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RESEARCH ARTICLE

A PROCEDURE TO DETERMINE THE GERMINATION PERIOD FOR OPTIMUM AMYLASE ACTIVITY IN MAIZE MALT CRUDE EXTRACTS FOR THE ARTISANAL PRODUCTION OF MALTOSE SYRUP FROM FRESH CASSAVA STARCH

Edmund AMEKO*, Sylvester ACHIO, Saeed ALHASSAN, George GYASI and Rhodaline SACKEY

Department of Science Laboratory Technology, Accra Polytechnic, Box 561, Accra, Ghana

Abstract

The study describes a timed colorimetric coupled enzyme assay for determination of amylase activity using starch as substrate. The method is simple for use by artisans to determine germination period for optimum amylase activity in maize malt crude extracts for maltose syrup production. The activity of amylase enzyme which released Glucose from standard starch solution is estimated by timing the decolourisation of standard acid permanganate solution by the released glucose. The germination period for optimum amylase activity in maize malt crude extracts from batches of Obaatanpa-Pokuase, Obaatanpa-Fumesua, Obaatanpa-MOFA, Mamaba and Golden Jubilee local maize having germinative capacities ≥ 90% were determined. Peak periods (P_1 and P_2) of amylase activity occurred; on germination days 4 and 9 for Obaatanpa-Pokuase, and 5 and 10 for the other varieties. Decolourisation rates at P_2 were 1.51 – 2.01 faster than at P_1 with Obaatanpa-MOFA having the fastest decolourisation rate at P_2. Obaatanpa-MOFA malt crude extract was subsequently used to process fresh Cassava (*Esiaba var.*) to brown maltose syrup with pH 4.60 – 5.30, 0.45% sulphate ash, DE 40%, and 33.4% water content. Syrup yield was 23.59% (mL/g wet starch) with a malty aroma and flavour.

Key words: Starch, Malt, Amylase, Hydrolysis, Maltose syrup

Introduction

There has been for over thirty years now in South East Asia an artisanal process for producing maltose sweetener from starch. The process uses amylases in cereal seedlings to break down starch to sugars (Quynh and Cecil, 1996). It is possible to replicate the East Asian success story in other countries, especially in Africa where cassava starch is readily available.

During the production process a suitable batch of rice seeds are germinated for about 10 days until the shoots are 10 cm long and the main root about 6 cm long. The whole germinated plant is crushed or chopped and used as a crude source of amylase for the conversion of starch into maltose. The resulting sweet liquid is heated and evaporated in open pans to obtain the maltose syrup which is then packaged in bottles and plastic bags (Quynh and Cecil, 1996).

*Corresponding author: edmundameko@yahoo.com

In order to minimize the quantity of malted cereals used it is necessary to determine the optimal stage of seedling development at which optimum amylase activity occurs.

According to Cecil (1995), Shaw and Chuang (1982) showed that rice amylases reached maximum activity after eight days. However, the time for optimal enzyme activity would vary between varieties. According to Rahman *et al.*, (2007) amylase and invertase activities of three Mungbean varieties increased at 24 hours and then decreased drastically at 96 hours of germination. However, protease activity increased at 48 hours of germination and then decreased. Gimbi and Kitabatake (2002) showed that the highest α-amylase activity occurred in malt flour of African finger millet (*Eleusine coracana (L) Gaertener*) when the cereal was germinated at 15°C for 9 days or at 20°C for 6 days, while the highest β-amylase activity occurred when the germination was for 5 days at 30°C. Mohamed *et al.,* (2009) indicated that a local Saudi Arabian wheat (*Triticum aestivum*) variety (*Balady*) showed high levels of amylolytic activities at different stages of germination and the activity of α- amylase increased from day 0 to day 6 of germination followed by decrease of activity till day 16. These reports from Cecil (1995), Rahman *et al.*, (2007), Gimbi and Kitabatake (2002), and Mohamed *et al.*, (2009), show that variations occur in the activities of various enzymes during seed germination and that optimal enzyme activity can occur at different stages of germination, and not necessarily when the roots and shoots are well developed. The lengths of roots and shoot lengths are therefore not valid indicators of the levels of amylase activity in germinating cereals and therefore cannot be used to determine the optimal stage of seedling development at which optimum amylase activity occurs.

