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STUDIES ON POTENTIAL OF FINGER MILLET (*ELEUSINE CORACANA* GAERTN. L.) AMYLASES FOR INDUSTRIAL APPLICATIONS

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ABSTRACT

*Amylases are of great significance especially in the food, brewing and detergent industry. An attempt has been made to study the characteristics of amylases produced during germination of finger millet (*Eleusine coracana Gaertn. L.*). Of the 25 genotypes studied RAU8 showed the maximum activity at pH 5 and temperature 60°C. This is the first report of optimum amylase activity at 60°C in finger millet. The best activity was observed in seeds geminated for 96 hours. The enzyme extract showed maximal activity till 90 minutes and 82.24% activity was retained even after 120 minutes. The thermostable character of the enzyme and retention of activity of the unprotected enzyme for 2 hours makes it suitable for industrial purposes. Further characterization of the different forms of the amylases may help in isolation of the most robust form which could then be cloned and expressed in microbes to enable large scale production for industrial purposes.*

Keywords: Maltose, Starch, Amylase, Germination, Finger millet, Thermostable.

Contribution/ Originality

The paper's primary contribution is finding that amylases derived from finger millet can be effectively used in the brewing, baking and detergent industries. This study documents the activity of amylases of different genotypes of finger millet and the optimal parameters for activity of amylases derived from RAU 8 genotype.

1. INTRODUCTION

Amylase is an enzyme which breaks down starch (the reserve carbohydrate in plants) and glycogen (the reserve carbohydrate in animals) into reducing fermentable sugars, mainly maltose, and reducing non fermentable or slowly fermentable dextrins (Burtis and Ashwood, 1999). Amylases are produced by a variety of living organisms, ranging from microbes to plants and

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humans. In plants, amylases are synthesized during the germination of seeds rich in starch and convert insoluble starch to soluble sugars (Burtis and Ashwood, 1999).

Amylases are classified as α-amylases and β-amylases. The α-amylases are found in all types of organs and tissues, whereas β-amylase is found almost exclusively in higher plants. α -Amylase (1, 4-α-D-glucan glucanohydrolase, glycogenase) are calcium metalloenzymes, unable to function in the absence of calcium. It acts at random locations along the starch chain which makes it faster acting than β-amylase. It breaks down long-chain carbohydrates, yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. β –Amylase (1,4-α-D-glucan maltohydrolase, glycogenase, saccharogen amylase) is synthesized by bacteria, fungi and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1, 4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into sugar, resulting in the sweet flavor of ripe fruit (Burtis and Ashwood, 1999).

Amylases are among the most important enzymes and are of great significance in present day biotechnology especially in bread baking process where addition of the enzyme increases the bread volume and helps to retain its softness (Kanwal *et al*., 2004). Amylases possess important applications in the food industry. They are mainly used for production of syrup with high glucose content, sweeteners and ethanol. Amylase rich energy food (AREF) which has a high density of nutrients is distributed as weaning food and to pregnant and lactating women for nutritional benefits (http://www.indiamart.com/christy-friedgram-industry/products.html). They are used in the brewing industry for malt preparation, liquefaction of additives, improved fermentability of grain and modification of beer characteristic. The paper industry uses amylase for liquefaction of starch. It is also used as an additive in detergent to increase their cleansing power (Crueger and Crueger, 2005). Commercially α-amylases are produced mostly from fungal sources, some bacteria and also plant sources like barely, millets like sorghum, wheat, and maize.

In view of its importance in industrial application, an attempt has been made to study the characteristics of amylases produced during germination of finger millet (*Eleusine coracana Gaertn.* L*)*. Finger millet was chosen for the present study as the activities of finger millet malting enzymes are very high and come next only to barley, the world's premier beer grain (National Research Council, 1996). Our endeavor is also aimed in the direction of making this crop commercially attractive so that its inherent properties of drought tolerance, capacity to grow on upland acidic soil etc. Haider *et al*. (2003) can be exploited.

