Isolation, screening and optimization of amylase producing *Bacillus* sp. from soil

V. Singh[1], Richa Sharma[2], Poonam Sharma[2]

[1] *Department of Microbiology, Himachal Institute of Life Sciences, Paonta Sahib (H.P.), India*
[2] *Department of Biotechnology, Himachal Institute of Life Sciences Paonta Sahib (H.P), India*

ABSTRACT

Amylases are one of the main enzymes used in industry. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. Studies were carried out to screen amylase producing microorganism from soil. Total 60 samples were collected from different locations of Paonta sahib. A sum total of 17 pure isolate of different amylase producing bacteria were isolated. Screening procedures adopted were plating of decimal dilutions of soil with incubation at 37°C, isolation of pure cultures and flooding of isolate grown on 1% starch agar with iodine. Amylase positive isolate was detected by the formation of a hallo against blue black background.

Keywords: Amylase, *Bacillus* sp. Submerged fermentation.

Introduction

Starch, a main component of our daily diet, is frequently found not only in food residues on dishes but also in food stains on clothes. Amylases are enzymes that break down starch or glycogen. Recent discoveries of starch degrading enzymes have led to increased application of amylases in various industrial processes Amylases [α-amylase, β-amylase and glucoamylase (GA)] are among the most important enzymes in present-day biotechnology in many biotechnological processes including starch degradation, detergent, foodstuff, pharmaceutical, textile, and paper manufacturing. Amylases constitute one of the most important groups of industrial enzymes and account for nearly 25% of the total sale of enzymes. The enzymes of amylase family have great significance due to its wide area of potential application. The spectrum of amylase application has widened in many other fields, such as clinical, medical and analytical chemistry. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (1, 2). The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands; a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry [2]. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes manipulate to obtain enzymes of desired characteristics [3]. Although many microorganisms produce this enzyme, the most commonly used for their industrial application are *Bacillus licheniformis, Bacillus amyloliquifaciens* and *Aspergillus niger* stand out as a class of enzymes, which are useful applications in the food, brewing, textile, detergent and pharmaceutical industries. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry [4]. Amylases stable to high temperature have been known for a long time and the commercial application of heat stable bacterial amylases in textile desizing. The capacity of *Bacillus* strains to produce large quantities of enzymes has placed them among the most important industrial enzyme producers. Indeed, they produce about 60% of commercially available enzymes [5].

**Correspondence*
Dr.V Singh
Department of Microbiology, Himachal Institute of Life Sciences, Paonta Sahib (H.P.), India.
E Mail: virender_micro83@yahoo.co.in

MATERIAL AND METHDOS

Isolation and identification of *Bacillus* sp.: A total of 60 soil samples (from Paonta sahib sirmour district)

were collected from 20 different places. A 1g of each soil samples were dissolved in 10 ml sterile distilled water, and mixed thoroughly. The supernatant of these suspensions was used for isolation of *Bacillus* species which can produce amylase by plating on starch agar at 37^0C for 24h for amylase producing bacteria. Enzyme production was identified by clear zone around colonies of amylase (after addition of iodine) producing bacteria. Isolated predominant, morphologically distinct colonies were selected and all isolates were identified on the basis of cultural, morphological and biochemical characteristics.

Screening of amylase producing organisms: This was carried out on starch agar. This was then sterilized by autoclaving at 121°c for 15 minutes in an autoclave and then allowed to cool. The glass Petri-dishes were placed in a canister and sterilized in a hot air oven at 160°C for 2 hours, the work bench was swabbed with 75% alcohol. After cooling, the efficient bacterial isolates were re-subcultured onto starch agar plates for detection of their enzyme production efficacies. Productions of these enzymes were studies at various pH (7, 8, 9, and 10) and temperature (25^0C, 30^0C, 35^0C, 40^0C). A clear zone of hydrolysis on starch (after addition of iodine) gave an indication amylase producing bacteria. The efficiencies of enzyme production were studied on the basis of diameter of zone of hydrolysis.

Control

AMYLASE PRODUCING BACTERIA

Extraction of Amylase from the Fermentation Medium: After incubation the fermentation medium was harvested by centrifugation at 5000 rpm for 20 minutes at 4°C. The supernatant was collected and subjected to estimate the amylase activity.

