

Production of alpha-amylase enzyme from grain sprouting

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Abstract: Alpha-amylase is considered to be one of the primary enzymes responsible for starch degradation in cereals. During grain germination, α -amylase initiates the conversion of starch into simple sugars to fuel embryo and coleoptile growth in the first few days of germination. The present study involves the estimation of Alpha amylase content in four different grains varieties, namely, wheat grain and barley up to 8 days after germination. It was observed that, alpha amylase content steadily increased, and found to be saturated after reaching the peak values. In all the samples, the peak values were observed in 7th day of germination. The enzymatic assay were done by estimation of maltose content by Spectrophotometry as starch degraded to maltose by alpha amylase enzyme.

Keywords: Alpha amylase, Grains, Spectrophotometry



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Introduction

In past, sprouting of grains was considered a negative aspect for consumption as it was believed that during sprouting, carbohydrate and protein reserves breaks down into smaller fractions, to support the metabolic need of developing seedlings while decreasing the functional quality of grain as food by means of increased hydrolytic and proteolytic activity. Recently, it has been shown that cereals sprouts are more nutritious in terms of vitamins, minerals, and phenolic compounds when compared with their native counterparts because grain's mobilized energy reservoir is readily available in its active form. The responsible enzyme for the said process is α -amylase (EC 3.2.1.1). It is an endo-hydrolase belonging to the glycoside hydrolase 13 family and is considered to be one of the primary enzymes responsible for starch degradation. The roles and number of these α -amylase isoforms vary across the plant kingdom.

Seed germination is a critical process ensuring the continuity of life in plants that depend on it as healthy seedling development; with seedlings that have the capacity to withstand biotic or abiotic Stresses and develop into high yielding crops. The major source of energy for seed germination and seedling establishment is the degradation of stored starch in the endosperm achieved by means of α -amylase enzyme. Grains are the potential sources of amylase and after germination in sprouted grains content of alpha amylase has been found to be increased for generation of more usable energy in the form of maltose. Such sprouted grains can be used to prepare various energy foods. Amylase enzymes are classified from the group of hydrolysis enzymes, and they claim this name because they work on the analysis of amylose Amylolytic Enzymes Alpha amylase is one of the oldest enzymes used by man in various fields, the most important of which was the field of food industries, as he used it in

the analysis of starch to produce a drink Glucose as used in the production of ethanol and its use in improving bread (1)..It is also considered one of the commercially

important enzymes due to its ability to decompose starchy substances into simple sugars such as dextrin's, maltose and glucose. Which are considered important sugars in the food industry and the fact that amylase enzymes increase greatly in germinated grains, many researchers have produced the alpha- amylase enzyme from different germinated grains such as barley, wheat and sorghum, and studied its properties and use in the food industry Such as the use of malt in the production of meals for infants as an aid in the analysis of starchy substances into simple, easily digestible substances (1). Studies tended to use malt as one of the plant sources rich in amylase enzymes, being practical Malt production and enzyme extraction are easy methods and do not require sophisticated devices. The possibility of using grains that are used as animal feed in the production of malt, and thus the production of alpha- amylase enzyme from it. In addition to not producing. Toxic during the grain germination process compared to the production of materials. The enzyme is from microbial sources

In the present study, α -Amylase has been estimated by spectrophotometric method. The reducing groups released from starch were measured by the reduction of 3,5-dinitrosalicylic acid. One unit releases from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25°C and pH 6.9 under the specified conditions.

The most widespread applications of α -amylases are in The starch industry, which are used for starch hydrolysis In the Starch liquefaction process that converts starch Into fructose And glucose syrups (16). The enzymatic conversion of all Starch includes: gelatinization, which involves the dissolution Of starch granules, thereby forming a viscous suspension; Liquefaction, which involves partial hydrolysis and loss In Viscosity; and saccharification, involving the production of Glucose and maltose via further hydrolysis (12, 17). Initially, The

