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A comparative kinetic study on β-amylase and its antioxidant property in germinated and non germinated seeds of *Glycine max*. *L*

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Enzyme activity plays a key role in each stage of plant development starting from the initial seed germination responses. A comparative analysis of beta amylase activity was performed, isolated from germinated and non germinated *Glycine max* seeds, which was partially purified by dialysis. The optimum pH and temperature for germinated seeds was found to be 4.5 and 37^0 C and that for non germinated seeds were 5.5 and 75^0 C respectively. $MnCl_2$ and $CoCl_2$ exhibited marked activating effect on the enzyme, while $HgCl_2$ was a potent inhibitor for both seeds. The K_m and Vmax value for β-amylase with soluble starch as substrate was found to be 3.03mg/ml and 6.6micromol/min/ml for germinated seed and for non germinated seeds it was found to be 5mg/m and 10micromole/min/ml respectively. The molecular weight of partially purified enzyme was 57±1kDa on SDS PAGE with 1.918 fold purification and 27.98% yield for germinated seeds and for non germinated seeds the purification fold was 1.507 with 26.65% yield. The enzyme showed highest amount of total antioxidant activity of 304µg/ml in partially purified germinated seed. For ABTS free radical scavenging activity, partially purified enzyme from germinated seed showed the highest of 76.09% activity with an IC_{50} value of 58.20µg/ml. Germinated seeds have highest enzymatic activity which can be considered for several industrial purposes.

Key words: *Glycine max,* β-amylase, antioxidants, enzyme activity.

Most species of the legume family (*Fabaceae*) have non-endospermic seeds. Dry legumes are good sources of protein, energy and other nutrients in developing countries. However, their use is limited because of high dietary bulk; presence of anti nutritional factors, mainly phytic acid in most of the legumes; and low protein and carbohydrate digestibility (Sumathi *et al*., 1995; Komal and Darshan, 2000; Mubarak, 2005; Negi *et al*., 2001). The cotyledons serve as sole food storage organs. During embryo development the cotyledons absorb the food reserves from the endosperm completely. In most seeds, the food is stored in the cotyledons, or seed leaves, as starch. Although starch is a storage form of glucose, it cannot pass through living membranes due to the large molecular structure, so before it can be used for energy it needs to be broken down to its constituent sugars by enzymes. Enzymes are the biological catalysts that allow the chemical

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reactions necessary for metabolism to occur. During germination it secretes the amylase enzyme that breaks down endosperm starch into sugars to nourish the growing seedling.

Amylase is classified into alpha, beta and gamma. Both α-amylase and β-amylase are present in seeds. β-amylase is present in an inactive form prior to germination, whereas α-amylase and proteases appear once germination has begun. Germination was shown to increase monosaccharide and decreased disaccharide contents of legumes due to α-amylase (Akinlosottu and Akinyele, 1991). Amylase are among the most important enzymes used for several biotechnological applications particularly employed in starch processing industries for the hydrolysis of polysaccharides such as starch into simpler sugar constituents (Mubarak, 2005). It is also found useful in a wide variety of industrial applications such as production of ethanol and high-fructose corn syrup, detergents, desizing of textiles, modified starches, hydrolysis of oil-field drilling fluids and paper recycling (Sumathi *et al*., 1995).

Antioxidants are the substances able to prevent or inhibit oxidation processes in human body as well as in food products. The natural antioxidants are a stable part of nutrition as they occur in almost all edible plant products (Aleksandra and Tomasz, 2007). An antioxidant may play a role in antioxidation as a free radical scavenger, reducing agent, chelator and/or singlet oxygen scavenger. Studies have demonstrated that plant phenolics are a major source of natural antioxidants (Emma and Nedyalka, 2003). They can be distributed in fruits, seeds, leaves, vegetables, barks, roots, and flowers of plants (Wanasundara *et al*., 1997, Wang & Lin, 2000).

