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# Extraction and Characterization of Alpha Amylase from *Phaseolus Aconitifolius.*

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## Abstract
The Alpha amylase breaks down long chain carbohydrates and that's why it is very useful in food and pharmaceutical industries. The aim of the present work is to extract and characterize the Alpha amylase from germinated *Mat beans*, for optimum pH and temperature for activity and stability, effect of metal ions, optimum substrate concentration, Km and $V_{max}$. *Mat beans* were germinated and then extracted with Acetate buffer (pH 5.0). The amylase assay was based on the reduction in blue color intensity resulting from enzyme hydrolysis of starch. For optimization of pH for stability, buffer solutions from pH range of 3.5 to 10.0 were prepared. For determination of optimum temperature, the range used was 35$^{o}$C to 65$^{o}$C. Substrate solutions in range of 0.1% to 4.0% were prepared for optimum substrate concentration. *Mat beans* germinated for 3 days show maximum enzymatic activity (65.79U/ml). 95% of the original activity was retained in the pH range 7.0 to 8.0. The maximum activity of an enzyme was found to be at 55°C. 10mM $Ca^{2+}$ stimulated the enzyme activity up to 123%. Optimum substrate concentration was found to be 1.5%. Km was found to be 2.0 mM, and Vmax 100.0 nm/min. Alpha amylase extracted from germinated *Mat beans* follows Michaelis-Menten equation. It can be concluded that *Mat bean* is a good source of Alpha amylase and this enzyme could find promising application of hydrolysis of starch.

## Key Words
Alpha amylase, *Phaseolus Aconitifolius*, mat beans, starch.

## Introduction
Alpha amylase (Alternate names: 1, 4-α-D-glucan glucanohydrolase, glycogenase, E.C.3.2.1.1) catalyze the random hydrolysis of amylose, amylopectin and related polysaccharides to smaller oligosaccharides and glucose. Alpha amylase, ubiquitous in nature, have been isolated, purified and characterized from a number of animal, plant, fungal, as well as bacterial sources[1]. The Alpha amylase is calcium metalloenzymes, many times completely unable to function in the absence of calcium[2]. Amylase is instrumental in starch digestion in animals resulting in the formation of sugars, which are subsequently used in various metabolic activities[3]. Cereal Alpha amylase has gained importance due to their suitability for biotechnological applications in supplementary foods, breweries and starch saccharification[4]. Cereal Alpha amylase plays a very important role in the starch metabolism in developing as well as germinating cereals. These highly expressed enzymes are getting synthesized under the influence of plant growth hormones such as gibberellic acid (GA3)[5]. The presence of Alpha amylase activity during barley, wheat and oat seed maturation[6], as well as during seed germination[7-9] has been extensively examined. It is approximately 30% of the total protein synthesized during germination. The site of amylase synthesis is reported to be either in aleurone layer[10] or scutellum[11,8]. The aim of the present work is to extract an Alpha amylase enzyme from germinated *Mat beans* (Phaseolus aconitifolius Family- Paillionaceae). In the Characterization of crude extract, various aspects of study include determining the pH optima for activity and stability, temperature optima for activity and stability, effect of metal ions on enzymatic activity, optimum substrate concentration, Km and $V_{max}$.

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## Materials and Methods

### Material

The beans of Phaseolus Aconitifolius were purchased from the local market of Otur, Dist–Pune.

### Germination of Mat bean and Extraction of enzyme

25 gm of *Mat bean* were soaked in water for 4-5 hr and then allowed to germinate for 1 day to 4 days at room temperature (28°C) in closed chamber; water was sprinkled on *Mat beans* twice a day. The germinated *Mat bean* were crushed in a mixture and then the slurry was dissolved in 4 fold acetate buffer of pH 5 containing sodium benzoate (4.8%) and methyl p-hydroxy benzoate (0.9%) as preservative. Slurry was stirred for 30 min. to ensure complete extraction of enzyme. Then the slurry was passed through the muslin cloth to separate the dissolved enzyme from the crushed Mat beans. It was kept at 8-12°C in a refrigerator. The large solid particles and starch were separated by centrifugation at 5000 rpm at 8-10°C for 15 min. To quantify the enzyme extraction, enzymatic assay was made to determine Alpha amylase activities[12,13].