α -amylase of *Bacillus amyloliquefaciens* was used but it Has been replaced by the amylase of *Bacillus Stearothermophilus* or *Bacillus licheniformis* (18). The Enzymes from the *Bacillus* species are of special Interest for Large-scale biotechnological processes due to their remarkable Thermostability and because efficient expression systems are Available for these enzymes.)(17). Detergent industries are the primary consumers of Enzymes, in terms of both volume and value. The use of Enzymes in detergents formulations enhances the detergents Ability to remove tough stains and making the detergent Environmentally safe. Amylases are the second type of Enzymes used in the formulation of enzymatic detergent, and %90of all liquid detergents contain these enzymes (12, 13)These enzymes are used in detergents for laundry and Automatic dishwashing to degrade the residues of starchy foods Such as potatoes, gravies, custard, chocolate, etc. to dextrans And other smaller oligosaccharides. Amylases have Activity at lower temperatures and alkaline pH, maintaining the Necessary stability under detergent conditions and the oxidative Stability of amylases Is one of the most important criteria for Their use in detergents where the washing environment Is very Oxidizing (13, 14). Removal of

starch from surfaces is also important in providing a whiteness benefit, since starch can be an attractant for many types of particulate soils. Examples of amylases used in the detergent industry are derived from *Bacillus* or *Aspergillus*. (15). Ethanol is the most utilized liquid biofuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (6). In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as α -amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae*. The production of ethanol by yeast fermentation plays an

important role in the economy of Brazil (8). In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* (6). Among bacteria, α -amylase obtained from thermoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions. (19). Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (7). The α -amylases have been widely used in the baking industry. These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of α -amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread. Besides generating fermentable compounds, α -amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products (12, 18). Currently, a thermostable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry (18). Amylases are also used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber (10,11,18). Amylases are used in textile industry for desizing process. Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be removed quite easily. Starch is later removed from the woven fabric in a wet-process in the textile finishing industry. Desizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. The α -amylases remove selectively the size and do not attack the fibres (4, 9, 12). Amylase from *Bacillus* stain was employed in textile industries for quite a long time.

The use of α -amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch (12, 18). The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper. In this application, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α -amylases in a batch or continuous processes. Starch is a good sizing agent for the finishing of paper, improving the quality and erasability, besides being a good coating for the paper. The size enhances the stiffness and strength in paper (5, 12). Examples of amylases obtained from microorganisms used in paper industry include Amizyme®

(PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and -amylase G9995® (Enzyme Biosystems, USA) Alpha amylase can be derived from several sources such as plants, animals and microbes. The microbial enzyme meets the industrial demand and a large number of them are available commercially (Pandey et al., 2000) [22]. Safey and Ammar (2004) [24] reported approximately 5.66 fold Purification of amylase from *Aspergillus Falsus* var. *Columnaris* by ammonium sulphate precipitation 60% Whereas 9.6 fold purification reported in case of gel filtration Chromatography. Similarly (Shih et al., 1995) [25] reported Approximately 20.5 fold purification of Amylase from *Clostridium perfringens* in case of gel filtration Chromatography. The purified alpha amylase exhibited an Isoelectric point of 3.7, and a molecular weight of 65,000. The Optimum enzyme activity was obtained at pH 5.5, and the Enzyme showed stability at 40°C. The main

end-products of Maltotetraose, hydrolysis were glucose and maltose. Although Amylase has been derived from several fungi, yeasts, bacteria And actinomycetes, however, enzymes from fungal and Bacterial sources have dominated applications in industrial Sectors. Fungal sources are mostly terrestrial isolates such as *Aspergillus* species. Amylases from plant and microbial Sources are employed for centuries as food additives (Mabel Et al., 2006) [21]. Barley amylases are used in Brewing Industry. Fungal amylases are widely used in preparation of Oriental foods (Popovic et al., 2009). Fungal and bacterial Amylases are mainly used for industrial applications due to Their cost effectiveness, consistency, less time and space Requirement for production and ease of process optimization And modification (Ellaiah et al., 2002) [5] Among bacteria *Bacillus* sp. Is widely used for the production Of amylases. Species like *B.subtilis*, *B. stearothermophilus*, *B. Licheniformis*, and *B. amyloliquefaciens* are known to be good Producers of alpha amylase. Similarly filamentous fungi have Been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of Extracellular proteins, they are widely exploited for the Production of different enzymes including alpha amylases (Juliana et al., 2011) [20]. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the Production of alpha amylase. Production of enzymes by solid State fermentation using these moulds turned a cost effective Production technique. Starch in cereal, root and tuber crops Has been extensively characterized, but little study has been Done on characterization of fruit starches and its impact on Fruit texture. Apples, like many other fruit crops, accumulate Starch at early stages of maturation and progressively degrade Starch to increase sweetness during ripening (Parveen et al., 2011).]23[