So, the present investigation was started with the perspective to analyze the activity of enzyme isolated from germinated and non germinated seeds and to check its antioxidant capacity with the prospective of increasing its importance at industrial level.

Materials and Methods

Soya Bean seeds (*Glycine max*), a common variety in India, was purchased from a local market in Bangalore and stored in dry place at room temperature for experimental set up.

Preparation and extraction of crude enzyme

Two sets of 25g Glycine max seeds were taken and washed thoroughly under running tap water and surface sterilized by 2% savlon, and tween20 for 20 minutes. It was then rinsed with 70% ethanol for 30 seconds and 0.05% $HgCl_2$ for 6minutes. The seeds were then rinsed with distilled water to remove the traces of $HgCl_2$. One set of sterilized seeds were soaked in 25mM sodium acetate buffer pH 5.5 overnight at room temperature and the other set of seeds were allowed to germinate by covering them in muslin cloth for 24 hrs. The germinated and non germinated seeds were homogenized in sodium acetate buffer using blender. The homogenate was filtered through muslin cloth. The filtrate was collected and clarified further by centrifugation at 5000rpm for 20minutes at 4^0C. The supernatant (crude precipitate) was collected and stored at 4^0C for further enzyme assays.

Enzyme and Protein assay

The enzyme activity for germinated and non germinated seeds were determined by Bernfeld method (Bernfeld, 1955). The crude enzyme extract was diluted in the ratio of 1:30, using sodium acetate buffer. 0.1ml of and the diluted sample was incubated in 0.1ml of 1% soluble starch at room temperature for 5minutes. To this, 1.0ml of DNS was added and kept in boiling water bath for 20minutes. The final volume was made up to 10ml using distilled water and absorbance was read at 540 nm using

distilled water as blank. A standard curve using 1mg/ml of maltose was prepared. One unit (U/ml) of amylase activity was defined as the amount of enzyme that releases 1µg of maltose per minute under standard assay conditions. The amount of protein in crude enzyme extract was determined by Lowry's method (Lowry *et al.*, 1951) for both germinated and non germinated extract using bovine serum albumin as standard.

Partial purification of amylase

The crude extract of germinated and non-germinated seeds (50ml) was precipitated using 100% saturated ammonium sulphate solution. The precipitation was carried out at 4°C by constant stirring and centrifuged at 10,000rpm (9615g) for 15minutes. The precipitate was dissolved in sodium acetate buffer pH 5.5 and subjected to dialysis. Specific activity for the three fraction was estimated. The dialyzed sample was used for further studies.

Starch hydrolysis test for amylase (Shelby, 1993)

The hydrolysis of starch by partially purified amylase was studied by preparing starch agar plates. The plates were prepared by adding 1ml of 1% soluble starch mixed with 1% liquid agar and poured on a clean glass slide and allowed to solidify. After solidification, a well was created in the plate using gel punch and 40µl of partially purified germinated and non germinated enzyme was added. Then slides were maintained in a moist condition and incubated overnight at room temperature. After overnight incubation 1ml of iodine solution was added.

TLC analysis (Bilal and Figen, 2005)

The hydrolysis of soluble starch with dialyzed sample of both germinated and non germinated seeds were determined by thin-layer chromatography on TLC plate. 0.5ml of partially purified enzyme was incubated with 0.5ml of 2% starch for 5 minutes. The sugars released after enzymatic hydrolysis of amylase were separated on TLC using glucose, maltose and starch as standard with the solvent system of butanol: acetic acid: water (4:1:5). Spots on the TLC plate were detected using DPA (diphenylamine) spray after drying at 70^0C.

Determination of molecular weight of β-amylase (Laemmli, 1970)

The molecular weight of partially purified β-amylase was analyzed by SDS-PAGE (12%) from Laemmli's method. The bands were visualized by coomassie blue staining in order to determine the molecular weight of β-amylase using standard protein marker of 2-212kDa.

