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PARTIAL PURIFICATION AND CHARACTERIZATION OF ENDO-B-GLUCANASES OF TWO NIGERIAN MALTED MAIZE VARIETIES

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ABSTRACT: *In this study, two Nigerian maize varieties (Farz 23 yellow and Farz 34 white) were malted at controlled experimental variables to determine the optimum conditions for the development of β-glucanases. The independent variable employed were steeping time, germination time and kilning temperature; and the measured (dependent variables) were diastatic power (DP), cold water extract (CWE) and hot water extract (HWE). Some properties of the two varieties were compared with those of sorghum (SK 5912). The properties of the grains were obtained as 1000-corn weight, (W) 241g and (Y) 248g; moisture content (%) 13.2, 12.8; germinative energy (%) 96, 92; germinative capacity (%) 99, 96; water sensitivity (%) 83, 82; broken kernel (%) 0.9, 1.1; protein (N×6.25%) 8.65, 9.02; and fat (ether extract, %) 3.70 and 4.14. The β-glucanases purified 2.59 and 0.56 folds for the yellow and white varieties respectively by a combination of 5M sucrose fractionation, ion exchange on Q-Sepharose and gel filtration on Sephadex G-200; with a yield of 0.8% (yellow) and 7.6% (white). The specific activity for the yellow maize enzyme was 0.312µ/mg and 0.381µ/mg for the white. The optimal condition for the glucanase activity for the white variety were 50°C and pH 5.0 and 7.0, while maximum stability was achieved at 40°C, pH 5.0 and 7.5 (16h); and for the yellow 60°C and pH 5.0 and maximum stability at 40°C, pH 6.0 (16h). The purified enzyme of the white variety was appreciably activated by Co^{2+}, Mn^{2+} and Zn^{2+} gave inhibitory effects. The yellow variety glucanase activity was only enhanced by Mn^{2+} and fairly by Co^{2+}. The yellow variety glucanase displayed remarkable wide substrate specificity and rapidly hydrolyzed amylose, amylopectin and starch. On substrate concentration the white maize enzyme indicated substrate enhancement on both Sigma cell type 20 and CMC. The K_m values as obtained from Lineweaver-Burk plots for the white maize glucanase were 0.119 and 0.102mg/ml for CMC and Sigma cell respectively, and 0.072 and 0.0451mg/ml in same order for the yellow maize glucanase. Their corresponding V_{max} were 3.7 and 3.5mg/ml/min for the white maize enzyme and 4.0 and 9.0mg/ml/min protein for the yellow maize enzyme. The very low K_m values Sigma cell type 20 and CMC for the yellow indicates a high affinity of the yellow glucanase to the substrates. On overall assessment, the glucanases presents a promising application in the mashing conditions of the brew house.*

Keywords: Malting, β-glucanase, characterization, purification, maize

INTRODUCTION

Barley is malted prior to brewing in order to develop the starch-degrading enzymes that will be employed in the brew house and also to allow synthesis and action of enzymes that remove the cell wall components enclosing the starch in the starchy endosperm (Briggs, 1998). It is favoured as the classical grain for the production of lager or ale beer as well as other non-alcoholic beverages (Aisien, 1988). Much research attention has been geared towards barley selection and breeding process resulting in many different barley varieties of high quality. Therefore, worts produced from barley malts are rich in simple peptide and free amino nitrogen which favour higher enzyme activity and higher extract yield.

Countries of the world that are not within the temperate region do not grow barley in commercial amount to support the brewing industry. It therefore becomes imperative for such countries to import barley, either malted or unmalted. Nigeria had in the past two and half decades, spent a colossal percentage of its foreign earnings to import malted barley. Economics shows that no nation of the world can be stable spending so much on importation. Several efforts have been made to improve and/or modify tropical grains as replacement for malted barley which is the classical grain in beer production. Following the ban on the importation of barley malt and wheat in 1988 by the Nigerian government, cereals such as maize, rice, sorghum etc are now used for the purpose of brewing (Emmanuel, 2002). Research into the improvement of some local cereals for malt production for Nigerian brewing industries is proceeding (Agu and Okeke, 1992).

Three tropical cereals malted and unmalted have been recommended for use in the Nigerian brewing industry: sorghum, maize and rice (Anon, 1987). Among these, sorghum has been much studied as a replacement for barley at the experimental and industrial levels (Okafor and Aniche, 1980). Concerted efforts have been put in place towards finding a possible substitute to barley. In January 1978, the Breweries Association of Nigeria (BAN) inaugurated a body to study the possibility of using any of the available tropical cereals in beer production against the imported barley malt. Prior to this, in 1975, Guinness Nigeria Plc had on its own, taken some initiative in the use of local raw materials, and in association with the School of Agriculture, Samaru Zaria experimented on barley growing (National Concord, 1983). Nigerian Breweries Plc in collaboration with the then military government of Nigeria attempted without success the cultivation of barley grain in Kadaro.

Okafor and Aniche (1980) reported that malted maize for brewing has similar properties with those of sorghum malt. Also, Okafor and Iwouno (1991) assessed the malting and brewing potentials of Nigerian rice varieties and gave a good indication for rice as a possible replacement for barley in beer production. It is impossible to secure gelatinization at temperatures slightly above 60°C with sorghum, millet and maize and for many starches, heating to temperatures above 60°C is necessary to achieve gelatinization and ideal filtration properties after subsequent saccharification (Hemmigsen and Norman, 1980). Agu and Palmer (1997) reported that yellow maize variety has a better quality malting performance than the white maize variety. In line with this, is a report from the same authors that the yellow maize variety has lower malting losses, higher diastatic enzyme activity and so produces more extract yield when compared with the white variety. The low diastatic power and low enzyme levels of these tropical cereals have presented difficulties in using these grains as sole sources of carbohydrate for brewing lager beer (Okolie and Ogunsula, 1987).

In well modified malt, the majority of the cell walls from the starchy endosperm of barley are removed. However, even in the most extensively modified malt there is some residual polysaccharide (β-glucan and arabinoxylan) derived from the walls, either in the form of undegraded structure in the very distal regions of the kernels or as solubilized but incompletely hydrolysed material (Bamforth, 1994; Bamforth, 1999). Brewers are greatly concerned by the phenomenon of unhomogeneity, wherein a batch of malt contains kernels displaying varying extents of cell wall degradation (Kanauchi and Bamforth, 2002). Therefore, the objectives of this work are to determine the treatment combination that will produce optimal β- glucanase of two Nigerian maize varieties (yellow and white), to compare the performance of two different varieties of maize for the production of β-glucanase and to isolate, purify and study the action and role of the β-glucanase in brewing.

MATERIALS AND METHODS

The Two Maize Varieties, White Farz 34 and yellow Farz 23 represented as Fw and Fy whose production year was 2005 were obtained from National Cereals Research Institute (NCRI), Badeggi, Niger State, Nigeria. The maize varieties were first malted using the protocols already applied for barley. However, the recommended methods of analysis of the Institute of Brewing (Anon, 1982, 1989), the European Brewing Convention (EBC) and American Society of Brewing Chemists (ASBC) were adopted in the malting and analysis of the malted grains in the study, with modifications where necessary.

Determination of Properties of Maize Grain

Thousand corn weight

Five hundred grams of each of the grain varieties were weighed out. The maize grains were reduced using a divider, into two portions of about 20g. Foreign matter and half corns were also removed. Each sample was weighed in a beaker. The number of grains in each portion was counted (Recommended Methods of Analysis of the Institute of Brewing) (Anon, 1982). The weight of 1000 grains of dry maize grain (G) was determined by

$$G = \frac{W \times 1000 \times DM}{N \times WD}$$

Where N - Total number of grains counted
DM - Dry matter percentage (%) of maize
W - Total weight of maize taken

Moisture Content

About 20g sample of maize was ground finely according to the *Recommended Methods of Analysis of Institute of Brewing* (Anon, 1982). Five grams of sample was thoroughly mixed and immediately placed in a moisture dish. The dish was closed and the weight of dish taken immediately with the sample. The cover was removed and dish placed in a pre-heated Gallenkamp Hot box oven, model Ovb-300-010) for 3 hours at 105°C. The lid was replaced and dish allowed to cool in a desiccator for 20 minutes, at room temperature The dish was re-weighed to 0.001 g. The moisture percentage (M) of the maize sample was determined by the expression.

$$M = \frac{W1 - W2 \times 100}{W1}$$

Where W_1 - Weight of sample before drying
W_2 - Weight of sample after drying

The average value gave the percentage moisture content of sample.

Germination Capacity

Rapid and complete germination are essential features of good malt. The *Recommended Method of Analysis of the Institute of Brewing* (Anon, 1982) was used. Two hundred corns of maize were steeped for 48 hours in 200ml fresh hydrogen peroxide at 18 - 21°C. The steep liquor was strained off and replaced with 200ml of fresh H_20_2 (aq) at the same temperature and left for additional 24 hours. The steep liquor was again strained off and germinated corns counted after separation. The germinative capacity in percentage (H_20_2) was determined using the formula.

$$GC\ (\%) = \frac{200 - n}{2}$$

Where n - number of corns which did not germinate.

Germinative Energy

Here, the method of the American Society of Brewing Chemists (ASBC, 1958) was applied. One hundred kernels of maize were placed in 15 cm test tubes and covered with distilled water at 28°C, and filling the tubes to within 20 mm from tip. Intermittently, floating kernels were forced below the surface of the water. The water was drained off after 24 hours, and replaced with fresh water. After 48 hours of steeping, the water was drained off and the kernels rinsed with distilled water and drained again. A sheet of Whatman No. 1 filter paper was moistened by dipping it in distilled water for few seconds and allowed to drain. The moistened filter paper was placed on a glass sheet and the steeped grains distributed in compact single layers of kernels in the centre of one half of the filter paper. The other half of the filter paper was pressed down finally on the layer of the maize kernels and this was placed in a desiccator containing water in the bottom to maintain humidity near saturation. The desiccator was kept on a bench at a temperature of 28°C. After 72 hours, the germinating maize grains were examined and those kernels which had roots or sprouts were removed. The percentage of sprouted kernels was reported as Germinative Energy Percent (ASBC, 1985).

