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ISOLATION, PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF *A*-AMYLASE FROM *BACILLUS CEREUS* BY SOLID STATE FERMENTATION USING WHEAT BRAN AS A SUBSTRATE

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ABSTRACT - In the present study α-amylase production from *Bacillus cereus* was done by solid state fermentation using wheat bran as substrate in aerobic and anaerobic conditions. The aerobic fermentation of *Bacillus cereus* gave the maximum enzyme activity of α- amylase *i.e.* 158.6 IU/g and least by anaerobic fermentation *i.e.* 80.514 IU/g. The best α-amylase production activity by *Bacillus cereus* using wheat bran as substrate shown in Bushnell Hass mineral broth at 37°C for 48 hours *i.e.* 130.8 IU/g and least at Nutrient broth at 37°C for 48 hours *i.e.* 14.4 IU/g. Hence the Bushnell Hass broth used as selective media for the production of α-amylase.The observations demonstrated *Bacillus cereus* as an efficient producer of a-amylase,that revealed maximum production at temperature 37°C *i.e.*95.9 IU/ml, optimum pH 7 i.e,101.12 IU/g, optimum incubation time 48 hours *i.e.* 53.57 IU/ml and optimum carbon source glucose *i.e*18.26 IU/g and optimum nitrogen sources tryptone 10.25 IU/g.

KEYWORDS: *Amylase, Bacillus cereus, enzyme activity, optimization, purification*

INTRODUCTION

Microorganisms perform their metabolic processes rapidly and with remarkendly specificity under ambient conditions, catalyzed by their diverse metabolic reaction. Enzyme alternation to harsh chemical technology has led to intensive exploration of natural biodiversity to discover enzyme which could function effectively and generate pollution free '**Dream Technology'** in the immediate future (Srinivasan*et al.*1999)

Amylases are the enzyme which hydrolyze starch molecule to give diverse products including dextrin and small polymers composed of glucose units. The history of amylase began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. Later in 1930 Ohisson suggested the classification of starch digestive enzyme in malt as alpha and beta according to their anomeric types of sugar produced by the enzyme reaction.*a-amylases* are universally distributed throughout the plant and animal kingdom. The major advantages of amylase production is economically viable and microbes are easy to manipulate the desired characteristics.(Ramesh &Lonsane1990)

Amylase constitute a class of industrial enzyme having 30% of enzyme market(Sindhu et al 2009). However enzyme from fungal and bacterial sources have dominated applications in industrial sectors (Pandey*et al* 2000) Agro industrial residue are generally considered best substrate for solid state fermentation process. Because the metabolite produced are concentrated and purification procedure are less costly(Nigam and Singh, 1995; Chadha*et al*, 1997)

Recently interest and demand for enzymes with novel properties are very high in various industries and it leads to the discovery of various types of the amylases with unique properties. Each application of amylases requires unique properties with respect to specificity.Among various extracellular enzymes, a-amylases ranks first in terms of commercial exploitation (Babu& Satyanaryan1995). The most effective amylases are those that are thermostable. They are generally preferred as their application minimizes contamination risk and reduces reaction time, thus enabling considerable energy saving(Nigam,P.1996). Thermo stable *a-amylase* are used for the liquefaction of starch at high temperature and thermo labile *a*-amylase are used or the saccharification of starch in baking (Shaw *et al* 1995). Babu and Satyanarayana (1995) have reported production of a-amylases by a thermophilic*Bacillus* sp. and optimization of culture conditions for maximum enzyme production. Suitability of thermophilic*Bacillus coagulans*for a-amylases production by solid-state fermentation in flasks.

The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products for the reduction of cost of the medium (Ikram- Ul- Haq 2003). The use of agricultural wastes makes solid--state fermentation (SSF) an attractive alternative method (Ellaiah et al 2002). Baysal*et al.* (2003) have reported a-amylases production in solid-state fermentation with wheat bran and rice husks substrates.

