Volume : 3 | Issue : 2 | Feb 2014 • ISSN No 2277 - 8160

Research Paper Microbiology

Isolation, Screening and Optimization of Microorganism producing Amylase

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ABSTRACT

The present work was done on "Isolation, screening and optimization of microorganisms for amylase production . Soil samples collected from Mandi district of Himachal Pradesh were used in present investigation. Isolation of bacteria was done with enrichment technique with serial dilution of soil sample which were made in enrichment media.Ten bacterial isolates for amylase production were obtained by enrichment cultures which were named as AILS-A1-AILS-A10. Most of these bacteria belonged to the genus Bacillus.During the screening of the isolates strain AILS-A4 showed (0.568U/ml) maximum amylase activity and selected for further study. The maximum growth was recorded at pH 7.0.Optimum temperature for the growth of isolate was 30°C.The maximum growth was obtained in media containing glucose (1%) as carbon source (59.674U/ml).The maximum growth was obtained in media containing peptone (0.15%) as nitrogen source (64.74%).Optimum pH for enzyme reaction was 80°C.The maximum stability of enzyme reaction was obtained at pH 9.0 (glycine NaOH) at room temperature.The maximum thermostability of enzyme was obtained at 70°C up to 3 hrs.In conclusion, amylases have wide spread applications in food and textile industry and the potential of amylase produced by the isolate AILS-A-4 could be explored in various industries like detergent, brewing & distilling industries etc.

KEYWORDS : Bacillus,Thermostability,Glycine,Temperature,Enzymes,Nitrogen source,Carbon source,Applications

INTRODUCTION

Starch is the most common polysaccharides (carbohydrate) in plants. It is used by plants them-selves, by microbes and by higher organisms. So there is great diversity of enzyme able to catalyze its hydrolysis. Major difference in type of starch present in various species of plants is the difference in the ratio of amylase to amylopectin; e.g. the cornstarch from waxy maize contains only amylase but that from amylopectin is about 80% amylases. Some starch,for instance from potato contain covalently bound phosphate in small quantities (0.2%) approximately which has significant effect on the physical properties of starch but it does not interfere with hydrolysis. One components of starch amylase is a linear polymer composed of α (1-4) linked glucose unit. The length of amylase polymer averaged about 1000 glucose residues. The other components of starch amylopectin is highly branched polymer of glucose with α (1-4) linkage along the back bone and α (1-6)linkage forming the branched points.The actual composition of starch (length of polymer branch points and the percentage of amylopectin, etc. depend upon the plant source from which it has been isolated. **(Ferrai *et al*, 1993).**

The most important member of starch-degrading group of enzyme is amylase, traditionally used in conjugation with the other enzyme for conversion of starch to high fructose syrup. There is other important application like addition in laundary or in dishwashing detergent, textile, brewing, sewage treatment, pharmaceuticals and medicine.Some starch degrading enzymes are used to make cyclodextrins,which has generated high interest in this category of molecules because of their peculiar shapes and internal hydrophobic region. These compound are studied as a vehicle for encapsulating and protecting compound used in variety of industrial application involving food, pharmaceuticals and cosmetics **(Hacking,1991).**

The acid catalyzed old process of starch hydrolysis has been replaced by enzymatic degradation **(Barfoed, 1976; Underkofler et al,1976).** Amylase degrades the starch and hydrolyse α -1, 4 bond of starch into maltose, glucose etc. **(Lama et al,1991).** The sugar industry employs the thermo stable microbial enzyme for the production of glucose, maltose, maltotriose and higher dextrin from the starch **(Beisbuizen,1982 & Norman,1979).** The metabolism of these substrate begin with their hydrolysis by a series of extra cellular amylotic enzymes comprising amylase and pollunase activities, which were further studies in crude form **(Brown et al,1990).**