There are several methods for the determination of amylase activity. The Falling Number Method for evaluating alpha-amylase activity is a viscometric procedure (AACC, 2013); the dinitrosalicyclic acid method (Mohammed *et al.*, 2009), Nelson's method for reducing sugars used to determine beta amylase activity (Morrison *et al.*, 1993), and the starch-iodine method for alpha amylase (Yoo *et al.*, 1987; Adejuwon *et al.*, 2012) are spectrophotometric; the ICC method for the Determination of Alpha-Amylase Activity (AACC, 2013) uses a colorimeter with a yellow filter for analysis.

However, an appropriate method which is suitable for use for artisanal maltose production in developing countries or less developed rural areas of developed countries must be simple, affordable, easy to maintain and partial to small-scale (Akubue, 2000). There is therefore the need to determine a valid technologically appropriate method to monitor amylase activity in germinating cereals for maltose syrup production. Cooked starch is hydrolysed by amylase enzymes to glucose. Glucose reacts with purple permanganate ions (MnO_4^-), and reduces it to colourless manganese ions (Mn_2^+). The time taken for decolourisation is related to the concentration of glucose in solution (SAPS, 2013) and the concentration of glucose in solution would be related to the activity of amylase enzyme used to hydrolyse the starch. Therefore, the time taken by the glucose to decolourise potassium permanganate solution would be related to the level of amylase enzyme activity used to hydrolyse the starch.

This study demonstrates the use of a timed colorimetric coupled enzyme assay which uses starch as substrate and permanganate as indicator to determine germination period for optimum amylase activity in maize malt crude extracts for maltose syrup production.

Materials and Methods

Maize samples. Batches of three local varieties of maize seeds were purchased from the following sources: *Obaatanpa* from the Crop Research Institute of Ghana at Pokuase in the Eastern Region of Ghana; *Obaatanpa*, *Mamaba* and *Golden Jubilee* from the Crop Research Institute of Ghana located at Fumesua in the Ashanti Region of Ghana; and *Obaatanpa* from the Ministry of Food and Agriculture (MOFA) at Accra, Ghana. The maize seeds were coded as follows: *Obaatanpa-Pokuase* - OP, *Obaatanpa-Fumesua* - OF, *Obaatanpa* - *MOFA* - OM, *Mamaba* - MB, and *Golden Jubilee* – GJ.

Standard curve for estimating levels of glucose in solution

A standard curve for estimating glucose in solution was obtained by determining the time taken for a standard acid potassium permanganate solution to be decolourised by standard glucose solutions (SAPS, 2013). Triplicate tests were done and the means used to plot the standard curve. The correlation between glucose concentrations and decolourisation times was determined. Statistical analysis using a two factor Anova was used to determine for significant differences ($p<0.05$) in decolourisation times for various glucose concentrations and for the replicates.

Screening of batches of maize seeds

The germinative capacities of the various maize types were determined by the method of Hudec and Muchova (2008) and the lengths of roots and shoots by the method of Al-Karaki, (2007). Statistical analysis using a two factor Anova was used to determine for significant differences ($p<0.05$) in germinative capacities of the various maize varieties and for the replicates.

Selection and germination of screened maize seeds

Batches of maize seedlings with germinative capacities > 90% was selected and was germinated according to the method of Quynh and Cecil (1996) for the preparation of malt crude enzyme extracts.

Preparation of malt crude enzyme extracts and hydrolysis of starch solutions

Beginning from the steeping stage, every 24 hours, three separate 5g samples of the sprouting seeds were finely crushed with a mortar and pestle to obtain malt crude enzyme extracts.

Determining the optimal stages of seed development for optimum amylase activity in malt crude enzyme extracts.

100 ml portions of 1.0 % (w/v) gelatinised starch solution were put in three separate 200 mL conical flasks and to each were added 5g of malt crude enzyme extract. The mixtures were stirred thoroughly and incubated at room temperature for 24 hours, and then filtered through a few layers of cheesecloth to obtain three separate filtrates. The times taken by the filtrates to decolourise standard solutions of acidified potassium permanganate solution were determined separately.

A graph was obtained by plotting mean decolourisation times (mins) against germination period (days). From the graphs, the germination period where the Least Decolourisation Time (LDT) occurred was determined for the various maize types.

