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Effect of Different Physico-Chemical Parameters on Production ofAmylase by *Bacillus Sp*ecies.

[1]Neeraj Kumar Lohani*,[2]P.D .Trivedi, [3]Divakar Joshi,
[1]Institute of Biomedical Education & Research, Mangalayatan University, Aligarh- Uttar Pradesh, India,*
[2]Central Drug Research Institute Lucknow-Uttar Pradesh,India ,[3]M. B. Govt. P. G. College Haldwani, Kumaun University Nainital- Uttrakhand, India.

Abstract: *The present study is concerned with the production of amylase by Bacillus species strain. In this study 12 bacterial strains were isolated and screened for their α-amylase activity. These strains were maintained on nutrient agar medium. Fermentation for the production of amylase was carried out in the enzyme production medium (EPM). All the 12 strains were tested for amylase production. On the basis of maximum amylase activity strain no.1 was selected for further studies. Different starch concentrations, 0.75,1.00,1.25%, pH labels 6.5,7.0,7.5,8.0, aeration (RPM), 100,120,140, temperatures 25^{0}C,28^{0}C,37^{0}C, and 40^{0}C and inoculums level 0.5%,1.0%, 1.5% and 2.0% were studied.*
Key Words*: Amylase, Bacillus species.*

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I. Introduction

Amylase is an extracellular enzyme degrades α, 1-4 glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin (Calik&Ozdamar., 2001).Which have been derived from plants, animals and several microorganisms like bacteria (Vidyalakshmi P *et al*., 2009 Busch JE *et al.*,1997)and fungi.But the *Bacillus* species such as *B. subtilis, B.licheniformis*and *B. sterothermophilis*can be used for thebetter production of amylase in shake flask (Mamo&Gessesse., 1999).Amylase has great significant in biotechnology and are commercially important in various starch processing industries (Guzman *et al*., 1995). The enzymes from microbial sources generally meet industrialdemands.

The production of amylase is dependent on the strains, composition of media, methods of cultivation, cell growth, nutrient requirement, inoculum size, pH, temperature, time of incubation. The effect of starch concentration on the relative activity of amylase from *B. species* was detected and starch concentration for optimum production was 0.5-2.0%. The effect of temperature on the relative activity of amylase from *B. species* was detected and temperature was optimized between 30ºC- 40ºC, for maximum activity (Kim *et al.,* 1995). The production of the amylase is effected by aeration rate (RPM) the aeration rate at the level of 100-140 was studied. The size of inoculum has marked effect on the growth of the bacteria and biosynthesis of amylase as reported by (Allan *et al*., 1996).the inoculum at the level of 1-2% was studied for theproduction of amylase enzyme. The inoculum size was increased; the production of enzyme was decreased. The production and stability of the enzyme is very sensitive to pH observed that amylase obtained from *B. species* was stable at pH 6.5 –8.0 (Ivanova *et al*., 1993).

II. Materials And Methods

2.1 Chemicals Used

Table 1. Chemicals Used

S.No.	Name Of The Chemicals & Reagents	Manufacturer
A.	Ammonium Sulphate	Sisco Research Laboratories Pvt. Ltd. (Mumbai).
B.	Starch	Qualigens
C.	Sucrose	Sisco Research Laboratories Pvt. Ltd. (Mumbai) 400099 India.
D.	3,5-Di Nitrosalicylic Acid(DNS)	Sisco Research Laboratories Pvt. Ltd. (Mumbai) 400099 India.
E.	Sodium Hydroxide	Thomas Baker
F.	Zinc Sulphate	LobaChemiPvt.Ltd. Mumbai-400 005

G.	Urea	Qualigens Fine Chemicals.
H.	Magnesium Sulphate	Qualigens Fine Chemicals
I.	Peptone	Qualigens Fine Chemicals
J.	D-Glucose	Qualigens Fine Chemicals
K.	Ferrous Sulphate	Qualigens Fine Chemicals
L.	Potassium Sodium Tartarte	Qualigens Fine Chemicals
M.	Di-Potassium Hydrogen Ortho Phosphate	Qualigens Fine Chemicals
N.	Calcium Chloride	Sds Lab Rasayan
O.	Cobalt Chloride	LobaChemiPvt.Ltd. Mumbai