Amylase have most widely been reported to occur in microorganism, although they are also found in plants and animals. There are presently a number of microbial including the α – amylase of *Bacillus circulans* **(Takasaki,1982)**, *B. amyloliquefaciens* **(Nornab et al,1979; Forgarty, 1983; Forgarty** and **Kelly,1980,1990)** Bacillus sp H-167 **(Hayashi et al,1988)** and mutant *Klebsiella pneumoniae* **(Monma et al, 1983)** that produce maltohexose in the yields of 25-40% (w/w).α-amylase have generally very widely range of pH from 3.5 **(Kamo,1986)** to 9.0**(Yang,1993).**

(Kanno,1986) had reported a highly heat stable α-amylase from *Bacillus acidocaldarius* which had optimum pH 3.5. (**Yang ,1993)** isolated halophilic bacteria from the costal spring in Latao, Taiwan, which tolerate pH range 6.0 to 9.0. The α-amylase from *Pyrococcous furiosus* was able to maintains about 30% of it activity, when incubated for 30 min. at 80. (**Koch et al,1987)** reported that α-amylase from *Bacillus licheniformis* TCRD-B-13 is quite active even at 110°C however, it has shown highest activity at 90°C and broad pH range 5.5-10 **(Bajpai and Bajpai, 1987).** The activity of α-amylase was simulated with 1 MM ca^{2}+ **(Srivastava, 1987).** The enzyme from *Sulphobus solfataricus*, which was stable up to 89°C after 5h. of incubation and the stability of enzyme,was independent of ca^{2}+ ions (20 min) **(Lama et al,1991)**

Various bacteria producing α–amylase are *Bacillus subtili* amylosaccharifus,*Bacillus flavothermus*, *Bacillus* sp., *B.amyloliquefaciens, B.licheniformis, Pyrococcous woesei,P. furious*,*Therm coccous celer,*etc

Amylase is more important enzyme of great significance, derived from various source such as plants, animals and microorganism. Microbial enzymes are used at a large scale of industries process. Amylases are used in starch saccharification, brewing, textile, distilling industries. Low calorie beer required a higher degree of saccharification at lower starch content to reduce the alcohol and total solid content of the beer. This may be achieved by the use of glucoamylase and fungal amylase during the fermentation. In the distilling industries, saccharification continues throughout the fermentation period. In some cases (scotch, malt, whiskey, manufactures uses barley malt the enzyme are naturally present but in other (grain sprits production) the more heat stable bacteria α-amylase may be used in saccharificaiton. There are three stages in conversion of starch.

1. **Gelation:** The starch granules are dissolved to produce viscous suspension.
2. **Liquefication:** The liquefication step involved the partial hydrolysis of starch leading to loss velocity.
3. **Saccharification:** Glucose and maltose produce by further hydrolysis.

A generalized process for enzymatic hydrolysis of starch to low molecular weight sugar was given by **Bhalla and Chatanta, 2000**. In this process the starch granules are suspended in cold water at pH 6.5 containing 40 ppm Ca^{++} to produce 30-40% (w/v) starch slurry. Adding bacteria α-amylase and subjecting the mixture to 105°C for five minute gelatinize the starch. The temperature is lowered to 90°C for two hours and the starch gets liquefied. Saccharification is liquefied starch is carried out at 4.5 by using glucoamylase and pullulanse at 60°C for 72 h and the final product is glucose syrup (equivalent to 97% maltose of the total carbohydrate) the liquefied starch is hydrolyzed by fungal amylase at pH 5.5, 50 ppm, ca++ and 55°C temperature for 48h.

Amylases have also been reported to be used in detergent industry. Alkaline amylases find its application during the manufacture and cleaning action of modern detergent. Microbial amylase could be specifically useful in pharmaceutical and medicine. The fungal amylase is used in the manufacture of taka-diastase commercially used an acid. The most novel application of amylase could be in the treatment of cancer with the discovery by some researcher that the outer coating of a cancer cell has human chronic gonadotropin as the suitable substrate for the attack of amylase, then protease. The carbohydrate portion is salivated, but the sugar portion is susceptible to attack. But the protease, like carboxypeptidases trypsin and chymotrypsin are unable to reach the protein amylase does it work. This is the rationale for using amylase in abundance. The molecules seen show that amylase makes trypsin or other proteinases able to access their protein substrate.

