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RESPONSE SURFACE METHODOLOGY APPROACH OF INDUCING AMYLOLYTIC ENZYMES FROM *Digitaria exilis* AND *Digitaria iburua* GRAINS

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ABSTRACT

In this study, amylolytic enzymes were induced and extracted from the grains of *Digitaria exilis* (Acha) and *Digitaria iburua* (Iburu) which are underutilized African cereals. These findings can aid in determining the suitability of enzymes from the grains as a suitable alternative to enzymes from other tropical cereals. Whole grains of *Digitaria exilis* and *Digitaria iburua* (100 g) were steeped in water and water containing phosphate salt for 24 h at room temperature and germinated in a locally constructed malting chamber for six (6) days. The most appropriate pH and temperature conditions for the induction of amylolytic enzymes in the grains were selected based on the central composite design of response surface methodology (RSM). The amylolytic activities of crude extracts from harvested malts of *D. exilis* and *D. iburua* were measured by assaying the reducing sugar released from starch the using dinitrosalicylic acid method and soluble proteins were quantified by the Bradford method. The highest amylolytic enzyme activities were 17,948 and 24,337 units/mg protein for *D. exilis* and *D. iburua* respectively on day two (2) of the malting process. RSM showed that the maximum induction of amylolytic activity in grains of *D. exilis* and *D. iburua* was achieved by steeping in water containing phosphate salt at pH 6.5 and temperature of 22.5°C.The study concluded that grains of *D. exilis* and *D. iburua* were rich sources of amylases and could be of valuable for various biotechnological applications through studies of their physicochemical properties.

Keywords: Amylolytic enzymes, Response surface methodology, *Digitaria exilis*, *and Digitaria iburua.*

INTRODUCTION

Amylolytic enzymes (E.C 3.2.1.0), are collectively referred to as α-amylase, β-amylase and γ-amylase. Proteases are the second largest group of enzymes available in the market and account for approximately 30% of the global enzyme market as a result of their broad range of applications. (Aiyer, 2015; Sivaramakrishnan *et al.*, 2016). Amylolytic enzymes, or amylases, are enzymes that catalyze the hydrolysis of 1,4-glycosidic bonds present in polysaccharides including glycogen, amylose, and amylopectin, as well as the breakdown products of these sugars. Amylases are found in all kingdoms of animals, plants, and microbes since the breakdown products of starch are the typical sources of nutritional carbon. (Dehkordi-Mobini and Javan, 2012; El-Fallal *et al.*, 2012; Hashemi *et al.*, 2014; Shah *et al.*, 2014).

Seeds contain both α and β-amylases; β-amylase is present in an inactive form before germination, whereas α-amylase occurs after germination has started. This unique enzyme has been isolated from barley and rice plants. Adewale *et al.*, (2006) and Egwim and Oloyede (2006) extracted α-amylase (which is the most abundant amylolytic enzyme) from common cereals such as maize, millet, rice and sorghum and very good yields of this enzyme were obtained. The search for an alternative source of amylases as a good substitute for microbial sources has led to increased studies on cereal malting which involves the development of hydrolytic enzymes via a three-step process namely: steeping, germination and drying (Saxena *et al.*, 2014). α-Amylases (E.C.3.2.1.1) are liquefied enzymes because they hydrolyze bonds located in the region of the substrate. Endoamylases are termed α-amylases because they cleave α-1,4-glycosidic linkages in amylose, amylopectin and related polysaccharides such as glycogen randomly resulting in a rapid decrease in the viscosity of the starch solution (Gupta *et al.*, 2003; Drauz *et al.*, 2010).

β-Amylase (E.C 3.2.1.2) is referred to as saccharifying enzyme, and is exo-hydrolase enzyme that acts on α-1,4-glucosidic linkages in amylose, amylopectin and glycogen from nonreducing ends to yield successive maltose units. β-Amylase hydrolysis of branched

polysaccharides such as glycogen or amylopectin is partial because it cannot save the branching linkages in these molecules, leaving dextrin units behind. Sweet potatoes and higher plant seeds are the main source of β-amylase.(Sivaramakrishna *et al*., 2006).