Nitrogen Estimation for Protein Determination

Twenty grams of ground maize was well mixed in a Stoppard bottle. About 1.5g (w) of the sample was qualitatively transferred into a dried Kjeldhal flask. Ten grams of mercury catalyst tablets were added together with 20ml of concentrated sulphuric acid, and gently swirled to mix and wet the content of the flask thoroughly. The flask was placed in an inclined position on a digestion rack and heated to boil, until the content of the flask became clear. The flask and its content were cooled to room temperature and 200ml of water were added and the content mixed. The mixture was cooled to 24°C, and 25ml of sodium thiosulphate solution added to precipitate the mercury. Anti-bumping agent was added, and then 70ml of 0.1N sodium hydroxide solution was slowly added so that two distinct layers were formed. The flask was connected to a condenser by the bulb trap and the content was swirled to ensure rapid mixing and was heated until all of the ammonia had distilled into an excess 2% boric acid solution (about 25ml) containing about 0.5ml of screened indicator (methyl red/bromocresol green solution) The ammonia was titrated with the standard sulphuric acid (y) to a grey end point. The moisture content of the sample had earlier been determined as described.

The percentage nitrogen (N) in dry maize sample

$= \frac{Y \times 14}{W \times DM}$ (to two decimal places)

Where Y - ml of 0.1N acid required to neutralize the ammonia after Subtracting reagent blank

W - Weight of sample (in gram) taken

DM - Percentage of dry matter

Percentage protein = N x 6.25

Determination of Water Sensitivity

The *Recommended method of Analysis of the Institute of Brewing* (Anon, 1982) was used. Two filter papers were placed in the bottom of a Petri dish and 8ml of water was added. Water (4ml) was also added in another Petri dish containing two filter papers. One hundred kernels of maize were placed in each of the Petri dishes, so that each was in good contact with the wet filter paper. In the 8 ml test, only the vertical side touched the paper to avoid drowning of the embryo. Each dish was covered with its lid. The dishes were incubated for 72 hours at room temperature (usually 28°C). The difference between the percentage in 4ml test and 8ml test was reported as water sensitivity percent.

Fat Content Determination

The fat content of the maize grains was determined by Rose- Gottlieb method as described by Pearson (1976). Ten grams of ground maize were quantitatively transferred to lower section of a Jacob's Singer separatory flask. Ammonium hydroxide (1.25ml) was added to the sample and mixed thoroughly. Ninety-five percent ethyl alcohol (11ml) was added and the sample well mixed. Ethyl ether (25ml) was added and the sample shaken vigorously for 30 seconds. Petroleum ether (25ml) was added, and the sample was shaken again for another 30 seconds.

The separatory flask was Stoppered at the upper section. The sample was kept standing until the upper layer became very clear. The mixed ether layer was drawn off into a weighed "fat" flask. The lipid remnant in the flask was extracted with 5ml of 95 percent alcohol and 15ml of ethyl ether, shaking vigorously for 30 seconds after each addition and allowing the mixture to settle. The clear solution was drawn off into the same tarred flask. Water was added to the separatory flask. The remaining ether solution was drawn into the same tarred flask as carefully as possible. The ether in the tarred flask was evaporated off after each extraction, slowly on a steam bath while the subsequent extraction was allowed to settle. The outside of the flask was wiped off and the flask placed in thermostatically controlled oven (Gallenkamp, model 046300) at 100° - 105°C for 5 min. The flask was weighed with a similar flask as a counter poise after cooling in a dessicator. The fat content of the maize was calculated by the following formula:

$$\text{Fat content (\%)} = \frac{\text{Weight of fat in tarred flask x 100}}{\text{Weight of maize sample}}$$

Malting

The maize grains (White and Yellow varieties) were separately sorted and manually cleaned using tray; impurities, broken grains and foreign matter were also screened out. About 1.5kg of the grain varieties were surface- sterilized in sodium hypochlorite solution for 30 min and washed several times in tap water.

Steeping

Steeping of each sample was done at a temperature of 30°C for different periods; 30, 36 and 42 hours. The steep cycle involved alternating 12 hour wet-steep with 30 min air-rest period. And the steep water temperature fluctuated between 25°C and 28°C.

Couching

After steeping, the grains were couched (heaped) on jute bags previously sterilized with dry heat.

Germination/ Kilning

Samples were germinated at fluctuating temperature 25° - 30°C, and samples were removed from the third day of germination to the fifth day and kilned in a hot air oven at different temperatures of 50, 55 and 60°C for 30 hours. Kilned samples were manually de-rooted by rubbing.

Malt Analysis

The following analyses were carried out on the maize malt samples.

Moisture Content

This analysis was as earlier described.

Malting Loss

The material lost as percentage (%) dry weight in converting maize grain into maize malt is known as the malting loss (ASBC *Method of Analysis*, 1958). One kilogram each of sample of malted maize grains was weighed at the end of malting. The difference in weight between

the resulting malt and the original grain as a percentage of the grain weight, gives the malting loss.

Cold Water Extract (CWE)

Ten grams of ground malt were digested with 200ml of distilled water containing 12ml of 0.1N ammonia for 3 hours at 20 ± 0.2°C and stirring at 30 minute intervals. The solution was filtered and the relative density of the filtrate measured at 15.5°C. The cold water extract percentage was assessed as follows:

$$CWE\ (\%) = \frac{G-1000 \times 20}{3.86}$$

Where G - Excess degree of gravity of the filtrate taking water at 15.5°C as 1000
i.e. G = 1000 (S.G. -1)

Hot Water Extract (HWE)

In this determination, the *Recommended Method of Analysis of the institute of Brewing* (Anon, 1982) was used. Fifty grams of ground maize malt were put in a 1000ml beaker. The beaker was placed in heated water bath (Gallenkamp, model 046300) at 65°C, and preheated for 10 - 15 minutes. Three hundred and sixty milliliters of distilled water were added into the beaker at a temperature not exceeding 68°C to ensure an initial mash mix of 65°C. All lumps in the mash were rapidly eliminated by stirring. The mash was kept at temperature of 65°C for exactly 1 hour after the initial mix. The beaker was immersed, at least to the level of the mash surface and the mash stirred at 10 minutes intervals. After 1 hour, the mash was removed from the water bath and quickly cooled to 20°C. The mash was then transferred to a 515ml flask through a funnel exactly 25 minutes after removal from the bath, washing out the beaker with distilled water and making up the contents of the flask with distilled water to 515ml of 20°C. The contents of the flasks were thoroughly mixed by vigorous shaking for five minutes. Thereafter, the volume of the prepared mash was adjusted to 515ml at 20°C as described above. The entire mash was filtered with the first 50 ml of filtrate returned to the filter and the filtrate collected determined for extraction at 15.5°C within one hour of collection.

The hot water extract (HWE) was calculated as follows:

Extract 'as is' = G × 10.13litre degree/Kg

Where G = Excess degree of gravity of the filtrate taking water at 15.5°C as 1000

i.e. G = 1000(S.G -1)

$$\text{Extract (dry)} = \frac{\text{Extract (as is) x 100}}{100-\mu}$$

Where μ = Moisture percentage of sample

Diastatic Power (°Lintner)

A malt infusion method was prepared as done in cold water extract CWE). The extract was not filtered but allowed to settle. Three milliliters aliquot of the supernatant was removed using pipette into 100ml of 2% buffered starch solution at temperate at 25°C and contained in a 200ml flask. The flask was shaken and left at this temperature for an hour. The reaction was stopped by adding 30ml of 0.1 N of sodium hydroxide and the volume made up to 200rm at 20°C with distilled water. Five milliliters of Fehlings solution was pipetted into a 150ml narrow-necked boiling flask. The digested solution was added to the cold Fehlings solution from a burette and the flask content was mixed and boiled with moderate heat for 2 minutes. The boiling and addition of starch solution was continued for another 2 minutes until the blue

colour of Fehlings solution was discharged. Three (3) drops of methylene blue indictor was added and the titration completed. The end point was indicated by the decolorization of the methylene blue indicator and reacting liquid, just became red. The Diastatic Power (D.P) expressed in °Lintner (IOB) was calculated from the expression.

$$D.P = \frac{2000}{Xy} - \frac{200}{Sx}$$

Where X - Number of ml of malt extract used to digest starch
Y - Number of ml of converted starch to reduce 5 ml of Fehling's solution
S - Titre of the starch blank

Enzyme Extraction

Two hundred millilitres of the extraction buffer consisting of 0.05M acetate (pH 5.7) containing 0.1M sodium chloride and 0.01 M $CaCl_2$ was added to 100g of ground malt. The mixture was agitated in a shaker at room temperature at 120 rpm for 1h. This was followed by centrifugation with a Centurion scientific centrifuge (model 2041, UK) at 5,000 x g for 20 min. The resultant supernatant was recovered as the crude glucanase.

β-Glucanase Activity Determination

Determination of β-glucanase activity was carried out using the dinitrosalicylic acid method described by Bernfeld (1955). The enzyme reaction mixture was composed in 0.2ml of 5mg/ml carboxymethyl cellulose (CMC) in 0.05M sodium/acetate buffer (w/v), pH 5.7 and 0.2ml of the crude glucanase. Incubation of the enzyme reaction mixture was at 40°C for 30min in a GFLD-30938 water bath (Germany). The reaction was stopped by adding 0.4ml of dinitrosalicyclic acid reagent (DNS and heated for 10min. in boiling water after which it was cooled rapidly in ruining tap waiter On cooling, 1.2ml of water was added and absorbance was measured at 540nm using a Jenway 6405 uv/vis spectrophotometer (EU) against a blank prepared by the above procedure but adding DNS before the enzyme. One unit of glucanase activity was defined as the amount of enzyme which liberated 1µM of glucose per minute under the assay conditions.

Enzyme Purification

Enzyme concentration

Enzyme concentration was performed at refrigeration temperature (4°C) according to Okolo *et al.*, (2000). The crude enzymes from the white and the yellow variety of maize were concentrated separately by dialysis using a dialysis tubing (cellulose membrane; size 33mm x 21mm, Sigma-Aldrich) in 5M sucrose solution overnight. The dialysates were separately collected and tested for enzyme activity as described above.

Determination of protein content of enzyme

This was done either by direct determination of the optical density at 280nm using an Eppendorf biophotometer 22331 (Hamburg, Germany) against a buffer blank or by using the method of Bradford (1976).The Bradford reagent was prepared by dissolving 50mg of Coomasie Blue 250G (Serva) in 47.5ml of ethanol. Fifty milliliters of 85% phosphoric acid were added and the volume made up to 500ml with deionized water. The reagent was filtered through Whatman no. 1 filter paper and stored frozen in a brown bottle. According to Bradford (1976), one volume of the Bradford reagent was diluted with four volumes of deionized water. To 0.05ml of the sample, 2.5ml of diluted Bradford reagent was added and shaken vigorously. Measurement was at OD 595nm using a Jenway 6405 uv/vis spectrophotometer against a blank with deionized water in place of sample. The amount of protein was then determined using a standard curve of bovine serum albumin (0- 1.0mg/ml).