MATERIAL AND METHODS

A) ISOLATION OF BACTERIA FROM SOIL SAMPLE

Soil samples collected from Mandi district of Himachal Pradesh were used in present investigation.

1. Media and Reagents:-

Gram's iodine:

Iodine	-	1.0 gm
Potassium iodine	-	2.9 gm
Distilled water	-	300 ml

2. Nutrient agar – gm/litre

Beef extract	-	3.0
NaCl	-	5.0
Peptone	-	5.0
Agar	-	15.0
Distilled water	-	1000 ml
pH	-	7.0(+0.2)

3. Starch agar – gm/litre

Beef extract	-	3.0
Peptone	-	5.0
Starch	-	20.0
Agar	-	15.0
Distilled water	-	1000 ml
pH	-	7.0(+0.2)

B) Enrichment of samples

Isolation of bacteria was done with enrichment technique with serial dilution of soil sample were made in enrichment media. One gram of soil sample was added in 9 ml of enrichment media and then serially 1ml was transferred in five tubes to obtain 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions. They were kept for shaking at 150 rpm for 48 hr at 30°C. Starch media composition was as follows:

1) Beef extract	-	3g
2) Peptone	-	5.0g
3) Starch	-	20.0g

Water to make 1000 ml and pH 7.0.
The enrichment step was repeated three times.

1) Plating and screening of bacterial colonies

One ml. solution was taken from the enrichment broth and serials dilution with normal saline (89%NaCl) was carried out. Solutions with 10^{-2}, 10^{-4}, 10^{-6} dilution were plated on Petri plates containing starch agar media (with 2% agar). The colonies showing significant growth were selected and streaked on another plates containing above media.

2) Pure culture

A single colony was selected from these plates and streaked on slants media with 2% agar. The slants were incubated at 30°C for 48 hr to obtain pure culture of the bacteria. The pure culture was stored in refrigerator for further use.

3) Screening of bacteria for the production of enzymes

Reagents:

* 1% starch in starch agar.
* Gram's iodine solution (1g iodine and 2g Potassium iodine dissolved in 300 ml of DW)

Procedure:

The medium used was starch agar. The pure culture of bacteria was inoculated in the plates and incubated at 30°C for 48 hrs. Then starch agar plates having growth of bacteria were flooded with Gram's Iodine Solution. The clear zone around the bacterial colonies was indication of starch utilization.

C) PRODUCTION OF AMYLASE

1) Preparation of seed culture

The seed culture (50 ml) was prepared by inoculating a loopful of pure culture. The flasks were incubated for 24 h with continuous shaking (150 rpm) at 30°C.

2) Production of enzyme

2 ml of seed culture was inoculated in 50 ml of production medium containing Beef extract (0.25%), peptone (0.15%) and starch (1%) and grown for 48 hrs in incubation shaker at temperature 30°C and at 150 rpm. Production media was centrifuged at 5,000rpm for 10 minutes. The supernatant was collected and stored in refrigerator till further use.

3) Amylase assay

Ress and Mendel (1963); and Milles (1959)

Reagents:

Phosphate buffer (0.1M, pH 7.0)
Starch solution (1%)
DNS Reagent:-
1 g Dinitrosalicyclic acid (DNSA)
1 NaOH
0.05g sodium sulphate
20g Potassium sodium tartarate
Distilled water to make 100 ml.

Procedure:

0.5ml of starch solution, 0.3ml phosphate buffer (0.1M, pH 7.0) and 0.2ml enzyme solution was added. The control was also run with all the components except the enzyme and added 3ml of DNSA reagent to each tube. Contents were incubated at 30°C for 30 minutes. Cooled to room temperature 0.2ml of denatured enzyme solution was added to control tubes after boiling water bath and absorbance (O.D.) was measured against a reagent blank at 540 nm. One unit of amylase was defined as, "The amount of enzyme that catalyses the release of one mol of reducing sugar (equivalent to maltose) per minute per ml of culture broth from starch under assay conditions".

