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Extraction of Enzymes from Improved Sorghum Cultivars (SSV98001, SSV98002 and SK5912) and their Applications in the Mashing Studies of a Nigerian Local White Sorghum Variety

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Abstract

Three improved Nigerian sorghum cultivars (SSV98001, SSV9800, and SK5912) were malted and their brewing qualities evaluated. The values for germinative capacity were 99, 97 and 69%, and germinative energy 99, 99 and 77% for SSV98001, SSV98002, and SK5912 respectively. The percentage protein decreased from 7.2, 7.8, 8.4 to 3, 3.6 and 4% for SSV98001, SSV98002 and SK5912 respectively after malting. Diastatic power, cold water extract and hot water extract values increased from 2.92(°L), 15.68(kg/°L) and 25.58(kg/°L) to 11.24(°L), 30.54(kg/°L) and 104.56(kg/°L) for SSV98001; 3.42(°L), 16.32(kg/°L), and 25.71(kg/°L) to 12.08(°L), 112.1(kg/°L) and 32.42(kg/°L) for SSV98002, 4.12(°L), 19.4(kg/°L), and 29.8(kg/°L) to 14.21(°L), 120.2(kg/°L) and 41.4(kg/°L) for SK5912. The wort produced from temperature programmed mashing using crude enzyme showed values of reducing sugars as 40mg/ml glucose, 51mg/ml maltose, for SSV98001, 42mg/ml glucose and 32mg/ml maltose for SSV98002; 68mg/ml glucose, and 54mg/ml maltose for SK5912. While worts produced without enzyme had values of 48mg/ml glucose and 39mg/ml maltose for SSV98001; 49mg/ml glucose and 40mg/ml maltose for SSV98002, 50mg/ml glucose and 41mg/ml maltose for SK5912. Amylase enzyme with optimum temperature of activity 60^{o}C and *p*H of 5.0 were extracted and purified from SSV98001 and SSV98002 using gel filtration. Effects of metal ions on the activity of purified enzyme showed that the enzyme had highest activity in the presence of Ca^{2+}. The use of purified enzyme from improved cultivars in mashing trial with Nigerian local white variety using different regimes showed that temperature programmed mashing with enzyme from the cultivars produce high value of glucose, maltose, and maltotriose and lower dextrins than without enzymes. But single decoction mashing with enzymes from the cultivars produced the highest value of sugars and dextrins. Multiple comparisons of sugars produced by the improved varieties and that from local variety showed a significant difference among the cultivars. This shows that effective beer brewing can be achieved using the local white variety in decoction mashing than in temperature programmed mashing.

Keywords

Mashing

Sorghum cultivars

Introduction

Barley malts have formed the chief and most dependable raw material for beer production (Agu and Obanu, 1991) as a result of development of wider array of enzymes during malting, low starch gelatinization temperature among others. In tropical brewing, the use of sorghum as a replacement for barley in beer brewing has been effective (Archibong et al., 2009).The grain sorghum, known as (*Sorghum bicolor* L.) Moench is the fifth most important cereal after rice, wheat, maize and barley, and it contributes significantly to the protein and energy requirements for millions of people, especially for the poor persons in Africa, Asia and semi-arid tropics (Elkhier and Hamid, 2008). It has distinct advantage compared to other major cereals for being drought resistant.