### Optimization of germination period

For optimizing the germination period of *Mat beans* for maximum activity of Alpha amylase, four batches of 25 gm of presoaked *Mat beans* were kept for germination in closed chamber at room temperature. After every day enzyme was extracted from one batch of germinated *Mat beans* and enzymatic assay was performed[12].

### Confirmatory tests for Alpha amylase

1. Prepare viscous solution of raw starch, add enzyme extract to it.
2. Take 1 ml enzyme extract, add 5 ml 1% starch solution, heat for 10 minutes at $40^0$C, add to it 1ml Iodine solution[14.]

### Assay of enzyme

The amylase assay is based on the reduction in blue color intensity resulting from enzyme hydrolysis of starch. The reaction contained 1 ml enzyme supernatant and 10 ml of 1% starch solution incubated at 50°C for 10 min. The reaction was stopped by adding 10 ml of 0.1N HCl. 1 ml of this acidified solution was added to 10 ml of 0.1N HCl. From this, 1 ml was added to 10 ml iodine solution (0.05% iodine in 0.5% KI). The optical density of the blue-colored solution was determined at 660 nm. The same procedure was repeated using 1 ml-distilled water instead of the enzyme sample in order to measure the optical density without the enzyme[15].

### Characterization of Alpha amylase

### Optimum pH for stability

For optimization of pH for stability for Alpha amylase, buffer solution from pH range of 3.5 to 10 were prepared by using Acetate buffer of pH 5.0; Phosphate buffer of pH 6.0; and alkaline borate buffer in the pH range of 8 to 10. For pH 4 and pH 7, buffer tablets were used; then, enzyme extract was added to each buffer solution in 1: 1 proportion these solutions kept in stand for 12hr. at room temp ($28\pm2^o$C), then the assay was carried out for each buffer solution[12].

### Optimum temp for activity

For determination of optimum temperature for activity of the enzyme, the assay was carried out as per the procedure from the incubation temperature of $35^o$C to $65^o$C at optimum pH with an incubation period of 10 min[12].

### Optimum temp for stability

The enzyme preparation was incubated in water bath at 55°C for the period of the 10min to 120min and for 4°C in refrigerator as well as for Room temperature for the period of 1 day to 60 days. After the every time duration, enzyme was assayed at optimum pH and temperature as per assay procedure[12].

### Optimization of substrate concentration

For the determination of optimum substrate concentration for maximum enzymatic activity, Substrate, solutions of soluble starch in the range 0.1% to 4.0% were prepared. Then assay was carried out as mentioned earlier by using 10 ml of substrate, 1ml of enzyme extract at optimum pH and optimum temperature with an incubation period of 10 min[12].

### Determination of Km and Vmax

It was calculated by plotting Lineweaver- Burke plot[12].

### Effect of metal ions and other compounds

The effect of various metal ions like $Mg^{2+}$, $Ca^{2+}$, and Ethidine Diamine Tetra Acetate (EDTA) on enzymatic activity carried out my adding various concentration from 1 to 10 mM. The volume taken was 1ml in reaction mixture described in the assay and the assay was carried out at optimum pH and

optimum temperature with an incubation time 10 min[16].

## Results

**Optimization of Germination period and Confirmation of Alpha amylase**

25 gm *Mat bean* presoaked for 4-5 hr and germinated at room temp in closed chamber showed fungal growth after 4 days. Hence germination was not continued beyond 4 days. The crude extract of 3 days germinated *Mat beans* shows maximum enzymatic activity (65.79U/ml). [Figure 1]

The enzymatic activity kept on increasing from day 1 to day 3. In the confirmatory tests, decrease in viscosity of starch solution and change in the color of starch-iodine color complex were observed.

**Optimization of pH for stability**

The maximum enzymatic activity was found to be at pH 8.0 (65.85U/ml). In the pH range of 7.0 to 8.0 more than 95% of the original activity was retained after 12hr at room temperature. [Figure 2, Table 1]

**Optimization of temperature for activity**

In the optimization of temperature for Alpha amylase as the temperature increased from $35^0$C to 55°C enzyme activity increased progressively. Above 55°C enzyme activity decreased gradually. Thus the maximum enzymatic activity was found to be at 55°C (63.90U/ml). [Figure 3, Table 2]