Ramachandran*et al*. (2004) have checked the potential of coconut oil cake as substrate for the production of a-amylases using *Aspergillusoryzae*, a GRAS strain. Ikram-ul-Haq*et al*. (2003) have described the selection of a suitable low cost fermentation medium for the production of a-amylases by using agricultural by-products. Glucoamylase production with an *Aspergillus*sp. has been reported using cheap rice flake manufacturing wastes as substrate (Anto et al 2006).

Bacteria, fungi and actinomycetes have been extensively screened for industrial enzyme production and numerous processes. The enzyme vary widely in their properties particularly in regard to temperature and ph optima for activity and stability. It is interested that even mesophylic strains secrete enzyme proteins which are active and stable at temperature and Ph.eg. Mesophylic strain *Bacillus* secrete *a*-amylase active at 90-100oc and find wide spread application in textile desizing (Soni*et al* 2003).Cellulolytic *Trichoderma* and *Penicillium* strains growing at optimum temperature of 28^0-30^0C secrete exoglucanases.

While enzyme production on semi-commercial or commercial scale is on, current research aim at improving enzyme quality performance under user conditions and cost effectiveness. Modern technology includes recombinant DNA technology and protein engineering have been applied to achieve the desired goal in several leading academics and industrial laboratory.

MATERIAL AND METHODS

Collection of Raw Material

The raw material was used for the production of amylase is agricultural waste such as wheat bran from local market.

Isolation and identification of culture

The 1g wheat bran sample was taken which collected from a local market. Isolate the bacterial cultureby using serial dilution method. The bacterial culture was incubated at 37°C° for 24 h. The isolated colonies were further confirmedby culture on Starch agar media at 37°C for 24 h. After incubation, 1% iodine solution over layered on the culture and the observation made to note the substrate utilized zone around the colony due to starch hydrolysis Only the positive and the better zone formed strain was taken for the further study. Based on the key provided by the Bergey's manual of Systematic bacteriology the isolated strain was identified as *Bacillus cereus*.

Solid state fermentation (SSF)

Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks that contained 10 g of wheat bran and 100 ml of distilled water (moistening agent) was taken and add 1.0 ml (v/w) of exponential phase culture of *Bacilluscereus* added, mixed well and incubated at 55 ˚C in a humidified incubator. Flasks will periodically mixed by gentle shaking. At the end of incubation period (24 h), the flasks willtaken out and the contents from the each flasks are filtered, centrifuged at 6000rpm for 10 min. The clear supernatant acts as a crude enzyme.

Development of the inoculum, enzyme production and extraction

For the development of inoculum, culture is transferred from stock to 100-ml nutrient broth and the inoculated flasks incubate overnight at (35±2)°C on rotary sheker (150 rpm) for 24 hrs. The cells were harvested from the broth and checked theirabsorbanceat 660 nm. Accordingly, cells with inoculums size of *A*660=0.5 (10 % inoculum (volume per mass))per 10 g of substrate will harvested, washed and resuspended the cells in sterile distilled water. Production media contained10 g of solid substrate and 10 ml of Bushnell Haas(BH) mineral salt medium in 250-ml Erlenmeyer flasks inoculated with the above inoculum. Inoculated production media incubate under static conditionsat (35±2) °C and enzyme production was checked out after every 24 h for 4 days.

Enzyme was extracted in 50ml of 0.1 M phosphate buffer (pH=7) on a rotary shaker at 250 rpm for 30 min. The content was filtered through muslin cloth, filtrate was centrifuged at 8325 × *g* for 10min and clear brown supernatant was used as the crude enzyme.

α-Amylase enzyme assay

α-Amylase activity was determined by incubating a mixture of 0.5 mL of aliquote of each enzyme source and 1 % soluble starch dissolved in 0.1 M phosphate buffer, pH=7, at 55 °C for 15 min. The reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid(DNS), then followed by boiling for 10 min. The final volume will made up to 12 ml with distilled water and the reducing sugar released measured at 540 nm . One unit(U) of *a*-amylases activity defined as the amount of enzyme that releases 1 mol of reducing sugar as glucose per minute, under assay conditions and expressed as U/g of dry substrate.