Sorghum plays a crucial role in world economy as it has contributed to rural food security. It has an emerging market for its use in the brewing industry as it constitutes cheaper source of extracts for that industry and therefore, offers potential employment to all stakeholders along the supply value chain (i.e.) farmers, marketers, maltsters, brewers for both alcoholic and non-alcoholic beverages. In Africa, the cereal sorghum is malted widely to provide an important raw material (amylolytic enzymes that hydrolyse the starchy endosperm of the sorghum grain into soluble sugars) used in brewing. In southern Africa, approximately 200 000 tonnes per annum of malted sorghum are used in the production of traditional (opaque) sorghum beer. World production of sorghum in 2007 was estimated at 64 million tons (FAO, 2009), In South Africa approximately 166 000 tons were planted in the 2007/2008 season (FAO, 2009). From 2003-2009, an average of 231 000 ton where produced annually in South Africa, which is approximately 2.6% and 12.6% of the average of maize and wheat production in south Africa respectively (FAO, 2009). There have also been growing interests in the use of malted sorghum as a close substitute in the brewing of clear lager type beers. The potential of sorghum as an alternative substrate for beer brewing has long been recognized over six decades ago (Odibo et al., 2002). Research studies with sorghum as a brewing raw material are progressing rapidly and making a great impart in brewing despite the earlier misunderstanding that malted sorghum produces insufficient hydrolytic enzymes. Varietal difference, malting and mashing temperatures employed in the studies of sorghum in the past were major contributory factor that complicated the understanding on the physiological behaviour of sorghum during malting and brewing.

Over 10,000 varieties of sorghum exist and more new varieties of sorghum are developed through continuous plants breeding research. Among these new varieties are some whose malts possess beneficial qualities for beer brewing, such as good diastatic power, alpha and beta-amylase activities and extract recovery (Owuama, 1999). During malting (i.e. the germination of cereal grain in moist air under controlled conditions) a number of hydrolytic enzymes develop and degrade the reserve nutrients of the endosperm. Since proper enzyme development ultimately leads to complete saccharification and high extract yield, the development of the enzymes α - and β –amylase during malting are of critical importance as they initiate the breakdown of starch granules during germination and further hydrolyse starches to fermentable sugars during the beer brewing process (Odibo et al., 2002). The aims of the present study are:

- To study the malt and wort properties of the three improved sorghum varieties (SSV98001, SSV98002 and SK5912) using different mashing regimes.

- To extract amylase enzyme from the improved sorghum varieties their application in the mashing studies of Nigerian local white sorghum varieties.

Materials and methods

Sample collection and reagents

The improved sorghum varieties (SSV98001, SSV98002 and SK5912) were obtained from Institute of Agricultural Research, Ahmadu Bello University, Samaru, Zaria, Nigeria. The Nigerian local white variety was obtained from Eke Awka market, Anambra State, Nigeria. The *Aspergillus carbonarius* was isolated from rotten cassava tubers and maintained on potato dextrose agar (PDA) slant at 4°C and used for the enzyme production. All reagents used were of analytical grades.

Steeping

Three hundred (300) grams of each grain sample (SK5912, SSV98002 and SSV98001) were washed several times in tap water. A 24h steeping regime was

used (12h water steep, 2h air-rest, 10h water steep) using a steeping ratio of 1:2 (grain : water) at 25ºC.

Germination

Germination was carried out at 30ºC for five (5) days in dark germination chambers with twelve (12) hourly spray of 10ml water to prevent drying out. Germinated samples were collected everyday for kilning and subsequent analysis.

Kilning

Germinated grains were kilned in an oven at 50ºC for 24h.

Grain analysis: Determination of germinative capacity

This was determined according to the Recommended Methods of Analysis of the Institute of Brewing (IOB), 1991 – Hydrogen Peroxide and Peeling Reference Method.

Determination of germinative energy/water sensitivity

This was determined according to the recommended methods of analysis of the Institute of Brewing (IOB), 1991.

Germinative energy = G.E. (4ml) %

Water sensitivity = W.S. (8ml) %

Determination of thousand (1000) corn weight

This was carried out according to the Recommended Methods of Analysis of the Institute of Brewing (IOB), 1991.

Determination of specific gravity

This was determined according to the official methods of Association of Official Analytical Chemists (A.O.A.C), 1980.

Determination of total nitrogen content (TSN)

According to the recommended methods of analysis of the Institute of Brewing (IOB), revised 1991.

Determination of cold water extract (CWE)

According to the recommended methods of analysis of the Institute of Brewing (IOB), 1991.

Determination of hot water extract (HWE)

This was carried out according to the decantation method of Etokakpan (1992).