2.2 Apparatus Uses

Table 2. Apparatus Used

S.No.	Name Of Equipment & Instrument	Manufacturer
A.	All Glass Ware (Sterilize Glass Pipette, Sterilize Test Tubes, Glass Rod, Conical Flask, Beaker)	Borosil.
B.	ALL PLASTIC WARE(Measuring Cylinder, Appendoff Tubes)	Tarson.
C.	Autoclave	Mac, Macro Scientific Work Lab.Equipments,10/Ua(Delhi)
D.	Digital Oven	Tanco An Iso 9001:2000 Co.
E.	Incubator Shaker	Lark, Innovative Fine Technolwledge 1037, 4o Th Street, Chennai. 600080 India.
F.	Laminar Air Flow	Mac, Macro Scientific Work, (10a/Ua) J.N. Delhi 7
G.	Microwave Oven	Kenstar
H.	UV Double Beam Spectrophotometer Model: Uv-2601	Beijing Rayleigh Analytical Instrument Corp. Bldg. A5, No.9 Jiuxianiao East Road, ChaoyangDistt. Beijing 10016, P.R. China
I.	Spin Win	Tarson Product Pvt. Ltd. Jasmin Tower,(Kolkata,700017)
J.	Vortex Shaker	Mac, Macro Scientific Work, (10a/Ua) J.N. Delhi 7

DNS reagent, sterilize medium, cotton, tissue paper, 70% ethanol are also used during experiment.

2.3 Method

2.3.1 Isolation of Bacillus Culture

This study was carried out at biotech department M.B. Govt. P.G. College Haldwani Nainital.Bacillus cultures were isolated from soil by serial dilution method.1gm soil sample from the college campus was suspended in 10ml sterilized distilled water the soil suspension was diluted to 10-7 and .1ml diluted suspension was speared on the nutrient agar plate containing starch and this volume spread by a triangular glass spatula evenly and incubated at 37°c for 24 hours. After 24 hour 12 bacillus colonies were picked up in nutrient agar slant which were morphologically identified by gram stain method and all were found gram positive bacilli.

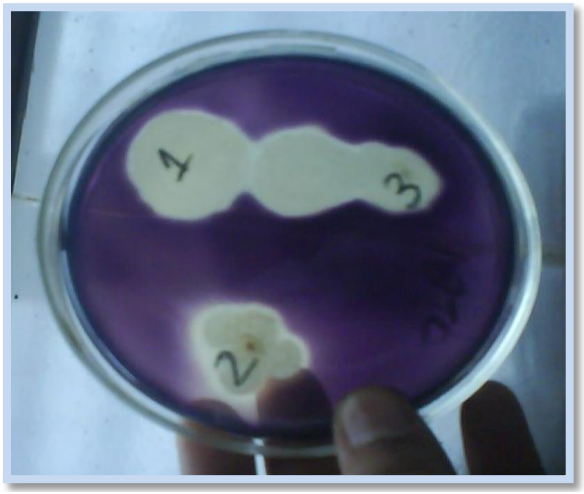

Figure 1.Bacillus Culture isolated from soil sample.

Table 3.Cmposition of Fermentation Medium

Bacteriological peptone	**6.0gm/l.**
Mgso4.7H2O	**0.5gm/l.**
KCL	**0.5gm/l.**
Starch	**1.0gm/l.**

The above ingredients were mixed and distributed in 100ml Eyrlen Meyer flask sterilized by autoclaving at 121°c for 15min and inoculated with the isolated strains. After 24, 48 and 72 hour incubation enzyme activity was carried out.

2.3.2 Strain No-1

Strain no.1 was found to produce maximum amylase at 48 hour. Therefore this strain was further studied for optimization of different growth and enzyme production parameters which are substrate concentration, organic and inorganic nitrogen concentration, different pH, inoculums size, aeration and temperature.

2.3.3 Enzyme Assay (Amylase Assay)

Enzyme activity was determined by dinitro salicylic acid method (DNS) using starch as a substrate. The reaction mixture contained 10mg starch 100µg or 0.1ml crude protein (broth) in 50mili molar sodium phosphate buffer pH 7.0. The reaction mixture was incubated at 37ºC for 30 minutes and the reaction was terminated by adding 3ml DNS solution. After stopping the reaction the tubes were placed in a boiling water bath for 5 minutes and then cooled and absorbance was taken at 540nm in a UV double beam spectrophotometer .The amount of glucose produced was calculated by referring to the standard curve using glucose as the reducing sugar in 1minute under assay conditions.