Effect of pH on β- amylase activity

β-Amylase activity from the dialyzed samples were determined using 1% soluble starch as substrate at a pH range from 4.5 to 7.8 using 25mM sodium acetate buffer. The optimum pH for enzyme activity was determined using DNS reagent.

Effect of temperature on β-amylase activity

A temperature gradient was employed in order to determine the β-amylase activity. Buffer, substrate solution, and dialyzed samples were incubated at different temperatures between 0°C to 100°C. The optimum temperature was determined using DNS reagent for enzyme activity.

Effect of metal ions on β-amylase activity

The effect of metal ions on the activity of β-amylase were determined by adding known concentrations of metal ions such as $CaCl_2$, $HgCl_2$, $CuCl_2$, SDS, $MgCl_2$, EDTA, $FeCl_3$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $AlCl_3$ and KCl. Each of these metal ions were added to the enzyme substrate reaction and incubated for 5minutes and its activation and inhibition property of each metal ion on enzyme activity was determined using DNS reagent.

Effect of Substrate Concentration for determination of *K*m and *V*max (Lineweaver and Burk, 1984)

The Michaelis-Menten kinetic constants, Km and *V*max, for partially purified β-amylase was determined by using varying concentrations of soluble starch. Enzyme activity (U/ml) against different concentrations of substrates (µM) were measured under standard assay condition and the kinetic constants, Km and Vmax, were estimated by the method of Line-weaver and Burk plot.

Determination of antioxidant capacity

Phosphomolybdenum assay (Prieto *et al*., 1999)

0.1ml of crude and dialyzed enzyme samples from germinated and non germinated seeds of *Glycine max* was taken and 1ml of total antioxidant reagent (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) was added and mixed. Different aliquots (20µg -100µg/ml) of standard ascorbic acid was taken and the volume was made up to 0.1ml with DMSO and 1ml of total antioxidant reagent was added and incubated in a thermal block at 95^{o}C for 90 minutes, cooled to room temperature and the absorbance was measured at 695nm against DMSO as blank.

ABTS (2, 2-azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid)) radical cation decolorization assay (Re *et al*., 1999)

ABTS radical cation ($ABTS^{+}$) was produced by reacting 7mM ABTS with 2.45mM ammonium persulfate and the mixture was allowed to stand in dark at room temperature for 12-16 hours before use. Enzymes samples of germinated and non germinated, crude and dialyzed, (2mg/ml) were taken at various concentrations (10-50µg/ml) and the volume was adjusted to 500µl with DMSO which also serves as blank. 0.3ml of ABTS solution was added, the final volume was made up to 1ml using ethanol and incubated in dark for 30 minutes at room temperature. The absorbance was read at 745nm. Radical cat-ion decolonization activity was expressed as the inhibition percentage of cat-ions by the samples and was calculated using the formula,

% ABTS radical scavenging activity = [(control OD – Sample OD)/ Control OD)] *100.

Statistical analysis:

The experiments were conducted in triplicates. All the values obtained from the mean replicates were averaged. The data were analyzed in relation to the variance and presented as mean+/-standard error (SE). Analysis of variance was conducted by two way ANOVA and all the statistical analysis was performed at 1% significance level using IBM SPSS Statistics (version 20) by IBM.

Results

Starch Hydrolysis test

The crude extract of germinated and non germinated soybean seeds confirmed the presence of amylase after overnight incubation on starch agar plate, a clear zone of starch hydrolysis was observed on addition of iodine solution. The zone of clearance was comparatively high in germinated than in non germinated seed extract.

Partial purification of enzyme

Crude extract was partially purified by ammonium sulphate precipitation and subjected to dialysis and purified to 1.918 fold with a yield of 27.98% for germinated seeds (Table 1) and non germinated seeds were purified to 1.507 fold with yield of 26.65% (Table 2).

Determination of molecular weight of β-amylase

The molecular weight of partially purified β-amylase in germinated and non germinated seeds were found to be approximately 57±1kDa by SDS-PAGE on staining with coomassie brilliant blue (figure 2).