Estimation of α-amylase and diastatic activity

These were determined according to the simple diamylase procedure described by Etokakpan and Palmer (1990).

Determination of reducing sugar

This was determined using the method of Miller (1959).

Determination of crude protein

The micro-Kjeldahl method for protein determination is employed for protein determination.

Enzyme extraction, purification and analysis

Ten (10) grams of grain sample was blended with 50ml of sodium acetate buffer, pH 5.5 at room temperature, filtered with muslin cloth to remove suspended particles, followed by centrifugation at 10,000g at -2^{o}C to remove insoluble materials. The supernatant was brought to 40 – 70% (NH_4) SO_4 saturation and left overnight in a refrigerator. Thirty percent (30%) was discarded and 70% was centrifuged and the precipitate collected was re-dissolved in 1ml of phosphate buffer (pH 6.5) containing 1mm $CaCl_2$ buffer. The concentrated crude enzyme was passed through a G-75 Sephadex column and was eluted with phosphate (pH 6.5, 50mm $CaCl_2$) buffer. Fractions were collected and assayed for protein and amylase activity.

The protein concentration was determined by Bradford method

The peaks of different fractions were pooled together. The protein concentration of the pooled fraction and other fractions was determined by Bradford method (the OD was extrapolated from a standard curve prepared using bovine serum albumin standard).The amount of Protein content in the enzyme extracts was estimated

using bovine serum albumin as the standard. Enzyme activity is expressed as specific activity which is equivalent to 1/mg protein. All the experiments were carried out in triplicates and the standard error was calculated. 1ml of Bradford was added to 0.1ml of sample or standard at room temperature and 0.1ml, of valued concentration of standard bovine serum albumin (BSA). OD was taken at 595nm, then the protein concentration of the sample was extrapolated from the standard curve of the BSA.

The amylase was determined by DNS method, according to Miller (1959).

Effect of temperature on enzyme activity: This was carried out by adding 0.1ml of the enzyme to 0.2ml phosphate buffer (6.5) followed by the addition of 0.5ml of starch, 0.1ml NaCl and shaken. Then, incubated for 20mins at various temperatures,(40°C, 50°C, 60°C, 65°C, 70°C, 80°C) with the addition of 0.1ml NaOH. The reaction is stopped by the addition of Dinitrosalicylic acid (DNS) and heated in boiling water for 10mins, then the absorbance checked at 540 nm.

Effect of pH on enzyme activity

This was carried out by incubating the enzyme in 0.2ml phosphate buffer at various pH (4.0, 5.0, 6.0, 6.5, 7.0, 8.0) followed by the addition of 0.5ml of starch and shaken. Also 0.1ml NaCl was added and the enzyme mixture incubated for 20mins, followed by the addition of 0.1ml NaOH and the reaction stopped by the addition of Dinitrosalicylic acid (DNS). This is heated in boiling water for 10 mins and allowed to cool, the absorbance was taken at 540 nm.

Effect of metal ions on enzyme activity

The substrate was prepared using 0.1g in 10ml (1%) phosphate buffer and heated to gelatinize, 5mM of each metal salt was prepared dissolving (molar mass of salt/1000 X 5)g in 1000ml of water at different concentration (2g/l, 5g/l and 7g/l) were prepared. The effect of salts ($CaCl_2$, $CuSO_4$, $MgSO_4$, $FeCl_2$ and $MnCl_2$) on enzyme activity was determined. A control was set up with 0.2ml of substrate plus 0.2ml of enzyme. 0.2ml of enzyme was added to 0.2ml of substrate plus 0.2ml of 5mM of salt. This was incubated at 55^{o}C for 10 mins, reaction was stopped with 0.4 DNS, boiled for 10min, cool and 2ml of water added. Absorbance was taken at 540nm.

