Research Article

Modulation of steeping conditions influence the diastatic enzymes and protein profile in pearl millet malt.

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ABSTRACT: Malting is targeted at getting the optimum point of enzymatic induction without losing much energy during the embryo metabolism and growth. Successful production of malt includes production of various hydrolytic enzymes and controlled degradation of the grain endosperm structure. Attention is at the centre stage of using Pearl millet as a substitute for barley, wheat and sorghum due to the cost of importation of barley and wheat to tropical countries. This study seeks to understand the effect of different steeping conditions with respect to varying pH, temperature and time on key enzymes associated with malting processes. Activities of α-amylase, β-amylase, β-glucanase, β-glucan content, protein profiles were monitored with respect to the varying steeping conditions. There was a steady increase (from 0 to 96 h) in the α-amylase activity at 30 °C under all the pH stress conditions with the exception of acidic pH malted pearl millet where the enzyme activity decreased from 191.04 ± 1.5 U/g to 142.50 ± 2.20 U/g between the 72nd and 96th hour. Optimal activity (248.04 ± 0.20 U/g) was observed at 96 h for alkaline pH steeped pearl grains germinated at 30 °C. However activity decreases as germination days prolong. Optimal activity was recorded at the 96th hour for malted pearl millet grains subjected to alkaline pH stress (2.73 ± 0.20 U/g) as compared with the control. β-glucanase activities of the malted pearl millet grains were high especially under the 30 °C heat stress. Peak activity was observed at the 96th hour for the pearl millet grains subjected to alkaline pH stress (892.34 ± 0.20 U/kg). β-glucan content under the alkaline pH stress, acidic pH stress and control conditions at 30 °C were within the same range of approximately 4-8 % w/w malt flour.

KEYWORDS: Steeping, Pearl millet malt, Enzymes, Protein profile.
INTRODUCTION
The name millet is used to describe a number of small seeded cereals, most of which are native to the tropics or sub-tropics. The major species in terms of area and total yield is pearl millet (Pennisetum glaucum). Across south Asia and Africa several indigenous foods and drinks are made from flour/meal and malt of these millets. Millets are valuable foods for monogastric animals including man due to high levels of methionine and cystine which are vital amino acids to human health and are reportedly deficient in maize, wheat, rice and sorghum. About 80 % of the world's millet is used as food, with the remaining being used for feed, beers and bird seed. (Okoh et al., 1985).

In various cereals, research has shown that nutrients such as iron, calcium, zinc and B vitamins are not available due to the presence of anti-nutritional factors or low digestibility. However, simple and low-cost processing techniques, such as malting or germination, have made life easier by meeting the nutrient needs of cereal-consuming populations. Malting is the controlled germination of cereal grain in moist air, the process involves three steps: steeping (soaking of the seeds in water), germination and lastly, kilning otherwise known as drying (Laithia et al., 2006). During malting, the nascent seed embryo triggers the induction of enzymes capable of degrading the grain macromolecules into soluble compounds. Germination increases endogenous phytase activity which leads to phytate degradation. Germination also decreases the levels of phytic acid and condensed tannins present in cereals (Iyang and Zachari, 2008). In fact, germination has been claimed to improve the nutritive quality of cereals (Ochanda, 2009; Tatala et al, 2007).

The joint amylolytic activity of primarily β-amylase and α-amylase, termed diastatic power, is a major malting quality parameter of malted products (Clancy et al., 2003; Badau et al., 2006). In the same vein, the extent of endosperm cell wall degradation by the β-glucanase enzymatic activity and the β-glucan content left after processing are vital determinant factors of malting quality especially in the friability of the malt product (Wang et al., 2004).

