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RESEARCH ARTICLE

ISOLATION, SCREENING AND CHARACTERIZATION OF AMYLASE AND CELLULASE PRODUCING BACTERIAL ISOLATES FROM DIFFERENT SOIL SAMPLES.

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Key words:-
Amylase, Cellulase, Bacillus species, Starch hydrolysis, Cellulase hydrolysis, Enzymes activity.

Abstract

Amylases and cellulases are amongst most widely used enzymes in industries such as food, fermentation, starch processing, textile and paper. In the present investigation, bacteria were isolated from municipal dump sites of different regions of Telangana, screened for the production of amylase and cellulase their optimum growth conditions were determined. A total of 15 bacterial colonies were isolated from collected soil samples.Three bacterial isolates, displayed zones of clearance in starch hydrolysis test. And one bacterial isolate displayed zone of clearance in cellulase hydrolysis test. The isolates displaying maximum amylase and cellulase activity on quantitations was selected. Characteristic feature of the strains indicates that it belongs to the genus Bacillus and will be later used for further characterization. Maximum yield of amylase and cellulase was obtained after 48hours of incubation. The first three optimum pH for enzymes activity was found to be at pH 7.0 and the next optimum pH for enzyme activity was found to be at pH 5.0 and also the four optimum temperatures for the activity was found to be at 40 C

Introduction:-

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells (Smith et al., 1997, Grisham, 1999). Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell. All enzymes which have been purified are protein in nature, and may or may not possess a non-protein prosthetic group. The bio-catalytic uses for enzymes have grown immensely in recent years since they are ecologically correct, have a high specificity, present chemo-regioenantio selectivity, and have a wide diversity of reactions. The main industries that apply microbial enzymes are the food, textile, leather, pharmaceutical, cosmetics, fine chemicals, energy, biomaterials, paper, cellulose and detergent industries (Adams, E. C.,1957, Bernfeld P., 1951). Enzymes occur in every living cell, hence in all microorganisms. Each single strain of organism produces a large number of enzymes, hydrolyzing, oxidizing or reducing, and metabolic in nature individual enzymes produced vary markedly between species and even between (Bode H. E., 1954). Hence, it is customary to select strains for the commercial production of specific enzymes which have the capacity for producing highest amounts of the particular enzymes desired. Moreover, the conditions, to obtain and optimize the production of enzymes in terms of nutrients, pH, temperature, and aeration are easily controlled in bioreactors. Microoraganisms can also be manipulated genetically to improve the desirable characteristics of a bio-catalyzer. Additionally, the substrates used in the cultural medium are sustainable and industrial residuals can be used to

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produce value-added products. All these characteristics together have encouraged the ever-growing search for bio-catalytic processes. Immobilization processes allow the reuse of these enzymes and increase stability. In the present study, we report the isolation, screening and characterization of amylase and cellulase producing bacteria from the soil samples collected from different municipal dump sites. Production conditions were optimized (temperature, pH, etc.) to achieve high enzymes production and better enzymes activity.

Materials and Methods:-

Collection of soil samples:-

Soil samples were collected from different municipal dump sites of surroundings regions, Warangal district of Telangana, with the help of sterile spatula. Collected samples were transferred to sterile plastic bags in aseptic conditions.

Medium for isolation:-

Medium used for isolation of bacterial species was Starch agar and CMC (Carboxy methyl cellulose) agar. The starch agar medium containing per liter (5g starch ; 5g peptone ; 5g Nacl ; 3g yeast extract ; 20g agar ; pH 7) and CMC agar medium containing per liter (2g CMC ; 1g NaNo3 ; 1g K2HPo4 ; 1g Kcl ; 0.5g Mgso4 ; 0.01g Feso4 ; 5g Yeast extract ; 20g agar ; pH 7.2).

Isolation of bacterial species:-

Serial dilution technique was used for the isolation of bacteria. 1g of soil was added to the glass tube containing 9ml sterilized distilled water, samples were serially diluted up to 10-1 to 10-7 and spread on starch and cmc agar plates followed by incubation at 37 C for 24 hours.

Identification of isolated organisms:-

The isolated bacteria identified by using morphological and biochemical tests as per procedures described in Bergey's Manual of systematic Bacteriology (Buchanan R. E. and Gibbons N. E.,1974). For the identification of enzyme producing bacteria, the isolates were observed under the microscope and the bacterial colonies were recorded in respect to their color, size, shape, margins, and pigmentation.