Table 1: Summary of purification of β-amylase in germinated soybean seed extract

Step	**Volume (ml)**	**Total Activity (µmol /min)**	**Total Protein (mg)**	**Specific activity (units /mg of protein)**	**Purification fold**	**Yield (%)**
Crude enzyme	50	23.3	15.0	1.416	1.0	100
Purified enzyme by chilled ammonium sulphate precipitation method	10	11.748	4.5	2.610	1.843	50.4
Dialysed product	8.0	6.52	2.4	2.716	1.918	27.98

Table 2: Summary of purification of β-amylase in non germinated soybean seed extract.

Step	**Volume (ml)**	**Total Activity (µmol /min)**	**Total Protein (mg)**	**Specific activity (units/mg of protein)**	**Purification fold**	**Yield (%)**
Crude enzyme	50	21.25	9.3	2.505	1	100
Purified enzyme by chilled ammonium sulphate precipitation method	10	10.98	3.0	3.66	1.46	51.6
Dialysed product	8.0	5.66	1.5	3.776	1.507	26.65

Table 3: Percentage of scavenging activity and IC_{50} value of different enzyme extracts

Extracts	**ABTS% of scavenging**	**ABTS IC_{50} (µg/ml)**	**Total antioxidant (µg/ml)**
Ascorbic acid	52.082^{c}	55.66^{a}	-
Non germinated crude	27.97^{e}	160.74^{e}	20.02^{d}
Non germinated dialyzed	54.00^{b}	76.33^{c}	90.27^{c}
Germinated crude	43.73^{d}	125.46^{d}	239.3^{b}
Germinated dialyzed	76.09^{a}	58.20^{b}	304.0^{a}

Note: Mean of triplicates. Mean values with different superscripts ($^{a, b, c, d, e, f, g, h, I, j}$ and ^{k}) differ significantly at P<0.01 by Tukey (HSD) test.

TLC analysis of partially purified amylase

The partially purified enzyme was analyzed on TLC plate using maltose and glucose as standard. Both the germinated and non germinated sample fractions correspond with standard maltose which confirmed it to be β-amylase (figure 1).

Effect of pH on the β-amylase activity

To determine the effect of pH on enzyme activity, different pH ranges- 4.5, 5.5, 6.5, 7.5, 7.8 of sodium acetate buffer was taken and soluble starch was used as substrate. β-amylase activity was found to be maximum at pH 5.5 in germinated seeds and

for non germinated seeds the activity was found to be maximum at pH 4.5 (figure 3). Results showed a considerable decrease in amylase activity as the pH increased.

Figure 1: Thin Layer Chromatography of enzyme hydrolyzed product of non-germinated (NG) and germinated (G) seed samples. (Glu- Glucose, Mal-Maltose)

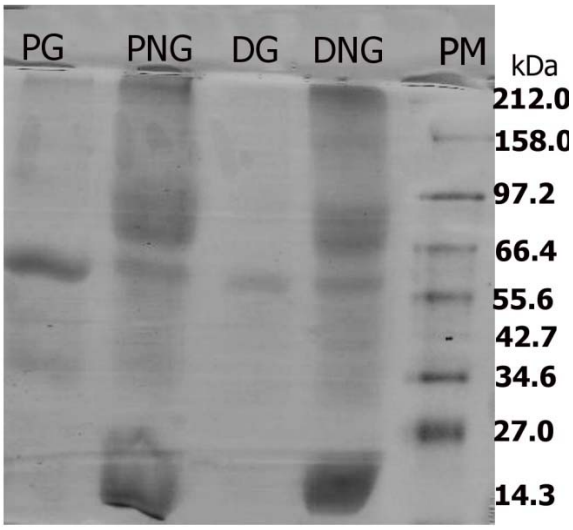

Figure 2: Molecular weight of β-amylase determined on SDS-PAGE (PM-Protein marker, DNG- Dialyzed Non Germinated, DG- Dialyzed Germinated, PNG-Precipitated Non Germinated, PG- Precipitated Germinated, seed enzyme samples)

Effect of temperature on β-amylase activity

The enzyme was subjected to various temperatures ranging from 0^0C - 100^0C and its activity was analyzed. The activity of β-amylase in germinated and non germinated seeds were found to be maximum at temperature 37^0C and 75^0C (figure 4) respectively. The activity of enzyme gradually decreased with the increase in temperature.