In plants, starch is the most abundant type of stored carbohydrate. Microorganisms and higher organisms use it, as do the plants themselves. Thus, a wide variety of enzymes are capable of catalyzing its hydrolysis. All plant sources of starch are in the form of granules, which vary greatly in size and physical properties among species. Differences in composition are less pronounced. The primary distinction is the proportion of amylose to amylopectin; for example, waxy maize corn starch has only 80% amylose. In cereals, the endosperm is the primary site of starch synthesis and storage. Cereals (40–90%), roots (30–70%), tubers (65–85%), legumes (25–50%), and some young fruits (bananas, mangos, etc.) are the main sources of starch. These fruits also contain approximately 70% starch by dry weight. (Santana and Meireles, 2014). Hydrolytic enzyme systems are particularly limited by amylopectin, one of the two components of starch. This is because the presence of α-1,6-glycosidic branched residues, which account for approximately 4-6% of the total amount of glucose. The majority of hydrolytic enzymes exhibit specificity for the α-1,4-glycosidic link; however, to fully hydrolyze amylopectin and produce glucose, the α-1,6-glycosidic links also need to be broken. One of the most remarkable recent experiments has focused on new enzymes capable of hydrolyzing this bond. Amylases have great commercial value in biotechnological applications ranging from the bakery, alcohol, textile and paper industries (Parmar and Pandya, 2012). They can breakdown the glycosidic bonds found in both starch and glycogen molecules. and are widely utilized in the brewing and detergent industries (Khan and Priya, 2011). Nonetheless, amylolytic enzymes are found in many different kingdoms, including microbes, plants, and animals, and their modes of action vary depending on the source. (Padhiar and Konmu, 2016).

The search for an alternative source of enzymes has led to increased studies on cereal malting (Hammond and Ayernor, 2000). The range of applications for amylolytic enzymes has expanded with the introduction of new biotechnological frontiers. There is a need for other cereals that can tolerate a wider range of soil and climatic conditions (Doss and Anand, 2012) and that can possibly replace the import of wheat and barley. According to Adefila *et al*., 2012, sorghum would have been a very good substitute but research has shown, it contains a very small quantity of beta amylase and a good mixture of α-amylase and β-amylase; however this mixture is required for the complete saccharification of starch, hence further studies on *D.exilis* and *D.ibura* which are African unpopular grains and could possibly serve as a good substitutes, are needed.

MATERIALS AND METHODS

Materials

Digitaria exilis and *Digitaria iburua* grains were purchased from the Sabon Gari Market in Zaria, Kaduna State, Nigeria. These grains were authenticated at the Herbarium of the Department of Biological Science, Kaduna State University, Kaduna, Nigeria. The voucher number is 6/6

Chemicals

3,5,-Dinitrosalicylic acid (DNSA), sodium hydroxide pellet, sodium potassium tartrate (Rochelle Salt), blue dextran, glucose, bovine serum albumin (BSA), 2-mercaptoethanol, acetic acid, sodium phosphate dibasic (Na2HPO4), anhydrous sodium phosphate monobasic (NaHPO4), soluble starch, ammonium persulphate, Coomassie brilliant blue G-250, phosphoric acid and calcium chloride dihydrate were obtained from Sigma Chemical Company, St Louis, Mo, U.S.A. Glycerol, sodium chloride and methanol were obtained from British Drug House Chemicals Limited, Poole, England. All other reagents were of analytical grade.

