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Thermophilic and Alkalophilic Amylase from Strain Bacillus Marinus MG 12

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ABSTRACT

A new bacterial strain MG12 with high amylase activity was characterized as Bacillus marinus by morphological characteristics, 16S rRNA sequence homology and molecular phylogeny. Maximum production of amylase by strain MG12 was found in pH 7.0 and 30°C with an incubation time of 48h. SDS-PAGE and characterization of enzyme showed 57.24 KDa with highest activity at pH 8 and 55°C. The Km and Vmax value for the enzyme activity was found using Line weaver-Burk plot.

Keywords : Amylase, Bacillus, SDS-PAGE, TLC, Kinetics, Specific activity

I. INTRODUCTION

Amylases are group of enzymes have the ability to degrade glycosidic linkage between linked glucose molecules with variety of applications in different industries were isolated (Gupta et al 2003, Mc Tigue et al.,1995). Thermophilic amylases will be useful in different industries (Leveque et al., 2000). Thermo stability is the desired characteristics. High temperature tolerant amylases are available from the mesophilic *Bacillus licheniformis* (Morgan and Priest, 1981), *Bacillus sp.ASMIA* -2 (Teodoro and Martin, 2000). Improvement in strategies to isolate amylases with higher amylase activity leads to the discovery of new α - amylases with industrial applications. The advantage of thermophilic α - amylases are cooling cost can be reduced, solubility of substrates can be increased, lowering of viscosity allows enhanced mixing and pumping and decreases the risk of contamination. The wide range of amylases are acidophilic or neutral. But detergent industry is in a greater need of alkalophilic amylases.

Present study we have isolated and characterized a novel thermophilic alkaline α- amylase producing bacteria designated as MG 12

II. Material and Methods

Microorganism:

In order to isolate amylolytic bacteria, soil samples were collected from the Pichavaram mangrove forest located near Chidambaram, Tamil Nadu. To isolate extracellular amylase producing bacteria, 1g of soil suspension was dissolved with 100ml saline water. Deca times dilutions of soil suspension were plated onto amylase agar, which contain all the components described in Asha eta al 2012 with Starch soluble 0.2%, agar 1.5% at neutral pH was incubated for 72h at 30˚C and observed for zone of inhibition surrounding the bacterial colony due to amylase activity. Using Lugols iodine amylase producing colonies were selected. The physical, morphological and biochemical characterization of strain was determined based on Senath et al., 1986. 16srDNA

sequencing was done to construct phylogenetic tree to find the strain.

Optimization of media for the enzyme production:

Initially the organism was incubated in 1% starch, 0.05% $MgSO_4.7H_2O$, 0.02% $0MgCl_2.6H_2O$, 0.1% K_2HPO_4 at pH 7.0 and 30^{0}C for 48h. The influence of different pH values (5-10) and temperature (25- 40^{0}C) for α - amylase production was investigated. Optimization parameters were set based on Asha etal 2012.

Enzyme Assay

The assay was done by Bernfeld method at 50^{0}C.

Protein Estimation:

Lowry Method with BSA as standard used for protein concentration calculation (Lowry et al., 1971)

Fermentation media, culture conditions and Purification of enzyme:

Enzyme was produced using compositions 1.5% starch, 0.75% Yeast extract, 0.5% $0MgSO_4$. $7H_2O$, 0.2 %$MgCl_2.6H_2O$, 1.0 % K_2HPO_4 at pH 7.0 and 30^{0}C for 30h. Centrifugation has been done to collect supernatant at 15000g for 20min and was used for further studies.

Salt precipitation:

80% (w/v) Ammonium sulphate precipitation was done and kept at 4^{0}C for 12hand the procedure was as same as Asha et al 2012.

Size exclusion chromatography and HPLC

Size exclusion chromatography and HPLC was done same as in Asha eta al. 2012.

Determination of molecular mass

The purified enzyme molecular weight was measured by performing the procedure of Laemmli (Laemmli, 1970).

Biochemical characterization of purified enzyme:

The influence of pH and temperature on the stability of α -amylase was assayed by using 1.5% soluble starch in a range of different pH and temperature using the methods of Asha et al 2012.