Effect of Temperature: To study the effect of temperature on amylase production the submerged fermentation was carried out at different temperatures (25°C, 30°C, 35°C and 40° C)

Effect of pH: The fermentation medium was prepared by varying the pH values for the production of amylase, pH in the range of 7.0–10.0 were examined for their effect on amylase production by the selected isolate grown in production media. The pH of the medium was adjusted using 1 N HCl or 1 N NaOH. The flasks were incubated at 37ºC for 24 h. Samples were taken at regular time intervals for protein estimation and amylase activity.

Enzyme production: The inoculum was prepared by inoculating the loopful of strain in to nutrient broth and it was Incubated in shaker for 24 hrs. 1.5ml of this 24 hr old inoculum was transferred aseptically to 250 ml production medium [g/l] 6.0 g Bacteriological peptone; 0.5 g MgSO4 .7H O; 0.5 g KCl; 1.0 g Starch and incubated in incubator shaker at 180 rpm for 14 hrs. The Amylase production was carried out in shake flask fermentation using production media.

Enzyme assay

Plate assay: The plate assay was performed using agar plates amended with starch. The agar plates were prepared amended 2% of starch with 1.5% of agar. After agar solidification, around 10 mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with the culture filtrate and incubated at 37°C for overnight. 1% of iodine solution was over layered on the agar and the observation was made to see the hydrolytic zone around the well (shown in Figure). The negative control was maintained by adding sterile water in the separate well.

Chemical assay: In this method three flasks of 250ml capacity were taken and labeled with 1(starch control), 2(enzyme control), 3(test).After that 5 ml of phosphate buffer was transferred to each of the flask. Then add 2ml of 0.1% starch solution in the flask 2 and 3, while 2ml of distilled water in flask 1, and then add of the 2ml of 0.01N HCL in each flask and incubate all the three flasks at 37^0C for 3 min. After the incubation, 1ml of active enzyme filtrate was added in the flasks 1 & 3 and 1ml of the inactivated (heat killed) enzyme in flask 2.incubate at 37^0 C in water bath for 15, 30, & 45 min. Thereafter add 80 ml of distilled water in each flask. Then 4 ml of 0.01N iodine in each flask was added, mix well and measure absorbance at 578nm.And the enzyme activity was calculated by the formula

$$\text{Volume activity} = \frac{E_0 - E_t \times A \times 1000 (V/L)}{E_0 \times T \times V}$$

$E_0 = OD_2 - OD_1$

$E_t = OD_3 - OD_1$

T= Incubation time (minutes)

A= 12.35 (constant)

V= Volume of starch

The amylase activity was determined in IU/mL/min by applying the following formula (6).

$$\text{IU/ml/min} = \frac{\text{Activity of enzyme} \times 1000}{\text{Molecular wt. of maltose} \times \text{time of incubation}}$$

Purification of Amylases: After 24 hrs of growth, the culture was centrifuged at 5,000 rpm for 5 min at 4°C and the cell free supernatant was used .Amylase produced was partially purified by precipitation with ammonium sulphate and followed by dialysis. Ammonium sulphate precipitation technique was performed by mixing culture filtrate and ammonium sulphate (75%, w/v) solution at 1:3 ratios [7]. The mixture was then stored in cold room for 24 h to precipitate all the proteins present in the sample. Precipitate was removed by centrifuging sample in an ultra centrifuge at 10000 rpm for 10 min. The supernatant was discarded and precipitate obtained was dissolved in 5 ml of 1 M-citrate phosphate buffer (pH.5) (8). Then the mixture was subjected to dialysis. The enzyme was dialysed in same buffer overnight at 4°C.