Microscopic observation:-

Attempts have been made to identify the bacterial strains on the basis of Gram staining, endospore staining, capsule staining and motility test.

Biochemical characterization:-

Biochemical characterization of bacterial isolates were done by performing various biochemical tests like Indole test, MR-VP test, Citrate utilization test, Nitrate reduction test, Catalase test, Urease test, Deaminase test, H2S production test, Sugar fermentation test (Glucose / Mannitol / Lactose / maltose / Sucrose), Gelatin hydrolysis test, Starch hydrolysis test, Cellulase hydrolysis test.

Screening For Enzyme Activity:-

Many distinctive enzyme activities can be demonstrated by observing the product resulting from the action of enzymes on specific prepared media. For determination of enzyme producing activity (amylase, cellulase) of the isolates screening tests are conducted. In order to observe enzymatic activities of microorganisms, pure cultures of microorganisms were inoculated in selective media.

Screening for Amylase Activity (Starch Iodine Test):-

Bacterial cultures were screened for amylolytic activity by starch hydrolysis test on starch agar medium (composite of soluble starch-5g; peptone-5g; Nacl-5g; yeast extract-3g; agar-20g; distilled water-1000ml; pH 7) (Shaw, J. F., Lin, F.P., Chen, S.C. and Chen., H.C.,1995). The pure isolated colonies were streaked on starch agar plates with starch as the only carbon source. After incubation at 37 C for 24 hours, the individual plates were flooded with Gram's iodine (Gram's iodine-250mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is the basis of the detection and screening of an amylolytic strain. The amylase producers displaying maximum diameter of zone of clearance, were further investigated (Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B 2003). The pure cultures were sub cultured at regular intervals and starch-nutrient agar slants were maintained at 4 C.

Screening for Cellulase Activity:-
Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1% congo red and allowed to stand for 15 min at room temperature. One molar Nacl (0.58g of Nacl in 100 ml of water) was thoroughly used for counterstaining the plates, clear zones were appeared around growing bacterial colonies indicating cellulase hydrolysis (Andro T, Chambost JP., 1984). The bacterial colonies having the largest clear zone were selected for identification and cellulase (Vipul Verma., 2012, Muhammad Irfan., 2012) production in submerged system.

Amylase production medium:-
A loop full of bacterial culture was transferred from starch-nutrient agar slants to starch-nutrient broth at pH 7 for activation and incubated in a shaker at 40 C at 120 rpm for 24 hours. Fermentation medium contained soluble starch (5g/L), peptone (5g/L), Nacl (5g/L), yeast extract (3g/L), (NH4)2SO4 (2g/L), KH2PO4 (1g/L), K2HPO4 (2g/L), Mgcl2 (0.01g/L) at pH 7. The fermentation medium was inoculated with the activated culture (20% v/v) and incubated in shaker at 37 C for 24 hours. At the end of the fermentation period, the culture medium was centrifuged at 10,000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Amylase Activity Assay:-
Amylase activity was assayed as described (Bernfeld P 1955) with some modifications. Briefly 1.5 ml of 1% starch in 2ml, (0.1)M phosphate buffer (Ph 6.5) And 0.5ml of diluted enzyme (1 to 10) were incubated for 15 min at room temperature 37 C. The reaction was arrested by adding 1ml of DNS reagent and kept in a boiling water bath, for 10 min and diluted with 8ml of distilled water.
The absorbance was measured at 540nm against blank prepared as above without incubation. One unit of alpha-amylase activity was defined as the amount of enzyme that liberates 1umole of reducing sugar (Maltose equivalents) per minute under the assay conditions (Miller, G.L. 1959). Unless otherwise stated all experiments were carried out in triplicate.

Determination of optimum PH:-
1% Starch was used as a substrate. Substrate solution was prepared in sodium phosphate buffer at pH 6, 6.4, 6.8, 7, 7.4, 7.8 and 8.0 in different test tubes. 0.5ml each of diluted crude enzyme solution was added into buffer tubes. Then the mixture was incubated at room temperature for 15 min reactions were terminated by adding 1ml DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12ml with distilled water and the activity of enzymes was determined by taking the absorbance at 540nm.