Influence of effectors (metal ions) on the stability of amylase

The influence of 10 mM concentration of different metal ions Mn^{2+} ($MnCl_2$), Fe^{3+} ($FeCl_3$), Mg^{2+} ($MgCl_2$), Zn^{2+} ($ZnSO_4$), Cu^{2+} ($CuCl_2$) and Hg^{2+} ($HgCl_2$) was determined at 55˚C in pH 8.5 for 60 min (Miller et al. 1959).

Kinetics parameters determination

The enzyme (5.58 U/ml) was treated with different concentration of soluble starch (0.5-3.0%) in pH 8.5 at 55˚C.

III. Results

Characterization of bacterial strain:

The morphological and physiological properties of the isolated bacteria is in (Table 1). Presence of Bacillus sp. was based on Bergys *Manual of Systematic Bacteriology*. By 16srDNA sequencing and phylogenetic tree analysis the strain was affiliated to *Bacillus marinus*. (Fig.1 & 2)

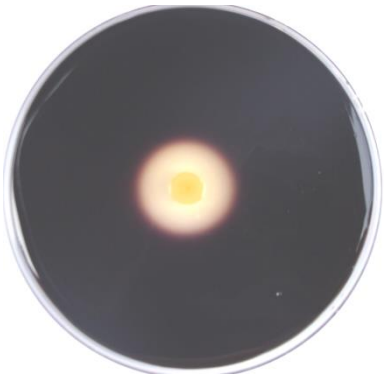

Fig: 1.Amylolytic activity of strain MG12 on starch agar plate.

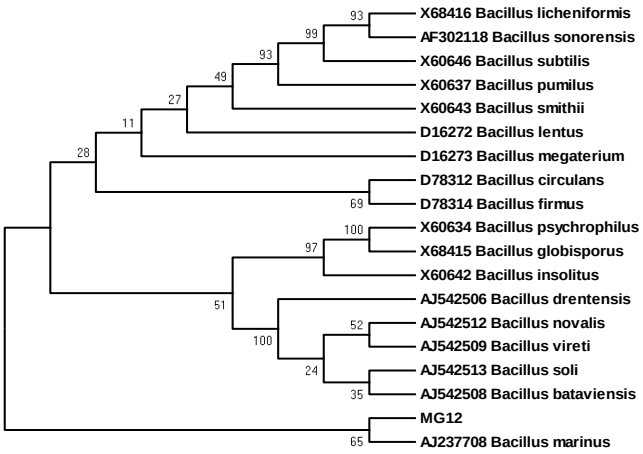

Fig: 2. Phylogenetic tree analysis of strain MG12

Table 1 Identification of microorganism

Physical	
Shape	Gram positive, rods
Biochemical characteristics	
Catalase	Positive
Citrate	Positive
TSI	Positive
Urea test	Positive
Methyl Red	Negative
Oxidase	Negative
Indole test	Negative
Nitrate reduction	Reduced to nitrite
VP test	Negative
Carbohydrate	Acid positive
Presence of NaCl	up to 7%
Growth at temperature	25^0C-65^0C

Hydrolysis of polymer substrates

Starch	Positive
Carboxymethylcellulose	Positive
Lipid	Negative
DNA	Negative
Proteins	Negative

Fermentation and production of enzyme:

The result on amylase production and growth of strain MG 12 with 1% starch as substrate is shown in (Fig 1). The enzyme production and growth were maximum (11.66U/ml) at 30h and was gradually decline up to 48h (Fig 3). Readily available carbon sources are depleted at stationary phase and so effective induction of enzyme was not observed in this phase. (Huang et al;2003, Wanderely et al;2004). α -amylase production by *Bacillus flavothermus*, *Bacillus amyloliquefaciens* and *Bacillus* sp ANT 6 the biomass and enzyme production was increased double fold and highest activity was observed after 24h (Kelly 1997.Hillier 1997,Burhan 2003).But incase of *Bacillus subtilis* AX20 highest enzyme activity was observed in 34-46h (Najafi et al,2005).