Protein Assay

The protein assay was determined by Lowery method using BSA as a standard.

Partial purification of amylase enzyme

Bacillus cereus was cultured for 24- 48hours in optimized starch broth at 37^0C. After incubation, the culture centrifuged at 10,000 rpm for 15 min at 37^0C.The

supernatant separate from the pellet containing cell debris. The ammonium per sulfate will added to the culture supernatant to get 75 %saturation level from initial zero concentration. Then the precipitated solution wasrecentrifuged at 6000rpm for 10-15 min at 37^0C. The pellet obtained after centrifugation dissolved in minimum volume of50mM Phosphate buffer, pH 7.0. Overnight dialysis was performed using 10,000 MW cutoff dialysis bag for the precipitate against 0.001 phosphate buffer with respective three changes of same buffer, dialysate will centrifuged to 10,000 rpm at 37^0C.

The pellet was separated and freeze dried. The lyophilized enzyme dissolve in minimum amount of 0.001 M phosphate buffer and applied on to the sepharosecolumn(1.5 X 25 cm)previously equilibrated with 20mM Phosphate buffer. The fractions collected by eluting the enzyme using by 50mM phosphate buffer. The active fractions collected by measuring the optical density at 280nm.The small aliquots collected fraction was tested for enzyme activity by iodine micro plate method. Fractions showing amylase activity pooled and again applied to (1 X 20 cm) DEAE-Cellulose matrix column (Bangalore genei).The column previously equilibrated with50mM phosphate buffer pH 7.0. and the enzyme bound to the column eluted with a linear gradient of 2ml/ hr. fractions collected and checked for enzyme activity.

Purification by SDS-PAGE Electrophoresis

SDS-PAGE was performed using 12%polyacrylamide gel for purified crude enzyme. The protein bands was visualized by staining comassine brilliant blue and molecular weight of the crude enzyme was determined by comparing with Rf values of standard molecular weight markers of glucose.

Characterization of crude amylase

Effect of temperature on enzyme activity

To determine temperature activity profile for α-amylase enzyme, assay was carried out at different temperature i.e 25^0, 35^0,$45^0$$55^0$Cand 65^0C.

Effect of pH on enzyme activity

For determination of suitable pH range for enzyme activity on various pH i.e. 4,5,6,7,8,9 of 0.1M acetate buffer and 0.1M phosphate buffer was used.

Effect of Incubation Time on enzyme activity

For determination of suitable incubation time range for enzyme activity on various incubation time period i.e. 0,8,12,24,48,72 and 96hrswas used.

Effect of carbon sources on amylase production

Various carbon sources such as fructose, lactose, sucrose, maltose and glucose was evaluated for their effect on amylase production by replacing starch in the production medium. The flasks was inoculated with 2% inoculum and incubated at 37ºC for 48 h. The optimum carbon source will found by analyzing the results of amylase production**.**

Effect of nitrogen sources on amylase production

Different nitrogen sources such as Tryptone, Yeast extract, Peptone, beef extract. Casein,were use to find out the bestnitrogen sources for the high production of *a*-amylase.The mediawas inoculated with 2% inoculum and incubated at 37ºC for 48 h.

Analysis of hydrolysis products of α-amylase by TLC

The hydrolysis products of the α-amylase from the isolated strain was analyzed by thin-layer chromatography (TLC) using Aluminum coated TLC silica gel plates(Kieselgel 60 F254; Merck, Darmstadt, Germany). After development of the products in a solvent system of water-acetonitrile 2:8 (v/v), the spots will visualized by spraying with methanol-sulfuric acid 1:1 (v/v)reagent and then baking it.

Effect of incubation temperature

This experiment was performed by incubating a-amylase at different temperatures viz.: 10, 25, 30, 35, 40, 50 and 60 ºC respectively.

