Optimization of Cultural Condition and Partial Characterization of Alkaline Amylase from *Bacillus sp*.

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***Abstract*: Alkaline amylase producing *Bacillus sp.* was isolated from Mangrove soil sample. Optimization of cultural conditions revealed that the maximum amylase production by the isolate cultivated in basal media containing soluble starch reached at 72 hrs, at pH 9.0 and 50^{0}C the levels of the amylase production detected in culture supernatants varied greatly with the type of carbon and nitrogen sources. Starch, lactose and maltose stimulated amylase production, with highest at 2% starch concentration. 1% peptone was found to be best for amylase production. Partially purified amylase exhibited specific activity of 2776.35 U/mg which corresponds to 5.40 purification fold and 60% yield. The optimum activity of partially characterized amylase was found at temperature 70^{0}Cand at pH 9.0. $CaCl_2$ and $MgSO_4$ had a stimulatory effect on the amylase activity and Tween 40 was found to stimulate the activity. Such alkaliphilic amylase can be exploited for industrial application.**

***Keywords*: alkaliphilic amylase, *Bacillus sp.*, partial purification, enzyme characterization.**

I. INTRODUCTION

Amylase is an extracellular enzyme, degradesα-1, 4-glucosidic linkage of starch and related products and produce oligosaccharides. These enzymes have a great significance with potentially biotechnological applications in bread making, food, textile, detergents, paper, industrial starch liquefaction, waste water treatment [1, 2], which make amylases major product in the enzyme market [3, 4, 5].Amylases are obtained from several sources such as plant, animals and microbes [6].The microbial amylases source is preferred to other sources because of its plasticity and vast availability [7]. Enzymes from microbes that thrive in extreme conditions have drawn attention for isolation of alkaliphilic bacterium to obtain alkaline enzyme production [8]. The alkaline amylases can be effectively used as additives in laundry and automatic dishwashing detergents operating under high alkalinity [9,10].*Bacillus* species is heterogeneous forms of organisms and they are very versatile in the adaptability to the extreme environments. The first alkaline amylase of an alkaliphilic *Bacillus* strain was reported by Horikoshi [11]. Alkaline amylase has been reported from a variety of species [2, 11-21].

The present study deals with the isolation identification and optimization of cultural condition for optimum alkaline amylase production from bacterium isolated from mangrove soil sample and characterization of partially purified α-amylase.

II. MATERIALS AND METHOD

Isolation and screening of microorganism

Bacillus sp. was isolated from the mangrove soil sample collected from Diva-Thane creek, Mumbai. The primary screening was done on starch agar plate. Strains capable of producing α-amylase were screened by allowing them to grow for 24 hrs on nutrient agar plates containing 1% (w/v) starch with initial pH 8.0 at 25^{0}C. The plateswere stained with Gram's iodine solution and a largest halo-forming zone was considered as the most promising strain [13] and was selected for further investigation. The culture was maintained at 4^{0}C on nutrient agar slant for further studies.

Media composition

The basal medium used for amylase production was composed of (%):0.2yeast extract, 0.2peptone, 0.25 Na_2HPO_4, 0.2 $(NH_4)_2SO_4$and0.005 $MgSO_4$. $7H_2O$, 0.1NaCl, and 0.005 $CaCl_2$, 1.0 soluble starch [16].The pH of the medium was adjusted to8.0 and was autoclaved.

Enzyme Assay

A spectrophotometric amylase assay was carried out based on the measuring residual maltose content after the enzymatic reaction. In the assay, the reaction mixture consisted of 0.5ml enzyme extract and 0.5 ml of 1% (v/v) starch in phosphate buffer at pH 9.0 and incubating for 3 minutes at50^{0}C, with 1 ml of DNS reagent reaction was stopped and kept in boiling water bath exactly for 5 minute.After cooling in ice bath8ml of distilled water was added and mixed thoroughly. O.D was measured at 540 nm. One unit of amylase activity is defined as the amount of enzyme that released one micromole of reducing equivalent under the assay condition [22].