This germination period corresponds to the optimal stage of seed development for optimum amylase activity in the respective malt crude enzyme extracts. The correlations between germination periods (days) and decolourisation times (mins) were determined. Statistical analysis using a two factor Anova was used to determine for significant differences ($p < 0.05$) in decolourisation times for the various maize varieties and germination periods.

Selection of malt crude enzyme extract for production of maltose syrup

The malt crude enzyme extract with the highest optimum amylase activity was determined to be from the OM maize type.

Production of maltose syrup

Starch extracted from fresh cassava (Esiaba var.) by the method of Ashveen *et al.* (2008) was processed according to the method of Quynh and Cecil (1996) into maltose syrup by the malt crude enzyme extract obtained from the OM maize.

Analysis of maltose syrup

pH was determined electrometrically with a pH meter (Cyberscan PC 6000) from a solution containing 50g of the sample dissolved at 50^{o}C in 50ml of distilled water and measured at 25^{o}C. Moisture content was determined by the AOAC method (AOAC, 2000) using a hot air oven at temperature of 105 ^{o}C.

The Dextrose Equivalence (DE) was measured by determining reducing sugars using Fehling's solution (International Starch Inst., 1999). Sulphated ash was determined according to the method of the International Starch Institute (1997).

Percent yield of maltose syrup

This was calculated from the formula:

$$\% \text{ yield of maltose syrup (mL/Kg starch)} = \frac{\text{Volume of syrop (mL)}}{\text{Weight of starch (Kg)}} \cdot 100$$

Results and discussions

Increasing glucose concentrations gave decreasing decolourisation times and this is observed in the Standard plot from the decolourisation of a standard acidified permanganate solution by standard glucose solutions (Figure 1). Standard deviations for decolourisation times ranged from 0.04 - 0.29.

Correlations between glucose concentrations and decolourisation times were negative and very strong. Statistical analysis indicated that decolourisation times for various glucose concentrations were significantly different ($p < 0.05$) but not significantly different for the replicates. This indicates a strong dependency of decolourisation time on glucose concentration.

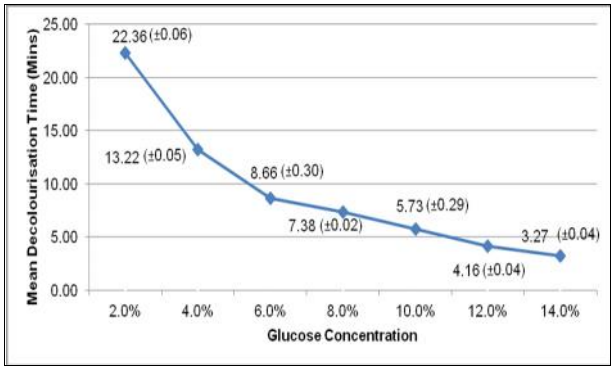

Figure 1. *Standard plot for the decolourisation of a standard acidified permanganate solution by standard glucose solutions*

A germinative capacity of at least 90.0% and preferably over 95.0% of the seeds is required for the cereal grains to be accepted for use as an enzyme source for hydrolyzing starch to maltose. Germinative capacities ranged between 85.0% - 97.0%, while mean germinative capacities ranged between 89.0% - 95.4% (Figure 2).

The mean value for the three batches of Obaatanpa was 91.5% which was greater than the mean germinative capacities of MB and GJ. OM had the highest germinative capacity of 95.4%. OP and GJ had the same germinative capacity of 90.0% while OF recorded the least value of 89.0% which is just below the limit.

Germinative capacities of the various maize varieties were significantly different ($p < 0.05$), but not significantly different among the replicates.

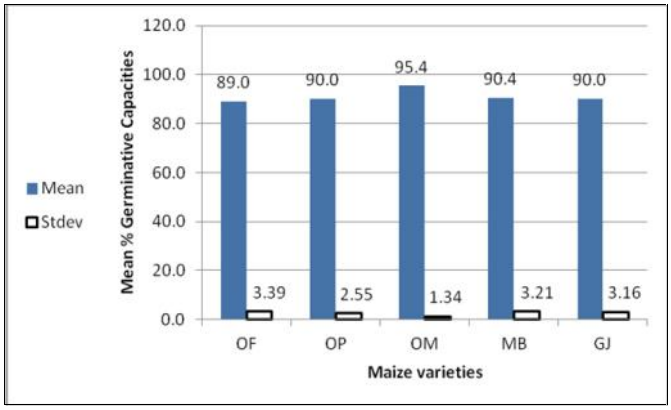

Figure 2. *Mean Germinative Capacities of various maize types*

The cereals were germinated for 11 days. Root lengths increased with germination period.