Effect of metal ions on β-amylase activity

Various metal ions such as KCl, $CaCl_2$, $HgCl_2$, $CuCl_2$, SDS, $MgCl_2$, EDTA, $FeCl_3$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $AlCl_3$ (at concentration of 2mM) were tested for activation or inhibition effects on the enzyme. There was a significant increase in enzyme activity in germinated seeds with $MnCl_2$, $CoCl_2$ (figure 5) and metals ions such as $CaCl_2$, $CuCl_2$, SDS, $MgCl_2$, $FeCl_3$, $ZnCl_2$, $AlCl_3$, KCl showed a negligible increase in the activity whereas $HgCl_2$ and EDTA inhibited the activity of enzyme. In non germinated seeds the activity was inhibited by $HgCl_2$ and metal ions such as $FeCl_3$, $ZnCl_3$, $MgCl_2$, EDTA, $CaCl_2$ showed a slight increase in enzyme activity, whereas $CoCl_2$, $AlCl_3$ and KCl showed the highest enzyme activity which acts as a potent activator.

Effect of substrate concentration and determination of Km and Vmax

The enzymatic activity for germinated and non-germinated seed was determined using soluble starch as the substrate. The kinetic constant Km and Vmax was determined using Lineweaver Burke plot. The substrate concentration (Km) for germinated and non-germinated seeds was 3.03mg/ml and 5mg/ml respectively. The maximum velocity (Vmax) for germinated and non-germinated was found to be 6.6µmol/min/ml and 10µmol/min/ml respectively (figure 6 and figure 7).

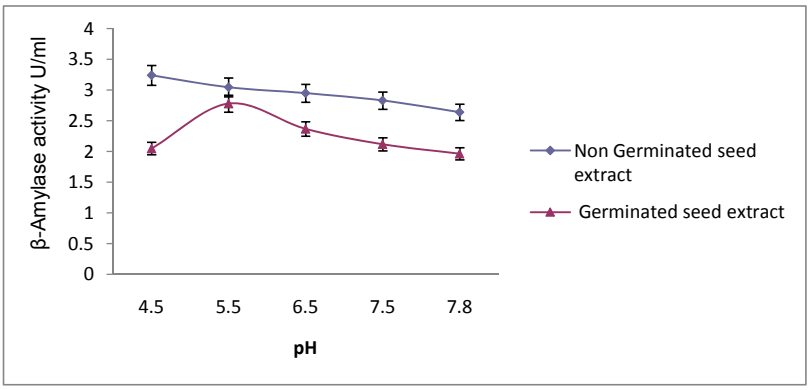

Figure 3: Effect of pH for β-amylase activity in germinated and non germinated seed.