Effect of different pH values

This experiment was planned to investigate the effect of different pH values on purified
a-amylase activities. The purified a-amylase was incubated at different pH values viz.: 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0 using phosphate buffer (0.2 M), using pH meter

Effect of incubation period

The purified a-amylase was incubated for different incubation time's viz.: 1, 6, 12, 18, 24, 30 and 36 h at 35 ºC, respectively.

RESULT

Isolation of *Bacillus cereus from* wheat bran

Toward the goal of identifying enzyme various strains were isolate from the wheat bran sample collected from local market of Dehradun (Uttrakhand). 8 bacterial strains were isolated on Nutrient Agar media. The isolated strains were named as WB1, WB2, WB3, WB4, WB5, WB6, WB7, and WB8. The isolated strains were characterized by morphological and biochemical identification (Table 1). The isolated strain WB7 was confirmed as *Bacillus cereus* on the basis of biochemical tests.(Table 2).

Solid State fermentation in aerobic and anaerobic condition

After screening of *Bacillus cereus* (WB7*) strain solid state fermentation was carried in aerobic and anaerobic conditions. The aerobic fermentation gave maximum production of α-amylase in 72 hours at 55°C *i.e* 158.6 IU/g. The anaerobic fermentation gave maximum production of α-amylase in 72 hours at 55°C i.e. 80.514 IU/g. (Figure1). The comparative study of enzymatic

activity of α-amylase using wheat bran as a substrate from *Bacillus cereus* strain (WB7*) gave maximum production in aerobic condition *i.e* 158.6 IU/g at 540 nm absorbance and least production in anaerobic condition 80.514 IU/g.

Media optimization for Solid State fermentation

Theproduction of α-amylase was carried in nutrient broth gave maximum production at 48 hours *i.e.* 14.4 IU/g and least in 96 hours *i.e.* 10.4 IU/g. (Figure 2). The production of α-amylase was best in selective media (Bushnell Hass mineral salt broth) at 37°C for 72 hours.

Characterization of crude amylase

The maximum production of α-amylase was produced at 48 hours *i.e* 130.8 IU/g and minimum at 24 hours *i.e*7.0 IU/g. The production of α-amylase at 72 hours was 40.5 IU/g and at 96 hours was 32.4 IU/g (Figure2). The enzymatic activity was also compared with the standard curve of maltose concentration (200 µg-2000 µg) at absorbance of 540 nm (Figure 3). Hence the Bushnell Hass mineral salt broth was used as selective media for the production of α-amylase.

It shows that the optimum enzymatic activity was found at temperature 37°C *i.e.*95.9 IU/ml, optimum pH 7 *i.e.* 101.12 IU/ml, optimum incubation time 48 hours *i.e.* 53.57 IU/ml (Figure 8.9,10).

The hydrolysis of α-amylase by TLC shows Rf values of crude enzyme was 47 and standard of maltose was 75.

Effect of carbon sources on amylase production

It was show very poor enzyme activity when starch (2.52 IU/g) was used as carbon source. Lactose (12.31 IU/g) and xylose(10.54IU/g) based production media also showed good enzyme activity and the highly enzyme activity was achieved when glucose (15.36 IU/g) was the carbon supplement(Figure 5).

Effect of nitrogen sources on amylase production

The highest enzyme activity was found when tryptone used as the nitrogen source. Tryptone showed (10.25 IU/g) enzyme activity. Yeast extract used as nitrogen source also show (8.62IU/g) high enzyme activity and Peptone (5.57IU/g) beef extract (4.80IU/g) casein (6.32IU/g) show average enzyme activity (Figure 6).