METHODS

Induction of amylolytic enzymes

Amylolytic enzymes were induced by steeping 100 g of screened *D. exilis* and *D. iburua* grains in water and water containing phosphate salts (10 mM sodium phosphate adjusted to pH 6.5) for 24 h at room temperature. Steeped grains of *D. exilis* and

D. iburua were blotted to remove excess water after 24 h and were spread out in a locally constructed malting chamber at room temperature. The optimum day of germination was determined for each grain by harvesting malts on each day of germination, homogenizing to obtain a crude extract and assaying for amylolytic enzymes until a decline in induced amylolytic activity was obtained. The optimum pH and temperature for amylase induction were investigated using response surface methodology (RSM) which computes a set of experimental conditions based on two factors (pH and temperature in oC). The steps were conducted based on the experimental design of the RSM, The grains of D. *exilis* and D. *iburua* were steeped at a designated pH and temperature according to RSM, and germination was performed on the optimum day established from the previous experiment. Grains were sprinkled with water and water containing phosphate salts at 12 h intervals (twice a day) for optimization of the germination conditions.

Extraction of amylolytic activity

Induced amylolytic enzymes from *D. exilis* and *D. iburua* malts were extracted by preparing 30% homogenate of the malted grains using water and water containing phosphate salt (10 mM sodium phosphate buffer pH 6.5 containing 1 mM CaCl2), following the method of Adefila *et al.* (2012). A 30% homogenate was prepared by homogenizing 100 g of malted *D. exilis* and 170 g of malted *D. iburua* in cold 10 mM sodium phosphate buffer pH 6.5 containing 1 mM CaCl2. The crude homogenates were centrifuged at 13,000x*g* for 30 min at 4 oC using a Hitachi High speed Refrigerated Centrifuge Himac CR21G H. The pellets were discarded and the supernatants were collected. The amylolytic activity and protein concentration in each supernatant were determined and the supernatants were stored at -20 oC until further use.

Standard procedures for the amylase activity assay

The amount of reducing ends released upon starch hydrolysis by amylolytic enzymes was estimated using the modified method of Bernfeld (1951). A unit of amylolytic activity was defined as the amount of enzyme that liberated reducing sugars equivalent to 1 μg of D-glucose per minute at 25 oC under standard assay conditions.

The final concentration of the assay mixture (2mL) consisted of 10 mM sodium phosphate buffer, pH 6.5 containing 1 mM CaCl2, 0.2 mL of +1 % soluble starch and 0.01 mL of the enzyme. The assay mixture was incubated for 5 min at room temperature for the enzymatic reaction to occur, after which the reaction was terminated with 1 ml of 0.5 mM 3,5-dinitrosalicylic acid. The solution was boiled for 5 min for color development and the yellow color of 3,5-dinitrosalicylic acid turned i reddish brown and was cooled under a running tap and diluted with distilled water to 10 ml. The optical density was measured at 470 nm using a spectrophotometer. Two blanks were set up for the experiment; the first blank consisted of all assay components except the enzyme while the second contained denatured (boiled at 100 oC) enzyme. Glucose was used to prepare the standard curve from which the amount of reducing sugars liberated was estimated.

Protein concentration determination

The protein concentration in the crude supernatants was determined using a Coomassie dye binding assay, following the method of Bradford (1976) using bovine serum albumin as the standard protein. The method measures the increase in absorbance of Coomassie Brilliant Blue G-250 dye at 595 nm upon binding to protein.

Determination of the optimum pH and temperature for inducing amylases using RSM

The optimum pH and temperature were studied with the aid of response surface methodology (RSM), which was used to design the experiment at pH 4.0 – 8.0 and temperatures ranging from 0-30oC. 0 - 30°C. The pH and temperature were chosen based on preliminary experiments (pilot study). The experimental design considered two factors in which grains were steeped at various pH and temperature values but were germinated under the same conditions that were previously established. Amylolytic activity induced in the malts of *D. exilis* and *D. iburua* was then determined in the crude extracts obtained after the malts were homogenized. The specific activity

of the enzyme from each grain malts was calculated and slotted into the experimental table as the response and was used to plot the 3D surface.