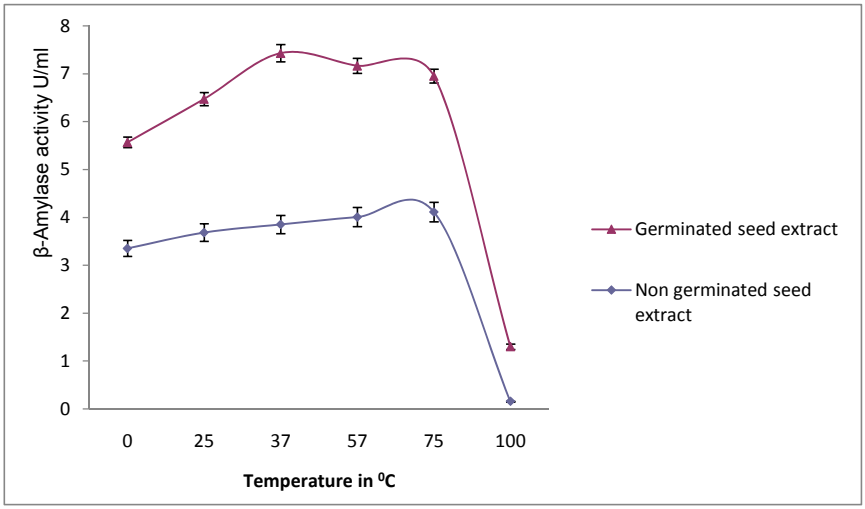

Figure 4: Effect of temperature for β-amylase activity in germinated and non germinated seed.

Antioxidant studies

The enzyme showed highest amount of total antioxidant content of 304µg/ml in dialyzed enzyme sample from germinated seeds where as non germinated seeds showed 90.24µg/ml. ABTS free radical scavenging activity showed 76.09% which was higher with an IC_{50} value of 58.20µg/ml for germinated seed enzyme when compared to non germinated seed with 54% of scavenging activity and IC_{50} value of 76.33µg/ml (table 3) (figure 8).

Discussion

During seed germination various enzymes are involved in the growth of seedling such

as amylase, which is an important enzyme employed in the starch processing industries for the hydrolysis of polysaccharide (Sanni, 2000). Amylase has vast industrial applications. The enzymes in germinated and non germinated seeds are affected by various factors such as pH, temperature and metal ions. The present investigation was carried on to check if any variation in the above factors during germination and before germination. pH affect the activity of the β-amylase , whose activity is mostly high in acidic pH in both germinated and non germinated G*lycine max* seeds which supports the previous studies (Nitta *et al*., 1979; Toda and Svensson, 2000; Shen *et al*., 1988). The most important factor is the temperature, which is essential for all seed germination, also affects the enzyme activity. Our studies revealed that β-amylase are highly active at 37^0C to 40^0C which justifies from the work by lizotte *et al*, 1990, in pea epicotyls. It has also been reported that beta amylase is active at 70^0C (Shen, 1988; Kirti, 2012), this agrees with the results obtained in the present study. Beyond this range there is decline in the activity because of the Structural unfolding trasition at high temperature (Duy and fitter, 2005).

Various metal ions play a major role either as activator or an inhibitor for enzyme activity. $Mncl_2$ and $CoCl_2$ enhanced enzyme activity in germinated and non germinated seeds. This is in line with the findings of (Dahot *et al*., 2001) in *Moringa oleifera* seeds and (Dutta *et al*., 2006) in *Heliodiaptomus viduus*. Most of the findings have shown that $HgCl_2$ is a potent inhibitor for enzyme activity. Lin *et al*., 1998 and Gupta *et al*., 2003, reports the inhibitory activity by Hg+ in microbial amylase which coincides with our present finding. As per the studies on beta amylase in plants, it shows that the molecular mass is between 50 to 60kDa (French, *1961;* Thoma *et al*., 1972; Mikami *et al*.,1993; Joyce *et al*., 1998, Motoyasu *et al*., 1988), and the reports by Yoshiki Yamasaki, 2003 closely relates with our present findings but doesn't supports the findings of Gertler and Yehudith, 1965; Sarowar *et al*., 2009**.**