Ion exchange chromatography
The enzyme concentrate from the maize of the white variety (10.6ml) was applied to a 2cm x 13cm Q-Sepharose Fast flow (Pharmacia, Sweden) column equilibrated with several volumes of acetate buffer (pH 5.7). The enzyme was eluted with the same buffer (60ml) and then with a linear gradient of 0.0 - 1M NaCI in buffer (200ml). Fractions were collected a: a flow rate of 90ml/h. The active fractions (no. 4-10) were pooled and concentrated in 5M sucrose solution. The enzyme activity and protein cone-: of the concentrate were determined.

Gel filtration chromatography
Six milliliter of the concentrate from the last purification step for the white variety was applied on a Sephadex G-200 column (1 x 36 cm) equilibrated with acetate buffer (pH 5.7) and eluted (10 ml fraction size) with the same buffer at a flow rate of 1.5 ml/h. On the other hand, 10.6ml of the concentrate from ion exchange for the yellow variety was also further purified by applying on a Sephadex G-200 column (1 x 29 cm). The active fractions for the two glucanases were pooled and concentrated by dialysis as previously described.

Gel Electrophoresis
This was done according to the method of Laemli (1970). The purified glucanase was subjected to a homogeneity test based on electrophoresis on 300g/l polyacrylamide slab gel containing 100µl of 10% sodium dodecyl sulphate (SDS- PAGE, Sigma Aldrich). Twenty microlitre of the enzyme sample in 20µl of sample buffer, consisting of 1ml Tris-HCI buffer (0.5M, pH 6.8), 0.8ml glycerol, 1.6ml SDS (10% w/v). 0.4ml bromophenol blue (0.5% w/v) and 0.4ml 2-mercaptoethanol were placed in an Eppendorf tube and heated for 5mins in a water bath with boiling water to dissociate the enzyme. SDS-PAGE was then performed with a stacking gel consisting of 3.4ml distilled water, 0.63 Tris buffer (1.5M, pH 6.8), 0.88ml acrylamide mix (30% w/v), 100µl SDS (10% w/v), 50µl ammonium persulphate (APS, 10w/v) and 5µl Temed, and a separating gel consisting of 3.3ml distilled water, 2.5ml Tris buffer (1.5M, pH 8.8.), 4ml acrylamide mix (30%,w/v), 100µl SDS (10% w/v), 100µl APS (10%, w/v) and 5µl Temed. A tracking dye of 0.5% bromophenol blue in sample buffer was used. A current of 40mA and 180volts was used to run the electrophoresis at room temperature until the tracking dye emerged using a FB Fisher Scientific electrophoresis system (Germany).
Protein was visualized by staining with silver nitrate solution consisting of 60ml water, 0.6ml formaldehyde (37%) and 0.8ml $AgN0_3$ (20%) after pretreatment of the gel. A solution consisting of 60ml water, 25ml formaldehyde (37%), 25pl formaldehyde (37%), $Na_2S_20_3$ (10% w/v) and 1.2g of Na_2C0_3 was used to develop the gel until the bands appeared. The reaction was then stopped with 5% acetic acid in water and the gel stored in water.

Estimation of Relative Molecular Weight
The molecular weight of the purified enzyme was determined by SDS-PAGE. Molecular mass markers used were an assortment of proteins of various molecular weights (Sigma colour burst electrophoretic markers). Proteins were detected using a calibration curve drawn on the basis of the relative mobility values and molecular weights of the reference proteins.

Kinetic Procedures
Temperature activity and stability determination
The temperature activity and stability profiles were carried out according to a modification of the method of Okolo *et al.*, (2000). The temperature activity profile of the glucanase enzymes

were determined over the range of 30 - 90°C by adding 50µl of the purified amylase preparation to 50µl of CMC in buffer (0.5% w/v and incubating at the test temperature for 30min. The enzyme activity was then determined using DNS reagent as described above. To determine the thermostability, the enzyme was pre-incubated at the various temperatures between 30 and 90°C for 30min and then promptly chilled on ice. The residual activity was then determined under normal assay conditions.

pH activity and stability determination

The effect of pH on the activity of the purified glucanase was studied over the pH range of 3.0 - 7.0 using 0.1 M citrate/phosphate buffer and at pH 8.0 - 9.0 using 0.2M Tris/HCI buffer (Okolo *et al.*, 2000). The reaction mixture consisted of 50µl of the purified enzyme preparation and 50µl of 0.5% CMC in the appropriate buffer at pH 3.0 - 9.0. The mixture was incubated for 30 minutes at 50°C for white variety and 60°C for yellow variety. The amount of reducing sugar released was assayed as described above.

The pH stability profile of the glucanase was determined by dissolving the enzyme solution in appropriate buffer at pH 3.0 - 9.0 and pre-incubating it for 16h at room temperature (30°C). Thereafter, the residual enzyme activity was determined using the above substrate in 0.05M acetate buffer (pH 7.0) for white variety and 0.05M acetate buffer (pH 5.0) for yellow variety.

Effect of metal ions on enzyme activity

The effect of various cations (Fe^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Ba^{2+} Hg^{2+} Co^{2+}, Sr^{2+} and Pb^{2+}) on β-glucanase activity was evaluated. The glucanases and the individual metal chloride, sulphate or nitrate (5mM) in buffer (pH 7.0 for white variety and pH 5.0 for yellow variety) were incubated with an equal volume of substrate in buffer at 50°C for white variety and 60°C for yellow variety.

Relative Rate of Hydrolysis of Different Substrates

Different glucans [CMC and Cellulose (sigma cell) type 20] were used to determine their relative rates of hydrolysis by the different glucanases. Each substrate (0.5% w/v) in 0.05M acetate buffer was separately incubated with the enzymes at the optimal conditions for the individual enzyme activity. The reducing sugar released was determined according to the method of Bernfeld (1955) using the DNS reagent. The relative rates of hydrolysis were then evaluated.

Effect of substrate concentration on enzyme activity

Purified β-glucanase activity was assayed in reaction mixtures containing different concentrations (0-1.0mg/ml) of the test polysaccharide substrates, CMC and Cellulose (Sigma cell type 20) in 0.05M acetate buffer under the optimal condition for enzyme activity. The kinetic constants (K_m, V_{max}) were estimated by double reciprocal plots of the data according to Lineweaver and Burk (1934).

RESULTS

Determination of the properties of the maize grain varieties

Table 1 gives the results of some chemical and physical properties of the unmalted maize varieties and compares them with those of sorghum. Hence the yellow maize variety had larger 1000 corn values, though not very much larger than the white, the sorghum had correspondingly lower figure.

Table 1: Properties of unmalted maize grain varieties studied and sorghum (SK-5912)

Item	White	Yellow	Average for maize varieties	Sorghum
Thousand corn weight (g)	241	248	244.5	36.2
Moisture (%)	13.2	12.8	13	7.4
Germinative energy (%)	96	92	94	79
Germinative capacity (%)	99	96	97.5	81
Water sensitivity (%)	83	82	82.5	-
Broken kerne! (%)	0.9	1.1	1.0	-
Protein (N x 6.25) (%)	8.65	9.02	8.84	9.1
Fat (ether extract) (%)	3.70	4.14	3.92	4.4

Determination of the effect of germination and steeping time on maize grain varieties

Table 2 shows the effect of different germination and steeping times on the malting loss of the maize grain varieties. It is interesting to observe from the results of the malting loss that up to day 3 germination period at different steeping times, the malting losses through the roots and shoots growths were similar. After day 3, except for the 30 hour steep period at day 4 where the malting loss of the white variety was higher than the yellow, the malting losses from day 4 to 6 were higher in yellow variety.

Table 2: Effect of numbers of days of germination and steeping time on malting loss

Days of germination	**Steeping Time**					
	30 hours		**36 hours**		**42 hours**	
	White	**Yellow**	**White**	**Yellow**	**White**	**Yellow**
2	8.28	8.05	8.15	8.24	8.20	8.15
3	10.19	10.17	10.64	10.71	10.80	10.73
4	14.22	11.88	14.13	14.31	14.04	14.36
5	17.46	19.87	17.75	20.16	18.09	20.16
6	18.54	20.84	16.56	21.10	20.41	21.01

Cold and hot-water extracts values of the maize varieties

Table 3 gives the cold water extract of the two maize varieties studied at different malting conditions. The result from the maize varieties showed that the cold water values increased with longer period of steeping and germination time, reaching maximum value after 5 days germination and 42 hours of steeping in the two varieties. The hot water extract values as seen in Table 4 also show increasing trends with the increase in steeping time and germination period and reaching the maximum value at the 5th day of germination.

Diastatic power of maize grain varieties

Table 5 shows the results of the Diastatic power measured in Lintner degrees (°Lintner)

Table 3: Cold Water Extract

		Yellow Variety	White Variety
	K_{50}	29.60	29.62
$S_{30}G_3$	K_{55}	29.31	29.30
	K_{60}	31.10	31.13
	K_{50}	30.56	30.58
$S_{30}G_4$	K_{55}	30.54	30.51
	K_{60}	30.55	30.56
	K_{50}	35.02	35.03
$S_{30}G_5$	K_{55}	35.05	35.05
	K_{60}	35.15	35.17
	K_{50}	33.84	33.86
$S_{36}G_3$	K_{55}	33.87	33.85
	K_{60}	33.75	33.79
	K_{50}	37.70	37.79
$S_{36}G_4$	K_{55}	37.67	37.68
	K_{60}	37.88	37.86
	K_{50}	37.11	37.11
$S_{36}G_5$	K_{55}	39.26	39.29
	K_{60}	39.34	39.37
	K_{50}	37.17	37.19
$S_{42}G_3$	K_{55}	34.22	34.20
	K_{60}	34.20	34.21
	K_{50}	38.09	39.10
$S_{42}G_4$	K_{55}	38.42	38.45
	K_{60}	38.39	38.38
	K_{50}	44.50	44.59
$S_{42}G_5$	K_{55}	43.84	43.86
	K_{60}	43.48	43.47

Key: S = Steeping period (hr); G = Germination period (days); K = Kilning temperature (°C).