Estimation of glucose, maltose and maltotriose using Merck silica G60 TLC

The wort was estimated for approximate soluble sugar content by measuring the refractive index using a refractometer. This estimate was used to determine the amount of carbohydrate to be spotted on the TLC plates for quantitative purposes which is in range of 1- 25 µg. De-fatting was done by mixing 50µl with 100l of chloroform in a micro-tube and capped. It was placed on a vortex shaker for one minute and centrifuged at 6,500rpm for 5min to separate the chloroform from the wort. The lower chloroform layer was discarded. Dilution or concentration of wort was carried out to acceptable spotting concentrations of sugars within the volume of 0.5 to 1.0 µl. Spotting points on Merck G60 silica TLC plates were designated at intervals 0f 1.5cm and 2cm from plate edge. Appropriate standard sugar solutions of expected sugars in the range 1 to 25 µlg were spotted to give at least four concentration variables which will be used to prepare a standard calibration curve. The dried spotted sugars were developed and used to estimate concentration of unknown sugars.

Statistical tool used: Results were analysed using the Two Way ANOVA.

Results

Table 1 shows the characteristics of un-malted sorghum grains. SSV98001 has the highest moisture content while SSV98002 had the least. The grains had high germinative capacity, germinative energy and water sensitivity. SSV98002 had the highest tannins, 1000 corn weight, cold and hot water extract while SSV98001 had the lowest. SK5912 also had the highest value for diastatic power and protein while SSV98001 had the least values.

Table 2 shows the characteristics of malted grains (SSV98001). The cold water extract, hot water extract and diastatic power increased gradually from the first day to the fifth day. Table 3 shows the characteristics of malted grains (SSV98002). The moisture content, protein, cold water extract, hot water extract and diastatic power gradually increased from the first day of germination to the fifth day. Table 6.0 shows the characteristics of malted grains (SK5912). The moisture content, protein, cold water extract, hot water extract and diastatic power gradually increased from the first day of germination to the fifth day.

Table 1. Characteristics of un-malted sorghum grains (SSV98001, SSV98002 and SK5912 varieties).

Parameters	Sorghum varieties		
	SSV98001	SSV98002	SK5912
Moisture content (%)	14.4	12.8	13.4
Germinative capacity (%)	99	97	69
Germinative energy (%)	99	99	77
Water Sensitivity (%)	98	93	86
Presence of tannins (%)	5	8	6
1000 Corn weight(g)	34.1	42.7	35.5
Protein (%)	7.2	7.8	8.4
Cold water extract (Kg/°L)	15.32	16.12	15.86
Hot water extract (Kg/°L)	25.21	25.51	25.19
Diastatic power (°L)	2.88	3.11	3.24

Table 2. Malt characteristics of SSV98001.

Parameters	Days of germination				
	1	2	3	4	5
Moisture Content (%)	35.2	26.4	18.8	17.8	16.2
Protein Content (%)	7.2	5.4	4.1	3.2	3
Cold Water Extract (%0	15.68	16.24	18.98	26.82	30.54
Hot Water Extract (%)	25.58	28.88	48.64	84.96	104.56
Diastatic Power(°L)	2.92	3.1	4.62	9.32	11.24

Table 3. Malt characteristics of SSV98002.

Parameters	Days of germination				
	1	2	3	4	5
Moisture content (%)	32.8	24.6	18.1	16.8	16.4
Protein content (%)	7.8	6.7	4.4	4.1	3.6
Cold water extract (%)	16.32	18.64	27.84	30.11	32.42
Hot water extract (%)	25.71	29.2	50.46	90.12	112.1
Diastatic power (°L)	3.42	3.86	5.84	10.21	12.08

Table 4. Malt characteristics of SK5912.

Parameters	Days of germination				
	1	2	3	4	5
Moisture content (%)	34.4	25.8	18.6	18.4	16.4
Protein content (%)	7.9	7	4.8	4.4	4
Cold water extract (%)	19.4	24.6	30.7	38.5	41.4
Hot water extract (%)	29.8	36.2	58.4	98.6	120.2
Diastatic power (°L)	4.12	4.62	6.81	11.43	14.21

Table 5. Wort Analysis of sorghum worts (SSV98001, SSV98002 and SK5912) from temperature programmed mashing using fungal α-amylase 'termamyl'.