OM and OP had the longest root lengths of 26.92 cm and 23.92 cm respectively while GJ had the shortest of 18.39 cm at the end of the germination period (Figure 3).

Root lengths were significantly different ($p < 0.05$) among the various maize varieties and germination periods.

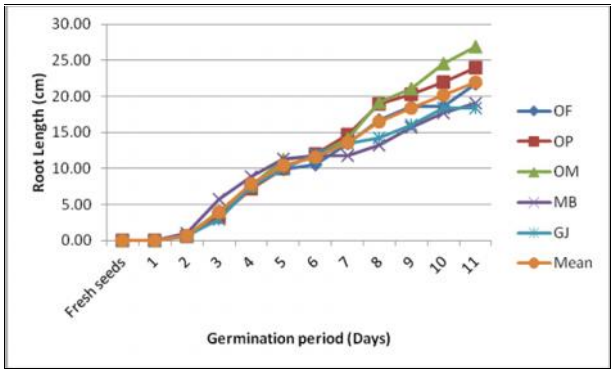

Figure 3. *Root lengths (cm) of seeds of various maize types at various periods of germination*

There were corresponding increases in shoot lengths over the same germination period.

OM and OF maize varieties grew fastest and had the longest shoot lengths of 18.69 cm and

13.59mm respectively, while GJ had the shortest shoot length of 12.29 cm (Figure 4) at the end of 11 days.

The shoot lengths were significantly different ($p < 0.05$) among the various maize varieties and germination periods.

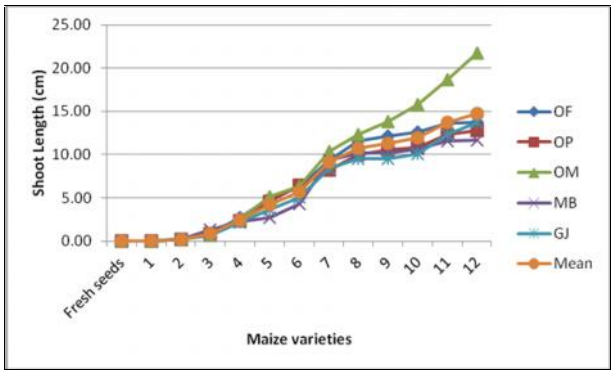

Figure 4. *Shoot lengths (cm) of germinating maize seeds of various maize types*

Clear watery solutions were obtained from the hydrolysis of cooked starch by malt crude extracts obtained from the germinating maize. Glucose present in the solutions was oxidized by the permanganate MnO_4^- anion which in turn was gradually reduced from pink to the colourless MnO_3^- (Naz, 2008). The kinetics of oxidation of glucose by alkaline and acid permanganate anion shows that the rate of the reactions is enhanced by increase in pH, ionic strength, and temperature as well as the concentrations of reactant (Odebunmi and Owalude, 2008). The rate of permanganate decolourisation is positively dependent on the concentrations of $KMnO_4$, sugar, and H_2SO_4 respectively, and on reaction temperature. The main oxidation products of maltose are formic acid, arabinonic acid, and malturic acid (Naz, 2008).

DTs were significantly different ($p < 0.05$) among the various maize types as well as at various germination periods. There were very strong negative correlations of (-0.86) - (-0.90) between germination period and DTs. The results indicate that the coupled "enzymatic starch hydrolysis–permanganate decolourisation" reaction is a valid method for monitoring amylase activity in malt crude extracts from germinating maize seeds.

Two distinct phases of growth Phase 1 and Phase 2, with separate LDT's (LDT 1 and LDT 2) corresponding to peak periods (P_1 and P_2) of amylase activity were observed over the 11 day germination period for all the maize types. For OF, OM, MB and GJ, LDT 1 occurred on day 5 during Phase 1 and LDT 2 on day 10 during Phase 2 (Figures 5, 6, 7 and 8). After day 5, DT increased steadily up to day 7, and then decreased gradually to a minimum on day 10.