1-1 Enzymes in developing wheat grain

Mature wheat grain contains a wide range of enzymes, including α -amylases, β - amylases, α -glucosidase, proteases, lipases, lipoxygenases, polyphenol oxidases and peroxidases, that are distributed not only in the living tissues (i.e. embryo, scutellum, and the aleurone) but also in the dead tissues (i.e. seed coat and the starchy endosperm) (Carver, 2009). During normal wheat grain development, α - amylase activity in developing grain increases in the early stages following anthesis and then decreases slowly towards grain maturation and ripening (Sandstedt and Beckford, 1946). In immature wheat grain, the major portion of α -amylase activity is in the pericarp and only a small portion is found in the seed coat and endosperm. In addition to this, Marchylo et al. (1980) observed

negligible α -amylase activity in embryos of immature wheat grain. However, occasionally a high level of α -amylase is found during the later stages of grain development in the case of PHS or PMA. Thus, α -amylases are present at very low levels in dry, ripe grain, but are induced during germination to mobilize storage macromolecules in the endosperm. The degradation of starch in the endosperm of germinating cereals is a key process that involves the action of hydrolytic enzymes. Initially, starch granules are hydrolyzed by α -amylase to release branched and linear dextrins, which are then subsequently hydrolyzed to maltose and glucose by the combined action of β -amylase, α -glucosidase, and limit dextrinase (Carver, 2009).

Methods

Evaluation of the effectiveness of amylase determination enzymes
Activity of Amylases
The activity of the amylase enzyme was estimated using method (3) with some modifications to it. The enzymatic unit is defined as the amount of enzyme that hydrolyzes 0.1 mg of the subject and starch within ten minutes at a temperature of 40°C when the concentration of the subject is (4 mg ml). The protein concentration in the different stages of the study was estimated based on to the way (2)

Samples:

Wheat and barley of grains were purchased from local market of Al-refaay city.

Chemicals and reagents:

α -amylase enzymes, Sodium phosphate buffer, Sodium Chloride, Ethyl alcohol, Sodium potassium tartarate tetrahydrate, Sodium hydroxide, 3, 5 dinitrosalicylic acid, starch and maltose were gifted from Wassit university.

Instrument /equipment:

Shimadzu UV1900 spectrophotometer, thermostatic digital Water Bath and analytical balance were used for this analysis.

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Procedure:

Sprouting Technique

The sprouting method was based on that used by Tian et al (2010). To inhibit microbial Growth in sprouts, wheat and barley (100g) were soaked in a 2% hypochlorite solution for 15 min, followed By constant rinsing with tap water for 20 min. wheat and barley were then soaked in 2 parts 20-22°C water to One part wheat and barley (w/w) for 6-8 h. The soaking and Sprouting took place in a controlled atmosphere chamber with 98 percent humidity at 16°C. After Soaking, seeds were wrapped in cheesecloth and placed on perforated, aluminum pans in the Control atmosphere chamber. This

point was considered the beginning of sprouting time. Every 24 h, the wheat and barley were watered and aerated by hand (Donkor et al 2012).

Preparation of standard curve with Maltose: In numbered tubes, maltose standard ranging from 0.3 to 5.0 micromoles per ml has been prepared including two blank tubes with water. Into a series of corresponding numbered tubes, 1 ml of each dilution of Maltose (0.3-5.0 micromoles/ml) has been added followed by addition of 1 ml of dinitrosalicylic acid color reagent. The test tubes were incubated in boiling water bath for 5 minutes and cooled to room temperature. 10 ml distilled water was added to each tube and mixed well. Absorbance was taken at 540 nm. The standard curve was given in Figure 1.

Enzyme assay: 0.5 ml of respective enzyme dilutions (1-10 micrograms/ml) were added into a series of numbered test tubes including a blank prepared with 0.5 ml reagent grade water.

Sample preparation:

2 to 3 gm of grounded grain samples were weighed and dissolved in 50 ml of buffer solution by means of vortex. The solutions were centrifuged at 4 deg C for 10 minutes at 8000 rpm. 0.5 ml of aliquot from each tubes was pipetted out in another set of test tubes. All the test tubes were incubated at 25°C for 3-4 minutes to achieve temperature equilibrium. 0.5 ml starch solution (at 25°C) were added followed by incubation for exactly 3 minutes and at timed intervals 1 ml dinitrosalicylic acid color reagent has been added to each tube. All the test tubes were incubated in a boiling water bath for 5 minutes followed by cooling to room temperature.