Samples	Parameters		
	Glucose (mg/ml)	Maltose (mg/ml)	Protein
SSV98001			
With enzyme	40	51	1.3
Without enzyme	48	39	1.8
SSV98002			
With enzyme	42	32	1.4
Without enzyme	49	40	1.8
SK5912			
With enzyme	68	54	1.4
Without enzyme	50	41	2.1

Fig. 1 shows the temperature-programmed mashing regime, with and without enzymes, for SSV98001, SSV98002 and SK5912. Table 5 shows the wort analysis of SSV98001, SSV98002 and SK5912 from temperature-programmed mashing with exogenous enzyme (fungal α-amylase 'termamyl')

Fig. 1: Temperature programmed mashing regime for sorghum malts (SSV98001, SSV98002 and SK5912).

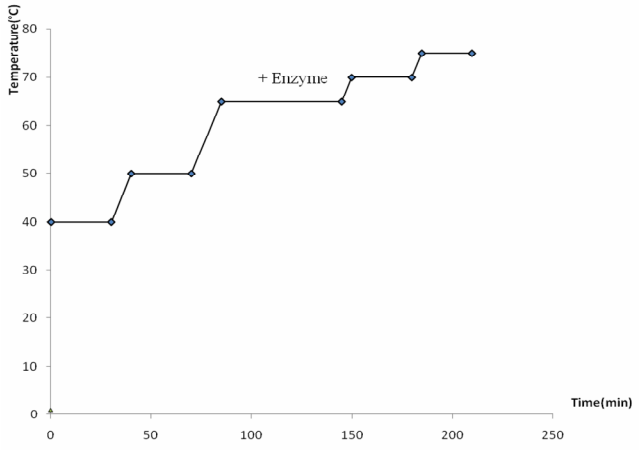

Fig. 2: Single decoction mashing regime for sorghum malts (SSV98001, SSV98002 and SK5912).

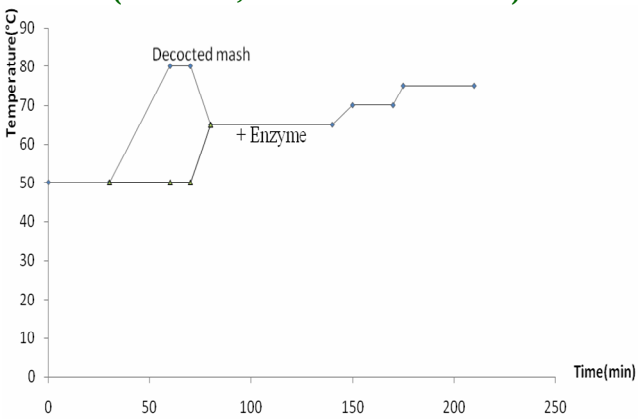

Fig. 3: Protease activity profile for SSV98001.

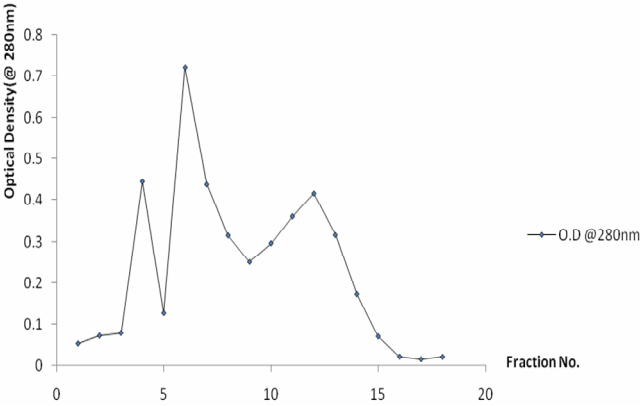

Fig. 4: Amylase activity profile for SSV98001.