RESULTS

Levels of amylolytic activity in *Digitaria exilis* and *Digitaria iburua* grains

Figure 1 shows a summary of the levels of amylolytic activity in the crude homogenates of malted *D.exilis* and *D.iburua* in water and water containing phosphate salts. A greater amount of amylolytic enzyme was detected in grains steeped with water containing phosphate salts than in those steeped with water for both grains. The obtained results provide the preliminary information for further studies.

Effect of days of germination on malted *D.exilis* and *D.iburua* grains

Grains of *D.exilis* and *D.iburua* were steeped in water and water containing phosphate salts and were germinated for several days in a locally constructed malting chamber. Harvested malts were subjected to homogenization and a specific activity of 17,948 U/mg protein and 24,337 U/mg protein for *D.exilis* and *D.iburua* respectively on day two (2) of germination produced the greatest amount of amylolytic enzymes for both grains as summarized in Figure 2.

Response surface methodology approach of inducing amylolytic enzymes

Tables 1 and 2 show the experimental designs for *D.exilis* and *D.iburua* grains respectively. The central composite design of the response surface methodology was used to design different steeping conditions for *D.exilis* and *D.iburua* grains. The experimental results of these experiments were used to construct the 3D graph of the amylolytic enzymes from *D.exilis* and

Table 1: RSM experimental design for the malting of *D. exilis* grains.

Standard	RUN	BLOCK	FACTOR 1A:pH	FACTOR 2B: Temperature
12	1	Block 1	6.00	15.00
2	2	Block 1	8.00	0.00
7	3	Block 1	6.00	0.00
10	4	Block 1	6.00	15.00
5	5	Block 1	3.50	15.00
13	6	Block 1	6.00	20.00
1	7	Block 1	4.00	0.00
6	8	Block 1	9.00	15.00
3	9	Block 1	4.00	30.00
4	10	Block 1	8.00	30.00
11	11	Block 1	5.00	20.00
8	12	Block 1	6.00	30.00
9	13	Block 1	5.00	20.00

Table 1 shows the computed set of conditions for the induction of amylolytic enzymes by RSM. The table was used to determine the optimum conditions for the induction of amylolytic enzymes.

Table 2: RSM experimental design for the malting of *D. iburua* grains.

Standard	RUN	BLOCK	FACTOR 1A:pH	FACTOR 2B: Temperature
8	1	Block 1	3.00	20.00
1	2	Block 1	4.00	10.00
3	3	Block 1	4.00	30.00
16	4	Block 1	4.00	30.00
12	5	Block 1	4.00	10.00
11	6	Block 1	5.00	20.00
4	7	Block 1	6.00	20.00
7	8	Block 1	6.00	30.00
10	9	Block 1	6.00	0.00
15	10	Block 1	6.00	20.00
13	11	Block 1	5.00	20.00
14	12	Block 1	8.00	30.00
5	13	Block 1	8.00	10.00
6	14	Block 1	8.00	10.00
2	15	Block 1	8.00	30.00
9	16	Block 1	9.50	20.00

Table 2 shows the computed set of conditions for the induction of amylolytic enzymes by RSM in *D. iburua* grains.