2. MATERIALS AND METHODS

Seeds of twenty five genotypes of finger millet were used in the present study. Sixteen genotypes were obtained from Department of Plant Breeding and Genetics, Birsa Agricultural University, Ranchi. These are A 404, AKE 1033, BM 1, BM 2, GGP, GPU 28, GPU 45, GPU 48,

GPU 67, HR 374, IE 7, JWM 1, R^+, OEB526, OUAT 2 and VR708. Five genotypes viz. IE 3693, IE 4671, IE 5066, IE 5165 and IE 5870 were obtained from ICRISAT, Patancheru. Three genotypes were obtained from VPKAS, Almora, Uttaranchal. These are VL149, VL315 and VL347. One genotype i.e. RAU 8, was obtained from Igatpuri, Maharashtra.

The total amylase was extracted in 0.1M phosphate buffer or 10mM $CaCl_2$. Starch unconsumed was calculated from a standard curve of starch. Starch unconsumed was found to be higher for amylase extracted in phosphate buffer whereas it was lower for amylase extracted in $CaCl_2$. It is known that calcium is required for α-amylase activity (Burtis and Ashwood, 1999). Based on these results further extractions were carried out in $CaCl_2$.

Maltose assay was used to estimate the breakdown of starch. Levels of amylase were determined by measuring the mg of maltose liberated when incubated with 1% starch. The initial experiments were conducted on the genotype A 404. One gram of dry, dehusked seeds were taken and placed in a 250 ml beaker. A little water was added to aid germination. The beakers were then covered with aluminium foil and kept in an incubator set at 28°C for 72 hr. No illumination was provided. The germinated seeds were ground in 10ml of ice-cold 10mM $CaCl_2$ in a pre cooled mortar. The solution was added gradually till a smooth paste was obtained. This mixture was then transferred to an oak ridge tube and kept at 4^0C overnight for proper extraction of amylase. The extract was centrifuged at 7,500rpm in Hermle table top centrifuge (rotor 220.80 VO2) for 15 min. at 4^oC. The supernatant was used as the enzyme source. It was kept at 4^0C or on ice and used on the same day. A standard curve of maltose was made by taking varying volumes of 0.2% maltose solution. Colour reagent (Bernfeld, 1955) was added and the tubes were capped and placed in boiling water bath for 15 minutes and then cooled in ice water. The OD was read at 540nm in UV-Visible Spectrophotometer (Spectrascan UV 2600) after addition of 9ml distilled water against a blank which had no maltose. Samples were prepared in duplicates and average of two reading was taken. A standard curve of maltose was prepared by plotting mg of maltose on X axis and OD_{540nm} on Y axis. This curve was used for deduction of maltose released by different extracts.

Amylase was assayed as per standard procedure (Bernfeld, 1955). The following were added in the order : 1 ml buffer, 400 µl 1% starch, 500 µl distilled water (DW) and 100 µl extract (100 µl DW for Blank). The tubes were capped and incubated at 50^0C for 15 min. 1ml of color reagent was added to each tube and kept in boiling water bath for 15 min and then cooled in ice water. Finally 9ml of DW was added to each tube. The absorbance was measured at 540nm.

Amylase activity was determined at various pH values using different buffers such as sodium acetate buffer (0.2 M, pH 3.6-5.6) and sodium phosphate buffer (0.1 M, pH 5.8-8.0). For each pH, set of 3 test tubes (1 blank & test sample in duplicate) was prepared. Once the optimal pH had been determined the amylase activity was determined at different temperatures ranging from

25^{0}C to 75^{0}C (with an interval of 5^{0}C). The optimum germination time was checked from the seeds germinated for different durations. The seeds were germinated for 24, 48, 72, and 96 hours.

3. RESULTS AND DISCUSSION

The A 404 extract was found to be active over a broad range of pH viz. 3.8 to 7.8 (pH 3.8 – 5.4 in acetate buffer and pH 5.8 – 7.8 in phosphate buffer). The optimum pH was found to be 4.6. Below pH 4.6 the activity of amylase decreased drastically, whereas there was gradual drop in activity above pH 4.6. Similar results have been obtained by other workers (Nirmal and Murali Krishna, 2003), (Gimbi and Kitabatte, 2002), (Mar *et al*., 2003) and this appears to be characteristic of several cereal α-amylases (Nirmal and Murali Krishna, 2003), (Rao *et al*., 2005). It is known that the endosperm environment where starch is primarily stored and broken down is acidic in nature (Dicko *et al*., 2000), therefore, amylases have their optima in acidic pH.