D) OPTIMIZATION OF CULTURE CONDITIONS

1) Growth at different pH

pH in the range of 6-10.0 is examined for its effect on growth of microorganism by the selected isolate grown in production media. The pH of the medium was adjusted using 1 N HCl or 1N NaOH. The flasks were incubated at 30°C for 24 hrs. The production medium of different pH was harvest at 5,000 rpm for 10 min. Pellet was discarded and supernatant was taken for assay of amylase.

2) Growth at different temperatures

The effect of temperature was evaluated by incubating the 50ml of sterile production medium inoculated with 2ml seed culture at different temperature (20-60°C) for 24 hrs. (pH was adjusted as selected in above step). The medium was harvested at 5,000rpm for 10min. Then amylase assay was done.

3) Effect of carbon sources on amylase production

Various carbon sources such as Glucose, lactose, Xylose, and Starch were evaluated for their effect on amylase production by replacing starch in the production medium.The flasks were inoculated with 2ml

seed culture and incubated at 30°C for 24hrs. Then it was harvested at 5,000 rpm for 10min. The optimum carbon source was found by analyzing the results of amylase production**.**

4) Effect of nitrogen source on amylase production

The various nitrogen sources like yeast extract, peptone, beef extract, casein, tryptone were evaluated for the growth of microorganism. The flask were inoculated with 2ml seed culture and incubated for 24hrs at 150rpm (temperature, pH, carbon source were adjusted as selected above).

5) Growth studies

Growth studies were carried out in shake flask fermentation using production medium. 200ml of sterile production broth containing beef extract, peptone, and glucose at (pH 7) was prepared in 500 ml conical flask and 5ml seed culture was transferred aseptically into production medium and incubated at 30°C for 48 hr in a rotary orbital shaker at 150 rpm. Samples were withdrawn at regular time intervals and analyzed for culture growth and enzyme activity. The crude enzme was obtained by centrifugation at 5000 rpm for 10 min and assay monitored with UV-VIS Spectrophotometer at 540 nm. Growth curve was plotted for OD versus growth and enzyme activity versus time.

E) OPTIMIZATION OF REACTION CONDITIONS

1) Optimization of pH

To determine the optimize pH for amylase activity; the enzyme reaction was carried out at different pH ranging from 6.0 to10.0.

2)Optimization of temperature

To find out the optimum temperature for amylase activity, reaction mixture was incubated at various temperatures from 30°C to 70°C.

3) pH stability of amylase

The pH stability of enzyme was determined by incubating the enzyme fraction at various pH between 6 to 10 without the substrate, for 2hrs at room temp. Then aliquots of 0.2ml of the incubated enzyme were assayed for activity.

4) Thermostability of amylase

The thermo stability of the enzyme was determined by incubating the enzyme fraction at various temperatures between 30°C - 80°C, without substrate, for 1 hr. At 10 min intervals, aliquots of 0.2ml of the incubated enzyme were assayed for activity.

RESULTS AND DISCUSSION

1) ISOLATION OF BACTERIA FOR AMYLASE PRODUCTION

Ten bacterial isolates for amylase production were obtained by enrichment cultures which were named as AILS-A1, AILS-A2, AILS-A3, AILS-A4… AILS-A10. Most of these bacteria belonged to the genus *Bacillus*.

2) SCREENING OF ISOLATES FOR AMYLASE ACTIVITY

During the screening of the isolates or amylase activity strains AILS-A4 showed significant amylase activity out of strains.

2.1) Screening of isolates for amylase activity by starch utilization test

Samples AILS-A4 displayed maximum clear zone around the bacterial colonies when flooded with iodine solution indicating maximum starch utilization among the bacterial isolates.These samples were considered for further investigation. The clear zone is developed around the bacterial colony due the utilization of the starch present in the media by the amylase produced by the bacterium. In the rest of region the iodine solution from a complex with the starch forming a dark zone on rest of the plate (Fig.4.1)



Figure. 4.1. Clear zone around the streak culture of isolate AILS-A4 after flooding with iodine solution

2.2) Screening of isolates by evaluating the enzyme activity

The value of enzyme activity obtained from various isolates was used as screening factor for further studies. Table 1 shows enzyme activity produced from various isolates during screening. The isolates AILS -A4 (0.568 U/ml) showed maximum enzyme activity from various samples and hence selected for further study. This organism was found to be Gram positive rod shaped, spore former and tentatively identified as *Bacillus* sp.