Determination of optimum Temperature:-
1.5 ml of substrate was taken into six different test tubes and 2ml of phosphate buffer pH 7 was added in each test tubes. Tubes were marked with different temperature (at 30, 35, 40, 45, 50, 55 C). 0.5 ml of diluted enzyme solution was added in each tube. Then tubes were incubated at specific temperature for 10 minutes. Reactions were terminated by adding 1ml DNS reagent and the mixture incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12 ml with distilled water and the activity of enzymes were determined by taking the absorbance at 540nm.

Cellulase production medium:-
A basal media (1% CMC, 0.1% K2HPO4, 0.1% K2HPO4, 0.04% Mgso4, 0.005% Nacl, and 0.000125% Feso4, pH 7.0) was used for production of cellulase . For seed culture, a fresh isolated colony was inoculated in 5 ml basal media and incubated at 37 C and 120 rpm for 24 hours. The seed culture (5%) was then inoculated in 50 ml production media in a 250 ml conical flask and incubated at conditions as indicated. The cell free supernatant obtained by centrifugation at 8,000 rpm for 15 min at 4 C was used for determining the cellulase activity or further investigations.

Cellulase Activity Assay:-
The carboxy methyl cellulose (CMC ase) activity was assayed using a method described by miller (Miller GL 1959), with some modifications (Bhat MK 2000, Wood TM, Bhat KM 1988). A 0.5 ml of diluted enzyme (1 to10) was added to 0.5 ml of 1% CMC prepared in 50MM sodium citrate buffer (pH 4.8) in a test tube and incubated at 60 C for 30 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) and subsequently placing the reaction tubes in a water bath at 100 C for 15 minutes. One ml of Rochelle salt solution (40gm Rochelle salt in

100ml distilled water) was then added to stabilize the color. The absorbance/optical density (OD) was recorded at 575nm wave length against a blank of 50MM sodium citrate buffer. One unit of CMCase activity was defined as the amount of enzyme that liberated 1umol of reducing sugar (glucose) in 1min at 37 C and pH 7.0 (Zhang YH, Hong J, Ye X 2009)

Determination of optimum pH:-

1% carboxy methyl cellulose, was used as a substrate. Substrate solution was prepared in sodium citrate buffer at pH 6, 6.4, 6.8, 7, 7.4, 7.8, and 8 in different tubes. 0.5 ml each of diluted crude enzyme solution was added into buffer tubes.Then the mixture was incubated at room temperature for 15 min, reactions were terminated by adding 1 ml DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12 ml with distilled water and the activity of enzyme was determined by taking the absorbance at 540nm.

Determination of optimum Temperature:-

1.5 ml of substrate was taken into six different test tubes and 2 ml of sodium citrate buffer pH 7 was added in each test tubes. Tubes were marked with different temperature (at 30, 35, 40, 45, 50, 55 C). 0.5 ml of diluted enzyme solution was added in each tube. Then tubes were incubated at specific temperature for 10 minutes. Reactions were terminated by adding 1 ml DNS reagent and the mixture incubated in boiling water for10 min. After cooling at room temperature, final volume was made to 12 ml with distilled water and the activity of enzyme were determined by taking the absorbance at 540nm.

Results:-

Sample collection and isolation of Gram positive and negative bacteria:-

Soil samples were collected from 5 different areas of Warangal, Telangana. 15 different organisms were isolated from these samples. Morphological and biochemical characteristics revealed 4 isolates belonging to genus Bacillus. First one isolate was Gram positive, rod shaped spore former and starch hydrolyzers. Another three isolates were Gram negative, rod shaped, spore former and cellulase hydrolyzer. (Table 1)

Table 1:- Morphological characteristics of bacterial isolates

S.NO	Name of the test	Strain code NB-1	Strain code NB-2	Strain code NB-3	Strain code NB-4
1	**Culture Medium**	Starch medium	Starch medium	Starch medium	CMC medium
2	**Growth**	Excellent	Good	Good	Excellent
3	**Form**	circular	Round	Circular	Round
4	**Elevation**	Flat	Convex	Convex	Raised
5	**Margin**	Entire	Entire	Entire	Entire
6	**Opacity**	Translucent	Translucent	Translucent	Translucent
7	**Color**	Cream	Cream	Cream	Cream
8	**Surface**	Smooth	Smooth	Smooth	Smooth
9	**Gram staining**	Positive	Negative	Negative	Negative
10	**Shape of vegetative cell**	Rod	Rod	Rod	Rod
11	**Motility**	Motile	Motile	Motile	Motile
12	**Starch hydrolysis**	Positive	Positive	Positive	Negative
13	**Cellulase hydrolysis**	Negative	Negative	Negative	Positive