**Optimization of temperature for stability**

Alpha amylase was not stable for 70 minutes at $55^0$C with the loss of almost 40% relative activity. For 20 minutes at $55^0$C, 100% relative activity was observed. More than 80% relative activity of Alpha amylase was retained after 30 days at $4^0$C. [Figure 4, 5, Table 3, 4]

**Effect of metal ions and other compounds**

Of various divalent metal ions examined, 5mM $Mg^{++}$ stimulated the enzyme activity up to 97%. 1mM and 10mM $Mg^{++}$ stimulated the enzyme activity by 83% and 93% respectively. 10mM $Ca^{++}$ stimulated the enzyme activity up to 123%. 1mM and 5mM $Ca^{++}$ stimulated the enzyme activity by 83% and 108% respectively. The 1mM EDTA inhibited the enzyme activity drastically. The activity with 10mM EDTA was lost up to 81%. [Table 5]

**Optimization of substrate concentration**

As the substrate concentration was increased above 0.1%, rate of reaction increased progressively up to 1.5%. When concentration increased above 1.5%, the rate of reaction remained almost constant. [Figure 6, Table 6]

**Determination of Km and Vmax**

Km and Vmax were determined from the Lineweaver Burke plot $K_m$ was found to be 2.0 mM and $V_{max}$, 100.0 nm/min. [Figure 7]

## Discussion

***Mat bean* Germination period and Confirmation of Alpha amylase**

Crude extracts of wheat at different stages of germination showed amylolytic activity when starch was used as a substrate. The activity of Alpha amylase increased from day 0 to day 3 of germination, where it exhibited its highest level ((65.79U/ml), followed by fungal growth on day $4^{th}$. Several articles has been studied the Alpha amylase activity during germination. Alpha amylase synthesized *de novo* and its level increased several hundred folds over a 4 or 5 day period[17], in barley aleurone, it is approximately 30% of the protein synthesized during germination[18]. Alpha amylase from germinated safflower seeds showed maximum activity after 5 days of growth[19]. Alpha amylase from wheat local verity (balady) showed maximum activity at $6^{th}$ day of germination[20]. Change in viscosity of starch solution and change in intensity of blue color complex formed by starch- iodine solution confirms presence of alpha amylase[14] and testifies the existence of glucidic reserve, particularly starch in the *Mat beans*[19].

**Effect of pH**

Alpha amylase was found to have pH optima at 8.0 which is alkaline. Slightly alkaline pH optimum was detected for Alpha amylase from *P*. erosus tuber (pH 7.3)[21]. However neutral optimum pH (7.0) was observed for Alpha amylase from vine shoot inter-nodes[22] and wheat isoenzyme AV[20]. Acidic pH optima ranged from 4.5 to 6.5 were reported for Alpha amylases from wheat Sakha 69 [23], finger millet[24], shoots and cotyledons of pea (*Pisum sativam* L.) seedlings[25], mung beans[26] and wheat isoenzymes AI to AIV[20].

**Effect of temperature**

The optimum temperature for *Mat beans* Alpha amylase activity is $55^o$ C. This value is consistent with data reported for other plant amylases (wheat Alpha-1 ($55^o$.C), pearl millet Alpha-1 ($55^o$.C) [4], safflower seeds ($55^o$ C)[19] and smaller than that for azuki bean amylase ($70^o$.C)[27].

*Mat bean* alpha amylase lost 40% of its activity at 70 $^{o}$ C. Wheat Balady isoenzymes showed only 30% activity at 70 $^{o}$ C[20]. The *P. erosus* tuber Alpha amylase was stable at temperature up to 40°C for 30 min incubation followed by rapid inactivation above 40°C[21]. Sorghum, millet and maize Alpha amylase was stable to heat denaturation at lower temperature (30°C)[28].

**Effect of substrate concentration**

Starch is apolysaccharide which is used by plant and animals for storing glucose for future use. Starch consists of amylose (Alpha-1,4-linkage ) and may be amylopectin (Alpha-1,6-linkage).Results indicated that *Mat bean* Alpha amylase had good affinity towards starch as a substrate. Wheat Alpha amylase isoenzymes had highest activity toward Alpha-1, 6-linkage. Glycogen was better substrate than starch[20].