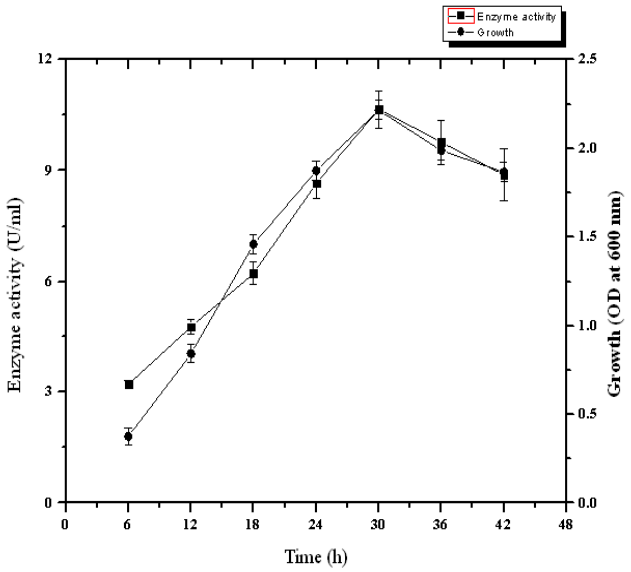

Fig. 3 : Time course induction of amylase production by MG12

The microbe has minimum characteristics in acidic and alkalophilic conditions. The highest production of enzyme and bacterial growth was observed at 7.0. α -amylase have an optimum pH between 6.0 and 9.0 for most of the *Bacillus* strain (Burhan 2003; Castro 1993; Van-Leeuwen and Patel, 1999). In this study the strain MG12 showed higher activities α -amylase (13.89U/ml) at pH 7.0.

Temperature between 25^0C and 35^0C will give production of enzymes (Fig 4). Even though the tested bacteria has the capability to grow in all the temperature but maximum enzyme activity was attained at 30^0C. Different temperature range were reported (Bajpai and Bajpai, 1989; Burhan et al, 2003; Castro et al, 1992; Lin et al, 1998).In this study the highest growth and production of α -amylase (13.11U/ml) was at a temperature of 30^0C.

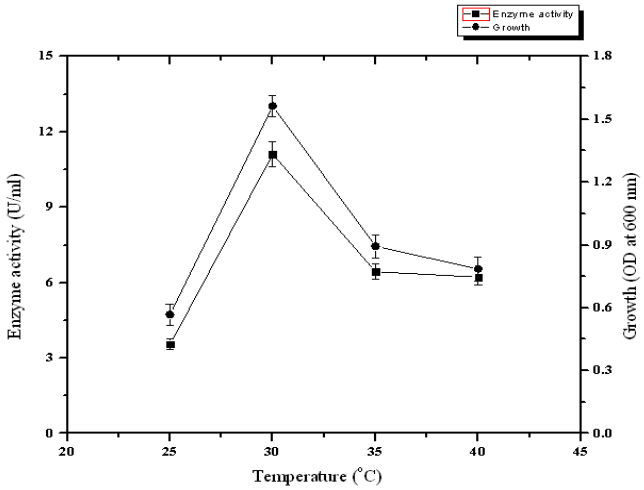

Fig: 4 : Influence of Temperature on enzyme production by MG12

Starch was replaced by readily available carbon.Presence of starch induced the production of enzyme (Fig 5). All other carbon source decreased the production of enzyme. Glucose will suppress the production of enzyme (Lin et al.1998). In 1.5% concentration of starch the strain MG12 showed highest growth (11.66U/ml).