Fig 1: Isolate NB-1 showing Positive starch hydrolysis test

Fig 2: Isolate NB-2 showing Positive starch hydrolysis test

Fig 3: Isolate NB-3 showing Positive starch hydrolysis test

Fig 4: Isolate NB-4 showing Positive cellulase hydrolysis test

Identification of Bacillus species:-

All 4 isolates were found to be Indole , Nitrate , Citrate , Gelatinase , and Deaminase positive capable of producing acid from glucose, maltose, sucrose, and mannitol. All the isolates showed motility using SIM medium.The results are presented in Table 2

Table 2:- Biochemical characteristics of bacterial isolates

S.NO	*Name of the Tests*	*Strain code NB-1*	*Strain code NB-2*	*Strain code NB-3*	*Strain code NB-4*
1	Indole Test	+	+	+	+
2	Methyl Red Test	-	-	-	-
3	Voges-Proskauer Test	-	-	-	-
4	Citrate Utilization Test	+	-	-	-
5	Nitrate Reduction Test	+	+	+	+
6	Catalase Test	+	+	+	+
7	Gelatinase Test	+	+	+	+
8	Urease Test	-	-	-	-
9	Deaminase Test	+	+	+	+
10	Production of Acid Glucose	+	+	+	+
11	Maltose	+	+	+	+
12	Sucrose	+	+	+	+
13	Lactose	-	-	-	-
14	Mannitol	+	+	-	-
15	H2S Test	-	-	-	-

Fig 1: Isolate NB-1 showing Positive nitrate test **Fig 2 : Isolate NB-2 showing Positive nitrate test** **Fig 3 : Isolate NB-3 showing Positive nitrate test** **Fig 4 : Isolate NB-4 showing Positive nitrate test**

Fig 1 : Isolate NB-1 showing Positive indole test **Fig 2 : Isolate NB-2 showing Positive indole test** **Fig 3 : Isolate NB-3 showing Positive indole test** **Fig 4 : Isolate NB-4 showing Positive indole test**

Fig 1 : Isolate NB-1 showing **Fig 2 : Isolate NB-2 showing** **Fig 3 : Isolate NB-3 showing** **Fig 4 : Isolate NB-4 showing**

Positive catalase test Positive catalase test Positive catalase test Positive catalase test

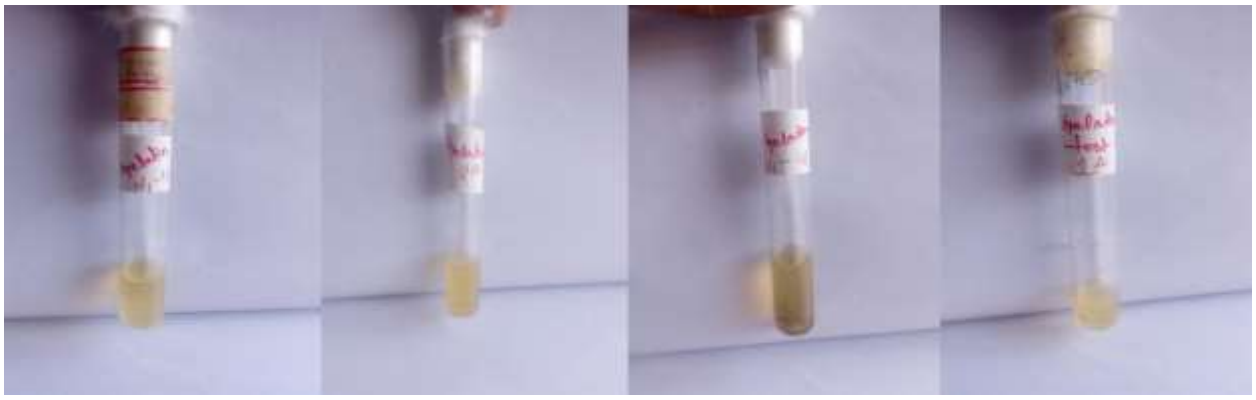


Fig 1 : Isolate NB-1 showing Fig 2 : Isolate NB-2 showing Fig 3 : Isolate NB-3 showing Fig 4 : Isolate NB-4 showing

Positive gelatinase test Positive gelatinase test Positive gelatinase test Positive gelatinase test