RESULTS & DISCUSSION: Total 60 samples were collected from 20 different places of Paonta sahib. Out of 60 samples 17 isolates screened tested positive. The isolates were characterized and identified on the basis of Morphological characteristics such as Gram staining rxn, Colony characteristics and Biochemical test Holt *et al.*1994.Bergey's manual of determinative Bacteriology, 9th Ed. Based on the Morphological & Biochemical characterization. In present study On the basis of level of productivity of the amylase, an isolate producing a maximum of amylase activity was screened from soil and used for detailed investigation. Screening of the amylase producing organisms was carried out on the starch agar. Productions of these enzymes were studies at various pH (7, 8, 9, and 10) and temperature (25^0C, 30^0C, 35^0C, 40^0C). A clear zone of hydrolysis on starch (after addition of iodine) gave an indication amylase producing bacteria the efficiencies were studied on the basis of the zone of hydrolysis. The isolate showing the maximum zone of hydrolysis was selected for identification. The use of starch nutrient agar and iodine for detecting amylase (hydrolytic enzyme) producing Microorganisms have been reported by Forgarty and Kelly, (1979) and also by Iverson and Millis, (1974) that starch hydrolysis can be detected on plates as a clear zone surrounding a colony. This procedure employed showed a positive result for the *Aspergillus* strain isolated. The mechanism of clear zone observed was due to the fact that the amylase produced during the growth of the organisms has hydrolysed the starch around the colony, thereby testing negative when flooded with iodine. The un-hydrolysed part of the plate tested positive to the presence of starch (amylose), hence the blue-black appearance. According to Akpan *et al.* (1999) screening for amylase producing microorganism by the

method described above is time consuming and inconvenient for direct isolation of intact cells, as the cells die after flooding with iodine, therefore a rapid screening method such as Remazol Brilliant Blue (R.B.B) will be more effective. The former method was adopted for this research which involves screening through the use of starch agar and iodine solution. The formation of clear zones indicated that the organisms test positive for 17 isolates screened.

Table 1: Biochemical Characterization

S. No.	Indole	MR	VP	Citrate utilization	Lactose	Dextrose	N R	Sucrose	Catalase	Identified Organism
1.	-	+	+	+	-	+	+	+	+	*Bacillus subtilis*

Effect of incubation temperature

The data of tables shows the effect of different incubation temperatures on the production of amylase by *Bacillus* sp. In the present study 17 isolates of *Bacillus* species produced enzymes over the large range of temperature however the maximum zone of starch hydrolysis observed at temperature 40^{0}C. Below 35^0 C showed decrease in the zone of starch hydrolysis. Fig. 5.4 shows the activity of enzyme was gradually increased and found maximum at temperature 40^{0}C (30mm zone of starch hydrolysis) of isolate I-8. On the basis of the maximum zone of starch hydrolysis I-8 was selected for the quantitative analysis of enzyme. The fermentation was carried out at different temperatures 25^{0}C 30^{0}C 35^{0}C and 40^{0}C in rotary incubator shaker. The maximum production of amylase was obtained with pH 7 at 40°C (36 IU/mL). Biosynthesis of amylase was significantly decreased with the decrease in the incubation temperature below 35ºC.The production of the enzyme was greatly inhibited at 25°C. Thus the incubation temperature 40°C was selected for maximum production of enzyme.

Effect of incubation pH

These 17 isolates of *Bacillus* species produced enzymes over the large range of pH investigated (7 to10). In present study, the different pH (7-10) of starch solution was tested for the activity of amylase. However the maximum production observed at pH 7 and 8. At pH 7 and 8, zone of starch hydrolysis was more than pH 9 and 10. The activity of enzyme was gradually increased and found maximum at pH 7 (30mm zone of starch hydrolysis) of isolate I-8. Further increase in the initial pH resulted decrease in the activity of amylase. The maximum zone of starch hydrolysis of the enzyme was obtained at slightly alkaline pH 7. With the increase in pH the results were extremely low. The organism did not grow at pH 9.0, and 10.0. This may be due to the fact that bacteria required slightly alkaline pH for the production of amylase. Increasing the initial pH of the medium up to pH 9.0 resulted in a reduction in amylase production. Enzyme synthesis and bacterial growth of *Bacillus* sp. KCPSS-12ss was observed between pH 4.0 to 11.0. The results suggest that there is a spur in enzyme synthesis at pH 7.0 and the higher enzyme production at this pH was considered, probably, as a result of increased cell growth [12]. The data of the tables shows the effect of initial pH of reaction mixture (enzyme substrate complex) for the activity of amylase. The zone of starch hydrolysis was optimum at pH 7.0. Further increase in the initial pH resulted decrease in the activity of amylase. The production and stability of amylase depends upon temperature. In present study, the fermentation was carried out at different incubation temperatures. The maximum production of enzyme was observed at 40ºC. However, the pH of reaction mixture for the hydrolysis of starch was found to be optimum at 7 pH.