Table 4: Hot Water Extract

		Yellow Variety	White Variety
	K_{50}	126	123
$S_{30}G_3$	K_{55}	120	116
	K_{60}	118	115
	K_{50}	111	108
$S_{30}G_4$	K_{55}	111	108
	K_{60}	115	111
	K_{50}	226	219
$S_{30}G_5$	K_{55}	221	216
	K_{60}	230	226
	K_{50}	148	144
$S_{36}G_3$	K_{55}	148	147
	K_{60}	156	152
	K_{50}	186	181
$S_{36}G_4$	K_{55}	188	252
	K_{60}	196	277
	K_{50}	260	261
$S_{36}G_5$	K_{55}	266	119
	K_{60}	270	122
	K_{50}	123	129
$S_{42}G_3$	K_{55}	126	228
	K_{60}	133	226
	K_{50}	235	237
$S_{42}G_4$	K_{55}	233	237
	K_{60}	243	289
	K_{50}	300	294
$S_{42}G_5$	K_{55}	300	301
	K_{60}	306	305

Key: S = Steeping period (hr); G = Germination period (days); K = Kilning temperature (°C).

Table 5: Diastatic Power of the Maize Varieties

		Yellow Variety	White variety
	K_{50}	19.22	18.81
$S_{30}G_3$	K_{55}	19.15	18.64
	K_{60}	19.42	18.73
	K_{50}	23.45	22.91
$S_{30}G_4$	K_{55}	23.42	22.88
	K_{60}	23.36	22.74
	K_{50}	25.34	24.75
$S_{30}G_5$	K_{55}	24.30	16.74
	K_{60}	23.81	23.25
	K_{50}	22.15	21.97
$S_{36}G_3$	K_{55}	22.15	21.55
	K_{60}	22.15	15.56
	K_{50}	28.13	27.38
$S_{36}G_4$	K_{55}	28.10	27.45
	K_{60}	28.22	27.53
	K_{50}	29.01	27.61
$S_{36}G_5$	K_{55}	29.32	28.63
	K_{60}	29.04	28.37
	K_{50}	23.12	22.66
$S_{42}G_3$	K_{55}	23.10	22.41
	K_{60}	23.32	22.70
	K_{50}	26.92	25.73
$S_{42}G_4$	K_{55}	26.78	25.98
	K_{60}	26.69	26.06
	K_{50}	30.15	29.35
$S_{42}G_5$	K_{55}	30.22	29.41
	K_{60}	30.08	29.39

Glucanase Development

The result of the crude glucanase development in the maize varieties malted at 42- hour steep cycle, germinated at different days and kilned at 50°C is given in Table 6. From the result, both varieties showed progressive increase in glucanase development until after the fifth day. The maximum development of glucanase activity for the yellow and white at the fifth day were 0.448 and 0.615 units for the yellow and white respectively. At the sixth day the activity decreased to 0.402.and 0.546 units.

Table 6: Glucanase activity of the maize varieties malted at 42h steep, different days of germination at 50^{0}C.

	Glucanase activity (Units)	
Days	Yellow	white
1	0.356	0.407
2	0.430	0.444
3	0.437	0.452
4	0.444	0.456
5	0.448	0.615
6	0.402	0.546

Purification of the Glucanase

Tables 7 and 8 give the summary of the glucanase purification of the white and yellow varieties, respectively. The glucanase from both varieties were purified from the crude supernatant to obtain a final yield of 1.5% and 0.8% and purification factor of 0.56 and 1.50-fold (specific activity, 0.528 u/mg and 1.21 u/mg protein) for the white and yellow varieties, respectively. These were achieved by a combination of 5M sucrose fractionation, ion exchange chromatography (Figures 1 and 2) and gel filtration on Sephadex G-200. Concentration of the glucanases using sucrose solution proved very successful with minimal loss of activity.

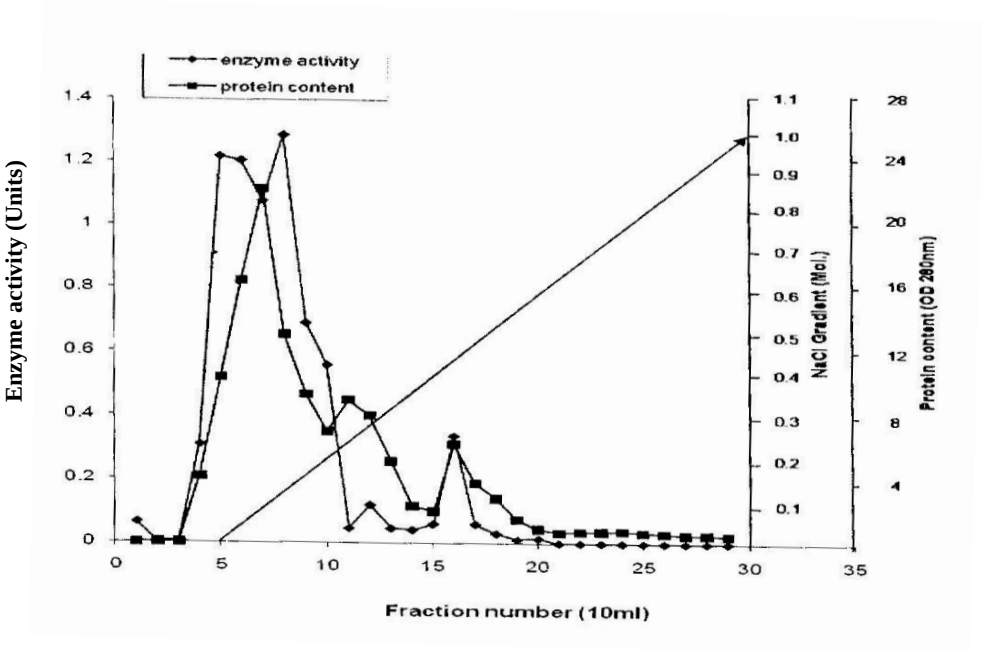


Figure1: Elution pattern of the glucanase from the white variety (glucanase W) of maize on Q- Sepharose F F column.

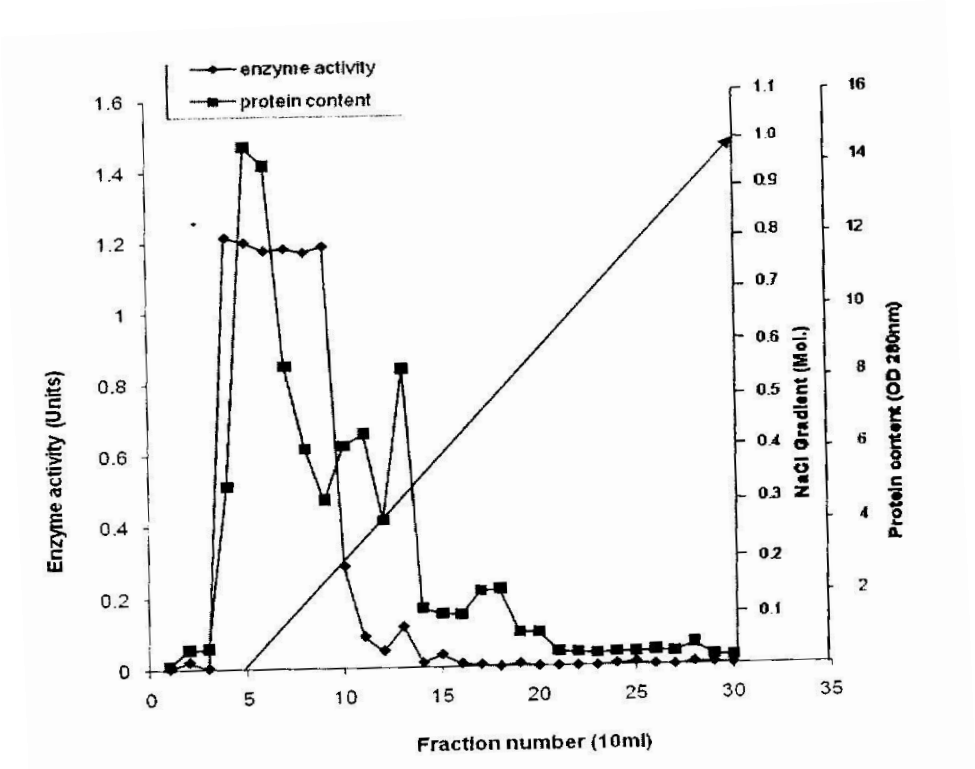


Figure 2. Elution pattern of the glucanase from the yellow variety (glucanase Y) of maize on Q - Sepharose F F column.

Table 7: Summary of purification for glucanase W

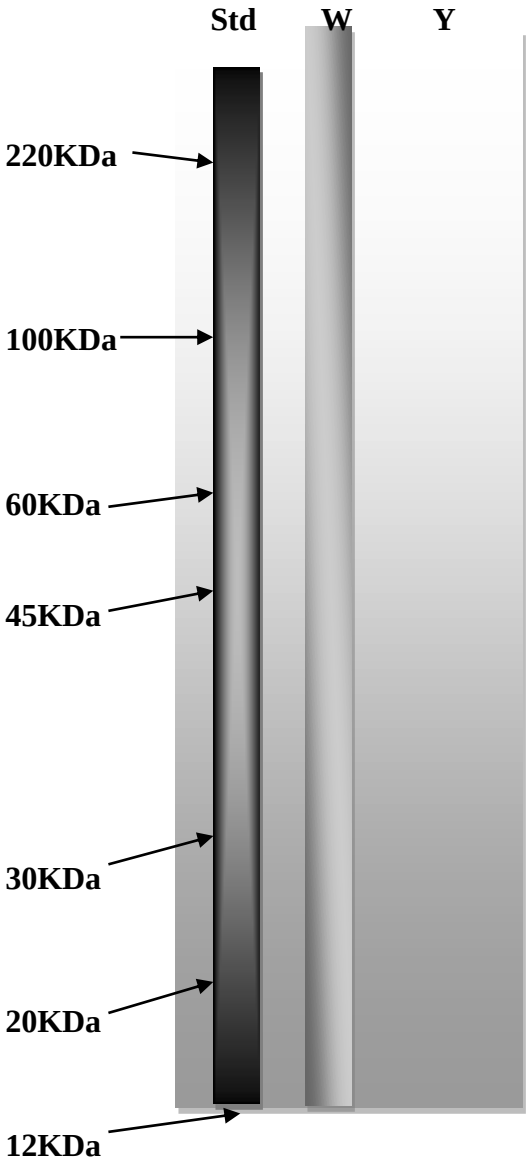
Steps	Vol. (ml)	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Malt Extract	180	110.7	117.9	0.939	100	1
Dialysis	19.5	13.17	30.71	0.429	11.9	0.457
Ion Exchange Gel Filtration on Sephadex	6	4.578	10.62	0.431	4.1	0.457
G-200	10	1.692	3.204	0.528	1.5	0.562

Table 8: Summary of purification for glucanase Y

Steps	**Vol. (ml)**	**Total activity (Units)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Yield** (%)	**Purification fold**
Malt Extract	250	112	240	0.467	100	1
Dialysis	18	12.006	34.02	0.353	10.7	0.76
Ion Exchange Gel Filtration on Sepahdex	10.3	9.785	14.42	0.679	8.7	1.454
G-200	3.5	0.903	2.89	1.21	0.8	2.59

Electrophoresis and Molecular Weight Determination

From the result of the electrophoresis in Figure 3, the molecular weight of the glucanase was estimated to be about 30 KDa (yellow) as calculated from the migratory distance in relation to reference proteins. The low molecular weight suggests that the enzyme presumably possesses a short peptide chain.