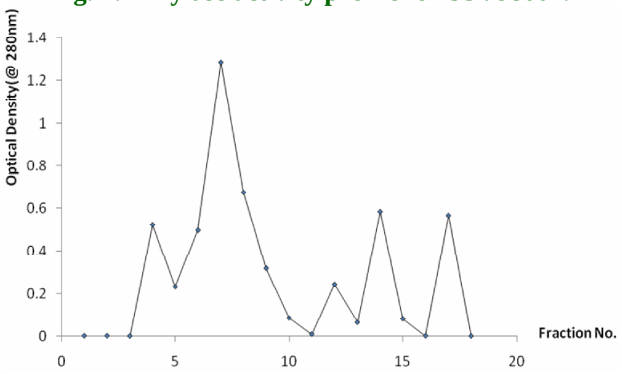

Fig. 5: Protease activity profile for SSV98002.

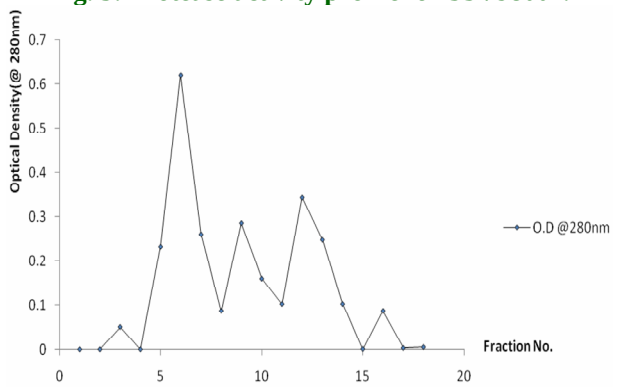

Fig. 6: Amylase activity profile for SSV98002.

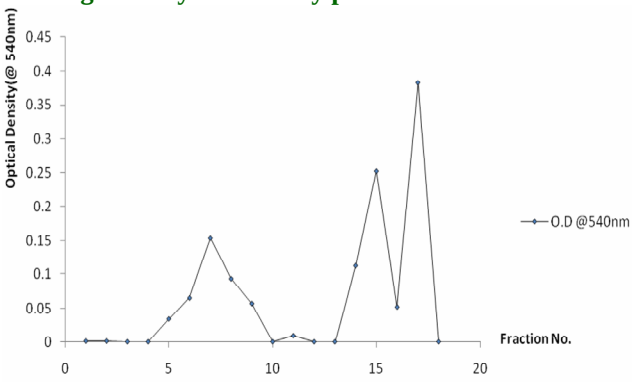

Fig. 7: Effects of pH on enzyme activity from SSV98001 and SSV98002.

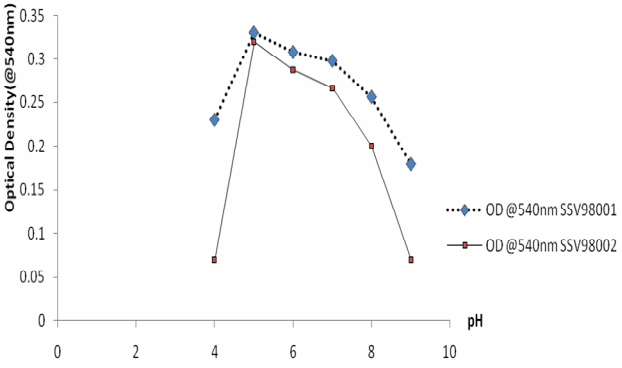

Fig. 8: Effects of temperature on enzyme activity from SSV98001 and SSV98002.

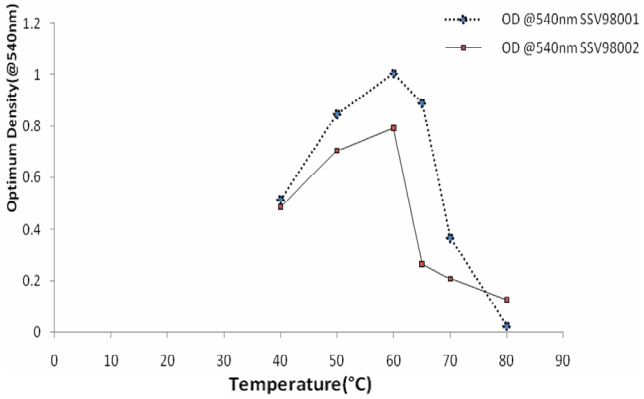

Fig. 9: Effects of metal ions on enzyme activity for SSV98001 at different concentrations.