Table 2: Effect of pH on enzyme activities (Zone of hydrolysis in mm) at 25^{0}C

No. of isolates	pH = 7	pH =8	pH = 9	pH =10
I-2	20	5	10	9
I-4	22	10	20	12
I-5	15	14	11	-
I-8	21	23	16	15

I-12	12	15	12	-
I-14	20	13	18	-
I-16	18	20	9	-
I-17	21	10	15	-
I-19	15	18	20	10
I-21	23	22	15	9
I-24	16	18	-	-
I-26	15	21	16	5
I-28	10	19	20	8
I-30	21	22	21	14
I-31	20	19	18	16
I-32	15	10	12	5
I-33	18	22	20	-

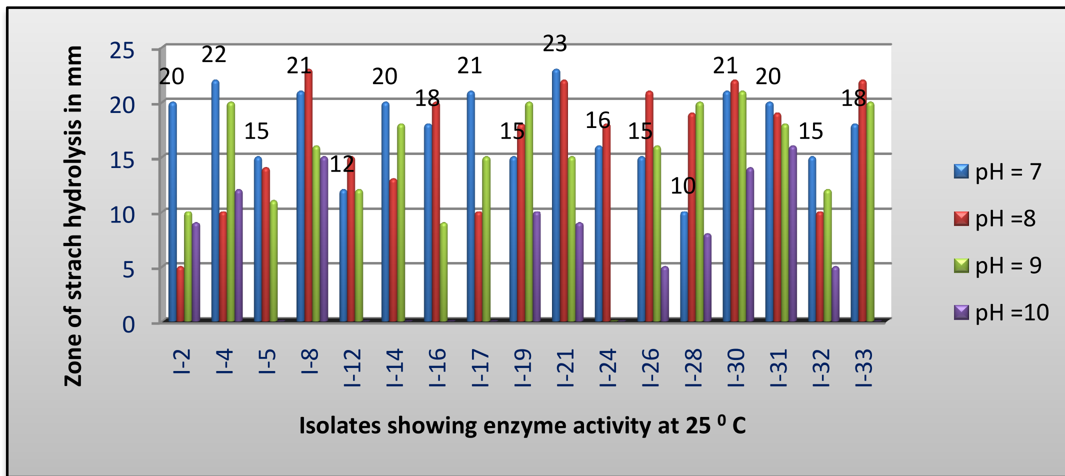


Fig 1: Effect of different pH on the production of amylase by *Bacillus sp.* (temp =25^{0}C, Incubation time period =24 hrs)

Table 3: Effect of pH on enzyme activities (Zone of hydrolysis in mm) at 30^{0}C

No. of isolates	**pH = 7**	**pH =8**	**pH = 9**	**pH =10**
I-2	20	12	14	16
I-4	19	16	6	17
I-5	21	15	10	15
I-8	23	22	17	20
I-12	16	19	20	12
I-14	17	17	18	11
I-16	10	12	14	-
I-17	15	19	21	14
I-19	22	21	20	10
I-21	22	9	11	-
I-24	21	20	18	-
I-26	23	19	16	15
I-28	17	18	19	20

I-30	14	14	18	-
I-31	18	19	17	9
I-32	22	18	18	15
I-33	22	20	22	18

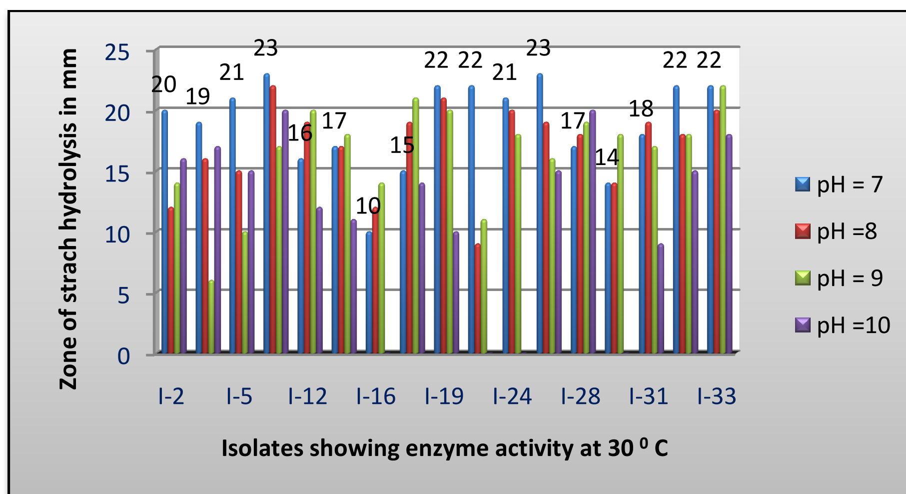


Fig 2: Effect of different pH on the production of amylase by *Bacillus sp.* (temp =30^{0}C, Incubation time period =24 hrs)