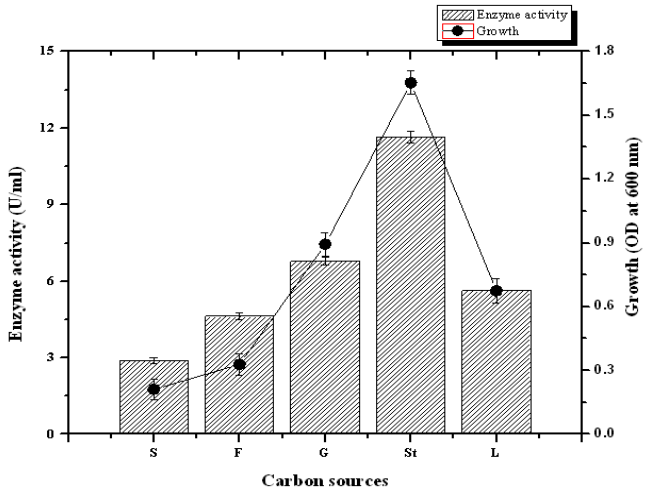

Fig: 5. Influence of carbon sources on enzyme production by MG12

The presence of nitrogen sources significantly influenced the production of enzyme. Presence of 0.75% yeast extract showed maximum growth and production (13.89U/ml). Nitrogen sources like yeast extract and peptone usually have stimulatory effect on enzyme production (Forgatty and Kelly.1980; Hamiltton et al.1999; Hewitt and Solomons.1996). In the Presence of 0.2% yeast extract induced the production of Enzyme in *Bacillus* sp BKL 20(Kubrak et al.,2010)

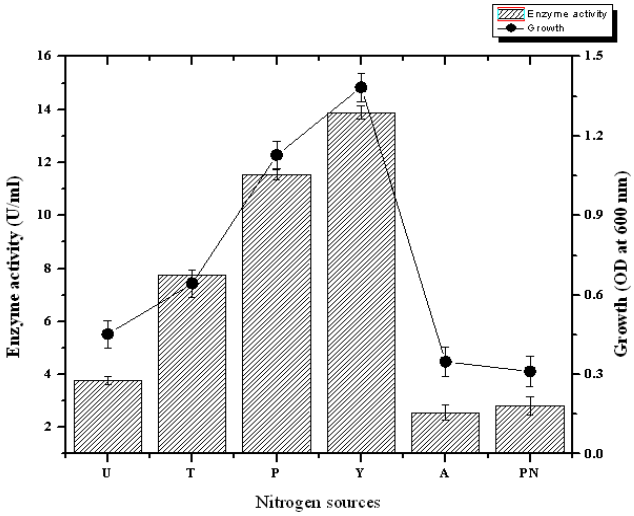

Fig: 6. Influence of Nitrogen sources on amylase production by MG12

Purification and Characterization of amylase enzyme:

MG12 was grown in fermentation media (1000ml) with all the optimized conditions for 48h.The supernatant was collected and partial purification by ammonium sulphate (80%) yielded 26.01%. Purification results are summarized in (Table2).

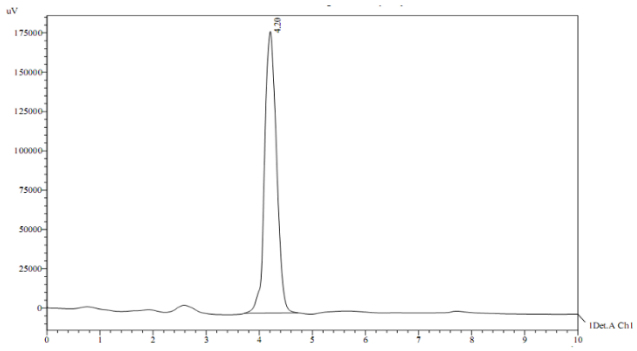

Fig.7.High performance liquid chromatogram of amylase from strain MG12

Table 2: Results of purification from strain MG12

Purification Step	Volume (ml)	Enzyme activity (U/ml)	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Crude Extract	960	12.66	12153.6	7872	1.54	100	1
$(NH_4)_2SO_4$ Fractionation	425	7.44	3162	1020	3.06	26.01	1.98
Sephadex G-50	125	6.33	791.25	112.5	7.033	6.51	4.58
HPLC	40	5.58	223.2	18	12.04	1.83	7.93

The relative molecular mass of the enzyme was found to be 57.26kDa. The molecular weight of amylase enzyme produced by the strain *Bacillus* sp YX was found to be 56kDa (Liu et al., .2008). The raw starch degrading enzyme from *Bacillus amyloliquefaciens* was found to be 58kDa (Gangadharan et al., 2009).