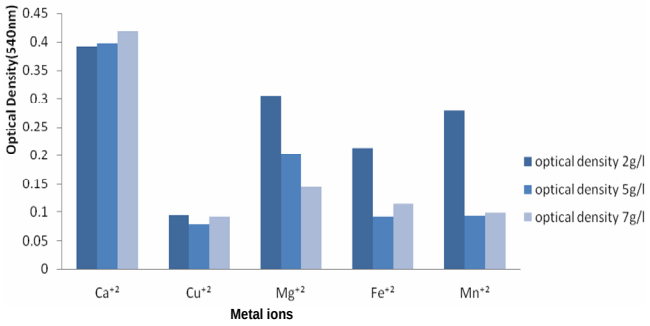

Fig. 10: Effects of metal ions on enzyme activity for SSV98002 at different concentrations.

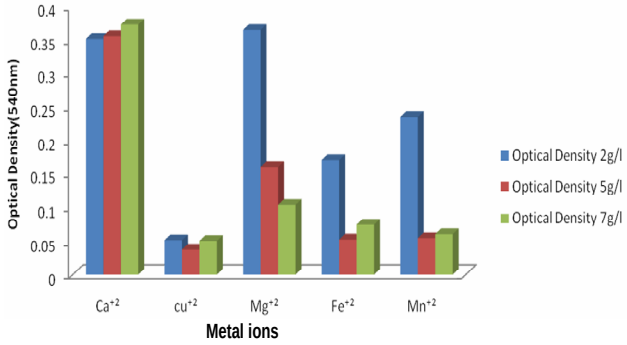

Fig. 11: Temperature programmed mashing regime for sorghum malts.

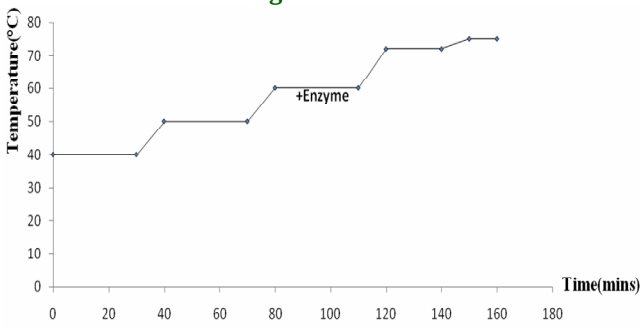

Fig. 12: Single decoction mashing regime for sorghum malts (white local variety).

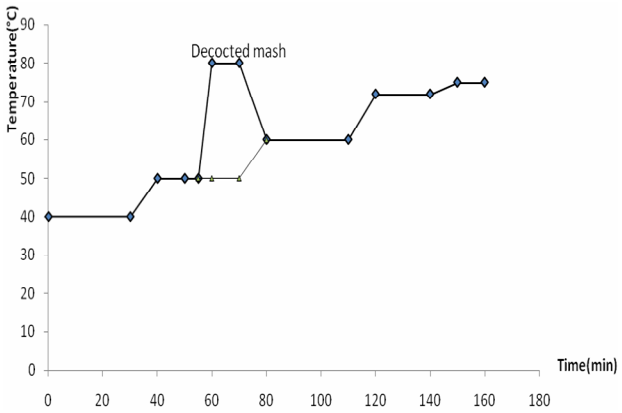

Fig. 2 shows the decoction mashing regime with and without the produced enzyme addition. Table 6 shows the wort analysis from the decoction mash without the produced enzyme. SSV98002 produced the highest glucose, while SSV98001 produced the highest maltose. Figs. 3, 4, 5 and 6 show the elution profiles of the enzyme purification processes from the improved varieties using G-75 Sephadex column while the data obtained on pH and temperature effect are provided in Figs. 7, 8, 9 and 10; Fig. 11 shows the temperature-programmed mashing regime used for the mashing process of local white sorghum malt. Fig. 12 shows the single decoction mashing regime used for the mashing process of local white sorghum malt.