Purification of *a*-amylase

The enzyme analysis of α-amylase from *B. cereus* was carried out by SDS-PAGE electrophoresis method and Molecular weight was determined by comparing the relative mobility of the protein with the molecular weight markers.Theelectrophoresis was also carried out to distinguish protein bands capable of starch hydrolysis. Molecular weight of extracellular α-amylase from *B. cereus* was determined as 56,000 KDa. Five protein bands were detected in the partially purified sample (Fig. 7, Lane 2). After purification, SDS-PAGE profile shows a single protein band of extracellular α-amylase (Fig. 7, Lane 1) confirming that the α-amylase has been purified to homogeneity. and homogeneity of the purified enzyme was confirmed (Figure 7, Lane 1). The molecular weight of extracellular α-amylase from *B. cereus* was quite closed to the molecular weight of purified α-amylase as compared with protein molecular weight markers and found between 55 KDa and 57 KDa(Fig. 7, Lane 3).

Table 1: Isolation of *Bacillus cereus* from wheat bran

Strain No.	**Morphological characterization of colonies**		
	Color	**Shape**	**Gram Staining**
WB1	Cream	Circular	Gram +veCocci
WB2	Cream	Circular	Gram +veCocci
WB3	Off-white	Irregular	Gram -ve Rod
WB4	Yellow	Circular	Gram +veCocci
WB5	Pale yellow	Round	Gram -ve Rod
WB6	Grey	Rough &wrinkle	Gram +ve Rod
WB7*	Dusty & grey	Rough &irregular	Gram +ve Rod
WB8	Light orange	Circular Small	Gram +ve Rod

Table 2: Biochemical Characterization of isolated strains

Biochemical test	**WB6**	**WB7**	**WB8**
H_2S Production	+ve	-ve	-ve
Oxidase test	-ve	+ve	+ve
MR Test	+ve	-ve	-ve
VP Test	-ve	+ve	-ve
Indole Test	+ve	-ve	-ve
Starch hydrolysis	-ve	+ve	-ve
Fermentation Lact.,Dext.,Sucr.	-ve	-ve	-ve
Citrate Test	-ve	-ve	+ve
Urease activity	+ve	-ve	+ve
Catalase activity	-ve	+ve	-ve

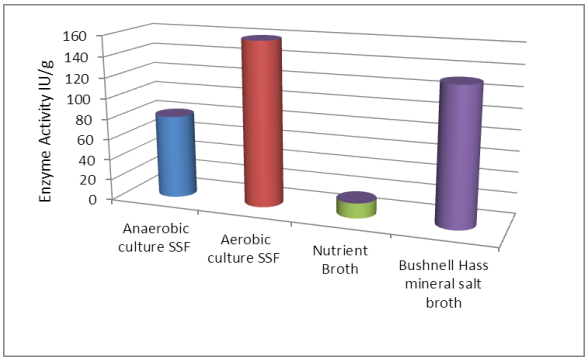

Figure1. Comparative study of production α-amylase from *Bacillus cereus* (WB7*) strain in aerobic and

anaerobic condition of solid state fermentation Bushnell Hass mineral salt broth and Nutrient broth.

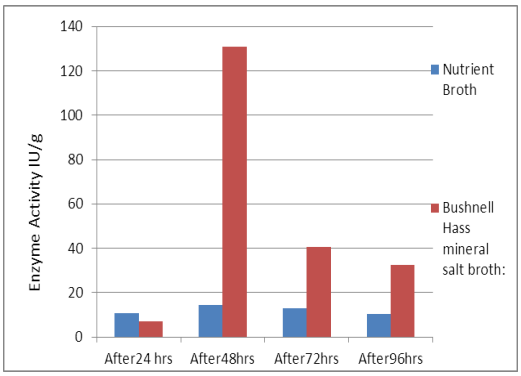

Figure2. Comparative study of enzymatic activity of *a*- amylase produced by *Bacillus cereus* (WB7*) strain in Bushnell Hass mineral salt broth and Nutrient broth.