Std. = Standard, **Y** = β-Glucanase Y and **W** = β-Glucanase W.

Figure 3. Electrophoretic determination of the molecular weight of the glucanases from the white and yellow variety of maize.

In the case of the white variety, three different protein bands were detected on the SDS. The molecular weight of the glucanase of the white variety were estimated to be about 240 KDa, 30 KDa and 17 KDa (Figure 3) as also calculated from the migratory distance in relation to reference protein.
The very high molecular weight of 240 KDa suggests that the enzymes presumably possess many polypeptides which were not separated by the sod urn dodecyl sulphate. Alternatively, it might be a different enzyme that was not separated by the processes. The other corresponding reference protein of 30 KDa and 17 KDa as in the yellow may not contain many polypeptides.

Physicochemical Properties of the Glucanases
Figures 4 and 5 shows the physicochemical properties of the glucanases for the white and

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yellow maize varieties, respectively. The optimum temperature of activity for the glucanase of the white maize variety was 50°C with 65% activity still present at 60°C for the white variety. For the yellow, the activity was optimum at 60°C with just about 30% activity present at 70°C.The glucanase of the yellow maize variety was highly unstable to temperature increase with about 42% drop in the original enzyme activity at 90°C. The glucanase from the white maize variety, however, was slightly more stable than the glucanases from the yellow variety. The enzymes had activation energies (Ea) of 3.707kcal for white variety and 7.85kcal for yellow variety to hydrolyze the substrate (carboxyl methyl cellulose).

The studies of the pH effect on the activity and stability was done at the optimum temperature for activity of the glucanases. For the white variety, the optimum activity was observed at pH 5 and pH 7.5 (Figure 6). At pH 4 towards the acid side, there was a sharp drop in activity of the glucanase. The glucanase was optimally stable at pH 3, with a drop in stability with increase in pH. Increase in pH from 6 to 9 had minimal effect on the stability of the glucanase. However, it is noteworthy that the glucanase from the white maize variety showed stability of above 70% for the pH range used. The glucanase from the yellow maize variety however had an optimum pH of 5 and was maximally stable at pH 6.

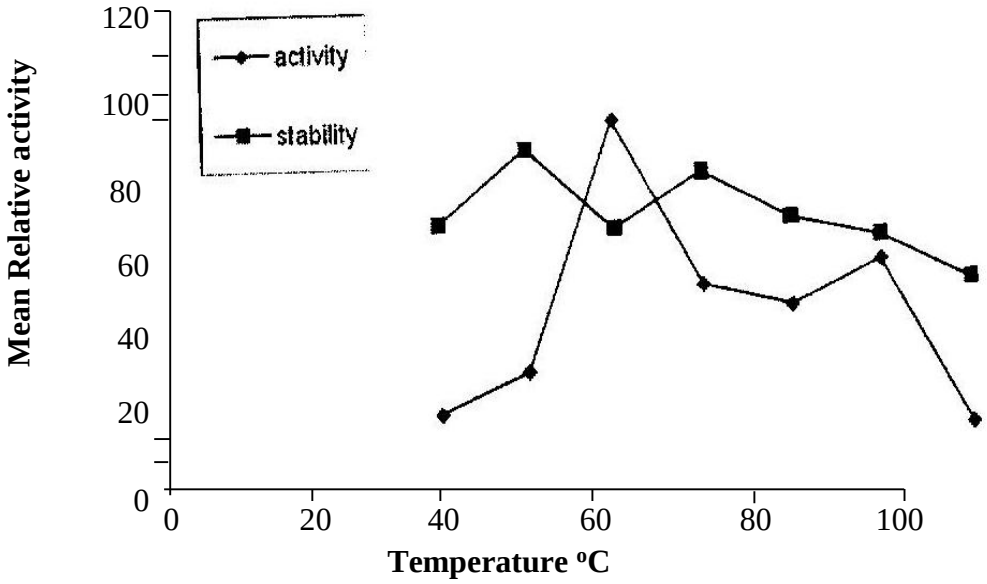

Figure 4: Effect of temperature on the activity and stability of glucanase W

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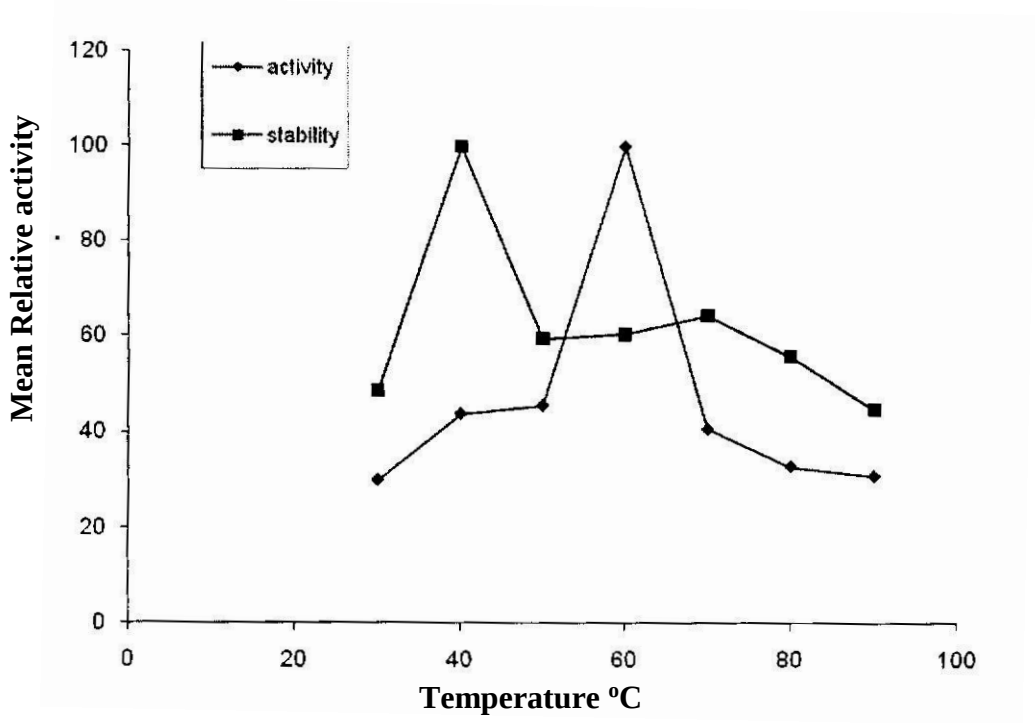

Figure 5: Effect of temperature on the activity and stability of glucanase Y

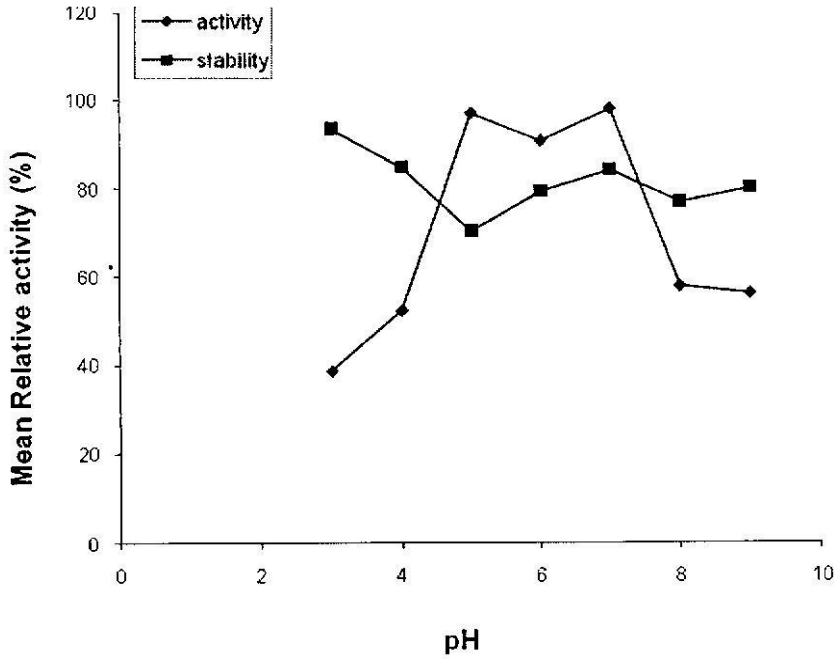

Figure 6: Effect of pH on the activity and stability of glucanase W

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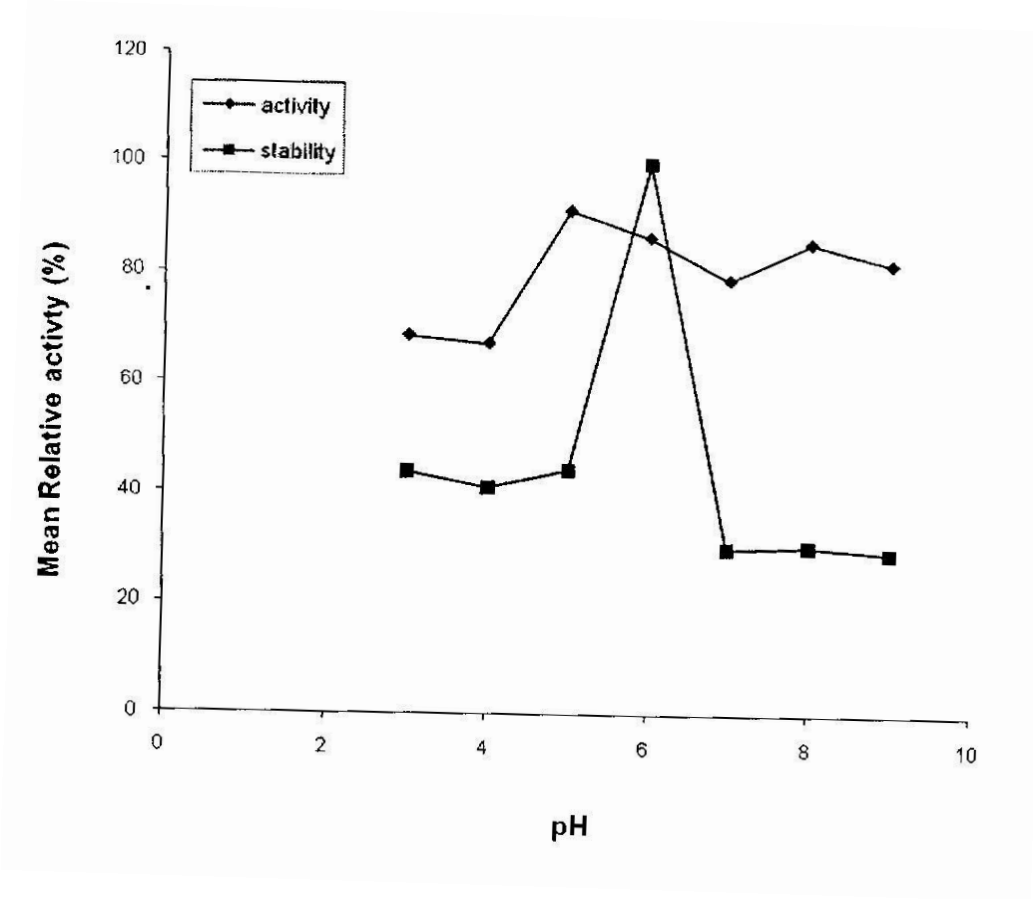