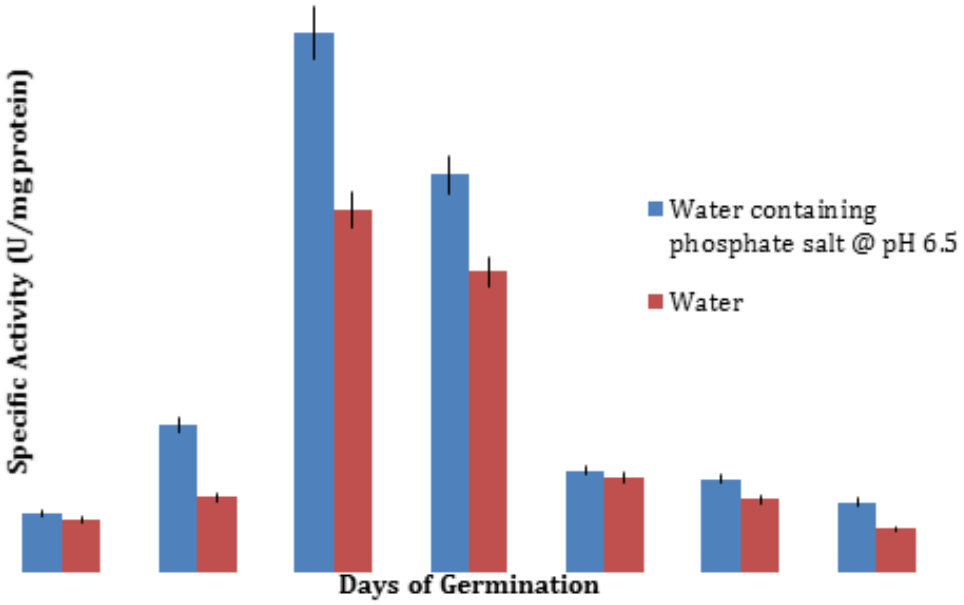
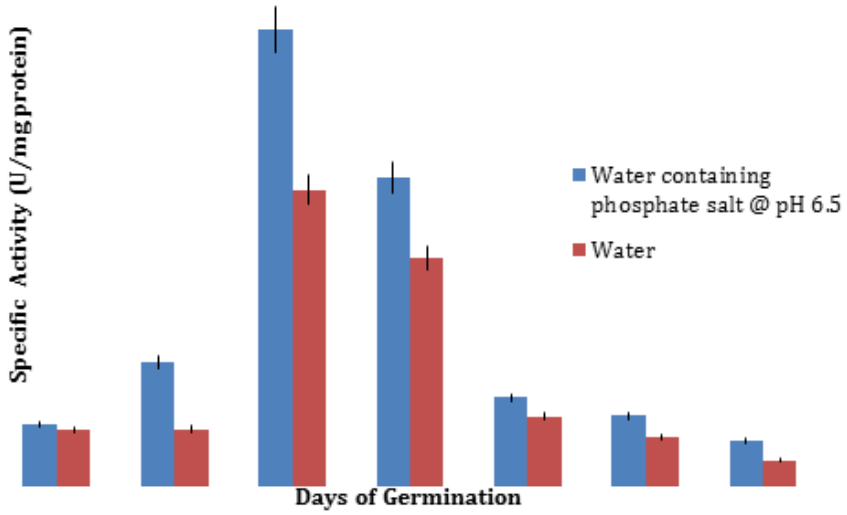


Figure 1: Germination profile of malted *Digitaria exilis* grains.