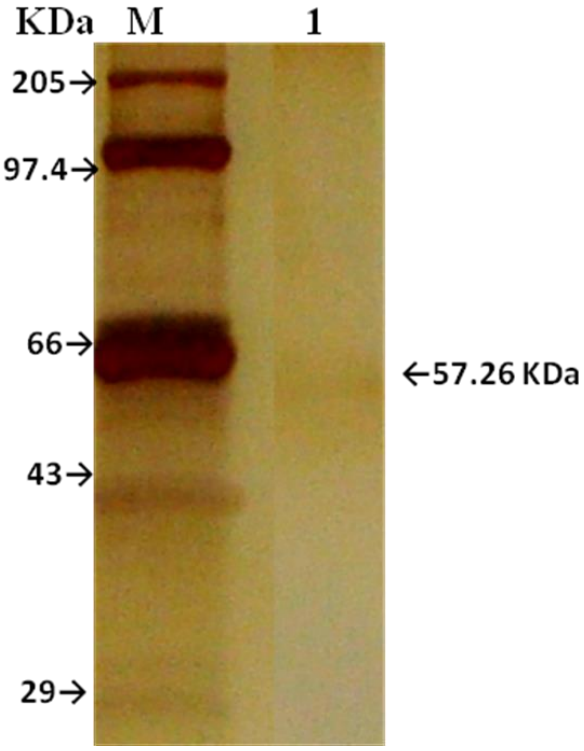

Fig: 8. SDS PAGE of amylase from MG123

Activity of pH tolerance of amylase enzyme

Optimum activity was at a pH of 8.0. The activity of enzyme gives an inference to its alkalophilic nature. Alkalophilic amylase enzymes have proved several industrial applications.

Activity of temperature tolerance of amylase enzyme

Optimum activity at 55^{0}C at a pH of 8.0. The activity of enzyme gives an inference to its thermophilic nature. Alkalophilic and thermophilic amylase enzymes have proved several industrial applications. (Fig 9)

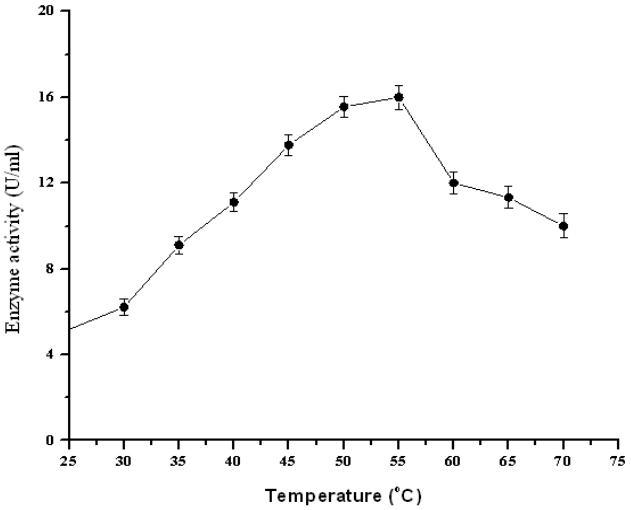

Fig: 10. Influence of temperature on amylase activity

Activity of cations and anions in amylase activity

The influence of cations and anions on activity of enzyme was measured in 10mM concentration, and presence of Fe^{3+} ion gave more activity. This result indicates that the amylase enzyme produced by MG12 is a metalloenzyme (Fig 10).

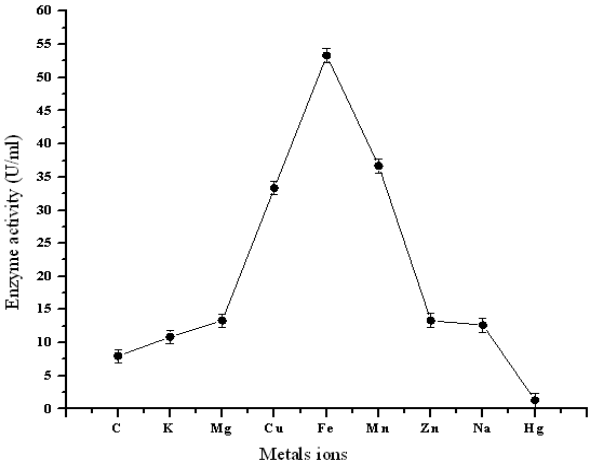

Fig: 11. Effect of metal ions in amylase activity

Calculation of kinetics constant

(Fig. 12).