III. Results And Discussion

Table 4.Effect of Different Concentrations of Starch on Amylase Production

Concentration of starch (%)	Absorbance at 540nm	Glucose concentration (µg/ml/min)
0.75	**0.792**	**10**
1.0	**0.910**	**12.83**
1.25	**0.104**	**9.12**

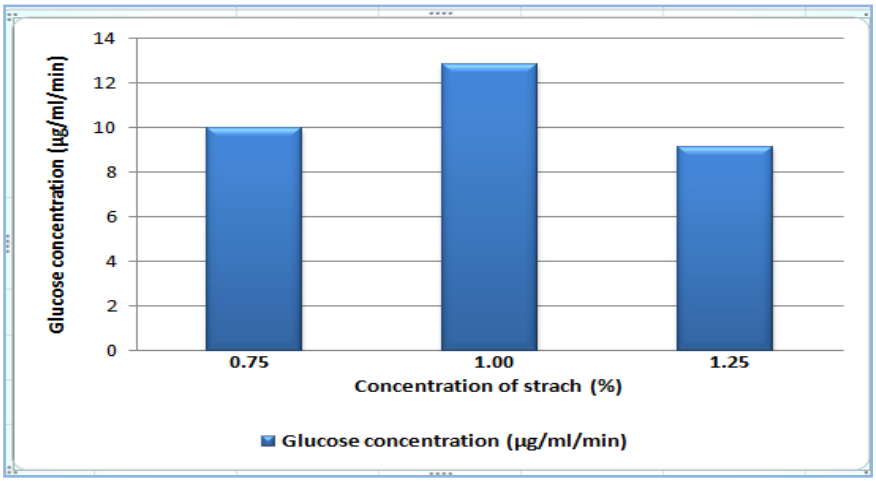

Figure 2. Effect of Different Concentrations of Starch on Amylase Production

Table 5.Effect of Temperature onAmylase Production.

Temperature ˚C	Absorbance at540nm (24hrs)	Glucose conc.(µg/ml/min)	Absorbance at540nm (48hrs)	Glucose conc. (µg/ml/min)
25	0.786	10	0.769	8.83
28	0.100	1.16	0.109	1.33
32	0.104	1.23	0.102	1.20
37	0.773	9.88	0.765	9.33
40	0.737	9.43	0.729	8.92

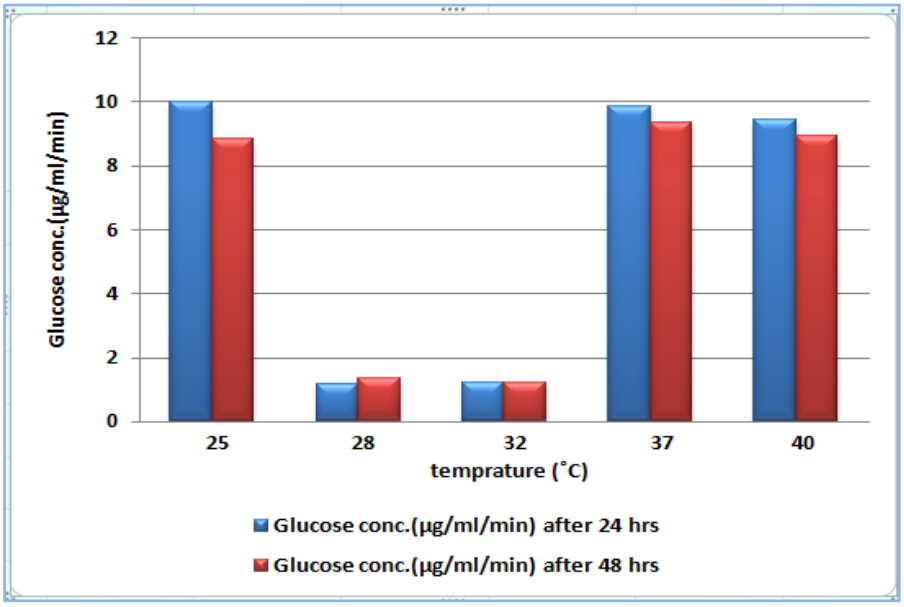

Figure 3. Effect of Temperature on Amylase Production

Table 6.Effect of Aeration onAmylase Production.