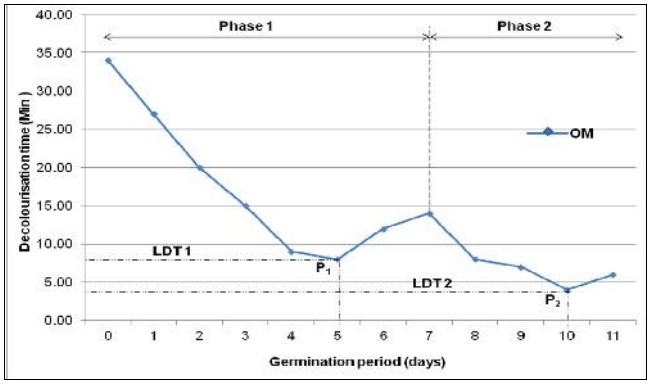

Figure 5. *Time (min) taken by hydrolysates obtained from the breakdown of standard cassava starch solution by malt crude enzyme extracts of Obaatanpa-MOFA maize to decolourise standard acidified permanganate solution*

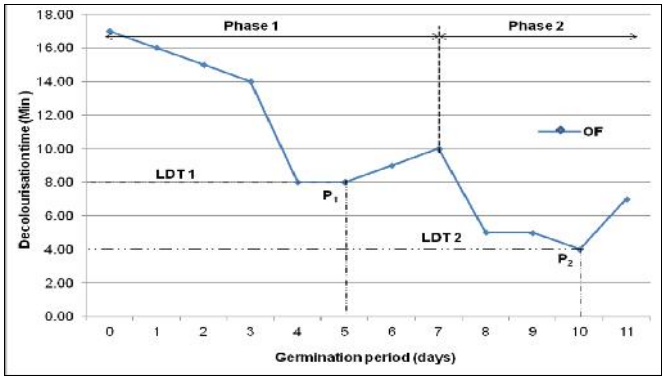

Figure 6. *Time (min) taken by hydrolysates obtained from the breakdown of standard cassava starch solution by malt crude enzyme extracts of Obaatanpa-Fumesua maize to decolourise standard acidified permanganate solution*

However, for the OP maize, LDT 1 occurred on day 4 during Phase 1. The DT increased steadily thereafter up to day 7, and then decreased gradually to give LDT 2 on day 9 during Phase 2 (Figure 9). It was therefore concluded that after imbibition of water by the maize endosperms, amylase activity reached its peak in the germinating seeds on days 4 – 5 and again on day 10.

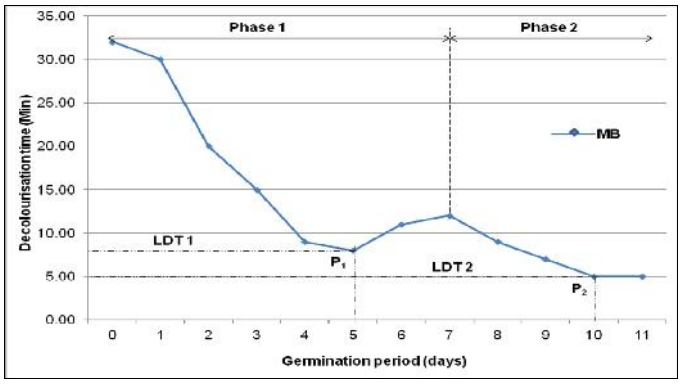

Figure 7. *Time (min) taken by hydrolysates obtained from the breakdown of standard cassava starch solution by malt crude enzyme extracts of Mamaba maize to decolourise standard acidified permanganate solution*

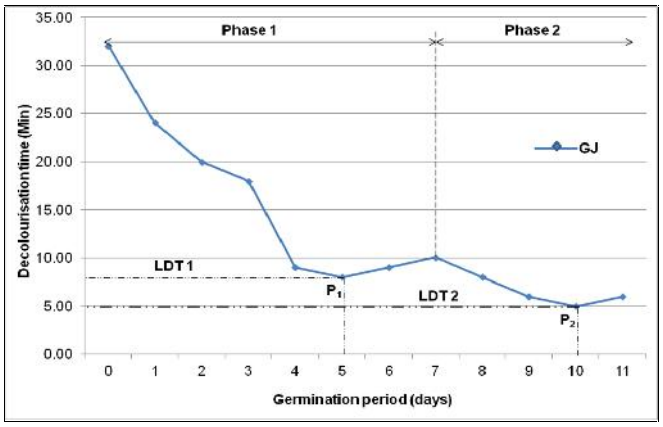

Figure 8. *Time (min) taken by hydrolysates obtained from the breakdown of standard cassava starch solution by malt crude enzyme extracts of Golden Jubilee maize to decolourise standard acidified permanganate solution*

The results of this study agree with those of Lenz and Oaks (1978) and Mohammed *et al.*, (2009). According to Lenz and Oaks (1978) amylase activity in intact maize seeds began 2-3 days after imbibition of water and reached its maximum at 6 days.