After reaching the room temperature, 10 ml reagent grade water were added in each tube. The contents of the tubes were mixed well and absorbance was read at 540 nm versus blank by means of UV-Visible spectrophotometer. Micromoles of maltose released from standard curve were calculated from the standard curve of Maltose. The results were depicted in Table 1.

Evaluation of the effectiveness of amylase determination enzymes

Activity of Amylases The activity of the amylase enzyme was estimated using method (20) with some modifications to it. The enzymatic unit is defined as the amount of enzyme that hydrolyzes 0.1 mg of the subject and starch within ten minutes at a temperature of 40°C when the concentration of the subject is (4 mg ml).. The protein concentration in the different stages of the study was estimated based on to the way (9)

Thermal stability of the enzyme in the presence of calcium chloride

ml of imidazole buffer solution (5) containing 0.01 M of calcium chloride was added to 2 ml of purified enzyme solution and incubated in a water bath for (10, 20, 30 minutes) at temperatures ranging between (190-60°C). The enzyme activity was estimated according to the method. (19) A relationship was drawn between the percentage of residual activity at different temperatures to find out the stability of the enzyme in the presence of the calcium ion

Determining the optimal PH for the activity of the enzyme.

The pH was set for the stability of the activity as mentioned (14), as the purified enzyme was incubated at a temperature of 40. for 30 minutes with buffer solutions ranging from

Set the optimal PH to stabilize enzyme activity

The optimum pH for effectiveness was set according to what was mentioned (14), as the purified enzyme was incubated at a temperature of 40 °C for 30 minutes with buffer solutions with a range of pH ranging from (4-9). The solutions of the subject matter were prepared as stated in (14).

Calculation and Expression of Results:

α -Amylase was calculated using the following equation:

units/min/mg of protein : Micromoles maltose released mg enzymes in reaction mixture x 3 min

Results and discussion

Results and Discussion

During the first 24 h of germination of wheat seeds, starch is hydrolysed by free β -amylase. In the next 24 h, some amount of inactive form of β -amylase is converted into active form and this together with α -amylase synthesized *de novo* brings about the hydrolysis of starch. The amount of α -amylase is greater in seeds with embryo intact than with embryo excised after 24 h hydration. However, at later stages of seed germination α -amylase becomes predominant and the activity of β -amylase steadily diminishes.



Barely seed

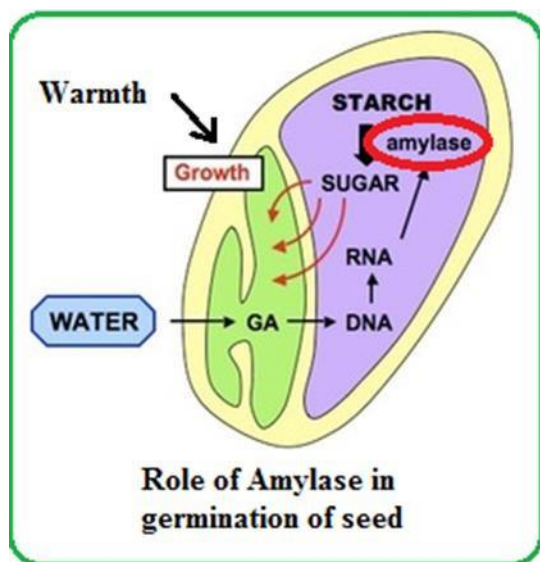


wheat seed

During cereal seed germination, α -amylase in the aleurone layer plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and shoot.