Rpm	Absorbance at 540 nm (24 hrs)	Conc. of glucose (µg/ml/min)	Absorbance at 540 nm (48hrs)	Conc. of glucose (µg/ml/min)
100	0.797	10.13	0.995	12.83
120	0.972	12.46	1.025	13.16
140	0.925	11.86	0.944	12.00

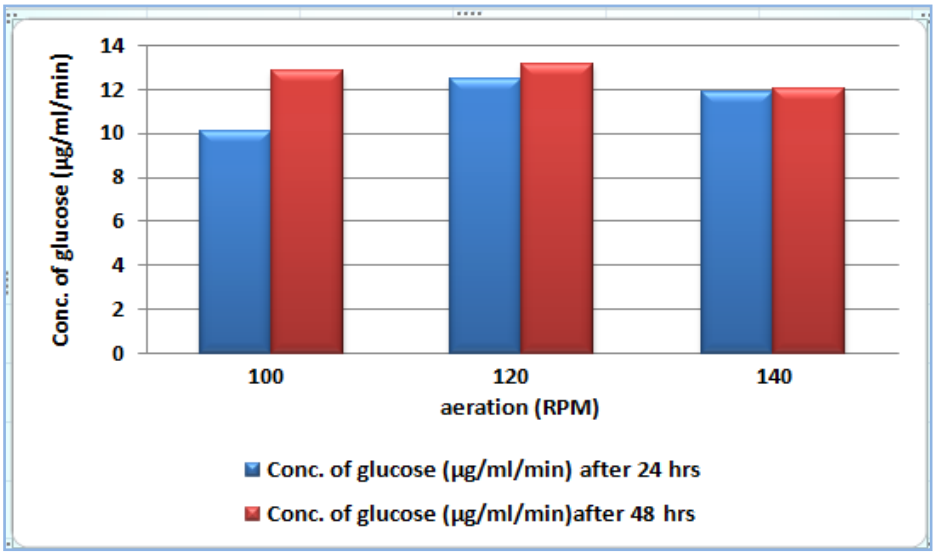

Figure 4.Effect of Aeration on Amylase Production.

Table 7.Effect ofInoculums Size on Amylase Production

Inoculum Size %	Absorbance at 540 nm (24 hrs)	Conc. of glucose (µg/ml)	Absorbance at 540 nm (48 hrs)	Conc. of glucose (µg/ml)
0.50	0.882	11.33	0.888	11.33
1.0	0.774	9.83	0.858	1083
1.5	0.886	11.33	0.896	12.33
2.0	0.656	8.16	0.799	10.16