Fig 1 : Isolate NB-1 showing Fig 2 : Isolate NB-2 showing Fig 3 : Isolate NB-3 showing Fig 4 : Isolate NB-4 showing Positive deaminase test Positive deaminase test Positive deaminase test Positive deaminase test

Fig 1 : Isolate NB-1 showing Positive producing acid maltose

Fig 2 : Isolate NB-2 showing positive producing acid maltose

Fig 3 : Isolate NB-3 showing Positive producing acid maltose

Fig 4 : Isolate NB-4 showing Positive producing acid maltose

Fig 1 : Isolate NB-1 showing Positive producing acid sucrose

Fig 2 : Isolate NB-2 showing Positive producing acid sucrose

Fig 3 : Isolate NB-3 showing Positive producing acid sucrose

Fig 4 : Isolate NB-4 showing Positive producing acid sucrose

Fig 1 : Isolate NB-1 showing Positive producing acid glucose

Fig 2 : Isolate NB-2 showing Positive producing acid glucose

Fig 3 : Isolate NB-3 showing Positive producing acid glucose

Fig 4 : Isolate NB-4 showing Positive producing acid glucose

Fig 1: Isolate NB-1 showing Positive producing acid mannitol

Fig 2 : Isolate NB-2 showing Positive producing acid mannitol

Fig 3 : Isolate NB-1 showing Positive producing acid mannitol

Starch Hydrolysis Test:-

Starch hydrolysis was performed with the first three isolates, zone of hydrolysis was measured and enzyme index was calculated for each colony (Table 3). Two isolates showing high values include NB-1 (1.12), NB-2 (1.62) as shown in table 3.

Cellulase hydrolysis Test:-

Cellulase hydrolysis was performed with another one isolate, zone of hydrolysis was measured and enzyme index was calculated for one colony NB-4 (1.2) as shown in table 3.

Table 3:- Amylase and Cellulase production by bacterial isolates

Isolate number	Diameter of colony (mm)	Diameter of halo (mm)	Enzyme index
NB-1	10	12	1.2
NB-2	18	24	1.62
NB-3	16	9	1.05
NB-4	8	9	1.12

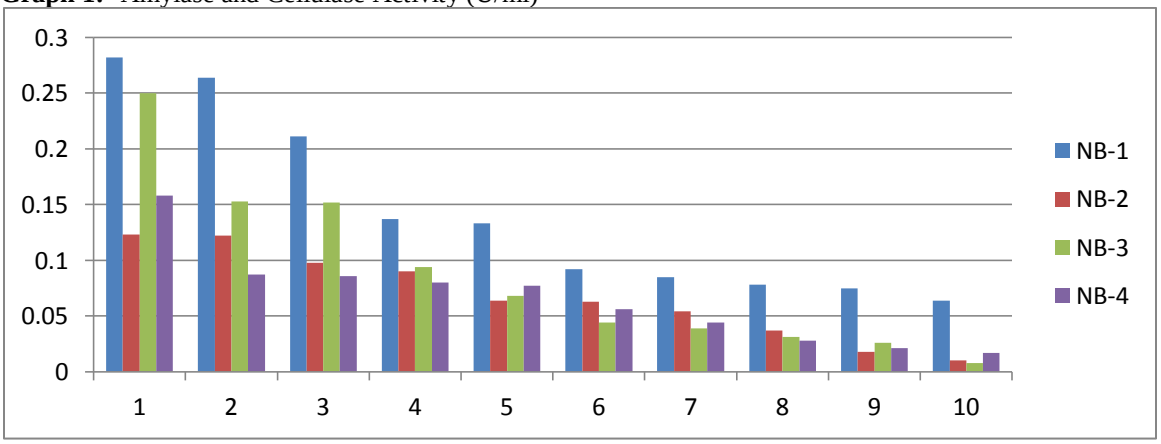
Enzyme Assay:-

Four isolates with high enzyme index were analyzed for enzyme activity. Among the four, NB-1, NB-2, NB-3, NB-4 showed good enzyme activity as shown in Table 4 and graphs 1.