For tuber Alpha amylase, high-molecular-mass substrates containing the Alpha-1, 4-linkage was better substrates for the enzyme. The relative rate of hydrolysis of the polymeric substrate decreased with decreasing percentage of Alpha 1,4-linkages and increasing percentage of Alpha 1,6-linkages in the substrate, suggesting that the enzyme prefers high molecular-mass, amylose type material as the substrate. It hydrolyzed amylose at rates similar to those obtained with soluble starch, but it was considerably less active on amylopectin and showed no effect on maltose and maltotetraose[26]. It was observed that all the three amylases from finger millet were found to have a high affinity towards its natural substrate i.e., ragi starch. The Alpha 3 was found to be most efficient followed by Alpha 1 and Alpha 2.The affinity for cereal starches were found to be in the order of ragi > rice > wheat > maize for Alpha 1 and Alpha 3 whereas for alpha 2 it was in the order of ragi> wheat > rice > maize[29].

**Effect of metal ions and other compounds**

$Ca^{2+}$ ions imparted activating effect on the alpha amylase. $Mg^{2+}$ ions showed slight activation and EDTA drastically inactivated the enzyme. Inactivation due the EDTA is may be because of chelating effect of the EDTA. Alpha amylase is known to be calcium- metalloenzymes containing at least one $Ca^{2+}$ per molecule[30] and its number may go up to 10[31]. Role of $Ca^{2+}$ and $Mg^{2+}$ in maintaining the stability and structure of the alpha amylase is well documented[32]. Enhancement of amylase activity of $Ca^{2+}$ ions is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which resulted in stabilization as well as maintenance of enzyme conformation.

In addition, calcium is known to have a role in substrate binding[33]. It has also been documented that binding of Ca to amylase is preferred over other cations such as $Mg^{2+}$ [34].

**Kinetic parameters**

$K_m$ was found to be 2.0 mM and $V_{max}$, 100.0 nm/min. It shows good affinity of alpha amylase towards substrate.

Km- Vmax for wheat balady isoenzymes for hydrolyzing starch were 1.42 mg/0.5 ml (0.28 %) and 0.83 ìmol reducing sugar for AI, 2.0 mg/0.5 ml (0.4%) and 0.625 ìmol reducing sugar for AII, 1.1 mg/0.5 (0.22%) ml and 0.3 ìmol reducing sugar for AIII, 2.5 mg/0.5 ml (0.5%) and 0.66 ìmol reducing sugar for AIV, and 1.7 mg/0.5 ml (0.34%) and 0.33 ìmol reducing sugar for AV. These values were similar to Km values reported for Alpha amylase from ragi *Eleusine coracana* (0.59% - 1.43% starch) and tuber *Pachyhizus erosus* (0.29% starch). The lower km value was reported for á-amylases from wheat Sakha 69 (0.57 mg and 1.33 mg starch/ml) and Mung beans (1.6 mg/ml). However, maize and millet Alpha amylase had the higher Km values (12.5 mg and 5.8 mg/ml, respectively)[20].

## Conclusion

*Mat beans* are extensively cultivated in all parts of India especially in Maharashtra, Karnataka and North Gujarat. Results show good affinity of the enzyme for substrate i. e. starch. So the *Mat beans* can be a cheaper source for the alpha amylase which is extensively used in food, textiles and Pharma industries. This study opens many horizons to us to comprehend the germination metabolisms of these types of seeds, the physiological significance of which calls for study in future works. Partial purification and enzyme immobilization can be the future plans for further studies on this enzyme.

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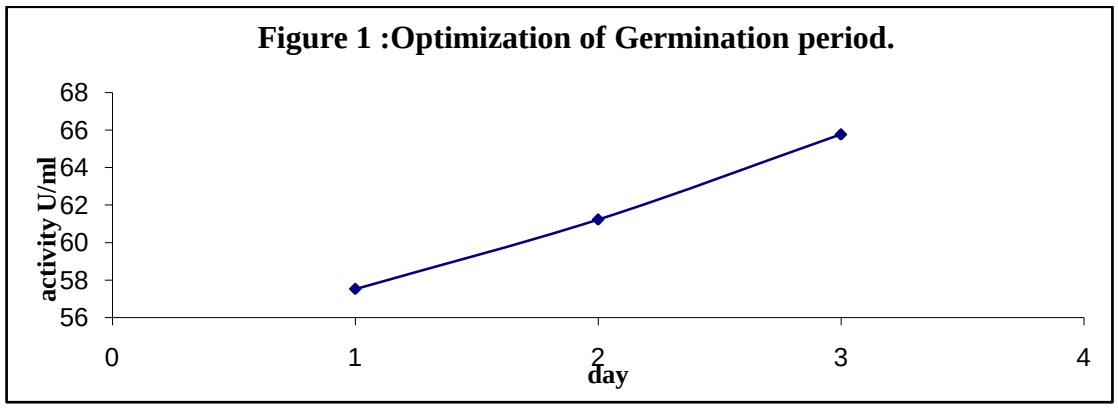