Table 1: Enzyme activity of enzyme produced from various isolates.

Serial Number	Isolate	Enzyme Activity*(U/ml)
1	AILS-A1	0.101
2	AILS -A2	0.107
3	AILS -A3	0.102
4	AILS -A4	0.568
5	AILS -A5	0.201
6	AILS -A6	0.101
7	AILS -A7	0.525
8	AILS -A8	0.205
9	AILS -A9	0.106
10	AILS -A10	0.108

*** One unit of amylase activity was defined as the amount of enzyme which released one µ mol glucose under assay condtions.**

3) OPTIMIZATION OF CULTURE CONDITIONS

3.1) Effect of different pH

Maximum amylase production was achieved at neutral pH (7) (9.00 Units/ml) by isolate AILS-A4 of *Bacillus* sp. (Fig.4. 2). Although pH 6-10 also supported amylase production and the organism did not grow in production medium adjusted at pH 10.0 (0.04 Units/ml). Result suggests that there is stimulation of enzyme at neutral pH. As the maximum enzyme activity was observed at neutral pH 7.0 thus showing the wide applicability of amylase produced by isolate AILS-A4 in food and beverage industry. (**Arikan, 2008)** reported production of amylase by *Bacillus* sp. A3-15 at pH 8.5 and in between pH 7-11.0 b *Bacillus* sp. isolate ANT-6 **(Arikan et al, 2003).**

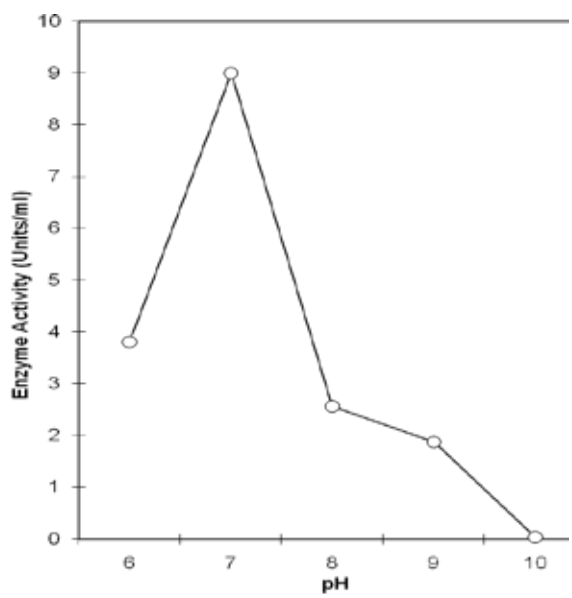

Fig. 4.2. Effect of pH on production of amylase by isolate AILS-A4

3.2) Effect of temperature

Environmental parameters showed high influence on the growth of the organism. Maximum growth was recorded at 30°C. After 30°C the growth of isolate AILS-A4 decreased sharply up to 60°C (Fig.4.3). Increase in temperature resulted in lowering the growth of organism showing the mesophilic nature of organism. (**Arikan et al ,2003)** reported production of amylase by *Bacillus* sp. isolate ANT-6 with in pH range 7-11 and with optimum pH of 9.0.

3.3) Effect of carbon source

Maximum growth of organism was achieved when glucose (59.67 U/ml) was the carbon supplement. Growth was inhibited when sucrose (1.675U/ml) was used as carbon source. Lactose (49.30 U/ml) and xylose based production media also showed comparable enzyme activity production by the isolate AILS-A4 (Table 2). (**Lynn et al ,1999)** also reported optimum amylase production by *Bacillus* sp. IMD 435 using lactose as a carbon source.