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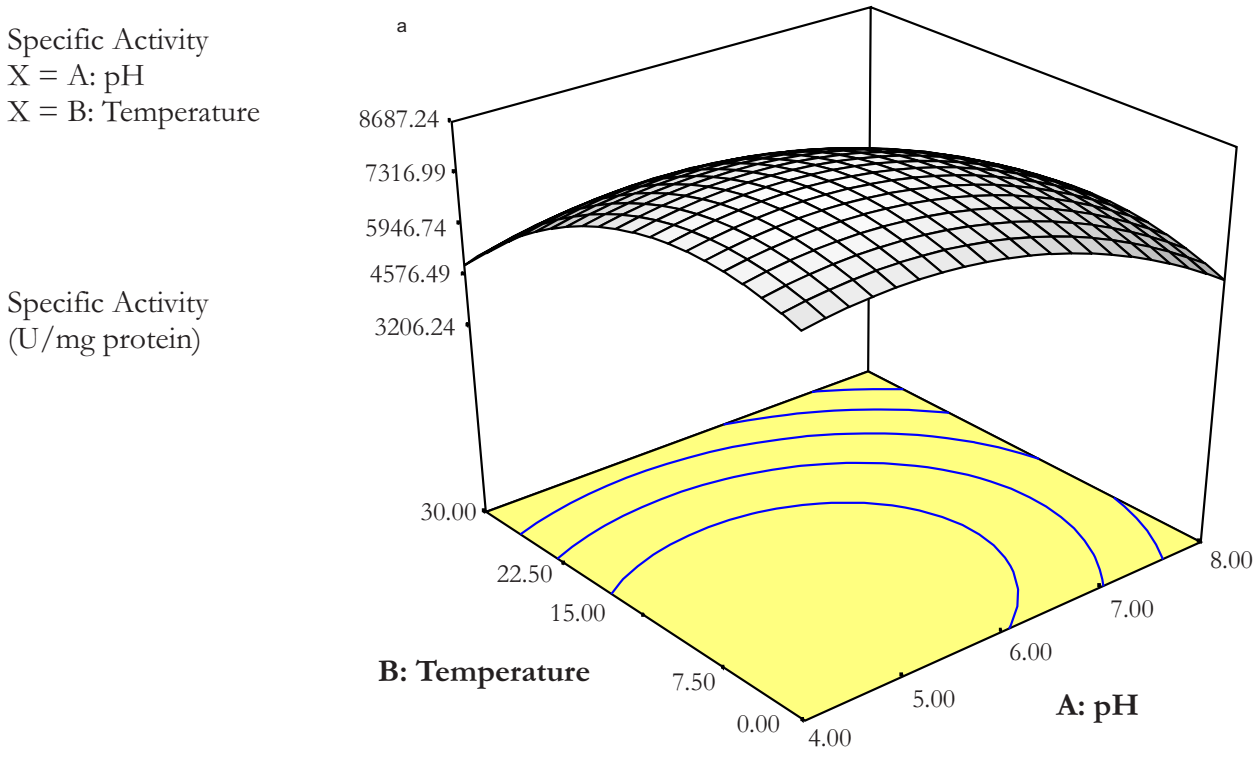