Optimization of Culture Condition for Production of Amylase

Fermentation studies were done in basal medium broth for 96 hours at 25^0C on shaker at 200 rpm. An aliquot of the cultural broth was withdrawn after every 24 hours for analysis of enzyme activity.The effect of different carbon source [1% (w/v) glucose, lactose, maltose, sucrose, xylose, arabinose, fructose, starch] and nitrogen sources [1%(w/w) $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$, urea, yeast extract, beef extract, peptone] on the production of extracellular amylases was carriedout. The optimum starch concentration (0.15, 0.5, 1.5, 2.0, 2.5 and 3.0 %) and peptone concentration (0.5, 1.5, 2.0, 2.5 and 3.0 %) on the production of extracellular amylases by the isolate was studied by carrying out amylase assay. The study of effect of temperature ranging from 25^0 C to 80^0 C with 5^0C difference and at pH 3.0 to 11.0 with difference of1 pH unit, was carried out on the production of extracellular amylases by incubating at various temperature and in buffered basal medium at different pH [100mM acetate buffer (pH 3.0-6.0), 100mM potassium phosphate buffer (pH 7.0-8.0),100mM glycine-NaOH buffer (pH 9.0-11)].

Production and extraction of crude extracellular amylase

1m1 of 1 $X10^5$ cell density adjusted at 660 nm of 18 hours old culture was inoculated in 100ml sterile enzyme production broth (pH-9.0) in 250ml Erlenmeyer flasks and incubated at 50^0C on rotatory shaker at 150 rpm for 72 hours. After incubation the crude amylase was extracted by cold centrifugation (4^0C) at 3000 rpm for 30 minutes, the supernatant was used for further work.

Partial Purification of Enzyme

The entire purification step was carried out at 4^0C.The supernatant was subjected to fractionated ammonium sulfate precipitation for enzyme purification. Ammonium sulfate crystals were added to the supernatant at 20, 30, 40,50,60,70 and 80% saturation in an ice bathovernight. The precipitate was collected by centrifugation at 3000 rpm at 4^0C, for 30 min.The enzyme was recovered by re-suspending the precipitate in 100mM phosphate buffer at pH9.0. Then the suspension was dialyzed against the same buffer for 3days with several changes of buffer for desalting [8].

Effect of pH and temperature eon amylase activity

Temperature and pH effects for amylase activity were assayed at different temperature ranging from 10^0C-100^0C and at pH values from 3-11 for 1 hr. The following buffers were used in the reaction: 100mM acetate buffer (pH-3-6), 100mM potassium phosphate buffer (pH 7.0-8.0), 100mM glycine-NaOH buffer (pH 9.0-11).

Effect of metal ions and additives on amylase activity

The effect of metal ions and additives was studied on amylase activity by pre-incubating the enzymes in presence of the metal ions (NaCl, $FeCl_2$, $MnCl_2$, $CaCl_2$, $MgSO_4$, $ZnSO_4$) and additives (H_2O_2**,** perchloric acid**,** SDS, Tween20, Tween40, Tween80, Triton-X-100) with the final concentration of 1mM for 1hour at 70^0Cand pH 9.0. The activity in the absence of any metal and additive was taken to be 100%as control.

III. RESULTS AND DISCUSSION

The organism isolated from Mangrove soil, was identified as *Bacillus* sp. according to Bergey's manual of determinative bacteriology on the basis of morphological and biochemical characteristics and thoroughly investigated for amylase production. Data presented in (fig.1) showed that the maximum amount of enzyme activity (1789 U/ml) was obtained after an incubation of 72 hours. Further increase in the fermentation period did not improve the rate of enzyme synthesis. Incubation temperature has very critical influence on the production ofamylase. The production of enzyme was maximum (1854 U/ml) at50^0C [16, 25] (fig.2) beyond which gradual reduction in enzyme activity was observed. As the activity was found maximum at 50^0C thus, this temperature was selected for amylase production.