The protein content of malted products is also pivotal to the determination to their quality (Jamar et al., 2011). The full knowledge on these parameters and conditions which bring about their improvement is very important to industrialists in food and brewing industries. The quality of the malt is of primary significance in the manufacture of brewed products of excellent quality. To increase the brewing yield and efficiency, malts with high extract values, high enzymatic activities, and good modification are essential. To produce malt that meets these requirements, the grains should be able to germinate rapidly and uniformly (Riss and Bang-Olsen, 1991; Woorton et al., 2005a), producing a correspondingly high diastatic power, high β-glucanase activity and low protein content. Attention is at the centre stage of using Pearl millet as a substitute for barley, wheat and sorghum (Gimbi and Kitabake, 2002) due to the cost of importation of barley and wheat to tropical countries. With respect to these parameters, this study seeks to bring the pearl millet to limelight as a form of positive contribution to the world’s fast growing biotechnology. The main objective of this study is, therefore, to investigate the effect of different malting conditions of Pearl millet on key enzymes linked with production of quality malt for world’s fastest growing biotechnology.

MATERIALS AND METHODS
Materials: Pearl millet grains (Pennisetum typhoideum) (local variety) was acquired from the Farin Gada market, Jos, Nigeria in September 2015. The seeds were identified and authenticated in the Department of Crop, Soil and Pest Management, The Federal University of Technology Akure, Nigeria and stored at ≤4 °C until used. Enzyme activity kits of α-amylase (K-CERA), β-amylase (K-BETA), endo- (1,3) (1,4)-β-D-glucanase (K-MBGL) and β-glucan (K-BGLU) were obtained from Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland. All other materials used were of analytical grade from authorized commercial suppliers.

Malting: Micro-malting was done as reported by Macnicol et al., (1993). The malting process consists of three stages-the steeping stage, the germination stage and the drying stage. Before initiating the steeping stage, grains were manually sorted out to remove broken grains and other extraneous materials. The grains were then weighed (100 g per group), washed (in distilled water) and sterilized using 1% sodium hypochlorite solution for 20 minutes at room temperature to prevent microbial growth (Adewale and Oladejo, 2008).

Design of simulated acidic, alkaline and salt conditions: The sterilized grain samples, in their respective groups were steeped overnight at 25 °C under acidic pH stress condition (25 mM Glycine-HCl Buffer, pH 3.0), alkaline pH stress condition (25 mM Tris-HCl, pH 9.0) and salt stress condition (0-200 mM NaCl, a neutral salt, in deionized water). The grains steeped in distilled water serve as a control for the experiment. The grains were allowed to germinate at these stress conditions and water was constantly sprinkled to maintain the moisture content during germination stage for 96 hours at room temperature (set at 25 °C). Sampling was done at every 24 h and immediately kept at -20°C until used.
Heat stress treatment

Another sterilized grain samples, steeped overnight with autoclaved distilled water were micro-malted incubator set at respective temperatures of 30 °C, 35 °C and 40 °C for 96 h. The grains were sprinkled with distilled water regularly to maintain their moisture content and also to allow germination to occur. Sampling was done at every 24 h and immediately kept at -20 °C until used. Before use, samples were properly dried to a constant weight at room temperature, the germinated seeds with their vegetative parts were pulverized using a thermoline® blender.

Measurement of diastatic enzymes indices

α-Amylase activity was measured in the malted millet using the Ceralpha alpha-amylase assay kit (Megazyme International Ireland Inc., Bray, Ireland). α-Amylase activity was measured spectrophotometrically using Amylase HR reagent, B-PNPG7 (Ceralpha methods; Megazyme International, Wicklow, Ireland Ltd) as a substrate. 10 mL of extraction buffer, pH 5.4 (50 mM sodium malate, 50 mM NaCl, 2 mM CaCl₂) was added to 0.05 g Pearl millet flour to extract α-amylase. The extraction was carried out at room temperature (25 ± 2 °C) for 20 min while being stirred continuously and centrifuged at 10,000 g for 20 min at 4 °C. Diluted crude extract (10 μL) was added in an equal volume of B-PNPG7 at room temperature, and the absorbance of the reaction mixture was immediately measured using a spectrophotometer. The activity of the enzyme was recorded as absorbance at 410 nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μM of p-nitrophenol from BNPG7 (in the absence of excess thermostable α-glucosidase and glucoamylase) in 1 min at 40°C per gram of malted flour. The results are expressed as U/g.