According to Mohammed *et al.*, (2009) local Saudi Arabian wheat (*Triticum aestivum*) variety (Balady) showed high levels of amylolytic activities at different stages of germination, increasing from day 0 to a maximum at day 6, followed by a decrease of activity till day 16.

According to Ritchie *et al.*, (1993) corn growth can be divided into two stages; the vegetative stage where the seedling emerges from the soil and the roots and shoots develop; and the reproductive stage involving development of the kernel and its parts.

Under adequate conditions, the maize endosperm absorbs water and begins growth. The radicle is first to begin elongation, from the swollen kernel, followed by the coleoptile, and then the three to four lateral seminal roots.

The plant emerges from the soil within 4 to 5 days after rapid mesocotyl elongation pushes the growing coleoptile to the soil surface.

Growth of the lateral and seminal roots, however, slows soon after the emergence of the coleoptile and is virtually non-existent at the latter stages of the vegetative stage. In this study DT decreased up to day's 4 - 5 and this corresponded to increased amylase activity required for breaking down starch to release energy for the coleoptile to emerge.

The increase in DT after day 5 corresponds to a decrease in amylase activity at the end of the vegetative stage. The decrease in DT after day 7 signals an increase in amylase activity for the start of the reproductive stage.

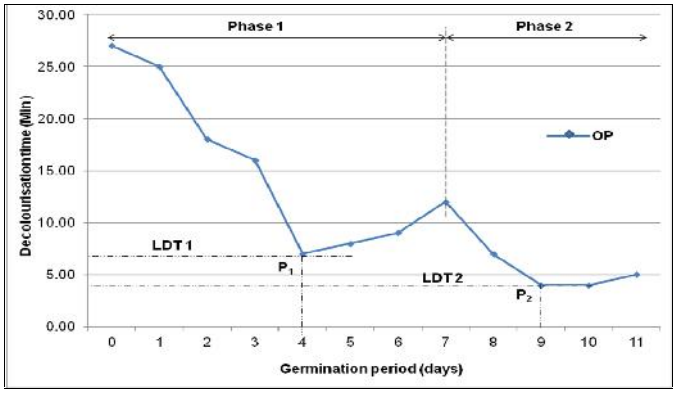

Figure 9. *Time (min) taken by hydrolysates obtained from the breakdown of standard cassava starch solution by malt crude enzyme extracts of Obaatanpa-Pokuase maize to decolourise standard acidified permanganate solution*

Enzyme activity was 1.51 – 2.01 faster at P_2 than at P_1 and this was indicated by the ratio of LDT2: LDT 1 (Table 1).

Table 1. *Ratios of LDT 2:LDT 1 for various maize types*

Maize types						
OF	**OP**	**OM**	**MB**	**GJ**	**Mean**	**Stdev**
1.85	1.88	2.01	1.51	1.55	1.76	0.22

The LDT 2: LDT 1 ratio indicates the level of amylase activity at P_2 relative to that at P_1

OM which had the highest LDT2: LDT 1 ratio was selected as the most suitable among the available maize types. A batch of OM was consequently germinated for 10 days and malt crude extracts prepared and used to process fresh Cassava (*Esiaba var.*) starch to maltose syrup.

The OM malt crude extract to wet starch ratio (w/w) used for the production of the syrup was 1: 3.3. This yielded 3000 ml of clarified juice which was subsequently reduced by concentration to approximately one-fourth the volume.