**Figure 1 :Optimization of Germination period.**

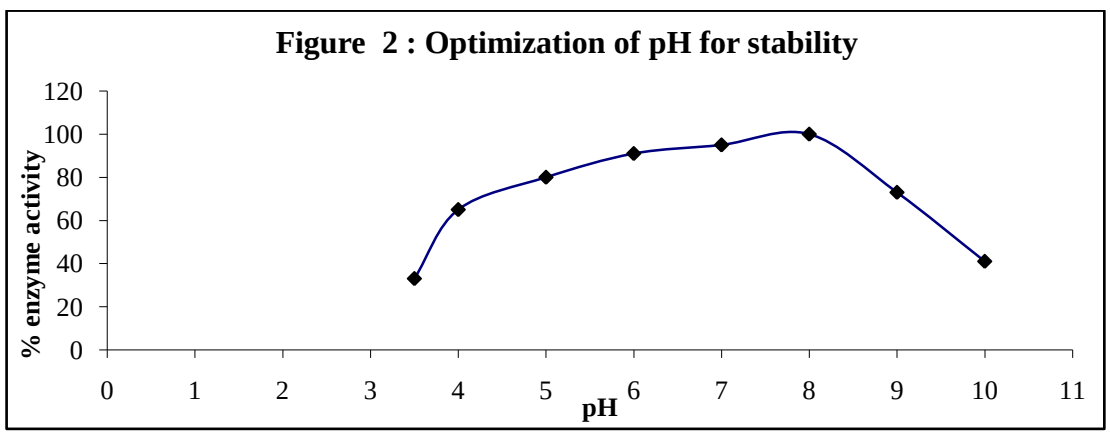



**Figure 2 : Optimization of pH for stability**

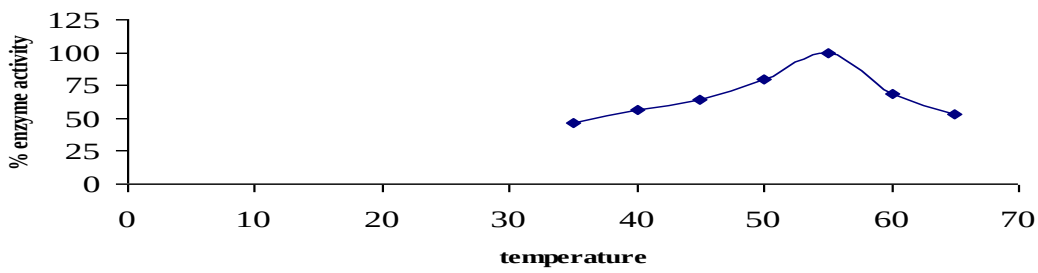



**Figure 3 : Optimization of temperature