β-Amylase activity was done using the Betamy assay kit (Megazyme International Ireland Ltd., Bray, Ireland). Crude extracts for the β-amylase assay were prepared in an extraction buffer, pH 8.0 (50 mM Trizma base and 1 mM disodium EDTA) at room temperature. The malt millet flour samples were thoroughly stirred with the extraction buffer and centrifuged for 10 mins at 10,000 g at 4 °C. The supernatant was used for an enzyme activity assay. The β-amylase activity was determined based on the release of p-nitrophenol from the specific substrate p-nitrophenyl maltopentaoside (Betamy reagent, PNPG5 substrate from Megazyme International, Wicklow, Ireland). This enzyme extract was incubated with pre-equilibrated PNPG-5 substrate mixture (0.2 ml), for 10 mins in a 40 °C water bath. The reaction was terminated by the addition of 1% Trizma base (1 ml, pH 10). Color development was monitored by taking each sample’s absorbance at 400 nm. One unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of p-nitrophenol from non-reducing-end of the blocked β-nitrophenyl maltopentaoside under the defined assay conditions.

Malt β-glucanase enzyme activity in malted sample was measured according to the enzymatic method of a commercial assay kit (Megazyme International Ireland, Bray, Ireland). Crude extracts were prepared using a buffer solution containing 40 mM acetate/phosphate and 70 % industrial methylated spirit, pH 6 serving as an extraction medium. The endo-(1,3) (1,4)-β-D-glucanase assay is based on the conversion of the dye-labelled azo-barley β-glucan polymers into dye-labelled azo-barley fragments. Extracts were incubated with the substrate for 30 mins at 30 °C, a precipitant solution was introduced and the tubes were centrifuged for 10 mins at 1000 g. The characteristic colour development of the supernatant solutions of each sample was measured at 590 nm. One unit of β-glucanase activity was defined as the amount of enzyme in U/g of malt flour that converts the dye-labelled azo-barley β-glucan polymers into dye-labelled azo-barley fragments.

Total β-glucan content in malted finger millet was measured according to the enzymatic method of McCleary and Shamer [22] using a commercial assay kit (Megazyme International Ireland, Bray, Ireland). Soluble β-glucans were measured using a new method of extraction described in the manufacturer’s manual. Polysaccharides of D-glucose monomers linked by β-glycosidic bonds were estimated, specifically, the mixed linkage (1,3)(1,4)- β-D-glucan using the Megazyme enzyme kit in which purified lichenase and β-glucanase enzymes have been included. These enzymes hydrolyse the mixed linkage (1,3)(1,4)-β-D-glucan- the purified lichenase enzyme was incubated with the sample for partial hydrolysis while the β-glucanase enzyme hydrolyzes to completion to produce a D-glucose solution which is assayed for using a glucose oxidase/peroxidase reagent (McCleary and Nurthen, 1986). The procedure requires repeated ethanol-washing steps before the introduction of the enzymes into the reaction system. At the end of the time measured for the enzymes to react, a stopping reagent was incubated with the samples for 20 minutes and the samples' absorbance were measured at 510 nm and β-glucan content was expressed in % w/w. The calculation of the β-glucan content of the malt was carried out using the Megazyme Mega-Calc™ (www.megazyme.com). The kit procedure, based on 1 g sample weight was modified for use with a 100 mg sample weight.
All spectrophotometric assays were monitored using Shimadzu UV 1800 double beam UV–visible spectrophotometer. All measurements were performed thricely unless otherwise stated.