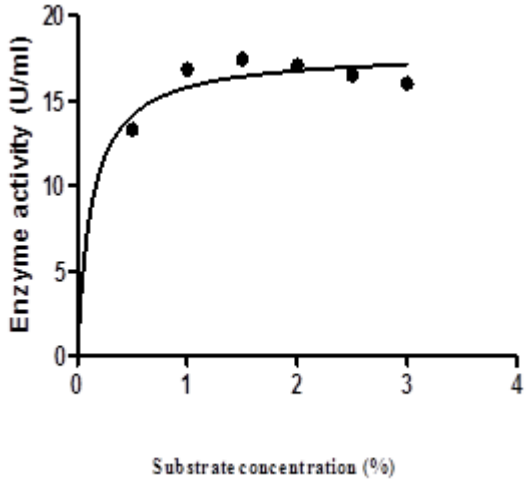

Fig. 12 Michaelis-Menten plot

Km: 0.1327, Vmax: 17.89 and Slope: 134.815 (Vmax/Km)

Km and Vmax were calculated from Lineweaver-Burk plot using initial reaction rates for different soluble starch concentrations (0.5% - 3.0%) at 55˚C (Fig. 13).

Best fit values: **Slope: 0.0038**

$X_1 = 1.0$, $X_2 = 0.5$, $Y_1 = 0.0546$, $Y_2 = 0.0527$, 1/Slope (X/Y): **263.15**

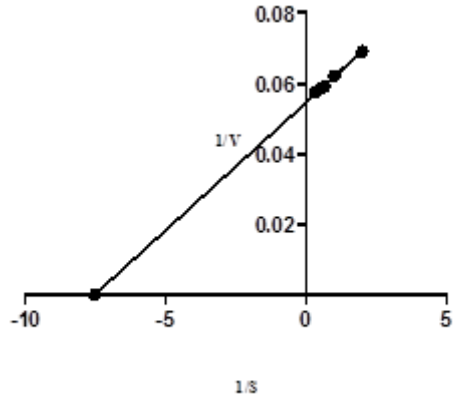

Fig. 13 Lineweaver-Burk plot

IV. REFERENCES

[1]. Asoodeh,A.Chamani, J., Lagzian, M. 2010. A novel thermostable, acidophilic α-amylase from a new thermophilic "Bacillus sp. Ferdowsicous" isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization.International Journal of Biological Macromolecules.46.289-297.

[2]. Al-Quadan.F., Akel.H. And NAtshi.R.2009. Characteristics of a novel highly thermostable and extremely thermophilic alkalitolerant amylase from a hyperthermophilic Bacillus strain HUTBS71.Online Journal of Biological Sciences.9.67-74

[3]. Arikan.B. Highly thermostable,thermophilic,alkaline,SDS and chelator resistant amylase from a thermophilic Bacillus sp.isolate.A3-15.2008.Bioresourcr technology.99.3071-3076.

[4]. Asger,M. Asad,M.J.Rahman,S.U and Legge,R.L.2007.A thermostable alpha amylase from a moderately thermophilic Bacillus subtilis strain for starch processing.Journal of food Engineering .79.950-955.

[5]. Liu,B. Wang,Y and Zang,X.2006.Characterization of recombinant maltogenic amylase from deep sea thermophilic Bacillus sp.WPD 616.Enzyme and Microbial Technology.39.805-810.

[6]. Hamilton.L.M., Kelly,C..T and Fogarty, W.M.1998. Carbohydrate Research, 314,251-257.

[7]. Gupta.R., Gigras.P.,Mohapatra.H., Goswami..V.K and Chauhan.B(2003).Microbial alpha amylase:a biotechnological perspective .Process Biochemistry.38.1599-1616.

[8]. Laemmli.U.K. 1970.Cleavage of structural proteins during the assembly of the head of bacteriophageT4.Nature 227.680-685.

[9]. Lowry.O.H., Rosebrough.N.J., Farr.A.L.,Randall,R.J.1951.Protein measurement with the Folin-Phenol reagent.Jornal of Biological Chemistry.193.265-275.