The optimum temperature for amylase activity was found to be 50^{0}C. Maltose release was observed up to 75^{0}C, with some loss of activity. Such high temperatures for optimal enzyme activity have been observed in other grains also (Nirmal and Murali Krishna, 2003), (Rao *et al*., 2005), (Terashima *et al*., 1995) Also, seeds were germinated for 72 hours were found to possess highest amylase activity which points towards peak breakdown of stored starch.

Twenty five genotypes were then tested for amylase activity at the optimized conditions for A 404. The incubation time was increased to 30 min. Fig. 1. shows the difference in activity of different genotypes.

One gram dehusked seeds of each genotype had been taken for germination and amylase extract preparation. The variation in amylase activity observed was very significant so it was investigated whether the number of seeds per gram had any correlation with amylase activity. Fig. 2 shows a correlation graph between the number of seeds per gram and amylase activity.

The scatter diagram shows that there is no correlation between number of seeds/1g from which the extract was prepared and the amylase activity/g of seeds. Thus, amylase activity may be a genotype specific trait.

Since RAU 8 showed the best amylase activity (Fig. 1) it was taken up for further studies to obtain the optimum conditions for its activity. Studies on effect of different pH showed the presence of two peaks of activity (Fig. 3). The first peak is at pH 5.0 and second much lower peak at pH 7.0. Amylases are known to exist in different forms (www.gmo-compass.org/eng/databases/enzymes/80.amylase.html, 29.11.2012) like α, β and γ and exhibit maximal activity at different pH. In our study the complete extract has been taken. It is expected that multiform of the amylases would be present and they would exhibit their maximal activity at different pH. However, the maximal activity was obtained at acidic pH corroborating our findings with A 404.

Generally, the optimal pH for α-amylase is 6.7 to 7.0, for β-amylase it is 4.0 to 5.0 and γ-amylase has the most acidic pH optimum at around pH 3.0 (www.wikipedia.org/wiki/Amylase, 29.11.2012) The optimum pH for pure soy bean β-amylase has been shown to be in the range 5.0 to 6.0 (Gertler and Birk, 1965). It has also been shown that α-amylase is inactivated at a low pH whereas β-amylase remains stable at a low pH of 4.8 (Bilderback, 1973). The optimum pH of plastidic α-amylase was found to be 6.2 and that of β-amylase was 4.6 in pearl millet leaves (*Pennisetum americanum*) (Vally and Sharma, 1995). The α-amylase of African finger millet has been shown to display maximum catalytic activity at pH 5.4, whereas β-amylase was active at pH 6.0 (Gimbi and Kitabatte, 2002). Thus, it appears that β-amylases which are almost exclusively found in higher plants are generally more active in acidic environments.

The optimum temperature of the amylase activity of the RAU 8 extract was studied at optimum pH 5.0. Fig. 4 shows that the enzymes are equally active at 40, 50 and 60°C. There is a sudden drop in activity at 70°C which indicates that denaturation of the major enzyme might have taken place. However, about 50% activity was observed even at 80°C. This indicates that some forms of amylases present in the extract may be tolerant to such high temperatures. It would be interesting to isolate these forms as they would have wide application in the brewing and detergent industry, where high temperatures are encountered. It also raises the question whether the internal microenvironment where amylase activity takes place reaches such temperatures or not.

It has been reported that in African finger millet the α-amylase displays maximum catalytic activity at around 45°C whereas the optimum temperature for β-amylase activity is between 50°C and 55°C (Gimbi and Kitabatte, 2002),. Another study (Kolawole, 2009) showed that showed that β-amylase from African finger millet seed malt had temperature optima of 50°C. In yet another study (Nirmal and Murali Krishna, 2003) the optimum temperature for amylases α-1, α-2 and α-3 from finger millet was found to be in the range 45°C to 50°C. The α and β-amylase enzymes obtained from maize (*Zea mays*) malt had optimal temperature 50°C and 90°C, respectively (Biazus *et al*., 2009). The α and β-amylases, isolated from *Sorghum bicolor* cv. (Feterita) malt, had maximum activity at temperature 70°C and 50°C, respectively (Mawahib *et al*., 2010).