Table 4:- Enzyme activity of bacterial isolates

S.NO.	Enzyme dilutions	Strain code NB-1	Strain code NB-2	Strain code NB-3	Strain code NB-4
1	**1**	0.282	0.123	0.250	0.158
2	**2**	0.264	0.122	0.153	0.087
3	**3**	0.211	0.098	0.152	0.086
4	**4**	0.137	0.090	0.094	0.080
5	**5**	0.133	0.064	0.068	0.077
6	**6**	0.092	0.063	0.044	0.056
7	**7**	0.085	0.054	0.039	0.044
8	**8**	0.078	0.037	0.031	0.028
9	**9**	0.075	0.018	0.026	0.021
10	**10**	0.064	0.010	0.008	0.017

Graph 1:- Amylase and Cellulase Activity (U/ml)



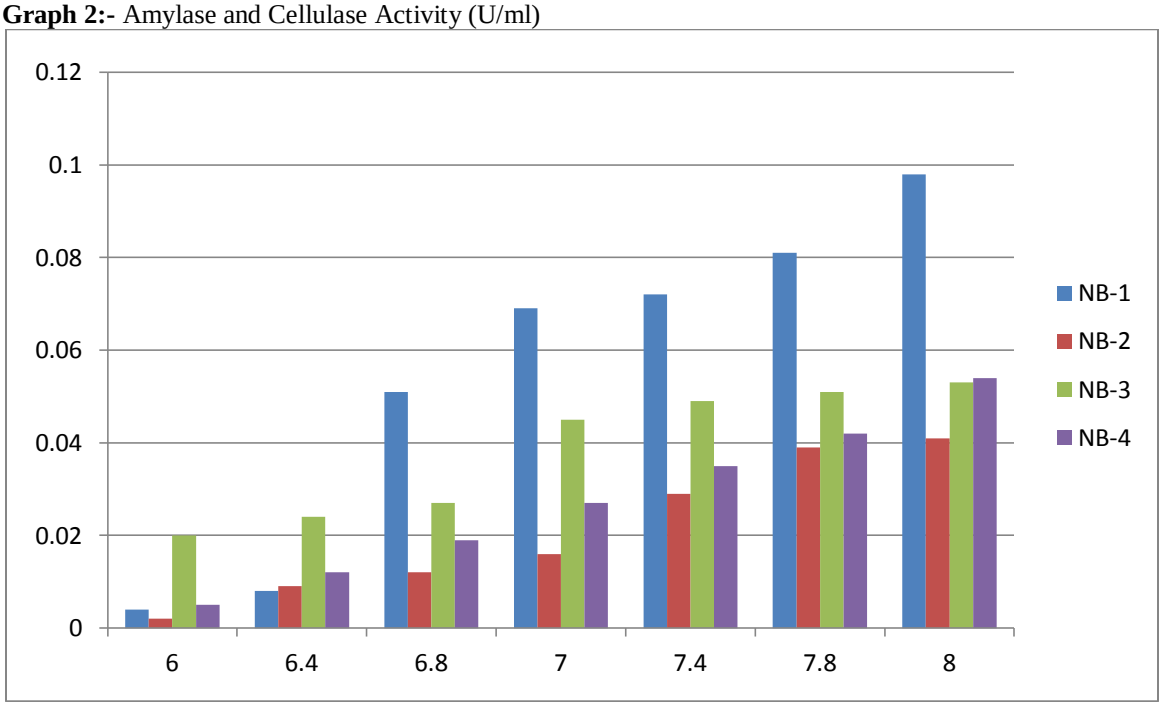
Enzyme dilutions

Enzymes optimum PH:-
Four isolates with high enzyme index were analyzed for enzyme activity . Among the four NB-1, NB-2, NB-3, NB-4 showed good enzyme activity as shown in Table 5 and graph 2

Table 5:- Effect of varying PH on Amylase and Cellulase Activity

pH	Amylase Activity(u/ml) NB-1	Amylase Activity(u/ml) NB-2	Amylase Activity(u/ml) NB-3	Cellulase Activity(u/ml) NB-4
6.0	0.004	0.002	0.020	0.005
6.4	0.008	0.009	0.024	0.012
6.8	0.051	0.012	0.027	0.019
7.0	0.069	0.016	0.045	0.027
7.4	0.072	0.029	0.049	0.035
7.8	0.081	0.039	0.051	0.042
8.0	0.098	0.041	0.053	0.054

Graph 2:- Amylase and Cellulase Activity (U/ml)