Discussion

The diastatic power of the improved sorghum grains was consistent with other reports of Eneje et al. (2012), Agu and Palmer (1998). The result of wort analysis of improved sorghum varieties using temperature programmed mashing regime presented yielded higher values of reducing sugars. The mash with and without the crude enzyme yielded more reducing sugars in SK5912, while SSV98001 produced lower sugars. However, the maltose content of wort from SSV98001 was higher.

The difference in reducing sugars during malting and mashing can be attributed to different diastatic activities of exogenous enzymes and the amount of starch available for hydrolysis according to Okafor and Aniche (1980). Wort from SSV98001 single decoction mashing without enzyme produced more maltose than glucose, this was probably brought about by increased mash maltogenic amylase activity (Novellier, 1960).

Table 6. Analysis of sorghum worts (SSV98001, SSV98002 and SK5912) from single decoction mashing using enzyme complex.

Samples	Parameters		
	Glucose(mg/ml)	Maltose(mg/ml)	Protein
SSV98001			
With enzyme	56	45	1.8
Without enzyme	40	51	2.5
SSV98002			
With enzyme	52	42	2.2
Without enzyme	50	40	3.5
SK5912			
With enzyme	68	54	2.1
Without enzyme	48	39	2.8

Table 7. The analysis of local sorghum wort from the single decoction and temperature-programmed mashing regimes.

Samples	Parameters (in mg/ml)			
	Glucose	Maltose	Maltotriose	Dextrins
White variety				
Temperature programmed mashing				
Purified enzyme from SSV98001	84	24.4	13.6	11.5
Purified enzyme from SSV98002	77.8	26.3	14.7	10.7
Without enzyme	59.5	27	14.5	19.2
Single decoction mashing				
Purified enzyme from SSV98001	121.2	72.3	41.6	26.6
Purified enzyme from SSV98002	77.5	49.8	22.6	12.4
Without enzyme	60.4	38.4	16.4	10.2

Ca^{2+} and Mg^{2+} showed better activity than other ions. Amylase is a metalloenzyme which contains at least one activating Ca^{2+} ion. The affinity of Ca^{2+} to amylase is much stronger than other ions (Gupta et al., 2003). The influence of temperature on the produced alpha amylase shows that the enzyme activity increased progressively with increase in temperature from 40°C with its optimum activity at 60°C. The effect of pH on the purified enzyme showed that the enzyme specific activity reached a maximum value at pH 5.0. Some studies have found barley malt worts to contain more maltose than glucose (Briggs et al., 1981) while others have reported that sorghum malt worts contain similar levels of glucose and maltose. The difference observed in the proportions of glucose and maltose sugars in sorghum and barley malt worts has been attributed to the low levels of β-amylase in sorghum malt. Other authors (Taylor and Robbins, 1993) have attributed the high level of glucose found in sorghum malt wort to the catalytic activity of α-glucosidase. The main reason for the limitation of maltose production in sorghum malt wort is likely to be inadequate gelatinization of sorghum starch rather than inadequate levels of hydrolytic enzymes (Agu and Palmer, 1998). Results obtained by Agu and Palmer (1998) showed that different sorghum varieties malted and mashed under similar conditions presented wide variations in their sugar profiles due to seasonal and processing differences. However, in this research, the use of enzymes derived from the improved varieties gave high values of reducing sugars when used in the mashing of local white variety.

Conclusion

The use of improved sorghum cultivars in brewing is recommended as these cultivars have been shown to improve reducing sugar production, reduced protein content and enhanced brewing operation. Results obtained in this study showed that those improved sorghum cultivars can serve as a good sources of hydrolysing enzymes, and with the use of suitable mashing regime can produce improved results in brewing operations using local varieties.

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