Thus, it appears that amylases are generally thermostable with β-amylase being more tolerant to higher temperatures than α-amylases. However, this is the first report of optimum amylase activity at 60°C in finger millet. Further work to characterize the form of amylase exhibiting such high heat tolerance is warranted.

Germination entails breakdown of complex carbohydrates to simple sugars. Different forms and levels of amylases may be present at different stages of germination. The effect of germination duration on amylase activity in RAU 8 was studied at the optimum pH 5.0 and two incubation temperatures viz. 50°C and 60°C. The amylase activity observed after 24, 48 and 72 hour of germination was almost the same (Fig. 5). The best activity was observed in seeds

geminated for 96 hours and the activity declined thereafter. This indicates that maximal starch breakdown is completed by 96 hr and the energy requirements thereafter might be met by photosynthetic products in natural conditions. Earlier studies have found the highest α-amylase activity in African finger millet malt flour germinated at 15ºC for 9 days and at 20ºC for 6 days, while the highest β-amylase activity was displayed in the malt flour germinated for 5 days at 30ºC (Gimbi and Kitabatte, 2002). Finger millet seeds germinated for 72 hours were found suitable for amylase activity in yet another study (Nirmal and Murali Krishna, 2003) β-amylase was found to be catalytically active in African finger millet seed malted for 96 hours (Kolawole, 2009). Other reports exist showing varying degree of germination time (3-5 days) for best amylase activity in different crops (Mawahib *et al*., 2010), (Nerkar *et al*., 2011), (Usha *et al*., 2011), (Prakash and Deshwal, 2013).

Thus, not only does the germination time affect the production of amylase but it is also affected by the temperature of germination which in the present study was 28°C. The knowledge of the most suitable duration of germination and temperature of germination are important for generation of high quality malts during the brewing process.

Once it was established that extract from seeds germinated for 96 hour showed maximum activity at pH 5.0 and incubation temperature of 50°C/60ºC, the length of incubation for amylase activity was studied. The extract was incubated with the substrate (1% starch) for different time periods at 50ºC. The results obtained are shown in Fig. 6.

Maximal activity was obtained after incubation for 15 min. Thereafter, the activity was almost constant till 90 min. There was a gradual decline till 105 min and a sharper decline thereafter. Enzymes exhibit their activity *in vivo* in ideal microenvironments. Their activity *in vitro* is affected by many factors such as pH, incubation, salts etc. The pH (5.0) and incubation temperature (50°C) had been standardized for *in vitro* conditions in the present study. Such high temperatures are not normally experienced by plants and it is not known whether germination of finger millet takes place at 50°C. Therefore, the activity pattern reported is a reflection of the stability of the enzyme *in vitro*. In the cell the products formed are removed constantly and the reaction moves in the forward direction. This does not occur *in vitro*. The accumulation of the end products i.e. maltose and maltodextrins, may have an inhibitory effect on the amylases which is reflected by the decline in activity after 90 min. The decline in activity could also be due to the degradation of the enzyme *in vitro* after a certain period. It has been reported that African finger millet malt extracts incubated for up to 4 hours retained about 84 and 64% of α-amylase activities (Gimbi and Kitabatte, 2002). In the present study 82.24% activity was retained even after 2 hours (Table 1). The α-amylase isolated from *Heliodiaptomus viduus* (Gurney) has been shown to retain its full activity at 30ºC for 2 hours. However, it became inactive at 60ºC after 2 hours and at 70ºC after 1 hour (Prakash and Deshwal, 2013). Therefore, it is possible that incubation at lower temperature would have shown higher activity levels for a longer time in the present study. In the

same study cited above (Prakash and Deshwal, 2013) it was found that the enzyme activity was retained at 60% in 2 M NaCl after 24 hour incubation, while full activity was found in 0.5 M NaCl for the same duration of incubation. Therefore, changes in salt concentrations could also be studied to check for longer activity retention.

The results presented in this study would greatly help the brewing, detergent and baking industry in using RAU 8 seeds as a source of amylase. Further characterization of the different forms of the amylases present may help in isolation of the most robust form which could then be cloned and expressed in microbes to enable large scale production for industrial purposes. However, since different forms of amylases exhibit different properties it would be better to use the whole extract of germinated seeds. A demand from the industry for finger millet grains would translate into a valuable source of income for marginal farmers as the plant grows well on degraded soils in poor moisture conditions, thus requiring little or no input in terms of fertilizers and irrigation – the two major financial constraints of poor farmers.