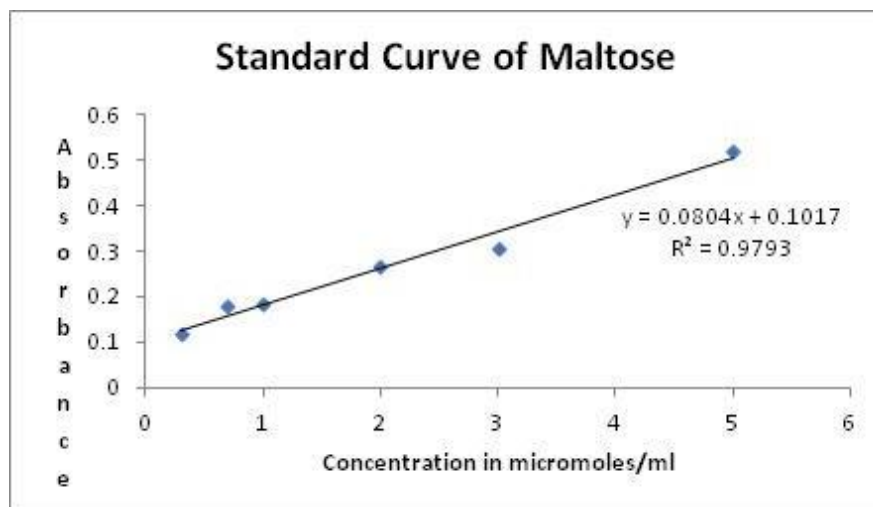
1. In the presence of H₂O, Gibberellin or gibberellic acid (GA) stimulates the production of amylase.

2. Amylase breaks down starch to maltose, allowing for the formation of ATP (via glucose).
3. The energy produced in the embryo is used to facilitate germination.
4. The glucose produced may also be used to synthesis cellulose - for cell wall formation.
5. Warmth helps speed up the process.



Standard curve with Maltose: The standard curve of maltose was prepared from 0.3-5.0 micromoles/ml. The regression co-efficient was found to be 0.979.

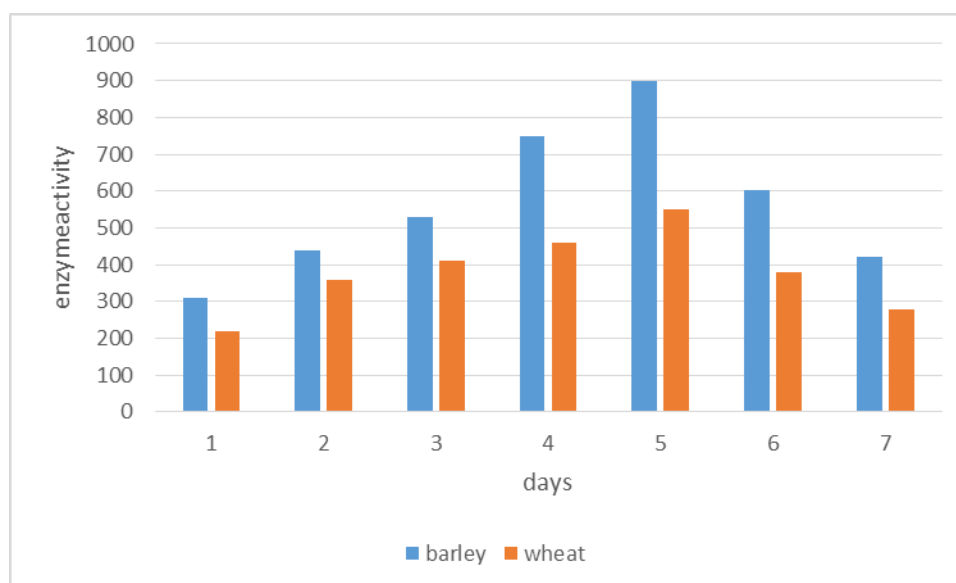
Figure 1: Standard Curve of maltose



Estimation of α -amylase content:

The α -amylase contents of different grain samples, namely, wheat and barley were carried out each day (from day 1 to day 7). It was observed that, during sprouting, α -amylase contents were found to increase till day 5. The results are incorporated in figure 1. The use of tables and figures should be mentioned in the text by mentioning table 1; figure 1 and so on.

Fig. 2 maximum activity of each day



Activity of enzyme

In this study it was found that α -amylase from germinating seeds showed maximum activity after 5 days of growth and then declined rapidly (Fig. 1). From this information, we decided to purify this enzyme activity 5 days following seed imbibitions. The existence of amylase activity in oleaginous seeds testifies the existence of the glucidic reserves, particularly starch. The origin of this starch is to be checked. Does this starch exist in dry seed or is it synthesized during germination starting from other precursors, particularly the breakdown products of the lipids storage. Earlier work showed that the oleaginous seeds accumulate starch at the beginning of their germination [19,20]. Thus, it was shown that the content of starch in endosperm tissue of castor seed (*Ricinus communis*) increases from 0.5 to 1.1 mg per seed in 5-