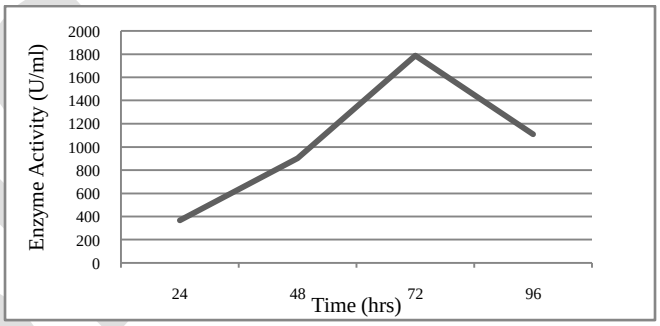

Fig. 1 Determination of optimum incubation period.

Microbial growthand production are very sensitive to any change in pH, the pH of fermentation medium plays a very profound effect on the enzyme production [11]. The result presented in (fig 3)showed that the maximum activity (2100 U/ml) was obtained at pH 9.0. The other coworkers Krishnan and Chandra [19] and Hagihara *et al*, Horikoshi [10, 11], have obtained optimal amylase synthesis at neutral pH and pH 8.0-9.5 respectively by *Bacillus* sp. However, in the present study pH 9.0 was found to be optimum. It might be due to the fact that the organism required alkaline pH for its growth. Therefore, alkaline pH of fermentation medium was selected for maximum production of amylase.

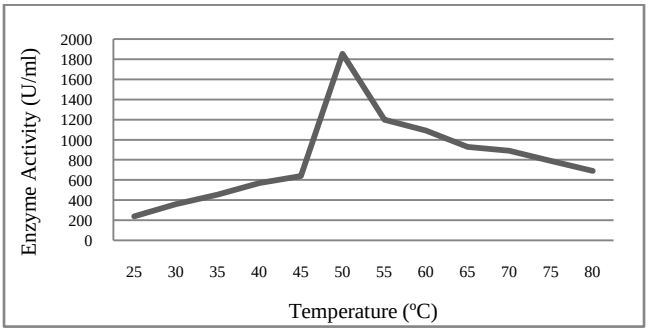

Fig. 2 Determination of optimum incubation temperature.

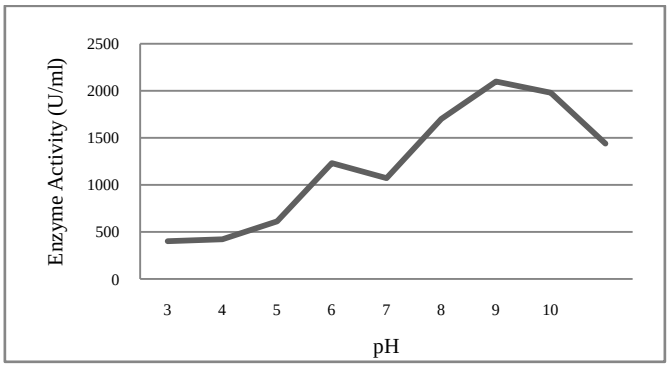

Fig. 3 Determination of optimum incubation pH.

The productivity of amylase by the bacterium can be further improved by the addition of different carbon and nitrogen sources to the fermentation medium as reported by Mc Tigue et al [14]. So, different carbon and nitrogen sources were investigated for the maximum production of amylase. Of all the sugars tested, starch gave maximum production of amylase (2030 U/ml) (fig.4) followed by lactose (1933 U/ml) and maltose (1893 U/ml).

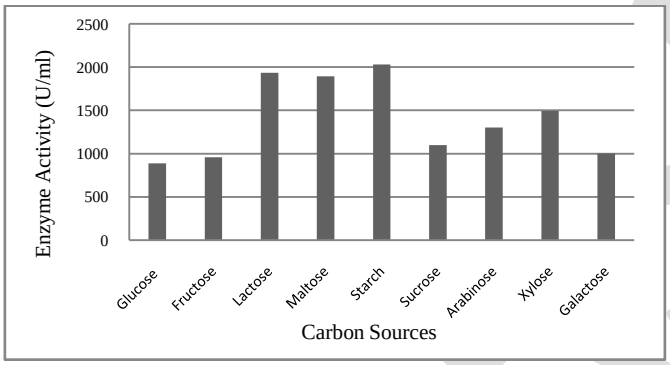

Fig. 4 Determination of effect of different carbon sources.