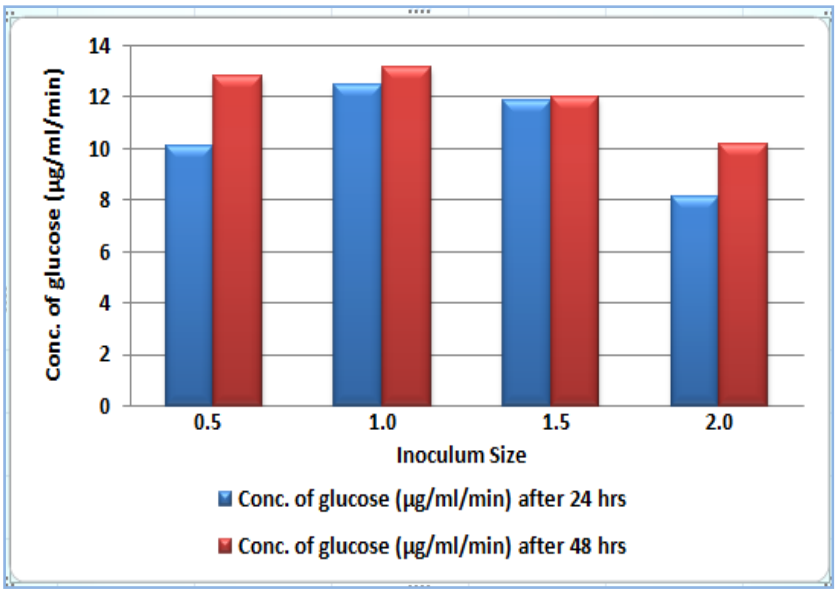

Figure 5.Effect of Inoculums Size on Amylase Production

Table 8.Effect ofpH on Amylase Production

pH	Absorbance at 540 nm 24 hrs	Conc. of glucose µg/ml/min	Absorbance at 540 nm 48 hrs	Conc. of glucose µg/ml/min	Absorbance at 540 nm 72 hrs	Conc. of glucose µg/ml/min
6.5	0.201	2.5	0.217	2.83	0.145	1.66
7.0	0.222	2.83	0.215	2.76	0.186	2.33
7.5	0.197	2.18	0.165	1.93	0.181	2.16
8.0	0.147	1.02	0.151	1.16	0.169	1.18

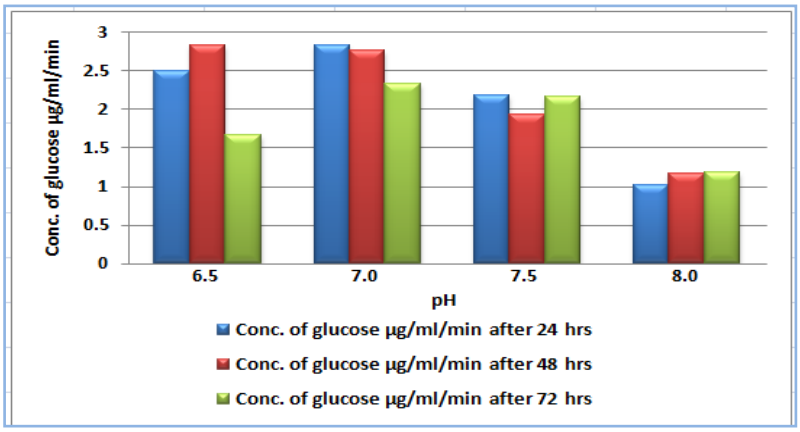

Figure 6.Effect of pH on Amylase Production

The occurrence of amyloytic organisms from the soil agrees with earlier reports. Reportedthat the soil is known to be a store house of amylase producing bacteria and fungi (Rehana and Nand., 1989, Adebiyi *et al*., 1998).

The amylase producing bacterial colonies were grown on nutrient agar plate containing 1% starch which after growth of 24 hrswereflooded with grams iodine showed a sharp zone of hydrolysis of starch with the blue black background (A.K.Pon*et al*., 1999). A number of reports are available in the literature regarding the influence of various environmental conditions of growth parameters like effect of pH, optimum temperature, inoculation incubation period, carbon source & their concentration, nitrogen sources and their concentrations etc. on the production of amylase.

Increase in the incubation period resulted decreases in the amylase production. It may be due to the fact that after maximum production of the amylase the production of other enzymes as well as by products and depletion of the nutrients. These by-products inhibited the growth of bacteria and enzyme formation. The starch concentration is a factor of amylase production. In this experiments we found that 1% starch concentration is appropriate for amylase production (Figure.2).

The temperature of incubation at 37^0C was found most suitable for amylase production among the various incubation temperatures studied(Figure.3).

Aeration or supply of oxygen is another important parameter of growth and production which can be provided in shake flask studies either by reduction in volume of medium or by increasing the shaker speed or by both. In our studies we tried to provide oxygen to the culture by increasing the shaker speed at different RPM. It was observed that maximum amylase was produced at 120 RPM (Figure.4).The size of inoculum is another factor of amylase production. In this experiments for the detection of an appropriate inoculum volume we found that 1% v/v inoculum is sufficient for amylase production (Figure.5).

The enzyme is very sensitive to pH therefore a selection of optimum pH is very essential. Amylase production was therefore studied at pH 6.0, 6.5, 7.0, 7.5 and 8.Highest amylase production was noted at pH 7.0. It was observed that both organic & inorganic nitrogen were essential for growth of bacillus as well as the production of amylase (Figure.6).

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