Figure 7: Effect of pH on the activity and stability of glucanase Y

Relative Rates of Hydrolysis of Various Substrates by the Glucanases

The hydrolytic properties of the enzymes were studied at optimal conditions on the substrates CMC and Sigma cell type 20. For the yellow variety glucanase, hydrolytic activity was highest towards Sigma cell type 20 at 297%. For the white variety glucanase, the enzyme hydrolytic activity was also highest towards Sigma cell type 20 at 180% (Table 10).

Table 9: Relative rates of hydrolysis of various substrates by the glucanases from the white and yellow varieties of maize.

	Mean Relative activity (%)	
Substrate	White	Yellow
CMC	100	100
Sigma cell type 20	180.55	297.15

Effect of Metal Ions on the Enzyme Activity

The effects of some divalent cations on the purified glucanases were evaluated and results are shown in Table 13. The glucanase of the white maize variety was appreciably activated by Co^{2+}, Mn^{2+}, Ca^{2+} and Sr^{2+} by 87%, 74%, 37% and 23%, respectively. Barium ion, Cu^{2+}, Pb^{2+}

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and Fe^{2+} showed only a slight increase in the enzymatic activity of the glucanase. Mercury ion, Mg^{2+} and Zn^{2+} had inhibitory effect on the enzyme, with Zn^{2+} having only a relative activity of 3.2%. On the other hand, activity of the glucanase of the yellow variety was only enhanced 44% and 5%, Mn^{2+} and Co^{2+} respectively. All the other ions appreciably inhibited the glucanase the white variety.

Table 10: Effect of metal ions on the glucanase activity for glucanase W and Y

Metal ions (5mm)	Mean Relative activity (%) White	Yellow
None	100	100
S^{2+}	123.25	89.95
Fe^{2+}	100.65	97.65
Cu^{2+}	113.4	63.6
Mg^{2+}	43.73	83.1
Ca^{2+}	137.4	70.9
Zn^{2+}	3.2	77
Mn^{2+}	174.85	144.05
Ba^{2+}	118.95	56.8
Hg^{2+}	90.2	73.6
Co^{2+}	187.05	105.85
Pb^{2+}	103.95	92.8

Dependence of the Glucanase Activity on Substrate Concentration

The effects of substrate concentration on the glucanases were determined for both glucanases using CMC and Sigma cell type 20 (Figures 8 and 9). The glucanase from the white variety displayed an initial turnover rate from 0.195 to 0.3 when the concentration of Sigma cell type 20 was increased from 0.2 to 1.0 mg/ml indicating substrate enhancement. This was also

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observed when CMC was used as a substrate; however, substrate saturation occurred at 0.6 mg/ml of substrate after which there was drop in activity suggesting substrate inhibition. Increase in the CMC resulted in increase in activity till a substrate concentration of 0.8mg/ml (0.235 units) after which activity remained stable with further increase in concentration. When Sigma cell type 20 was used as a substrate, however, the glucanase (Y) displayed a peak activity at 0.6 mg/ml substrate concentration.

The kinetic parameters Km and Vmax of the glucanases from the yellow and white varieties of maize were determined with CMC and Sigma cell type 20 with Lin ewe aver-Burk plots (Figures 10 and 11). For glucanase (white), the km values for CMC and CSC were 0.119 mg/ml and 0.102 mg/ml respectively. The Vmax for CMC and Sigma cell type 20 were 3.7 mg/ml/min and 3.5mg/ml/min respectively. Glucanase (Yellow) had Km values of 0.072 mg/ml and 0.041 mg/ml for CMC and Sigma cell type 20, respectively (Table 11). The Vmax values for CMC and Sigma cell type 20 were 4.0 and 9.0mg/ml/min, respectively. The very low Km values for Sigma cell type 20 and CMC of the glucanase from the yellow variety when compared to that of the glucanase from the white variety of maize indicates a higher affinity of the glucanase (Yellow) to these substrates than the glucanase from the white maize variety.

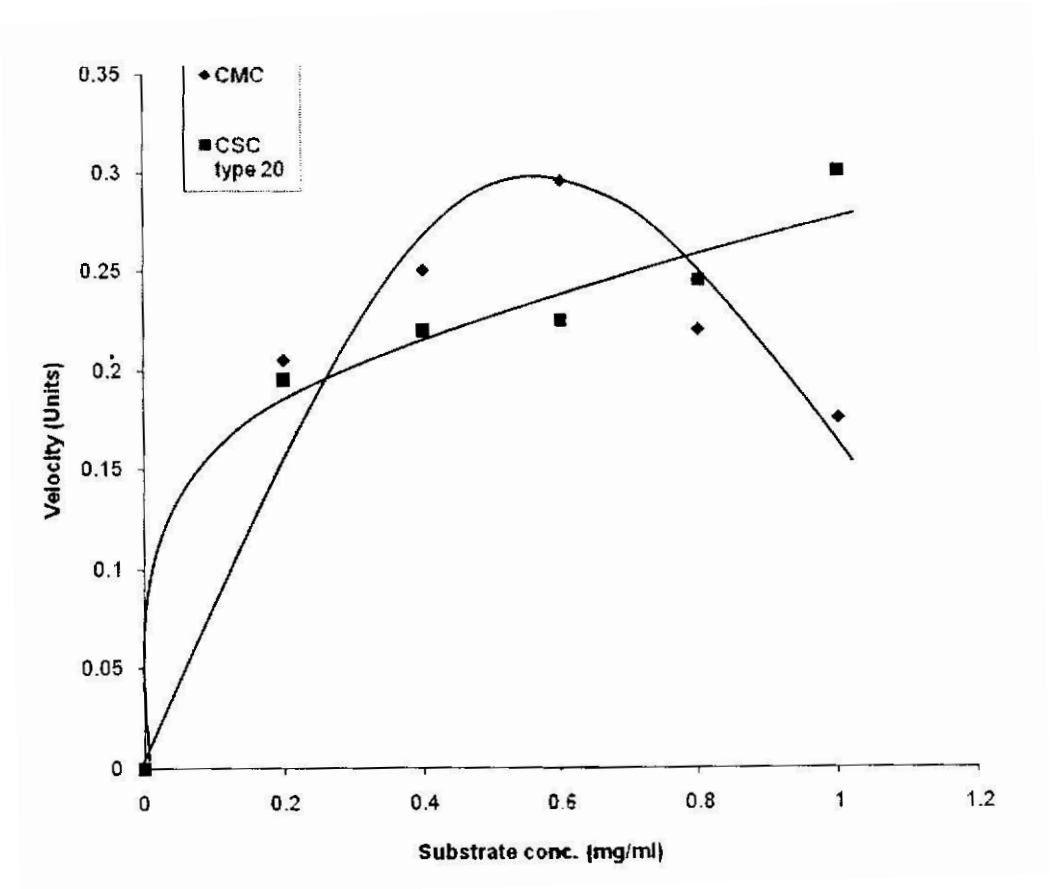

Figure 8: Effect of substrate concentration on the activity of glucanase W

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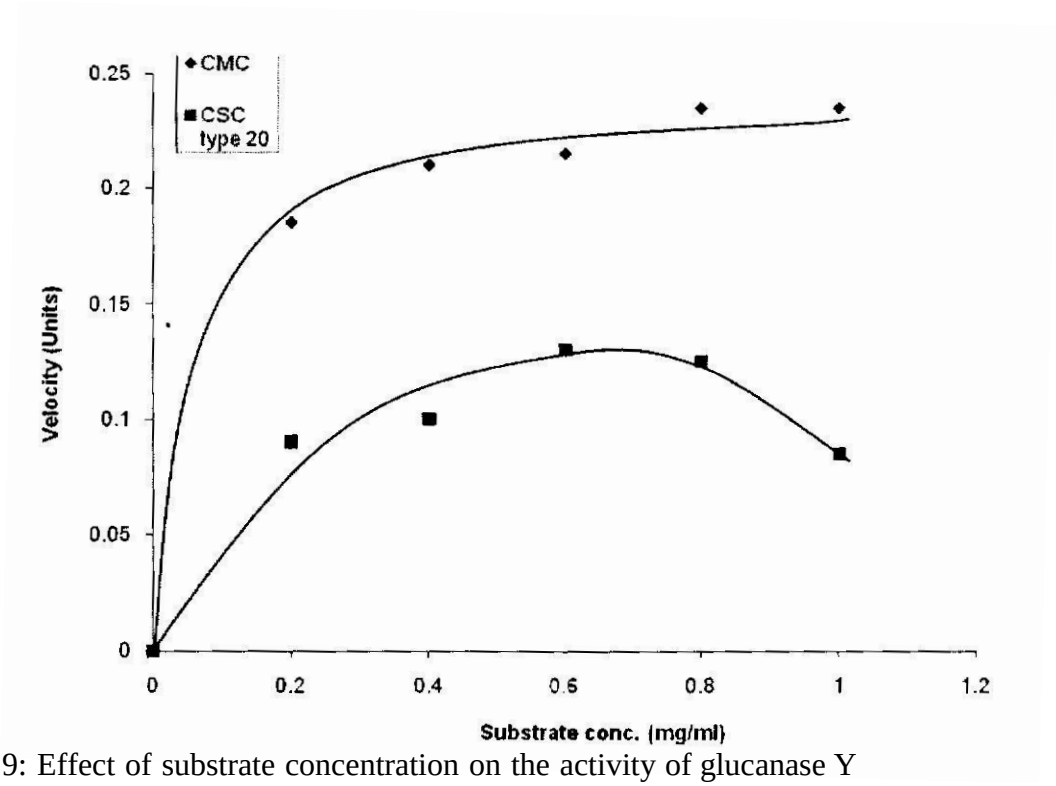