Table 2 Effect of carbon sources on amylase production by isolate AILS-A-4

Carbon Source	Enzyme Activity (Units/ml)
Sucrose	1.67
Lactose	49.30
Xylose	26.18
Starch	4.04
Glucose	59.67

3.4) Effect of nitrogen source

Maximum growth and enzyme production (64.74 U/ml) was achieved when peptone was used as the nitrogen source. Yeast extract (2.40 U/ml) and beef extract (2.19U/ml) showed minimum amylase production and the production reached almost nil in the presence of tryptone (Table 4.3). However, the yeast extract was reported best nitrogen source for amylase production by *Bacillus* sp. IMD 435 **(Lynn et al, 1999)**

3.5) Thermostability

The thermostability of the enzyme was evaluated by incubating the enzyme at different temperatures ranging from 30°C-80°C and subsequent assay after intervals of time at different temperatures separately (Fig. 4.8). The enzyme was stable at all the temperatures up to 1 hr and thereafter the stability varied with different temperatures. The enzyme was most stable at the 50°C and retained 81% of activity up to 4h of incubation. Similarly the enzyme retained 75% of activity after 4 hr of incubation at 40°C.The The stability of enzyme decreased with increase in temperature up to 80°C and only 54% of the activity was retained after 4h. However the stability was 66 and 76 % respectively at 30°C and 40°C up to 4h. The amylase of *Bacillus* sp. isolate A3-15 was approximately 70% stable at 60°C after 24 h. **(Arikan, 2008).**

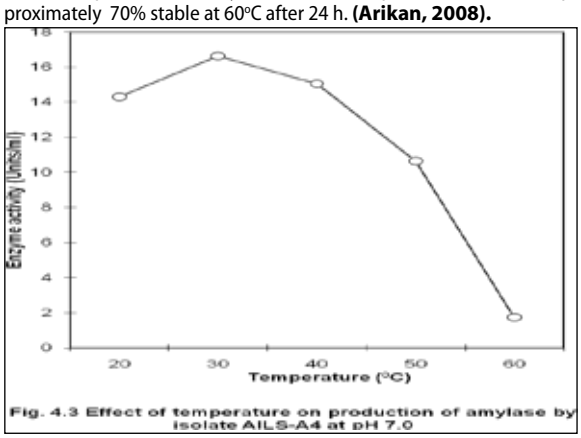

Fig. 4.3 Effect of temperature on production of amylase by isolate AILS-A4 at pH 7.0

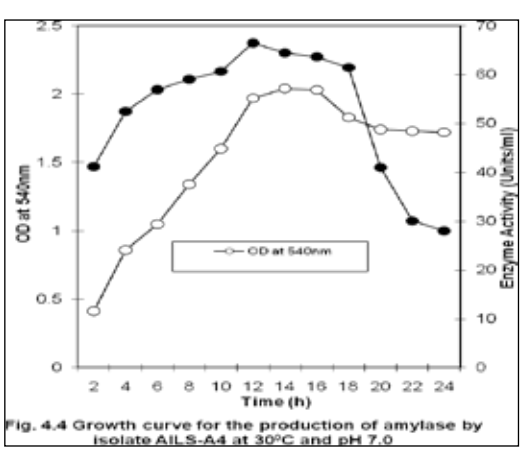

Fig. 4.4 Growth curve for the production of amylase by isolate AILS-A4 at 30°C and pH 7.0