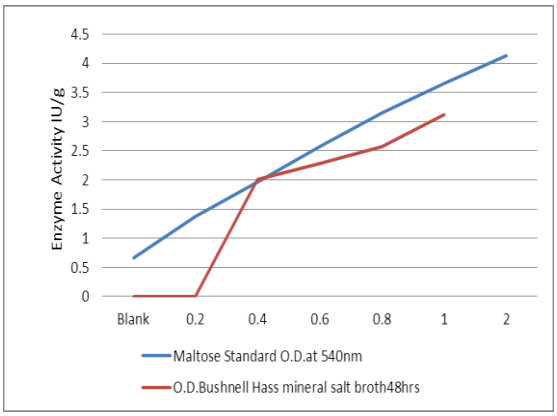

Figure 3.Comparative study of α-amylase produced by *Bacillus cereus* (WB7*) in selective media with Standard curve of maltose (concentration 200ug/ml)

Lane 1 *a*-amylase Lane 2 Maltose

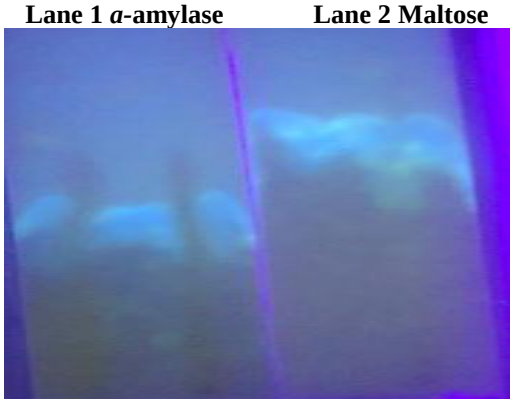

Figure 4.Analysis of hydrolysis products of α-amylase by thin layer chromatography (TLC)under UV light

Rf value of *a*- amylase produce by isolates*Bacillus cereus* (WB7*) (Table 1).

Sl. No	Sample	Distance travelled by solute	Distance travelled by solvent	Rf value
1	α-amylase	8.5	18	47
2	Maltose	13.5	18	75

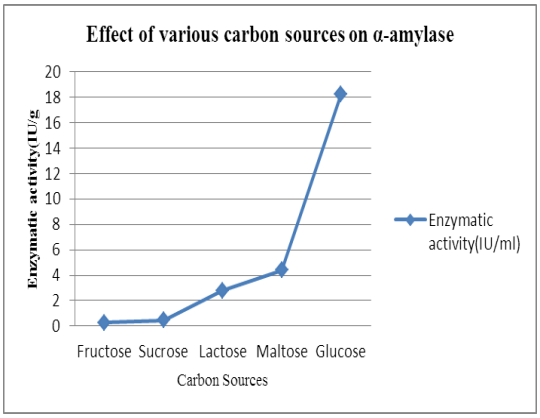

Figure 5.Effect of carbon source on amylase production by isolates*Bacillus cereus*

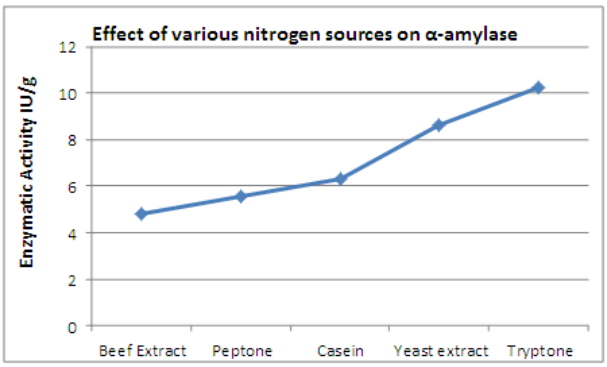

Figure 6.Effect of nitrogen source on amylase production by isolates *Bacillus cereus*