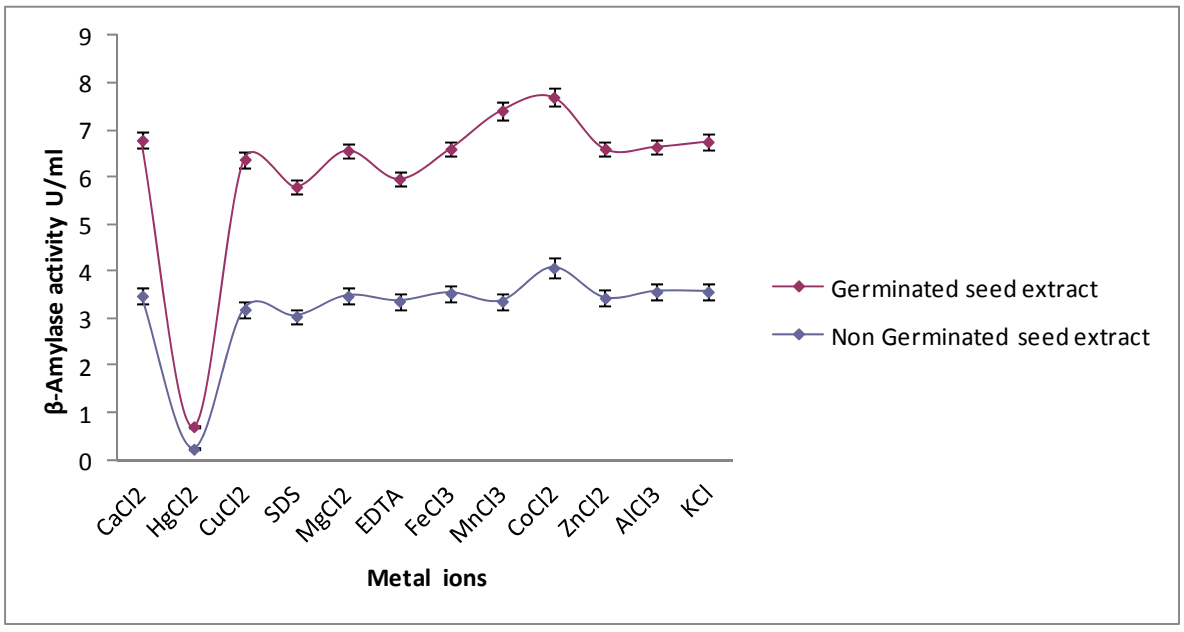

Figure 5: Effect of metal ions on β-amylase activity in germinated seeds and non germinated seed.