**Protein content assay and SDS-PAGE**

Total Protein concentration was determined according to the dye binding method of Bradford [23] with bovine serum albumin as standard. This was done by preparing extracts from 0.1 mg of each day’s germinated millet powdered sample using 0.1 mL of 20 mM Sodium phosphate buffer (pH 6.5) under mechanical stirring for 1 h. The mixtures were centrifuged at 10, 000 × g for 10 mins at room temperature. Supernatants containing total soluble proteins were obtained and 20 µL of the supernatant was mixed with 780 µL distilled water. The same were assayed for their respective protein content by adding 200 µL of the Bradford reagent and the tubes were allowed to stand for 5-10 mins. Simultaneously, to 200 µL of standard solution (Bovine serum albumin 0.1 mg/mL), 200 µL of Bradford reagent was added and kept for 5-10 minutes (Bradford, 1976). The absorbance of the samples was measured at 595 nm using a Biochrome 4060 UV-visible Spectrophotometer. The Protein content was subsequently calculated using a standard graph made with known amount of protein and the protein amount expressed in mg/mL extract.

**SDS-PAGE**

Aliquots of the millet protein extract were taken from the 96 h germinated samples for SDS-PAGE. This was produced by mixing 500 mg of each germinated millet sample with 1.0 mL of 20 mM Sodium phosphate buffer (pH 6.5) under mechanical stirring for 1 h. The mixtures were centrifuged at 10, 000 x g for 10 mins at 4°C. 200 µL of the supernatants containing protein extract were precipitated with 100 % trichloroacetic acid (TCA) in cold acetone 1 g/ 1 ml (w/v) and stored overnight at 0°C. Thereafter, they were centrifuged in a refrigerated centrifuge at 10, 000 x g to collect the pellets formed. The pellets were washed repeatedly with cold acetone to remove all TCA and then air-dried.

The obtained residue was dissolved in electrophoretic sample buffer containing 20 gL⁻¹ SDS, 0.0625 mol L⁻¹ Tris, 100mL L⁻¹ glycerol and 0.1 gL⁻¹ bromophenol blue (pH 6.8). Vertical acrylamide Slab gels of 1 mm thickness prepared with 10 ml resolving gel and 3 ml stacking gel and used with disassociating discontinuous buffer system as described by Laemmli, 1970. 10 µL of the extracted protein was loaded in each well. A low range protein molecular weight marker containing lysozyme (20.0 kDa), soya bean trypsin inhibitor (29.2 kDa), carbonic anhydrase (37.5 kDa), ovalbumin (54 kDa), bovine serum albumin (97.3 kDa) and phosphorylase b (100 kDa), obtained from Amersham Bioscience (GE healthcare). This marker was also run along with protein samples as standard. Electrophoretic separation was allowed to keep running at 100V until the tracking dye, bromophenol blue, reached the bottom of the resolving gel. Gels were stained with Coomassie Blue; the stained gels were unstained in another solution containing 50 % (v/v) methanol, 40 % (v/v) distilled water and 10 % (v/v) acetic acid to make the background clear.

**Statistical analysis**

Data from triplicate results were presented as mean values ± standard deviation (SD). The results were statistically evaluated by variance analysis (ANOVA) using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) statistical package. LSD test as post hoc tests with a p = 0.05 significance, to compare the treatment effect.

**RESULTS**

**α-amylase**

The result of α-amylase activity of malted pearl millet during varying stress conditions is shown in Fig 1. There was a steady increase (from 0 to 96 h) in the α-amylase activity at 30°C under all the pH stress conditions considered except for acidic pH malted pearl millet where the enzyme activity decreased from 191.04 ± 1.5 U/g to 142.50 ± 2.20 U/g between the 72nd and 96th hour. Highest activity (248.04 ± 0.20 U/g) was observed at 96 h for alkaline pH steeped pearl grains germinated at 30 °C. However activity decreases as germination days prolong.