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TABLES

Table-1. Retention of amylase activity at pH 5.0 and temperature 50°C by the extract of 1g seeds of genotype RAU 8 germinated for 96 hrs for different incubation periods.

Incubation time (min)	% reduction in activity	Activity retained (%)
15	0.00	100.00
30	3.73	96.27
45	3.58	96.42
60	4.03	95.97
75	4.03	95.97
90	4.48	95.52
105	8.66	91.34
120	17.76	82.24

FIGURES

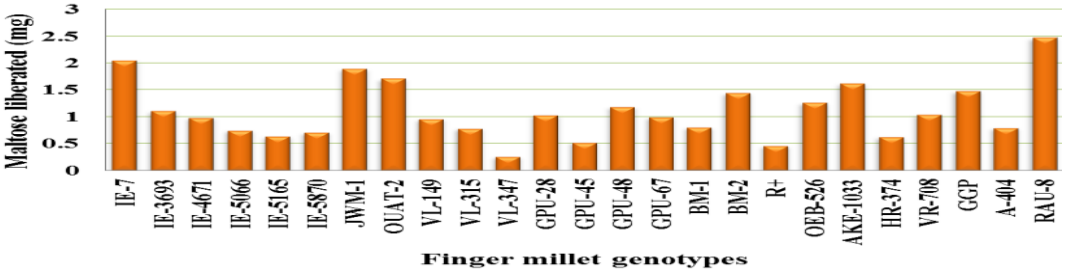

Fig-1. A histogram showing the maltose liberated from 1% starch solution in 30 minutes by amylase extracted from germinated seeds of different finger millet genotypes.

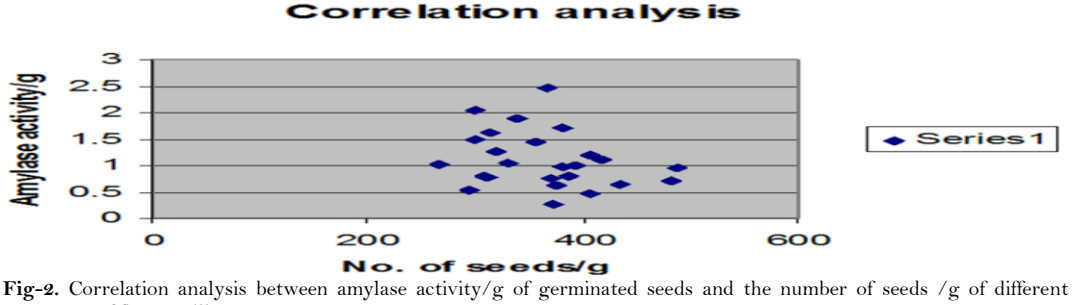

Fig-2. Correlation analysis between amylase activity/g of germinated seeds and the number of seeds /g of different genotypes of finger millet.

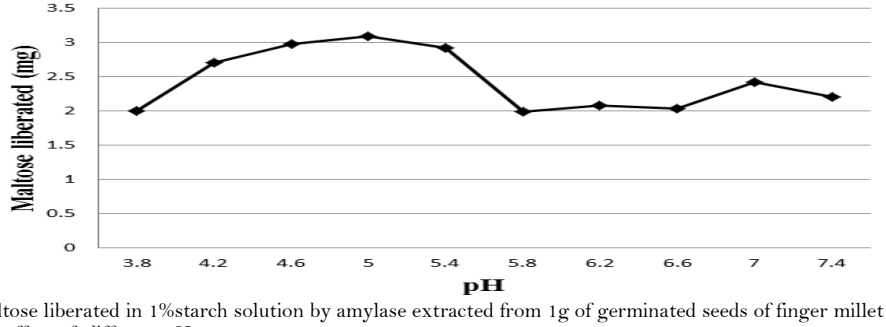

Fig-3. Maltose liberated in 1%starch solution by amylase extracted from 1g of germinated seeds of finger millet genotype RAU 8 in buffers of different pH.