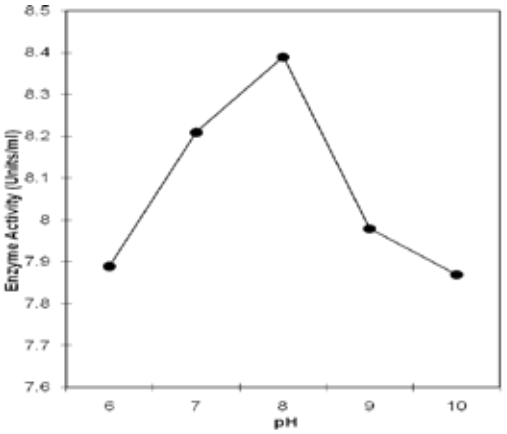

Fig. 4.5 Effect of pH on the activity of amylase under assay conditions

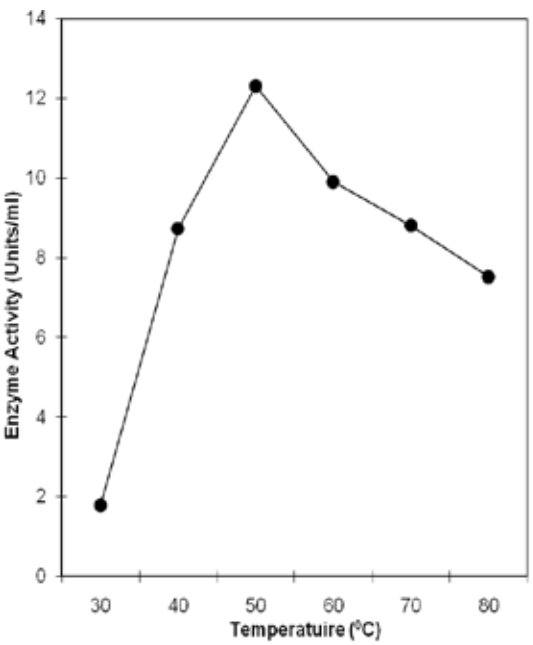

Fig. 4.6 Effect of temperature on activity of amylase under assay conditions

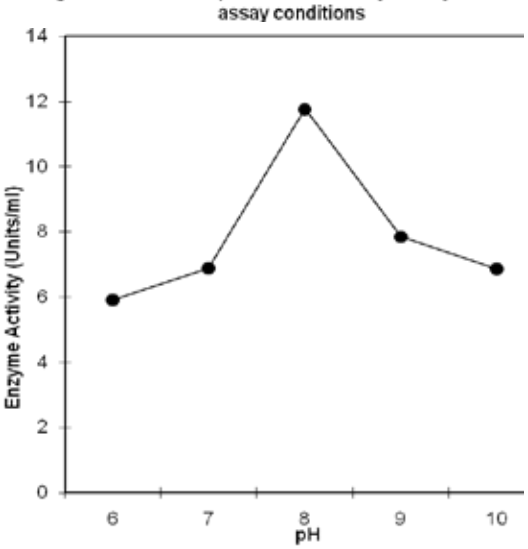

Fig. 4.7 Stability of amylase after incubation at different pH of buffer

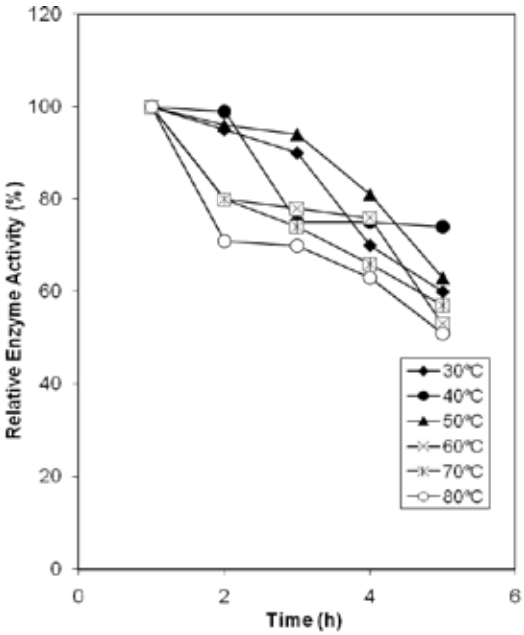

Fig. 4.8 Thermostabilty profile of amylase after incubation at different temperatures

ACKNOWLEDGEMENT

As a prefatory exercise, we owe our heartiest gratitude and unflinching thankfulness to Dr. Dinesh Kumar Chatanta, Head of Department of Life Science,for their guidance, planned execution guidance throughout our project. We are very thankful to the lab attendant who lends a helping hand during our project work. We are equally grateful to our parents and all family members who bore with solemn patience when the work was in progress.

Their help valued me a lot at all time. We express our sincerest thanks to all our friends and classms for their programatic suggestions.

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