Figure 9: Effect of substrate concentration on the activity of glucanase Y

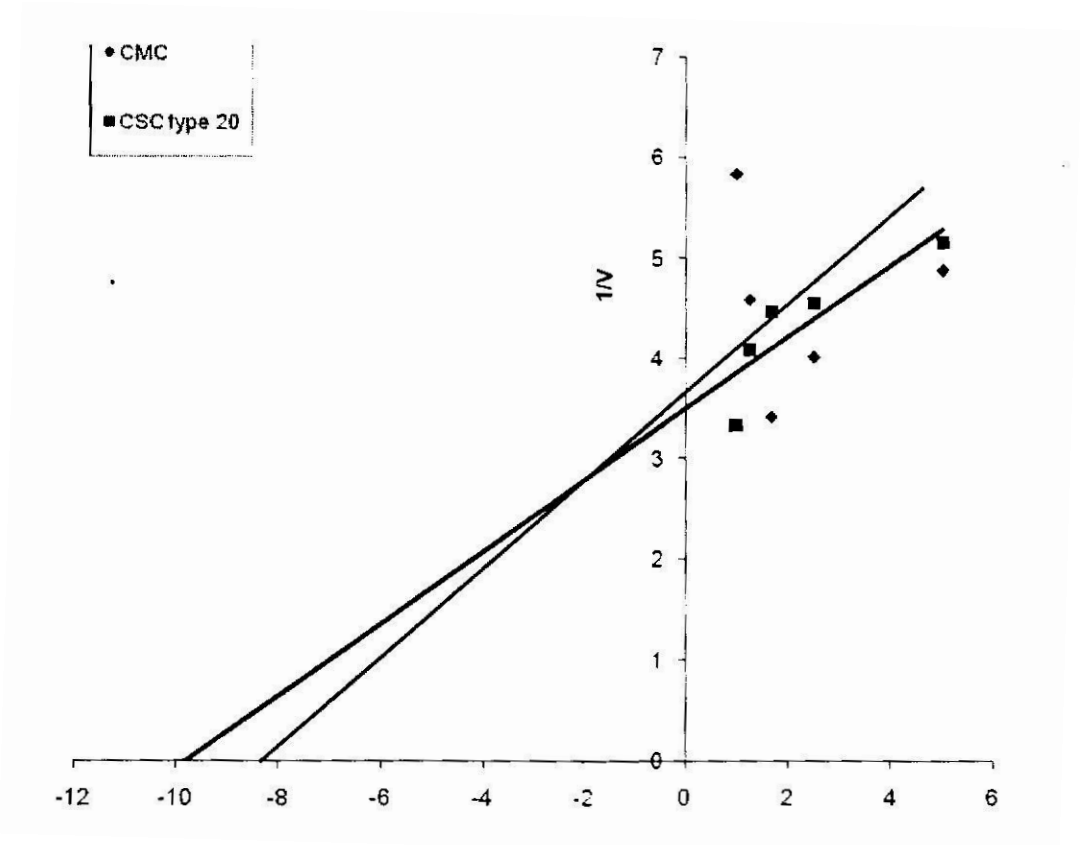

Figure 10: Lineweaver plot of the β-glucanase W for various substrates (CMC, CSC and Starch)

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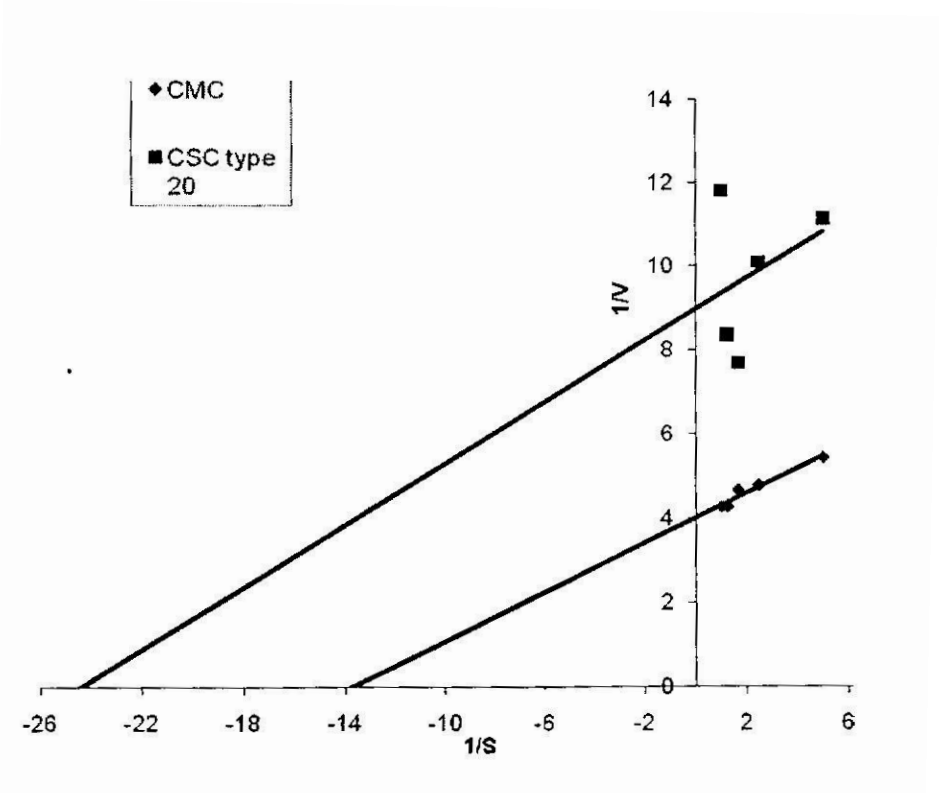

Figure 11: Lineweaver plot of the β-glucanase Y for various substrates (CMC, CSC (type 20)

Table 11: Kinetic Parameters of the Glucanases

Substrate	K_m(mg/ml)		V_{max}(mg/ml/min)	
	Yellow	**White**	**Yellow**	**White**
CMC	0.072	0.119	4.0	3.7
Sigma Cell Type-20	0.041	0.102	9.0	3.5

DISCUSSION

Table 1 shows the results of some chemical and physical properties of the unmalted maize varieties and compares them with those of sorghum (SK 5912). In barley the 1000 kernel weight is a measure of the size of the grains and hence of their extract potential. It is therefore expected that the larger the grain, the greater the 1000-corn weight (Schneider, 1953). Hence the yellow variety had larger 1000-corn value; though not much larger than the white, while the sorghum had correspondingly lower value. The moisture levels of the maize varieties were within the same range of 12 - 14%, but higher than in sorghum (Table 1).The determination of the germinative percentage for barley is to ensure good germination of the grains to be malted. The germinative energies and capacities noted for the maize varieties in the study were about the same as several varieties of sorghum studied by Okon and Uwaifo (1985). However, the average germinative energy and capacities of the varieties had higher values than sorghum (SK 5912). The germinative percentages of both varieties are at variance

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to the findings of Okafor and Aniche (1980) for sorghum, at 98% and that of barley 99.9% (Anon, 1982). The water sensitivity values were similar for both maize varieties, with the yellow maize variety being 82% and the white, 83%.

The findings also show that the maize samples have tolerable levels of broken kernels. Broken kernels are major sources of microbial infection during malting of grains (Agu and Palmer, 1999). The average protein content of the maize grains is similar to that of sorghum (about 9%). The protein percentage for the maize grains in this study is high when placed against the general accepted figure for cereals; about 7.5% (Adair, 1972). It may be that these varieties have been specially bred for high protein content since they are used for food.

The comparatively low fat values of the grains may be important because lipids can destroy foaming potentials of beer. They can accelerate staling. The average fat values for the two varieties of maize are 3.92 %(w) and 4.14 %(y), compared to the value of 4.4% for sorghum. The low values of yellow farz 23 and white farz 34 make them promising candidates for use in the production of brewers' grits.

Table 2 shows the effect of different germination and steeping times on the malting loss of the two varieties. It is interesting to observe from the results of the malting loss, that up to the 3rd day of germination period at different steeping times, the malting losses through the roots and shoots growths were similar. After day 3, except for the 30h steep cycle period of day 4, the malting loss of the white variety was higher than that of yellow. The malting losses for days 4 to 6 were, however, higher in yellow variety. In any case, this is at variance with the report of Eneje *et al.,* (2004). However, it could be concluded that the effect of the steeping periods made no significant difference on the malting loss, but rather day of germination.

Studies on sorghum show that the optimum seedling growth period lies between 4 and 5 days (Okafor and Aniche, 1980; Okon and Uwaifo, 1985). With barley, it can be 5 to 6 days (Hough *et al.,* 1981). Since similar information does not seem to be generally available for maize, a series of malting experimental variables were examined to determine the best conditions on the maize varieties as regards β- glucanase development. To this end, maize malts derived from grains at different steeping cycles were allowed to germinate for 3, 4 and 5 days and analyzed.
Table 3 gives the cold water extract (CWE) of the two maize varieties studied at the same malting conditions. The cold water extract of the maize varieties show that the values increased with longer periods of steeping and germination time, reaching maximum values after 5 day germination and 42 hours of steeping in the two varieties.

The hot water extract values as seen in Table 4 also show increasing trends with the increased steeping time and germination period, and reaching the maximum value at the 5th day of germination. This agrees with the findings of Jayatissa *et al.,* (1980), who observed that the hot water extract obtained from sorghum malt, was greater after 6 days of germination than after 4 days. Similar results were also obtained by Morall *et al.,* (1986), who reported that the extract yield in sorghum malt increased with germination time, reaching maximum value after 6 days germination. The different kilning temperatures had little or no significant contribution on the hot water extract values.

Table 5 has the results of the diastatic power measured in Lintner degrees (%). Diastatic power is not meaningful when malt is rich in β-amylase (Lyons, 1987; Rose, 1987). Tropical

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cereal malts are, however, low in β-amylase when compared with carley malt (Okon and Uwaifo, 1985). From the result, the yellow variety which has cheater embryo growth also exhibited better diastatic values than the white variety. This observation is in agreement with that obtained in sorghum where the yellow or red sorghum variety developed higher enzyme levels than the white sorghum variety Agu and Palmer, 1997).