**β-amylase analysis**

Figures 2 shows the effect of heat stress and pH stress on β-amylase activity in Pearl millet. The malted grains subjected to alkaline and acid stress showed an increase in β-amylase activity from 0 to 96 h, with the optimal activity recorded at the 96th hour for malted Pearl millet grains subjected to alkaline stress (2.73 ± 0.20 U/g) as compared with the control which has its peak activity (2.38 ± 0.02 U/g) at 48 h after which it fell at 30 °C. Optimal β-amylase activity for Pearl millet at 40 °C was observed for the control condition at 72 h (1.61 ± 0.20 U/g) though the activity fell to 1.42 ± 0.20 U/g at the 96th hour. Similar activity (1.42 ± 0.20 U/g) was observed for the malted Pearl millet grains subjected to alkaline pH stress conditions from 48 to 96 h, while slight changes in the β-amylase activity were observed for the malted Pearl millet grains subjected to acid pH stress conditions as the germination stage moved from the 48th to 96th hour.
Figure 1: α-amylase Activity In malted pearl millet grains at 30 °C and 40 °C. Each bar represents mean ± SD values of independent experiment performed in triplicate. Alkaline pH: the grains subjected to the steeping process carried out in Tris-HCl Buffer (25 mM, pH 9). Acidic pH: the grains subjected to the steeping process carried out in Glycine-HCl Buffer (25 mM, pH 3). Control: the grains subjected to the steeping process carried out in distilled water.

β-glucanase analysis

The result in Figure 3 shows the effect of Heat stress and pH stress on β-glucanase activity in Pearl millet malt. Generally, the β-glucanase activities of the malted Pearl millet grains were high especially under the 30 °C heat stress. In Figure 5, the results show that a steady and gradual increase was recorded for both pH stress conditions from 0 to 72 h. Peak activity was observed at the 96th hour for the Pearl millet grains subjected to alkaline pH stress (892.3±0.2 U/kg). This was followed closely by the malted Pearl millet grains subjected to acidic pH stress (725.9±0.2 U/kg). The enzyme activity for malted Pearl millet at 40 °C showed gradual increase under both test and control conditions as the germination stage moved from 24 to 72 h. However, peak β-glucanase activity (597.3±0.2 U/kg) was observed for acidic stress at 96 h.

β-Glucan Content Estimation

Figures 4 show the results of the effect of Heat stress and pH stress on the β-glucan content of the malted Pearl millet grains. Results of the effect of heat stress and pH stress on the β-glucan content of the malted Pearl millet grains subjected to alkaline pH stress, acidic pH stress and control conditions at 30 °C were within the same range of approximately 4-8 % w/w malt flour. Exceptions to this were the malted Pearl millet grains produced under the control condition which was as high as 9.98 % w/w malt flour at 72 h of germination and those of the alkaline stress at 96 hours which had β-glucan content as low as 3.79 % w/w malt flour. These results also showed neither a consistent nor definite pattern in the β-glucan content from germination time 0 h to 96 h.
On the other hand, the β-glucan content of malted Pearl millet grains at 40 °C showed decreases as the germination time moved from 0 h to 24 h. This was followed closely by an increase from the 24th to the 72nd hour. The lowest β-glucan content value (1.00 % w/w malt flour) was obtained for the control malted grains at 96 h while the highest β-glucan content value (12.37 % w/w malt flour) was obtained at 72 h among the malted grains subjected to alkaline stress. At the same germination stage, 72 h, the results show that the β-glucan concentration for the other conditions were at their peaks.