Table 4: Effect of pH on enzyme activities (Zone of hydrolysis in mm) at 35^{0}C

No. of isolates	pH = 7	pH =8	pH = 9	pH =10
I-2	16	15	12	-
I-4	18	10	20	-
I-5	19	19	25	15
I-8	27	25	24	20
I-12	23	16	20	10
I-14	15	22	20	-
I-16	18	20	24	-
I-17	21	26	20	-
I-19	27	25	27	21
I-21	22	20	28	12
I-24	16	21	17	-
I-26	25	20	22	-
I-28	21	26	24	10
I-30	18	25	17	-
I-31	19	22	20	-
I-32	24	25	21	16
I-33	27	14	22	12

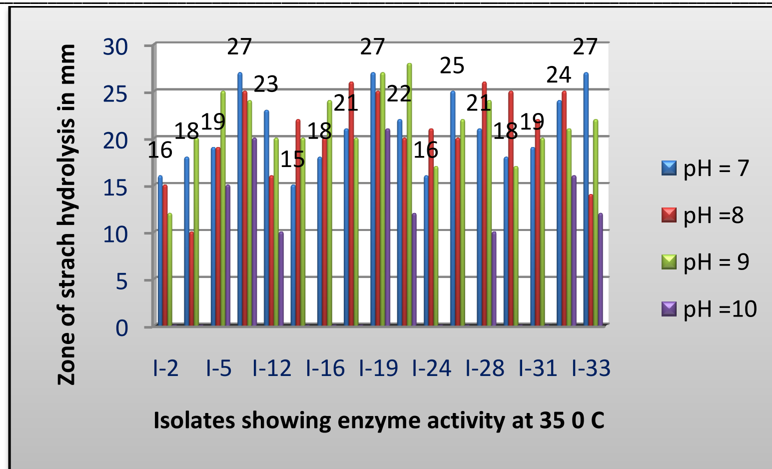


Fig 3: Effect of different pH on the production of amylase by *Bacillus sp.* (temp =35^{0}C, Incubation time period =24 hrs)

Table 5: Effect of pH on enzyme activities (Zone of hydrolysis in mm) at 40^{0}C

No. of isolates	pH = 7	pH =8	pH = 9	pH =10
I-2	22	16	15	12
I-4	18	20	10	-
I-5	20	12	21	15
I-8	30	23	19	15
I-12	24	22	20	19
I-14	21	19	15	-
I-16	21	19	16	11
I-17	23	24	20	17
I-19	28	16	16	18
I-21	27	16	15	-
I-24	22	20	17	12
I-26	28	19	16	-
I-28	27	17	17	-
I-30	21	17	12	10
I-31	22	20	17	15
I-32	28	17	15	-
I-33	25	16	20	-