These observations for sorghum or maize (present study) are at variance with those reported by Daiber (1975) for sorghum, where it was suggested that pigmented coloured) grain sorghum tend to have low enzyme and extract potential because of :.heir high content of polyphenols.
However, the evidence provided by high nitrogen of the yellow variety, declined to support the findings of Agu and Palmer (1999), that a high nitrogen content of the grain is not always linked to high enzyme levels. From the table also, it is observed that the steeping time and germination period, caused corresponding increase in the values of the diastatic power. It can therefore be concluded that there are significant improvements in the diastatic power values as the period of steeping and germination increased.

The development pattern of the glucanases of the maize varieties showed that at an early stage of the germination process both varieties had developed the enzyme (glucanases). The values of the glucanase activity from the first day of germination to the 5^{th} day were closely related (Table 4). This is consistent with the study of the changes in β-glucan content and β-glucanase activity in barley before and after malting. The β-glucanase activity was much lower but detectable in grains and it dramatically increased after malting. It was also discovered that there was a positive and significant correlation between grain and malt β-glucanase activity (Weng *et al.*, 2003). Planas (2000) also reported that the enzymatic depolymerization of 1,3-1,4 β- glucans is an early event in the germination process.

The crude enzymes were developed in this study on the conflicting compromises of the independent experimental variables (steeping time, germination time and kilning temperature), and on the measured dependent variables (diastatic power, cold water extract and hot water extract).
In barley grain, β-glucanase is produced during germination under controlled conditions. The report of the study by Gibson *et al.*, (1999) indicates 1,3-1,4-β- glucanase gene expression indicated in the scutellar epithelium. As germination proceeds, expression is observed in the aleurone layers and progress from the proximal to distal end of the grain (Gibson *et al.*, 1999). The same study also reported that the production of p-glucanase by the barley grain during malting is of particular importance since the endosperm cell walls has a major constituent of the 1,3-1,4-p-glucan and arabinoxylans (Gibson *et al.*, 1999).

The β-glucanase from the white and yellow varieties of maize were both purified by a combination of 5 M sucrose fractionation, ion exchange on Q-Sepharose and gel filtration on Sephadex G-200. The glucanase from yellow maize variety had a specific activity of 1.21 U/mg and was found to be 2.59 times purer than the original crude enzyme and a yield of 0.8% (Table 16). The glucanase from the white maize variety on the other hand had a specific activity of 0.528 U/mg and purification fold of 0.562. The recovery percentage of the β-glucanase (white) was 7.6% (Table 11).

The glucanase of yellow variety appeared on SDS-PAGE as a single migrating protein band clearly corresponding to 30 kDa molecular mass (Fig 3). For the white variety, the purified

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enzyme revealed three (3) migrating protein bands on the SDS- PAGE (Fig 3). The relative molecular weights of the enzyme isoform were 240 kDa, 30 kDa and 17 kDa, as calculated from their migrating distances in relation to the reference proteins. This observation suggests that the enzyme probably has three isoforms, with different charges, or that the glucanase possessed many polypeptides, which were separated by sodium dodecyl sulfate (Okolo *et al.*, 2000).

Woodward and Fisher (1982) were able to characterize two isoforms of 1,3-1,4-β- glucanases produced from a germinated barley grain. The iso enzymes differed in the isoelectric points and molecular weights but have similar substrate specificities and action pattern. Planas (2002) reported the isolation from *Bacillus*, 1, 3-1,4-|B- glucanases (mono domain proteins) with molecular masses of 25 - 30 kDa, basic isoelectric point (pH 7.5 - 9.1), with pH optimum around neutrality (6 - 7.5).

The white variety glucanase demonstrated optimal activity at 50°C and maximal stability between 40°C and 80°C, but retained over 75% of its original activity after 30 min incubation at 80°C at pH optimum of 3.0. However, on both sides of the pH, the activity never fell below 60%. This is similar to that of a *Bacillus sp*. which maintained > 80% activity in the pH range of 7.0 - 12, and temperature optimal activity varying from 45° - 65°C (Planas, 2000). Also a novel 1,3-1, 4-β-glucanase has been produced from *Bacillus halodurans* 125. The glucanase showed pH optimum between 6 and 8 and the temperature optimum was 60°C. After 16h incubation at 50°C and 60°C, the residual activity remained 100% and 50%, respectively (Akita *et al.*, 2005).

The *B. macerans* enzyme is one of the most thermostable *Bacillus* glucanase at neutral pH, with a temperature for optimal enzyme activity at 65°C, and retaining 80% of its activity at 75°C. But it is rather labile in an acidic environment, while the corresponding enzyme from *B. amyloliquefaciens* is less affected by acidic condition with a pH optimum around 6.0 (Planas, 2000). The temperature for hydrolysis of lichenam by a 1, 3- 1, 4-p-glucanase from *Bacteriodes succinogenes* at pH 6.0 was 50°C, as reported by Erfle *et al.,* (1988).

For the yellow variety purified glucanase, it demonstrated optimal activity at 60°C and maximal stability at 40°C, but retained about 60% of its original activity after 30min incubation at 70°C. Its optimum temperature of activity was similar to a β- glucanase produced by *Rhizopus microsporus* var. *microsporus* (Celestino *et al.*, 2006). Maximal enzyme activity was observed in the pH range of 4 to 5; however, no enzyme activity was detected at 45°C and 50°C, indicating that the optimal temperature for the glucan hydrolysis is 60°C.
Other non-Bacillus 1,3-1,4-β-glucanases which are usually larger than *Bacillus* enzymes because of additional domains with different functions have similar biochemical properties. Among the fungal β-glucanases, those of the *Orpinomyces* (26 kDa, pH opt 6, Temp. opt. 45°C) and *Talanomyces emensonii* (40.7 kDA, pH opt 4.8, Temp, opt 80°C) have been characterized (Planas, 2000). The purified glucanase of the white variety demonstrated optimal activity of pH 5.0 - 7.0, but maximal stability at pH 3.0, but retained over 70% of its original activity after 16 hours at pH 3.0 - 9.0 while a glucanase produced by *Rhizopus microsporus* var. *microsporus* (a fungus) had pH optimum at 4.0 to 5.0 (Celestino *et al.*, 2006).

Incidentally, the pH conditions of the mash tun are in the acid range making the purified

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glucanases from maize malts (yellow and white) likely candidates in the breakdown of glucans and possibly other hemicelluloses and their products during both malting and mashing.

On the effects of the metal ions (Table 9), the glucanase activity of the white variety was increased by Co^{2+}, Mn^{2+}, Ca^{2+} and Sr^{2+}, whereas Ba^{2+}, Ca^{2+}, Pb^{2+} and Fe^{2+} were only slightly stimulatory to the purified glucanases. Hg^{2+}, Mg^{2+} and Zn^{2+} had inhibitory effect on the enzyme with Zn^{2+} having the most effect at 3.2%.

The yellow variety was only enhanced by Mn^{2+} and fairly by Co^{2+}. All the other ions appreciably inhibited the glucanase of the yellow variety. There is a striking difference in the preference of the divalent ions or otherwise by the purified glucanases of the varieties. They only seem to agree on the choice of Mn^{2+} and Co^{2+} for the enhancement of the glucanases. Celestino *et al.*, (2005) reported the purification of a fungal glucanase that was highly sensitive to Cu^{2+} (12pM) and fairly sensitive to zinc and manganese, but insensitive to magnesium, calcium and aluminum. Glucanases produced by *Rhizopus oryzae* (Murashima *et al.*, 2002) and *Trichoderma harzianum* (Rena *et al.*, 2003) show similar sensitivity to the divalent metal ion copper.

The yellow variety glucanase exhibited better multifunctional properties as against the glucanase of the white variety. It (yellow) was hydrolytically active to two of the tested substrates, Sigma cell (type 20) and CMC (Table 11). Several multifunctional glucanases (having multiple substrate specificity) have been reported (Kumar and Deobagkar, 1996). The multifunctionality of these enzymes provides cells with a very efficient mechanism to utilize substrates. They help by channeling the reaction intermediate between consecutive enzymes and reducing ne transient time for establishing a steady state (Easterby, 1989).

Multifunctional enzymes (proteins) may result due to gene sharing, gene fustion or exon shuffling and therefore have been a topic of study (Chaudhary *et al.*, 1997). Several bacteria and fungi have produced multifunctional glucanases (Kumar and Deobagkar, 1996), while certain bacteria produce multienzyme complexes such as the "cellulosome" synthesized by *Clostridium thermocellum* (Lamed *et al.*, 1984) and a cellulase-xylanase-β-glucosidase complex produced by *Trichoderma reesei* (Sprey and Lambert, 1983). The multifunctionality of the yellow glucanase may perhaps be due to the presence of a carbohydrate binding domain (CBD) in the glucanase (Kumar and Deobagkar, 1996; Shoseyov *et al.*, 2006). While raw starch substrate oinding domain (SBDs) has been identified in many amylases (Petricek *et al.*, 1992), the presence of CBD is rare (Shoseyov *et al.*, 2006).

Attempts have been made to construct multifunctional glucanases via gene fusion; enzymes in which such artificial genetic engineering has been successfully carried out include an exoglucanase-endoglucanase (Warren *et al.*, 1987), as well as a xylanases-endoglucanase (Tomme *et al.*, 1994) and a chimeric cyclodextrin glucotransferase (Kaneko *et al.*, 1990).

CONCLUSION AND RECOMMENDATION

Glucanases are immensely important in brewing industry as a result of the role they play in reducing mash viscosity and turbidity, thereby increasing filtration rates and decreasing the appearance of gelatinized precipitates in the finished beer (Celestino *et al.*, 2006). β-Glucanases purified and characterized for yellow and white varieties of maize showed a peak

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production profile of glucanases on the 5th day of germination, 42 hour steep cycle and 50°C kilning temperature. The glucanases from the white and yellow varieties showed optimum temperature for activity at 50°C and 60°C respectively. This is advantageous since during mashing the enzyme will be able to remain active.

In this study, it could be concluded that none of the varieties, either white or yellow should claim exclusive better quality. Proper manipulations of some experimental variable (independent) during malting will enhance β-glucanase development in either of the varieties. Malting maize at the conditions stated above will definitely improve the β-glucanase content. Due to poor enzyme development generally during malting, large-scale brewing with maize malt definitely becomes difficult. But more effort should be intensified using the relatively new science of recombinant DNA technology to produce transgenic maize grains.

One important aim of genetic engineering is the production of hybrid enzymes with improved thermostability. Genetic engineering therefore can also be applied to improve β-glucanase content of maize and it becomes a biotechnological target both to broaden their usefulness in beer production and to enhance the development of other equally important brewing enzymes. If and when this is achieved, using maize malt in brewing therefore will reduce the production cost as well as capital outlay. Also large scale farmers of maize will have good bargain for their usually bumper produce - maize.

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