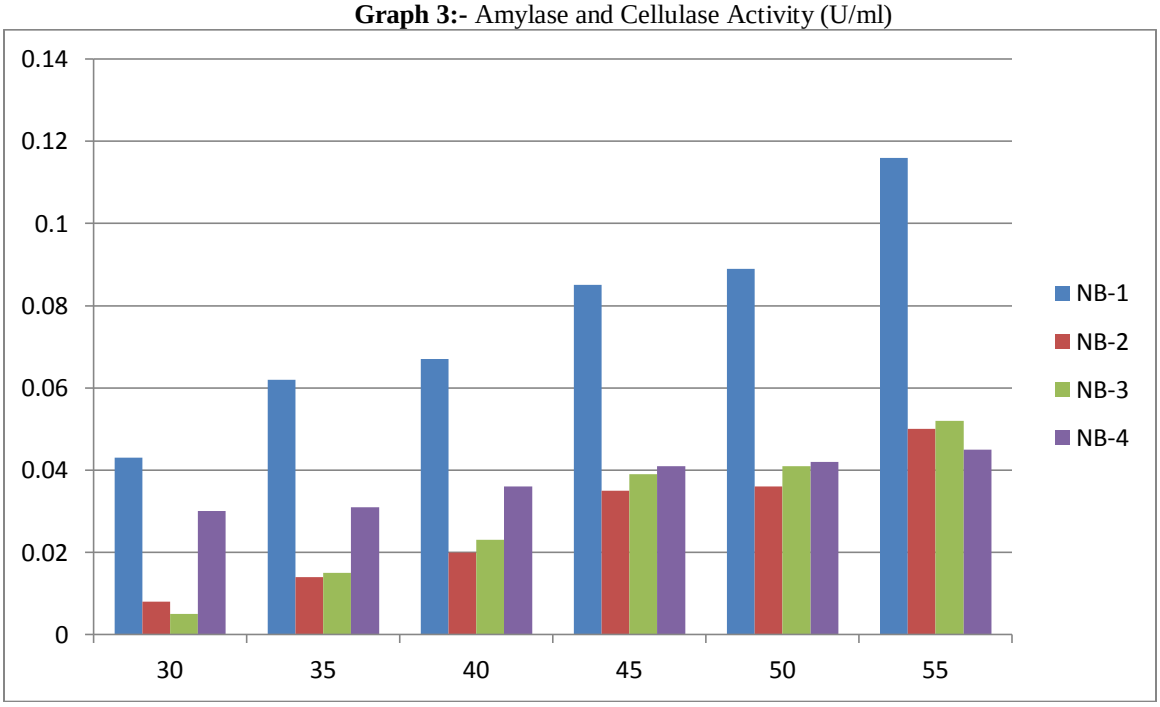
pH

Enzyme optimum Temperature:-
Four isolates with high enzyme index were analyzed for enzyme activity . Among the four (NB-1, NB-2, NB-3, NB-4) showed good enzyme activity as shown in Table 6 and Graph 3.

Table 6:- Effect of varying temperature on Amylase and Cellulase Activity

Temperature	Amylase Activity(u/ml) NB-1	Amylase Activity(u/ml) NB-2	Amylase Activity(u/ml) NB-3	Cellulase Activity(u/ml) NB-4
30	0.043	0.008	0.005	0.030
35	0.062	0.014	0.015	0.031
40	0.067	0.020	0.023	0.036
45	0.085	0.035	0.039	0.041
50	0.089	0.036	0.041	0.042
55	0.116	0.050	0.052	0.045

Graph 3:- Amylase and Cellulase Activity (U/ml)



Temperature

Discussion:-

The occurrence of amylolytic and cellulolytic organisms from the soil agrees with the earlier report that the soil is an enriched repository of amylase and cellulase producers (Madhav 2011). Various species of bacteria, fungi and actinomycetes are most prominent enzyme producers. Among various amylase and cellulase producers Bacillus species are most prominent (Pandey et al.,2000). Present investigation shows the potential of soil organisms to produce highly commercially important enzyme capable of starch in Table 3 . Enzyme assay for all two high enzyme producing strains showed that NB-1 gives highest enzyme activity 0.282IU/ml/min. NB-3 is the second highest enzyme producer (0.250 IU/ml/min). Enzyme activity for NB-4, NB-2 is 0.158IU/ml/min, 0.1223IU/ml/min respectively as shown in Table 4. The study revealed that soil harbors amylolytic and cellulolytic bacilli and that the amylase and cellulase production by these bacteria may in future, be used in different industrial sectors as well as other research areas. For further studies, the Bacillus specie NB-1 with highest enzyme activity has been selected for optimization, characterization and purification of enzyme.

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