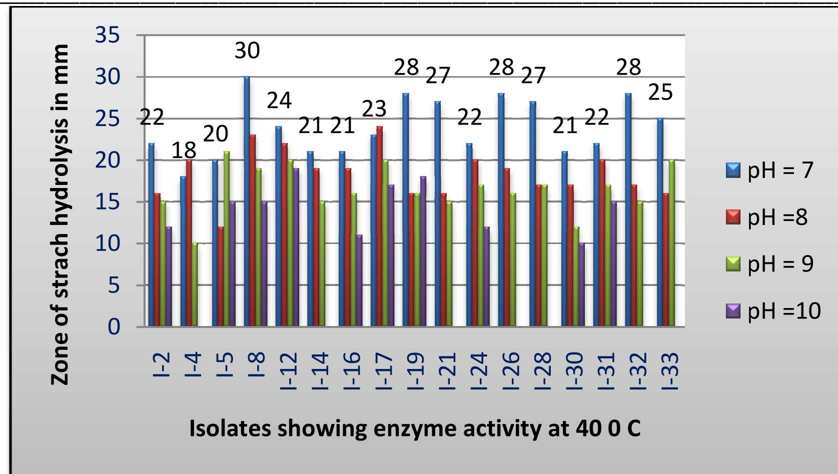
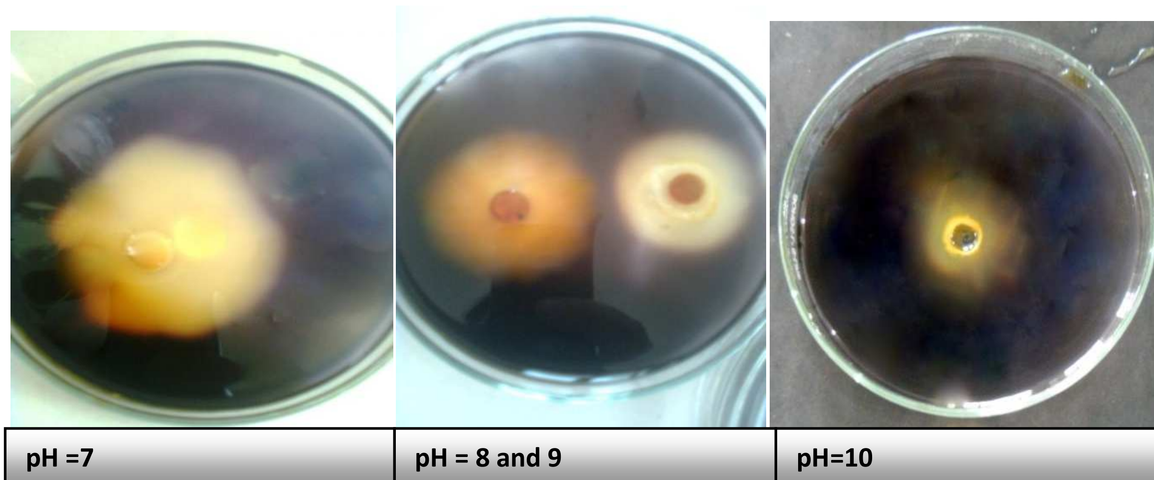


Fig 4 :Effect of different pH on the production of amylase by *Bacillus sp.* (temp=40^{0}C, Incubation time period =24 hrs).

Rate of amylase fermentation

Slide.5.7 shows the maximum zone of starch hydrolysis by isolate I-8 on the starch agar at temperature 40^0 C and 7 pH. Thus the incubation temperature 40°C was selected for maximum production of enzyme. In the present study 17 isolates of *Bacillus* species produced enzymes over the large range of temperature however the maximum zone of starch hydrolysis observed at temperature 40^{0}C (30mm zone of starch hydrolysis) of isolate I-8. The amylase fermentation by *Bacillus subtilis* was carried out in shake flask. The culture was incubated at different temperature 25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C for 14 h at pH 7. The production of enzyme was reached maximum (36 IU/mL) at 14 h after inoculation. Thus optimum time of enzyme synthesis was found to be 14 h after inoculation. Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased. In present study, the rate of enzyme was increased with the increase in the fermentation period and reached maximum 14 h after inoculation. It might be due to the organism entered in the incubation period resulted in the decreased production of amylase. It may be due to the accumulation of other by products in the fermentation medium. The size of inoculum has marked effect on the growth of the bacteria and biosynthesis of α-amylase as reported by Allan *et al.* (1996).



pH =7	pH = 8 and 9	pH=10

Slide 5.7: Zone of starch hydrolysis for Isolate I-8(*Bacillus subtilis*) at 40^0 C, pH=7, 8, 9, and 10:

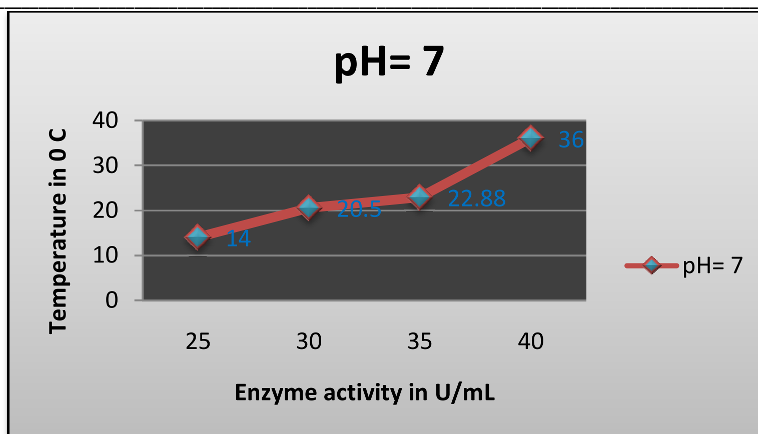


Fig 5: Effect of Temperature on the production of amylase by *Bacillus subtilis* (pH=7 Incubation time period =14 h