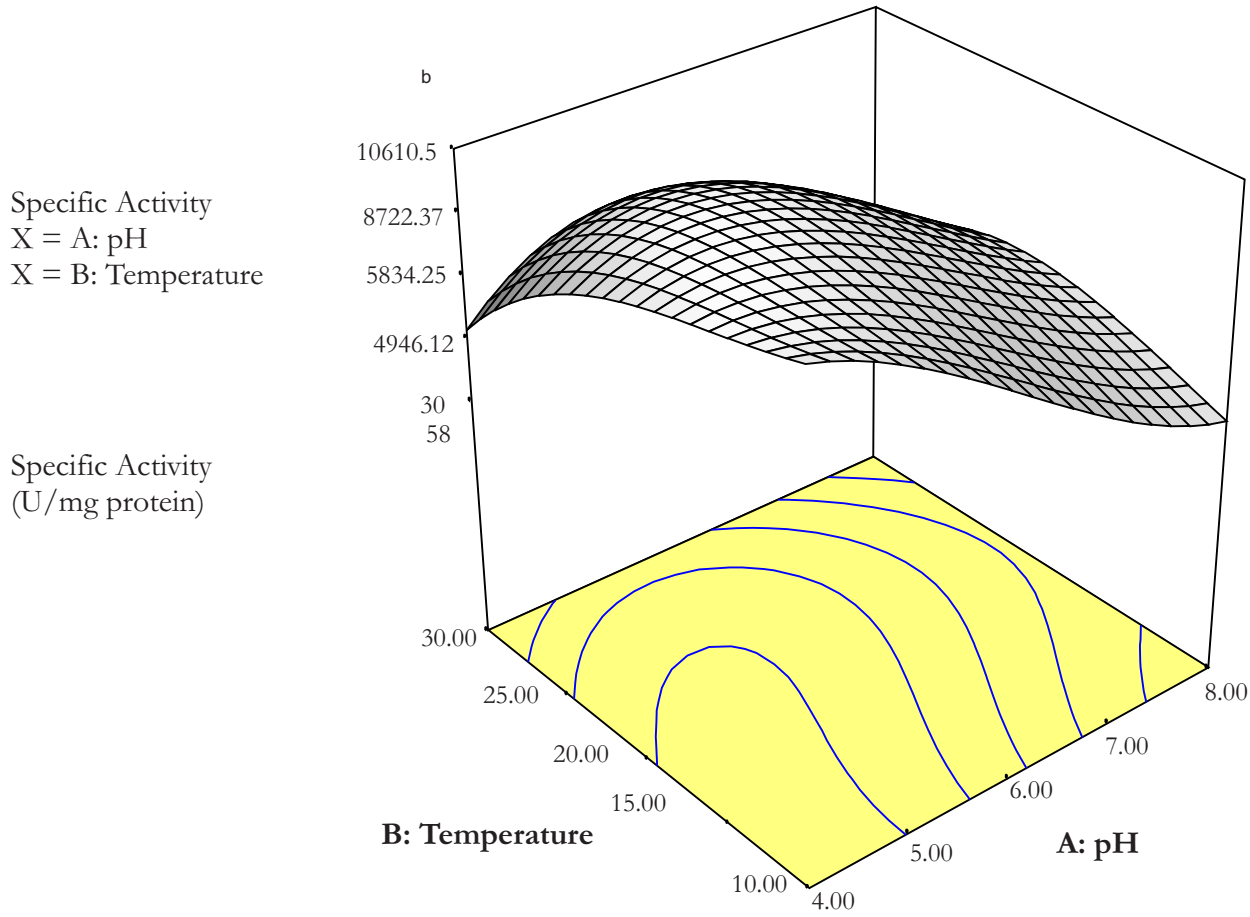

Figure 2: 3D surface model amylolytic enzymes induced by response surface methodology approach. (a) Amylolytic enzymes from *Digitaria exilis* grain (b) Amylolytic enzymes from *Digitaria iburua* grain

Figure 3: Most suitable conditions for the induction of amylolytic enzymes from *D. exilis* and *D. iburua* grains. (a) pH 6.5 at 22.5 °C is the optimum condition for *Digitaria exilis* (b) pH 6.0 at 20 °C is the optimum condition for *Digitaria iburua*

DISCUSSION

This study optimized the germination conditions for the induction of amylolytic enzymes in *D. exilis* and *D. iburua* grains. High malting loss has previously been reported for these grains, which was demonstrated in the preliminary experiments in this work. High malting loss in *D. exilis* and *D. iburua* grains was prevented by steeping grains a large volume of water (1:20 (w/v) of water). After 24 h *D. exilis* and *D. iburua* grains became swollen which could be a result of the higher water absorption capacity of these grains, probably because of their thin seed coats. As such, *D. exilis* and *D. iburua* require high moisture content for germination. To ensure uniform germination grains were sprinkled at 12 h intervals contrary to the 6 h usually employed for other grains such as sorghum. However, studies have shown that the

rate of water diffusion in grains depends on factors such as steeping duration, water temperature, grain dimension, protein content of grains and possibly the quantity of available oxygen (Francis, 2013).

The maximal enzyme activity was obtained after 2 days which is lower than the number of days required to achieve the same result in other grains (Adewale *et al*., 2006; Adefila *et al*., 2012). The enzyme was extracted and the resulting supernatants were assayed for amylolytic enzyme activity. The amylolytic enzyme induced is a function of the days of germination as the highest amylase activity was obtained on the second day (48 h) of germination with 17498 U/mg protein and 24337 U/mg protein for *D. exilis* and *D. iburua* respectively (Figure 1 a and b) for grains steeped in water containing phosphate salt. Approximately 5000 U/mg protein was the difference in the amylolytic enzyme activity induced under the same conditions but with only water as the medium for steeping. Water is traditionally the steeping medium in most industries. The observed reduction in amylolytic enzyme activity after 48 h is an indication that the induced enzymes may have been degraded to produce other biomolecules required by the growing plant.