**Estimation of malted millet protein content**

Figures 5 shows the results of the effect of Heat stress and pH stress on the protein concentration of the malted Pearl millet grains as estimated using the Bradford method. Results of the effect of heat stress and pH stress indicate decreases (0 h to 24 h) in the protein concentration of the malted Pearl millet grains subjected to alkaline pH stress, acidic pH stress and control conditions at 30 °C. Thereafter, increases were observed in each group till the 96th hour but their respective protein contents never rose above the values recorded at 0 h (1.4 mg/mL, 1.5 mg/mL and 1.4 mg/mL for the Pearl millet grains subjected to alkaline pH stress, acid pH stress and control conditions respectively). Similar results were also observed for the malted Pearl millet grains at germination temperature of 40 °C the only malted Pearl millet grains which had protein concentrations higher than those obtained at 0 h were those subjected to the alkaline pH stress conditions especially at 24 h and 48 h; thereafter, a decline in their protein content was observed at 72 h and then an increase at 96 h which never rose above that of 0 h. These values were higher than the control from 24 h to 96 h.
Effect of steeping parameters on key enzymes and protein profile of pearl millet malt

Figure 5: Estimation of Protein content In malted Pearl millet grains at 30 °C and 40 °C. Each bar represents mean ± SD values of independent experiment performed in triplicate. Alkaline pH: the grains subjected to the steeping process carried out in Tris-HCl Buffer (25mM, pH 9). Acidic pH: the grains subjected to the steeping process carried out in Glycine-HCl Buffer (25mM, pH 3). Control: the grains subjected to the steeping process carried out in distilled water.

As for the grains subjected to acidic pH stress in this group, there was a decrease in the protein concentrations (Figure 5) just as there was among the control grains but it is worthy of note that the test (i.e. acidic pH stress) protein content values were lower than those of the control grains at each germination stage.

Protein profile of malted Pearl millet using SDS-PAGE

Figure 6 shows representations of the pattern of the electrophoretic mobility of proteins contained in the malted millet grains understudied with respect to three different germination temperature conditions (30 °C, 40 °C, and 50 °C) with preferences given to the grains whose germination was stopped at 96 hours. These were also compared with those of the millet grains sampled just before they were incubated at the various temperatures considered.

Figure 6 shows that every group had expression of the proteins and three major bands were seen, though at different intensities. Lanes A-F and lanes M-R have rather low intensities while lanes G-K and lanes S-X showed higher band intensities. By estimation, in comparison with the standard used, the first band (which was expressed as a bulky loci) have molecular weights within the range of 45-66 kDa; next to that band is one estimated to have a molecular weight of 25-27 kDa while the last band there would have a molecular weight that is less than 25 kDa.
DISCUSSION

Grain Malt that meets industrial requirements are expected to be able to germinate rapidly and uniformly (Riss and Bang-Olsen, 1991; Woonton et al., 2005a), with a correspondingly high diastatic power, high β-glucanase activity and low protein content. The result of α-amylose activity of malted pearl millet during varying stress conditions is shown in Figure 1. There was a steady increase (from 0 to 96 h) in the α-amylose activity at 30 °C under all pH stress conditions considered except for acidic pH malted pearl millet where the enzyme activity decreased from 191.04 ± 1.5 U/g to 142.50 ± 2.20 U/g between the 72nd and 96th hour. Highest activity (248.04±0.20 U/g) was observed at 96 h for alkaline pH steeped pearl grains germinated at 30 °C. This agrees with Kanensi et al., (2013) that alkaline pH stress-influenced malt could be a substantial source of α-amylose activity, among other amylolytic activity. This was also highlighted in previous studies which show that the maximum development of α-amylose activity usually occurs after 96 h in germinating millet (Mbithi-mwikya et al., 2000). Hence, condition of steeping, germination time, as well as temperature are important factors in obtaining a high α-amylose activity in malted pearl millet. Ukwuru, 2009 reported that steeping conditions are highly determinant factors for amylolytic development.

There was a consistent increase in the β-amylase activity throughout the period of germination from 0 to 96 h at both acidic and alkaline pH conditions, with the optimal activity recorded at the 96th hour for malted Pearl millet grains subjected to alkaline stress (2.73 ± 0.20 U/g) as compared with the control which has its peak activity (2.38 ± 0.02 U/g) at the 48 h after which a decrease was observed at 30 °C.