for activity**

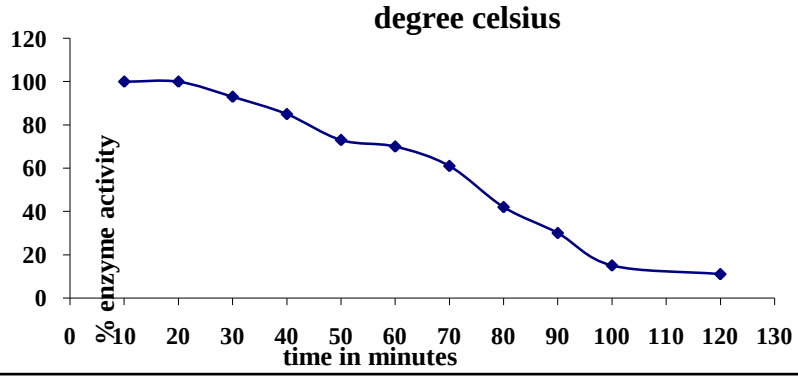



**Figure 4 : Optimization of temperature for stability at 55 degree celsius**

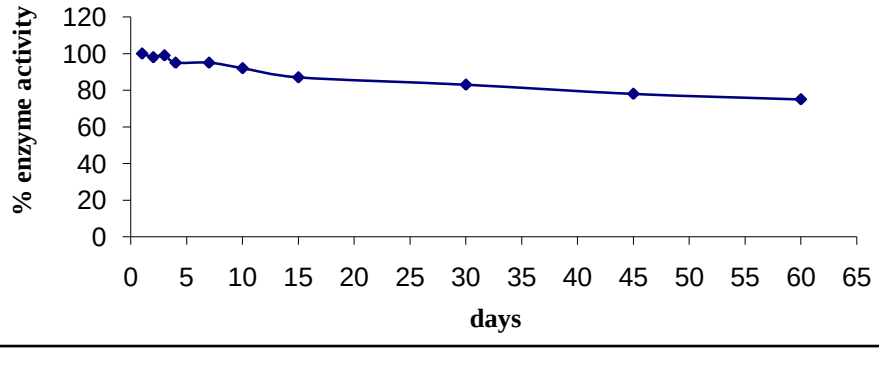
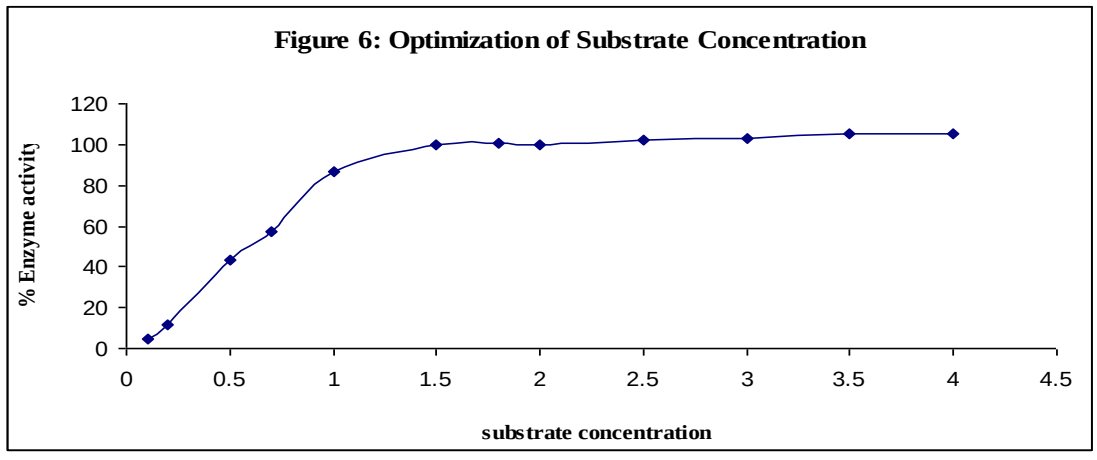