This study therefore established that more amylolytic enzymes were induced in *D. exilis* and *D. iburua* grains within a very short germination period than in other grains such as sorghum and millet which require 3-5 days for maximal amylolytic activity (Egwim and Oloyede, 2006). This implies that *D. exilis* and *D. iburua* grains would generate far larger quantities of amylolytic enzymes which will invariably increase the economic value of these underutilized African grains.

The effects of pH and temperature on amylolytic enzyme induction were investigated using a response surface methodology (RSM) tool, in which the pH and temperature were optimized (Tables 1 and 2). RSM allows the verification of variables individually and their interactions. It is a statistical model that correlates variables and permits the optimization of the hydrolysis process. The experimental design for the variables used here was based on a preliminary study that established that an optimum amylolytic enzyme could be induced within the range of parameters studied. Malts from *D. exilis* and *D. iburua* grains were steeped at designated pH and temperature values and were germinated under the same atmospheric conditions taking into consideration the best day of germination. However, the pH and temperature ranges were based on previously established studies by Adefila *et al*. (2012). Therefore, a pH of 4.0-8.0 and a temperature of 0 - 30 °C were fed into the RSM for the design; after the grains were malted according to the design, amylolytic enzymes induced from *D. exilis* and *D. iburua* grains were extracted using 10 mM sodium phosphate buffer pH 6.5 containing 1 mM CaCl2 and the calculated specific activity was used as a response to the experimental design of the RSM. The methodology suggested a cubic and quadratic model and the range of values given was significant. Hence, factors A (pH) and B (temperature) are limiting factors because any variation in these two factors will greatly affect the induced amylolytic enzymes which is the response. The specific activity (response) for both *D. exilis* and *D. iburua* grains increased with increasing in both pH and temperature until a further increase in these two factors resulted in a decrease in their response (Figure 2a and b). With respect to RSM, pH values of 6.5 and 22.5 °C were the best conditions for amylolytic enzyme induction in *D. iburua* grains while pH values of 6.0 and 20 °C were obtained for *D. exilis* grains (Figure 3a and b). A similar result was obtained by Adefila *et al*. (2012) who reported that pH values of 6.5 and 20 °C resulted in optimum amylase induction in S*orghum bicolor*. The amylolytic enzymes used for further studies in this report were therefore obtained based on the optimum conditions established by RSM.

This study therefore revealed that amylolytic enzymes were increased approximately 7-fold when malts were produced from *D. exilis* grains steeped in water containing phosphate salts at pH 6.0 at 20°C and pH 6.5 at 22.5°C for malts of *D. iburua* compared to water. The visual assessment of the germinated grains did not correlate well with the amount of amylolytic enzymes induced, as some malts that visually appeared to be uniformly germinated tended to have lower amylolytic enzymes.

Amylolytic enzymes are important in many industrial processes and the search for amylase from new sources is a continuous process. This study has thus demonstrated conclusively that malts of *D. exilis and D. iburua* could be suitable replacements for imported barley and wheat..

CONCLUSION

D. exilis and D. iburua are good sources of amylolytic enzymes. Amylolytic enzymes from *D. exilis and D. iburua* could be alternative sources to those currently utilized in most starch-based industries.

Further studies however could be performed on the purification and kinetic properties and how the stability of amylases from *D. exilis* and *D. iburua* could be further enhanced by chemical modification and protein engineering. This could increase the economic value of these underutilized grains.

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AUTHORS CONTRIBUTIONS

The laboratory works were done by A.O.A and supervised by A.O.I. in his laboratory at the Department of Biochemistry and Molecular Biology. Obafemi Awolowo University, Ile-Ife. Osun State.

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