Table 2. *Physicochemical properties of maltose syrup from Cassava starch (Esiaba var.)*

Parameters	
pH	4.6 - 5.30
Moisture content	33.4%
Total dissolved solids	66.6%
Dextrose equivalent	40.0%
Sulphate ash	0.45%
Colour	Golden brown
Taste	Strong malty
Volume (ml)	720 ml
% Yield (ml/g wet starch)	23.59%

The enzymatic conversion of starch to maltose involved three steps; gelatinization, which involved the dissolution of cooked starch granules into a viscous suspension; liquefaction, which involved partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis (Souza and Magalhães, 2010). During liquefaction, gelatinised starch was converted by alpha amylases to maltodextrins which contained mainly oligosaccharides and dextrin's (Maps Enzymes, 2010). Maltodextrins were further converted during saccharification into maltose units by beta amylase enzymes (Sigma Process Technologies, 2012). The saccharified liquid was filtered through cheesecloth to obtain a sweet filtrate. During the concentration of the sweet filtrate into syrup, caramelisation and melting of glucose and maltose began at 160 °C and at 180 °C respectively, followed by boiling and foaming. During caramelisation there was loss of water from the maltose and glucose molecules followed by isomerisation, fragmentation and polymerisation.

Flavour production occurred during the fragmentation reactions and colour production during polymerization. Some flavour compounds formed during caramelisation are diacetyl which is mainly responsible for a buttery or butterscotch flavour, furans like hydroxymethylfurfural and hydroxyacetylfuran, furanones such as hydroxydimethylfuranone, dihydroxydimethylfuranone and maltol from disaccharides and hydroxymaltol from monosaccharide's (Food-Info.net, 2012).

The resulting sweet viscous syrup had a pH of 4.60 – 5.30 (Table 2) but this was lower than the values of 5.5 - 6.5 reported by Dziedzoave *et al.,* (2004). The 0.45% sulphate ash of the syrup was lower than the 0.9% obtained by Bello-Perez *et al.,* (2002) for syrup prepared from Banana starch. The DE value of 40% was lower than the 50-70% reported by Arasaratnam *et al.,* (1998) for sugar syrups obtained from corn starch and comparable to the 38% reported by Bello-Perez *et al.,* (2002) from banana starch. The 33.4% water content of the syrup from this study was above the 15-20% reported by Dziedzoave *et al.,* (2004). The colour of the syrup obtained in this study was golden brown and it had a strong malty taste.

The yield of syrup (mL/g wet starch) was 23.59% which is far lower than the results obtained by Zainab *et al.,* (2011) on the hydrolysis of gelatinized starch from maize, millet and sorghum starch with Commercial Amyloglucosidase where a glucose syrup yield of 86.71% (4.34 ± 0.37g) from the maize starch, followed by the 65.94% (3.30 ± 0.25g) from millet starch and a yield of and 64.71% (3.23 ± 0.09g) from sorghum starch respectively were obtained, and a dextrose

equivalent of 78.28 ± 0.57%, 73.50 ± 0.66% and 65.66 ± 0.61% for sorghum, yellow maize and millet syrups, respectively.

The enzymes used by Zainab *et al.*, (2011) were purified and this could account for their higher performance than that obtained from the malt crude enzyme source used for this project. The set up for this project was a batch system in a plastic container and there was minimal mixing. This limited the contact between the enzymes and starch substrate leading to a lower yield. Also, some amount of non-gelatinised corn starch was released from the malt crude extract into the sweet saccharified liquid. This fine mass of uncooked starch trapped a significant portion of the saccharified liquid and made it difficult to extract the sweet liquid during filtration. The fine particles of uncooked starch also blocked the pores of the cheesecloth during filtration, reduced the amount of filtrate obtained, and ultimately resulted in the low yield of syrup.

Sarmidi and Barker (1993) achieved for soluble potato starch, maltose conversions of up to 79% at feed flow rates of up to 400 cm^3 h^{-1} and 15–5% w/v concentrations in a continuous rotating annular chromatograph (CRAC) by saccharifying the liquefied starch to maltose using the enzyme maltogenase, which is a thermostable, exo- alpha-amylase.

Conclusions

The coupled enzymatic starch hydrolysis – permanganate decolourisation reaction is a valid procedure for monitoring amylase activity in malt crude extracts prepared from germinating cereals. It can be used to determine the period for optimum amylase activity during germination in order to ensure maximum degradation of starch to maltose.

This will ensure savings on time and money. The procedure is technologically appropriate for the artisanal production of maltose syrup especially in developing countries because it is very simple and the main instrument needed is a timer. The solution is initially pink when the permanganate is added and this makes the visual observation of decolourisation easy.

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