[10]. Miller.G.L. 1959.Use of dinitrosalicylic acid reagent for determination of reducing sugar.Analytical Chemistry.31.426-428.

[11]. Morgan.F. and Priest.F.1981.Characterization of a thermostable alpha amylase from Bacillus licheniformis NCIB 6346.Journal of Applied Bacteriology.50.107-114.

[12]. Seneath.P.H.A. Mair.N.S., Sharpe.E.M. Holt.J.G.1986.Bergeys Manual of Systematic Bacteriology,Ninth ed., Williams and Wilkins ,Baltimore,1986.

[13]. Lineweaver.H.. and Burk.D.1934.Journal of American Chemical Society.56.658-666.

[14]. Ivanova.V., Dobreva .E.,Emanuilova.E.1993.Purification and Characterization of of thermostable alpha amylase from Bacillus licheniformis. Journal of Biotechnology.28.277-289.

[15]. Mc Tigue..M.A.,Kelly.T.C.,Doyel.M.E.,Forgatty.M. W.1995.The alkaline amylase of the alkaophilic Bacillus sp IMD 370.Enzyme and Microbial Technology.17.570-573.

[16]. Mamo.G and Gesseses.A.1999. Purification and characterization of two starch digesting thermostable alpha amylase from thermophilic Bacillus.Enzyme and Microbial Technology.25.433-438.

[17]. Huang.H.,Ridgwan.D.,Tingyue.GU.,MurrayMo o.Y.(2003).A segregated model forheterologus amylase production by Bacillus subtilis.Enzyme and Microbial Technology.37.407-413.

[18]. Bernfeld, P., 1955. Amylases Alpha and Beta. In: Methods in Enzymology, Colowick, S.B. and N.O. Kaplan (Eds.). Academic Press, New York, pp: 149-153.

[19]. Wanderely.K.J.,Torres.F.A.G.,Moraes.L.M.P.,U lhoa.C.J.2004.Biochemical characterization of alpha amylase from the Yeast Cryptococcus flavus.FEMS Microbiolagy Letters.231.165-169.

[20]. Kelly.C.T.,Bolton.D.J and Fogarty.W.M.1997.Biphasic production of alpha amylase of Bacillus flavothermus in batch fermentation. Biotechnology Letters.19.675-677.

[21]. Hillier, P., D. Wase and A. Emery, 1996. Production of α-amylase by Bacillus amyloliquefaciens in batch and continuous culture using a defined synthetic medium. Biotechnol. Lett., 18: 795-800.

[22]. Burhan.A.,Nisa.U.,Gokhan.C.,Ashabil.A.,Osma ir.G.2003.Enzymatic properties of novel thermostable thermophilic alkaline and chelator resistant amylase from an alkaphilic Bacillus sp Isolate.ANT-6.Process Biochemistry.38.1397-1403.

[23]. Najafi.M.F and Kembhavi.2005.One step purification and characterization of an extracellular alpha amylase from marine Vibrio sp. Enzyme and Mcrobial Technology.36. 535-539.

[24]. Castro.G.A.,Ferrero.M.A.,Mendez.B.S and Sineriz.F.1993.Screening and selection of bacteria with high amylolytic activity. Acta Biotechnol.13.197-201.

[25]. Van-Leeuwen.J.H.,Jin.B and Patel.B.999.Mycelial morphology and fungal protein production from starch processing waste water in submerged cultures of Aspergillus oryzae. Process Biochemistry.34.335-340.

[26]. Pratima Bajpai, Pramod K. Bajpai.1989. High-temperature alkaline -amylase from Bacillus iicheniformis TCRDC-B13

[27]. Castro.PML.,Hayter.P.M,Ison.A.P and Bull.A.T.1992.Application of statistical design to the optimization of culture medium for recombinant interferon gamma production by Chinese hamster ovary cells.Applied Microbiology and Biotechnology.38.84-90.

[28]. Lin.L.L.,Chyau.C.C and Hsu.W.H.998.Production and properties of a raw starch degrading amylase from the thermophilic and alkaliphilic Bacillus sp.TS-23