The β-amylase activity levels in the malted millet grains were not as high as the α-amylose (Figure 2). Optima β-amylase activities in the present study were observed among the grains germinated at 30 °C as against those of 40 °C. This is consistent with the report of Taylor and Robbins (1993) that a low temperature is optimal to obtain high β-amylose activities. This requirement of a relatively low germination temperature may be related to the fact that the β-amylase is thermostable. The alkaline pH stress steeping conditions appears to have a stronger enhancing effect on the β-amylase activity in the malted Pearl millet than the acidic pH stress steeping conditions at 30 °C and 40 °C (Figure 2). The same observations were made regarding malt β-amylose activity by Okolo and Ezeogu, 1996 and Okungbowa et al., 2002 who stated that steeping at alkaline pH generally enhanced this enzyme’s activity. However, the control steeping condition appears to be better at enhancing the β-amylase activity of the malted millet grains than both acidic and alkaline pH.

The significance of the results obtained in the present study is that the α-amylose activity levels indicate the possibility of being a good indicator of the diastatic activity in the malted millets due to its higher contribution. It can be inferred that the Pearl millet grains which were subjected to alkaline pH stress at 30 °C will most likely have a correspondingly high diastatic activity when compared to the acidic and control due to their respective α-amylose activity levels. Hence, an industrialist seeking for malt products of high diastatic activity may consider producing them under the alkaline pH stress steeping condition and 30 °C heat stress germination condition. In consonance with these observations, reports have it that diastatic activity increase is caused by an increase in activity of the amylase enzymes during pearl millet germination (Mbithi-mwikya et al., 2000; Kanensi et al., 2013). The endo-(1,3)(1,4)-β-D-glucanase enzyme activities were high in the Pearl millet grains subjected to alkaline and acidic pH stress and germinating at 30 °C, especially those sampled after 96 hours of germination (Figure 3). This is an indication of a high rate of β-glucan hydrolysis. From their work, Gupta et al., 2010, observed that better malting performance is expected to be associated with lower levels of β-glucan in grains and higher levels of β-glucanase in malt. β-glucanase activity has been reported to be responsible for the degradation of endosperm cell walls and subsequent changes in β-glucan levels during malting, which depolymerizes β-glucan (Etokakpan, 1993; Gupta et al., 2010). This attempts to solve the problem of inadequate malt enzyme activity as raised by Agu and Palmer, 1997 and could be of use in industries which have demands for malts of low β-glucan content due to its viscous effects. The quality (in terms of its viscosity) of the malt produced in the present study could be predicted from the results obtained on the β-glucan content of the malted Pearl millet (Figure 4).

Generally, decreases in the protein content of the malted Pearl millet (Figure 5) was observed 24 h after the germination process was initiated under both stress (pH and heat) conditions applied. The decrease in protein as a result of heat stress conditions applied is due to hydrolysis of native proteins to low molecular weight proteins or peptides and allowing a corresponding increase in enzyme activity. Thereafter, an inconsistent pattern in the protein content values was observed at 48 h, 72 h and 96 h after the initiation of the germination period. This may be attributed to denaturation of proteins as a result of heat stress conditions (Gimbi and Kitabatake, 2002). Moreover, as it has been reported by Mbithi-Mwikya et al., 2000, the points of increases in millet protein could be attributed to dry matter loss, particularly
carbohydrates, through respiration (during germination) and the action of the carbohydrate degrading enzymes, causing an apparent increase in other nutrients such as protein.