Carbon sources greatly influences amylase production and the most commonly used substrate is starch [21]. As starch gave optimum results therefore, its various concentrations were also tested for the production of amylaseshowed that the production of amylase was increased with increase in the starch concentration and was found optimum at 2% (1990 U/ml) (fig.5) concentration, above whichproduction was reduced. One of the reason may be due to end-product repression of amylase production [30], also it might be due to the high viscosity of the medium affecting availability of oxygen concentration as reported by G. Mamo *et al* and Bajpai P *et al* [20, 26]. Among inorganic nitrogenous source only $(NH_4)_2SO_4$ showed 1560 u/ml activity. However, of all the nitrogen sources studied peptone (1980 u/ml) produced the best results, followed by yeast extract

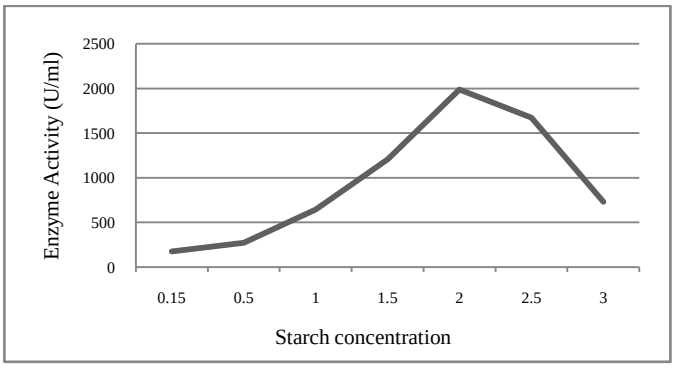

Fig. 5 Determination of optimum starch concentration.

and beef extract, presented in (fig.6). 1% peptone (1974 u/ml) concentration was optimum for maximum amylase synthesis. Beyond 1% peptone concentration enzyme synthesis was found to bedecreasing this maybe due to viscosity of the medium.This finding is accordance with Bajpai *et al.* [21]

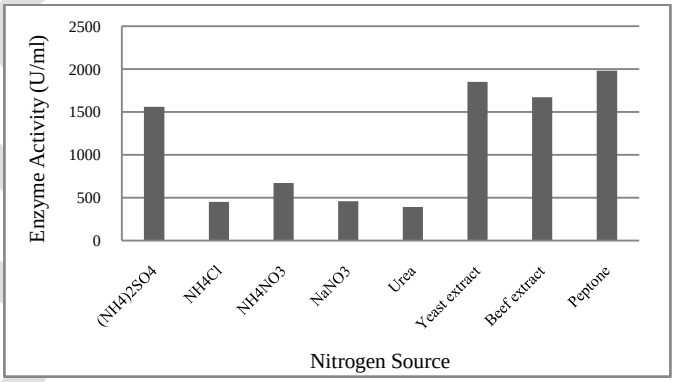

Fig. 6 Determination of effect of different nitrogen sources.

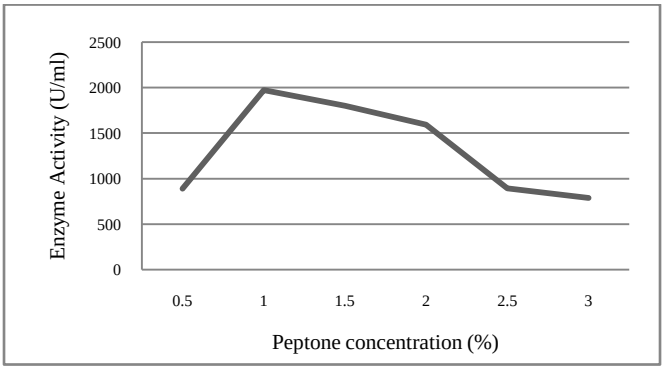

Fig. 7 Determination of optimum peptone concentration.