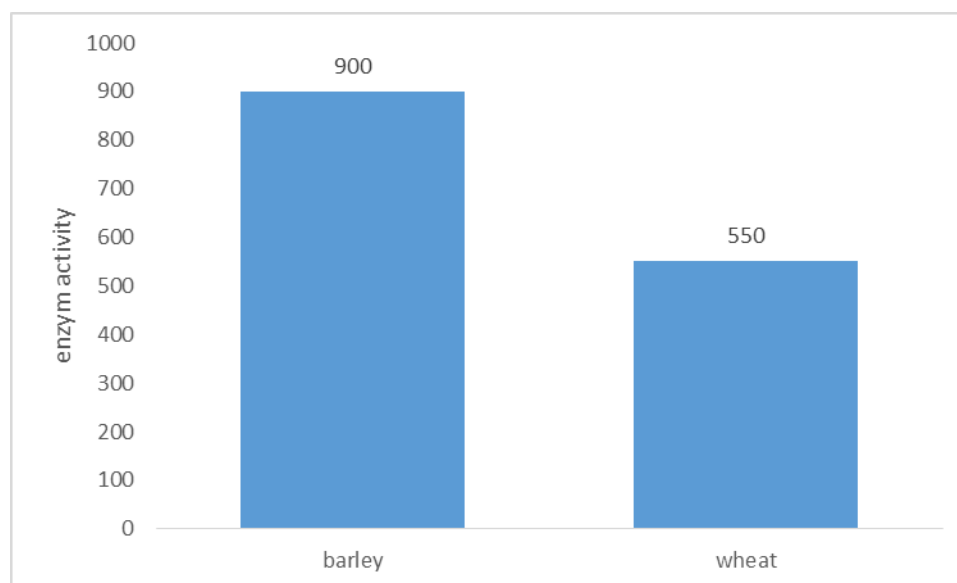
day-old seedlings [21]. Recently, another study showed, by proportioning and electronic microscopy, that the cotyledons of yellow lupine seeds (*Lupinus luteus* L.), which are regarded as leguminous seeds rich in lipids, accumulate starch and that the content of starch increases from 8 to 22 mg/g of dry matter in 4-day-old seedlings [22]

Screening for hydrolytic enzymes in barley and wheat grains

The enzyme extracts from barley and wheat were tested for enzyme activities (Fig. 2). Different temperatures were chosen for these screening experiments in order to cover a broad temperature range. A straight comparison of the results was done with the afterwards obtained main results.

By far the most abundant enzyme activity present in both barley and wheat extracts was that of α -amylase, with an almost twelvefold higher α -amylase activity in the germinated barley extract (roughly 900 unit per kg of grain) than in the wheat extract (about 550 unit per kg of grain).

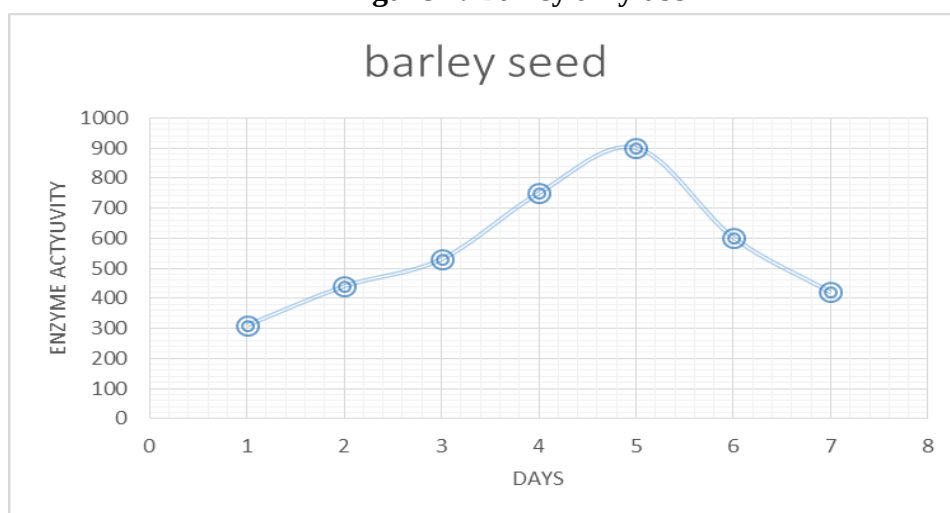
Figure 3: Hydrolase activity found in the extracts from barley and wheat



The amylases of developing barley seed were investigated by colorimetric methods. Maxima of amylolytic activity appeared in the aleurone layers and starchy endosperm at 1 to 7 days and maximum was at 5 day. Amylase from 5-day-old aleurone layers could be separated into four rapidly moving bands with α -amylase activity.