The protein profile of the malted grains subjected to pH stress and heat stress conditions as shown in Figure 6 revealed bands of proteins firstly at approximately 20 kDa with their level of expression varying from one group to the other. This band confirmed the expression of β-glucanase present in the protein extracts of the malt. Olsen and Thomsen (1990) reported similar protein profiling expression for β-glucanase at an apparent molecular mass of about 24 kDa. The solubilisation of β-glucan requires the involvement of several enzymes among which is β-glucanase, whose enzymatic activity is triggered at the inception of the malting process and increases rapidly until the 4th day of the process (Ginaininetti et al., 2007). β-glucan is a cell wall polysaccharide, which accounts for approximately 70% (w/w) of the endosperm cell in some cereals (Wang et al., 2004).

The polyacrylamide gel electrophoresis in Figure 6 revealed bands around 45 kDa. Speculation about this band is that it could be made up of the amylase enzymes; their expression appeared to be higher by proportion when compared to the other bands, indicating its abundance in the malted grains. The molecular weight of α-amylose in the malted millet grains may be estimated to be within the range, 45-50 kDa and the molecular weight of β-amylose in the malted millet grains, estimated to be between 50-60 kDa, which are close to the literature data. Previous studies by Chakraborty et al., (2012) revealed that the purified enzyme (α-amylose) from a fungal source, showed a single band of about 45 kDa on SDS-PAGE while Liu et al., 2003, reported the SDS-PAGE pattern of β-amylose in soya-bean reveal its molar mass of about 57 kDa. In the hydrolysis of starch, the role of α -amylose is primarily to generate oligosaccharides that are then hydrolyzed by other enzymes such as β-amylose, to produce maltose. The biotechnological application of β-amylose includes the production of high maltose syrups and maltose which is widely applied in food and pharmaceutical industries, since its properties are represented by mild sweetness, good thermal stability and low viscosity in solution (Ramachandran et al., 2004). From this study, it can be deduced that presence of α and β amylases released from these grains during germination will aid the breakdown of the stored starch in the malted millet grains to simpler sugars.

Separations of hordein fractions extracted from the malted millet samples are also shown on the polyacrylamide gel (Figure 6). According to the findings made by Qi et al., 2005, hordein is the main storage protein fraction in cereals and accounts for up to half of the total protein in the mature grains, and has recently been classified into three groups named A (or γ), B and C hordeins based on their electrophoretic mobilities on SDS-PAGE (Echart-Almeida and Cavalli-Molina, 2001). Different analyses have indicated that C-hordein can be separated into polypeptides with molecular weight ranging between 67 kDa and 86 kDa, B-hordein, between 30 kDa and 60 kDa, and A-hordein, between 13 kDa and 20 kDa. Among these fractions, the visible bands expressed in this study were the A (approximately 15-20 kDa) and B (approximately 35-40 kDa) fractions which indicated differences in their proportions in the malted Pearl millet grains.

The A-hordein comprises only 1-2% of the total of the three hordein fractions and apparently is not a true hordein. Hence, the visible band around 20 kDa which happens to be the same band estimated for A-hordein and β-glucanase expression may be more of the enzyme than the A-hordein fraction. On the other hand, the expression of B fraction bands was quite visible on the lanes represented except the decreased expression on lane J which appeared very faintly on the gel. The significance of these fractions and their expression on the gel plates is their ability to influence the malt quality by adjusting the diastatic power of the malted millet grains. Reports show that B hordein fraction has more influential effect on the malt quality in terms of diastatic power than the A and C fractions (Peltonen et al., 1995; Qi et al., 2005). This implies that, in this study, there may be limited influence of the B hordein fractions on the diastatic power of the malted grains due to the proportion of the expressed bands of the fractions to those of the amylases which are the major determinants for high diastatic power of malted grains.

In summary, There was a significant influence on the effect of pH and temperature on the varying enzymes studied. Pearl millet has great potentials in competing well with malt of other cereal sources and can be a good substitute for barley, wheat and finger millet malt if it is produced under properly controlled temperature and pH. However, further optimization of the conditions may be necessary if considering pearl millet for malt production on Industrial scale.

REFERENCES