The specific activity of enzyme was increased with the purification of amylase. It is due to the fact that the activity of the enzyme increases after the purification of enzyme [31]. So, after optimization of cultural conditions the crude amylase was partially purified by ammonium sulphate precipitation. The results of partial purification studies are summarized in Table. 1

Purification Step	Total Enzyme Activity (Units)	Total Protein (mg)	Yield (%)	Specific Activity	Fold Purification
Supernatant	126000	245	100	514.28	1
Ammonium Sulphate Precipitation	91350	68.92	72.5	1329.31	2.58
Dialysis	75600	27.23	60	2776.35	5.4

Table-1 Partial Purification of Amylase.

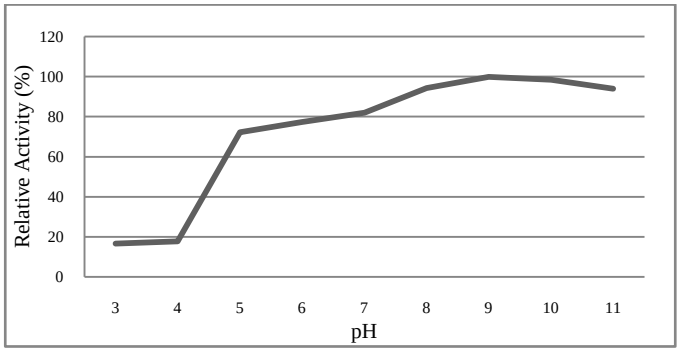

Fig. 8Effect of pH on partially purified amylase.

The hydrolytic activity of partially purified amylase is sensitive to pH and temperature as reported by Ashabil et al, Hiroshi et al [8, 9]. The effect of pH on optimum relative activity at pH 9.0 (fig.8) was obtained. It may due to inactive nature of enzyme at acidic mediumpartially purified amylase activity wascarried out and due to change in the hydrogen ion concentration. Any change in hydrogen ion concentration inhibits the active site of the enzyme resulting in the decreased activity of amylase. The optimal relative temperature of the partially purified amylase was found at 70^0C (fig.9) and the amylase activity decreased rapidly at temperatures higher than. Thisis of concern, since starch liquefaction is generally carried out at high temperatures of between 70^0C – 90^0C, and the thermo stability of amylase is of great significance for the efficient liquefaction of gelatinized starch. The amylase from *T. lanuginosus* 34626 displayed 70^0C and 55^0C for optimum temperature and thermal stability characteristics by Ngyuen et al [24] and amylase from *Bacillus sp.* was reported by Soni et al, [29], to have an optimum temperature at 70^0C and thermal stability at 60^0C.

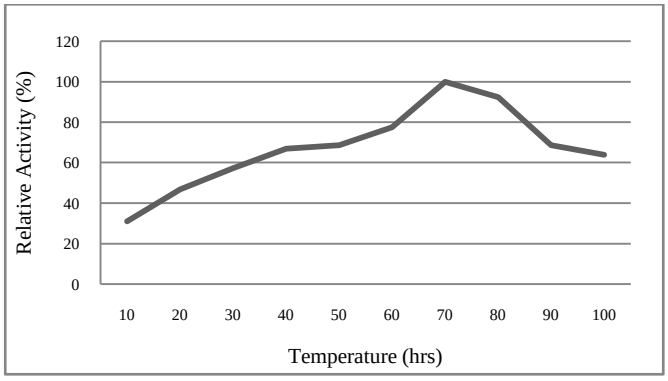

Fig. 9 Effect of temperature on partially purified amylase.

The results of study of different metal ions and additives that influence amylase activities are summarized Table.2.$CaCl_2$ and $MgSO_4$ showed a stimulatory effect on the amylase activity. Tween 40 was found to stimulate the activity and SDS marginally inhibited the activity. The role of Ca^{+2}ions was investigated by Mishra and Maheshwari[23], and it was shown that Ca^{+2} had a positive effect on enzyme activity. It was also reported by Lin L-L, *et al*[12] in the presence of Ca^{+2} ions the thermostability of other amylolytic enzymes, including the amylases from *B. licheniformis*, *P. furiosus* and *T. litoralis*are improved. This phenomenon was confirmed by Pandey A. *et al* [1] for α-amylase from other organisms. The reasons for these effects are not clearly understood, although metal ions often act as salt or ion bridges between two adjacent amino acid residues, resulting in the stimulatory effect that Ca^{+2} ions have on enzyme activity [12].