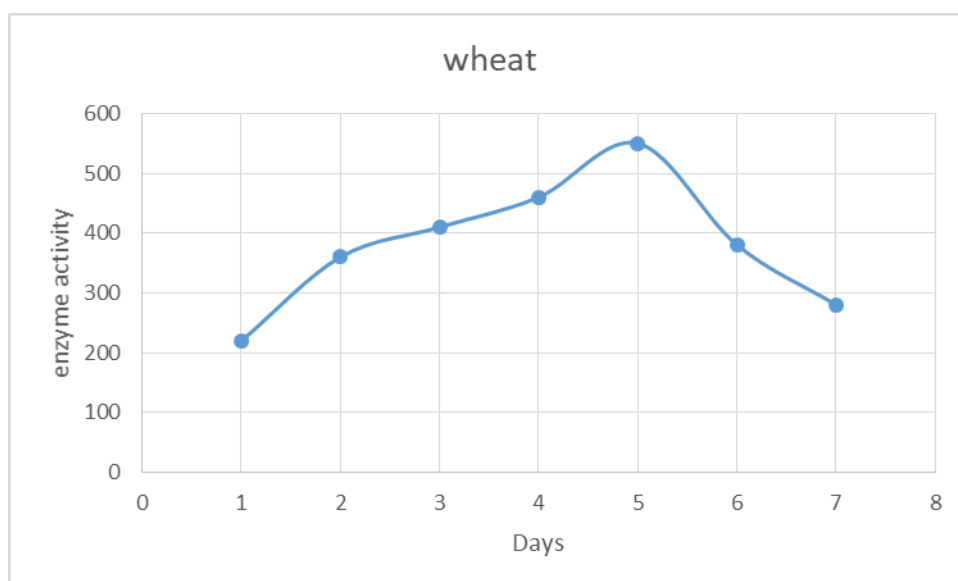
Barley seems to be resistant to high pretreatment temperatures, which could be due to the grain structure. Contrary to wheat grains, barley seeds are encapsulated by husks, which could have this protective effect on the grains.

Figure 4: Barley amylase



In contrast, the purified wheat α -amylase was found to be stable enzyme over 2 months at 4 C. Having a high kcat, this demonstrates its ability to hydrolyse starch efficiently. Its thermal stability further indicates its potential for industrial applications, especially for SDS based detergent industries.

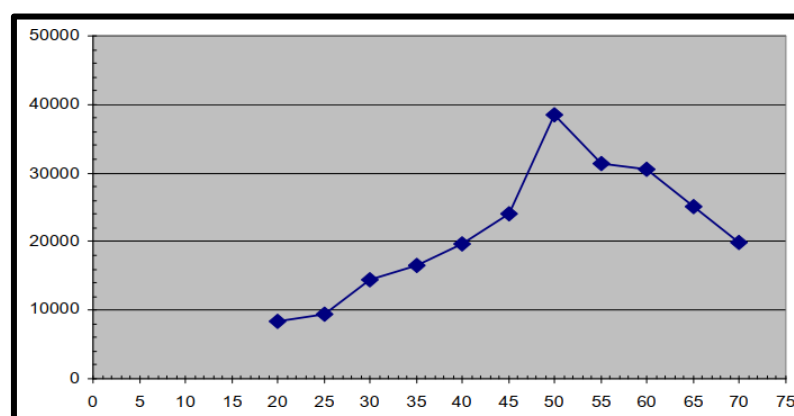
Figure 5: wheat amylase



Determination of the optimum

Temperature for the enzyme the effect of temperature was studied on the activity of the purified alpha-amylase from barley malt of IPA-99 variety, within a temperature range of 20-70 °C. The results shown in Figure (4) showed an increase in the activity of the enzyme with a higher temperature reached its maximum at 50°C when it reached 39000 units/ml, then it decreased with increasing temperature until it reached the temperature of 70°C when it reached 20000 (units/ml). The reason for the increase in the speed of enzymatic reactions with a rise in temperature to a certain extent is attributed to the increase in collisions between the enzyme molecules and the subject matter, as a result of the increase in the kinetic energy of the molecules due to the effect of increasing the temperature (14), while the high temperatures cause a decrease in the effectiveness of the enzyme due to its effect on the deformation of the enzyme as a result of the effect of Heat in the enzyme synthesis, and change the configuration of the active site, which leads to the loss of its effectiveness (9)