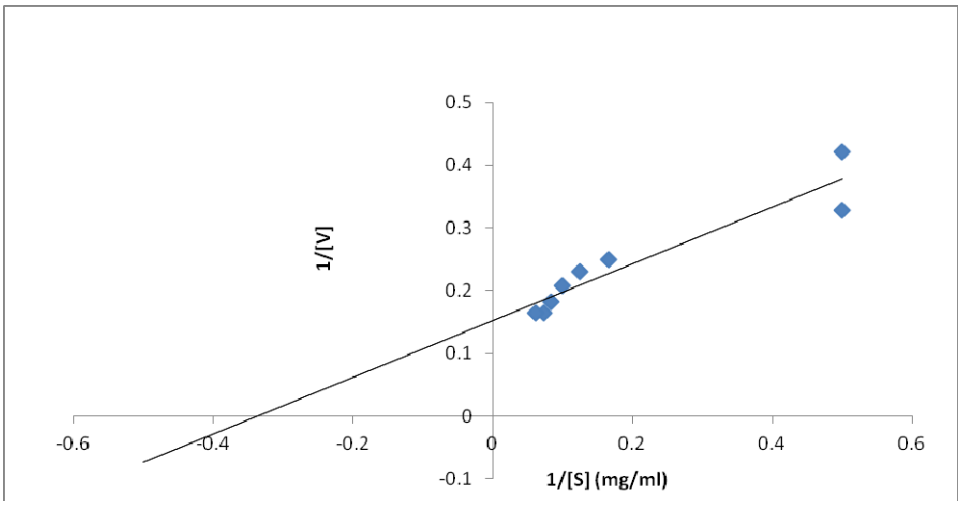

Figure 6: LB plot using soluble starch as substrate for germinated seed extract

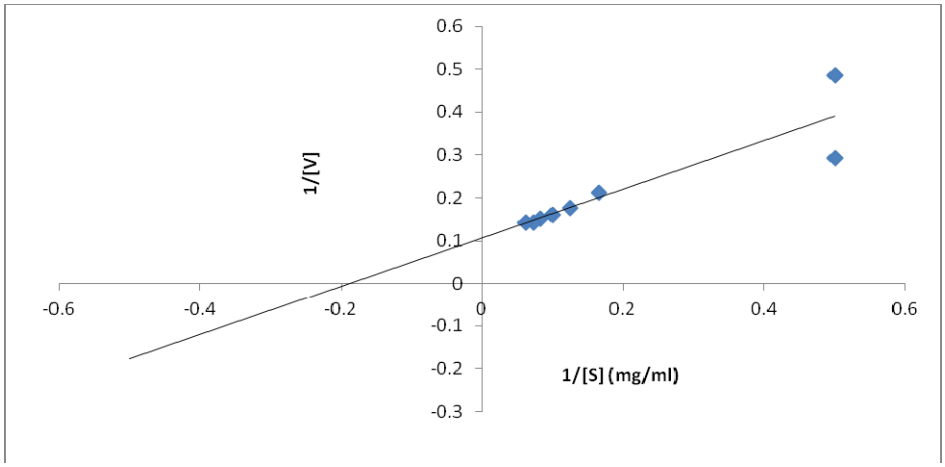

Figure 7: LB plot using soluble starch as substrate for non germinated seed extract

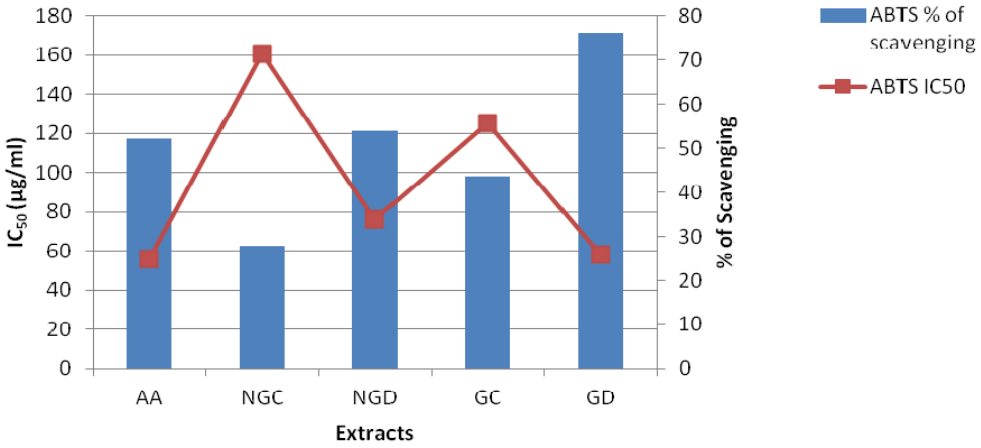

Figure 8: ABTS scavenging activity of different extracts and its IC_{50} (µg/ml) (AA- Ascorbic acid, NGC- Non Germinated Crude, NGD- Non Germinated Dialyzed, GC- Germinated Crude, GD- Germinated Dialyzed extracts).

Enzyme activity is also based on the substrate concentration (Km) and its maximum velocity (Vmax), the result obtained in the present research closely relates with the work on kinetic study by Femi-Ola and Ibikunle, 2013, and this also supports with the work by Deshwal Sapna, 2012; Sanni *et al*., 2000. Low Km activity indicates high affinity of the enzyme for the substrate (Hamilton *et al*., 1998). Antioxidants has a potent capacity of inhibiting oxidation processes in human body as well as in food products, which was analyzed on our isolated enzyme from germinated and non germinated seeds, shown to be better in germinated seeds which agrees with the findings of Fernandez- Orozco *et al* ., 2008. Based on the present work, it can be speculated that β-amylase and antioxidants from germinated seeds of *Glycine max* can be employed for industrial purpose

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