Metal ions and Additives	Relative Activity (%)
Control	100
NaCl	96±2.29
$FeCl_2$	46±2.30
$MnCl_2$	52±1.72
$CaCl_2$	137±1.92
$MgSO_4$	107±1.28
$ZnSO_4$	73±1.52
H_2O_2	64±1.99
PERCHLORIC Acid	59±2.01
SDS	82±1.59
Tween 20	73±1.83
Tween 40	103±1.45
Tween 80	35±1.99
Triton-X 100	45±2.21

Table-2 Effect of metal ions and Additives on amylase activity

IV. CONCLUSION

The results obtained in present study showed that there is appreciable high production and activity of the amylase at alkaline pH and high temperatures and enhanced activity was found in presence of Ca^{+2} and Mg^{+2} metal ions and Tween 40.Thus *Bacillus* sp.can be exploited for industrial alkaline amylase synthesis and various biotechnological applications.

REFERENCES

[1]. Pandey A. Soccol C.R. and Mitchell D (2000), New developments in solid state fermentation: I-bioprocesses and products. Process Biochemistry, 35:1153-1169.

[2]. Prasanna V. Aiyer, (2005) Amylases and their applications,4:1525-1529.
[3]. Burhan A., Nisa U., Gökhan C., Ömer C., Ashabil A. And Osman G, (2003). Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from analkaliphilic *Bacillus sp*. Isolate ANT-6. 38:1397-1403.
[4]. Rani Gupta, Paresh Gigras, Harapriya Mohapatra, Vineet kumar Goswami, Bhavna Chauhan, Microbial amylases: a biotechnological perspective. Process Biochem. 38 (2003) 1599-1616.
[5]. R.S Desai, N K Krishnamurthy, S Mavinkurve & Bhosle, (2004). Alkaliphiles in estuarine mangrove regions of Goa (central west coast of India), Indian J Marine Sci, 33(2):177-180.
[6]. K. Kathiresanand S. Manivannan,(2006). Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. Afr. J. of bacterio. 5:829-832.
[7]. Sashmita Mishra and Niranjan Behera,(2008). Amylase activity of a starch degrading bacteria isolated from soil receiving kitcten wastes, Afr J Biotech, 7:3326-3331.
[8]. AshabilAygan, Burhan Arikan, HaticeKorkmaz, SadikDincer,OmerColak,(2008). Highly thermostable and alkaline α-amylase from a halotolerant- alkaliphilicBacillussp.AB68, BrazilianJMicrobio, 39:547-553.
[9]. Hiroshi Hagihara, Kazuaki Igarashi, Yasuhiro Hayashi, Keiji Endo, Kaori Ikawa-Kitayama ,Katsuya Ozaki, Shuji Kawai and Susumu Ito,(2001) Novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic Bacillusisolate KSM-K38, Appl&Env Microbio,67 :1744-1750.
[10]. Hiroshi Hagihara, Kazuaki Igarashi, Yasuhiro Hayashi, Kaori Kitayana, Keiji Endo, Tadahiro Ozawa, Katsuya Ozaki, Shuji Kawai and Susumu Ito,(2002). Improvement of Thermostability of a Calcium-free α-amylase from an Alkaliphilic *Bacillus sp*.By Protein Engineering, J. Appl. Glycosci. 49:281-289
[11]. Horikoshi, K, (1971) Production of alkaline amylase by alkalophilic microorganisms II Alkaline amylase produced by *Bacillus no A-40-2*, Agric. Biol.Chem, 35 : 1783-1791.
[12]. Lin L-L., Chyau C-C.and Hsu W-H, (1998) Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus sp.* TS-23,J Biotechnol Appl Biochem, 28:61-68
[13]. Bhutto M. Aqueel and Dahot M. Umar, (2010). Effect of alternative carbon and nitrogen sources on production of alpha-amylase by *Bacillus megaterium*, World Appl Sci J 8(Special issue of Biotech& Genetic engg) . 85-90.