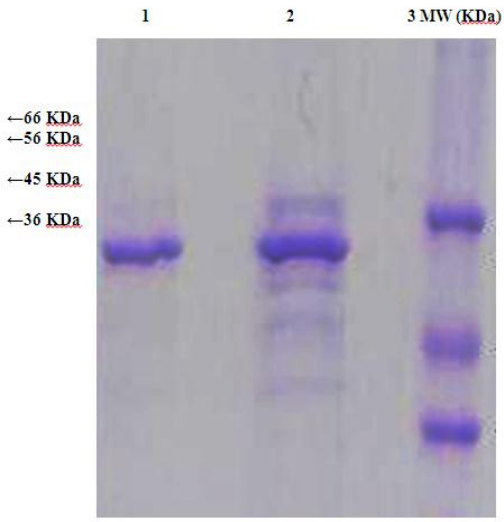

Figure 7.SDS-PAGE of α-amylase from *B. cereusLane 1* purified alpha amylase (Coomassie blue staining); *Lane 2* partially purified alpha amylase; *Lane 3MW* molecular weight markers

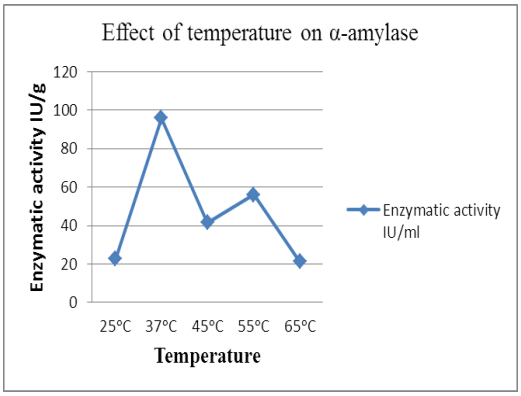

Figure 8.Effect of temperature on enzymatic activity of α-amylase by *Bacillus cereus*

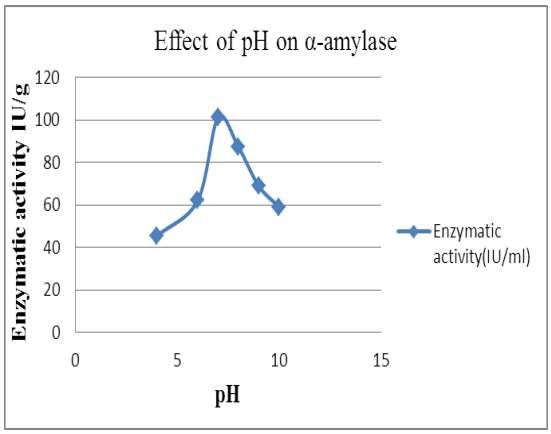

Figure 9. Effect of pH on enzymatic activity of α-amylase by *Bacillus cereus*

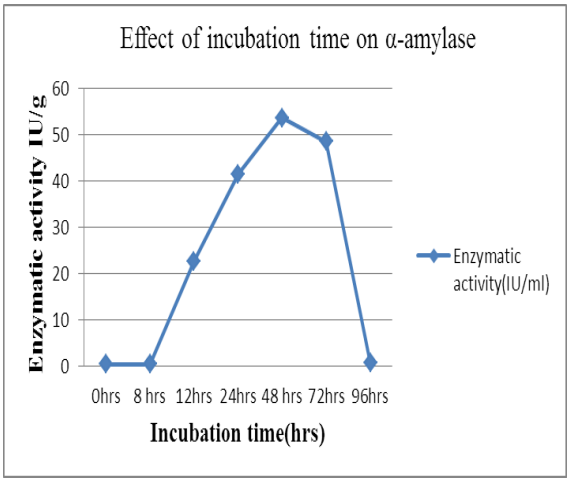

Figure 10. Effect of incubation time on enzymatic activity of α-amylase by *Bacillus cereus*

CONCLUSION

The present study conclude that theaerobic solid state fermentation using wheat bran as substrate by *Bacillus cereus* strain WB7* produce high rate of α- amylase, theBushnell Hass mineral salt broth was found best media for the production of α- amylase in control environment. Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, solid-state fermentation (SSF) constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly. SSF is preferred as compared to SmF because of simple technique, low capital investment, lower levels of catabolic repression and end product inhibition, low waste water output, better product recovery, and high quality production.

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