**Figure 5 : Optimization of Temperature for stability at 4 degree celsius.**



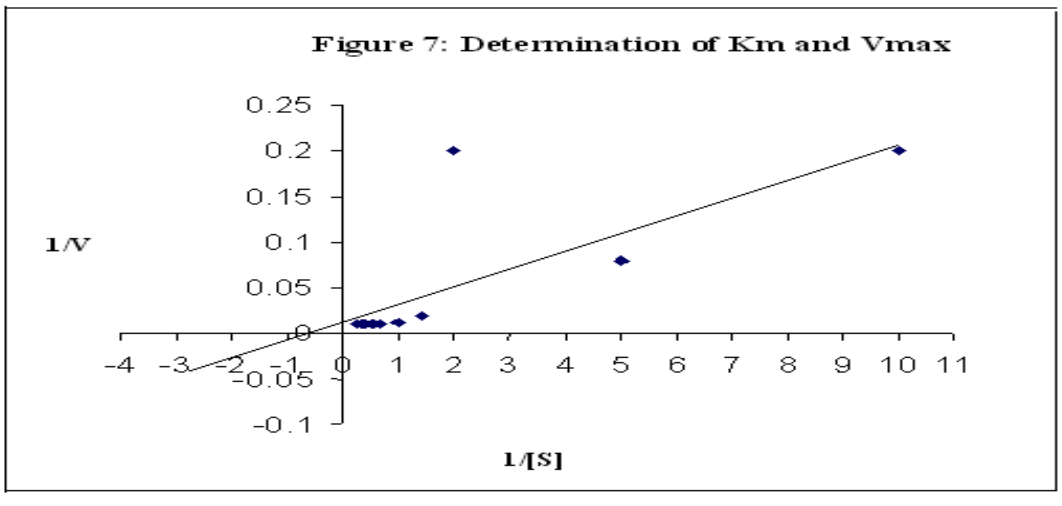




**Table 1: Optimization of ph for stability.**

| Sr. No | pH | Activity (U/ml)* | % Relative Activity |
|---|---|---|---|
| 1 | 3.5 | 21.73 | 33 |
| 2 | 4 | 39.51 | 65 |
| 3 | 5 | 52.68 | 80 |
| 4 | 6 | 59.92 | 91 |
| 5 | 7 | 62.55 | 95 |
| 6 | 8 | 65.85 | 100 |
| 7 | 9 | 48.07 | 73 |
| 8 | 10 | 26.99 | 41 |

*** Average value of 3 determinations**

**Table 2: Optimization of temperature for activity.**

| Sr. No | Temp. $^0$C | Activity (U/gm)* | % Relative Activity |
|---|---|---|---|
| 1 | 35 | 30.03 | 47 |
| 2 | 40 | 35.56 | 56 |
| 3 | 45 | 40.25 | 64 |
| 4 | 50 | 56.86 | 80 |
| 5 | 55 | 63.90 | 100 |
| 6 | 60 | 44.09 | 69 |
| 7 | 65 | 33.86 | 53 |

*** Average value of 3 determinations**

**Table 3: Optimization of temperature for stability at 55°c.**

| Sr. No. | Time (Min) | Activity (U/ml)* | % Relative Activity |
|---|---|---|---|
| 1 | 10 | 65.71 | 100 |
| 2 | 20 | 65.71 | 100 |
| 3 | 30 | 61.11 | 93 |
| 4 | 40 | 51.94 | 85 |
| 5 | 50 | 47.96 | 73 |
| 6 | 60 | 45.99 | 70 |
| 7 | 70 | 40.08 | 61 |
| 8 | 80 | 27.59 | 42 |
| 9 | 90 | 19.71 | 30 |
| 10 | 100 | 9.85 | 15 |
| 11 | 110 | 7.22 | 11 |

*** Average value of 3 determinations**

**Table 4: Optimization of temperature for stability at $4^0$c.**

| Sr.No | Duration (Days) | Activity (U/ml)* | % Relative Activity |
|---|---|---|---|
| 1 | 1 | 64.20 | 100 |
| 2 | 2 | 62.90 | 98 |
| 3 | 3 | 63.55 | 99 |
| 4 | 4 | 60.99 | 95 |
| 5 | 7 | 60.99 | 95 |
| 6 | 10 | 59.06 | 92 |
| 7 | 15 | 55.85 | 87 |
| 8 | 30 | 53.28 | 83 |
| 9 | 45 | 50.07 | 78 |
| 10 | 60 | 48.15 | 75 |

**Table 5: Effects of metals and inhibitors on activity of Alpha amylase.**

| Sr. No | Concentration (mM) | % Relative activity | | |
|---|---|---|---|---|
| | | $Mg^{++}$ | $Ca^{++}$ | EDTA |
| 1 | 1 | 83 | 83 | 63 |
| 2 | 5 | 97 | 108 | 44 |
| 3 | 10 | 93 | 123 | 19 |

**Table 6: Optimization of substrate concentration.**

| Sr. No. | Substrate conc. | Activity (U/ml)* | % Relative Activity |
|---|---|---|---|
| 1 | 0.1 | 3.27 | 5 |
| 2 | 0.2 | 7.85 | 12 |
| 3 | 0.5 | 28.14 | 43 |
| 4 | 0.7 | 37.30 | 57 |
| 5 | 1.0 | 56.94 | 87 |
| 6 | 1.5 | 65.45 | 100 |
| 7 | 1.8 | 66.10 | 101 |
| 8 | 2.0 | 65.45 | 100 |
| 9 | 2.5 | 66.75 | 102 |
| 10 | 3.0 | 67.41 | 103 |
| 11 | 3.5 | 68.72 | 105 |
| 12 | 4.0 | 68.72 | 105 |

*** Average value of 3 determinations**