Discussion

In present study On the basis of level of productivity of the amylase, an isolate producing a maximum of amylase activity was screened from soil and used for detailed investigation. Screening of the amylase producing organisms was carried out on the starch agar. Productions of these enzymes were studies at various pH (7, 8, 9, and 10) and temperature (25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C). A clear zone of hydrolysis on starch (after addition of iodine) gave an indication amylase producing bacteria the efficiencies were studied on the basis of the zone of hydrolysis. The isolate showing the maximum zone of hydrolysis was selected for identification. The strain was Gram +ve, rod shaped aerobic, Catalase +ve, MR +ve, CU +ve and VP +ve. The bacterial isolate was identified as *Bacillus subtilis.* Cordeiro et al., 2003 showed *Bacillus* species produce a large variety of extra cellular enzymes, such as amylases, which have significant industrial importance. The use of starch nutrient agar and iodine for detecting amylase (hydrolytic enzyme) producing microorganisms have been reported by Forgarty and Kelly, (1979) and also by Iverson and Millis, (1974) that starch hydrolysis can be detected on plates as a clear zone surrounding a colony. In present study maximum production of amylase by *bacillus subtilis* was obtained (36 IU/mL) with pH 7 at 40°C for 14 hrs. Studies reported by Sekar Sudharhsan, *et al*., Amylase producing *Bacillus* sp. was isolated from spoiled food waste, which yielded 30 U ml-1 of amylase in medium containing 4% starch and 2% yeast extract at 37°C, pH 7.0 after 20 h of incubation. Maximum amylase activity was at pH 7.0 and 37°C. The enzyme retained 70% activity at pH 9.0. Nadia Riaz, *et al.*, 2003 reported the maximum production of enzyme was optimized at the pH 7.5, while the incubation temperature investigated was 40ºC, the volume of basal medium at 25 mL and inoculum size at 4% were also optimized. The hydrolytic action of α-amylase is greatly affected by pH. In present study, the different pH (7-10) of starch solution was tested for the activity of amylase. The maximum activity of the enzyme was obtained at slightly alkaline pH 7. At acidic pH the results were extremely low. It might be due to the enzyme was inactive in the acidic medium. Anyangwa *et al*., 1993; Castro *et al.,* 1993 reported the different pH (4-8) of starch solution was tested for the activity of α-amylase. The maximum activity of the enzyme was obtained at slightly alkaline pH 7.5. The production of the enzyme was obtained maximum at 48 hours after incubation (535 IU/mL/min). Isolation and screening of *Bacillus* species were carried out from soil samples of saline belt of Purna River for multiple extracellular enzymatic activities. Submerged fermentation for production of alpha amylase was done earlier by (Riaz, N. *et al*., 2003). Production medium [g/l] 6.0 g Bacteriological peptone; 0.5 g MgSO4 .7H O; 0.5 g KCl; 1.0 g Starch previously used by (R. Vidyalakshmi, *et al.*, 2009) for the production of alpha amylase. Partial purification of the crude amylase was done by ammonium sulphate precipitation & dialysis similar techniques have been used earlier by (Yandri. *et al*., 2010).

Conclusion

Extra cellular amylase was extracted from *Bacillus* sp. isolated from soil samples. The various factors affecting amylase production was assayed which include pH; temperature. Results showed that pH 7.0 and 40ºC temp was found to be optimum values for both the growth of the isolate and maximum enzyme production. Further experiments will be done to purify the secreted amylase and stability studies will be performed to enhance the application of enzyme to commercial level. The above study clearly revealed new and interesting perspectives showing that bacterial strains isolated from soil of various

places from Paonta sahib represents a source of amylase that can be exploited potentially for various industries for enzyme production, mainly in detergent industry.