[14]. McTigue, M. A., C.T.Kelly, E. M. Doyle, and W. M.Fogarty, (1995). The alkaline amylase of the alkaliphilic *Bacillus sp* IMD 370. Enzyme Microb.Techno,17: 570–573.
[15]. P. V. Dharani Aiyer, (2004). Effect of C: N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. African Journal of Biotechnology, 3:519-522.
[16]. M. Asgher, M. Javaid Asad, S .U. Rahman, R. L .Legge, (2007). Athermostable α-amylase from a moderately thermophilic *Bacillussubtilis* strain for starch processing, J Food Engg ,79: 950-955.
[17]. Farouk Al-Quadan, Hazem Akel and Rasha Natshi,(2009) Characteristics of a novel highly thermostable and extremely thermophilic alkalitolerant amylase hyperthermophilic *Bacillusstrain* HUTBS71,Online J Biol Sci,9: 67-74.
[18]. E.W. Boyer, M. B. Ingle, and G. D.Mercer ,(1973*),Bacillusalcalophilussubsp. haloduranssubsp.nov.*: an alkalineamylase producing, alkaliphilic organism, Internat J Sys Bacterio, 23238-242.
[19]. T. Krishnan and A. K. Chandra, (1982). Effect of oilseed cakes on α-amylase production by *Bacilluslicheniformis* CUMC305, Appl & Env Microbio,44 : 270-274.
[20]. G. Mamo and A. Gessesse,(1999) Effect of cultivation conditions on growth and α-amylase production by a thermophilic *Bacillus sp*, Lett in Appl Microbio, 29 : 61-65.
[21]. Bajpai P and Bajpai P, (1989). High-temperature alkaline alpha amylase from *Bacilluslicheniformis* TCRDC-B13. Biotech. Bioeng.33: 72-78.
[22]. Bernfeld, P, Amylases α- and β-methods. Enzymology.I, 149-158.
[23]. Mishra R. S. and Maheshwari R,(1996). Amylases of the thermophilic fungus *Thermomyceslanuginosus*: their purification, properties, action on starch and response toheat,Journal of Bioscience, 21:653-672.
[24]. Nguyen D.Q., Rezessy-Szabó J.M. and Hoschke Á, (2002). Purification and characterization of amylolytic enzymes from thermophilic fungus T*hermomyceslanuginosus* strain 3426, Enzyme and Microbial Techno,31: 345-352.
[25]. S Kar and R C Ray, (2008). Partial characterization and optimization of extracellular thermostable Ca^{+2} inhibited α-amylase production by *Streptomyceserumpens* MTCC 7317, 67:58-64.
[26]. Saad A. Almari, Isolation, (2010). Phylogeny and characterization of new α-amylase producing thermophilic *Bacillus sp*. from the Jazanregion , Saudi Arabia, International J Biotech & Biochem, 6(2010) 537-547,
[27]. Burhan Arikan, (2007). Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic Bacillus sp., Isolate A3-15, Bioresourcetechno, *articleinpress*.
[28]. S.Dhingra, M Khanna & C S Pundir,(2006). Immobilization ofα-amylase onto glass beads affixed inside a plastic beaker: Kinetic properties and application, Indian J Chem techno, 13 : 119-121.
[29]. Soni S.K., Kaur A. and Gupta J.K., (2003). A solid-state fermentation based baterial α-amylase and fungal glucoamylase system abd its suitability for the hydrolysis of wheat starch. Process Biochemistry. 39:185-192.
[30]. Omidiji, O., O.O. Braimoh and A.A. Llori. (1997). Production of alpha-amylase in a corn steep liquor-soya bean meal medium by strain of *Bacillus stearothermophilus*. Microbio. 90:155-162.
[31]. Fisher, E. and I. Stein, (1961).α–amylase from human saliva. Biochem. Prep., 8: 27