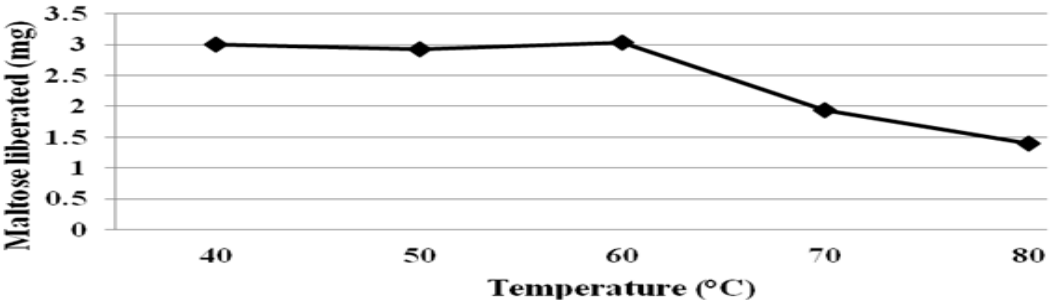

Fig-4. Maltose liberated in 1%starch solution by amylase extracted from 1g of germinated seeds of finger millet genotype RAU 8 at pH 5.0 and different temperatures, viz. 40°C, 50°C, 60°C, 70°C and 80°C.

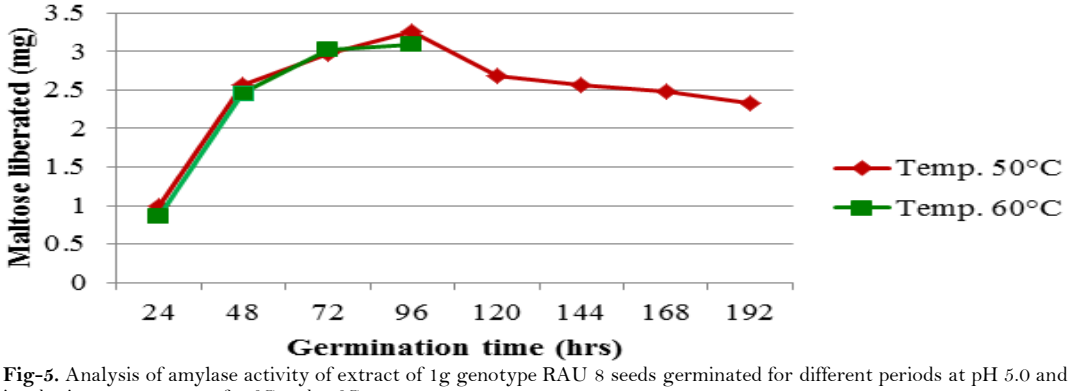

Fig-5. Analysis of amylase activity of extract of 1g genotype RAU 8 seeds germinated for different periods at pH 5.0 and incubation temperature of 50°C and 60°C.

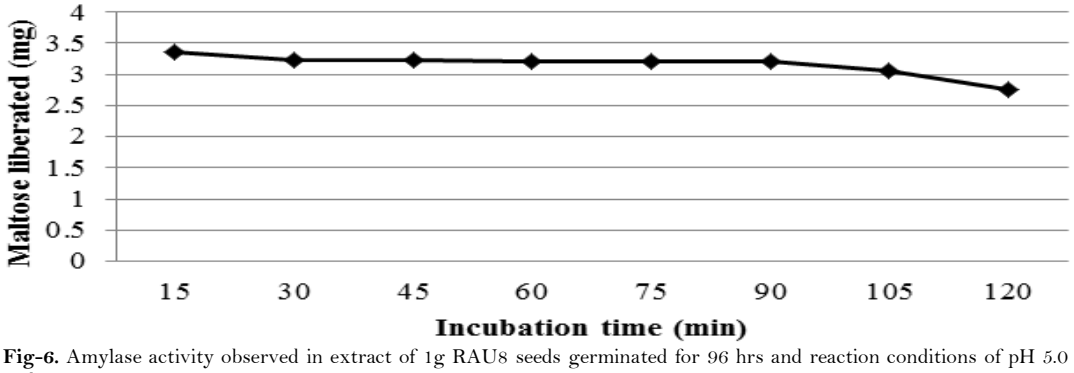

Fig-6. Amylase activity observed in extract of 1g RAU8 seeds germinated for 96 hrs and reaction conditions of pH 5.0 and temperature 50°C.