References

1. Crueger, W. and Crueger, A. Industrial Microbiology, Sinauer Associates, Sunderland, MA, 189–218,1989.
2. Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh D, Mohan, R. Advances in microbial amylases. Biotechnol. Appl. Biochem. 2000; 31: 135-152.
3. Aiyer, P.V., 2005. Amylases and their applications. Afr. J. Biotechnol., 4(13): 1525-1529.
4. R. Vidyalakshmi, R. Paranthaman and J. Indhumathi, Amylase Production on Submerged Fermentation by *Bacillus* spp.Indian Institute of Crop Processing Technology, Thanjavur- 613 005, Tamil Nadu, IndiaWorld Journal of Chemistry 4 (1): 89-91, 2009 ISSN 1817-3128 © IDOSI Publications, 2009.
5. Burhan, A., Nisa, U., Gokhan, C., Omer, C., Ashabil, A. and Osman, G., 2003, "Enzymatic properties of a novel thermophilic, alkaline and chelator resistant amylase from an alkalophilic *Bacillus* sp. Isolate ANT- 6". Proc. Biochem.,38, pp.1397-1403
6. Haq, I., N. Riaz, H. Ashraf, M.A. Qadeer and J. Iqbal, 2002. Effect of inorganic salts on the biosynthesis of aslpha amylase by *Bacillus subtilis. Indus. J. Plant Sci.,* 1: 115–21
7. Ganesh C, Seung Joo H, Koo YM, Paik SR, Chang C (2004).Thermostable Alkaline Protease from a Novel Marine Haloalkalophilic *Bacillus Clausii* Isolate, World J. Microbiol. Biotechnol. 20:351-357.
8. Stamford T.L, Stamford N.P, Coelho L.C, Araujo J.M (2001) Production and *Nocardiopsis* sp. endophyte of yam bean. Bioresour Technol 76:137-141.
9. Forgarty W and Kelly, C (1979) Starch degrading enzymes of microbial origin. J of progress in Industrial Microbiology 15:87-150.
10. Iverson W. and Millis, N A (1974) method for the detection of starch hydrolysis by bacteria. Journal of applied bacteriology 37:443-446.
11. Akpan I, Bankole M.O, Adesemowo A.M, Latunde-Dada G.O (1999) Production of amylase by *aspergillus niger* in chaep solid medium using rice bran and agricultural materials. Journal of Tropical Science 39:77-79
12. Suman. S[*1] and K. Ramesh[1] (2010) Production of a thermostable extracellular amylase from thermophilic *Bacillus* species, J. Pharm. Sci. & Res. 2(2):149-154
13. Allan S, B.F. Henrik and B. Torbenvedel (1996) A method of designing alpha mutants, with predetermined properties alpha amylase varients and detergents containing the variants. Process Birchem 31: 110–210.
14. Holt JG, Krieg NR, Sneath PH, Stanley JT and Williams ST (1994). Bergey's Manual of Determinative Bacteriology. Williams and Wilkins Baltimore: 529-550
15. Cordeiro CAM, Martinas MLL, Lucaino A, (2003) Production and Properties of alpha amylase from thermophylic *Bacillus* species. Braz. J. Microbiol 33:1-3
16. Castro G.R, M.A Ferrero, B.S. Mendez and F. Sineriz (1993) Screening and selection of bacteria with high amylolytic activity. Acta Biotechnol 13: 197–201
17. Anyangwa E.M, C. Mapsev, P. Musanage and M. Elemva (1993). The effect and removal of starch in the sugar refining industry. J. Int. Sugar 95: 210–3.
18. Sekar Sudharhsan[1], Sivaprakasam Senthilkumar[2] and Karunasena Ranjith[3] Physical and nutritional factors affecting the production of amylase from species of *bacillus* isolated from spoiled food waste.
19. Nadia Riaz, Ikram ul-Haq and M.A. Qadeer (2003) Characterization of α-Amylase by *Bacillus subtilis.* Int J Of Agriculture & Biology 1560–8530/05–3–249–252.
20. Riaz AN, Haq I and Qadeer M A (2003) Int. J. Agri. Biol 5 (3): 23- 28.
21. R. Vidyalakshmi, R. Paranthaman and J. Indhumathi (2009) Amylase Production on Submerged Fermentation by *Bacillus* sp. World Journal of Chemistry 4 (1): 89-91.
22. Yandri. S Tand Hadi S (2010) European. J. Scientific Res 39 (1): 64-74.

Source of Support: Nil
Conflict of Interest: None