PAGE separation of sorghum kafirins. Lane 1, standard proteins (Mwt 14.4-97.0 kDA), lane 2 and 3, spontaneous fermentaiton after 4 and 8 h respectively, Lane 4 and 5 W.confusa MINF8 (S+) after 4 and 8 h respectively,lane 6 and 7, L. plantarum MINF227 (S-) after 4 and 8 h respectively.

Figure 3(b). Lane 1, spontaneous fermentation after 48 h. Lane 2, W. confusa MINF8 (S+) after 48 h, lane 3, L. brevis MINF226 after 48 h, lane 4 L. plantarum MINF227 (S-) after 48 h, lane 5 L. paracasei subsp. paracasei MINF98 after 48 h, lane 6, L. fementum MINF99 after 48 h.

N.B. All other electrophonetogram at different intervals of fementation had simialr banding patterns. The above were chosen for their density; S+ = starch degrading strain, S- = non-starch degrading strain.

and El-Tinay, 1994). Rose (1961) as quoted by Abdelmoneim and El-Tinay (1994) in their study of microbial foods reported that microbial cell matter contains appreciable amounts of protein thus accounting for the protein content increase observed. During fermentation, some proteins will be hydrolysed as reported by Ikemefuna and Atti (1994). This may improve the digestibility of the proteins, but will not change the total amino acid content or the amount of nitrogen (as measured by Kjeldahl analysis). This means that synthesis of new proteins from amino acids resulting from proteolytic activity does not increase the total protein content unless an external nitrogen source is added to fermentation mixture.

Other researchers have indicated that fermentation has a slight or no effect on protein content of cereal-based fermented foods. Usha *et al.* (1996) reported that protein content of the millet was unaltered during fermentation. Banigo and Muller (1972) indicated that there was no protein increase during *ogi* fermentation. Hounhouigan *et al.* (1993) also showed that fermentation had only a slight effect on the crude protein content of *mawe* (maize sour dough). Kazanas and Fields (1981) found no significant difference in crude protein of unfermented and fermented sorghum meals.

Tiisekwa *et al.* (2000) reported that TSS is an important parameter, which can be used to monitor the rates of fermentation and alcohol production. This is in agreement with the results for spontaneously fermented *bushera*. However, our study suggested that TSS cannot be relied on as a parameter for monitoring controlled fermentation rates when single starters of lactobacilli are used since only small amounts of fermentable sugars are produced and reduced in relation to the amounts of lactic acid. Yousif and El Tinay (2000) reported an increase in TSS during fermentation of maize dough. Padhye and Salunkhe (1979) also reported an increase of TSS in *idli* prepared from rice and black gram. The results obtained in our study are contradictory to these findings. The contradiction may be attributed to the nature of the product and the raw material used.

The SDS-PAGE showed no differences in the banding patterns for proteins between or within the spontaneous or starter culture fermented *bushera* during the fermentation. The results suggest that there was little degradation if any, of proteins affected by the strains and during spontaneous fermentation. Similar results were reported by Mugula (2001) during *togwa* fermentation. Akinrele (1970) also reported that the predominant microorganisms isolated during the fermentation period of *ogi* showed very little degradation of maize protein. During germination, β-kafirin and γ-kafirin have been shown to be extensively degrade due to their peripheral location (Mazhar and Chandrashekar, 1993). Protein bodies are progressively hydrolysed from their outside surface (Mazhar and Chandrashekar, 1993). It seems that most of the changes in protein bands occurs during the germination of the grains. It may also be suggested that microorganisms use for their growth the easily available nutrients rather than the complex compounds, which have to be hydrolysed to simpler forms.

Conclusion

The study has shown that fermentation of *bushera* using starter cultures has little or no effect on the dry matter, total soluble solids and protein content. The reported increased protein content as observed during spontaneous fermentation of *bushera* is only apparent not absolute and is due to loss of carbohydrate dry matter as a result of their utilisation. It seems yeasts play a major role in this loss of dry matter. This study has shown that little or no protein degradation occurs during spontaneous fermentation of *bushera* and fermentation with starter cultures. The study has shown that the use of starter cultures retains the dry matter content. This is of great importance as far as energy density of the fermented foods are concerned.

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