Figure (6) The optimum temperature for the activity of purified alpha-



Stability of the enzyme towards temperature

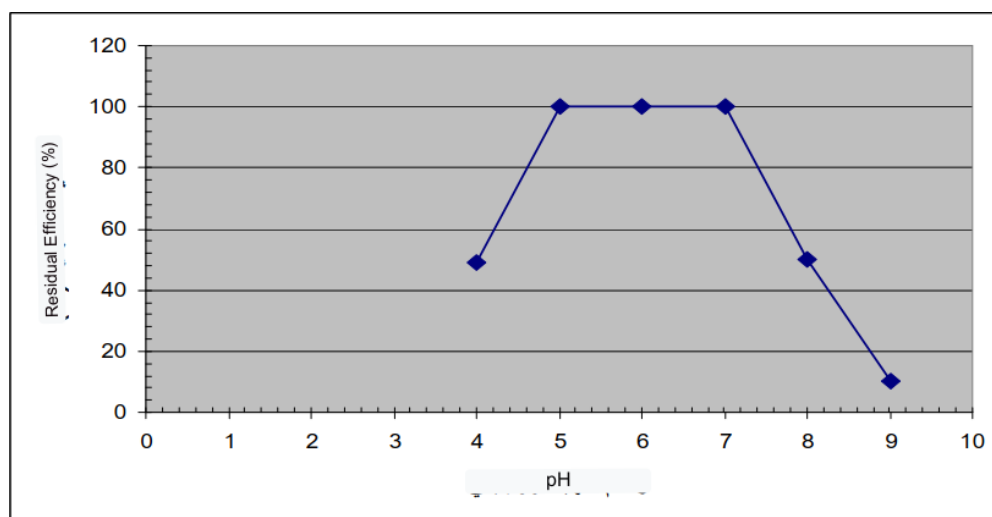
Figure (5) shows the stability of purified alpha-amylase from barley malt of IPA 99 variety when it was treated with different temperatures and for different periods of time, as the enzyme was incubated at temperatures ranging between (45-75°C) for periods of (10, 20, and 30 minutes). It is

noted from the results obtained that the enzyme retained its full effectiveness between the two temperatures (45-60) °C and for the three incubation periods, and the stability of the enzyme decreased when it was treated at a temperature of 65 °C in a period of 20 and 30 minutes, as the remaining effectiveness percentage reached 95% and 70%, respectively, while the enzyme lost its effectiveness significantly when it was treated at a temperature of 75 °C for 30 minutes, when it reached 10%.

Determination of the pH to stabilize the activity of the enzyme

The results shown in Figure (7) showed that the enzyme retains its full effectiveness 100% at the pH values (5-7). This means that the enzyme is stable in this range. And the stability of the enzyme decreases in the acidic pH values (4), as the remaining enzyme activity is 49.9%.

Figure (7) showed that the enzyme PH



Also, the activity of the enzyme decreases at the basic pH value (9), as the remaining enzyme activity reaches (12.6%). Hence the loss of enzymatic activity (14), and these results came close to the results mentioned by (15), as it was mentioned that the optimal pH for the stability of alpha-amylase purified from barley malt is within the range of (6.5), as it was close to what was reached by (4) when studying it. The stability of the alpha-amylase enzyme purified from green pea seeds towards a pH ranged between -4 (8) and it was found that the enzyme is stable at 6 (6.5) and that the enzyme retains about 90% of its activity at the pH (5) as it was noted that The enzyme retains 85% of the enzyme activity at pH (7).

Conclusion

Thus, the method is successfully carried out for the estimation of α amylase content of different grains after germination for various days. After sprouting α amylase contents were found to be increased upto 7th day, after that amount of alpha amylase got saturated. The potential for preparation of high energy food thus may be substantiated from the view point of higher enzymatic activity of α amylase resulting in breaking down complex starch to more energy producing usable maltose.

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